Mechanistic Studies on *Azospirillum brasilense* Glutamate Synthase†

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ABSTRACT: The reaction mechanism of *Azospirillum brasilense* glutamate synthase has been investigated by several approaches. ^{15}N nuclear magnetic resonance studies demonstrate that the amide nitrogen of glutamine is reductively transferred to 2-oxoglutarate in an irreversible manner with no release of the transferred ammonia group into the medium. Identical results were obtained using thio-NADPH and acetylpyridine–NADPH, which are shown to be less efficient substrates of the enzyme than NADPH. Similarly, no exchange of the ammonia group being transferred with exogenous ammonium ion was observed during catalysis. The glutamate formed as the product of the iminoglutarate reduction was determined to be in the L configuration. The enzyme was also found to catalyze, under anaerobic conditions, the exchange of the 4*proS* H of NADPH with solvent both in the absence and in the presence of 2-oxoglutarate and glutamine. The reductive half-reaction is therefore a reversible segment of the overall irreversible amidotransferase reaction. ^{15}N NMR studies also showed that the enzyme does not catalyze glutamate dehydrogenase/oxidase reactions or any observable glutaminase activity under neutral (pH 7.5) conditions. Glutaminase activity was also not observable with the reduced enzyme alone or in the presence of D-glutamate (a competitive inhibitor of glutamate synthase with respect to 2-oxoglutarate, with a K_i of about 11 μM) or with the oxidized enzyme in the presence of 2-oxoglutarate, D-glutamate, or $NADP^+$. These data confirm species-dependent differences of *A. brasilense* glutamate synthase with respect to the enzyme from other sources.

Glutamate synthase [L-glutamate:$NADP^+$ oxidoreductase (transaminating); EC 1.4.1.13; GltS[1]] plays a key role in ammonia assimilation in plants and in bacteria by catalyzing the transfer of the amide group of glutamine to 2-oxoglutarate (and subsequent reduction of the imine intermediate) to yield two molecules of glutamate. The reducing equivalents for this reaction are provided by NAD(P)H in bacteria (eq 1) and either NAD(P)H or reduced ferredoxin in plants. The enzyme isolated from *Azospirillum brasilense* is similar to other bacterial glutamate synthases in that it contains 1 FAD, 1 FMN, ≈8 non-heme iron atoms, and ≈8 acid-labile sulfur atoms per protomer (Ratti et al., 1985; Vanoni et al., 1991b). The $\alpha\beta$ protomer consists of two dissimilar subunits of ≈140 and ≈55 kDa, respectively. Bacterial glutamate synthases have been reported to catalyze several other activities in addition to the physiological glutamine-dependent reaction (eq 1). The enzymes isolated from *Klebsiella aerogenes* and from *Escherichia coli* have been demonstrated to catalyze, at neutral pH, a glutaminase reaction at a rate 10% that of the glutamate synthase reaction (Trotta et al., 1974; Mantsala & Zalkin, 1976a) (eq 2). The *Klebsiella* enzyme, as well as other bacterial GltS, also catalyzes L-glutamate formation when ammonia is substituted for glutamine (eq 3). The rate for this reaction is found to increase at alkaline pH values (Trotta et al., 1974; Mantsala & Zalkin, 1976a,b). Slow L-glutamate-dependent $NADP^+$ reductase activities of GltS have been reported. With the *Klebsiella* enzyme, however, the rate observed at pH 7.8 was only 0.3% that of the physiological reaction (Trotta et al., 1974). Whether this activity leads to the formation of L-glutamine and 2-oxoglutarate (the reverse of the reaction in eq 1) or represents a glutamate dehydrogenase activity of glutamate synthase (the reverse of the reaction in eq 3) has not been investigated.

$$\text{L-glutamine} + \text{2-oxoglutarate} + \text{NADPH} + H^+ \rightarrow \text{NADP}^+ + 2(\text{L-glutamate}) \quad (1)$$

$$\text{L-glutamine} + H_2O \rightarrow \text{L-glutamate} + NH_3 \quad (2)$$

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADPH} \rightarrow \text{L-glutamate} + \text{NADP}^+ \quad (3)$$

We have recently shown that *Azospirillum* GltS catalyzes both L-glutamine- and ammonia-dependent reactions by a two-site uni–uni bi–bi ping-pong kinetic mechanism (part A of Scheme I; Vanoni et al., 1991a). Although the *Azospirillum* enzyme is very similar to GltS from other sources for the catalysis of the glutamine-dependent reaction (eq 1), the steady-state kinetic properties of the ammonia-dependent reaction differ significantly from those reported for glutamate synthases from other bacteria (Vanoni et al., 1991a,b). Moreover, the comparison of the amino acid compositions and N-terminal sequences of the isolated subunits of *Azospirillum* GltS with those from *E. coli* showed that the α subunits are

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[1] Abbreviations: GltS, glutamate synthase; NMR, nuclear magnetic resonance; NADP(H), (reduced) β-nicotinamide adenine dinucleotide phosphate; acetylpyridine–NADP(H), (reduced) 3-acetylpyridine–adenine dinucleotide phosphate; thio-NADP(H), (reduced) thionicotinamide adenine dinucleotide phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetracetic acid; 2-OG, 2-oxoglutarate.

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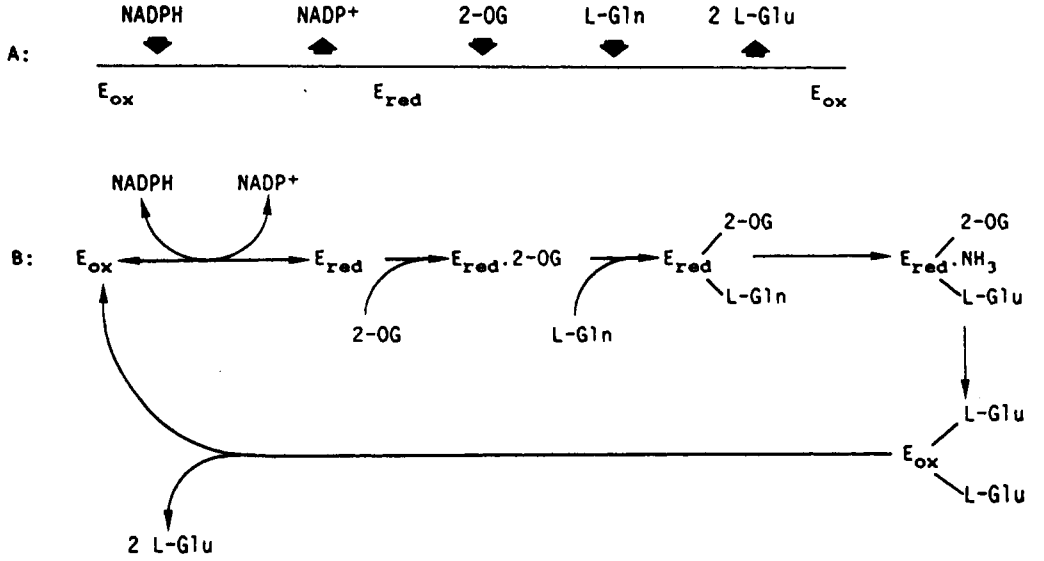
Scheme I



conserved while the β subunits exhibit a small degree of similarity (Vanoni et al., 1990a). Thus, for a better understanding of GltS from *Azospirillum* as compared to other glutamate synthases, a mechanistic study of the *Azospirillum* enzyme was undertaken.

Although considerable information exists on the reactions catalyzed by GltS isolated from various sources, there are still a number of unknown aspects regarding their respective catalytic mechanisms. For example, it is unknown whether the physiological reaction catalyzed is reversible or irreversible, and the mechanism of the amide transfer from L-glutamine to 2-oxoglutarate has not yet been elucidated. With the various activities described for glutamate synthase, such studies might be impossible or at least difficult to investigate using standard approaches. For these reasons, we decided to investigate the reactions catalyzed by *A. brasilense* GltS using ^{15}N NMR spectroscopy in addition to other techniques. Since the chemical shift value of the amide nitrogen resonance of glutamine is well-separated from those of the α-amino group of glutamate and of free ammonium ion, the relative distributions of these reaction substrates and/or products can readily be determined without lengthy separations and analyses (Blomberg & Rueterjans, 1983). If the GltS-catalyzed reaction were found to be reversible, it might be possible to estimate rate constants for the forward and reverse reactions (Campbell & Dwek, 1984). Similarly, the presence (or absence) of other activities such as glutaminase, glutamate dehydrogenase/oxidase, etc., can readily be monitored by NMR. Finally, insights regarding the transfer mechanism from the amide of glutamine to 2-oxoglutarate might be forthcoming from this approach.

The results presented here demonstrate that *A. brasilense* GltS, under conditions of physiological pH, (a) exhibits a reversible NADPH-dependent reductive half-reaction, but overall catalysis is essentially irreversible; (b) catalyzes the reduction of 2-iminoglutarate to form L-glutamate; (c) does not catalyze any glutaminase and/or glutamate dehydrogenase activities; and (d) does not release and/or exchange NH_4^+ with the medium during catalysis.

Experimental Procedures

Materials. GltS was purified from *A. brasilense* Sp6 (Ratti et al., 1985). Bovine liver glutamate dehydrogenase (type III), *E. coli* L-glutamate decarboxylase (type V), and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIII or type XXIV) were from Sigma Chemical Co. The following compounds with 99% enrichment were purchased from Cambridge Isotope Laboratories: L-[*amide*-^{15}N]glutamine, L-[*amino*-^{15}N]glutamic acid, and [^{15}N]ammonium nitrate. NMR tubes (10-mm diameter), equipped with a Teflon seal cap for anaerobiosis, were purchased from Wilmad. L-[U-^{14}C]Glutamate was from Amersham, and 2-oxo[U-^{14}C]glutarate was from New England Nuclear. All other reagents were from Sigma Chemical Co. (St. Louis, MO). Thio-NADPH and acetylpyridine-NADPH were prepared from their oxidized forms with glucose-6-phosphate dehydrogenase and glucose 6-phosphate as described (Vanoni et al., 1990b; Orr & Blanchard, 1984).

NMR Spectroscopic Methods. ^{15}N NMR spectral data were obtained using a Bruker CXP-300 spectrometer operating at 30.4 MHz. A spectral width of 8 kHz was used with 8K data points. Before Fourier transformation, all FID's were exponentially filtered using a 20-Hz line broadening. All chemical shifts were relative to an external standard of 100 mM [^{15}N]ammonium nitrate in water. A 90° pulse, 0.68-s acquisition time, 1-s delay between pulses, and a broad band proton decoupling power of 2 W were used in all experiments. Under these conditions, an acceptable signal-to-noise ratio was achieved after 2000–4000 acquisitions, and the lower limit of detection of any ^{15}N-enriched species is estimated to be approximately 5% of the concentration of the starting material. Typically, solutions containing 10 mM each of the substrates of the reaction under study were made up in 100 mM Hepes/K^+ buffer, pH 7.5, and 10 mM EDTA in a 10-mm NMR tube. Control experiments showed that the enzyme activity was not affected by the buffer concentration or the presence of 10 mM EDTA. Anaerobiosis was achieved by bubbling oxygen-free argon (Williams et al., 1979) in the solution for at least 10 min. The cap was replaced on the NMR tube under a stream of argon. All further additions were made through the Teflon seal with a gas-tight Hamilton syringe. A first NMR spectrum was taken prior to the addition of the enzyme. Additional spectra were recorded at different times while the solution was incubated at room temperature in the dark. At the end of each experiment the enzyme activity was measured and found to be at least 80% retained.

Determination of GltS-Catalyzed Exchange of Tritium from NADPH with the Solvent. (4*S*)-[4-^3H]NADPH was prepared from D-[1-^3H]glucose, ATP, and $NADP^+$ using

hexokinase and glucose-6-phosphate dehydrogenase as described (Vanoni et al., 1990b). Glutamate synthase was incubated with 100 μM (4*S*)-[4-^{3}H]NADPH (17 000 dpm/nmol) in 10 mM Hepes/K^+ buffer, pH 7.5, under anaerobiosis, in the absence or presence of 5 mM L-glutamine and 1.5 mM 2-oxoglutarate. Anaerobiosis was obtained by bubbling oxygen-free nitrogen (Williams et al., 1979) in the reaction mixture for 20 min prior to the addition of the enzyme. All additions and withdrawals were made with gas-tight syringes. Aliquots (110 μL) were withdrawn before the addition of the enzyme and at different time intervals after the addition of glutamate synthase (0.5–11 milliunits). Each aliquot was immediately diluted 5-fold with cold water, and 500 μL was analyzed chromatographically as described in Vanoni et al. (1991a). The fractional quantities of the ^{3}H disintegrations per minute associated with water and with residual NADPH, relative to the total radioactivity eluted from the column, were calculated. These values were correlated with the fractional conversion of NADPH as monitored spectrophotometrically at 340 nm.

Determination of the Stereochemistry of Glutamate Formed from 2-Oxoglutarate by GltS. GltS (17.8 milliunits) was incubated with L-glutamine (5 mM), NADPH (0.2 mM), and 2-oxo-[U-^{14}C]glutarate (0.25 mM, 57 000 dpm/nmol) in 50 mM Hepes/K^+ buffer, pH 7.5, at 25 °C, in a final volume of 1 mL. After complete oxidation of NADPH (45 min from enzyme addition as monitored at 340 nm), GltS was removed by filtration through a Centricon 30 microconcentrator. The filtrate was diluted to 10 mL with water and loaded on a 1 × 10 cm Dowex 1-X8 (acetate form) column, and glutamate was purified as described by Geary and Meister (1977). Glutamate-containing fractions were pooled, lyophilized, dissolved in H_2O, and neutralized. The concentration of glutamate was calculated by taking into account the 50% dilution of label due to the formation of glutamate from glutamine (28 500 dpm/nmol). Glutamate was also prepared by incubating GltS (375 milliunits) with 2-oxo-[U-^{14}C]-glutarate (0.25 mM, 57 000 dpm/nmol), NH_4Cl (0.8 M), and NADPH (0.2 mM) in 50 mM Ches/K^+ buffer, pH 9.3, at 25 °C. The concentration of glutamate, purified as described above, was calculated from the specific radioactivity of 2-oxo-[U-^{14}C]glutarate. As a control, L-glutamate was also synthesized by incubation of bovine liver L-glutamate dehydrogenase (1.8 units) with NH_4Cl (0.2 M), NADPH (0.2 mM), and 2-oxo-[U-^{14}C]glutarate (0.25 mM, 57 000 dpm/nmol) in 50 mM Hepes/K^+ buffer, pH 7.5 at 25 °C in a final volume of 1 mL. The final concentration of L-glutamate was calculated using a specific radioactivity of 57 000 dpm/nmol. The stereochemistry of the glutamate produced enzymatically was determined by conversion to 4-aminobutyrate using L-glutamate decarboxylase and analyzed by ion-exchange chromatography as described (Geary & Meister, 1977). During these experiments 2.5–6 nmol/100 μL [U-^{14}C]-glutamate (26 000–57 000 dpm/nmol depending on the source) in 0.1 M sodium acetate, pH 5.0, was equilibrated at 37 °C. A 10-μL aliquot was counted to determine the total ^{14}C content. A 100-μL aliquot was withdrawn, neutralized by the addition of 30 μL of 0.1 M NaOH, diluted to 500 μL with H_2O, and analyzed on a 0.5 × 1.5 cm Dowex 1-X8 (acetate form) column. L-Glutamate decarboxylase (20–40 μg/100 μL) was added to the remaining reaction mixture. A 100-μL aliquot was withdrawn after 30–60 min and treated as described above. The specific radioactivity of 4-aminobutyrate was calculated by correcting the specific radioactivity of the starting glutamate for loss of 20% of ^{14}C as CO_2 during the L-glutamate decarboxylase reaction.

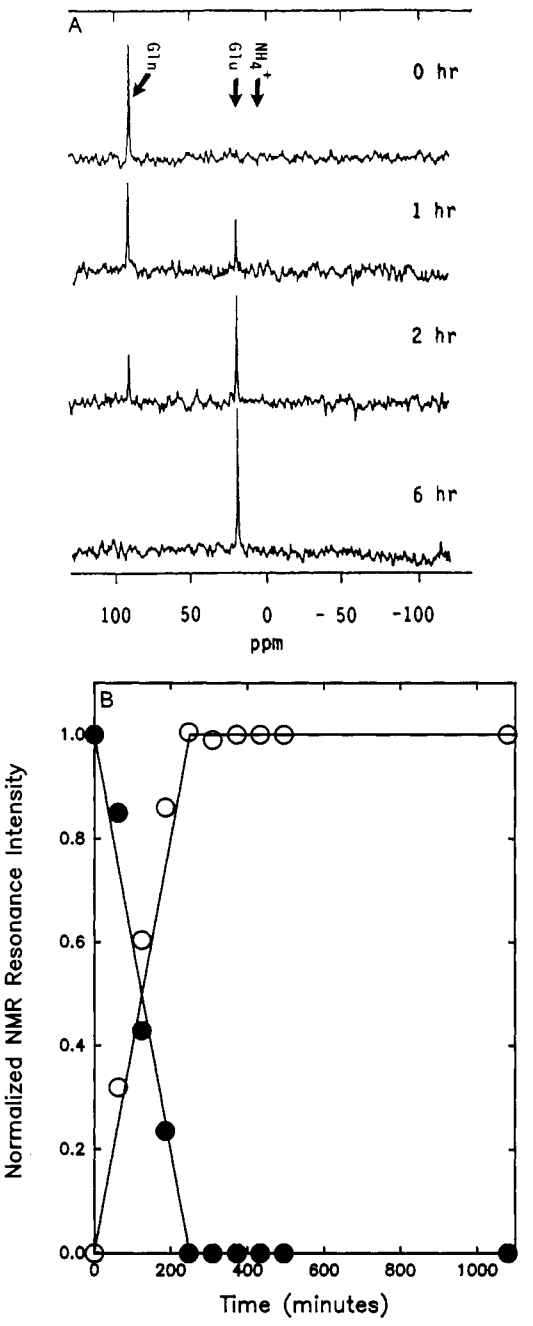


FIGURE 1: Time course of the L-glutamine-dependent GltS reaction as monitored by ^{15}N NMR. A solution containing 10 mM each L-[*amido*-^{15}N]glutamine, 2-oxoglutarate, and NADPH in 100 mM Hepes/K^+, pH 7.5/10 mM EDTA was made anaerobic. Spectra were recorded before addition of the enzyme and at several time intervals after GltS addition (0.15 unit). NMR spectra were recorded over a period of 18 h, and 62 min was required to acquire each spectrum. In both panels the 0-h spectrum was recorded before the addition of the enzyme. Panel A: Representative NMR spectra recorded during the experiment. Panel B: Relative intensities of the signals due to L-[*amido*-^{15}N]glutamine (●) and [*amino*-^{15}N]glutamate (○), respectively, versus the time at which each acquisition was started. The lines drawn are based on the assumption that the rate of L-glutamine loss is identical with the rate of L-glutamate formation.

RESULTS

L-Glutamine-Dependent Activity of GltS As Monitored by ^{15}N NMR. With the activities reported for other glutamate synthases (eqs 1–3), our initial expectation was that a number of ^{15}N-labeled products might form on incubation of L-[*amide*-^{15}N]glutamine with *A. brasilense* GltS, NADPH, and

2-oxoglutarate. Figure 1A shows ^{15}N NMR spectra observed when glutamate synthase was added to an anaerobic solution containing equimolar concentrations (10 mM) of all substrates. A gradual conversion of the ^{15}N resonance of the isotopically enriched amide nitrogen of L-glutamine (91.6 ppm) to the α-amino nitrogen of glutamate (20.5 ppm) was observed. No ammonium ion (5.7 ppm) was detected during the conversion or at times after completion of the reaction (Figure 1B).

Several conclusions can be drawn from this experiment. The total loss of the ^{15}N resonance from the amide nitrogen of glutamine to form the α-amino group of glutamate when the substrates are at equimolar concentrations suggests the reaction catalyzed to be essentially irreversible. Similarly, the failure to observe any resonance from ammonium ion, either during or after conversion of glutamine to glutamate, suggests any glutaminase activity to be quite low relative to amidotransferase activity and that any glutamate dehydrogenase activity is also very slow. Alternatively, the absence of any detectable ammonium ion during the catalyzed reaction may be due to its reutilization in the ammonia-dependent reaction (eq 3) to form glutamate at a rate equal to or faster than its formation. This latter possibility is considered unlikely since previous steady-state kinetic work (Vanoni et al., 1991a) has shown that the ammonia-dependent reaction is undetectable at neutral pH values and that the K_M for ammonia is very high ($\approx$0.5 M, at pH 9.3). In agreement with steady-state data, incubation of GltS with 10 mM each ^{15}N-enriched ammonia, 2-oxoglutarate, and NADPH, at pH 7.5, did not result in any [*amino*-^{15}N]glutamate formation even after 2–3 days (data not shown).

In separate NMR experiments where the NADPH concentration was kept constant at 1 mM using a NADPH-regenerating system (20 mM glucose 6-phosphate and 3.5 units of glucose-6-phosphate dehydrogenase), results identical to those shown in Figure 1A were observed.

Steady-state data showed that thio-NADPH and acetylpyridine–NADPH can serve as reductants of *A. brasilense* GltS. In the presence of thio-NADPH (2.5 mM 2-oxoglutarate and 5 mM L-glutamine in 50 mM Hepes/K^+ buffer, pH 7.5, at 25 °C), the apparent maximal velocity was 54% that obtained in the presence of NADPH with an apparent K_M value of 10 μM. Using acetylpyridine–NADPH, the observed maximal velocity under the above conditions was only 3.7% that calculated with NADPH; the apparent K_M value was 17 μM. With these slower substrates, possible competing reactions might be observable in ^{15}N NMR experiments. When the higher potential NADPH analogues (Scharschmidt et al., 1984), thio-NADPH and acetylpyridine–NADPH, were tested in the presence of the reduced pyridine nucleotide regenerating system, only the conversion of L-glutamine to glutamate was again observed. The progress of the respective reactions proceeded at a slower rate than that observed with NADPH, in agreement with steady-state data.

^{15}N NMR Experiments To Detect L-Glutamate Dehydrogenase/Oxidase Activities of GltS. L-Glutamate dehydrogenase activity (the reversal of the reactions in eqs 1 or 3) has been reported for *K. aerogenes* (Trotta et al., 1974) and *E. coli* (Mantsala & Zalkin, 1976b) glutamate synthases. With the *K. aerogenes* enzyme, this reaction occurs at a rate 0.3% that of the glutamine-dependent reaction, at pH 7.8, whereas for the *E. coli* GltS, at pH 9.2, the reported rate is 12% that of the physiological reaction. Thus, it was of interest to determine if *A. brasilense* GltS could catalyze L-glutamate oxidation which might result in ammonia formation and would be detectable by NMR on prolonged incubation of the enzyme

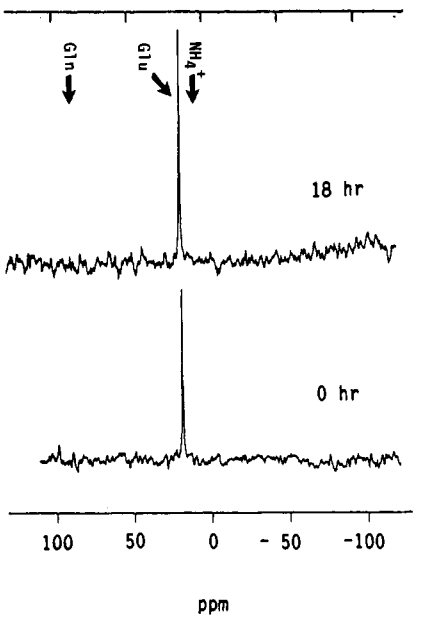


FIGURE 2: Glutamate dehydrogenase reaction of *A. brasilense* GltS. A solution containing L-[*amino*-^{15}N]glutamate (10 mM) and $NADP^+$ (10 mM) in 100 mM Hepes/K^+, pH 7.5/10 mM EDTA was made anaerobic. A NMR spectrum was recorded prior to the addition of GltS (0.15 unit) and 18 h after incubation of the reaction mixture at 25 °C in the dark.

with L-[*amino*-^{15}N]glutamate in the presence of an oxidant such as O_2, $NADP^+$, or its higher potential analogues (thio-$NADP^+$ or acetylpyridine–$NADP^+$). GltS (0.15 unit) was anaerobically incubated with L-[*amino*-^{15}N]glutamate (10 mM) alone or in the presence of $NADP^+$ (10 mM, Figure 2), thio-$NADP^+$ (10 mM), or acetylpyridine–$NADP^+$ (10 mM) in 100 mM Hepes/K^+, pH 7.5/10 mM EDTA. No ^{15}N-enriched L-glutamine or ammonia could be detected even 48 h after enzyme addition. Identical results were obtained when the reaction was carried out with oxygen as electron acceptor, thus ruling out a significant rate of glutamate dehydrogenase or oxidase activity at pH 7.5 and supporting the concept of irreversibility of the overall glutamate synthase reaction.

During separate experiments, $NADP^+$ reduction could be observed when GltS was incubated with L-glutamate (but not D-glutamate) at pH values above 9 (0.2 mM $NADP^+$ and 40 mM L-glutamate). At the apparent optimum pH value for this activity (pH 9.8) the rate of $NADP^+$ reduction was 4–5% that measured for the standard NADPH-dependent glutamine amidotransferase activity. The product of the L-[U-^{14}C]-glutamate oxidation was found by ion-exchange chromatography to be 2-oxoglutarate either in the presence or in the absence of oxygen. No NMR experiments were performed at alkaline pH values due to the instability of the enzyme to prolonged incubations under these conditions.

GltS-Catalyzed Tritium Exchange between NADPH and Solvent. Using GltS preparations isolated from *E. coli* and *K. aerogenes*, Geary and Meister (1977) demonstrated that each enzyme specifically removes the 4*S* H of NADPH during catalysis. They also found the 4*S* ^3H of NADPH to be transferred to solvent in the glutamine-dependent reaction. However, with ammonia as the nitrogen donor, the 4*S* ^3H was directly transferred to the iminoglutarate intermediate to form [2-^3H]glutamate. *A. brasilense* GltS is also found to stereospecifically oxidize NADPH at the 4*S* position (Vanoni et al., 1991a). The time course of tritium release into the solvent from (4*S*)-[4-^3H]NADPH catalyzed by GltS in the presence or absence of the other substrates is shown in Figure 3. The reactions were carried out anaerobically to prevent any

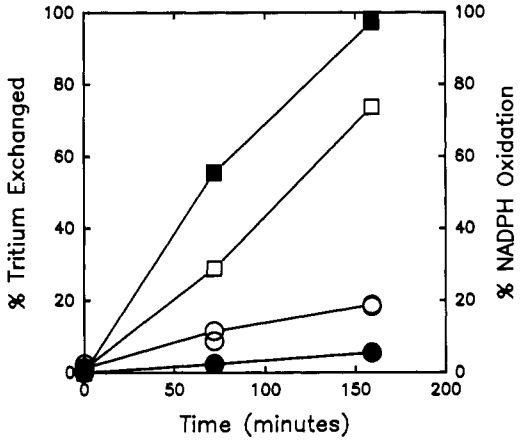
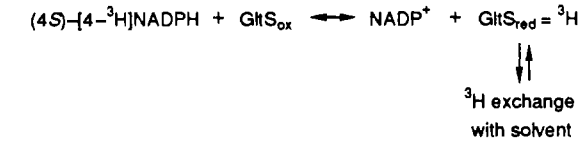


FIGURE 3: GltS-catalyzed tritium exchange with the solvent during NADPH oxidation. A reaction mixture containing 5 mM L-glutamine, 1.5 mM 2-oxoglutarate, 100 μM (4*S*)-[4-^{3}H]NADPH (28 000 dpm/nmol) in 10 mM Hepes/K^+ buffer, pH 7.5, was made anaerobic. A 110-μL aliquot was withdrawn and analyzed as described under Experimental Procedures before the addition of the enzyme. A second 110-μL aliquot was withdrawn, supplemented with 4 units of GltS to obtain complete conversion of NADPH for subsequent analysis. In order to reach NADPH conversion values between 50 and 100% at times convenient for the chromatographic analysis of the reaction components, 0.5 milliunit of GltS was added at times 0, 60, and 72 min to vary the reaction rate (monitored spectrophotometrically at 340 nm) (□, ■). An identical reaction mixture lacking L-glutamine and 2-oxoglutarate was set up, and all additions and withdrawals were done as detailed above (○, ●). On each aliquot the percentage of oxidation of NADPH was determined spectrophotometrically (closed symbols), and the corresponding percentage of tritium exchanged from (4*S*)-[4-^{3}H]NADPH with the solvent was determined by separation on an ion-exchange column and scintillation counting (open symbols).

NADPH oxidase activity. In the absence of L-glutamine and 2-oxoglutarate, NADPH oxidation is barely above control levels, while ≈18% of the 4*S* ^{3}H is exchanged into the solvent after 160 min of incubation. These data are consistent with the following process:



in which the enzyme is reversibly reduced by NADPH. The tritium transferred to the enzyme-bound flavin is exchangeable with solvent, which leads to a greater level of ^{3}H exchange than NADPH oxidation. In the presence of the other substrates, NADPH oxidation proceeds at a measurable rate as does the exchange of the 4*S* ^{3}H from NADPH to solvent. The relatively faster rate of NADPH oxidation relative to ^{3}H exchange results from a tritium kinetic isotope effect on NADPH oxidation in the amidotransferase reaction of GltS, which is estimated to be ≈2 [from the data of Vanoni et al. (1991a)]. Of interest is the finding that no ^{3}H incorporation into glutamate was observed in either the glutamine- or the ammonia-dependent reaction of glutamate synthase from *A. brasilense* (Vanoni et al., 1991a), at variance with the results of Geary and Meister (1977) on the *E. coli* and *K. aerogenes* enzymes.

Stereochemistry of Glutamate Produced from 2-Oxoglutarate. Previous work with *E. coli* and *K. aerogenes* GltS preparations (Geary & Meister, 1977) demonstrated that L-glutamate is formed from 2-oxoglutarate by dithionite-reduced enzyme in the presence of glutamine. During the course of this study we found that D-glutamate is a good competitive inhibitor with respect to 2-oxoglutarate with a K_i value of 11 μM. D-Glutamate is also noncompetitive with L-glutamine ($K_i \approx 56$ μM) and uncompetitive with NADPH ($K_i \approx 44$ μM). The affinity of the D isomer of glutamate for the enzyme is approximately three orders of magnitude higher than that of L-glutamate (Vanoni et al., 1991a). Thus, it was important to determine if *A. brasilense* GltS also catalyzes the formation of L-glutamate. If D-glutamate were a product of the reductive amination of 2-oxoglutarate in *A. brasilense* GltS, the apparent irreversibility observed for the reaction could result from inhibition. Using 2-oxo-[U-^{14}C]glutarate and L-glutamate decarboxylase (Table I), the glutamate formed from 2-oxoglutarate was determined to be >95% in the L configuration for both the glutamine- and the ammonia-dependent reactions. These data therefore establish the stereochemistry of imine reduction by the enzyme in both reactions and eliminate the possibility of product inhibition as contributing to the observed irreversibility of the glutamine-dependent reaction.

Table I: Determination of the Stereochemistry of Glutamate Formed from 2-Oxoglutarate in the Glutamate Synthase Reaction

glutamate preparation	glutamate (nmol)	4-aminobutyrate (nmol)
commercial L-glutamate	3.3[a,c]	3.4
	4.4[a,c]	4.5
	6.0[b,d]	6.1
GDH reaction[e]	2.6[b,d]	2.6
GltS, L-Gln-dependent reaction[e]	4.4[a,c]	4.4
	4.5[b,d]	4.3
	5.1[b,d]	4.9
	2.5[b,d]	2.4
	2.6[b,d]	2.5
GltS, NH_3-dependent reaction[e]	5.2[b,d]	5.0
	2.6[b,d]	2.5

[a] 30-min incubation time with L-glutamate decarboxylase. [b] 60-min incubation time with L-glutamate decarboxylase. [c] 20 μg/100 μL L-glutamate decarboxylase in incubation mixture. [d] 40 μg/100 μL L-glutamate decarboxylase in incubation mixture. [e] See Experimental Procedures for conditions.

Attempt To Detect Exchange of Ammonia during the Amidotransferase Reaction of GltS with Exogenous Ammonia by ^{15}N NMR. The results in Figure 1 show no detectable release of ammonia into the solvent during the glutamine-dependent reaction of GltS. If an enzyme–ammonium ion intermediate is present in the reaction, the possibility exists for exchange of bound ammonia with exogenous ammonia in the medium (part B of Scheme I), even though no ammonia-dependent reaction (eq 3) is observable at pH 7.5.

To test for this exchange reaction, GltS was incubated with L-glutamine (10 mM), 2-oxoglutarate (10 mM), and NADPH (10 mM) in 100 mM Hepes/K^+ buffer/10 mM EDTA, pH 7.5, in the presence of 10 mM [^{15}N]ammonium nitrate for 18 h (a time sufficient for complete conversion of glutamine into glutamate). No ^{15}N-enriched glutamate was observed in the spectrum of this reaction mixture (Figure 4). This result is in agreement with the absence of ammonia leakage from the active site observed in Figure 1A and suggests either a tight binding of the "transferred" ammonia to a group on the enzyme or a concerted mechanism for ammonia transfer from L-glutamine to 2-oxoglutarate. Further work is required to distinguish the two possibilities. These results also suggest that the imine of the putative iminoglutarate intermediate in the GltS reaction does not exchange readily with exogenous ammonium ion. This is probably a result of its being tightly bound to the enzyme and of having only a transitory lifetime in the steady-state. Again, further work is required to probe this aspect of the enzyme-catalyzed reaction.

^{15}N NMR Tests for Glutaminase Activity in A. brasilense GltS. GltS preparations from *E. coli* and *K. aerogenes* exhibit (as do a number of other glutamine-dependent amido-

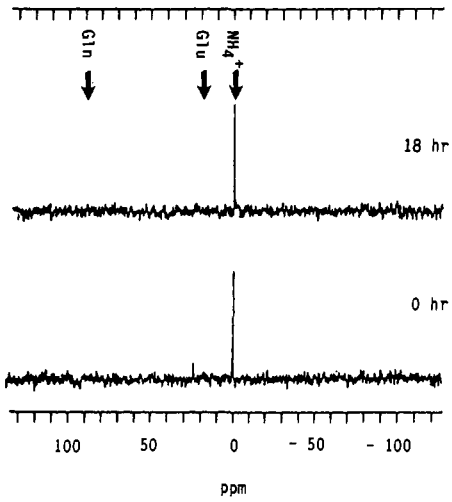


FIGURE 4: Attempt to detect exchange of ammonia during GltS reaction with exogenous ^{15}N-enriched ammonium ion. A solution containing 10 mM each L-glutamine, 2-oxoglutarate, NADPH, and ^{15}N-enriched ammonium nitrate in 100 mM Hepes/K^+, pH 7.5/10 mM EDTA was made anaerobic. NMR spectra were recorded before and 18 h after the addition of GltS (0.15 unit).

transferases; Buchanan, 1973) a low but detectable glutaminase activity (Trotta et al., 1974; Mantsala & Zalkin, 1976a; Geary & Meister, 1977). This activity appears to be dependent on the pH of the medium and on the term of storage for the above GltS preparations (Geary & Meister, 1977). To test whether *A. brasilense* GltS exhibits any glutaminase activity, L-[*amido*-^{15}N]glutamine (10 mM) was incubated with the enzyme (0.15 unit) in 100 mM Hepes/K^+ buffer/10 mM EDTA, pH 7.5, for 18 h. Any glutaminase activity would result in the formation of a resonance due to ^{15}N-enriched ammonium ion in the NMR spectrum. As shown in Figure 5, no detectable levels of NH_4^+ are present, thus demonstrating the absence of any significant glutaminase activity under the above conditions for *A. brasilense* GltS. Mantsala and Zalkin (1976a) have observed an increase (60–70%) in L-glutaminase activity in *E. coli* GltS on addition of NADPH or 2-oxoglutarate. To test whether this stimulation is operative for *A. brasilense* GltS, NMR experiments were carried out under several conditions. As shown in Figure 5, no glutamine hydrolysis is observed in the presence of 2-oxoglutarate or its analogue D-glutamate (10 mM), $NADP^+$ (10 mM), NADPH (1 mM in the presence of the NADPH-regenerating system), or NADPH plus D-glutamate. The latter experiment was designed to optimize the binding of glutamine, which has been shown to bind subsequent to 2-oxoglutarate to the reduced enzyme in the catalytic mechanism (Vanoni et al., 1991a). If the level of glutaminase activity of *A. brasilense* GltS approached the values reported for the enzymes from other sources, it should have been detected in the NMR studies presented.

Discussion

Previous steady-state kinetic studies from our laboratory (Vanoni et al., 1991a) have shown that *A. brasilense* GltS operates via a two-site ping-pong uni–uni bi–bi kinetic mechanism (part A of Scheme I), the communication between the sites being provided by flavins and/or iron–sulfur centers. By virtue of the known properties of other glutamate synthases (Rendina & Orme-Johnson, 1978; Geary & Meister, 1977), and based on preliminary absorption spectral data from this laboratory obtained on the *Azospirillum* enzyme (Ratti et al., 1987; Vanoni et al., 1991c), we have proposed that NADPH reduces the flavin located at one of the subsites in a reversible

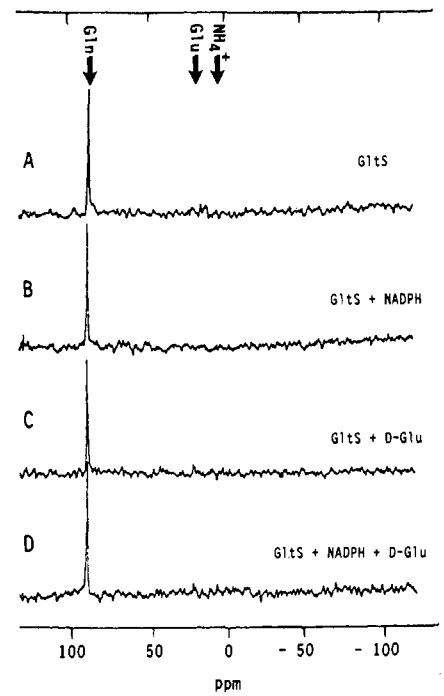


FIGURE 5: Glutaminase activity of *A. brasilense* GltS. GltS (0.15 unit) was incubated for 18–24 h at 25 °C in the dark with L-[*amido*-^{15}N]glutamine (10 mM) in 100 mM Hepes/K^+ buffer, pH 7.5/10 mM EDTA alone (panel A) or in the presence of 1 mM NADPH and the NADPH-regenerating system (panel B); 10 mM D-glutamate (panel C); 1 mM NADPH (plus NADPH regenerating system) and 10 mM D-glutamate (panel D).

reaction. As found with other GltS, the *A. brasilense* enzyme stereospecifically removes the 4*proS* H of NADPH and catalyzes exchange of this hydrogen with solvent in the absence of other substrates. These results are consistent with the view that NADPH reduction is a reversible process. The second half-reaction (part B of Scheme I) consists of the transfer of the amide group of L-glutamine to 2-oxoglutarate and subsequent reduction of the 2-iminoglutarate to form glutamate. The ^{15}N NMR studies presented here demonstrate this to be essentially an irreversible process as found with other amidotransferases (Buchanan, 1973). The product formed on reduction of the 2-iminoglutarate is demonstrated here to be L-glutamate rather than the D isomer, regardless of the ammonia donor (L-glutamine or free ammonia). No evidence was obtained for any leakage into the medium of the ammonia group being transferred or for any exchange of this group with exogenous ammonium ion. Thus, the ammonia transfer step is tightly coupled as well as irreversible. Whether the ammonia transfer from L-glutamine to 2-oxoglutarate is a concerted or a stepwise reaction remains a question for future work. The failure to detect any glutaminase activity with *A. brasilense* GltS in either its oxidized or reduced form suggests that binding of 2-oxoglutarate to the reduced enzyme is required for the hydrolytic cleavage of the amide group of glutamine. Binding of the competitive inhibitor D-glutamate to the 2-oxoglutarate site is not sufficient to effect glutamine hydrolysis. In contrast to other GltS studied, the *A. brasilense* enzyme seems to exert strict control on the amide hydrolytic reaction as well as on the ammonia transfer. Thus, under neutral pH conditions, this enzyme is efficient in committing the nitrogen of glutamine amide group toward the L-glutamate-dependent pathways and does not appear to give rise to a futile cycle where ammonia is released in the medium. This property may be particularly relevant to GltS preparations from nitrogen-fixing organisms where nitrogenase activity and *nif* gene ex-

pression are very sensitive to cellular ammonia levels (Postgate, 1982). Indeed, future comparative studies on GltS from nitrogen-fixing and non-fixing organisms may be useful in order to define the role(s) of GltS in nitrogen fixation.

References

Blomberg, F., & Rueterjans, H. (1983) in *Biological Magnetic Resonance* (Berliner, L. J., & Reuben, J., Eds.) pp 21–73, Plenum Press, New York.

Buchanan, J. M. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol. 39*, 91–183.

Campbell, I. D., & Dwek, R. A. (1984) in *Biological Spectroscopy*, pp 127–177, Benjamin/Cummings Publishing Co., Menlo Park, CA.

Geary, L. E., & Meister, A. (1977) *J. Biol. Chem. 252*, 3501–3508.

Mantsala, P., & Zalkin, H. (1976a) *J. Biol. Chem. 251*, 3294–3299.

Mantsala, P., & Zalkin, H. (1976b) *J. Biol. Chem. 251*, 3300–3305.

Orr, G. A., & Blanchard, J. S. (1984) *Anal. Biochem. 142*, 232–234.

Postgate, J. R. (1982) in *The Fundamentals of Nitrogen Fixation*, pp 103–137, Cambridge University Press, Cambridge.

Ratti, S., Curti, B., Zanetti, G., & Galli, E. (1985) *J. Bacteriol. 163*, 724–729.

Ratti, S., Vanoni, M. A., Grandori, R., Zanetti, G., & Curti, B. (1987) in *Flavins and Flavoproteins 1987* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 385–389, W. de Gruyter, Berlin.

Rendina, A. R., & Orme-Johnson, W. H. (1978) *Biochemistry 17*, 5385–5393.

Scharschmidt, M., Fisher, M. A., & Cleland, W. W. (1984) *Biochemistry 23*, 5471–5478.

Trotta, P. P., Platzer, K. E. B., Haschemeyer, R. H., & Meister, A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4607–4611.

Vanoni, M. A., Negri, A., Zanetti, G., Ronchi, S., & Curti, B. (1990a) *Biochim. Biophys. Acta 1039*, 374–377.

Vanoni, M. A., Wong, K. K., Ballou, D. P., & Blanchard, J. S. (1990b) *Biochemistry 29*, 5790–5796.

Vanoni, M. A., Nuzzi, L., Rescigno, M., Zanetti, G., & Curti, B. (1991a) *Eur. J. Biochem.* (in press).

Vanoni, M. A., Zanetti, G., & Curti, B. (1991b) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. 3, pp 309–317, CRC Press, Boca Raton.

Vanoni, M. A., Zanetti, G., Curti, B., & Edmondson, D. E. (1991c) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., & Zanetti, G., Eds.) pp 749–753, W. de Gruyter, Berlin.

Williams, C. H., Jr., Arscott, D. L., Matthews, R. G., Thorpe, C., & Wilkinson, K. D. (1979) *Methods Enzymol. 62*, 185–198.