The Recombinant α Subunit of Glutamate Synthase: Spectroscopic and Catalytic Properties†

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Received September 22, 1997; Revised Manuscript Received November 5, 1997

ABSTRACT: As part of our studies of *Azospirillum brasilense* glutamate synthase, a complex iron−sulfur flavoprotein, we have overproduced the two enzyme subunits separately in *Escherichia coli*. The β subunit (53.2 kDa) was demonstrated to contain the site of NADPH oxidation of glutamate synthase and the FAD cofactor, which was identified as Flavin 1 of glutamate synthase, the flavin located at the site of NADPH oxidation. We now report the overproduction of the glutamate synthase α subunit (162 kDa), which is purified to homogeneity in a stable form. This subunit contains FMN as the flavin cofactor which exhibits the properties of Flavin 2 of glutamate synthase: reactivity with sulfite to yield a flavin-*N*(5)-sulfite addition product ($K_d = 2.6 \pm 0.22$ mM), lack of reactivity with NADPH, reduction by L-glutamate, and reoxidation by 2-oxoglutarate and glutamine. Thus, FMN is the flavin located at the site of reduction of the iminoglutarate formed on the addition of glutamine amide group to the C(2) carbon of 2-oxoglutarate. The glutamate synthase α subunit contains the [3Fe-4S] cluster of glutamate synthase, as shown by low-temperature EPR spectroscopy experiments. The glutamate synthase α subunit catalyzes the synthesis of glutamate from L-glutamine and 2-oxoglutarate, provided that a reducing system (dithionite and methyl viologen) is present. The FMN moiety but not the [3Fe-4S] cluster of the subunit appears to participate in this reaction. Furthermore, the isolated α subunit of glutamate synthase exhibits a glutaminase activity, which is absent in the glutamate synthase holoenzyme. These findings support a model for glutamate synthase according to which the enzymes prepared from various sources share a common glutamate synthase function (the α subunit of the bacterial enzyme, or its homologous polypeptide forming the ferredoxin-dependent plant enzyme) but differ for the chosen electron donor. The pyridine nucleotide-dependent forms of the enzyme have recruited a FAD-dependent oxidoreductase (the bacterial β subunit) to mediate electron transfer from the NAD(P)H substrate to the glutamate synthase polypeptide. However, it appears that the presence of the enzyme β subunit and/or of the additional iron−sulfur clusters (Centers II and III) of the bacterial glutamate synthase is required for communication between Center I (the [3Fe-4S] center) and the FMN moiety within the α subunit, and for ensuring coupling of glutamine hydrolysis to the transfer of the released ammonia molecule to 2-oxoglutarate in the holoenzyme.

Glutamate synthase (EC 1.4.1.13, L-glutamate:$NADP^+$ oxidoreductase (transaminating), GltS[1]) catalyzes the reductive transfer of the glutamine amide group to the C(2) carbon of 2-oxoglutarate (2-OG). The reducing equivalents are provided by NADPH in the bacterial enzyme (NADPH-GltS), by reduced ferredoxin in the ferredoxin-dependent form of GltS (Fd-GltS) found in photosynthetic tissues, and by NADH in the yeast and plant NADH-dependent GltS (NADH-GltS). The enzyme plays an essential role in nitrogen metabolism in all organisms where the presence of this enzyme has been demonstrated. Interestingly, the distribution and role of glutamate synthase in higher eukaryotes is still unclear (*1*, *2*).

† This work was carried out with funds from Ministero dell'Università e la Ricerca Scientifica e Tecnologica, Consiglio Nazionale delle Ricerche (Contracts CNR-9302092 to G.Z., CNR-9502988CT14 to B.C., and CNR-93.00441CT04 and CNR-94.02504.CT04 to M.A.V.).

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[1] Abbreviations: GltS, glutamate synthase; NAD(P)H- or Fd-GltS, nicotinamide adenine dinucleotide (phosphate) dependent or ferredoxin dependent GltS, respectively; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; Taps, *N*-[tris(hydroxymethyl)-methyl]-3-aminopropanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); 2-OG, 2-oxoglutarate; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; AADP, 3-aminopyridine adenine dinucleotide phosphate; MV, methyl viologen; 5-deazaflavin, 5-deaza-5-carbariboflavin; FPLC, fast protein liquid chromatography; EPR, electron paramagnetic resonance; *K* indicates, depending on the context, the Michaelis constant or the dissociation constant (K_d) of the compound indicated as the subscript; K_i is the inhibition constant.

S0006-2960(97)02342-8 CCC: $15.00
Published on Web 01/30/1998

Studies from this laboratory on the GltS of *Azospirillum brasilense*, a Gram-negative nitrogen-fixing bacterium, have demonstrated that the bacterial enzyme is composed of two dissimilar subunits (α subunit, 162 kDa; β subunit, 52.3 kDa) which form the active αβ protomer (*3*−*5*). The latter contains two flavin cofactors (one FAD and one FMN) and three different iron-sulfur centers: one [3Fe-4S]$^{0;1+}$ cluster (Center I) and two [4Fe-4S]$^{1+;2+}$ centers (Centers II and III) (*6*). We have proposed a model for the active center of GltS, based on a series of kinetic and equilibrium spectroscopy studies on the enzyme from *Azospirillum* cells (Scheme I of ref *6*). According to the model, NADPH binds at Site 1 of GltS, and it is oxidized on electron transfer to Flavin 1 located at this site. The electrons flow through Centers I and II to Flavin 2 at Site 2 where reduction of the iminoglutarate, formed from addition of the amide group of L-Gln to 2-OG, takes place. The role of Center III of GltS is still unknown; its potential seems too low for it to be certainly involved in the intramolecular electron transfer process.

The cloning of GltS structural genes, *glt*D encoding the β subunit and *glt*B encoding the α subunit, allowed us to deduce the enzyme primary structure (*5*). Through comparison with those of other GltS's, we reached the conclusion that GltS from various sources is very conserved (*7*). Furthermore, sequence comparison of GltS and enzymes using the same substrates and cofactors allowed us to tentatively identify functional regions within the enzyme subunits (*5*). The overproduction of *Azospirillum* GltS β subunit in *Escherichia coli* allowed its purification and its kinetic and spectroscopic characterization (*8*). This subunit contains the GltS NADPH binding site and one FAD, which was identified with Flavin 1 of GltS. We demonstrated that the β subunit of GltS contains the functional Site 1 of GltS. Thus, it acts as a FAD-dependent NADPH oxidoreductase and serves to catalyze electron transfer from NADPH to the α subunit where the actual synthesis of L-Glu from L-Gln and 2-OG should take place. On the basis of these results, a common model for all GltS's could be proposed: they all share a polypeptide harboring the glutamine amidotransferase and glutamate synthase activities (the bacterial α subunit, the single polypeptide that forms the Fd-GltS or the corresponding part of the NADH-GltS). Vice versa, the enzyme species differ in the electron donor chosen. Reduced ferredoxin interacts reversibly with Fd-GltS, while the pyridine nucleotide dependent GltS has recruited a FAD-dependent NAD(P)H oxidoreductase. This is the small subunit of bacterial GltS, which in yeast and plants is fused at the C-terminus of the actual glutamate synthase yielding the single long polypeptide chain of NADH-GltS.

We report here the overproduction of the *A. brasilense* GltS α subunit in *E. coli*. A purification procedure is described that yields homogeneous preparations which allowed kinetic and spectroscopic characterization of the protein. The results are discussed in light of the known properties of the GltS holoenzyme and of the Fd-GltS, which should be evolutionary related to the bacterial GltS α subunit.

MATERIALS AND METHODS

Construction of pESTA4 for the Expression of the GltS α Subunit in E. coli. p1605 plasmid, the pUC18 derivative containing the 10 kb *Eco*RI fragment of *Azospirillum* DNA comprising *glt*D and *glt*B genes (*5*) was used as the starting material for the construction of an expression system for the *glt*B gene encoding the GltS α subunit. The 1.3-kb *Eco*RV−*Sca*I fragment was subjected to a site-directed mutagenesis experiment using the Sculpture in vitro mutagenesis system produced by Amersham. As a result a *Nde*I restriction site was introduced within the ATG codon of *glt*B. The *Eco*RV−*Sca*I fragment of one of the clones obtained from the mutagenesis experiment, which exhibited the correct nucleotide sequence, was substituted for the corresponding fragment of p1605. The 10.2-kb *Nde*I−*Bam*HI fragment of this plasmid, containing *glt*B, was ligated into pET11a (*9*) digested with the same enzymes. In this way, *glt*B is suitably positioned downstream from the strong promoter and the ribosome binding site of pET11a. The resulting plasmid (pESTA4) was transferred to *E. coli* BL21(DE3). All DNA manipulations were carried out using standard protocols (*10*, *11*), and sequencing was carried out with the T7 sequencing kit (Pharmacia) using 7-deaza-dGTP and 7-deaza-dATP to resolve band compressions in sequencing gels due to the high GC content of *Azospirillum* DNA (*5*).

Overproduction of the GltS α Subunit in E. coli BL21-(DE3) Harboring pESTA4. E. coli BL21(DE3) cells harboring pESTA4 were stored at −80 °C as glycerol stocks (*9*). Cells were plated on solid LB medium containing 100 μg/mL ampicillin and were allowed to grow overnight at 30 °C. A single colony was used to inoculate 100 or 500 mL of LB medium containing 100 μg/mL ampicillin. When the absorbance at 600 nm of the cultures reached 0.6−1.0, cells were harvested by centrifugation in the cold, resuspended in fresh culture medium, and used to inoculate 1.5 or 12 L of LB medium supplemented with ampicillin in 1.5 or 12 L fermentors (New Brunswick Scientific Co., NJ). When the absorbance of the culture reached 0.8−1.3, isopropyl β-D-thiogalactopyranoside (IPTG) was added for a final concentration of 0.1 mM. Growth was carried out at 25 or 30 °C, and aeration was set at 1.5−2 L/min and stirring at 400 rpm. Before and at different times after IPTG addition, aliquots of cells were harvested and then lysed by incubation at 100 °C for 5 min in 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% (v/v) 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue. These total cell extracts were analyzed by SDS electrophoresis on 10% minigels. During preliminary experiments, 0.5−1 g of cells was also harvested before and at different times after IPTG addition. They were homogenized with glass beads (see below), and after centrifugation at 40000*g* for 60 min, the supernatant was used to determine the specific activity of the GltS α subunit in cells and the electrophoretic pattern in the presence of SDS.

Purification of the Recombinant GltS α Subunit. Cells (8−20 g) were resuspended in 50 mM imidazole/HCl buffer, pH 7.5, 1 mM EDTA, 0.1 M KCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.01 mg/mL deoxyribonuclease (buffer A, 2 mL/g of cells). The suspension was mixed with glass beads (0.25−0.3 mm OD, 6 g/g of cells), and cells were disrupted by agitation. For 1−2-g aliquots of cells, the homogenization was carried out in glass test tubes and cells were disrupted with five 1-min cycles on a Vortex. For larger quantities a Vibrogen Zellmuehle (Bueler, Tuebingen) thermostated at 4 °C was used. The homogenate was diluted with buffer A (6 mL/g of cells) and centrifuged

at 5 °C at 40000*g* for 60 min. In the final procedure, the supernatant was diluted 2-fold with 10% glycerol and directly loaded on a 1 × 32 cm Q-Sepharose fast-flow column (bed size, 27 mL) equilibrated with buffer B (25 mM Pipes/KOH, 1 mM EDTA, 10% glycerol, 1 mM 2-OG), pH 7.5. Unbound proteins were removed by washing with the equilibration buffer, followed by the same buffer at pH 7.0. Weakly bound proteins were then removed using 5 volumes of buffer B, pH 7.0, containing 50 mM NaCl. The elution of the GltS α subunit was then achieved by applying a 50−500 mM NaCl gradient in buffer B, pH 7.0, in 200 mL. The GltS α subunit was eluted in a single peak between 150 and 250 mM NaCl. Fractions containing α subunits were pooled on the basis of their activity content and their spectral properties. The pooled fractions were concentrated by ultrafiltration in an Amicon apparatus equipped with a YM 30 membrane and chromatographed on an Ultrogel AcA 34 (LKB) column (1.5 cm × 120 cm; bed size 210 mL) equilibrated with buffer B, pH 7.5, lacking 2-OG. Fractions from the gel filtration were pooled on the basis of the quality of the absorbance spectrum in the visible and UV region and their specific activity. After concentration by ultrafiltration, aliquots were frozen in liquid nitrogen and stored at −80 °C.

Activity Assays. Glutamate synthase activity assays were carried out as described (*12*). The activity of the GltS α subunit was assayed routinely by measuring the initial velocity of reduction of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, 0.1 or 0.5 mM) at 490 nm in the presence of L-Glu (20 mM) in 50 mM Caps/KOH buffer, pH 9.5, at 25 °C. An extinction coefficient for INT at 490 nm of 18.5 mM^{-1} cm^{-1} was used.

Protein Determination. The biuret method (*13*) was used to determine protein concentration in crude extracts. The Bradford protein assay reagent (Amresco, *14*) was used in all other instances. Bovine serum albumin was the standard protein.

Electrophoretic Techniques. SDS electrophoresis was carried out using 10% acrylamide/bis(acrylamide) minigels (*15*) in a Hoefer apparatus. Protein samples were denatured by diluting them in 4X sample buffer (0.5 M Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 20% (v/v) 2-mercaptoethanol) and by incubating them at 100 °C for 5 min. Gels were stained with Coomassie Blue (0.1% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol, 10% (v/v) acetic acid) and thoroughly destained by diffusion on immersion in 40% (v/v) methanol, 10% (v/v) acetic acid.

Steady-State Kinetic Measurements. The kinetic parameters V, K_{L-Glu}, and K_{INT} were determined by measuring the initial velocities of reaction mixtures containing varying concentrations of L-Glu (1−20 mM) in the presence of constant levels of INT (0.03−0.5 mM) in 50 mM Caps/KOH, pH 9.5. After visual analysis of double reciprocal plots, which were linear, the steady-state kinetic parameters were determined by fitting the primary data to eq 1 (*16*).

$$v_0 = (VS)/(K + S) \qquad (1)$$

where v_0 is the initial reaction velocity; V is the apparent maximum velocity, K is the apparent Michaelis constant for the varied substrate; and S is the concentration of the varied substrate.

Increasing the INT concentration above 0.1 mM led to inhibition, and only data obtained at lower INT concentrations were used for further analysis. Double reciprocal plots showed that the data at INT concentrations below 0.1 mM intersected in the upper left quadrant. Data were thus fitted with eq 2, which describes a ternary complex mechanism:

$$v_0 = (VAB)/(K_{iA}K_B + K_BA + K_BA + AB) \qquad (2)$$

where v_0 is the initial reaction velocity; A and B are the concentrations of the varied substrate and of the substrate held at constant levels, respectively; K_A and K_B are the Michaelis constants for A and B, respectively; and K_{iA} is the calculated dissociation constant of the enzyme−A complex, assuming that A binds first.

In order to determine the effect exerted by 2-OG on L-Glu oxidation, initial velocities were measured on reaction mixtures containing L-Glu (20 mM), INT (0.1 mM), and increasing concentrations of the effector in 50 mM Caps/KOH, pH 9.5. For the determination of the inhibition constant exhibited by 2-OG, initial velocity measurements were carried out by varying L-Glu (1−20 mM) in the presence of INT (0.1 mM) and different levels of the inhibitor. The linearity and pattern of double reciprocal plots were determined visually, and then the data were fitted to eq 3,

$$v_0 = (VS)/(K(1 + I/K_i) + S) \qquad (3)$$

where the symbols used are the same as those defined for eq 1, I is the inhibitor concentration held constant at different levels, and K_i is the inhibition constant.

In order to determine the effect of L-Gln and 3-aminopyridine adenine dinucleotide phosphate (AADP, a nonreducible analog of $NADP^+$, *17*) on the L-Glu:INT oxidoreductase activity of the GltS α subunit, activity measurements were carried out using L-Glu (5 mM), INT (0.1 mM), and increasing concentrations of the effector (up to 20 mM) in 50 mM Caps/KOH, pH 9.5, or 50 mM Taps/KOH, pH 8.5. Similar measurements were carried out using a solution of GltS holoenzyme.

Characterization of the Reaction Products of the GltS α Subunit. Aliquots of the α subunit or of the GltS holoenzyme were incubated under anaerobiosis with ^{14}C-labeled L-Gln or 2-OG under various conditions. In all cases, a 250-μL reaction mixture containing L-Gln and 2-OG in 50 mM Tris-HCl buffer, pH 8.0, was prepared in a Reacti-Vial equipped with a Teflon septum. NADPH or methyl viologen (MV) was added as specified in Tables 3 and 5. When anaerobiosis was required, needles were inserted in the Teflon septum to allow bubbling of oxygen-free nitrogen gas into the solution. After 30 min, an aliquot of an anaerobic dithionite solution (100 mM in 100 mM Tris-HCl buffer, pH 8.0) was added, followed by an aliquot of enzyme solution. The reaction vessel was protected from light with aluminum foil, and a nitrogen stream was maintained on top of the reaction mixture during the time of the assay. After 20 min of incubation at room temperature (25 °C), the vessel was opened to air and the reaction mixture was directly loaded onto a 1-mL Dowex 1-X8 column (acetate form) equilibrated with water (*18*, *19*). Glutamine was eluted by washing the column with five 1-mL aliquots of water; glutamate was

eluted with five 1-mL aliquots of 0.3 M acetic acid. Under these conditions, 2-OG remains bound to the resin. Each fraction was directly collected in a scintillation vial, and after addition of 4 mL of Ultima Gold (Packard) scintillation fluid, radioactivity was counted in a PackardTri-Carb 2, 100TR scintillation counter. Counting efficiency was always 94%. Experiments were carried out using L-[U-^{14}C]glutamine (26 300 dpm/nmol, Amersham) or 2-oxo-[U-^{14}C]glutarate (26 300 dpm/nmol, New England Nuclear), as described later (see legend of Table 3). The identification of the glutamate product of the GltS holoenzyme and of its α subunit was carried out as described previously (*19*) as specified in the legend to Table 4 using *E. coli* L-glutamate decarboxylase (type V, Sigma) to convert L-glutamate to 4-aminobutyrate. In the quantitation of the reaction product, a correction was made for the loss of $^1/_5$ of the radioactivity of 2-oxo-[U-^{14}C]glutarate as CO_2 if 2-OG had been indeed converted to L-[U-^{14}C]glutamate by GltS holoenzyme or the GltS α subunit.

N-Terminal Sequence Analyses. The N-terminal sequence of the α subunit was determined as previously described on samples of the α subunit (*4*).

Flavin and Iron Analyses. Aliquots (300−500 μL) of the α subunit solution were gel-filtered through a Sephadex G-25 (medium) column equilibrated with 10 mM Tris-HCl buffer, pH 7.5. Fractions containing the α subunit were pooled, and aliquots were used to determine the protein concentration, the activity in the L-Glu:INT oxidoreductase reaction, and the iron or flavin content. If necessary, the protein solution was concentrated by centrifugation in a Centricon-30 prior to analysis. For the iron assays the method described before (*6*) was used. For the identification of the flavin bound to the α subunit, the emission spectrum of ∼2 μM solutions of α subunit was recorded between 450 and 600 nm with excitation at 440 nm in a Jasco FP-777 spectrophotofluorometer at 15 °C. A 250-μL aliquot of the protein solution was denatured by heating in the dark at 100 °C for 5 min. The denatured protein was removed by centrifugation in a microcentrifuge. The supernatant was transferred to the 200-μL fluorescence cuvette, and the emission spectrum of the solution was recorded again. Finally, snake venom phosphodiesterase was added (3 milliunits) in order to detect any FAD present (*8*, *20*). For the determination of the stoichiometry of the FMN bound to the GltS α subunit, the absorbance spectrum of the α subunit solutions used for the fluorimetric analysis and that of the flavin extracted by heat denaturation were also recorded in a Hewlett-Packard HP9153A diode array spectrophotometer. An extinction coefficient of 12.2 mM^{-1} cm^{-1} was used for free FMN (*21*).

Spectroscopic Techniques. UV−vis absorbance spectra were recorded with Cary 219 or Hewlett-Packard HP8452A or 9153A diode array spectrophotometers thermostated at 15 °C. When necessary, anaerobiosis was obtained as described previously (*6*, *8*) by subjecting samples to cycles of equilibration with oxygen-free nitrogen and evacuation (*22*). Anaerobic solutions of other reagents were prepared in the same way and transferred with gas-tight syringes (Hamilton). All experiments were carried out using 5−10 μM solutions of the α subunit that had been gel-filtered through a Sephadex G-25 column equilibrated with 25 mM Hepes/KOH buffer, pH 7.5, containing 1 mM EDTA and 10% glycerol. For the sulfite titration of the GltS α subunit, 1-μL aliquots of a 0.1 M and subsequently 1 M solution of sodium sulfite were added. Spectra were recorded at different times from each sulfite addition. Whenever a faint turbidity developed, the solution was centrifuged in a microcentrifuge in order to remove it prior to spectral measurements. The fractional changes of absorbance at 440 nm at the different sulfite concentrations ($(A_x - A_0)/(A_f - A_0)$) were used to determine the K_d of the flavin−sulfite complex (eq 4):

$$(A_x - A_0)/(A_f - A_0) = L/(K_d + L) \qquad (4)$$

where A_x is the absorbance after a given addition of the ligand; A_0 is the initial absorbance of the solution; A_f is the absorbance value at the end of the titration; L is the ligand concentration; and K_d is the dissociation constant of the enzyme−ligand complex.

The sulfite was displaced from the enzyme flavin on addition of aliquots of 25 mM and later 1 M 2-OG solution to the α subunit (5 μM) in the presence of 58 mM sulfite. An apparent dissociation constant for the enzyme−2-oxoglutarate complex ($K_{2-OG,app}$) in the presence of 58 mM sulfite was calculated using eq 4. The values of the dissociation constant of the enzyme−sulfite complex (K_S) determined during the enzyme titration with sulfite (see above) and of $K_{2-OG,app}$, determined during the back titration, were used to calculate an estimate of the dissociation constant of the enzyme−2-oxoglutarate complex (K_{2-OG}) using eq 5 (*6*):

$$K_{2-OG,app} = K_{2-OG}(1 + S/K_S) \qquad (5)$$

where S is sulfite concentration.

The reactivity of the GltS α subunit with L-Glu was determined by adding 500 nmol of L-Glu from the side arm of an anaerobic cuvette that contained 4 μM α subunit (1.1 mL). The spectrum of the solution was recorded before and at different times after the L-Glu addition at 15 °C.

Photoreduction of the GltS α subunit was carried out by irradiating anaerobic solutions of α subunit (∼5 μM) in the presence of 5 mM EDTA, 1 μM 5-deazaflavin, in 25 mM Hepes/KOH, pH 7.5, 10% glycerol (*23*). Reduction of the GltS α subunit was also obtained using dithionite. Aliquots of an anaerobic solution of sodium dithionite (1−5 mM, whose concentration was determined as described in ref *22*) were added to anaerobic solutions of α subunit (≅5 μM) by using a gas-tight syringe fitted on a side arm of the anaerobic cuvette. Spectra were recorded before and at different times after each dithionite addition. In different experiments, aliquots of solutions containing L-Gln (500 nmol) and/or 2-OG (500 nmol) were added from a side arm of the anaerobic cuvette. At the end of the experiment the cuvette was opened to air, and the spectrum of the air-reoxidized enzyme was recorded.

EPR spectroscopy was performed as described previously (*6*) on 12−24 μM solutions of the α subunit, as isolated, or reduced by irradiation after addition of 5 mM EDTA and 2 μM 5-deazaflavin or after anaerobic addition of 100-fold excess L-Glu. Measurements were carried out using a Bruker ER-200-D-SRC spectrometer equipped with an Oxford Instruments continuous flow cryostat in Atlanta or a Bruker

Table 1: Purification of the GltS α Subunit[a]

sample	act. (unit)	total protein (mg)	sp act. (unit/mg)	yield (%)	purifn factor (fold)
crude extract	11.3	535[b]	0.021	100	1
Q-Sepharose concentrate	7.7	46[c]	0.17	68	8.1
Ultrogel AcA34 concentrate	4.2	7.3[c]	0.57	37	27

[a] The table summarizes the results of the purification of *Azospirillum* GltS α subunit from *E. coli* BL21 (DE3) cells harboring pESTA4 plasmid (8 g). One unit of activity is the amount of enzyme that reduces 1 μmol of INT/min in the standard assay (0.1 mM INT, 20 mM L-Glu in 50 mM Caps/KOH, pH 9.5) at 25 °C. [b] The biuret method was used to determine the protein concentration (*13*). [c] The Bradford method was used to determine the protein concentration (*14*).

ER 200 D equipped with a home-built helium-flow cryostat in Wageningen. Quantification by double integration was versus external Cu^{II} standard solutions.

Data Analysis. The Grafit (Erythacus Software Ltd) and Excel (Microsoft) programs installed on a Zenith Z-425/SX personal computer were used for data analysis.

RESULTS AND DISCUSSION

Overproduction and Purification of the Recombinant GltS α Subunit. In a preliminary series of experiments, the recombinant *E. coli* BL21(DE3) strain harboring pESTA4 was grown in LB medium supplemented with 100 μg/mL ampicillin at 25 or 30 °C. IPTG was added to a final concentration of 0.1 mM when the culture was in the exponential phase ($A_{600} \cong 0.7-1.5$). Cells harvested before and at different times after the IPTG addition were analyzed by SDS electrophoresis of whole cell extracts and their soluble fractions and by measuring the L-Glu:INT oxidoreductase specific activity in the soluble fraction. Maximal specific activity was obtained with cells grown at 30 °C, IPTG was added when the culture A_{600} was ≅ 1, and cells were harvested after 9−13 h. Cells grown under these conditions were used for the preparation of the GltS α subunit.

Preparations of electrophoretically homogeneous GltS α subunits were obtained by ion-exchange and gel-filtration chromatographies, as described in Materials and Methods (Table 1). The major obstacles encountered in the purification of the protein were, initially, the instability of α subunit preparations and the presence of a heme-containing contaminant, which chromatographed very close to the α subunit. Instability of the α subunit preparations was circumvented by (a) abolishing initial ammonium sulfate precipitation steps, and subsequent dialysis; (b) inclusion of 10% glycerol in all buffers; and (c) equilibration of all buffers used with nitrogen. The heme-containing contaminant eluted from the Q-Sepharose column right before the α subunit when the column was developed using buffer at pH 7.5. A series of experiments using a MonoQ column in the FPLC allowed us to determine that, in order to obtain good resolution between the contaminant and the α subunit, it was sufficient to lower the pH of the buffer used for this chromatography from 7.5 to 7. In any case, in both the ion-exchange and the gel-filtration chromatographies, fractions were pooled on the basis of their absorption properties, thus sacrificing yield for purity.

The identity of the overproduced protein with the GltS α subunit was assessed in several ways. The N-terminal sequence of the isolated protein is identical with that of the mature α subunit of GltS prepared from *Azospirillum* cells, starting with Cys1 (*4*). This demonstrates that *E. coli* is able to correctly process the *glt*B gene product by removing the 36-residue propeptide and exposing Cys1: this residue is within the *PurF*-type glutamine amidotrasferase domain of GltS (*5*) and is essential for GltS catalytic activity with its physiological substrate, L-Gln (*24*). The mass of the recombinant α subunit was also identical to that of the α subunit resolved from the GltS holoenzyme, as judged by SDS electrophoresis (not shown). Furthermore, the recombinant α subunit eluted from the Ultrogel AcA34 column well after the void volume of the column. Given the fractionation interval of this resin (20 000−340 000), we can conclude that the GltS α subunit is isolated as a monomer. Also the GltS β subunit behaved as a monomer (*8*). Therefore, it appears that the ability of the GltS holoenzyme to aggregate to form an $(\alpha\beta)_4$ tetramer of approximately 800 kDa at protein concentrations above 1 μM (0.2 mg/mL) is a property of the αβ protomer rather than of one of the two subunits[2] (*4*). Another property that seems to depend on specific interactions between the α and β subunits in the GltS holoenzyme is the binding of GltS to 2′,5′-ADP-Sepharose. GltS is purified by affinity chromatography on such a resin (*3*). We have observed that the isolated recombinant β subunit does not interact with ADP-Sepharose, although it binds to Reactive Red, a ligand specific for the NADP(H) binding site of enzymes (*8*). During the present study, we also observed that the recombinant α subunit of GltS does not bind to ADP-Sepharose.

Cofactor Content of the GltS α Subunit. The purified α subunit of GltS exhibits the absorption spectrum of an iron−sulfur flavoprotein (Figure 1) and a A_{280}/A_{440} ratio of 7. The extinction coefficient at 440 nm was calculated to be 21 200 ± 1100 M^{-1} cm^{-1} (8 determinations), when protein content was estimated using the Bradford method. The α subunit also exhibits fluorescence properties typical of a flavoprotein. The flavin cofactor can be extracted by heat denaturation in the dark, under conditions that preserve the integrity of the flavin present. The solution containing the released flavin exhibits a 12.7-fold higher fluorescence with respect to that of the native protein. Addition of snake venom phosphodiesterase does not cause any change in the flavin fluorescence, leading us to the conclusion that the flavin in the α subunit is FMN, not FAD. This was anticipated by the finding of a potential FMN binding site in the GltS α subunit (*5*) and by the presence of the GltS FAD cofactor in the GltS β subunit (*8*). Furthermore, these data independently support the presence of only one type of flavin (FMN) also in the Fd-dependent GltS from spinach which was initially proposed to contain both FAD and FMN (see discussion in refs *8* and *25*). Measurement of the absorbance spectra of the FMN released from the α subunit also allowed us to determine a stoichiometry of 0.94 ± 0.12 FMN bound per α subunit (3 independent experiments), a value consistent with the presence of 1 FMN per α subunit. Solutions of the GltS α subunit were also analyzed by EPR spectroscopy at low temperature. As shown in Figure 2, the GltS α subunit, as

[2] The concentration of the α subunit before gel filtration was always greater than 5 μM, and that of the β subunit was always well above 10 μM.

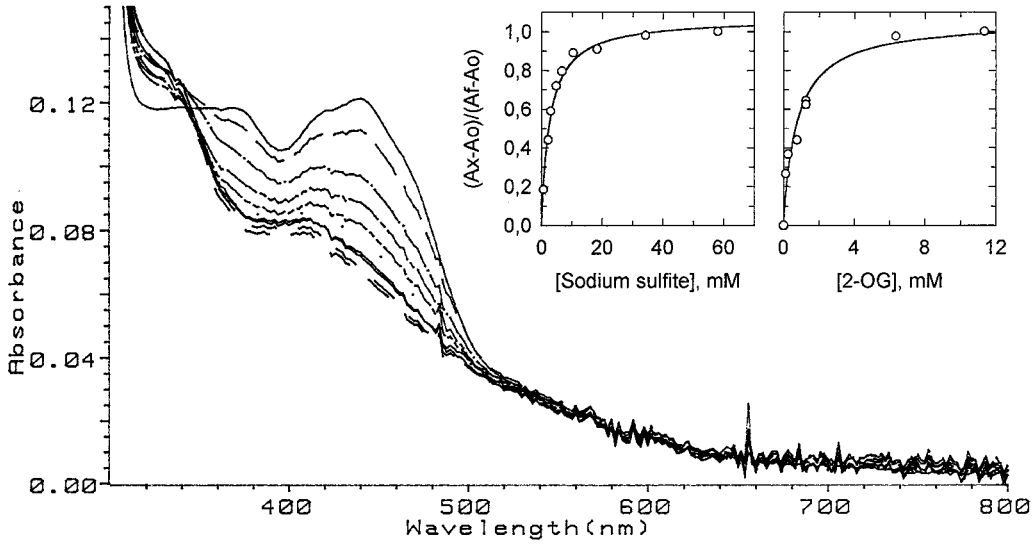

FIGURE 1: Sulfite reactivity of the FMN cofactor of the GltS α subunit. The spectrum of a 5 μM solution of the GltS α subunit in 25 mM Hepes/KOH, pH 7.5, 1 mM EDTA, 10% glycerol was recorded before and after the addition of increasing amounts of sulfite, yielding (from top to bottom) the final concentrations shown in the left inset. The left inset shows the plot of the fractional change of absorbance at 440 nm measured after each sulfite addition as a function of sulfite concentration. The curve is the best fit of the data to eq 4, which allowed the calculation of a dissociation constant of 2.7 ± 0.3 mM for the covalent complex between FMN bound to the GltS α subunit and sulfite. To the solution obtained at the end of the sulfite titration, which contained 58 mM sulfite, increasing amounts of a 2-oxoglutarate solution were added. Recovery of absorbance to yield a spectrum in the visible region indistinguishable from that of the free α subunit was obtained with 11.4 mM 2-OG, once dilution was taken into account. The right inset shows the dependence of the fractional changes of absorbance at 440 nm observed after each 2-OG addition, as a function of 2-OG concentration. The curve shows the best fit of the data to eq 4, which allowed us to calculate an apparent dissociation constant for the enzyme−2-oxoglutarate complex of 0.76 ± 0.18 mM. Since both the K_d of the enzyme−sulfite complex and the sulfite concentration in the solution are known, eq 5 allows the calculation of a K_d for the enzyme−2-OG complex of 34 μM.

isolated, exhibits an $S = {}^1/_2$ EPR spectrum, which is characteristic for a [3Fe-4S] cluster in the 1+, oxidized state. The spectrum is essentially identical to that of the [3Fe-4S] center of the GltS holoenzyme (*6*). The low-field absorption feature peaks at $g = 2.020$; it has a remarkably small line width, and relaxational broadening is observed even at temperatures below 10 K. Quantification of the 5.5 K spectrum versus a Cu^{II} standard shows 0.72 spins per α subunit. This value is in agreement with the analytical iron determination, which yielded 2.9 iron atoms per α subunit, and allows us to reach the conclusion that one 3Fe-4S cluster is present in the GltS α subunit. Anaerobic photoreduction of the protein preparation led to the loss of the signal in the $g = 2$ region, as expected for the reduction of the cluster. Furthermore, no other signal appeared in this region, ruling out the presence of other iron−sulfur centers in the GltS α subunit. This result agrees well with the fact that the spinach Fd-GltS contains a single Fe-S cluster, a [3Fe-4S] center (*26*), and that the primary structure of the GltS α subunit contains only a conserved cluster of cysteines (residues 1102, 1108, and 1113 in the *Azospirillum* α subunit), with a spacing similar to that of cysteines involved in the formation of cluster 3 of fumarate reductase and succinate dehydrogenase, the [3Fe-4S] cluster of these enzymes (*5*).

Properties of the FMN Cofactor Bound to the GltS α Subunit. The FMN cofactor in the α subunit of GltS should exhibit the properties of Flavin 2 of GltS holoenzyme (*6*). This hypothesis was confirmed experimentally. Figure 1 shows the spectral changes obtained on addition of sulfite to a 5 μM solution of α subunit. From a plot of fractional absorbance changes at 440 nm as a function of sulfite concentration, a dissociation constant for the enzyme−sulfite complex of 2.7 ± 0.3 mM was determined. Such a value is of the same order of magnitude as that determined for the GltS holoenzyme (1.3 mM, *6*). Addition of 2-OG to the α subunit−sulfite complex displaced sulfite from the flavin, leading to recovery of an absorbance spectrum indistinguishable from that of the initial α subunit preparation, once dilution of the sample was taken into account. A dissociation constant for the α subunit−2-OG complex of ≅34 μM was calculated. Again this value is only slightly higher than that calculated for the GltS holoenzyme (6 μM, *6*).

The involvement of the FMN cofactor in the oxidation of glutamate catalyzed by the GltS α subunit was determined at pH 7.5 by absorption spectroscopy. A 4 μM solution of the α subunit was made anaerobic, and an aliquot of an anaerobic solution of L-Glu was added from the side arm of the cuvette (Figure 3). Absorbance changes were complete within 5 min of L-Glu addition and were consistent with the reduction of the FMN cofactor but not of the 3Fe-4S center of the enzyme. A similar result was obtained with the GltS holoenzyme during early experiments (unpublished). That the 3Fe-4S cluster of the GltS α subunit is not reduced on addition of L-Glu was confirmed in a similar experiment in which the EPR spectrum of a 25 μM solution of the α subunit was measured before and after the anaerobic addition of 100-fold excess L-Glu (data not shown).

The 3Fe-4S center and the FMN cofactor of the GltS α subunit could be reduced by irradiation of the enzyme in the presence of EDTA and deazaflavin (not shown). During reduction no evidence for formation of flavin semiquinone was observed. However, when photoreduced α subunit was reoxidized on exposure to air, the recovery of the initial spectrum and of enzyme activity was partial. This result was unexpected because both the GltS holoenzyme and the recombinant β subunit can be photoreduced with full

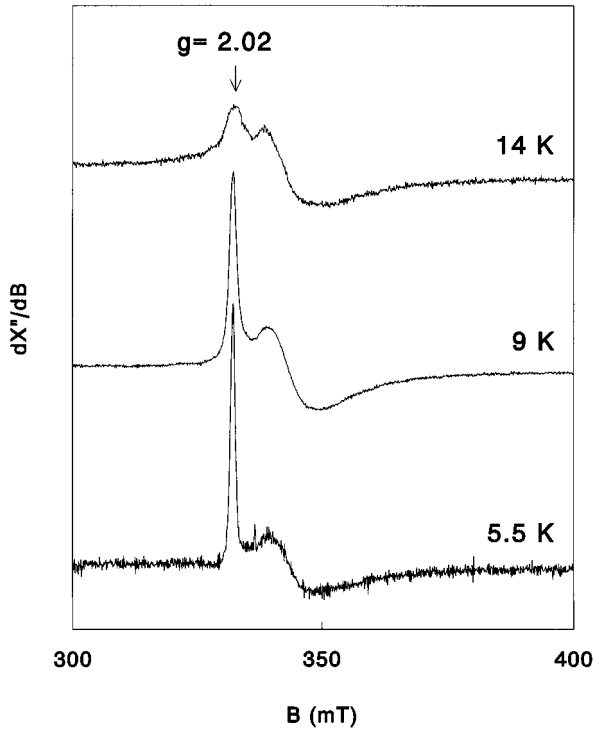

FIGURE 2: EPR spectrum of the GltS α subunit as a function of temperature. The "as isolated" (oxidized) GltS α subunit was 25 μM in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 10% glycerol. The amplitudes of the spectra have been normalized with respect to microwave power, amplification, and temperature. EPR conditions: microwave frequency, 9396 MHz; microwave power, 0.5, 5, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 0.4, 1.0, 1.2 mT; and temperature, 5.5, 9, 14 K, respectively.

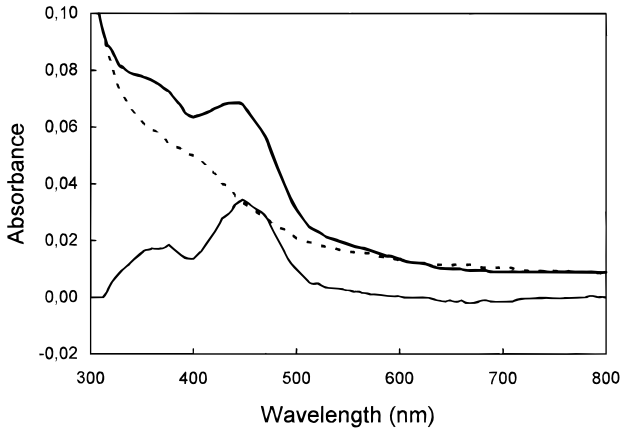

FIGURE 3: Reactivity of the GltS α subunit with L-glutamate. A 4 μM solution of the GltS α subunit in 25 mM PIPES/KOH buffer, pH 7.5, 1 mM EDTA, 10% glycerol was made anaerobic (thick line). A 50-μL aliquot of a 10 mM solution of L-glutamate was added from the side arm of the anaerobic cuvette and allowed to react with the GltS α subunit (dashed line). The difference spectrum between the oxidized and the L-glutamate-reduced GltS α subunit (thin line) is consistent with the reduction of the enzyme-bound flavin but not of the iron−sulfur center.

recovery of the initial absorbance and activity at the end of the experiment. The α subunit could instead be reduced chemically using dithionite, with full recovery of absorbance and catalytic activity at the end of the experiment. The absorbance changes (Figure 4) observed at different wavelengths did not indicate the formation of flavin semiquinone species, nor that the redox potentials of the flavin cofactor and of the 3Fe-4S center are very different. A 1.7-fold molar excess of dithionite was calculated to be sufficient to fully reduce the enzyme, in agreement with the cofactor content of this protein. Dithionite-reduced α subunit was not reoxidized when either 2-OG or L-Gln alone was added. However, when the two compounds were added together, absorbance changes in the visible region corresponding to the oxidation of the enzyme flavin were immediately observed (Figure 5). This result demonstrates that the FMN cofactor of the GltS α subunit is directly involved in iminoglutarate reduction. Thus, we can conclude that the α subunit of GltS contains Site 2 of the enzyme and that Flavin 2 of GltS is the FMN cofactor of the enzyme. The lack of reactivity of the reduced α subunit with 2-OG is in agreement with the proposed reaction mechanism of GltS: the iminoglutarate, formed on amide transfer from L-Gln to 2-OG, is the acceptor of electrons from FMN at Site 2 of GltS. In light of the high degree of similarity of the bacterial GltS α subunit and Fd-GltS, one would expect a similar behavior for the two enzymes with respect to the reactivity of the dithionite-reduced enzyme with the L-Gln and 2-OG substrates. Instead, it is reported that dithionite-reduced Fd-GltS from various sources (e.g., ref *27*) is reoxidized following anaerobic addition of 2-OG alone. These enzymes thus appear to possess a 2-OG reductase activity, a fact that is puzzling.

In our reoxidation experiment of the dithionite-reduced GltS α subunit with L-Gln and 2-OG, only exposure of the enzyme solution to air allowed recovery of the absorbance due to the 3Fe-4S center of the enzyme. Also during reaction of the protein with L-Glu only the FMN moiety was reduced. Thus, in the isolated α subunit electron transfer between the iron−sulfur center and the FMN cofactor in the α subunit does not take place. At the present stage, this observation can be explained by remembering that in no case did the reduction of the α subunit of GltS (this work) and of the Fd-GltS from spinach (*25*) lead to formation of detectable amounts of flavin semiquinone. Thus, one electron transfer between the iron−sulfur center and the FMN moiety may not take place due to thermodynamic reasons. Interestingly, when the photoreduced GltS holoenzyme was reacted with L-Gln and 2-OG, the spectrum of the fully oxidized enzyme was immediately obtained (*12*). Thus, it appears that the GltS β subunit is required to allow communication between the redox centers on the α subunit. This may be effected through conformational changes due to protein−protein interaction, which may alter the redox potential of the iron−sulfur center and flavin cofactor and/or their relative positioning. The GltS β subunit is also necessary to allow the formation of Centers II and III of the GltS holoenzyme. Center II, which appears to be involved in the intramolecular electron transfer process (*6*), may be required to ensure electron transfer between the redox active centers of GltS.

Catalytic Properties of the GltS α Subunit. As expected, the purified GltS α subunit exhibited no NADPH-dependent GltS activity in the presence of L-Gln (or ammonia) and 2-OG. In search of a suitable activity assay for the GltS α subunit, we remembered that GltS activity staining in nondenaturing polyacrylamide gels is easily obtained by incubating the gel slabs in a solution containing L-Glu and INT at pH 9.5. With this information, we set up a rapid and sensitive spectrophotometric assay of GltS α subunit,

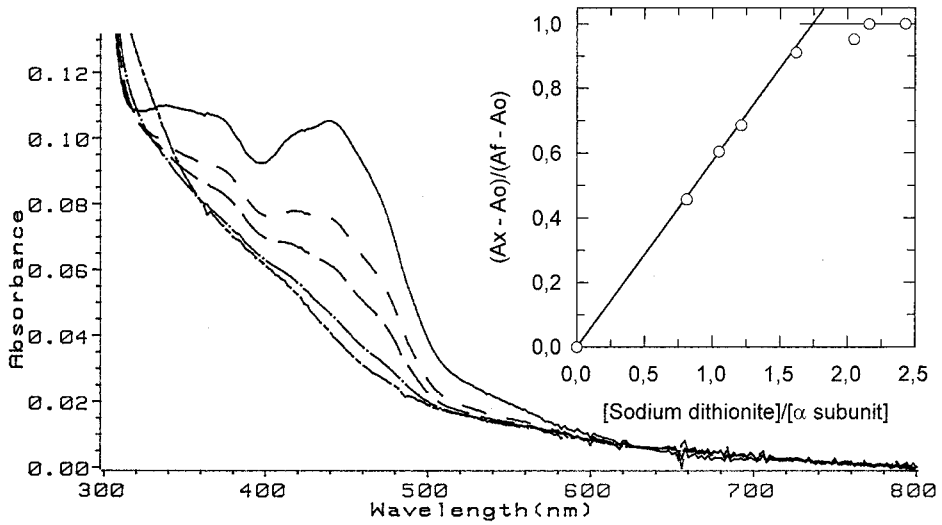

FIGURE 4: Dithionite reduction of the GltS α subunit. A 5.4 μM solution of the GltS α subunit in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 10% glycerol was made anaerobic. The absorbance spectrum was recorded before and after the addition of aliquots of a 1.32 mM dithionite solution corresponding to (from top to bottom) 0.81, 1.2, 1.62, 2.02, and 2.43 molar excess with respect to the GltS α subunit. The inset shows the plot of the fractional absorbance changes at 440 nm as a function of the dithionite/α subunit molar ratio.

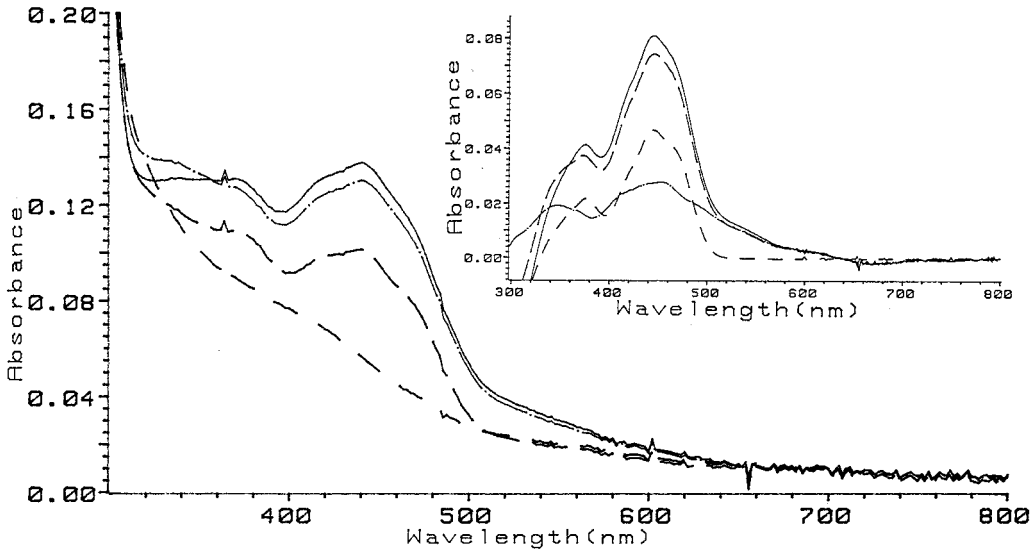

FIGURE 5: Reactivity of the dithionite-reduced GltS α subunit with L-glutamine and 2-oxoglutarate. A 6.5 μM α subunit solution (1.2 mL) in 25 mM Hepes/KOH, pH 7.5, 1 mM EDTA, 10% glycerol was made anaerobic (—). Up to 4 μL of a 4.2 mM dithionite solution were added anaerobically to achieve full reduction of the enzyme solution (— —). A 64-fold molar excess each of L-glutamine and 2-oxoglutarate (in 50 μL) was added from the side arm of the anaerobic cuvette. Immediate partial recovery of the absorbance spectrum was observed (— —). The cuvette was then opened to air, and the spectrum of the oxidized enzyme was recovered (—•—). The inset shows from top to bottom the difference spectrum between the oxidized α subunit and the dithionite-reduced α subunit (—); the difference spectrum between the air-reoxidized α subunit, at the end of the experiment, and the dithionite-reduced enzyme (— —); the difference between the spectrum of the α subunit after addition of L-Gln and 2-OG and the spectrum of the dithionite-reduced enzyme (— —); and the difference between the spectrum of the air-reoxidized enzyme and the spectrum of the enzyme reoxidized with L-Gln and 2-OG (—•—).

exploiting INT as the acceptor of the electrons released during oxidation of L-Glu. The L-Glu:INT oxidoreductase activity of the isolated GltS α subunit was found to increase from pH 7.5 to 9.5, and to decrease above it, when L-Glu and INT concentrations were held constant at 20 and 0.5 mM, respectively. At pH 9.5, the steady-state kinetic parameters of the L-Glu:INT oxidoreductase activity catalyzed by the GltS α subunit were determined (Table 2). For comparison, similar assays were carried out using the GltS holoenzyme (Table 2). With the GltS α subunit, L-Glu concentration was varied in the presence of constant concentrations of INT. This experiment revealed that INT concentrations above 0.1 mM are inhibitory. Thus, the data obtained at INT concentrations below it were used to determine both the kinetic mechanism of the reaction and the extrapolated values of V, K_{L-Glu}, and K_{INT}. Double reciprocal plots intersected in the upper left quadrant, and the data were best fitted assuming a ternary complex mechanism with L-Glu binding first. Since the FMN cofactor of the α subunit is indeed reduced by L-Glu (Figure 3), a ping-pong mechanism was expected for the L-Glu:INT oxidoreductase activity of the GltS α subunit. However, intersecting lines may derive from the fact that flavin reduction by L-Glu is unfavored, and thus an equilibrium may be established in the enzyme reductive half reaction, which abolishes the irreversible step between the binding of L-Glu and the binding of INT responsible for the parallel double reciprocal plots typical of a ping-pong mechanism

Table 2: Steady-State Kinetic Parameters Exhibited by the GltS α Subunit and the GltS Holoenzyme in the L-Glutamate:INT Oxidoreductase Activity Assay[a]

L-Glu (mM)	INT (mM)	effector (mM)	effect	eq	V (min^{-1})	K_{L-Glu}, (mM)	K_{INT} (μM)	K_i (mM)
			GltS α Subunit					
1−40	0.1			1	73 ± 0.9	1.05 ± 0.03		
1−40	0.03−0.1			2	79 ± 3	0.73 ± 0.13	9.3 ± 2.0	
1−40	0.1	2-OG, 0−0.5	C	3	109 ± 2	1.1 ± 0.1		0.11 ± 0.01
3−30	0.1	2-OG, 0−1	C	3	154 ± 6	3.1 ± 0.5		0.26 ± 0.05
3−30	0.1	2-OG, 0−1; L-Gln, 5	C	3	191 ± 8	3.0 ± 0.4		0.18 ± 0.03
			GltS Holoenzyme					
1−40	0.1			1	196 ± 5	0.65 ± 0.11		
2−20	0.1	2-OG, 0−0.5	C	3	236 ± 2	1.1 ± 0.08	0.19 ± 0.02	
5	0.l	L-Gln, 0−20	none		188[b]			

[a] All assays were carried out in 50 mM Caps/KOH, pH 9.5, at 25 °C. The substrates concentrations or concentration ranges are indicated. C stands for competitive inhibition. [b] Activity value measured in the absence of L-Gln.

(*16*). The maximal velocity of the reaction of the α subunit is approximately 2-fold lower than that measured using the GltS holoenzyme (Table 2), while the K_{L-Glu} value is of the same order of magnitude. AADP, a NADP(H) analog, is a good inhibitor of GltS holoenzyme in the physiological reaction, and it is competitive with respect to NADPH, with a calculated K_i of 2.4 ± 0.4 μM (Vanoni, M. A., and Stabile, H., unpublished). AADP had no effect on the L-Glu:INT oxidoreductase reaction of the α subunit or of the GltS holoenzyme at concentrations up to 1 mM. This result demonstrated that the L-Glu:INT oxidoreductase activity of GltS holoenzyme takes place at Site 2, which is within its α subunit. In full agreement with this model, 2-OG was a competitive inhibitor with respect to L-Glu, using both the GltS holoenzyme and the recombinant α subunit. The K_i values determined with the two enzymes are similar to each other and of the same order of magnitude of the dissociation constant of the α subunit−2-oxoglutarate calculated from the back titration of the enzyme−sulfite complex. Also the effect of L-Gln on the L-Glu:INT reaction was tested. With GltS holoenzyme, no effect of up to 10 mM L-Gln was observed in assays containing 5 mM L-Glu and 0.1 mM INT in 50 mM Caps/KOH buffer, pH 9.5. When assays were repeated in 50 mM Taps/KOH buffer, pH 8.5, a small increase (approximately 15%) of activity was observed at L-Gln concentrations above 1 mM. With the isolated α subunit, the activation effect of L-Gln was approximately 20% at both pH 9.5 and 8.5. This result prompted us to determine the effect of the presence of L-glutamine on both the K_{L-Glu} and the K_i exhibited by 2-OG with the α subunit. As shown in Table 2, the effect of L-Gln (5 mM) on the reaction maximal velocity is confirmed, while both the K_{L-Glu} and the K_i for 2-OG are similar within the experimental error. Thus, binding of L-Gln to the GltS α subunit appears to have no effect on the binding of 2-OG.

Glutamate Synthesis by the GltS α Subunit. In order to assess the ability of the GltS α subunit to catalyze the synthesis of L-Glu in the presence of L-Gln and 2-OG, we carried out a series of experiments using ^{14}C-labeled L-Gln or ^{14}C-labeled 2-OG under various conditions. In a first series of assays we wished to determine if L-[^{14}C]glutamate could be formed from L-Gln and 2-oxo-[U-^{14}C]glutarate in the presence of NADPH (Table 3). As expected, GltS holoenzyme converts 2-OG to L-Glu in the presence of L-Gln and NADPH, while the α subunit does not. A second series of experiments was carried out anaerobically using sodium dithionite as the reductant. As shown in Table 3, dithionite alone is unable to support the glutamate synthase activity of either the GltS holoenzyme or the α subunit. Inclusion of methyl viologen greatly increased the conversion of 2-oxo-[U-^{14}C]glutarate to glutamate. The activity of the GltS holoenzyme is much greater than that exhibited by the α subunit. The ^{14}C-labeled compound produced by the two enzymes was identified with L-[U-^{14}C]glutamate since it was quantitatively converted to 4-aminobutyrate by L-glutamate decarboxylase (Table 4). The requirement for methyl viologen to support glutamate synthase activity in these assays has been observed also with the *Synechococcus* Fd-GltS (*27*), but the reason is unclear. In the static spectrophotometric experiments (Figure 4), we observed a facile reduction of the enzyme with dithionite, and the FMN

Table 3: Glutamate Synthase Activity of the GltS α Subunit[a]

enzyme (pmol)	L-Gln (mM)	reducing syst	^{14}C dpm in 0.3 M acetic acid eluate (%)
GltS, 12.6[b]	5	NADPH, 0.4 mM	67.3
GltS, 12.6[b]	5	none	0.07
GltS, 12.6[b]	0	NADPH, 0.4 mM	0.16
α, 12.3[b]	5	NADPH, 0.4 mM	0.09
α, 12.3[b]	5	none	0.07
α, 12.3[b]	0	NADPH, 0.4 mM	0.06
none	5	none	0.1
GltS, 12.6	5	dithionite, 4 mM	0.78
GltS, 12.6	5	dithionite, 4 mM; MV, 0.12 mM	46
GltS, 12.6	0	dithionite, 4 mM; MV, 0.12 mM	0.17
GltS, 12.6	5	MV, 0.12 mM	0.07
GltS, 21.8	5	dithionite, 4 mM; MV, 0.12 mM	61
GltS, 33	5	dithionite, 4 mM; MV, 0.12 mM	61
α, 247	5	none	3.5
α, 247	5	dithionite, 4 mM	3.4
α, 247	5	dithionite, 4 mM; MV, 0.12 mM	18
α, 247	0	dithionite, 4 mM; MV, 0.12 mM	0.12
α, 247	5	MV, 0.12 mM	3.0
α, 247	0	MV, 0.12 mM	0.1

[a] All assays were carried out by incubating the enzyme in 50 mM Tris-HCl, pH 8.0, at 25 °C in the presence of 5 mM L-Gln and 0.3 mM 2-[U-^{14}C]oxoglutarate (26 300 dpm/nmol). Anaerobiosis was achieved by bubbling oxygen-free nitrogen in the assay mixture, prior to the addition of 10 μL of the anaerobic solution of dithionite (100 mM in 100 mM Tris-HCl, pH 8.0) and of 1−10 μL of the enzyme solution. In control experiments, L-Gln and/or the reducing system were omitted. After 20 min the reaction was stopped by opening the vessel to air, and the reaction components were separated by ion-exchange chromatography on a 1-mL Dowex 1 X8 column (acetate form) equilibrated with water. Unbound material was removed by washing the column with five 1-mL aliquots of water (always less than 1% of the total radioactivity loaded). L-Glu was eluted with 0.3 M acetic acid. [b] The reactions were carried out in the presence of air.

Table 4: Identification of the Reaction Product of the GltS α Subunit[a]

source of L-[U-^{14}C] glutamate	L-[U-^{14}C]glutamate (nmol)	4-aminobutyrate (nmol)
commercial	6	5.9
GltS reactn	5.4	5.2
α subunit reactn	1.77	1.76

[a] Parallel assay mixtures (750 μL) containing 5 mM L-Gln, 0.3 mM 2-oxo-[U-^{14}C]glutarate (26 300 dpm/nmol), and 0.12 mM MV in 50 mM Tris-HCl buffer, pH 8.0, were set up and made anaerobic by bubbling oxygen-free nitrogen. An aliquot (30 μL) of an anaerobic solution of dithionite (100 mM) was added along with either a 2-μL aliquot of GltS solution (65 pmol) or a 12-μL aliquot of a solution of the α subunit (740 pmol). After 20 min of incubation, the components of the reaction mixtures were separated on 1-mL Dowex 1 X8 columns. The ^{14}C-containing fractions eluted with 0.3 M acetic acid were pooled, lyophilized, and resuspended in water. Aliquots were incubated in 0.1 M $NaC_2H_3O_2$ buffer, pH 4.8, at 37 °C for 1 h in the presence or absence of 0.3 unit L-glutamate decarboxylase. After neutralization with the addition of 40 μL of 1 M NaOH to the 100-μL reaction mixtures, the samples were brought to 500 μL with water and loaded on a 1-mL Dowex 1 X8 column (acetate form) equilibrated with water. 4-Aminobutyrate formed by L-glutamate decarboxylase elutes, under these conditions, by washing the column with water, while residual glutamate (or radioactive material different from L-glutamate) is eluted with 0.3 M acetic acid. A control experiment was carried out using commercial L-[U-^{14}C]glutamate (22 719 dpm/nmol). In the absence of L-glutamate decarboxylase, all radioactive material was recovered, as expected, in the 0.3 M acetic acid eluate.

Table 5: Glutaminase Activity of the GltS α Subunit[a]

enzyme (pmol)	2-OG (mM)	reducing syst	^{14}C dpm in 0.3 M acetic acid eluate (%)
none[b]	2.5	NADPH, 0.4 mM	1.8
GltS, 33[b]	2.5	NADPH, 0.4 mM	48
GltS, 33[b]	2.5	none	3.5
GltS, 33[b]	0	none	3.6
α, 247[b]	2.5	NADPH, 0.4 mM	34
α, 247[b]	2.5	none	27.5
α, 247[b]	0	none	41
none	2.5	dithionite, 4 mM; MV, 0.12 mM	0.8
GltS, 33	2.5	dithionite, 4 mM; MV, 0.12 mM	67
GltS, 33	0	none	5.3
GltS, 229	2.5	dithionite, 4 mM; MV, 0.12 mM	66
GltS, 229	0	none	12
α, 247	2.5	dithionite, 4 mM; MV, 0.12 mM	77
α, 247	0	dithionite, 4 mM; MV, 0.12 mM	73
α, 247	0	none	62
α, 31	2.5	dithionite, 4 mM; MV, 0.12 mM	12.5
α, 31	0	none	9.3

[a] GltS or the GltS α subunit was incubated for 20 min at 25 °C in the presence or absence of 0.62 mM L-[U-^{14}C]glutamine (26 300 dpm/nmol) and/or 2.5 mM 2-OG in 50 mM Tris-HCl buffer, pH 8.0. In a series of assays that were carried out in the presence of air, 0.4 mM NADPH was used as the reducing system. In a second series of assays, dithionite (4 mM) and MV (0.12 mM) were included as the reducing system, and the assays were carried out anaerobically as described in the legend of Table 3. At the end of the incubation period, reaction components were analyzed as described before (see Table 3). [b] Experiments carried out in the presence of atmospheric oxygen.

cofactor appears directly involved in the reaction with the iminoglutarate intermediate/L-glutamate product couple (Figures 3 and 5). Thus, in the absence of additional information, it can be suggested that, during catalytic turnover, access of dithionite to the FMN cofactor may be prevented, while methyl viologen is able to effect electron transfer to the enzyme cofactor(s) under these conditions.

Glutaminase Activity of the GltS α Subunit. We have shown that the *A. brasilense* GltS holoenzyme is devoid of glutaminase activity, a fact that is at variance with what has been reported earlier for other glutamate synthases (*19*). We wished to determine if also in the recombinant GltS α subunit, release of the glutamine amide group is tightly coupled to its transfer to 2-oxoglutarate and reduction of the postulated iminoglutarate intermediate to yield L-glutamate. Toward this goal, a series of experiments were performed using L-[U-^{14}C]glutamine. As shown in Table 5, it was confirmed that the GltS holoenzyme does not exhibit significant glutaminase activity, while it is able to convert L-[U-^{14}C]glutamine into L-glutamate, provided that 2-oxoglutarate and a reducing system (NADPH or dithionite plus methyl viologen) are present. The small increase of glutamate produced in the presence of GltS holoenzyme with respect to the value obtained in the absence of enzyme, in fact, is not proportional to the amount of GltS holoenzyme added (it only doubles when GltS is increased 7-fold). The same measurements were also carried out using the recombinant GltS α subunit. In contrast with what was observed with the holoenzyme, a large proportion of L-glutamine was converted into glutamate in the absence of 2-OG and/or the reducing system. Such activity is high, proportional to the amount of α subunit present in the assay, and independent of the presence of 2-OG or the reducing system. Therefore, it can be proposed that coupling between amide transfer to 2-OG and reduction of the iminoglutarate intermediate to yield the L-glutamate product is much looser in the isolated α subunit with respect to the holoenzyme. Interestingly, with the α subunit, a lower percent conversion of 2-oxo-[U-^{14}C]-glutarate was observed in comparison to that obtained with the GltS holoenzyme in the experiments summarized in Table 3. This may be due to a lower reaction velocity of the α subunit under the experimental conditions we adopted or to the strong glutaminase activity: hydrolysis of glutamine may produce significant amounts of L-glutamate product, which may inhibit the glutamate synthase reaction.

CONCLUSIONS

Analysis of the properties of the *Azospirillum* GltS β subunit, overproduced in *E. coli*, demonstrated that it contains the functional Site 1 of GltS (*8*). The present study of the recombinant GltS α subunit leads to the conclusion that it contains the site of glutamine binding and hydrolysis and Site 2 of the enzyme, the site where the actual glutamate synthesis takes place. As predicted, the GltS α subunit contains the FMN cofactor of GltS, which exhibits the properties of Flavin 2 of the holoenzyme, and the 3Fe-4S center of GltS. The model proposed for the distribution of substrates' and cofactors' binding sites on the GltS subunits is thus fully confirmed (see introduction). However, it appears that the β subunit of the enzyme is responsible for many of the properties of the GltS holoenzyme: it not only provides reducing equivalents for the overall oxidoreduction reaction but also ensures both communication between the 3Fe-4S center and the FMN cofactor within the α subunit, and the tight coupling between amide transfer from glutamine to 2-oxoglutarate and reduction of the iminoglutarate intermediate to form the L-glutamate product. It may be proposed that (a) the β subunit induces conformational changes of the α subunit through protein−protein interaction and/or (b) the

β subunit is required for the incorporation of centers II and III of GltS, and that they are responsible for communication between the redox cofactors on the α subunit and/or control of the glutaminase/glutamate synthase activity. Evidence for long-range conformational changes taking place within the α subunit on binding of the pyridine nucleotide to the β subunit was indeed gathered from a series of limited proteolysis experiments on the GltS holoenzyme (*28*).

ACKNOWLEDGMENT

Dr. M. Coppola of this laboratory is thanked for providing the sample of purified GltS holoenzyme used in this work. We are also grateful to Dr. A. Negri for performing the N-terminal sequence analyses and to Dr. B. H. Huynh, Department of Physics, Emory University, and members of his laboratory for helpul discussions.

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BI972342W