Characterization of the Flavins and the Iron–Sulfur Centers of Glutamate Synthase from *Azospirillum brasilense* by Absorption, Circular Dichroism, and Electron Paramagnetic Resonance Spectroscopies†

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ABSTRACT: *Azospirillum brasilense* glutamate synthase has been studied by absorption, electron paramagnetic resonance, and circular dichroism spectroscopies in order to determine the type and number of iron–sulfur centers present in the enzyme $\alpha\beta$ protomer and to gain information on the role of the flavin and iron–sulfur centers in the catalytic mechanism. The FMN and FAD prosthetic groups are demonstrated to be nonequivalent with respect to their reactivities with sulfite. Sulfite reacts with only one of the two flavins forming an $N(5)$-sulfite adduct with a K_d of approximately 1 mM. The enzyme–sulfite complex is reduced by NADPH, and the complexed sulfite is competitively displaced by 2-oxoglutarate, which suggests the reactive flavin to be at the imine-reducing site. These data are in agreement with the two-site model of the enzyme active center proposed on the basis of kinetic studies [Vanoni, M. A., Nuzzi, L., Rescigno, M., Zanetti, G., & Curti, B. (1991) *Eur. J. Biochem. 202*, 181–189]. Each enzyme protomer was found, by chemical analysis, to contain 12.1 ± 0.5 mol of non-heme iron. Electron paramagnetic resonance spectroscopic studies on the oxidized and reduced forms of glutamate synthase demonstrated the presence of three distinct iron–sulfur centers per enzyme protomer. The oxidized enzyme exhibits an axial spectrum with g values at 2.03 and 1.97, which is highly temperature-dependent and integrates to 1.1 ± 0.2 spin/protomer. This signal is assigned to a $[3Fe\text{-}4S]^{1+}$ cluster $(Fe\text{-}S)_I$. Reduction of the enzyme with an NADPH-regenerating system results in reduction of the $[3Fe\text{-}4S]^{1+}$ center to a species with a $g \approx 12$ signal characteristic of the $S = 2$ spin state of a $[3Fe\text{-}4S]^0$ cluster. The NADPH-reduced enzyme also exhibits an [Fe-S] signal at g values of 1.98, 1.95, and 1.88, which integrates to 0.9 spin/protomer and is due to a second cluster $(Fe\text{-}S)_{II}$. Reduction of the enzyme with the light/deazaflavin method results in a signal characteristic of [Fe-S] clusters with g values of 2.03, 1.92, and 1.86 and an integrated intensity of 1.9 spin/protomer. This signal arises from reduction of the $(Fe\text{-}S)_{II}$ center and from that of the third, lower potential iron–sulfur center $(Fe\text{-}S)_{III}$. Circular dichroism spectral data on the oxidized and reduced forms of the enzyme are more consistent with the assignment of $(Fe\text{-}S)_{II}$ and $(Fe\text{-}S)_{III}$ as [4Fe-4S] clusters rather than [2Fe-2S] centers.

Glutamate synthase [L-glutamate:NADP$^+$ oxidoreductase (transaminating); EC 1.4.1.13; GltS[1]] catalyzes the reductive transfer of the amide group of L-glutamine to the C(2) carbon of 2-oxoglutarate (2-OG) to form L-glutamate (eq 1). The

$$\text{NADPH} + \text{L-Gln} + \text{2-OG} \leftrightarrow 2(\text{L-Glu}) + \text{NADP}^+ \quad (1)$$

reducing equivalents are provided by NAD(P)H in the enzyme from microorganisms and plants, although a reduced ferredoxin-dependent form of GltS is also present in photosynthetic microorganisms and tissues. The enzyme participates, with glutamine synthetase, in the major pathway for ammonia assimilation. This pathway is essential in diazotrophs, which rely on glutamine synthetase and GltS to efficiently assimilate ammonia, produced by nitrogenase, into glutamine and glutamate [for a recent review, see Vanoni et al. (1991c)]. Bacterial GltS have been isolated from several enteric organisms (i.e., *Escherichia coli* and *Bacillus subtilis*) but only from two diazotrophs (namely, *Klebsiella aerogenes* and, more recently, *Azospirillum brasilense*) (Vanoni et al., 1991c). The enzyme from *A. brasilense*, a Gram-negative, nitrogen-fixing, root-growth-promoting bacterium (Okon, 1985), has been isolated in our laboratory (Ratti et al., 1985).

Azospirillum GltS shares several properties with GltS from other bacteria. It is an iron–sulfur flavoprotein which was reported to contain 1 mol of FAD, 1 mol of FMN, ~8 mol of non-heme iron, and ~8 mol of acid-labile sulfur per mol of $\alpha\beta$ protomer of ~200 kDa (α subunit, ~140 kDa; β subunit, ~54 kDa). Preliminary structural studies (Vanoni et al., 1990) and DNA sequencing data from our laboratory (Pelanda et al., unpublished) indicate an overall similarity among bacterial GltS α subunits, while the β subunits seem less conserved. The sequence similarity among a subunits extends to the single polypeptide chain of the ferredoxin-dependent GltS from maize (Sakakibara et al., 1991) and is

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[1] Abbreviations: GltS, glutamate synthase; EPR, electron paramagnetic resonance; CD, circular dichroism; NADP(H), (reduced) nicotinamide 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; 2-IG, 2-iminoglutarate; deazaflavin, 5-carba-5-deazariboflavin.

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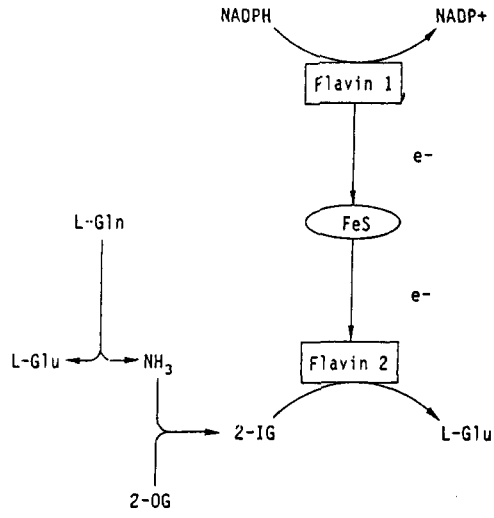
Scheme I



particularly high in regions which have been postulated to be part of the L-glutamine and the FMN binding sites.

The kinetic properties of the physiological reaction are similar for GltS from different bacteria (Rendina & Orme-Johnson, 1978; Matsuoka & Kimura, 1986; Vanoni et al., 1991b). The reaction is described by a two-site uni–uni bi–bi pong-pong mechanism and involves flavins and/or iron–sulfur centers for the electron transfer process from NADPH to the site at which the reductive amide transfer reaction takes place. Kinetic studies of the ammonia-dependent activity of the *Azospirillum* enzyme have shown that this activity is not likely to occur in vivo since it is undetectable at pH 7.5 (Vanoni et al., 1991a) and is characterized by a large K_m value for ammonia at alkaline pH values (Vanoni et al., 1991b). The mechanism of the ammonia-dependent reaction has been demonstrated to be identical to that found for the L-glutamine-dependent reaction and also involves flavin and/or iron–sulfur centers; a finding contrasting with conclusions reached from previous studies on the enzyme from other bacterial sources (Geary & Meister, 1977; Matsuoka & Kimura, 1986).

The kinetic scheme proposed for GltS (Rendina & Orme-Johnson, 1978; Vanoni et al., 1991b) suggests that the enzyme interacts with its substrates at separate catalytic sites (Scheme I). By analogy with other pyridine-nucleotide-dependent flavoproteins (Blankenhorn, 1976), one flavin is proposed to be at the NADPH site as the initial electron acceptor of reducing equivalents. Electron transfer occurs, possibly via the [Fe-S] centers to the second flavin where reduction of the imino acid takes place with production of L-glutamate. Other than the kinetic studies mentioned above, no data are available in the literature to support this model for the catalytic site(s) of GltS. If the FMN and FAD cofactors in GltS have differing roles such as suggested above, differences in their respective properties might be observable. In sarcosine oxidase, another multi-flavin-containing enzyme, Jorns (1985) showed that the flavin centers are readily distinguishable on the basis of their differing sulfite reactivity (Müller & Massey, 1969; Massey et al., 1969b; Massey & Hemmerich, 1980). Although GltS preparations from a number of bacterial sources are known to contain iron–sulfur centers [reviewed in Vanoni et al. (1991c)], little work has been done, with the exception of the *E. coli* and *Azotobacter vinelandii* enzymes (Rendina, 1980; Peisach et al., 1983), to characterize them with respect to type, number, and role in electron transfer between the flavin centers. As part of our studies on GltS isolated from *A. brasilense*, this paper demonstrates the flavin at the NADPH site to be distinguishable from that at the 2-OG site with respect to sulfite reactivity. In addition, EPR and CD measurements demonstrate the presence of three [Fe-S] centers in the enzyme. A $[3Fe\text{-}4S]^{1+}$ and a [4Fe-4S] center are shown to be reduced by NADPH, and a third center (possibly a [4Fe-4S] center) can only be reduced by using the light/EDTA/deazaflavin system, which demonstrates it to have a very low potential. Whether this low potential center, which is suggested to be a [4Fe-4S] center, functions in the intramolecular electron transfer or serves a different role in the GltS from *Azospirillum* is not known yet.

Materials and Methods

Enzymes. GltS was purified from cells of *A. brasilense* as previously described (Ratti et al., 1985) and stored at −80 °C or in liquid nitrogen in a solution of 25 mM Hepes/K^+, pH 7.5, 1 mM EDTA, 1 mM 2-oxoglutarate, 5 mM 2-mercaptoethanol, and 10% glycerol. Protein concentrations were determined spectrophotometrically using an extinction coefficient at 444 nm of 62 660 M^{-1} cm^{-1} (Vanoni et al., 1990). Activity measurements were carried out at 25 °C under conditions previously described (Vanoni et al., 1991b). Prior to each experiment, the enzyme was transferred to the appropriate buffer by gel filtration on a Sephadex G-25 column and concentrated using either Centricon-30 or Centricon-100 microconcentrators (Amicon).

For steady-state kinetic analyses of the inhibition caused by sulfite, initial velocity measurements were carried out in 50 mM Hepes/K^+ buffer, pH 7.5, by varying one enzyme substrate in the presence of different levels of sulfite and constant levels of the other two substrates. All spectrophotometric assays were carried out at 380 nm where the extinction coefficient of the $NADP^+$–sulfite adduct is negligible and the extinction coefficient of NADPH is 1.23 mM^{-1} cm^{-1} (this paper). Control experiments were carried out at both 340 and 380 nm. The inhibition patterns and the values of the kinetic parameters were calculated using the Hewlett Packard UV/vis Kinetic Software. The effect of sulfite on the ammonia-dependent reaction was studied in 50 mM Ches/K^+ buffer, pH 9.3, by varying sulfite concentration at two different concentrations of 2-OG (0.1 mM and 0.25 mM), in the presence of 0.1 mM NADPH and 0.4 M ammonium chloride. The data were analyzed using a Dixon plot (Dixon, 1953). Glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIII) was obtained from Sigma Chemical Co.

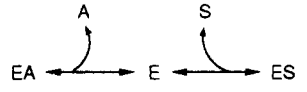
Iron Analysis. GltS (5–10 nmol) was denatured by boiling an aliquot of a concentrated solution for 5 min in the dark in the presence of 7.5 mM ascorbic acid and 10% trichloroacetic acid. This procedure caused protein denaturation, release of iron and flavins, and hydrolysis of FAD and FMN. After removal of the precipitated protein by centrifugation, the supernatant was diluted to 1 mL with 7.5 mM ascorbic acid. The spectrum of the solution was recorded, and the concentration of flavin was calculated using an extinction coefficient at 445 nm of 11.1 mM^{-1} cm^{-1} (Hinkson, 1968). Iron analyses of GltS supernatants were carried out using a modification (Salowe, 1987) of the method of Massey (1957). The iron-containing solution (3–26 nmol) was incubated with 200 μL of 75 mM ascorbic acid, 20 μL of 10 mM 4,4′-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bis(benzenesulfonic acid) (ferrozine), in a final volume of 420 μL for 5–10 min. Saturated ammonium acetate (380 μL) was then added to neutralize the solution and obtain full development of the color. The absorbance of the solution at 562 nm was then measured, and an extinction coefficient

of 28 mM^{-1} cm^{-1} was used to calculate the iron concentration. Control experiments were carried out using an iron solution (iron atomic absorption standard, Sigma Chemical Co.) as either an internal or an external standard as well as using milk xanthine oxidase as a reference iron–sulfur flavoprotein (Massey et al., 1969a). We found that inclusion of ascorbate in the GltS solution during trichloroacetic acid precipitation and in the diluted supernatant as well as the increase of ascorbate concentration in the colorimetric assay allowed greater iron recovery and did not interfere with the color yield of the iron standard.

Sulfite Titrations. Aliquots of concentrated solutions of sulfite (in 25 mM Hepes/K^+ buffer, pH 7.5, containing 1 mM EDTA and 10% glycerol) were added to 1–3 μM solutions of GltS. To determine the effect of enzyme substrates or substrate analogues on the reactivity of GltS with sulfite, the enzyme was first titrated with sulfite solution either until the endpoint of the reaction or until the desired sulfite concentration was reached. Either the compound whose effect was under study was added as a single aliquot or a back-titration was performed by the addition of several aliquots of the compound from a concentrated solution. The titration data were analyzed according to Scatchard (1949):

$$[S]_{bound}/[S]_{free} = -(1/K_S)[S]_{bound} + n([E_t]/K_S) \quad (2)$$

where $[S]_{bound}$ is the concentration of bound sulfite and is calculated from the ratio of the total decrease of absorbance after a given addition of sulfite over the maximum decrease of absorbance at 444 nm observed at the endpoint of the titration ($[A_{444(x)} - A_{444(0)}][E_t]/(A_{444(f)} - A_{444(0)})$); $[S]_{free}$ is the total sulfite concentration; K_S is the dissociation constant for the enzyme–sulfite complex; n is the number of sulfite binding sites per enzyme $\alpha\beta$ protomer; and $[E_t]$ is the total protomer concentration. The back-titration data were analyzed using the following equation, which assumes simple competition between sulfite (S) and the compound tested (A):



$$(([EA] + [E])([S]))/([ES]) = K_S + (K_S/K_A)[A]$$

In this equation, the ratio $([EA] + [E])/([ES])$ was calculated from the absorbance values at 444 nm as $(A_x - A_0)/(\Delta A_{max} - (A_x - A_0))$, where A_0 is the initial absorbance of the enzyme solution in the presence of a given amount of sulfite; A_x is the absorbance after the addition of a given amount of A; and ΔA_{max} is the maximum decrease of absorbance obtained at the endpoint of titrations of the enzyme with sulfite. [EA] is the concentration of the complex between the enzyme and the compound A; [E] is the concentration of the free enzyme; [ES] is the concentration of the enzyme–sulfite complex; [A] is the concentration of the compound A; K_S and K_A are the dissociation constants of the enzyme–sulfite and enzyme–A complexes, respectively.

Anaerobic Reduction of Glutamate Synthase. Samples for spectroscopic studies were placed in anaerobic EPR tubes or cuvettes and made anaerobic by several cycles of evacuation and filling of the apparatus with oxygen-free argon or nitrogen (Williams et al., 1979). Reduction of GltS was achieved by several methods: (1) anaerobic addition of NADPH to solutions of GltS in 25 mM Hepes/K^+ buffer, pH 7.5, containing 1 mM EDTA and 10% glycerol; (2) anaerobic addition of glucose 6-phosphate dehydrogenase (2.5 units) and $NADP^+$ (1 mol/mol of GltS) to a GltS solution containing a 50-fold molar excess of glucose 6-phosphate; (3) anaerobic addition of ~100-fold molar excess sodium dithionite in the presence of 1 μM methyl viologen as electron mediator; (4) irradiation for different periods of time of GltS solutions in buffer containing 30 mM EDTA and 5-deazaflavin (0.02 or 0.4 mol/mol of $\alpha\beta$ protomer for the EPR experiments and 0.1 mol/mol of $\alpha\beta$ for the absorption/circular dichroism spectroscopy experiments) (Massey & Hemmerich, 1978). During exposure to light the sample was maintained at 5–10 °C by immersion in a water bath. Enzyme reduction was monitored spectrophotometrically using either a Cary 14R or a Cary 219 spectrophotometer and was quantified relative to the maximal decrease at 444 nm, which can be achieved by photoreduction of the enzyme. When the sample was in EPR tubes, the spectrum was recorded in the Cary 14R equipped with a suitable adaptor in the cell compartment.

Electron Paramagnetic Resonance Spectroscopy. EPR measurements at X-band frequency of 30–80 μM solutions of *A. brasilense* GltS (0.2 mL) in 3-mm quartz EPR tubes were performed using a Bruker ER-200-D-SRC spectrometer equipped with an Oxford Instruments continuous flow cryostat. For all EPR measurements, the instrument settings were the following, unless otherwise specified: microwave frequency, 9.42 GHz; modulation amplitude, 8 G or 2 G; scan speed, 200 G/s. Gain settings, applied microwave power, and calculated g values are shown directly in the figures. The spectrometer was interfaced with an IBM XT personal computer for data acquisition. Data analyses (plotting, double integration of the EPR signals, calculation of g values, and subtraction of spectra) were performed using software written by Dr. B. H. Huynh (Department of Physics, Emory University). Quantitation of the [Fe-S] signals was performed using a solution of CuEDTA as a standard. Flavin semiquinone signals were quantified using a solution of one-electron-reduced *A. vinelandii* flavodoxin as the standard.

Circular Dichroism Spectroscopy. CD measurements were carried out using an AVIV 60DS circular dichroism spectrometer, at 25 °C in 1-cm-path-length anaerobic quartz cuvettes (Williams et al., 1979). The spectrometer was interfaced with an AT&T 6300 personal computer for data acquisition and storage. The data were analyzed using software supplied by AVIV and Associates.

Results

Reactivity of A. brasilense Glutamate Synthase with Sulfite. The reactivities of enzyme-bound flavins with sulfite have been exploited by several investigators to differentiate the coenzyme environments in flavoenzymes (Massey & Hemmerich, 1980; Jorns, 1985). From the mechanism proposed for the catalysis of the GltS reaction(s) (Scheme I), one would predict nonequivalence of the two flavin coenzymes with respect to their reaction with sulfite. The flavin at the NADPH site is predicted to exhibit poor or no reactivity with sulfite by analogy with the properties exhibited by other pyridine nucleotide-dependent flavoenzymes (Massey & Hemmerich, 1980). At the flavin 2 site, the transfer of reducing equivalents to the imine intermediate formed from 2-OG and ammonia occurs. This reaction is similar to the reverse reaction of an amino acid oxidase. Thus, the flavin at this site is expected to be reactive with sulfite with the reversible formation of a flavin–N(5)-sulfite adduct (Massey et al., 1969b).

The prediction of nonequivalence of the flavins with respect to their reactivity with sulfite is fully confirmed by the experimental data. A sulfite titration of GltS resulted in a decrease in the visible absorbance spectrum consistent with formation of a flavin–N(5) adduct (Massey et al., 1969b; Figure 1, spectrum 2). Spectral changes were complete during

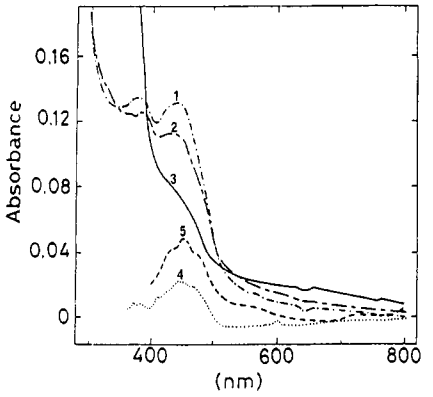


FIGURE 1: Spectral properties of the GltS–sulfite complex. GltS (2.1 μM) in 25 mM Hepes/K^+, pH 7.5, containing 1 mM EDTA, 1 mM DTT, and 10% glycerol (spectrum 1) was made anaerobic and titrated by addition of aliquots of a sulfite solution. At the end point of the titration (20 mM sulfite, spectrum 2), NADPH (100-fold molar excess) was added from the side-arm of the cuvette (spectrum 3). The difference spectra between oxidized and sulfite-bound GltS (spectrum 4) and between sulfite-bound and NADPH-reduced GltS (spectrum 5) are included in the figure.

the mixing time of sulfite with the enzyme solution. Analysis of the spectral data according to Scatchard (1949) showed a behavior consistent with the binding of only 1 mol of sulfite/$\alpha\beta$ protomer, with a K_d of 1.28 ± 0.07 mM. The level of spectral bleaching (difference spectrum 4 in Figure 1) also supports the conclusion that only one flavin reacts with sulfite.

To identify the functional role of the flavin reacting with sulfite, the effect of enzyme substrates and analogues on the enzyme–sulfite complex was examined. To test whether the sulfite-reactive flavin was at the NADPH site, the enzyme (2.1 μM) was made anaerobic and incubated with up to 20 mM sulfite (Figure 1, spectrum 2). NADPH (100-fold molar excess) was added from the side-arm of the cuvette. Within the mixing time, a further decrease of absorbance in the visible spectral region is observed with formation of a long-wavelength absorption band (Figure 1, spectrum 3). The changes observed on addition of NADPH (Figure 1, difference spectrum 5) are identical to those which are routinely observed when excess NADPH is added to free GltS, which suggests that the flavin at the NADPH site does not react with sulfite. Thus, the flavin at the imine-reducing site is the reactive species. To provide further evidence for this suggestion, the effect of those substrates and substrate analogues proposed to interact with the enzyme at the imine-reduction site were tested as perturbants of the enzyme–sulfite adduct. Addition of L-glutamine (5 mM) or L-methionine sulfone (1 mM), a glutamine analogue which exhibits a low K_i (0.1 μM) for the enzyme, had no effect on the absorption spectral properties of the enzyme–sulfite adduct even after prolonged incubation. Addition of 2-oxoglutarate (15 mM) to the enzyme–sulfite adduct led to rapid recovery of the native GltS spectrum. To determine whether these absorption spectral changes were due to displacement of sulfite from the enzyme because of 2-OG binding or to scavenging of sulfite by direct reaction with 2-OG, several back-titration experiments were performed. During different experiments the enzyme–sulfite complex (formed using varying concentrations of sulfite) was treated with aliquots of a 2-OG solution (25 mM). After each addition the absorption spectrum was recorded (Figure 2). Since only 0.7–1 mM 2-OG was sufficient to recover the spectrum of the free enzyme in the presence of 13.8 mM sulfite, we can conclude that the displacement of sulfite from the enzyme flavin is due to the interaction of 2-oxoglutarate with the enzyme. The data from each back-titration were analyzed using eq 3 (see Materials

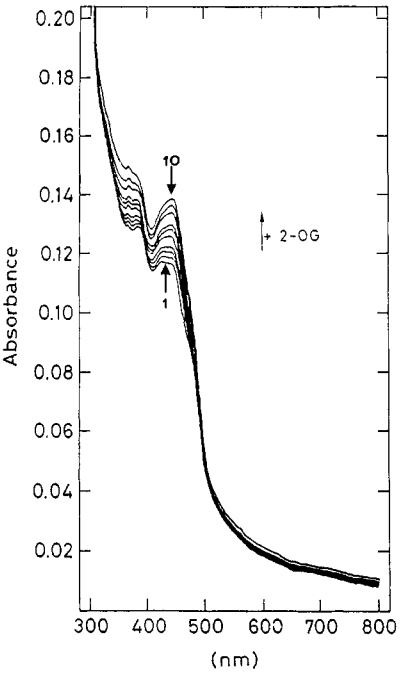


FIGURE 2: Back-titration of the GltS–sulfite complex with 2-oxoglutarate. GltS (2.2 μM) was titrated with sulfite until no further spectral changes were observed (13.8 mM sulfite final concentration, spectrum 1). Aliquots of a 25 mM solution of 2-oxoglutarate were added, and spectra were recorded after each addition. Spectrum 10 was obtained in the presence of 1 mM 2-oxoglutarate, and it corresponds to the spectrum of the starting enzyme solution.

and Methods). The data are consistent with the model, and a K_d for the enzyme-2-OG complex of (5.9 ±1.4) μM was calculated. This value should be compared with the K_m value of ≈40 μM calculated for this substrate during steady-state kinetic studies of the GltS reaction, at pH 7.5 (Vanoni et al., 1991b). The absorption spectral data presented above are further supported by steady-state kinetic studies of the inhibition patterns caused by sulfite during the L-glutamine-dependent reaction of GltS. Sulfite is a competitive inhibitor of the enzyme with respect to 2-OG (K_i = 11.5 ± 1.3 mM), noncompetitive with L-glutamine (K_i = 56.2 ± 2.1 mM) and uncompetitive with respect to NADPH (K_i = 52 ± 1.9 mM). These results both support the spectral data presented above and are consistent with the steady-state kinetic mechanism which has been determined previously (Vanoni et al., 1991b). The ammonia-dependent reaction of GltS was also found to be inhibited by sulfite. This result is in agreement with previous data on GltS from *A. brasilense*, which support the involvement of the enzyme-bound flavins in this reaction catalyzed by *A. brasilense* GltS (Vanoni et al., 1991b).

Determination of the Iron Content of Azospirillum Glutamate Synthase. As a prelude to performing low-temperature EPR studies on GltS to characterize the [Fe-S] centers present, the enzyme preparations used were analyzed for their iron content. While most bacterial GltS preparations have been reported to contain ~8 mol of non-heme iron/$\alpha\beta$ protomer (Vanoni et al., 1991c), EPR and Mössbauer spectroscopic studies on the *E. coli* and *A. vinelandii* enzymes suggested the number and type of iron–sulfur clusters/protomer to sum to a total iron content greater than that observed by chemical analysis (Rendina, 1980). Iron determinations were performed on supernatants from trichloroacetic acid precipitation of *A. brasilense* GltS. As shown in Table I, about 12 mol of iron/$\alpha\beta$ protomer were found when protomer concentration was calculated on the basis of the flavin concentration in the supernatants after trichloroacetic acid precipitation and with the

Table I: Iron Analysis on *A. brasilense* Glutamate Synthase

expt	GltS[a] (nmol)	iron (nmol)	iron/GltS (mol/mol)
1	0.4	4.6	11.5
	0.8	9.7	12.1
	0.4	4.7	11.7
	0.8	10.0	12.5
2	0.9	10.4	11.3
	1.8	22.3	12.1
	1.8	22.6	12.3
	0.9	11.5	12.5
	0.9	11.6	12.6
	0.9	11.8	12.8
av			12.1 ± 0.5 (4%)

[a] Based on the assumption that one $\alpha\beta$ promoter contains one FMN and one FAD.

assumption that the $\alpha\beta$ protomer contains two flavin cofactors. Since *Azospirillum* GltS has been found by us to bind both flavin cofactors tightly under nondenaturing conditions, the flavin concentration in the trichloroacetic acid supernatant provides a more accurate reference value for the calculation of the iron content of the enzyme protomer, rather than determination of the protein amount in the pellet by colorimetric methods or amino acid analysis. We believe the previously reported iron values for *A. brasilense* GltS (Ratti et al., 1985) to be low due to uncertainties in the determination of the protein content of samples subjected to colorimetric iron measurements. Ratti et al. (1985) did find the acid-labile sulfur content of *Azospirillum* GltS to be identical with the Fe content. Thus, by redefinition of the reference value, we conclude there are also 12 mol of acid-labile sulfur/$\alpha\beta$ protomer.

Low-Temperature EPR Studies on Oxidized Glutamate Synthase. Absorption spectra of GltS from *Azospirillum* in its oxidized and reduced forms have been reported (Ratti et al., 1987; Vanoni et al., 1991c). From these absorption spectral data, no information could be obtained on the identity and redox behavior of the iron–sulfur clusters, since the visible spectrum of the enzyme is dominated by flavin absorbance, and all attempts to obtain deflavo-GltS have been unsuccessful.

Low-temperature EPR experiments were performed in order to identify the type and number of iron–sulfur centers present and to correlate spectral changes produced by different reductants with the redox state of EPR active species. Previous EPR studies on *E. coli*, *A. vinelandii* (Rendina, 1980; Peisach et al., 1979), and *B. subtilis* (Matsuoka & Kimura, 1986) GltS preparations have resulted in spectra characteristic of [3Fe-4S]$^{1+}$ centers (Xavier et al., 1981). In a preliminary report (Vanoni et al., 1991d), we also presented EPR data supporting the existence of a [3Fe-4S]$^{1+}$ center in *Azospirillum* GltS. While the manuscript for this was in preparation, Knaff et al. (1991) published convincing spectroscopic data on the existence of a [3Fe-4S]$^{1+}$ center in the ferredoxin-linked GltS isolated from spinach. Figure 3 shows the EPR spectrum of native *Azospirillum* GltS. At 5 K, an axial spectrum is observed, which is characterized by a sharp peak at $g_{\parallel} = 2.03$, and a broader $g_{\perp}$ component at 1.97. Double-integration of the signal using a CuEDTA standard results in 1.1 ± 0.2 spin/$\alpha\beta$ protomer (three different preparations examined). The EPR spectrum is not saturated at microwave powers below 0.63 mW, and, consistent with known properties of other [3Fe-4S]$^{1+}$ centers (Teixeira et al., 1989; Beinert & Thomson, 1983), it rapidly relaxes as the temperature is raised so that it is not observed at 20 K or above (Figure 3). This signal is therefore identified as due to the presence of a [3Fe-4S]$^{1+}$ center in *A. brasilense* GltS. No other features are detected

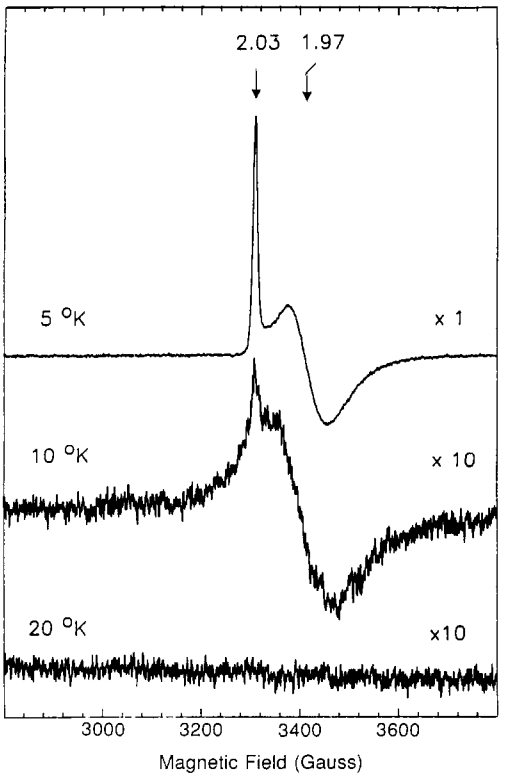


FIGURE 3: Temperature dependence of EPR spectrum of oxidized GltS from *A. brasilense*. The EPR spectrum of 36.7 μM native GltS in 25 mM Hepes/K^+ buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, and 10% glycerol is shown. Instrument settings: 9.42 GHz; modulation amplitude, 8 G; scan speed, 200 G/min; microwave power, 0.63 mW; gain, 1.25 × 10^5; the g values are indicated in the figure, as are the temperature values and the relative intensity scales.

in the EPR spectrum of oxidized GltS, beside a small $g = 4.3$ signal (Figure 7A).

Low-Temperature EPR Spectroscopy on NADPH-Reduced Glutamate Synthase. Anaerobic addition of a 20–40-fold molar excess of NADPH to GltS was found to cause a decrease of the visible absorbance of the enzyme consistent with reduction of only one enzyme flavin and the appearance of a long-wavelength absorption band (Vanoni et al., 1991c). These data suggest that NADPH reduction either leads to two-electron reduction of the enzyme, with formation of one reduced iron–sulfur center and flavin neutral semiquinone, or leads to two-electron reduction of 1 flavin/$\alpha\beta$ protomer and formation of a charge-transfer complex between the pyridine nucleotide and the flavin hydroquinone (Massey et al., 1966). Partial reduction of the enzyme by NADPH is consistent with the reversibility of the reductive half-reaction, which has been shown previously (Vanoni et al., 1991a). Anaerobic addition of a 33-fold molar excess of NADPH to oxidized GltS leads to >98% loss of the $g = 2.03$ EPR signal of the [3Fe-4S]$^{1+}$ center, to the appearance of an organic radical ($g = 2.00$) signal, and to a weak, broad signal with a positive peak at $g = 1.98$ and negative extrema at $g = 1.95$ and 1.88 (Figure 4). The free radical signal, which is assigned to a flavin neutral semiquinone on the basis of its line width of 19.5 G (Palmer et al., 1971), is readily quantified by raising the temperature to 70 K, where it is the only signal observable. Double-integration under nonsaturating conditions (<2 μW) using a flavodoxin semiquinone standard results in a value of 0.35 spin/$\alpha\beta$ protomer. Thus, the long-wavelength absorption band observed is assigned to the neutral flavin semiquinone species rather than a charge-transfer complex, in agreement with earlier studies on GltS from *E. coli* (MIller, 1974, Rendina,

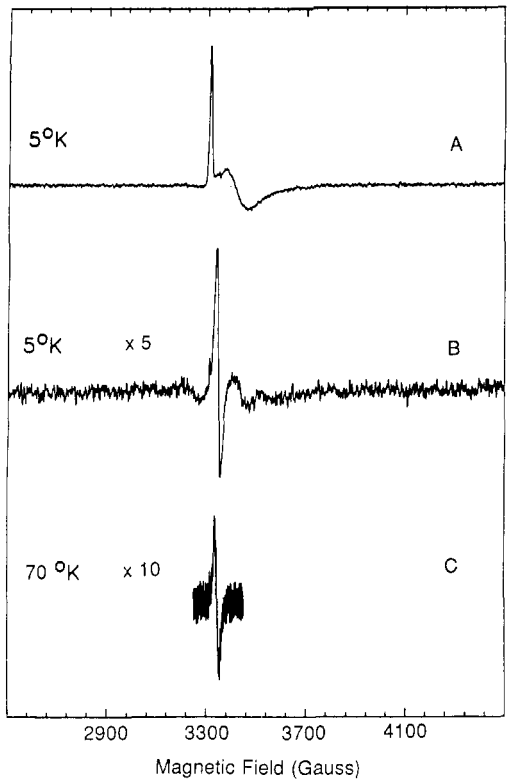


FIGURE 4: Reduction of GltS by NADPH. A comparison of EPR spectra of 30.1 μM GltS in 25 mM Hepes/K^+ buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, and 10% glycerol is shown before (A) and after the addition of 33-fold excess NADPH at 5 K (B) and 70 K (C). Instrument settings were as in Figure 3 except for microwave power, 0.02 mW; and gain, 8×10^4.

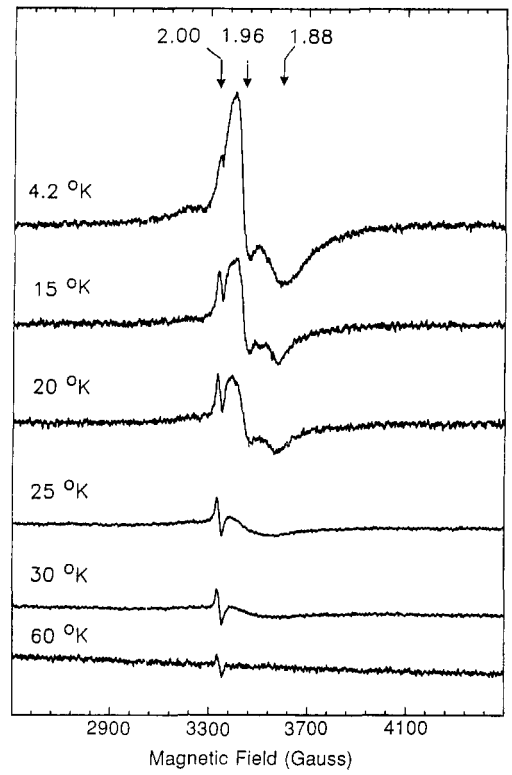


FIGURE 5: Temperature dependence of EPR spectrum of GltS reduced in the presence of a NADPH-regenerating system. GltS (47.85 μM) in 25 mM Hepes/K^+ buffer, pH 7.5, containing 1 mM EDTA and 10% glycerol was made anaerobic in the presence of 50 μM $NADP^+$ and 2.5 mM glucose 6-phosphate. The absorption and EPR spectra of the sample were recorded. Glucose 6-phosphate dehydrogenase (2.5 units, 10 μL) was then added anaerobically, at room temperature. Absorption spectra were recorded at different time intervals after the addition of the NADPH-regenerating enzyme. Within the first 10 min of reaction, a 74.7% decrease absorbance at 440 nm (with respect to the maximal absorbance decrease observed by photoreduction during several experiments) was obtained. After 5 h of incubation, 95% bleaching of the visible absorbance spectrum was obtained. The sample was then frozen in liquid nitrogen, and the EPR spectra were recorded at different microwave power settings and different temperatures. Instrument settings were as in Figure 3 except for gain, 8×10^4; and microwave power, 0.2 mW. Temperatures are as indicated in the figure. The spectra recorded at 25, 30, and 60 K are the average of five scans.

1980), spinach (Hirasawa & Tamura, 1984), and *Chlamydomonas reinhardii* (Marquez et al., 1986). The weak g = 1.96 signal, which is observed at 5 K, was quantified as 0.25 spin/$\alpha\beta$ protomer after subtraction of the flavin radical contribution. This signal is not saturated at power levels lower than 0.63 mW and broadens as the temperature is raised to 40 K, where it is barely detectable. Therefore, we assign this signal to an [Fe-S] center with temperature and saturation properties more similar to those observed for a [4Fe-4S] center rather than for a [2Fe-2S] center (Xavier et al., 1981). As it will be shown below, the visible circular dichroism spectral properties of the enzyme do not provide experimental support for the presence of a [2Fe-2S] center.

The relatively low degree of enzyme reduction by an excess of NADPH as monitored either by absorption or by EPR spectroscopy is readily explained by consideration of previous kinetic data (Vanoni et al., 1991b). Those data demonstrated $NADP^+$ to be a strong competitive inhibitor of NADPH with a K_i value of about 4 μM. Under conditions of spectroscopy experiments where enzyme concentrations are in the 50 μM range, the level of $NADP^+$ generated after reduction of the enzyme by the first mole of NADPH is sufficient to prevent further reduction of the enzyme. Thus, the data in Figure 4 are consistent with a two-electron reduction of GltS with substantial reduction of the $[3Fe\text{-}4S]^{1+}$ center and partial reduction of an additional [Fe-S] center and of flavin to its neutral semiquinone form.

To assess the level of enzyme reduction without complication by product inhibition, the enzyme was reduced by NADPH in the presence of a NADPH-regenerating system (glucose 6-phosphate dehydrogenase and glucose 6-phosphate). We have observed that the decrease of absorbance in the visible spectral region of GltS on anaerobic reduction with this system is similar to the degree of bleaching obtained by photoreduction of the enzyme (see below) in the presence of EDTA and deazaflavin. EPR spectral data of enzyme reduced by an NADPH-regenerating system under anaerobic conditions are shown in Figure 5. Spectral changes in the visible region were monitored spectrophotometrically. When maximum decrease of absorbance was obtained, the sample was frozen for EPR measurements. It is worth noting that during reduction of the enzyme by NADPH in the presence of the NADPH-regenerating system, no long-wavelength absorption band is observed. At low temperatures, the loss of the EPR signal due to the $[3Fe\text{-}4S]^{1+}$ center is evident as expected from the data in Figure 4. At 5 K, the EPR spectrum is dominated by a g = 1.96 signal, similar in shape but with a greater intensity than that observed in the absence of the NADPH-regenerating system. This increased intensity permits observation of a broad, weak signal at g = 2.08 (presumably a component of the Fe/S signal) that is not as apparent in the spectrum shown in Figure 4B. A small amount of g = 2.00 radical can be detected although its contribution to the overall intensity of the spectrum is not significant. Double-integration of the EPR signal at 5 K, under nonsaturating microwave power levels, yielded 0.9 unpaired electron/enzyme $\alpha\beta$ protomer. The g

= 1.96 signal becomes broader and eventually disappears at 60 K, where the quantification of the $g = 2.00$ signal yielded 0.01 unpaired electron/enzyme protomer. These data further support the identification of the long-wavelength absorption band in the NADPH-reduced enzyme as a flavin neutral semiquinone, since the presence/absence of the $g = 2.00$ signal in the EPR spectrum correlates with the presence/absence of the long-wavelength absorption band. Finally, by comparison of the g values, the power-saturation behavior, and the temperature dependence of the $g = 1.96$ signal with those of known [Fe-S] containing proteins, we infer that this center $(Fe\text{-}S)_{II}$ is more likely to be a [4Fe-4S] center than a [2Fe-2S] cluster (Xavier et al., 1981). Thus, *Azospirillum* GltS appears to contain a $[4Fe\text{-}4S]^{1+,2+}$ center $(Fe\text{-}S)_{II}$ as well as a $[3Fe\text{-}4S]^{1+}$ center $(Fe\text{-}S)_{I}$. Both centers are reducible by NADPH and therefore probably function in the intramolecular electron-transfer reactions during catalytic turnover. It also appears likely that the $[3Fe\text{-}4S]^{1+}$ center has a higher oxidation–reduction potential than the $[4Fe\text{-}4S]^{1+,2+}$ center since the latter is reduced stoichiometrically only in the presence of a NADPH-regenerating system while the former is reduced on partial reduction of the enzyme.

Low-Temperature EPR of Light/Deazaflavin-Reduced Glutamate Synthase. To confirm the presence of a second iron–sulfur center, and to detect the presence of additional iron–sulfur center(s) predicted by the iron content of the enzyme $\alpha\beta$ protomer beside the $[3Fe\text{-}4S]^{1+}$ cluster observed in the oxidized state of the enzyme, GltS was reduced by two different methods. In one experiment, dithionite (100-fold molar excess) and a catalytic amount of methyl viologen were added to an anaerobic solution of GltS (50 μM). Again reduction of the $[3Fe\text{-}4S]^{1+}$ center was observed as well as appearance of the $g = 2.00$ and $g = 1.96$ signals (data not shown). Double-integration of the $g = 1.96$ signal at 5 K gave 0.2 spin/protomer. Similarly, integration of the $g = 2$ flavin semiquinone signal observed at 70 K also indicated $\approx$0.2 spin/protomer. These data indicate that dithionite treatment of the enzyme only partially reduces the $(Fe\text{-}S)_{II}$ center but completely reduces the $[3Fe\text{-}4S]^{1+}$ cluster as well as the flavin cofactors. The failure of dithionite to completely reduce the $(Fe\text{-}S)_{II}$ center is probably due to its redox potential being below that of the dithionite/bisulfite couple (Mayhew, 1978). One conclusion apparent from this experiment is that the $[3Fe\text{-}4S]^{1+}$ cluster $(Fe\text{-}S)_{I}$ has a redox potential higher than that of $(Fe\text{-}S)_{II}$.

A method which has been exploited to reduce low-potential centers in biological systems uses the EDTA/light/deazaflavin system (Massey & Hemmerich, 1978). To monitor the level of reduced low-potential iron–sulfur centers in GltS, photoreduction was carried out by irradiating samples of the enzyme under anaerobic conditions, in the presence of 30 mM EDTA and 1.5–20% (mol/mol of $\alpha\beta$ protomer) deazaflavin. In two separate experiments, the enzyme was photoreduced at different rates as a result of changes in deazaflavin concentration. Both absorption and EPR spectra were recorded on the same sample at different stages of reduction. At a low molar ratio of deazaflavin/GltS (1.5%), reduction was slow enough to allow the recording of several spectra corresponding to different levels of enzyme reduction. No development of long-wavelength absorption was observed, and the absorbance decrease as monitored at several different wavelengths appeared to be monotonic (data not shown). EPR spectroscopy showed that the $[3Fe\text{-}4S]^{1+}$ center is initially reduced with parallel formation of a small amount of flavin semiquinone. Thus, as suggested by the NADPH experiments (Figures 4 and 5), the

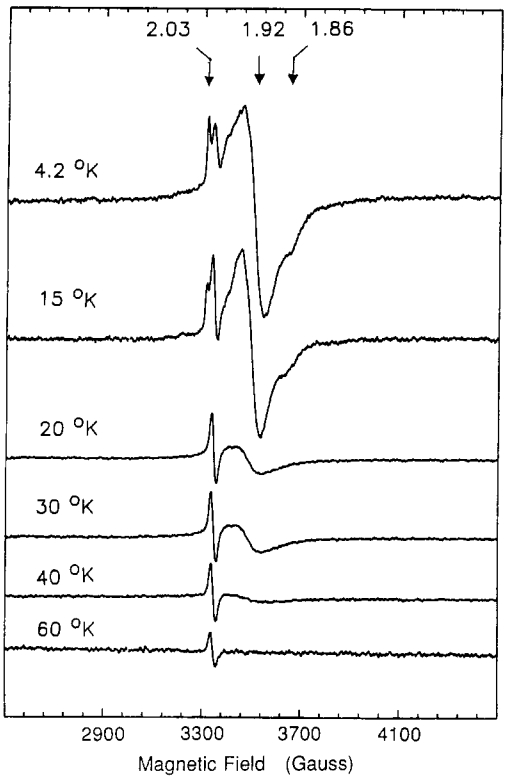


FIGURE 6: Temperature dependence of EPR spectrum of photoreduced GltS in the presence of deazaflavin. GltS (47.85 μ) in 25 mM Hepes/K^+ buffer, pH 7.5, containing 10% glycerol, 30 mM EDTA, and 19.8 μM deazariboflavin was made anaerobic in a 3-mm quartz EPR tube. The absorption spectrum of the sample was recorded at room temperature along with the EPR signal after freezing. After thawing, the sample was irradiated with a bank of fluorescent lamps in a water bath at 4 °C. The absorption spectrum of the solution was recorded at several time intervals. After 4 h of irradiation, the enzyme was greater than 95% reduced as judged by the absorption changes at 440 nm. The sample was then frozen in liquid nitrogen, and the EPR spectra were recorded at different microwave powers and different temperatures. Instrument settings were as described in Figure 3 except for gain, 8×10^4; and microwave power, 0.02 mW. Temperatures and g values are indicated in the figure. The spectra recorded at 30 K and 40 K are the average of five scans.

$[3Fe\text{-}4S]^{1+}$ center seems again to exhibit the highest oxidation–reduction potential relative to $Fe\text{-}S_{II}$ in GltS. Reduction of the $[3Fe\text{-}4S]^{1+}$ center also does not result in detectable features in the visible absorption spectrum of partially reduced GltS.

No other EPR spectral features appear up to $\approx$50% absorbance decrease, where only 29% $[3Fe\text{-}4S]^{1+}$ signal remains. When the enzyme is completely photoreduced, the EPR spectrum exhibits features not observed on reduction with NADPH or dithionite. This was studied in more detail in a separate experiment where the enzyme was reduced more rapidly by increasing the concentration of deazaflavin (0.2 mol/mol of $\alpha\beta$ protomer). A more intense EPR signal is observed with the photoreduced enzyme (Figures 6 and 7C) than with enzyme reduced in the presence of the NADPH-regenerating system (Figures 5 and 7B). Moreover, the shape of the spectra and observed g values differ from one another. The temperature and power saturation properties of this signal are consistent with those expected for an iron–sulfur center. Integration of the overall signal yield 1.9 unpaired electrons/enzyme $\alpha\beta$ protomer. On the basis of the spin quantitation, the signal observed is probably due to two overlapping [Fe-S] signals: one similar to that of the NADPH-reduced enzyme, and a new signal, only formed using the low-potential photoreduction system. Analysis of flavin radical content at

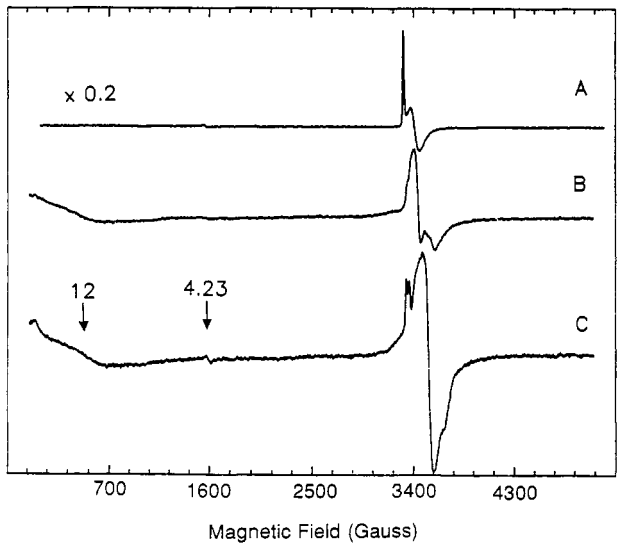


FIGURE 7: Comparative EPR spectra of oxidized and reduced forms of GltS using a wide field sweep. Spectrum A: Oxidized GltS (47.85 μM) in 25 mM Hepes/K^+ buffer, pH 7.5, 1 mM EDTA, 10% glycerol, containing 2.5 mM glucose 6-phosphate and 50 μM $NADP^+$. Spectrum B: 5 h after addition of glucose 6-phosphate dehydrogenase to complete the NADPH-regenerating system (see Figure 5). Spectrum C: Photoreduced GltS (see Figure 6). All spectra were recorded at 5 K and were normalized to 0.2 mW microwave power; gain was 8×10^4. Other instrument settings were as in Figure 3.

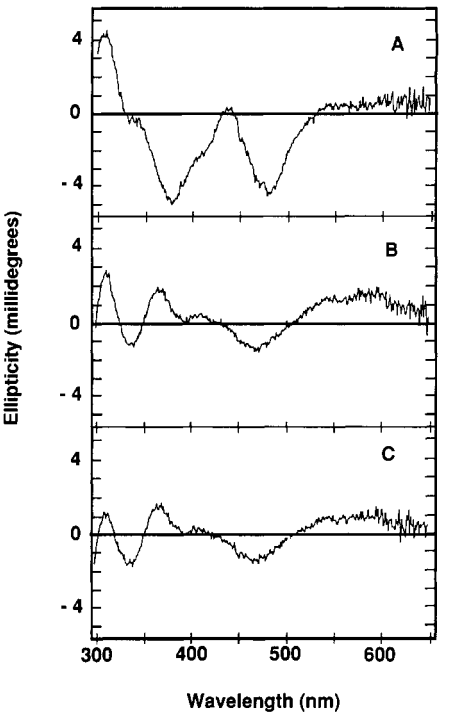


FIGURE 8: Circular dichroism spectra of oxidized and reduced forms of GltS. GltS (7.3 μM) in 25 mM Hepes/K^+ buffer, pH 7.5, 1 mM EDTA, 10% glycerol, containing 6 μM $NADP^+$ and 600 μM glucose 6-phosphate was made anaerobic in a 3-mL quartz anaerobic cuvette and was kept under an argon atmosphere. The circular dichroism spectrum was recorded at 25 °C (panel A). Glucose 6-phosphate dehydrogenase was then added anaerobically (10 μL, 2.5 units) and reduction was monitored by absorption spectroscopy at 25 °C. After 3 h of incubation, reduction of the enzyme as judged spectrophotometrically was complete. The CD spectrum of the sample was then recorded (panel B). In a separate experiment, GltS (7.3 μM) in 25 mM Hepes/K^+ buffer, pH 7.5, 30 mM EDTA, 10% glycerol, containing 0.9 μM deazaflavin was made anaerobic and irradiated until reduction was complete as judged spectrophotometrically (4 h). The CD spectrum of the photoreduced sample of enzyme is shown in panel C. Each spectrum is the average of 10 scans.

60 K shows 0.09 spin/$\alpha\beta$ protomer. These data suggest that *Azospirillum* GltS contains three rather than two iron–sulfur centers and that two of these centers are EPR-silent in their oxidized states.

The differential reduction properties of these two centers as well as the differing EPR signal lineshapes support the presence of two distinct EPR-detectable [Fe-S] clusters in reduced GltS. While EPR spectral data do not permit an unambiguous assignment of their structures as $[4Fe\text{-}4S]^{1+,2+}$ centers, the iron content determined for *A. brasilense* GltS (Table I) would certainly accommodate one $[3Fe\text{-}4S]^{1+}$ and two [4Fe-4S] centers. In addition, the relaxation properties of the EPR signals of these two centers are consistent with known properties of $[4Fe\text{-}4S]^{1+}$ clusters (Xavier et al., 1981).

EPR Properties of the Reduced [3Fe-4S] Center of A. brasilense Glutamate Synthase. Reduction of the $S = 1/2$ ground state $[3Fe\text{-}4S]^{1+}$ signal by one electron to a $[3Fe\text{-}4S]^0$ cluster has been shown by both magnetic circular dichroism and EPR spectroscopy to result in a $S = 2$ ground state for all $[3Fe\text{-}4S]^0$ clusters examined. *X*-band EPR signals have been detected near $g = 12$ for the $M_s = \pm 2$ states of $[3Fe\text{-}4S]^0$ clusters in *Desulfovibrio gigas* hydrogenase (Teixeira et al., 1989) and in spinach GltS (Knaff et al., 1991). The criterion for this detection is that the energy separation between the transition states must be smaller than the microwave energy ($\sim$0.3 cm^{-1}). The EPR spectrum in Figure 7A shows that no observable absorption occurs in the $g = 12$ region of oxidized *A. brasilense* GltS. On reduction of the enzyme by NADPH or by the light/deazaflavin system, absorptions are observed in this region corresponding to those transitions of a reduced $[3Fe\text{-}4S]^0$ center. These spectral data provide additional support for the assignment of $(Fe\text{-}S)_I$ as a $[3Fe\text{-}4S]^{1+}$ center in oxidized GltS and further demonstrate it not to be converted to a $[4Fe\text{-}4S]^{1+,2+}$ center on reduction by either method. Oxidation of NADPH- (or light/deazaflavin-) reduced enzyme by glutamine and 2-OG results in complete restoration of the spectrum shown in Figure 7A. Thus, substrate reduction and reoxidation does not lead to any observable breakdown of the [3Fe-4S] center. Furthermore, the redox state of the [3Fe-4S] center can be altered by oxidizing and reducing substrates of the enzyme which support its role in facilitating electron transfer between the two flavin centers.

Circular Dichroism Spectral Properties of Oxidized and Reduced Glutamate Synthase. With the observation of three distinct [Fe-S] centers in *Azospirillum* GltS by EPR spectroscopy, the question of whether one of the two low-potential centers [$(Fe\text{-}S)_{II}$ or $(Fe\text{-}S_{III})$] could be a $[2Fe\text{-}2S]^{1+,2+}$ center deserved further examination. Although the EPR relaxation properties of each of these centers were more similar to those known for $[4Fe\text{-}4S]^{1+}$ clusters in other systems studied (Xavier et al., 1981), the possibility of a [2Fe-2S] cluster cannot be ruled out. [2Fe-2S] clusters have been shown to be distinguishable from [4Fe-4S] centers on the basis of their characteristic intense positive CD bands in the 400–500-nm spectral region. For example, the visible CD spectrum of milk xanthine oxidase (containing two [2Fe-2S] centers and one FAD) is dominated by contributions from the [2Fe-2S] clusters while the bound FAD contributes only slightly (Palmer & Massey, 1969; Komai et al., 1969). On reduction of the $[2Fe\text{-}2S]^{2+}$ centers, the positive CD band observed for the oxidized form of the enzyme is lost and the reduced [2Fe-2S] cluster exhibits a much weaker dichroism in this spectral region. Circular dichroism spectra of oxidized, NADPH-reduced, and deazaflavin-reduced GltS were recorded (Figure 8). Difference spectra were also calculated (not shown). The CD spectrum

of oxidized *Azospirillum* GltS has a shape similar to the CD spectra reported for *E. coli* (Rendina, 1980), *B. subtilis* (Matsuoka & Kimura, 1986), and spinach GltS (Hirasawa et al., 1989), but it lacks the positive features at about 440 nm characteristic of $[2Fe\text{-}2S]^{2+}$ centers. Reduction by either NADPH (in the presence of the NADPH-regenerating system) or by irradiation in the presence of EDTA and deazaflavin leads to similar circular dichroism spectra for the respective reduced forms, which indicates that neither the NADPH-reduced nor the deazaflavin-reduced Fe-S centers of the enzyme show CD spectral properties similar to those reported for [2Fe-2S] centers. Further, these data also show that reduction of $(Fe\text{-}S)_{III}$, in addition to reduction of the $(Fe\text{-}S)_{II}$ center, by light/deazaflavin does not alter significantly the visible CD properties of the enzyme. The shapes and intensities of the CD spectrum of oxidized *Azospirillum* GltS is a composite of contributions from the three iron–sulfur centers and the two flavin centers. None of the iron–sulfur centers appear to exhibit CD properties that would dominate the observed visible CD spectrum.

Discussion

This work is an extension of previous kinetic and mechanistic studies on *A. brasilense* GltS to provide evidence for the involvement of flavins at the substrate-binding sites and to gain additional information on the structure and function of the iron–sulfur centers of the enzyme. The two-site model for the enzyme active site and the involvement of flavins in catalysis postulated from steady-state kinetic analyses of the enzyme reactions (Vanoni et al., 1991b) was confirmed by the study of sulfite reactivity with GltS. Sulfite reacts with only one of the two enzyme flavins to form a *N*(5)-sulfite adduct. The presence of sulfite does not affect enzyme reduction by NADPH, and the sulfite–enzyme complex is reversed only by 2-OG. These data provide evidence for one flavin interacting with NADPH, and the sulfite-reacting flavin being involved in reduction of the 2-iminoglutarate intermediate to form L-glutamate during turnover.

A reexamination of the iron content of the *Azospirillum* enzyme was prompted by the low-temperature EPR experiments presented in this study. While a previous communication on the purification and characterization of this enzyme reported a value of approximately 8 mol of iron/$\alpha\beta$ protomer with an equivalent amount of acid-labile sulfur atoms (Ratti et al., 1985), our current data show a value of about 12 mol of iron/$\alpha\beta$ protomer (an $\alpha\beta$ protomer taken as being equivalent to 2 mol of flavins). The difference in the level of iron content between the two studies is most likely due to an overestimation of the protein content of the GltS sample used for the early iron determinations. The iron content reported here is the result of multiple analyses in the presence and absence of internal standards and is also supported by the EPR data taken on the enzyme at differing oxidation/reduction levels.

EPR spectral analysis of oxidized enzyme as isolated provides strong evidence for the presence of a single $[3Fe\text{-}4S]^{1+}$ center in *Azospirillum* GltS. The *g* value and relaxation data presented are similar to those of $[3Fe\text{-}4S]^{1+}$ centers described for ferredoxins (Emptage et al., 1980; Huynh et al., 1980), hydrogenases (Teixeira et al., 1989), aconitase (Kent et al., 1982), and, more recently, spinach GltS (Knaff et al., 1991). Previous data on GltS isolated from *A. vinelandii*, *E. coli* (Rendina, 1980), and *B. subtilis* (Matsuoka & Kimura, 1986) are also consistent with the assignment of a $[3Fe\text{-}4S]^{1+}$ center in those enzymes. The only source of GltS examined by EPR spectroscopy which does not give a signal consistent with the presence of a $[3Fe\text{-}4S]^{1+}$ center is the GltS preparation from *C. reinhardii*, a green algae (Marquez et al., 1986).

The $[3Fe\text{-}4S]^{1+}$ center of *Azospirillum* GltS is reduced by NADPH by dithionite, and by photoreduction. This cluster is the only iron–sulfur center reduced initially by photoreduction and the only one completely reduced by dithionite, and it is suggested to exhibit the highest oxidation–reduction potential of the iron–sulfur centers present in the enzyme. EPR evidence for the cluster integrity on reduction by a variety of techniques suggests the cluster does not become converted to a [4Fe-4S] center upon reduction. Although specific experiments to critically test for this possibility of conversion of a "native" $[4Fe\text{-}4S]^{1+,2+}$ center to the observed $[3Fe\text{-}4S]^{1+}$ center in the purified *Azospirillum* enzyme have not been performed, the evidence accumulated with both plant (Knaff et al., 1991) and other bacterial GltS preparations (Rendina, 1980; Peisach et al., 1983) appears to be overwhelming for the designation of a $[3Fe\text{-}4S]^{1+}$ center as a native component of the enzyme. The observation of a [3Fe-4S] center in overexpressed *E. coli* fumarate reductase (Johnson et al., 1985) in whole cells is also supportive for the native functionality of $[3Fe\text{-}4S]^{1+}$ centers in complex flavoenzymes.

Reduction of GltS by NADPH results in the appearence of the EPR signal of a second iron–sulfur center with properties more similar to those of $[4Fe\text{-}4S]^{1+,2+}$ centers than to those of $[2Fe\text{-}2S]^{1+,2+}$ clusters. When $NADP^+$ is not removed by the NADPH-regenerating system, one electron equivalent equilibrates between the $(Fe\text{-}S)_{II}$ center and a flavin in the neutral semiquinone form. The observation of the formation of a free organic radical in NADPH-reduced GltS, which is assigned to a neutral flavin radical on the basis of the temperature, power-saturation dependence, and line width of 19.5 G of the EPR signal, correlates both with the appearence of a long-wavelength absorption band of the sample and with similar findings on the *E. coli* (Miller, 1972; Rendina, 1980) and *B. subtilis* (Matuoka & Kimura, 1986) enzymes. Photoreduction of GltS in the presence of EDTA and deazaflavin results in the expected reduction of the $[3Fe\text{-}4S]^{1+}$ cluster and the $Fe\text{-}S_{II}$ center, and in the appearence of the signal of a third Fe-S center, presumably also a $[4Fe\text{-}4S]^{1+,2+}$ center. These results led us to the conclusion that each protomer of GltS from *Azospirillum* contains two flavins, one $[3Fe\text{-}4S]^{1+}$ and one $[4Fe\text{-}4S]^{1+,2+}$ center, which are reduced by NADPH and therefore take part into the intramolecular electron-transfer process, and a third low-potential iron–sulfur center whose role in catalysis is not evident. The $(Fe\text{-}S)_{II}$ and $(Fe\text{-}S)_{III}$ clusters appear to be $[4Fe\text{-}4S]^{1+,2+}$ centers on the basis of their EPR properties. No evidence for the presence of $[2Fe\text{-}2S]^{1+,2+}$ centers in GltS was found by comparing the circular dichroism properties of the oxidized, NADPH- and deazaflavin-reduced enzyme. The iron content of the enzyme protomer can certainly accommodate one $[3Fe\text{-}4S]^{1+}$ and two $[4Fe\text{-}4S]^{1+,2+}$ centers.

Comparison of our results with those of Rendina (1980) on the *E. coli* and 4A. *vinelandii* enzymes suggests that a similar type and number of centers are present also in the GltS from these sources. In an effort to rationalize the complex EPR signal observed with deazaflavin-reduced *E. coli* GltS, Rendina (1980) proposed the presence of two interacting spin systems due to two distinct reduced $[4Fe\text{-}4S]^{1+,2+}$ clusters. While we feel confident in proposing a redox role in catalytic turnover for both the $[3Fe\text{-}4S]^{1+}$ and the $(Fe\text{-}S)_{II}$ clusters, it is not possible to establish whether the $(Fe\text{-}S)_{III}$ center, which is reduced only by the light/EDTA/deazaflavin system, is involved in the electron-transfer process or serves a different role in GltS. This center exhibits, under our experimental con-

ditions, a very low redox potential and is not reduced by NADPH. However, the presence of the other enzyme substrates (2-OG and L-glutamine) and/or temperature effects are factors that might significantly alter the redox behavior of this center. On the other hand, we cannot rule out that the $(Fe\text{-}S)_{III}$ center might serve a non-redox role in GltS. It has been shown that [4Fe-4S] clusters may play structural or regulatory roles (Grandoni et al., 1989) in enzymes as well as be involved in substrate binding or activation (Emptage et al., 1983). In the amidotransferase from *B. subtilis*, the presence of a low-potential $[4Fe\text{-}4S]^{1+,2+}$ center was demonstrated by Mössbauer, resonance Raman, and magnetic circular dichroism spectroscopies (Averill et al., 1980; Vollmer et al., 1983). The iron–sulfur cluster has been proposed to play a regulatory rather than a catalytic role in this enzyme. On the basis of site-directed mutagenesis experiments on the cysteine ligands of the cluster in *B. subtilis* amidotransferase (Grandoni et al., 1989), it has been suggested that the center acts as an oxygen sensor. In the case of *A. brasilense* GltS, mechanistic studies on the enzyme-catalyzed reactions have shown that the ammonia being transferred from L-glutamine to 2-OG is not released into the solvent or exchanged with exogenous ammonium ion (Vanoni et al., 1991a). While the detailed molecular basis for groups involved in ammonia transfer is obscure, one possible role for the low-potential $(Fe\text{-}S)_{III}$ center is to bind the substrate and/or transferred ammonia unit in the catalytic mechanism. As attractive as this hypothesis might be, an argument against this proposed function for the $(Fe\text{-}S)_{III}$ cluster is the apparent absence of such a center in plant GltS (Knaff et al., 1991). Alternatively, the $(Fe\text{-}S)_{III}$ center might function in the regulation of GltS activity. *A. brasilense* possesses both glutamate dehydrogenase and the glutamine synthetase/glutamate synthase pathway for ammonia assimilation. The levels of both glutamine synthetase and GltS are significantly lower in the presence of high ammonia concentrations in the growth medium than when cells are grown under low concentrations of the nitrogen source. Onset of nitrogen fixation and increases in the levels of glutamine synthetase and GltS activities are obtained in the absence of ammonia in the medium and at alow oxygen tensions (Zanetti et al., unpublished results). Therefore, although the isolated enzyme is stable to oxygen, it cannot be ruled out that the regulation of the activity levels of GltS in *Azospirillum* cells is exerted also through posttranslational modifications of the enzyme and may also involve its low-potential [Fe-S] center.

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Oxygen Evolution in the Absence of the 33-Kilodalton Manganese-Stabilizing Protein†

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ABSTRACT: There has been a considerable amount of controversy concerning the ability of photosystem II to evolve oxygen in the absence of the 33-kDa, manganese-stabilizing protein. Early reports indicated that some capacity for oxygen evolution existed in manganese-stabilizing protein-depleted membranes while more recent studies have suggested that the observed oxygen evolution activity arose from residual manganese-stabilizing protein present in the salt-washed preparations. In this paper, it is conclusively demonstrated that significant rates of steady-state oxygen evolution are observed in oxygen-evolving photosystem II membranes in the absence of detectable quantities of the manganese-stabilizing protein. More then 99% of the manganese-stabilizing protein was removed by either one $CaCl_2$ or two NaCl–urea washes. The amount of manganese-stabilizing protein removed was quantified immunologically using mouse polyclonal antibodies. Oxygen evolution rates of 115–140 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1} were observed in the NaCl–urea-washed preparations. These rates represent about 24% of the rate observed in untreated membranes [450–600 μmol of O_2 (mg of Chl)$^{-1}$ h^{-1}]. Somewhat lower, although still significant rates were observed in the $CaCl_2$-washed preparations. Optimal rates of oxygen-evolving activity in NaCl–urea-washed membranes which are devoid of the manganese-stabilizing protein required high concentrations of calcium and chloride.

Photosystem II (PS II)[1] is a multisubunit thylakoid membrane protein complex which catalyzes the light-driven oxidation of water to molecular oxygen and the reduction of plastoquinone to plastoquinol. This complex consists of both intrinsic and extrinsic protein subunits. Intrinsic polypeptides with apparent molecular masses of 49 (CPa-1), 45 (CPa-2), 34 (D1), 32 (D2), 9 and 4.5 (α and β subunits of cytochrome b_{559}), and 4 kDa (*psb*I gene product) in association with an extrinsic 33-kDa polypeptide have been assumed to form the minimum complex capable of photosynthetic oxygen evolution (Ghanotakis et al., 1987).

In higher plants, two additional extrinsic protein components with apparent molecular masses of 24 and 17 kDa are associated with the oxygen-evolving complex. Removal of these proteins by salt-washing (usually 1.0 M NaCl) dramatically lowers the oxygen-evolving capacity of PS II vesicles (Akerlund et al., 1982) and PS II membranes (Kuwabara & Murata, 1982). Much of the lost activity can be recovered by reconstitution with the 24- and 17-kDa proteins (Akerlund et al., 1982) or by the addition of moderate concentrations of calcium (Ghanotakis et al., 1984) and chloride (Andersson et al., 1984). These proteins are assumed to play a role in the regulation of calcium and chloride concentrations within the PS II complex.

The extrinsic 33-kDa protein is much more tightly associated with the intrinsic PS II proteins than are the 24- and 17-kDa proteins. Removal of this protein requires treatment with high concentrations of alkaline–Tris (Yamamoto et al., 1981), $CaCl_2$ (Ono et al., 1983), or NaCl–urea (Miyao & Murata, 1984). Treatment with alkaline–Tris also leads to the loss of the manganese cluster associated with the active site of PS II (Kuwabara & Murata, 1982). This was initially taken as evidence that the manganese cluster was associated with this

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[1] Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PS II, photosystem II; TMACl, tetramethylammonium chloride; Tris, tris(hydroxymethyl)-aminomethane.

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