Glutamate Synthase: Identification of the NADPH-Binding Site by Site-Directed Mutagenesis†

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ABSTRACT: To contribute to the understanding of glutamate synthase and of β subunit-like proteins, which have been detected by sequence analyses, we identified the NADPH-binding site out of the two potential ADP-binding regions found in the β subunit. The substitution of an alanyl residue for G298 of the β subunit of *Azospirillum brasilense* glutamate synthase (the second glycine in the GXGXXA fingerprint of the postulated NADPH-binding site) yielded a protein species in which the flavin environment and properties are unaltered. On the contrary, the binding of the pyridine nucleotide substrate is significantly perturbed demonstrating that the C-terminal potential ADP-binding fold of the β subunit is indeed the NADPH-binding site of the enzyme. The major effect of the G298A substitution in the GltS β subunit consists of an approximately 10-fold decrease of the affinity of the enzyme for pyridine nucleotides with little or no effect on the rate of the enzyme reduction by NADPH. By combining kinetic measurements and absorbance-monitored equilibrium titrations of the G298A-β subunit mutant, we conclude that also the positioning of its nicotinamide portion into the active site is altered thus preventing the formation of a stable charge-transfer complex between reduced FAD and $NADP^+$. During the course of this work, the *Azospirillum* DNA regions flanking the *glt*D and *glt*B genes, the genes encoding the GltS β and α subunits, respectively, were sequenced and analyzed. Although the *Azospirillum* GltS is similar to the enzyme of other bacteria, it appears that the corresponding genes differ with respect to their arrangement in the chromosome and to the composition of the *glt* operon: no genes corresponding to *E. coli* and *Klebsiella aerogenes glt*F or to *Bacillus subtilis glt*C, encoding regulatory proteins, are found in the DNA regions adjacent to that containing *glt*D and *glt*B genes in *Azospirillum*. Further studies are needed to determine if these findings also imply differences in the regulation of the *glt* genes expression in *Azospirillum* (a nitrogen-fixing bacterium) with respect to enteric bacteria.

Glutamate synthase (GltS)[1] is a complex iron−sulfur flavoprotein that catalyses the reductive transfer of L-glutamine (L-Gln) amide group to the C(2) carbon of 2-oxoglutarate (2-OG) to yield L-glutamate (L-Glu). The enzyme plays a key role in ammonia assimilation processes in microorganisms and plants, while its role in animal tissues is still unknown (for a recent review, see *1*). The bacterial GltS is NADPH-dependent (NADPH-GltS), is composed by two dissimilar subunits (α, 162 kDa and β, 52.3 kDa for the *Azospirillum brasilense* GltS, ref *2*) and contains one FAD and one FMN cofactors and three different iron−sulfur centers (one $[3Fe\text{-}4S]^{0,1+}$ center and two $[4Fe\text{-}4S]^{1+,2+}$ clusters).

The α subunit is similar to the single polypeptide chain that forms the ferredoxin-dependent GltS (Fd-GltS) found in photosynthetic tissues and to the N-terminal 3/4 of the eukaryotic-type pyridine nucleotide-dependent GltS. It was shown to contain the PurF-type (or Type II) glutamine amidotransferase site (*2*, *3*) and the site where 2-OG binds, is converted to the iminoglutarate intermediate (2-IG) on addition of ammonia from glutamine hydrolysis, and is reduced by the FMN cofactor to yield the L-glutamate product (*4*).

The small (β) subunit of bacterial NADPH-GltS has been shown to contain one FAD cofactor and the site where NADPH binds and is oxidized with parallel reduction of the bound FAD (*5*). This polypeptide is similar to the C-terminal region of the eukaryotic pyridine nucleotide-dependent GltS and appears to serve to make reducing equivalents available to the α subunit for reductive glutamate synthesis.

Interestingly, databank searches revealed that there are several proteins or protein domains, functionally unrelated to GltS, whose sequences are similar to that of the GltS β subunit (*1*). Alignment of sequences of the β subunits of GltS from different bacteria, of the C-terminal regions of NADH-GltS, and of the β subunit-like proteins or protein domains retrieved in databanks (*1*), allowed us to confirm the initial finding (*2*) of two conserved regions matching the

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[1] Abbreviations used are: GltS, glutamate synthase; NADPH-, NADH-, and Fd-GltS, NADPH, NADH, and ferredoxin-dependent GltS; 2-OG, 2-oxoglutarate; GR, glutathione reductase; INT, iodonitrotetrazolium; LB, Luria Bertani; IPTG, isopropyl β-D-thiogalactopyranoside; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); AADP, 3-aminopyridine adenosine dinucleotide phosphate; ADPRP, 2′-phosphoadenosine-5′-diphosphoribose; SDS, sodium dodecyl sulphate; ORF, open reading frame.

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consensus sequence for the formation of ADP-binding folds (residues 149−177 and residues 291−321 for the *Azospirillum* GltS β subunit, *6*) and one sequence matching the second FAD consensus sequence defined by Eggink et al. (*7*) (residues 432−442). In the bacterial NADPH-GltS, the C-terminal Gly-rich region shows deviations from the consensus sequence that are expected for binding of NADPH as opposed to that of NADH (*8*). Therefore, it was proposed (*2*) that the N-terminal potential ADP-binding region serves for FAD binding and that the C-terminal one corresponds to the NADPH-binding site. Interestingly, the overall arrangement of the two putative ADP-binding regions and of the second FAD consensus sequence in the GltS β subunit polypeptide is reminiscent of that found in enzymes of the disulfide reductase class (*9*). In these enzymes, the N-terminal ADP-binding fold interacts with FAD and that at the C-terminus with NAD(P)H as demonstrated by direct analysis of the enzymes' three-dimensional structures and by site-directed mutagenesis studies (*8*−*10*). In particular, a study of the effect of the substitution of the second glycine of the GXGXXG/A/P motif of the NADPH-binding site of *Escherichia coli* GR has been carried out (*11*). It was concluded that substitution of an alanyl residue for the second glycine of the motif is diagnostic for the identification of the ligand of an ADP-binding fold identified by sequence analysis. In fact, the G176A substitution in *E. coli* GR was reported not to alter overall protein folding and stability but to significantly affect the interaction between NADPH and the enzyme mutant species. The mutation caused a dramatic decrease of the enzyme catalytic efficiency (approximately 13000-fold) and a 18-fold increase of the K_M value for NADPH, which results from an approximately 1700-fold decrease of the rate of the enzyme reductive half reaction.

With this in mind, we mutagenised the *A. brasilense glt*D gene, encoding the β subunit of GltS, to produce the G298A-β subunit mutant. G298 is the second glycine of the GXGXXA motif part of the potential C-terminal ADP-binding region, which has been tentatively assigned to NADPH binding (*2*, *5*). It was expected that such mutant exhibited overall stability and FAD binding properties similar to those of the wild-type protein, but altered binding of the pyridine nucleotide. To test this hypothesis, the protein was overproduced in *E. coli*, purified and characterized with respect to its ability to interact with the flavin cofactor and the NADPH substrate.

As sequencing of DNA fragments was being carried out in relation to this work, the DNA regions flanking *glt*D and *glt*B genes in *Azospirillum* chromosome were also sequenced and analyzed with the aim to gain information on the structure of the *glt* operon in *Azospirillum*, an associative diazotroph, in comparison with the *glt* gene organization in enteric bacteria (*12*−*16*).

EXPERIMENTAL PROCEDURES

Complete Sequencing of the 10 kb DNA Region Containing Azospirillum brasilense gltD−*gltB Genes and Search for Genes Flanking gltD and gltB.* The 10 kb *Eco*RI *Azospirillum* DNA fragment containing *glt*D and *glt*B genes, encoding the β and α subunits of GltS, respectively, has been cloned in pUC18 to yield plasmid p1605 (*2*). The sequence of nucleotide 1 through 7376 can be accessed under GeneBank/EMBL number L04300. The sequence of the region between residues 7376 and 10155 was determined using subclones described in ref *2*. Sequencing was carried out using the T7 DNA polymerase, the T7 sequencing kit (Amersham), and 7-deaza-dGTP instead of dGTP to resolve band compressions in sequencing gels due to the high G+C content of *Azospirillum* DNA. The newly determined sequence can be retrieved under GenBank/EMBL accession number AF192408. The nucleotide sequence of the regions flanking *glt*D and *glt*B genes was analyzed for the presence of potential genes using programs of the GCG Wisconsin package (*17*). The deduced amino acid sequence of each potential open reading frame we found was compared with protein sequences deposited in databanks.

Production of pETG298A. p16054, a pUC18 derivative containing the *Eco*RI-*Sal*I 3 kb fragment of the *Azospirillum* DNA region cloned in p1605 (*2*), was used as the template in a mutagenesis experiment. In p1605, *glt*D spans from nucleotide 952 through nucleotide 2398. The 660 bp region between positions 1202 and 1862 of the cloned *Azospirillum* DNA fragment was amplified using the polymerase chain reaction and the following oligonucleotide primers.

Oligo GltS31: 5′-GTCTCCCAGGCACCAAC-3′

Oligo B:
5′-*CCATGG*CGGTGTCG**Ġ**CGCCGCCCAG-3′

Oligo GltS31 is identical to the *glt*D sequence between position 1202 and 1219 and is located upstream of the unique *Not*I site of the cloned DNA fragment. Oligo B is complementary to position 1838−1862 of the cloned *Azospirillum* DNA fragment, except for position 1848 (marked with a dot), which converts the GGC codon for Gly298 of GltS β subunit into the GCC codon for Ala. Oligo B also contains the site for *Nco*I (in italic) restriction enzyme. The following reaction mixture (100 μL) was set up: 30 ng p16504 plasmid, 100 pmol each oligonucleotide primers, 20 nmol each dNTP, 10 μL 10x Reaction buffer, 0.5 μL AmpliTaq Gold (5 U/μL, Perkin-Elmer), 100 nmol $MgCl_2$. PCR was carried out in a Perkin-Elmer Gene Amp PCR System Model 2400, and reaction conditions were as follows: Cycle 1, 10 min at 95 °C; cycle 2−36, 1 min at 95 °C, 1 min at 50 °C, and 4 min at 60 °C. A final extension for 15 min at 60 °C was also carried out. The product of the PCR amplification was purified after agarose gel electrophoresis using the BandPrep kit (Pharmacia) according to the manufacturer's instructions. The fragment was digested with *Not*I and *Nco*I. After purification, it was substituted for the *Nco*I−*Not*I site of pETβ9 plasmid carrying the wild-type *glt*D gene under the control of the T7/*lac* promoter of pET11a (Novagene, *18*, *19*). The *Not*I−*Nco*I fragment of one of the resulting plasmids (pETG298A) was sequenced in order to check the presence of the desired mutation and the absence of additional base changes. pETG298A was then used to transform *E. coli* BL21(DE3) cells for protein production experiments. pETβ9 was constructed after engineering a *Nde*I site on the start codon of *glt*D (see pBSC109 plasmid of Scheme 1 of ref *5*), several restriction sites in the *glt*D−*glt*B intergenic region, and a *Nde*I site on the start codon of *glt*B (*4*). The mutagenised *EcoR*V−*Sca*I fragment described in ref *4* was

substituted for the original fragment of pBSC109. The *Nde*I fragment of the latter plasmid, containing *glt*D, was cloned into the *Nde*I site of pET11a. All DNA manipulations were carried out using established procedures (*20*, *21*).

Production of the G298A-β Subunit. A few colonies of freshly transformed *E. coli* BL21(DE3) cells, harboring pETG298A or pET*β*9, were used to inoculate 50−100 mL Luria-Bertani (LB) medium containing 0.1 mg/mL ampicillin. Cultures were grown at 25−37 °C until a A_{600} of 0.5−1.0 was reached. Aliquots (0.1 to 5 mL) were used to inoculate 2 L flasks containing 0.5 L LB medium and 0.1 mg/mL ampicillin. Growth was carried out at temperatures between 20 and 37 °C. Induction of expression of the plasmid-encoded wild-type or mutant *glt*D gene was achieved by addition of 0.1 mM isopropyl *β*-D-thiogalactopyranoside (IPTG) to cultures exhibiting A_{600} between 1 and 2. Cells were harvested at different times after IPTG addition for analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS, *22*) of whole cell extracts or analysis of the cell soluble fraction with respect to NADPH:INT oxidoreductase activity content (*5*), protein concentration (*23*), and protein electrophoretic pattern. Whole cell extracts were obtained by incubating cells harvested at different times with SDS sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 5% (v/v) *β*-mercaptoethanol) for 5 min at 100 °C. To normalize the amount of protein loaded on polyacrylamide gels, cells harvested from 1 mL of a cell culture exhibiting A_{600} of 1 were resuspended in 100 *μ*L of SDS sample buffer; 10 or 20 *μ*L aliquots of the resulting solution were loaded on gels. To analyze the cell soluble fraction 0.5−1 g of cells harvested at various times during growth were homogenized with glass beads as described (*5*). The supernatant obtained after centrifugation at 39000*g* for 60 min at 4 °C was subjected to protein assay, NADPH:INT oxidoreductase activity assay, and SDS-electrophoresis on 10% or 12% polyacrylamide mini-gels.

Purification of the G298A-β Subunit. A minor modification of the procedure set up for the purification of wild-type GltS *β* subunit (*5*) allows to obtain nearly homogeneous preparations of G298A-*β* subunit, although with very low yields: cells are resuspended in 50 mM imidazole·HCl buffer, pH 7.5, 1 mM EDTA, 150 mM KCl, and 1 mM PMSF (buffer A, 1 mL/g cells) and sonicated with a Branson Sonifier Model 250 equipped with the microtip (five 1 min cycles, output 30 W and temperature maintained between 1 and 5 °C by immersion in a ice−salt mixture); the suspension is diluted with 6 mL buffer A/g cells and centrifuged at 39000*g* for 60 min. The supernatant is diluted 1.5-fold with water and glycerol to obtain a final concentration of 5% and loaded onto the Amicon Red column (Amicon). The rest of the procedure is similar to that described for the wild-type protein. The final protein solution was stored in aliquots at −80 °C after rapid freezing in liquid nitrogen. As an alternative, the diluted crude extract was loaded on a Q-Sepharose fast flow column (bed size 25 mL), equilibrated with 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 10% glycerol. Unbound and weakly bound proteins were eluted with the equilibration buffer and the same buffer containing 0.1 M NaCl, respectively. The G298A-*β* subunit could be eluted by applying a linear gradient from 0.1 to 0.5 M NaCl in 10 column volumes. Concentrated protein was then chromatographed on the Ultrogel AcA 54 (LKB) column as for the previous procedure. This second purification scheme yielded preparations that were contaminated with a cytochrome. Otherwise, the properties of various preparations did not differ significantly.

Protein and Activity Assays. Protein concentration was determined using the biuret method (*23*), on crude extracts, and the Bradford reagent (Amresco, *24*) in all other instances. Bovine serum albumin was used as the standard protein. The NADPH:iodonitrotetrazolium (INT) oxidoreductase activity of the GltS *β* subunit was determined as described previously (*5*). Under standard conditions, the assay mixtures contained 50 mM Hepes/KOH, pH 7.5, 0.1 mM NADPH, and 0.5 mM INT. Reactions were started with the addition of enzyme solutions to reaction mixtures equilibrated at 25 °C. The absorbance changes at 490 nm were measured and an extinction coefficient of 18.5 mM^{-1} cm^{-1} was used to calculate enzyme activity. For the determination of the steady-state kinetic mechanism of the NADPH:INT oxidoreductase reaction catalyzed by the G298A-*β* subunit mutant, the initial velocity of reactions (v) containing varying concentrations of INT (60−500 *μ*M) and fixed concentrations of NADPH (50−200 *μ*M) were measured. When necessary, the rate of nonenzymatic INT reduction by NADPH was subtracted. Data sets obtained at fixed NADPH concentrations were first individually analyzed using eq 1 and 2 where V_{app} and K_{Aapp} are the apparent maximum velocity and K_M values for the varied substrate and A is the varied substrate concentration (*25*).

$$\text{eq 1}\quad 1/v = 1/V_{app} + K_{Aapp}/(V_{app} \times A)$$

$$\text{eq 2}\quad v = (V_{app} \times A)/(K_{Aapp} + A)$$

After visual inspection of double reciprocal plots, the data were fitted to eq 3 that describes a ping-pong mechanism:

$$\text{eq 3}\quad v = (V \times A \times B)/(K_A B + K_B A + AB)$$

where V is the maximum velocity, A and B are the concentrations of INT and NADPH, and K_A and K_B are the K_M values for INT and NADPH, respectively.

Routinely, apparent V and K_M values for NADPH were determined by varying NADPH concentration (20−200 *μ*M) in the presence of 0.5 mM INT using eq 2. The apparent V and K_M values for NADH were determined under identical conditions, except for the fact that higher NADH concentrations were used. Both wild-type and G298A-*β* subunit were used for the experiment. The effect of $NADP^+$, 3-aminopyridine adenosine dinucleotide phosphate (AADP), and 2′-phosphoadenosine diphosphoribose (ADPRP) on the NADPH:INT oxidoreductase activity of the wild-type and the G298A mutant form of the GltS *β* subunit were also studied by measuring the initial velocity of reactions that contained varying concentrations of $NADP^+$, AADP, or ADPRP in the presence of 0.5 mM INT and fixed levels of NADPH. Dixon plots were constructed, were consistent with the fact that the pyridine nucleotide analogues were simple inhibitors of the reaction competitive with respect to NADPH, and the values of V, K_{NADPH} and K_i were calculated using eq 4

$$\text{eq 4}\quad 1/v = (K_A/(K_i VA))I + 1/V(1+(K_A/A))$$

where I is the inhibitor concentration and K_i is the inhibition constant.

Flavin Cofactor Identification, Quantification, and Determination of the Extinction Coefficient of the G298A-β Subunit. The identification of the flavin bound to the G298A-β subunit was performed fluorimetrically (*5*, *26*) using a homogeneous solution of G298A-β subunit (0.43 μM) that had been gel-filtered through a Sephadex G25 (medium) column (PD10 prepacked disposable columns, Pharmacia), equilibrated in 10 mM Tris·HCl, pH 7.5. The emission intensity at 524 nm of the native enzyme solution excited with light at 450 nm was compared to those of (a) the supernatant obtained after incubation at 100 °C for 5 min and removal of the denatured protein by centrifugation in a microfuge and (b) of the same supernatant after addition of snake venom phosphodiesterase (Boehringer Mannheim, 2 μL, 6 mU). The stoichiometry and extinction coefficient of the bound FAD cofactor was determined by absorbance spectroscopy (*5*, *26*). The spectrum of a 1 mL solution of G298A-β subunit (8.8 μM) that had been gel-filtered through a Sephadex G25 (medium) column, equilibrated with 10 mM Tris·HCl, pH 8.0, was recorded; 20 μL 10% SDS was added and absorbance changes, which were completed within 5 min after SDS addition, were recorded. An extinction coefficient of 11.3 mM^{-1} cm^{-1} was used to determine the concentration of FAD released from the G298A-β subunit. This value was used to calculate the FAD/G298A-β subunit molar ratio with the enzyme concentration calculated from the protein assay and the known mass of the G298A-β subunit (52 300). From the absorbance value at the maximum (454 nm) of the native enzyme solution and the known concentration of FAD released from the protein, the extinction coefficient of the bound FAD could also be calculated. Alternatively, the stoichiometry of bound FAD was determined by comparing the absorbance spectrum of the native protein (4.43 μM) in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 10% glycerol, and that of the supernatant obtained by heat denaturation and removal of the precipitated protein by centrifugation.

Absorbance Monitored Titrations of G298A-β Subunit. Solutions of G298A-β subunit were gel-filtered through G25 (medium) columns equilibrated in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, and 10% glycerol prior to each experiment. Aliquots of the concentrated titrating solution were added to the enzyme solution (5−10 μM), and spectra were recorded several times after each addition to make sure that absorbance changes were completed. Spectra were acquired with a Hewlett-Packard diode array spectrophotometer model HP-8453 connected to a thermostated bath. Instrument operation and data analysis was performed with the Hewlett-Packard UV−vis ChemStation run on a Vectra XA personal computer also from Hewlett-Packard. The titrating solutions were made up in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, and 10% glycerol. When necessary anaerobiosis was obtained by using cuvettes and vessels previously described (*27*) and by applying repeated cycles of evacuation and equilibration with oxygen-free nitrogen. Absorbance changes, which were observed at selected wavelengths in the presence of a given concentration of ligand, were used to calculate binding stoichiometries and dissociation constants of the enzyme−ligand complex using eq 5

$$\text{eq 5}\quad Y = \{-(K_d + L + n) + [(K_d + L + n)^2 - 4LN]^{1/2}\}/2$$

where: Y is the observed fractional absorbance change or absorbance change at a given ligand concentration (L), K_d is the dissociation constant of the enzyme−ligand complex, n is the number of binding sites per enzyme subunit if the ligand concentration is expressed as molar ratio with respect to the enzyme or is the maximal effect of the presence of the ligand on the value expressed by Y.

Data Analysis. Data were analyzed using the Grafit (Erythacus Software Ltd., UK) or Excel (Microsoft, USA) programs.

RESULTS AND DISCUSSION

Sequence Analyses of the DNA Regions Flanking gltD and gltB on Azospirillum Chromosome. During the course of this work, the approximately 1 kb DNA regions flanking *glt*D and *glt*B genes were analyzed in order to tentatively set the limits of the *glt* operon in *Azospirillum*. In *E. coli*, *glt*B and *glt*D form an operon with *glt*F that encodes a putative regulatory protein (*12*). Also in *Klebsiella aerogenes* the *glt* operon is formed by the B, D, and F genes (*13*). Finally, in *Bacillus subtilis* a regulatory gene, *glt*C, is upstream of *glt*B and is transcribed in the opposite direction (*14*). The approximately 1 kb region upstream of *glt*D was already available (*2*) and that downstream of *glt*B was determined during this work. The entire nucleotide sequence of the 10 kb *EcoR*I DNA fragment (the insert of p1605 of ref *2*) was translated in the possible six-frames. Putative ORFs, beside those corresponding to *glt*D and *glt*B genes, were identified on the basis of length (predicted product longer than 50 amino acyl residues) and properties (putative ORF preceded by a potential ribosome binding site, and codon usage reflecting that of *A. brasilense* within the ORF). The deduced amino acid sequence of each putative ORF was compared to sequences deposited in databases. None of the potential ORFs identified as described above encodes a protein similar to *E. coli* or *K. aerogenes* GltF nor a protein similar to *B. subtilis* GltC. Only two potential gene products were found to exhibit similarity with proteins whose sequences are deposited in databanks. The 5′-region of a gene encoding a protein similar to *E. coli bac*A gene product, which confers bacitracin resistance (*28*), was found at positions 1−561, with the gene running in the direction opposite to that of *glt*D and *glt*B. The second putative ORF (*orf*C) runs from nt 7874 through nt 8542 and it is transcribed in the same direction as *glt*D−*glt*B. It is separated from *glt*B (nt 2536−7084) by 790 bp that contain several short potential ORFs. The putative 222 residues gene product is similar (30% identity) to *Rhizobium leguminosarum gst*A gene product, which has been proposed to be a glutathionyl S-transferase (*29*). However, although we could produce such gene product in *E. coli* BL21(DE3) by overexpressing *orf*C under the control of a T7-promoter after transfer into a pET11d vector (*19*), the protein did not bind to glutathionyl-agarose (*30*) nor exhibited any detectable activity with reduced glutathione and 1-chloro-2,4-dinitrobenzene (*31*). While the identity of *orf*C gene product still needs to be determined, the absence of *glt*F- or *glt*C-like genes in proximity of *glt*D−*glt*B in *Azospirillum* suggests that the *glt* operon in this nitrogen

fixing bacterium is formed only by the GltS structural genes and could also indicate that regulation of *glt* gene expression in this diazotroph may be different from that found in enteric bacteria (*15*, *16*).

G298A-β Subunit Production, Purification, and General Properties. Pilot experiments of production of the G298A-β subunit mutant protein were carried out in 2 L flasks containing 0.5 L LB supplemented with 0.1 mg/mL ampicillin. *E. coli* BL21(DE3) cells freshly transformed with plasmid pETG298A were initially grown under conditions that yielded large amounts of soluble wild-type protein, namely, at 37 °C with addition of IPTG to induce expression of the heterologous gene when culture absorbance at 600 nm was approximately 1, and cell harvest 2−4 h after IPTG addition. The mutant protein form was indeed produced in large quantities but in a insoluble form as demonstrated by comparison of the electrophoretic analysis under denaturing conditions of total cell extracts with that of cell soluble fraction. Soluble protein was instead obtained when cells were grown at 30 °C until the culture reached an absorbance value at 600 nm of approximately 1, the temperature was then lowered at 25 °C, and IPTG was added. Cells harvested at various times showed that the G298A-β subunit was produced mainly in a soluble form in quantities apparently similar between 2 and 5 h after IPTG addition. In all cases, the amount of soluble G298A-β subunit was lower than that routinely obtained during production of the wild-type species under the same conditions. The latter conditions were chosen for growth of *E. coli* BL21(DE3) cells transformed with pETG298A on a larger scale in a fermentor containing 6−8 L LB medium with 0.1 mg/mL ampicillin. Cells were harvested 4 h after IPTG addition and stored frozen at −80 °C until they were used for G298A-β subunit purification.

The G298A-β subunit mutant was found to bind to Reactive Red (Sigma) or Amicon Red (Amicon) resins and to elute under conditions similar to those needed to elute the wild type protein (namely, two broad partially resolved peaks: one at approximately 0.5 M NaCl and a second peak at approximately 1 M NaCl, ref *5*). The two peaks were separately concentrated and chromatographed through the same Ultrogel AcA 34 gel-filtration column used for the wild-type enzyme.

The behavior of the mutant protein throughout the purification procedure was similar to that of the wild-type species, as judged by comparison of SDS-electrophoresis patterns, NADPH:INT oxidoreductase activity measurements and visible absorbance spectra of individual fractions. Taken together, it could be concluded that the G298A-β subunit mutant maintained the ability to bind NADPH and FAD, was catalytically active in the NADPH:INT oxidoreductase reaction, and had a monomeric state. In contrast with the wild-type species, the yield of each chromatographic step was low so that only a total of 0.1 mg of G293A-β subunit mutant could be obtained per g of *E. coli* cell paste, as opposed to 2 mg/g cells in the case of the wild-type form (*5*). This fact correlates with the low amount of G298A-β subunit mutant present in the soluble fraction of cells in comparison to that obtained with the wild-type protein and, presumably, with the lower stability of the mutant that caused production of insoluble protein during growth experiments at 37 °C. To increase the amount of G298A-β subunit mutant that could be prepared, we substituted a Q-Sepharose chromatography for the initial affinity column. The protein bound to the resin and eluted at approximately 0.3 M NaCl. The overall yield was greater than with the first purification scheme (approximately 0.26 mg β subunit/g cell paste) at the expenses of purity of the final preparation. Nevertheless, control experiments showed that the contaminant proteins present in the G298A-β subunit preparations did not seem to influence the results of the experiments provided the protein concentration was determined on the basis of flavin content.

Flavin Content of the G298A-β Subunit Mutant. FAD was identified as the bound flavin cofactor fluorimetrically; 0.86 mol FAD were found to be bound per mol of mutant β subunit, a value similar to that determined for the wild-type species (0.83 mol per mol β subunit, ref *5*). The absorbance spectra of the wild-type and mutant proteins were also similar with similar extinction coefficients at 454 nm (11385 ± 305 and 11300 ± 120, respectively). The properties of the bound cofactor were also determined fluorimetrically: as in the case of wild-type β subunit, the protein environment was found to enhance the flavin fluorescence (2.7- and 2.1-fold for the wild-type and G298A-β subunit mutant, respectively). Taken together these results demonstrate that the flavin environment is not perturbed by the G298A substitution.

Catalytic Properties of the G298A-β Subunit Mutant. The G298A-β subunit mutant catalyzed NADPH oxidation in the presence of INT as the electron acceptor. As in the case of the wild-type enzyme, it did not exhibit any detectable NADPH oxidase activity. The steady-state kinetic mechanism of the NADPH:INT oxidoreductase reaction was also found to be ping-pong (Table 1) with calculated turnover number and K_M for INT similar to those determined for the wild-type species, but a 10-fold higher K_M for NADPH. Since the K_M value for NADPH is by no means a direct measure of the dissociation constant of the enzyme−NADPH Michaelis complex, we determined the inhibition constants for a series of pyridine nucleotide analogues: $NADP^+$, AADP, and ADPRP. In all cases, the compounds acted as inhibitors of the NADPH:INT oxidoreductase reaction of the G298A-β subunit, competitive with respect to NADPH, and the K_i was 10−20-fold greater than that measured for the wild-type enzyme (Table 1). These results are consistent with the fact that the G298A substitution weakens the binding of the adenylate portion of the pyridine nucleotide to the enzyme with a moderate effect, if any, on the rate of the enzyme reductive half reaction. Whether such decreased affinity of the enzyme for the pyridine nucleotide was accompanied by a change in substrate specificity was tested by comparing enzyme activity with NADH as opposed to NADPH. Essentially no INT reduction is observed with the wild-type enzyme and 100 μM NADH (*5*). However, NADH was found to slowly reduce the GltS β subunit during an anaerobic equilibrium experiment with no formation of reduced enzyme−NAD^+ charge-transfer complex (unpublished observations). During the course of this work, we tested the ability of the wild-type and G298A-β subunits to catalyze INT reduction in the presence of high NADH concentrations. As shown in Table 1, a low activity could be detected in both cases. The K_M value for NADH measured with the mutant species is higher than that determined in a parallel experiment with the wild-type enzyme, confirming that the mutation affects binding of the pyridine nucleotide

Table 1. Comparison of the Steady-State Kinetic Properties of the Wild-type and G298A-β Subunit[a]

enzyme	NADPH (μM)	INT (μM)	eq	V (s^{-1})	K_{NADPH} (μM)	K_{INT} (μM)
G298A-β	50−200	60−500	3	44 ± 1.5	84 ± 5.8	122 ± 8.3
β	10−100	50−200	3	(32)	(3.5)	(50)

enzyme	NADPH (μM)	INT (μM)	inhibitor (μM)	eq	K_i (μM)
G298A-β	91−260	500	$NADP^+$, 0−200	4	37 ± 9.8[b]
G298A-β	50−100	500	AADP, 0−168	4	128 ± 16
β	30−100	500	AADP, 0−120	4	11.2 ± 5
G298A-β	43−162	500	ADPRP, 0−270	4	233 ± 43
β	9−100	500	ADPRP, 0−100	4	22.7 ± 6.8
β	10−100	500	ADPRP, 0−400	4	(21)

enzyme	NADH (μM)	INT	V_{app} (s^{-1})	K_{NADH} (μM)	V/K_{NADH} (s^{-1} mM^{-1})
G298A-β	95−278	500	2.3 ± 0.3	317 ± 76	0.00725
β	96−479	500	0.22 ± 0.02	113 ± 32	0.00195

[a] All assays were carried out in 50 mM Hepes/KOH buffer, pH 7.5, at 25 °C in the presence of the indicated substrates and inhibitors concentrations ranges. Data in parenthesis are from *5*. [b] For comparison, the K_i value determined with GltS was 1.8 μM (*35*) and that determined during a spectrophotometric titration of the GltS β subunit was 0.8 μM (*5*).

to the enzyme. The calculated turnover numbers were in both cases much smaller than those determined with NADPH, confirming that the β subunit is NADPH specific and demonstrating that the G298A substitution does not alter such specificity. However, the turnover number and the catalytic efficiency of the mutant species with NADH as substrate are higher than those of the wild-type. This observation can be explained assuming that the perturbation of the binding site of the adenylate portion of the pyridine nucleotide substrate at the same time loosens NADPH binding and offers less hindrance to the interaction with NADH, which, however, remains a very poor substrate of the GltS β subunit.

Spectrophotometric Titrations of the G298A-β Subunit with NADPH and AADP. To study the effect of the G298A mutation of the GltS β subunit in greater detail, anaerobic titrations of the enzyme with NADPH were carried out. NADPH was able to reduce the enzyme-bound FAD (Figure 1). No absorbance changes at long wavelengths were observed, as opposed to the increase of absorbance due to the formation of a stable charge-transfer complex between reduced FAD and $NADP^+$, obtained with the wild-type enzyme (Figure 1, and ref *5*). The absorbance changes at 450 nm as a function of NADPH/enzyme molar ratio were well-fitted with eq 5, leading to the conclusion that one NADPH is sufficient to reduce one enzyme molecule. The value of the apparent dissociation constant is 2.5 ± 0.6 μM, which is similar to that obtained with the wild-type β subunit (1.4 μM, *5*). This finding is not in contrast with the hypothesis that the G298A substitution in the GltS β subunit specifically weakens the interaction between the enzyme and the pyridine nucleotide. In fact, the dissociation constant calculated from the binding data reflects the reciprocal of the equilibrium constant of the overall enzyme-reductive half reaction. A minimal scheme for this reaction consists of: (a) formation of the E.NADPH complex, (b) oxidoreduction and formation of the stable charge-transfer complex between the reduced enzyme and $NADP^+$, which was observed with the wild-type enzyme, and (c) dissociation of the reduced enzyme−$NADP^+$ complex to yield the free products (eq 6).

$$\text{Eq. 6: } E + NADPH \underset{K_A}{\longleftrightarrow} E.NADPH \underset{K_B}{\longleftrightarrow} EH_2.NADP^+ \underset{K_C}{\longleftrightarrow} EH_2 + NADP^+$$

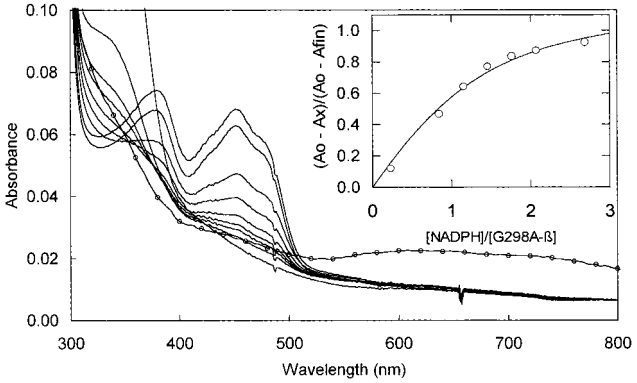

FIGURE 1: Anaerobic NADPH titration of G298A-β subunit. G298A-β subunit (5.4 μM) in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, and 10% glycerol was made anaerobic and titrated by adding aliquots of an anaerobic solution of NADPH (0.38 mM). Main panel: absorption spectra after addition of 0, 0.23, 0.84, 1.15, 1.46, 1.77, 2.1, and 2.7 mol NADPH/mol enzyme. The lower spectrum was obtained after addition of glucose 6-phosphate (100-fold excess) and *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase (2.5 U) from the sidearm of the cuvette. The line marked with circles shows the spectrum obtained for the wild-type β subunit in the presence of 1.73 mol NADPH/mol enzyme (from Figure 1 of ref *5*) after correction for the different enzyme concentration. Inset: plot of fractional absorbance changes at 454 nm as a function of NADPH/enzyme molar ratio. A_o, Initial absorbance; A_x, absorbance after a given addition of NADPH; A_{fin}, absorbance after addition of glucose 6-phosphate and glucose 6-phosphate dehydrogenase. The curve has been drawn using eq 5 with $n = 1$ and $K_d = 2.5$ μM.

The overall equilibrium constant of this reaction is the product of the individual equilibrium constants ($K_A K_B K_C$). If the G298A substitution decreases the affinity of the oxidized and reduced enzyme for NADPH and $NADP^+$, respectively, by approximately the same 10-fold factor, then the effects of the mutation on K_A (which decreases) and K_C (which increases) cancel each other out, leading to a calculated value of the overall equilibrium constant similar to that determined for the wild-type enzyme.

The similarity between the titration curve obtained with the wild-type and mutant β subunit species further supports the conclusion that the redox potential of the flavin bound to the protein species is not significantly affected by the mutation.

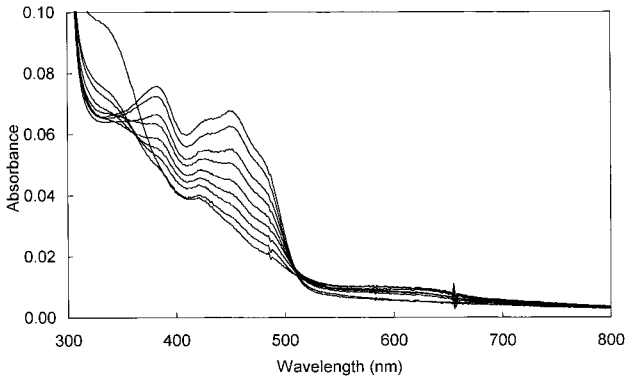

FIGURE 2: Photoreduction of G298A-β subunit in the presence of $NADP^+$. A G298A-β subunit solution (5.7 μM) that contained $NADP^+$ (5.3 mol/mol enzyme), deazaflavin (0.45 μM), EDTA (4.9 mM) in 25 mM Hepes/KOH, pH 7.5, 10% glycerol was made anaerobic in the dark. The figure shows the spectra obtained after 0, 19, 23, 24, 25, 26, 28, 30, and 41 min irradiation with a standard slide projector lamp.

To detect an effect of the mutation on the redox properties of the FAD bound to the G298A-β subunit mutant, we carried out its photochemical reduction in the absence or presence of $NADP^+$. Low amounts of flavin neutral semiquinone were observed during photoreduction of the wild-type β subunit in the presence of EDTA and deazaflavin, while higher concentrations of flavin semiquinone species could be obtained during photoreduction in the presence of 1.5 mol $NADP^+$/mol enzyme (see Figures 2 and 3 of ref *5*). Close inspection of the data of the latter experiment also revealed that some NADPH was indeed formed during the reaction. Irradiation of the G298A-β subunit solution containing EDTA and deazaflavin led to reduction of the flavin chromophore with formation of a maximum quantity of flavin neutral semiquinone comparable to that observed with the wild-type enzyme (see Figure 2 of ref *5*). Assuming that the extinction coefficient of the flavin semiquinone at 610 nm is 3900−4150 M^{-1} cm^{-1} (*32*, *33*), we could calculate that for both enzyme species a maximum of 7% of the enzyme is present as the flavin neutral semiquinone, which is reduced to the hydroquinone species at the end of the photoreduction. The experiment was repeated in the presence of 5.3 mol $NADP^+$/mol enzyme, which did not induce detectable spectral changes once dilution was taken into account. As shown in Figure 2, the absorbance changes observed at long wavelengths were much simpler than those observed with the wild-type enzyme (see Figure 3 of ref *5*). There was no indication of the formation of the charge-transfer complex between reduced flavin and $NADP^+$, in agreement with the data obtained during the NADPH titration of the enzyme (Figure 1), but it was observed that, also for the mutant enzyme, $NADP^+$ seems to cause a greater build up of the flavin neutral semiquinone as compared to the free enzyme: approximately 15% of the flavin is converted to the one-electron-reduced species in this experiment. Finally, the absorbance in the 340 nm region increased during irradiation. Most likely this is due to formation of NADPH from photoreduced enzyme and excess $NADP^+$ present. However, the spectrum of the solution clearly shows that the enzyme remains reduced at equilibrium. Taken together, these experiments establish the similar reactivity of the flavin cofactor of the mutant species as compared to the wild-type enzyme, with the exception of the lack of stabilization of

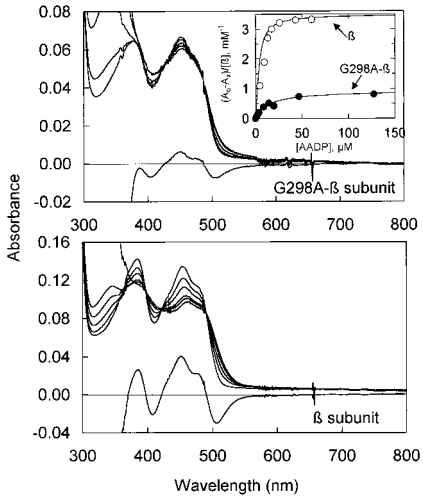

FIGURE 3: AADP titration of wild-type and G298A-β subunit. Upper panel: Absorption spectra of a G298A-β subunit solution (6.1 μM) after addition of 0, 0.5, 2.3, 7.6, and 21 mol AADP/mol enzyme. The difference between the spectrum of the enzyme obtained at the end of the titration and that of the free enzyme is also shown. Lower Panel: Absorption spectra of a β subunit solution (11.7 μM) after addition of 0, 0.4, 0.7, 1.1, 1.5, and 5.1 mol AADP/mol enzyme. The difference between the spectrum of the enzyme obtained at the end of the titration and of the free enzyme is also shown. The spectra in the two panels are scaled to allow direct comparison of the shape and extent of spectral changes. Inset: absorbance changes at 454 nm measured during the titration of the wild-type (open circles) and of the G298A-β subunit mutant (closed circles). To allow direct comparison the differences between the initial absorbance at 454 nm (A_o) and the absorbance after a given AADP addition (A_x) have been divided by the enzyme concentration (in mM) after correction for dilution. The curves have been drawn with eq 5. For the G298A-β subunit mutant, the K_d was 10.1 μM and the extrapolated apparent change of the extinction coefficient going from free enzyme to enzyme in complex with AADP ($\Delta\epsilon_{454,\beta/\beta-AADP}$) was 0.92 mM^{-1} cm^{-1}. For the wild-type form, a K_d of 1.1 μM and a $\Delta\epsilon_{454,\beta/\beta-AADP}$ of 3.85 mM^{-1} cm^{-1} were used.

the charge-transfer species between the bound reduced flavin and $NADP^+$. The data also contribute to support the hypothesis that the redox potential of the FAD bound to the G298A-β subunit is not significantly different from that of the cofactor bound to the wild-type enzyme.

The loss of ability to form the charge-transfer complex between the reduced flavin and $NADP^+$ in the mutant enzyme suggested to us that the mutation has not only an effect on the binding strength of the pyridine nucleotide but also that it may cause an altered positioning of the nicotinamide moiety of the substrate into the active center.

To test this hypothesis without the complication of flavin reduction, we wished to study the effect of binding of pyridine nucleotide analogues on the absorbance spectrum of the enzyme. With the wild-type β subunit, $NADP^+$ and ADPRP have been shown to give small or no perturbations of the flavin absorbance spectrum, respectively (*5*). $NADP^+$ was also found to give no significant absorbance changes on binding to the G298A-β subunit. However, during the course of this work, we determined that AADP is not only a good inhibitor of GltS β subunit reaction (Table 1), but that it also causes significant changes of the visible absorbance spectrum of the wild-type β subunit (Figure 3). Therefore, this compound was used to further characterize the effect of the G298A substitution in the GltS β subunit.

As shown in Figure 3, AADP determined absorbance changes of the spectrum of the G298A-β subunit mutant, which were qualitatively similar to those obtained with the wild-type protein. However, the calculated K_d of the enzyme−AADP complex was approximately 10-fold higher than that determined with the wild-type enzyme. Furthermore, at saturating concentrations of AADP, such absorbance changes were approximately 3-fold less intense with the mutant enzyme. Thus, this experiment confirmed the hypothesis that the substitution of G298 with a A residue in the GltS β subunit decreases the strength of the binding interaction between the enzyme and the pyridine nucleotide and that the G298A substitution most likely determines an altered positioning of the nicotinamide ring in the enzyme active site.

CONCLUSIONS

Sequencing and analysis of the *Azospirillum* DNA regions flanking the *glt*D and *glt*B genes, the genes encoding the GltS β and α subunits, respectively, showed that most likely the *glt* operon of *Azospirillum brasilense* is limited to the GltS subunits structural genes. Therefore, despite the strong similarity of GltS enzymes from different bacteria, their gene arrangement and, perhaps, their expression regulation is different.

The kinetic and spectroscopic characterization of the G298A mutant of the GltS β subunit allowed us to identify the potential ADP-binding site found at the C-terminus of the protein primary structure as its NADPH-binding site. This finding both contributes to our understanding of GltS structure and confirms the hypothesis of Rescigno and Perham (*11*) that substitution of an alanyl residue for the second G of the GXGXXG(A) fingerprint is diagnostic for the identification of the specific ligand. Indeed, the binding and environment of the flavin cofactor are not affected by the G298A substitution in GltS β subunit. However, an unexpected minor effect on protein stability was induced by the mutation leading to both (a) production of insoluble protein under conditions that yield large amounts of soluble wild-type enzyme and (b) the production of smaller amounts of enzyme, a fact that limited our ability to further characterize the protein. The mutation caused an increase of the K_M value for NADPH but, as expected, did not cause a switch in specificity toward NADH. The K_i values for $NADP^+$, ADPRP, and AADP also increased indicating a weakened binding of the pyridine nucleotide to the mutant enzyme. Therefore, the increase of the K_M value for NADPH is mainly due to an increase of the dissociation constant of the enzyme−NADPH complex rather than to kinetic factors (see below). The equilibrium titrations of the enzyme with NADPH and AADP together with the photoreduction experiments of the free or enzyme−$NADP^+$ complex also confirm and extend the conclusions of Rescigno and Perham with GR (*11*): the mutation does not seem to significantly alter the redox behavior of the bound FAD cofactor, but the weakened binding of the adenylate portion of the pyridine nucleotide seems accompanied by an altered relative positioning of the flavin isoalloxazine ring and of the substrate nicotinamide ring in the active center. As a result, no longer the stable charge-transfer complex between reduced flavin and $NADP^+$ is formed and weaker absorbance changes are observed on binding of AADP to the G298A-β subunit as opposed to the wild-type species. Such altered positioning of the substrate nicotinamide ring in the β subunit active site seems to have little or no effect on the rate of the enzyme reductive half reaction as deduced from no changes of both the G298A-β subunit turnover number and the K_M value for INT (*34*).[2] In GR (*11*), the effect of the corresponding mutation is markedly different from what we observed with the GltS β subunit. The G176A substitution in GR caused a greater than 2000-fold decrease of the rate of enzyme-reductive half reaction (*11*).[3] The different effect of the G-to-A substitution in the two enzymes could be rationalized keeping in mind that, at difference with the GltS β subunit, the formation of the charge-transfer complex between oxidized enzyme and the NADPH substrate is part of the mechanism of the GR reductive half reaction (*9, 10*). Thus, the altered positioning of the nicotinamide moiety of the substrate in the enzyme active site, detected by studying the GltS G298A β subunit mutant, may be responsible for the dramatic decrease of the rate of enzyme reduction by NADPH observed by Rescigno and Perham (*11*) with the corresponding GR mutant. Whether these experiments argue in favor or against a structural similarity between GltS β subunit (and β subunit-like proteins) and GR or other enzymes of the disulfide reductase family must await the resolution of the three-dimensional structure of GltS or of one of the β subunit-like proteins or protein domains.

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[2] With the wild-type β subunit, we have shown that flavin reduction by NADPH takes place at a rate of 900 s^{-1} at 25 °C (*5*). Thus, for the wild-type β subunit, the rate of enzyme reoxidation by INT is limiting the turnover rate (approximately 40 s^{-1}). With these values, and assuming that the mutation has no effect on the reaction between reduced enzyme and INT, only a decrease of the rate of flavin reduction by NADPH greater than 10-fold would be detected in turnover with the mutant enzyme. While the reviewing process of this manuscript was under way, we measured directly the rate of reduction of the G298A-β subunit mutant by NADPH in a stopped-flow apparatus under conditions similar to those described in ref *5*. Reduction of the enzyme-bound FAD took place in two phases, as judged by absorbance changes at 450 nm. The second minor phase of the reaction was essentially independent from NADPH concentration and took place at an observed rate (k_{2obs}) of approximately 23^{-1} s. On the contrary, the observed rate of the first phase of reaction (k_{1obs}) exhibited hyperbolic dependence on NADPH concentration: a maximum rate of 218.5 ± 12.8 s^{-1} and a $K_M(K_d)$ value for NADPH of 46.2 ± 12.7 μM were calculated, respectively. A transient increase of absorbance at 800 nm was also observed. The initial increase of absorbance paralleled the first phase of flavin reduction, as monitored at 450 nm, and was followed by a slow decrease of absorbance. This behaviour differs from that of the wild-type enzyme (*5*) in which the absorbance at long wavelengths increased until it reached a maximum value without subsequent decrease over a period of several minutes. Thus, it has been established that the G298A substitution only brings along a 4−5-fold decrease of the rate of reduction of the bound flavin by NADPH as compared to wild-type, and it is confirmed that the mutation causes (a) a decrease of the binding interaction between the mutant enzyme and the pyridine nucleotide substrate, and (b) a destabilization of the charge−transfer complex between reduced FAD and bound $NADP^+$, in full agreement with the reaction scheme presented in eq 6.

[3] The turnover number of *E. coli* G176A-GR mutant was found to decrease approximately 1700-fold (*11*) and the rate of the reductive half reaction in the wild-type enzyme is assumed to be approximately 2-fold faster than that of the oxidative half reaction as found for the yeast enzyme (*9, 10*).

REFERENCES

1. Vanoni, M. A., and Curti, B. (1999) *Cell. Mol. Life Sci. 55*, 617−638.
2. Pelanda, R., Vanoni, M. A., Perego, M., Piubelli, L., Galizzi, A., Curti, B., and Zanetti, G. (1993) *J. Biol. Chem. 268*, 3099−3106.
3. Zalkin, H., and Smith, J. L. (1998) *Adv. Enzymol. Relat. Areas Mol. Biol. 72*, 87−144.
4. Vanoni, M. A., Fischer, F., Ravasio, S., Verzotti, E., Edmondson, D. E., Hagen, W. R., Zanetti, G., and Curti, B. (1998) *Biochemistry 37*, 1828−1838.
5. Vanoni, M. A., Verzotti, E., Zanetti, G., and Curti B. (1996) *Eur. J. Biochem. 236*, 937−946.
6. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) *J. Mol. Biol. 187*, 101−107.
7. Eggink, G., Engel, H., Vriend, G., Terpstra, P., and Witholt, B. (1990) *J. Mol. Biol. 212*, 135−142.
8. Scrutton, N. S. Berry, A., and Perham, R. N. (1990) *Nature 343*, 38−43.
9. Williams, C. H. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. III, pp 121−211, CRC Press, Boca Raton, FL.
10. Palfey, B. A., and Massey, V. (1998) in *Comprehensive biological catalysis* (Sinnot, M. L., Ed.), Vol. 3, pp 83−154, Academic Press, Orlando, FL.
11. Rescigno, M., and Perham, R. N. (1994) *Biochemistry 33*, 5721−5727.
12. Castano I., Flores N., Valle F., Covarrubias A. A., and Bolivar F. (1992) *Mol. Microbiol. 6*, 2733−2741.
13. Kuczius, T., Eitinger, T., D'Ari, R., Castorph, H., and Kleiner, D. (1991) *Mol. Gen. Genet. 229*, 479−482.
14. Belitsky B. R., Janssen P. J., and Sonenshein, A. L. (1995) *J. Bacteriol. 177*, 5686−5695.
15. Reitzer, L. J. (1996) in *Escherichia coli and Salmonella. Cellular and molecular biology* (Neidhart, F. C., Ed.) pp 391−407, ASM Press, Washington, DC.
16. Newman, E. B., Lin, R. T., and D′Ari, R. (1996) in *Escherichia coli and Salmonella. Cellular and molecular biology* (Neidhart, F. C., Ed.) pp 1513−1525, ASM Press, Washington, DC.
17. Devereux J., Haeberli P., and Smithies O. (1984) *Nucleic Acids Res. 12*, 387−395.
18. Tabor, S., and Richardson, C. C. (1985) *Proc. Nat. Acad. Sci. 82*, 1074−1078.
19. Studier, W. F., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in *Molecular cloning: a laboratory manual*, 2nd ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Strhul, K. (1989) in *Current Protocols in Molecular Biology*, Wiley-Interscience, New York, NY.
22. Laemmli, U. K. (1970) *Nature 227*, 680−687.
23. Gornall, A. G., Bardewill, C. J., and David, M. M. (1949) *J. Biol. Chem. 177*, 751−766.
24. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
25. Segel, I. H. (1975) in *Enzyme kinetics*, Wiley & Sons, New York, NY.
26. Aliverti, A., Curti, B., and Vanoni, M. A. (1999) in *Methods in Molecular Biology: Flavoprotein Protocols* (Chapman, K. S., and Reid, G., Eds.) Vol. 131, pp 9−23, Humana Press, Totowa, NJ.
27. Williams, C. H., Arscott, L. D., Matthews, R. G., Thorpe, C., and Wilkinson, K. D. (1979) *Methods Enzymol. 62*, 185−207.
28. Cain, B. D., Norton, P. J., Eubanks, W., Nick, H. S., and Allen, C. M. (1993) *J. Bacteriol. 175*, 3784−3789.
29. Alkafaf, N. K., Yeoman, K. H., Wexler, M., Hussain, H., and Johnston, A. W. B. (1997) *Microbiology 143*, 813−822.
30. Smith, D. B., and Johnson, K. S. (1988) *Gene 67*, 31−40.
31. Habig, W. H., and Jacoby, W. B. (1981) *Methods Enzymol. 77*, 398−405.
32. Mayhew, S. G., Foust, G. P., and Massey, V. (1969) *J. Biol. Chem. 144*, 803−810.
33. Stankovich, M. T., Schopfer, L. M., and Massey, V. (1978) *J. Biol. Chem. 14*, 4971−4979.
34. Matthews, R. G. (1991) in *Flavins and Flavoproteins 1990* (Curti, B., Ronchi, S., and Zanetti, G., Eds.) pp 593−597, De Gruyter, Berlin.
35. Vanoni, M. A., Rescigno, M., Nuzzi, L., Zanetti, G., and Curti, B. (1991) *Eur. J. Biochem. 202*, 181−189.

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