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Review

Glutamate synthase: a complex iron-sulfur flavoprotein

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Abstract. Glutamate synthase is a complex iron-sulfur flavoprotein that forms L-glutamate from L-glutamine and 2-oxoglutarate. It participates with glutamine synthetase in ammonia assimilation processes. The known structural and biochemical properties of glutamate synthase from *Azospirillum brasilense*, a nitrogen-fixing bacterium, will be discussed in comparison to those of the ferredoxin-dependent enzyme from photosynthetic tissues and of the eukaryotic reduced pyridine nucleotide-dependent form of glutamate synthase in order to gain insight into the mechanism of the glutamate synthase reaction. Sequence analyses also revealed that the small subunit of bacterial glutamate synthase may be the prototype of a novel class of flavin adenine dinucleotide- and iron-sulfur-containing oxidoreductase widely used as an enzyme subunit or domain to transfer reducing equivalents from NAD(P)H to an acceptor protein or protein domain.

Key words. Glutamate synthase; flavoprotein; iron-sulfur clusters; ammonia assimilation; nitrogen metabolism; oxidoreductases; amidotransferases; electron transfer.

Introduction

Glutamate synthase (GltS) 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), yielding two molecules of L-glutamate (L-Glu, reaction 1).

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

The reducing equivalents are provided by reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced ferredoxin (Fd) depending on the type of glutamate synthase.

GltS has been purified from bacteria, yeast and plants and characterised to various extents. Work on microbial glutamate synthase has been reviewed by Miller [1], Meister [2], Vanoni et al. [3], and more recently by Zalkin [4], Curti et al. [5] and Zalkin and Smith [6]. The properties of plant GltS have been reviewed by Knaff and Hirasawa [7] and Temple et al. [8]. The genes encoding GltS have been cloned and sequenced from a growing number of organisms ranging from bacteria to lower animals. Comparison of the primary structures of GltS from various sources and of the known biochemical properties led to the definition of three classes of GltS, which differ for distribution among different organisms and tissues, subunit composition, cofactor content and physiological reductant:

1) NADPH-GltS (EC 1.4.1.13). The bacterial form of the enzyme is composed of two subunits (α subunit $\cong$ 150 kDa and β subunit $\cong$ 50 kDa) that form the active $\alpha\beta$ protomer ($\cong$ 200 kDa) containing one flavin adenine dinucleotide (FAD), one flavin mononucleotide (FMN) and three different iron-sulfur (Fe/S) clusters. The enzymes from *Klebsiella aerogenes*, *Escherichia coli*, *Bacillus subtilis* and *Azospirillum brasilense* are

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those that have been better characterised biochemically. With the exception of the *B. subtilis* enzyme, bacterial GltS is specific for NADPH; thus the abbreviation NADPH-GltS will be used throughout to indicate the bacterial form of GltS.

2) Fd-GltS (EC 1.4.7.1). Cyanobacteria and plants contain an Fd-dependent form of GltS. The enzyme is localised in plastids (in plants), is composed of a single polypeptide chain ($\cong$ 150 kDa) similar to the α subunit of bacterial NADPH-GltS and differs from the bacterial NADPH-GltS for a lower nonheme iron and acid-labile sulfur content. Early reports indicated a flavin content similar to that of the NADPH-GltS, whereas more recent data are consistent with the presence of only 1 mol FMN per mol Fd-GltS subunit [9, 10]. Most of the information available on the biochemical properties of Fd-GltS derives from studies of the enzyme prepared from spinach, although preparations of Fd-GltS from *Chlamydomonas*, *Synechococcus* and *Synechocystis* have also been characterised to different extents [7, 8].

3) NADH-GltS (EC 1.4.1.14). Yeast, fungi, plants and lower animals contain a pyridine nucleotide-linked form of GltS. The enzyme has been purified from *Medicago sativa* [11], *Saccharomyces cerevisiae* [12], and very recently a report describing an NADH-dependent GltS prepared from silkworm has appeared [13]. The genes or the complementary DNA (cDNA) encoding GltS in several eukaryotes have been cloned and sequenced. Direct biochemical evidence and sequence information available indicate that the eukaryotic pyridine nucleotide-dependent enzyme is formed by a single polypeptide chain of $\cong$ 200 kDa, which appears to derive from the fusion of polypeptides corresponding to the α and β subunits of bacterial GltS. This enzyme form was shown to be specific for NADH as the electron donor. Although it cannot be ruled out that in some instances the eukaryotic pyridine nucleotide-dependent enzyme may be NADPH-specific, we will refer to this form of GltS as NADH-GltS.

There is general agreement on the fact that, among enzymes of nitrogen metabolism, GltS is the one that has been studied the least. In particular, very little work has been done on the eukaryotic NADH-dependent GltS, presumably because of a combination of low levels and high lability. More data are available on the more abundant Fd-dependent GltS. However, most of the information available on GltS is that obtained through studies of the bacterial NADPH-dependent GltS. For this reason, we will focus on the properties of bacterial GltS in relation to those known for the other enzyme forms. The structural and functional information obtained in recent years in our laboratory during studies of the *Azospirillum brasilense* enzyme will be used to highlight similarities and differences with respect to other GltS. It is our aim to show how the study of the bacterial GltS holoenzyme, of its recombinant isolated α and β subunits and of mutant forms obtained by site-directed mutagenesis may provide complementary information for a better understanding of Fd-GltS and may represent a starting point for a thorough study of the poorly characterised eukaryotic-type NADH-GltS.

Physiological role of glutamate synthase

The early work of several groups with the bacterial enzyme, as reviewed in Reitzer and Magasanik [14] and Reitzer [15], was fundamental for the understanding of nitrogen metabolism in prokaryotes. Until the early 1970s it was thought that ammonia could be assimilated in bacteria either by the action of glutamine synthetase (GS, reaction 2) or by that of glutamate dehydrogenase (GDH, reaction 3).

$$\text{L-Glu} + NH_4^+ + ATP \leftrightarrow \text{L-Gln} + ADP + P_i \quad (2)$$

$$\text{2-OG} + NH_4^+ + NAD(P)H \leftrightarrow \text{L-Glu} + NAD(P)^+ \quad (3)$$

However, although efficient synthesis of glutamate is needed to support GS reaction and to allow synthesis of other amino acids for cell growth, it was found that the K_m values of GDH for ammonia were too high (3–4 mM for *K. aerogenes* GDH [16]) to justify cell growth in the presence of low ammonia concentrations. Furthermore, in some *Bacillus* species no glutamate dehydrogenase activity could be detected. Amino acid dehydrogenases other than GDH (e.g. alanine dehydrogenase, leucine dehydrogenase and valine dehydrogenase) were suggested to functionally replace GDH in these and other bacteria [1, 16]. The finding, in the early 1970s, of glutamate synthase, a novel L-glutamine-dependent amidotransferase, explained how bacterial cells could grow in the presence of low ammonia and led to the definition of a novel two-reaction pathway formed by GS and GltS (reaction 4, [16]).

$$\text{2-OG} + NH_4^+ + NAD(P)H + ATP \leftrightarrow \text{L-Glu} + NAD(P)^+ + ADP + P_i \quad (4)$$

The pathway is commonly known as the GS/GOGAT pathway, GOGAT being the acronym initially assigned to glutamate synthase. The enzyme was, in fact, erroneously classified as a glutamine:2-oxoglutarate aminotransferase. Due to the complexity of the reaction catalysed by GltS, the enzyme is indeed difficult to classify, and the systematic name glutamate:$NADP^+$ oxidoreductase (transaminating, glutamine forming) has also appeared in the literature. In any case, we will use the acronym GltS, which also reflects the commonly employed term (*glt*) used to define the genes encoding GltS (see below).

Ammonia assimilation through the GS/GltS pathway is made efficient by the low K_m values of both these enzymes for their substrates (*E. coli* GS: $K_{NH3} \cong 0.01$–0.06 mM; $K_{L\text{-}Glu} \cong 4$ mM; $K_{ATP} \cong 0.2$–2 mM, [17]; *A. brasilense* GltS, $K_{NADPH} \cong 1$–10 μM, $K_{2\text{-}OG} \cong 100$ μM, $K_{L\text{-}Gln} \cong 200$–500 μM [18]). The overall reaction catalysed by GS and GltS is similar to that of GDH except for the expenditure of 1 mol ATP, which has been defined as the price to pay to assimilate ammonia when its cellular level is low. In support of a role of the GS/GltS pathway for ammonia assimilation in bacteria is the fact that mutants defective in GltS activity are unable to grow in the presence of low ammonia levels, whereas GDH, when present, can support growth only when the level of ammonia is high [15]. For this reason, the GS/GltS pathway attracted much attention with respect to the physiology of nitrogen-fixing bacteria [19–21], which rely on the efficient glutamate synthesis catalysed by GS and GltS in order to produce amino acids for growth through the action of amino-transferases.

It should be kept in mind that, in bacteria, L-glutamate is not only the precursor of other amino acids, but also is involved in osmoregulation [22] and is, in some instances, the precursor of heme biosynthesis [23].

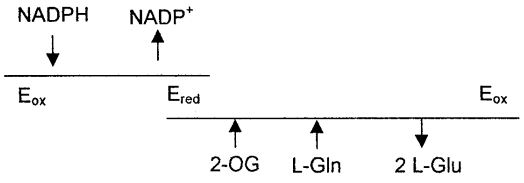
Studies of the regulation of the expression of the genes encoding the two enzymes of the GS/GltS pathway in bacteria and of the regulation of the activities of the enzymes have been mainly centered on GS [15]. Much less is known about the regulation of expression of *glt* genes, the genes encoding GltS, and the modulation of GltS activity. Several reviews have appeared on ammonia metabolism in enteric and nitrogen-fixing bacteria, and the reader is referred to them for details [14, 15, 21]. It has been proposed that the in vivo rate of the GltS reaction is limited by the rate of production of glutamine by GS, which in turn is tuned by the cellular levels of L-Gln and 2-OG. In this respect, GltS activity may play a role in modulating GS activity by influencing the 2-OG level. The interplay of *glt* gene expression, GltS activity and nitrogen and carbon metabolism in enteric bacteria is complex [15]. The structural genes encoding the GltS α and β subunits (*glt*B and *glt*D, respectively) form an operon with *glt*F, encoding a putative kinase [24]. In *E. coli*, expression of *glt*BDF genes is positively regulated by the leucine-responsive regulatory protein (LRP) in a leucine-independent fashion [25–27]. In addition, the levels of GltS activity and/or of *glt*F gene product have an effect on the induction of the Ntr response in enteric bacteria [15]. In *B. subtilis*, *glt* gene expression is positively controlled by the product of *glt*C, a member of the LysR family, unrelated to *E. coli* LRP or GltF [28]. However, *glt*C gene product may not be the only factor controlling *glt* gene expression in *B. subtilis* [29].

In nitrogen-fixing bacteria, in spite of the fundamental role of GltS, nothing is known about the mechanisms that regulate its gene expression. The levels of GltS are controlled by the ammonia level and are maximal under nitrogen-fixing conditions or in the presence of low ammonia levels, whereas GltS-defective mutants are unable to grow under nitrogen-fixing conditions [30]. No information is available on the presence of specific proteins that affect *glt* gene expression and no genes similar to *glt*F or *glt*C have been found in the DNA regions flanking *glt*D and *glt*B genes in *A. brasilense* (E. Verzotti and P. Morandi, unpublished). Finally, virtually nothing is known about regulation of bacterial GltS activity, if any, by posttranslational processing, effector binding or covalent modification.

Studies of nitrogen metabolism in plants led to the definition of a GS/GltS pathway also in cyanobacteria and plants [8, 31, 32] where it serves two main purposes: i) primary assimilation of ammonia derived from the action of associative or symbiotic nitrogen-fixing bacteria or of ammonia produced by nitrate and nitrite reductases using soil nitrate, and ii) secondary assimilation of ammonia derived from mobilisation of nitrogen storage compounds (glutamine, asparagine, arginine and ureides) or from photorespiration.

The Fd-dependent form of GltS was initially found in chloroplasts. Leaf Fd-GltS has been demonstrated to be essential for reassimilation of ammonia released during photorespiration since *glt* mutants are without exception photorespiratory mutants. In this respect, the elucidation of the mechanisms that allow the efficient assimilation of ammonia into glutamine and, subsequently, glutamate seems particularly challenging taking into account the compartmentalisation of photorespiratory enzymes producing ammonia (in the mitochondrion) and those using it (cytoplasmic or chloroplast GS, and Fd-GltS in the chloroplast). Recent studies revealed the presence of Fd-GltS also in root plastids, where reduced Fd may be formed from NADPH oxidation catalysed by the root-type ferredoxin-$NADP^+$ oxidoreductase [8]. Root NADH-GltS, presumably located in the plastids, is instead assumed to be responsible for primary ammonia assimilation. NADH-GltS has also been found in green tissues, but its level seems too low to support reassimilation of ammonia from photorespiration [8].

The mechanisms that regulate *glt* gene expression in plants still need to be elucidated. While it has been shown that the expression of the genes encoding Fd-GltS may be under the control of light through the phytochrome system, it appears that NADH-GltS is, as expected from its postulated function, regulated in a different but as yet unknown fashion.



Scheme 1. Steady-state kinetic mechanism of bacterial glutamate synthase.

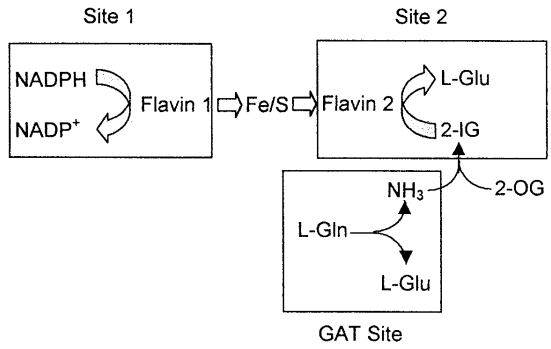
Catalytic mechanism of bacterial glutamate synthase

Multiple catalytic sites of GltS

Steady-state kinetic studies of GltS purified to homogeneity from *E. coli* [33], *B. subtilis* [34] and *A. brasilense* cells [18] demonstrated that the L-glutamine-dependent synthesis of L-Glu is well described by a two-site uni-uni bi-bi ping-pong mechanism (Scheme 1).

On the basis of the properties of the enzyme cofactors (one FAD, one FMN and several iron-sulfur clusters) and substrate/product couples (NADPH/$NADP^+$ and (L-Gln + 2-OG)/L-Glu), a scheme for the enzyme reaction was initially proposed [18, 33], and it is consistent with the presence of three distinct catalytic sites carrying out partial reactions (Scheme 2).

1) Site 1, the pyridine nucleotide site. At this site, NADPH binds to GltS and is oxidised with hydride transfer to one of the enzyme flavins, Flavin 1, located at this site. The reaction taking place at Site 1 is essentially a flavin-mediated NADPH:acceptor oxidoreductase reaction. The electron acceptor of the reduced flavin is postulated to be one (or more) of the iron-sulfur clusters that form the intramolecular electron transfer chain of GltS, leading to reduction of the second enzyme flavin (Flavin 2) at GltS Site 2.



Scheme 2. Two-site model and partial reactions of bacterial glutamate synthase.

2) Site 2, the iminoglutarate site. At this site 2-OG binds and is converted to the 2-iminoglutarate (2-IG) intermediate on addition of ammonia from the L-glutamine amide group. The 2-IG intermediate is reduced by the reduced flavin cofactor (Flavin 2 of GltS) located at this site. Site 2 thus catalyses a glutamate dehydrogenase-like reaction with Flavin 2 formally substituting for NAD(P)H as the hydride donor to the C(2) position of the imino acid. As mentioned above, reducing equivalents are postulated to derive from the intramolecular electron transfer chain formed by the enzyme Fe/S clusters, which mediate electron transfer from Flavin 1 to Flavin 2. Thus, both flavins of GltS act as switches between 1- and 2-electron donor/acceptor couples, a typical function of this cofactor [35]. However, the need of an intramolecular electron transfer chain formed by iron-sulfur clusters, with a different flavin cofactor at each end, in order to catalyse the simple NADPH-dependent reduction of an iminoacid was not evident until recently, as discussed below.

3) GAT site: the glutamine amidotransferase site. Steady-state kinetic analyses of the GltS reaction demonstrated that glutamine binding and hydrolysis to release L-Glu and ammonia takes place at a distinct site, following 2-OG binding to the reduced enzyme at Site 2 in the second segment of the reaction (schemes 1 and 2). Formally, a glutaminase reaction must take place at this site. Glutamine hydrolysis is followed by ammonia addition to 2-OG to yield the postulated 2-IG intermediate.

All data available so far on the *A. brasilense* GltS (Ab-GltS) are consistent with the above mechanism. However, for this enzyme as well as for the other GltS, the information on the kinetics and thermodynamics of the reaction sequence is far from complete.

The existence of distinct sites for NADPH oxidation and 2-IG reduction was confirmed both by studying the reaction of Ab-GltS with NADPH in the presence of synthetic electron acceptors [36] and by studying the reactivity of GltS with sulfite [37]. The reaction of GltS with NADPH and compounds such as iodonitrotetrazolium (INT), ferricyanide (FeCN), dichlorophenolindophenol (DCIP) and menadione was studied in the absence and presence of analogs of the enzyme physiological substrates (2′-phosphoadenosine 5′-diphosphoribose, ADPRP, L-methionine sulfone, and oxalylglycine as NADPH, L-Gln and 2-OG analogs, respectively). The inhibition pattern observed with the different synthetic acceptors and the GltS substrate analogs was consistent with INT and FeCN reacting at Site 1 and DCIP and menadione at Site 2 of GltS [36]. Furthermore, the calculated turnover number of GltS with NADPH and INT, FeCN or DCIP was two- to three-fold greater than that calculated with the physiological

substrates (57 s^{-1}). These results were consistent with the two-site model proposed for GltS (scheme 2) and indicated that steps following NADPH oxidation and intramolecular electron transfer from Flavin 1 to Flavin 2 may be limiting the overall turnover of GltS with L-Gln and 2-OG.

The study of the reactivity of GltS with sulfite during a series of equilibrium titrations of the enzyme not only confirmed the two-site model for the GltS active center but also provided a tool to distinguish between Flavin 1, the flavin at the NADPH site, and Flavin 2, the flavin at the 2-OG/2-IG site [37]. Sulfite was found to react with only one of the GltS flavins, leading to spectral changes consistent with the formation of a flavin N(5)-sulfite addition product. The presence of sulfite did not alter the reactivity of GltS with NADPH, and among the other enzyme substrates only 2-OG was able to displace sulfite from the enzyme flavin in a competitive fashion.

NADPH oxidation at Site 1 of GltS

Early work on *E. coli* and *K. aerogenes* GltS [38], later confirmed with the *A. brasilense* enzyme [18], demonstrated that the enzyme is specific for NADPH and specifically removes the 4 proS hydrogen of NADPH. Using [4S-^{3}H]-NADPH, it was shown that tritium is released into the solvent rather than being incorporated into the L-Glu product, in full agreement with the mechanistic scheme presented above (schemes 1 and 2). The strict specificity for NADPH is a property common to all GltS except for the *B. subtilis* enzyme [34], which was reported to exhibit activity with NADH at a rate of 20% of that measured under similar conditions with NADPH.

NADPH oxidation at Site 1 of GltS appears to be reversible based on the observation that i) during prolonged incubation of *K. aerogenes* [38] and Ab-GltS [39] with [4S-^{3}H]-NADPH the amount of tritium released into the solvent exceeded the (small) amount of NADPH being oxidised, and ii) anaerobic addition of NADPH to solutions of GltS from various sources led to only partial loss of absorbance in the visible region of the spectrum [18], whereas full reduction of both flavins of Ab-GltS was obtained by removing $NADP^+$ with an NADPH-regenerating system formed by glucose 6-phosphate and glucose 6-phosphate dehydrogenase [37].

The rate of reaction between Ab-GltS and NADPH was measured at 25 °C directly in a stopped-flow apparatus [40]. The absorbance changes that led to (partial) enzyme reduction on addition of NADPH took place at a rate ($\cong 950$ s^{-1}) much greater than the rate of overall turnover of GltS with its physiological substrates (57 s^{-1}) or synthetic electron acceptors (100–200 s^{-1}). These results confirmed that enzyme reduction by NADPH does not limit the rate of overall turnover of GltS [36].

Glutamate synthesis at Site 2 of GltS

According to the two-site model of GltS, Site 2 catalyses a GDH-like reaction using reduced Flavin 2 as a substitute for the reduced pyridine nucleotide and ammonia from L-glutamine. As predicted by the model shown in scheme 2, addition of L-Gln and 2-OG to photochemically fully reduced GltS led to recovery of the spectrum of the starting (oxidised) GltS solution [18]. Anaerobic addition of excess L-Glu indeed led to absorbance changes consistent with reduction of approximately one of the enzyme flavins (unpublished observation), suggesting the irreversibility of electron transfer between GltS catalytic sites.

The ability of GltS to catalyse glutamate synthesis from 2-OG and free ammonia has been tested with various bacterial GltS and different results as reviewed in [3]. With *E. coli*, *K. aerogenes* and *B. subtilis* enzymes, GltS was reported to catalyse the so-called ammonia-dependent reaction at a low rate, without the involvement of GltS redox cofactors, but with direct hydride transfer from NADPH to the postulated imino acid intermediate. With *A. brasilense* GltS no ammonia-dependent glutamate synthesis could be detected at neutral pH [39], whereas at high pH values it was found to proceed via the same ping-pong mechanism demonstrated for the glutamine-dependent reaction, and with the participation of the enzyme redox centers [18]. Although the rate of the ammonia-dependent reaction at its pH optimum of 9.3 was similar to that of the glutamine-dependent reaction at the same pH value, the K_m value for ammonia + ammonium ion was as high as 0.5 M. Thus, the ammonia-dependent reaction of glutamate synthase appears to have no physiological role. Rather, it likely represents a partial reaction of GltS overall turnover.

The iron-sulfur clusters of GltS

In the two-site model proposed for the GltS reaction (scheme 2), communication between Site 1 and Site 2 is established by an intramolecular electron transfer chain formed by some or all of the iron-sulfur centers of the bacterial enzyme. The number and type of Fe/S clusters present in NADPH-GltS has been elucidated with studies on the absorbance, circular dichroism and electron paramagnetic resonance (EPR) properties of 'as isolated' and reduced forms of Ab-GltS [37], which confirmed and extended previous studies with the *E. coli* [41] and *B. subtilis* [34] enzymes. Ab-GltS preparations exhibited an EPR signal consistent with the presence of one $[3Fe\text{-}4S]^{+1}$ cluster per $\alpha\beta$ protomer. Anaerobic addition of NADPH (33-fold excess) to Ab-GltS led to

reduction of one enzyme flavin and stabilisation of some neutral flavin semiquinone (0.35 spin/$\alpha\beta$ protomer), reduction of the $[3Fe\text{-}4S]^{+1}$ center to the EPR silent 0 state and partial reduction of a $[4Fe\text{-}4S]^{+1,+2}$ cluster (0.25 spin/$\alpha\beta$ protomer). Regeneration of NADPH with glucose 6-phosphate and glucose 6-phosphate dehydrogenase led to full reduction of the enzyme flavins as judged by absorbance spectroscopy and to reduction of the $[3Fe\text{-}4S]^{0,+1}$ cluster and of one $[4Fe\text{-}4S]^{+1,+2}$ center, as judged by EPR spectroscopy. Photochemical reduction of GltS in the presence of EDTA and deazaflavin led to absorbance changes similar to those obtained with the NADPH-regenerating system, but EPR spectroscopy measurements revealed that photochemically reduced GltS exhibited a signal consistent with two 4Fe-4S clusters being reduced. Thus, it can be proposed that bacterial GltS contains one $[3Fe\text{-}4S]^{0,+1}$ cluster (Center I or Fe/S_I) and one $[4Fe\text{-}4S]^{+1,+2}$ center (Center II or Fe/S_{II}) that most likely participate in the intramolecular electron transfer process from Flavin 1 to Flavin 2. A second $[4Fe\text{-}4S]^{+1,+2}$ center (Center III or Fe/S_{III}) is also present, but exhibits a potential too low for it to be likely involved in the intramolecular electron transfer chain connecting Flavin 1 to Flavin 2. In a growing number of proteins iron-sulfur clusters are being demonstrated to play roles in substrate binding and activation for catalysis or structure stabilisation rather than in electron transfer processes [42, 43]. The involvement of the low potential 4Fe-4S cluster of GltS in catalysis of amide transfer from glutamine to 2-oxoglutarate should be ruled out based on the fact that spinach Fd-GltS was shown to contain only one type of Fe/S cluster, one $[3Fe\text{-}4S]^{0,+1}$ center [44]. The studies on spinach Fd-GltS also showed that the cluster could not be converted into a 4Fe-4S center on treatment with excess iron and sulfide under conditions that would lead to reconstitution of 4Fe-4S centers in other proteins. The question of a structural role of Center III of NADPH-GltS still needs to be addressed. However, at this stage it cannot be ruled out that this center also participates in the intramolecular electron transfer process.

GAT site: GltS as a Ntn-amidotransferase

A property common to several L-glutamine-dependent amidotransferases is the presence of a detectable glutaminase activity [4, 6]. GltS preparations from *K. aerogenes* and *E. coli* exhibited glutaminase activity [45, 46] at a rate as high as 10% that of the physiological reaction. In contrast, no glutaminase activity was detected when Ab-GltS-catalysed release of ammonia from glutamine was determined by incubating the enzyme with L-(^{15}N-amino)-glutamine in the presence or absence of various combinations of substrates and/or analogs, and by monitoring the reaction by ^{15}N-nuclear magnetic resonance (NMR) spectroscopy [39]. These experiments also showed that no ^{15}N was incorporated into the L-Glu product on incubation of GltS with L-Gln, 2-OG, NADPH and excess ^{15}N-enriched ammonium. Thus, it was concluded that Ab-GltS has no detectable glutaminase activity at neutral pH values, and that the ammonia molecule released from glutamine during catalysis is either tightly bound to an enzyme site, which is shielded from solvent, or transferred directly to the 2-OG acceptor molecule.

The questions of the mechanism of ammonia release from glutamine and of a stepwise versus concerted ammonia transfer from glutamine to the acceptor molecule are common to all enzymes of the glutamine-amidotransferase class [4, 6].

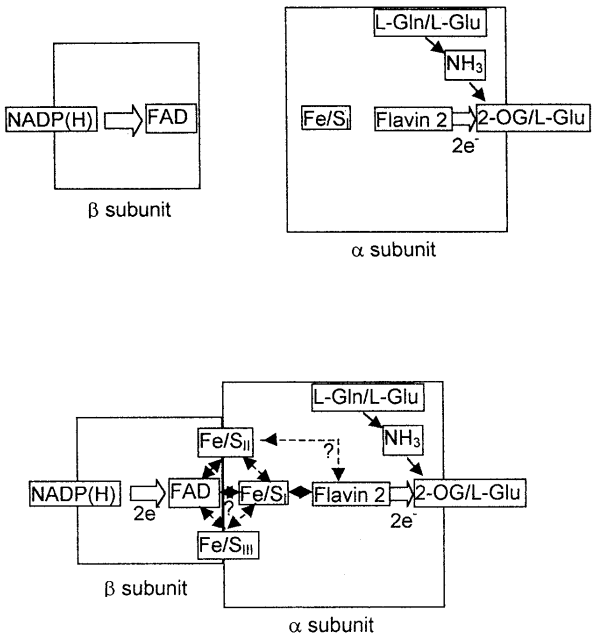
Work on GltS from *E. coli* and *K. aerogenes* demonstrated that the general mechanism of glutamine hydrolysis employed by well-characterised L-glutamine-dependent amidotransferases should apply to GltS [45–47]: release of ammonia from glutamine takes place on nucleophilic attack of the thiolate of a catalytically essential cysteine residue on the C(5) carbon of L-glutamine. Following the formation of a first tetrahedral intermediate, ammonia would be released, leaving an enzyme-glutamyl thioester. In the second part of the reaction hydrolysis of the thioester occurs with the transient formation of a second tetrahedral intermediate. The presence of the catalytically essential Cys residue at the N-terminus of the bacterial α subunit suggested that GltS was part of the PurF-type class of amidotransferases whose prototype is the bacterial phosphoribosyl pyrophosphate amidotransferase (PRPP-AT). This conclusion was confirmed by sequence analyses that led to the identification of a PurF-type amidotransferase domain at the N-terminus of GltS α subunit, conserved in all other GltS [48–50].

The recent analysis of the three-dimensional structures of several amidotransferases provided insights on the mechanism of ammonia transfer from glutamine to 2-OG in GltS.

According to the new nomenclature proposed [6], amidotransferases belong to one of two classes: class I, previously defined as the TrpG-class, and class II, corresponding to the PurF-class. The amidotransferases of the two classes use a different strategy for the activation of the cysteine thiol involved in ammonia release from glutamine, but a common mechanism to effect efficient coupling of ammonia transfer from glutamine to the nitrogen acceptor molecule. Class I amidotransferases use a Cys-His-Asp triad, part of the glutamine domain to catalyse the glutaminase segment of the reaction. Stabilisation of the tetrahedral intermediates is carried out by a suitably positioned oxyanion hole. PRPP-AT, encoded by the *pur*F gene, is the structural prototype of

class II amidotransferases, the class to which GltS glutamine domain seems to belong. This class of amidotransferases is now defined also as the Ntn-amidotransferase class [6], where Ntn stands for N-terminal nucleophile, in that enzymes such as PRPP-AT, penicillin acylase and the proteasome [51] exhibit a common structural fold and a common mechanism in which the N-terminal residue acts as the nucleophile in the reaction [Cys1 in amidotransferases of this class] and is assisted in acid-base catalysis directly by its free amino group. Again, the tetrahedral intermediate common to enzymes of this novel class of enzymes is stabilised thanks to an oxyanion hole.

Class I and class II amidotransferases instead use a common mechanism for transfer of ammonia from glutamine to the acceptor molecule. The binding sites of glutamine and of the acceptor are located in distinct domains or even different subunits of the amidotransferases. Moreover, the sites are from 20 (for PRPP-AT) to 96 Å away (for carbamyl phosphate synthetase), but they are connected by a solvent-shielded tunnel whose function is to funnel ammonia released from glutamine into the acceptor binding site [6]. Thus, it appears that in amidotransferases the mechanism of ammonia transfer is stepwise, and that coupling of the glutaminase reaction with the synthase reaction is made efficient via the intramolecular tunnel connecting the two sites.



Scheme 3. Distribution of substrates and cofactor binding sites and of catalytic activities on the α and β subunits of bacterial GltS and on the holoenzyme.

Localisation of Site 1 and Site 2 within bacterial NADPH-GltS subunits and identification of Flavin 1 and Flavin 2

The cloning of the genes encoding *A. brasilense* GltS opened the way to a number of studies concerning the biochemical properties of the enzyme [50]. While sequence analysis of GltS will be discussed below, we will here summarise the information on the GltS structure-function relationship that has been gained by studying the recombinant α and β subunits of Ab-GltS. Plasmids for the overexpression of the individual α and β subunits of Ab-GltS in *E. coli* have been constructed. The two proteins were overproduced in a soluble form in *E. coli*, purified to homogeneity and their characterisation was undertaken [40, 52].

Properties of the Ab-GltS β subunit

The GltS β subunit was found to exhibit no glutamate synthase or glutamate dehydrogenase activity, but to contain an NADPH binding site, and to catalyse its oxidation in the presence of synthetic electron acceptors such as INT, FeCN and DCIP [40]. The K_m for NADPH was in the μM range, as found with GltS holoenzyme. Furthermore, the kinetic mechanism was ping-pong, consistent with the presence of a reduced enzyme intermediate species. Accordingly, it was also found to contain stoichiometric amounts of FAD, which exhibited the properties of Flavin 1 of GltS: lack of reactivity with sulfite but reduction on anaerobic addition of NADPH. No iron-sulfur centers were associated with GltS β subunit. During reaction with NADPH, as measured under anaerobiosis conditions at 25 °C in a stopped-flow apparatus, FAD reduction took place at a rate ($\cong 900\ s^{-1}$) 10–20-fold higher than the rate of GltS turnover with its physiological substrates or synthetic electron acceptors ($57\ s^{-1}$ and $100–200\ s^{-1}$, respectively). Thus, it could be concluded that the functional Site 1 of GltS is within its β subunit. As a consequence it could be proposed that the protein acts as an FAD-dependent NADPH oxidoreductase, which serves to input electrons into the α subunit for reductive glutamate synthesis (scheme 3).

Properties of the Ab-GltS α subunit

As expected from the known properties of GltS holoenzyme and the novel information obtained by studying the isolated β subunit, the recombinant Ab-GltS α subunit had no glutamate synthase or glutamate dehydrogenase activities in the presence of NADPH as the electron donor [52]. However, it was found to catalyse

L-glutamate synthesis from 2-OG and L-Gln in the presence of a reducing system formed by dithionite and methylviologen. The isolated α subunit could also oxidise L-Glu at high pH values by transferring electron equivalents to INT. NADPH and L-Gln analogs had no effect on this activity, while 2-OG acted as an inhibitor of the reaction, competitive with respect to L-Glu. The kinetic parameters of the reaction were similar to those measured with Ab-GltS under similar conditions. The α subunit was also found to contain only one type of flavin, FMN, in stoichiometric amounts, and one [3Fe-4S]$^{0,+1}$ cluster. FMN exhibited the properties of Flavin 2 of GltS: reactivity with sulfite and competitive displacement of sulfite from the flavin by 2-oxoglutarate, reduction on anaerobic addition of excess L-Glu, oxidation on addition of L-Gln (or ammonia) + 2-OG to the dithionite-reduced protein, but not with each one of these compounds alone. Thus, the α subunit of Ab-GltS appeared to contain Site 2 and the amidotransferase site of GltS, and FMN could be identified with Flavin 2 of GltS (scheme 3).

Structural information on glutamate synthase

At the time of writing no three-dimensional structure of GltS is available. Therefore, information on the structure-function relationship of this enzyme is confined to that derived from the primary structure of enzyme subunits and complementary experiments. Only a few GltS have been purified to homogeneity and characterised. In contrast, the genes encoding GltS have been cloned and sequenced from a growing number of organisms, as shown by searches in protein and nucleic acid sequence data banks. In bacteria, the genes encoding the GltS α and β subunits were initially cloned and sequenced from *E. coli* chromosome [53], and later from *A. brasilense* [50]. The genes coding for the bacterial α and β subunits were named *glt*B and *glt*D, respectively, with the exception of those of *B. subtilis*, in which *glt*A corresponds to the structural gene for the α subunit and *glt*B encodes the β subunit [28]. We will use the prevailing definition of *glt*B and *glt*D to indicate the genes coding for the bacterial GltS α and β subunits, respectively. In all bacteria, *glt*D follows *glt*B with the exception of *A. brasilense*, where the gene order is reversed. The gene encoding Fd-GltS was cloned from maize [49] and later from cyanobacteria and various plants [8]. In the latter organisms it is nuclear-encoded, with the exception of red algae where it is found in the chloroplast genome. The Fd-GltS gene was given different names (*glt*F, *gls*F or *glt*S), a fact that generates confusion: probably, the denomination *gls*F is a reasonable compromise, which takes into account the ferredoxin-dependence of the gene product, and avoids confusion with the *glt*F gene found in the *glt* operon of enteric bacteria [24, 15]. Finally, genes encoding the eukaryotic NADH-GltS have been cloned from *M. sativa* [54] and *S. cerevisiae* [12]. Open reading frames encoding proteins similar to NADH-GltS were found during the sequencing of the genome of *Caenorhabditis elegans* and *Plasmodium falciparum*. The genes have been defined in various ways, and we will use *gls*N for consistency with the nomenclature adopted for the Fd-GltS gene and to indicate the NADH dependence of this form of glutamate synthase.

The primary structure of GltS deduced from the nucleotide sequence of the corresponding genes has been confirmed in several instances by N-terminal sequencing of the protein purified from the natural source, and, in

Figure 1. Sequence analysis of the NADPH-GltS α subunit, Fd-GltS and N-terminal part of NADH-GltS. The sequence of the *A. brasilense* GltS α subunit has been annotated to summarize the results of comparisons of the 22 complete sequences analyzed (see legend of fig. 2). In bold are the residues conserved in all 22 sequences. Residues numbering is that of the mature polypeptide starting with Cys1. -36 through -1, propeptide residues. GAT domain (blue): *Ntn*, N-terminal nucleophile. 26, 27: positions R31 and G32 of GltS that correspond to R26 and G27 of PRPP-AT, where they serve to position the Cys1 side chain and to increase the basicity of the free N-terminal nucleophile; L-Gln loop, region corresponding to the R73 through S79 segment of PRPP-AT, which forms part of the glutamine binding site; 87, P224 of GltS corresponds to P87 of PRPP-AT that is in the *cis* configuration and participates in the formation of the glutamine binding site; oxyanion hole, N231 and G232 of GltS are the counterparts of N101 and G102 of PRPP-AT, whose backbone forms the oxyanion hole in the glutamine site; 127, S272, D273 and S274 correspond to S126, D127 and S128 of PRPP-AT, which are also part of the glutamine binding site [6]. GltB insert, $\cong$ 120-residue insertion in all GltSs needed to optimise the alignment with the glutamine domain of PRPP-AT. End GAT, end of region of similarity between GltS α subunit and PRPP-AT. FMN, region involved in FMN binding (green); r, residues corresponding to those that in the enzymes of the flavocytochrome b2 class interact with the FMN ribityl moiety. Fe/S-I, region containing the Cys cluster proposed to participate in the formation of the 3Fe-4S cluster of GltS (purple); Gly, Gly-rich region matching the consensus sequence for the formation of an ADP-binding fold (red, [58, 59]). The stars indicate the positions that match the consensus sequence. The inserts present in *P. falciparum* NADH-GltS are indicated as **a** (125-residue insert), **b** (55-residue insert), **c** (99-residue insert), **d** (203-residue insert). At position **b**, also *M. sativa* and *C. elegans* NADH-GltS exhibit inserts of 20 and 55 residues, respectively. ***A*** indicates the 16-residue insert present in all Fd-GltS. The C-terminal HLH(or K)GL sequence is conserved in all Fd-GltS sequences examined. CR1 through CR8 are regions conserved in GltS resulting from the multiple alignment of the sequences of GltS α subunits, Fd-GltS and NADH-GltS listed in the legend of figure 2. The arrows indicate the sites of tryptic (Tr) and chymotryptic (Ct) cleavage of the Ab-GltS α subunit identified during limited proteolysis experiments using GltS holoenzyme [36].

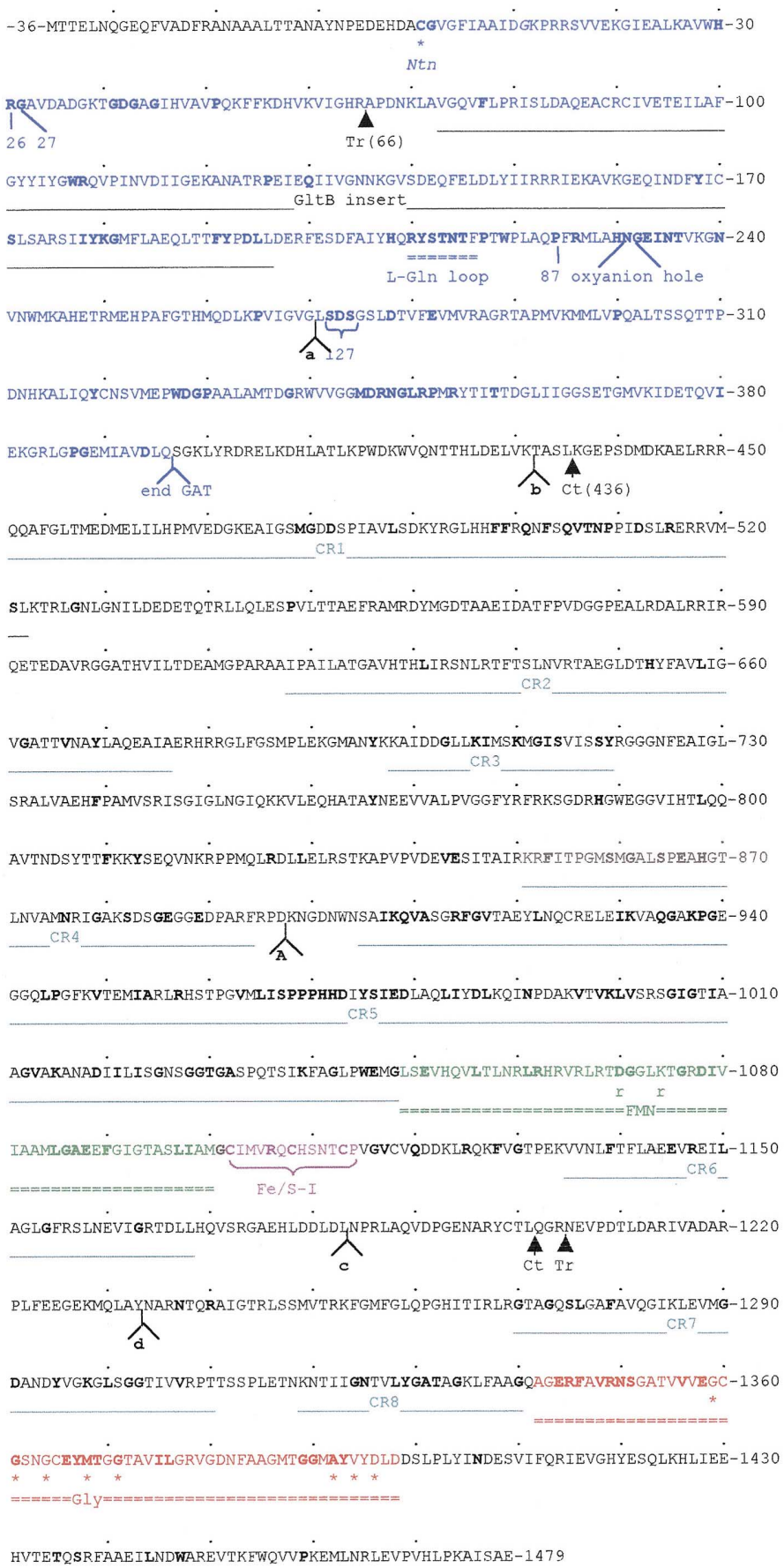
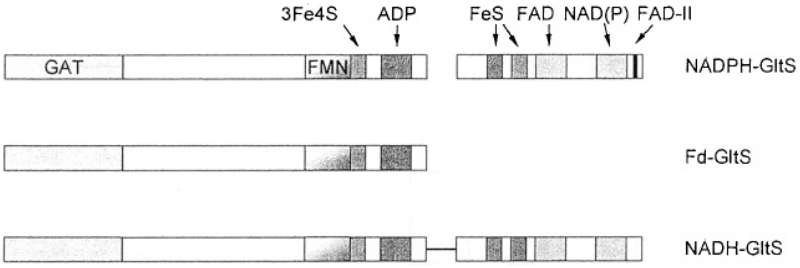

-36-MTTELNQGEQFVADFRANAAALTTANAYNPEDEHDACGVGFIAAIDGKPRRSVVEKGIEALKAVWH-30
Ntn
RGAVDADGKTGDGAGIHVAVPQKFFKDHVKVIGHRAPDNKLAVGQVFLPRISLDAQEACRCIVETEILAF-100
26 27
Tr(66)
GYYIYGWRQVPINVDIIGEKANATRPEIEQIIVGNNKGVSDEQFELDLYIIRRRIEKAVKGEQINDFYIC-170
GltB insert
SLSARSIIYKGMFLAEQLTTFYPDLLDERFESDFAIYHQRYSTNTFPTWPLAQPFRMLAHNGEINTVKGN-240
L-Gln loop
87 oxyanion hole
VNWMKAHETRMEHPAFGTHMQDLKPVIGVGLSDSGSLDTVFEVMVRAGRTAPMVKMMLVPQALTSSQTTP-310
a
127
DNHKALIQYCNSVMEPWDGPAALAMTDGRWVVGGMDRNGLRPMRYTITTDGLIIGGSETGMVKIDETQVI-380
EKGRLGPGEMIAVDLQSGKLYRDRELKDHLATLKPWDKWVQNTTHLDELVKTASLKGEPSDMDKAELRRR-450
end GAT
b
Ct(436)
QQAFGLTMEDMELILHPMVEDGKEAIGSMGDDSPIAVLSDKYRGLHHFFRQNFSQVTNPPIDSLRERRVM-520
CR1
SLKTRLGNLGNILDEDETQTRLLQLESPVLTTAEFRAMRDYMGDTAAEIDATFPVDGGPEALRDALRRIR-590
QETEDAVRGGATHVILTDEAMGPARAAIPAILATGAVHTHLIRSNLRTFTSLNVRTAEGLDTHYFAVLIG-660
CR2
VGATTVNAYLAQEAIAERHRRGLFGSMPLEKGMANYKKAIDDGLLKIMSKMGISVISSYRGGGNFEAIGL-730
CR3
SRALVAEHFPAMVSRISGIGLNGIQKKVLEQHATAYNEEVVALPVGGFYRFRKSGDRHGWEGGVIHTLQQ-800
AVTNDSYTTFKKYSEQVNKRPPMQLRDLLELRSTKAPVPVDEVESITAIRKRFITPGMSMGALSPEAHGT-870
LNVAMNRIGAKSDSGEGGEDPARFRPDKNGDNWNSAIKQVASGRFGVTAEYLNQCRELEIKVAQGAKPGE-940
CR4
A
GGQLPGFKVTEMIARLRHSTPGVMLISPPPHHDIYSIEDLAQLIYDLKQINPDAKVTVKLVSRSGIGTIA-1010
CR5
AGVAKANADIILISGNSGGTGASPQTSIKFAGLPWEMGLSEVHQVLTLNRLRHRVRLRTDGGLKTGRDIV-1080
r r
FMN
IAAMLGAEEFGIGTASLIAMGCIMVRQCHSNTCPVGVCVQDDKLRQKFVGTPEKVVNLFTFLAEEVREIL-1150
CR6
Fe/S-I
AGLGFRSLNEVIGRTDLLHQVSRGAEHLDDLDLNPRLAQVDPGENARYCTLQGRNEVPDTLDARIVADAR-1220
c
Ct Tr
PLFEEGEKMQLAYNARNTQRAIGTRLSSMVTRKFGMFGLQPGHITIRLRGTAGQSLGAFAVQGIKLEVMG-1290
CR7
d
DANDYVGKGLSGGTIVVRPTTSSPLETNKNTIIGNTVLYGATAGKLFAAGQAGERFAVRNSGATVVVEGC-1360
CR8
GSNGCEYMTGGTAVILGRVGDNFAAGMTGGMAYVYDLDDSLPLYINDESVIFQRIEVGHYESQLKHLIEE-1430
Gly
HVTETQSRFAAEILNDWAREVTKFWQVVPKEMLNRLEVPVHLPKAISAE-1479

Fig. 1.



Scheme 4. Proposed localisation of functional domains in glutamate synthases. GAT, Class II (PurF-type) amidotransferase domain; FMN, FMN binding region; 3Fe4S, Cys-rich region for the formation of the 3Fe-4S center of GltS; ADP, ADP-binding fold; FeS, Cys-rich regions potentially implicated in the formation of the 4Fe-4S centers of GltS; FAD, ADP-binding fold for FAD binding; NAD(P)H, ADP-binding fold for NAD(P)H binding; FAD-II, second FAD consensus sequence [60].

fewer cases, by sequencing several peptides obtained by proteolytic or chemical cleavage of the enzyme.

The bacterial α subunit is similar to the single polypeptide chain of Fd-GltS and to the N-terminal three quarters of NADH-GltS. The bacterial β subunits are similar to each other and to the C-terminal one quarter of NADH-GltS. The parts of the NADH-GltS corresponding to the bacterial α and β subunits are linked by a short polypeptide chain, which finds no counterpart in bacterial NADPH-GltS or Fd-GltS (scheme 4). In addition, *P. falciparum* GltS contains several inserts, which are located outside the regions conserved in all GltS with the exception of the first 125-residues one (figs 1 and 2). Mature GltS α subunits, Fd-GltS and NADH-GltS exhibit a conserved N-terminal Cys residue located 7–130 residues far from the predicted translation start site. Removal of the initial methionine seems to be the only maturation event for the β subunit.

Several regions are conserved in all GltS. Comparison of such conserved regions of GltS with the sequences available through databases, and in particular with those of well-characterised enzymes that use the same substrates and cofactors as GltS, allowed the identification of potential functional regions within the enzyme subunits [48–50, 55, 56].

Sequence analysis of the NADPH-GltS α subunit, the Fd-GltS and the N-terminal part of NADH-GltS

Glutamine amidotransferase domain. The N-terminal region spanning residues 1–390 of the Ab-GltS mature α subunit is similar to the class II (Pur-F-type) glutamine amidotransferase domain, now defined as the Ntn-amidotransferase domain, provided a 120–residue insertion in GltS is allowed for in the alignment (fig. 1 [6, 57]). Such an insert may replace the region of *E. coli* PRPP-AT between residues 40 and 60, a region that appears to be highly variable in different class II amidotransferases [6]. Several residues that in the *E. coli* PRPP-AT form the glutamine binding site appear conserved in GltS, as are the residues forming the oxyanion hole and the *cis*-proline conserved structural element, as detailed in figure 1 [6].

A feature of Ntn hydrolases is the proposed autocatalytic excision of a propeptide as part of the covalent posttranslational activation process that exposes the catalytic N-terminal nucleophile [51]. A propeptide has been found in some but not all class II amidotransferases [6]. The analysis of the sequence of the genes encoding GltS revealed that all GltS α subunits are synthesised as proproteins (fig. 2). Nuclear-encoded Fd-GltS actually have a long propeptide thought to be composed of a chloroplast-targeting peptide and the specific GltS propeptide that is removed on enzyme activation to expose Cys1. The amino acid sequence surrounding Cys1 of all types of GltS is highly conserved (fig. 2), and it is attractive to propose that the conserved residues determine correct protein maturation. The conserved sequence around Cys1 of GltS is not similar to that of class II amidotransferases, nor to that of other Ntn-hydrolases, which appear unrelated to each other. The fact that the Ab-GltS α subunit is correctly processed when the protein is expressed in *E. coli* cannot be taken as an argument in favor of or against autocatalytic processing. Rather, it indicates that the conserved region around Cys1 may be sufficient to determine correct processing, that is: the *E. coli* maturation enzyme able to process the *E. coli* GltS can recognise the corresponding (and similar) part of the *A. brasilense* enzyme. In any case, the fact that both the isolated α subunit and the α subunit coexpressed with the β to yield the $\alpha\beta$ Ab-GltS holoenzyme are correctly processed indicates that association of the two subunits in the protomer is not required for propeptide cleavage.

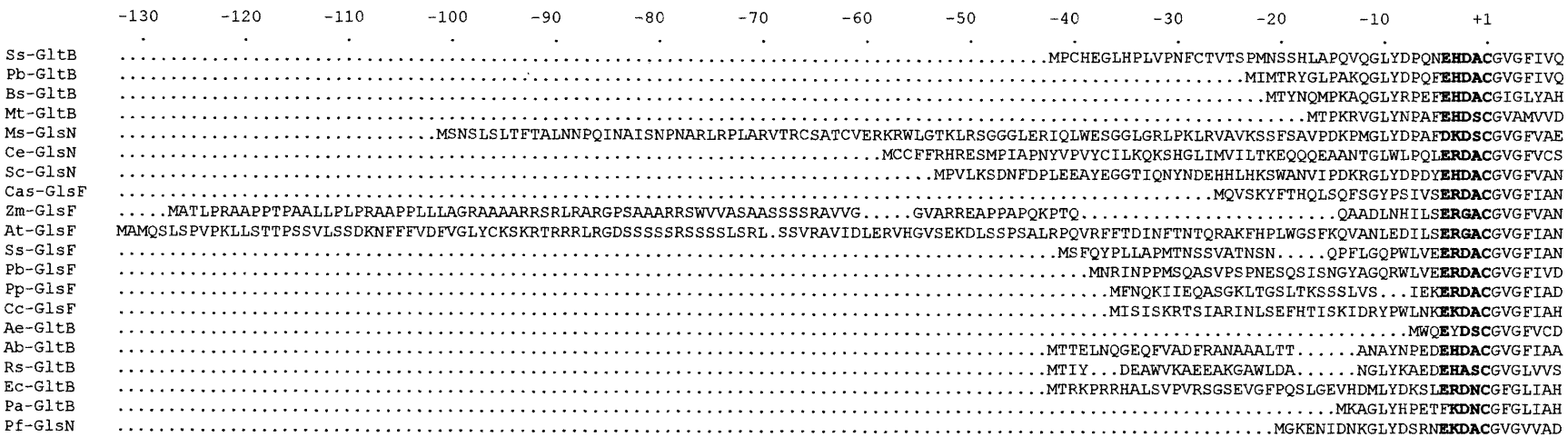


Figure 2. Alignment of the propeptide region of NADPH-GltS α subunits, Fd-GltS and NADH-GltS. In bold are the conserved residues at the C-terminus of the propeptide and the N-terminal Cys residue of the mature proteins. The source and accession number used to retrieve the sequences of the GltS α subunits (GltB), Fd-GltS (GlsF) and NADH-GltS (GlsN) from GenBank/EMBL and Swiss-Prot databases were as follows: Ss-GltB, *Synechocystis* sp. PCC6803, X80485; Pb-GltB, *Plectonema boryanum*, D85230; Bs-GltB, *Bacillus subtilis*, Z99114; Mt-GltB, *Mycobacterium tuberculosis*, MTCY1A6; Ms-GlsN, *Medicago sativa*, L01660; Ce-GlsN, *Caenorhabditis elegans*, Z49889: Sc-GlsN, *Saccharomyces cerevisiae*, X89221; Cas-GlsF, *Antithamnium* sp., chloroplast-encoded *gls*F gene product, CHASGLTB; Zm-GlsF, *Zea mays*, M59190; At-GlsF, *Arabidopsis thaliana*, Y09667; Ss-GlsF, *Synechocystis* sp. PCC6803, D78371; Pb-GlsF, *Plectonema boryanum*, D85735; Pp-GlsF, *Porphyra purpurea* chloroplast-encoded *gls*F gene product, U38804; Cc-GlsF, *Cyanidium caldarium* RK1 chloroplast-encoded *gls*F gene product, AF022186; Ae-GltB, *Aquifex aeolicus glt*B gene product, AE000746; Ab-GltB, *Azospirillum brasilense*, L04300; Rs-GltB, *Rhodobacter sphaeroides*, Y12482; Ec-GltB, *Escherichia coli*, ECOGLTB; Pa-GltB, *Pseudomonas aeruginosa*, PAU81261; Pf-GlsN, *Plasmodium falciparum*, PFALGLTS.

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As-GlsF      GEDSTRFKSI QDLDTSGVSR TFSHLKGLKI NDLASSAIKQ IASGRFGVTP
CAs-GlsF     GEDSTRFKSI QDLDTSGVSR TFSHLKGLKI NDLASSAIKQ IASGRFGVTP
Zm-GlsF      GEDPIRWNPL TDV.VDGYSP TLPHLKGLQN GDTATSAIKQ VASGRFGVTP
At-GlsF      GEDPIRWKPL TDV.VDGYSP TLPHLKGLQN GDIATSAIKQ VASGRFGVTP
Ss-GlsF      GEDVVRYLTL DDVDSEGNSP TLPHLHGLQN GDTANSAIKQ IASGRFGVTP
Ss-GltB      PEDVVRYLTL DDVDSEGNSP TLPHLHGLQN GDTANSAIKQ IASGRFGVTP
Pb-GlsF      GEDPVRYKIL NEVE.NGTSP LLPHLKGLQT GDTANSAIKQ VASGRFGVTP
Pp-GlsF      GEDPVRFKVL NDVNESGNSD LLPHLKGLRN GDTASSAIKQ IASGRFGVTP
Cc-GlsF      GEDSLRFTVL TDVDETGNSP SFPHLKGLKN GDSLSSAIKQ IASGRFGVTP
Ab-GltB 888-GEDPARF.RP DK ....... .........N GDNWNSAIKQ VASGRFGVTA-919
             **  *:      :   * *     **:**    *   ***** :********
```

Figure 3. Conserved 17-residue insert unique to Fd-GltS. The sequences of the conserved region unique to Fd-GltS (in bold) are shown in comparison to the sequence of the corresponding region of Ab-GltB. Sequences are identified as listed in the legend of figure 2. Stars indicate identical residues in all GlsF sequences and colons indicate conserved substitutions.

FMN binding region. The conserved region between residues 1049 and 1100 of the Ab-GltS α subunit is similar to the C-terminal part of the FMN binding region of enzymes of the flavocytochrome b2 structural class, as previously reported [49, 50]. The postulated FMN binding region follows two regions (marked as CR5 and CR6 in fig. 1) that are conserved in all GltS but do not exhibit evident similarity with regions of other proteins whose sequences are available through databases.

3Fe-4S cluster ligands. The proposed FMN binding region leads into the only part of the α subunit polypeptide that contains a cluster of three conserved cysteine residues. These cysteine residues (1102, 1108 and 1113 of the Ab-GltS α subunit) are the only conserved cysteine residues in all GltS beside Cys1 and Cys1365. The latter is however a Phe in *S. cerevisiae* GltS. Therefore, Cys1102, 1108 and 1113 are likely candidates for the formation of the 3Fe-4S cluster of GltS, which is the only Fe/S cluster of Fd-GltS and is most likely conserved also in NADH-GltS. This hypothesis was supported by the finding of a similarity between the spacing of these GltS Cys residues with that of the residues that are involved in the formation of the 3Fe-4S center of fumarate reductase and succinate dehydrogenase, as discussed in [50].

C-terminal Gly-rich region. The C-terminal region of GltS α subunit from residue 1220 through $\cong$1400 is conserved, and is flanked by poorly conserved stretches of amino acids. Such a conserved C-terminal domain contains only one region with features similar to those of well-characterised enzymes: residues 1353–1396 match well the consensus sequence for the formation of an adenosine diphosphate (ADP)-binding fold in FAD- and NAD(P)H-dependent proteins [58, 59]. At the time when the experimental evidence was in favor of the location of both FAD and FMN in the bacterial α subunit and in Fd-GltS, this sequence was proposed to serve for FAD binding [50]. Recently, the flavin content of *Synochococcus* [9] and spinach Fd-GltS [10] and of the recombinant GltS α subunit overproduced in *E. coli* [52] was established to be one FMN per polypeptide. Thus, the function of the C-terminal potential ADP binding region of GltS remains to be established. A possibility that needs to be tested is the location within this part of GltS of the binding site of a nucleotide that may serve for allosteric modulation of the properties of GltS. However, early studies on bacterial GltS, which have been confirmed recently with the recombinant Ab-GltS overproduced in *E. coli* (H. Stabile, unpublished), did not show any effect on enzyme activity by adenylate-containing nucleotides. Indeed, experiments should be carried out to establish the effect of other nucleotides on GltS activity or on other properties of the enzyme (e.g. aggregation state, sensitivity to proteases). Furthermore, it cannot be ruled out that the enzyme is isolated with the bound but as yet unidentified effector.

Other conserved regions of the α subunit. Other regions are conserved in GltS α subunit, Fd-GltS and in the corresponding part of the NADH-GltS (fig. 1). However, no regions of proteins other than GltS were found to exhibit significant similarity to them. Thus, no information on the role these sequences might play in GltS could be obtained. Interestingly, optimal alignment of NAD(P)H-GltS and Fd-GltS was obtained, assuming in the Fd-GltS sequences a 16-residue insert at position 97 of the Ab-GltS α subunit (fig. 3). This sequence is conserved in all Fd-GltS and contains several basic residues, but the question of its function (e.g. involvement in the interaction with Fd) has not been addressed experimentally. The domain structure proposed above for GltS α subunit, Fd-GltS and NADH-GltS N-terminal three quarters was confirmed indirectly by the recent finding that the *P. falciparum* GltS contains several inserts, but they are all (except for that at position 270) located outside the proposed functional regions of GltS (fig. 1).

Evidence for the presence of the structured regions of the GltS α subunit defined above was also given by a series of limited proteolysis studies on the Ab-GltS [36]. Limited tryptic and chymotryptic proteolysis was carried out with Ab-GltS holoenzyme alone or in the presence of its substrates and their analogs in various combinations. Cleavage sites that yielded polypeptides that could be analysed by N-terminal sequencing were all clustered in regions around residues 400–500, 1200 and 1400 of the α subunit. Thus, all cleavage sites fall within poorly conserved regions of GltS, outside the proposed substrate and cofactor binding sites. Only one site (position 65–66) was within the GAT domain. Interestingly, this position is within the insert of GltS needed to optimise the alignment with the GAT domain of class II amidotransferases (fig. 1). The sensitivity to proteolytic attack of some of these sites was influenced by the presence of the enzyme ligands: NADPH, which binds to the β subunit, was sufficient to induce such changes within sites of the α subunit, suggesting that substrate-induced long-range conformational changes may take place in GltS.

Sequence analysis of the NADPH-GltS β subunit and of the C-terminal part of NADH-GltS

The β subunit of GltS and the corresponding part of the eukaryotic NADH-GltS are less conserved than the α subunits, Fd-GltS and the N-terminal part of NADH-GltS. However, they do exhibit several conserved regions, which allowed their tentative assignment to substrates and cofactors binding.

FAD and NAD(P)H binding regions. Two regions (149–177 and 291–339) match the consensus sequence for the formation of ADP-binding folds in several well-characterised pyridine nucleotide-dependent and FAD-containing oxidoreductases [58, 59]. The N-terminal potential ADP-binding fold is very conserved in all GltS β subunit sequences. It could be assigned to the binding of the adenylate portion of FAD on the basis of the sequence itself, the evidence now available that the recombinant Ab-GltS β subunit overproduced in *E. coli* cells contains one FAD cofactor, and the fact that both the recombinant Ab-GltS α subunit and the Fd-GltS contain only one type of flavin cofactor, one FMN residue. The C-terminal putative ADP-binding fold was proposed to be responsible for binding NADPH [50] on the basis of the sequence analysis [50]. Site-directed mutagenesis of the 291–339 region of the *Azospirillum* GltS β subunit overproduced in *E. coli* is being carried out in our laboratory (P. Morandi and B. Valzasina, unpublished). The data available are fully consistent with the assignment of the potential ADP-binding fold at the C-terminus of the GltS β subunit to NADPH binding, and of that at the N-terminus to interaction with FAD.

FAD- and NADPH-binding interaction with the GltS β subunit may be similar to that found in the disulfide reductases class of flavoenzymes. The overall organisation of the proteins may also be similar: an N-terminal FAD binding domain is followed by a C-terminal NAD(P)H binding region. Furthermore, the second FAD consensus sequence identified by Eggink et al. [60] is found conserved in all GltS β subunits and in NADH-GltS. The sequence (1281–1291) is found in a limited number of FAD-containing enzymes but in all those related to the disulfide reductases. This region corresponds to a short β sheet ending with a D or E residue that establishes a hydrogen bond with the O-3 group of the FAD ribityl residue.

N-terminal Cys-rich regions. A distinctive feature of the GltS β subunit is the presence of two cysteine-rich regions at the N-terminus, which are conserved in the NADH-GltS. The alignment of GltS β subunits from a variety of bacteria and of the NADH-GltS demonstrates that in the first cysteine-rich region Cys47, 50 and 55 are all conserved and located in conserved regions, whereas Cys59, although conserved, is at a distance varying from 3 to 12 residues from Cys55. In the second Cys-rich region Cys94 is a T in several GltS whereas Cys98, 101 and 108 are conserved, with a spacing of three to five residues between C98 and C101 in various GltS. All or some of these Cys residues conserved at the N-terminus of the GltS β subunit have been proposed to participate in the formation of the two 4Fe-4S clusters of GltS, and this hypothesis is being tested by specific site-directed mutagenesis experiments on Ab-GltS. The identification of the 4Fe/4S clusters ligands is complicated by the fact that (i) the spacing of the cysteine residues within the N-terminal region of the GltS β subunit is not that typical of cysteines involved in the formation of 4Fe-4S centers in well-characterised ferredoxins; (ii) the first residue of the second cysteine cluster is substituted by a T in several GltS, supporting the possibility that residues other than Cys may be the ligands to Center II and/or Center III of GltS or that (iii) the clusters may be formed at the interface between the subunits with ligands provided by residues of the α subunit. The latter possibility is supported by the study of recombinant Ab-GltS. The Ab-GltS β subunit was overproduced in *E. coli* cells, and no Fe/S centers were found to be associated with it, whereas only the 3Fe-4S center of GltS was found within the α subunit. Overproduction of the active recombinant Ab-GltS in *E. coli* cells proved to be very sensitive to the growth conditions of recombinant *E. coli* cells. We actually found a correlation between lack of activity of the purified protein and absence of the 4Fe-4S centers [56]. This finding indicated that correct folding of the two subunits together to form the holoenzyme is needed to assemble the 4Fe-4S clusters of GltS. This may be due

to the fact that the α subunit provides the correct environment for cluster formation and stabilisation and/or because residues of the α subunits are direct ligands to the clusters.

A common model for GltS from various sources

Comparison of the known structural and functional properties of GltS from various sources and of the recombinant Ab-GltS α and β subunits suggests that L-glutamine-dependent reductive glutamate synthesis takes place within the NADPH-GltS α subunit, which is similar to the single polypeptide chain of the Fd-GltS and to the N-terminal three quarters of the NADH-GltS. In photosynthetic tissues, reducing equivalents are provided by reversible association of Fd-GltS with reduced ferredoxin. In nonphotosynthetic organisms reduced pyridine nucleotides are used. Thus, an FAD-dependent NADPH oxidoreductase has been recruited as the β subunit of bacterial GltS to input electrons into the glutamate synthase protein. In bacteria the β subunit is tightly associated with the α subunit in the GltS holoenzyme. In nonphotosynthetic eukaryotes, a protein corresponding to the β subunit has been fused at the C-terminus of the polypeptide corresponding to the bacterial α subunit and the Fd-GltS to form a single polypeptide chain. Also, the pyridine nucleotide specificity seems to have been switched from NADPH in bacteria to NADH in eukaryotes.

This common scheme for GltS from various sources, which implies a modular architecture of GltS, may explain the presence of two different flavin cofactors and of at least one of the three iron-sulfur clusters of NAD(P)H-GltS. FMN and the 3Fe-4S center belong to the common glutamate synthase protein (GltS α subunit or Fd-GltS polypeptide). FAD has been recruited as part the NAD(P)H oxidoreductase required in nonphotosynthetic tissues. The need for the additional 4Fe-4S centers of GltS has not been fully understood although they may be necessary to mediate electron transfer between Site 1 on the β subunit and Site 2 within the α subunit. It is possible that they functionally substitute for the Fd iron-sulfur cluster. In this respect it may be worthwhile to mention that it has been demonstrated that two Fd molecules bind per Fd-GltS subunit [7].

Is GltS α subunit a valid model for Fd-GltS?

The similarity between the GltS α subunit and the single polypeptide chain of Fd-GltS might suggest that the two proteins are functionally very similar. However, comparison of the reported properties of Fd-GltS with those of the recombinant Ab-GltS α subunit also revealed significant differences. The GltS α subunit and Fd-GltS exhibit a similar flavin and iron-sulfur content [10, 37, 44, 52]. They appear to be similar also with respect to the redox properties of the FMN cofactor and of the 3Fe-4S center. The redox potentials of the FMN cofactor and of the 3Fe-4S center of Fd-GltS have been measured for spinach Fd-GltS. Similar midpoint potentials for the two centers were determined ($\cong -225$ mV, [10]), and no flavin semiquinone species were observed during reduction. In agreement with the similarity between the GltS α subunit and the Fd-GltS, no flavin semiquinone species was observed during dithionite titrations of the GltS α subunit [52], and preliminary redox titrations of this protein also suggest similar midpoint potentials for the 3Fe-4S center and the FMN cofactor of the isolated Ab-GltS α subunit (S. Ravasio, unpublished).

In contrast with the postulated functional similarity of the GltS α subunit and Fd-GltS, a major difference concerns the involvement of reduced FMN in 2-IG reduction. For Ab-GltS α subunit, the flavin but not the iron-sulfur center is reduced by anaerobic addition of excess L-Glu. Recovery of the spectrum of oxidised flavin was observed on addition of L-Gln (or ammonia) + 2-OG to dithionite-reduced α subunit. No spectral changes were observed when L-Gln, ammonia or 2-OG were added individually to enzyme solutions that had been reduced photochemically or with dithionite under anaerobiosis conditions [52].

Similar results would be expected for the Fd-GltS. While L-Glu reactivity of Fd-GltS has not been reported, partial to full reoxidation of Fd-GltS that had been reduced with an excess of dithionite was observed by adding 2-oxoglutarate alone [7, 9]. Thus, Fd-GltS is reported to possess a 2-oxoglutarate oxidoreductase activity, which involves the Fd-GltS flavin. Indeed, this result is surprising and should deserve further work. In particular, the identification of the reaction product would be very much needed.

GltS $\alpha\beta$ holoenzyme is more than the sum of its α and β subunits

The lack of reduction of the 3Fe-4S center of the GltS α subunit by L-Glu was initially interpreted as due to a lower redox potential of this center with respect to that of the flavin cofactor [52]. However, the lack of reoxidation of the 3Fe-4S cluster of the dithionite-reduced α subunit with L-Gln and 2-OG was unexpected: full recovery of the spectrum of the oxidised enzyme was obtained during a similar experiment with the Ab-GltS holoenzyme. Therefore, we

had to conclude that the two centers in the isolated α subunit are simply not able to exchange electrons. A second unexpected property of the isolated α subunit was the presence of a significant glutaminase activity, absent in the Ab-GltS $\alpha\beta$ holoenzyme. In the absence of additional evidence we interpreted these data as due to the fact that the GltS β subunit not only serves to input electrons from NADPH into the α subunit for glutamate synthesis, but it is also needed to determine some of the properties of the α subunit itself, namely (i) establishing electronic communication between the 3Fe-4S center and the FMN cofactor within the α subunit and (ii) coupling of ammonia release from glutamine to its addition to 2-OG and formation of the L-Glu product.

How the β subunit modulates the properties of the α subunit remains to be established. Conformational changes within the α subunit may take place on association with the β subunit within the $\alpha\beta$ protomer, thus altering or creating the solvent-shielded site or tunnel required for ammonia transfer from glutamine to 2-OG as observed for other amidotransferases [6]. Such conformational changes could also have an effect on the redox properties and relative orientation of the 3Fe-4S cluster and the FMN cofactor, which would now be able to promote the correct electron transfer for catalysis.

The available experimental evidence, however, indicates that assembly of the α and β subunits to form the $\alpha\beta$ protomer does not only involve conformational changes: the striking difference between the combined properties of the two α and β subunits of Ab-GltS and those of the actual Ab-GltS $\alpha\beta$ holoenzyme is the presence in the holoenzyme of two 4Fe-4S centers. As outlined above, one of these centers (Center II) is reduced with NADPH along with the 3Fe-4S cluster and both of the flavin cofactors, provided $NADP^+$ is scavenged by an NADPH-regenerating system. Center III is reduced, along with the flavins and the other two Fe/S centers when GltS is reduced photochemically, thus exhibiting a low potential. Therefore, the second effect of the presence of both the α and β subunits in the GltS holoenzyme is the formation of Center II and Center III of the enzyme. The formation of such centers may trigger the conformational changes required to ensure coupling of the glutaminase and glutamate synthase activities and electronic communication among GltS redox centers. Most likely, at least one of these centers (Center II) or even both directly participate with the 3Fe-4S cluster in the α subunit, in the electron transfer process between Flavin 1 and Flavin 2 of GltS. As discussed before, the location of Center II and Center III of GltS is unknown, although there are indications that the centers may be at the interface between the subunits.

Is the GltS β subunit a member of a novel family of FAD-dependent NAD(P)H oxidoreductases?

Data bank searches of proteins similar to the GltS β subunit gave additional interesting results. In this section GltD will be used as a synonym of the GltS β subunit to simplify discussion, and we will define as GltD (i) the small subunits of GltS that have been identified biochemically or (ii) the proteins whose sequences have been deduced from gene sequences, provided the putative *glt*D is adjacent to a putative *glt*B gene on the chromosome. Other proteins similar to GltD but which do not meet the criteria defined above will be indicated as GltD-like proteins. Several proteins or domains of proteins whose sequences are now present in data banks are strikingly similar to GltD. The similarities between *E. coli aeg*A gene product (Ec-AegA, [61]) and *Rhodobacter capsulatus glt*X gene product (Rc-GltX) and GltD have been reported. We recently found that the N-terminal region of dihydropyrimidine dehydrogenase (DPD), another complex Fe/S flavoprotein found in animals, aligns very well with GltD [62]. Given the enzyme primary structure and its functional properties it was concluded that this GltD-like domain most likely serves in DPD to input electrons from NADPH into the dihydrorotate-like domain for uracil reduction. The finding of GltD-like proteins or protein domains in a number of proteins clearly different from GltS such as Ec-AegA, Rc-GltX and DPD leads to the attractive hypothesis that the GltS β subunit is a member of a novel family of FAD-dependent NAD(P)H oxidoreductases. They would serve to mediate electron transfer between a reduced pyridine nucleotide and a second protein or protein domain, using FAD as the flavin cofactor and the Fe/S cluster(s) encoded by its N-terminal sequence. This would be, for example, the α subunit of GltS or the dihydroorotate dehydrogenase-like domain of dihydropyrimidine dehydrogenase [62].

Open reading frames encoding proteins similar to GltD have also been found in *Aquifex aeolicus* [63], *Pyrococcus horikhoshii* and *Pyrococcus sp.* KOD1. As shown in figure 4, the sequences of GltD and GltD-like proteins or protein domains were aligned with the Pileup program of the GCG Wisconsin package [64], which takes into account the degree of similarity among the different sequences. GltDs cluster at the top of the alignment, whereas Ec-AegA, Rc-GltX and the *A. aeolicus* and *Pyrococcus* putative GltD-like proteins align lower in the list, with the N-terminal GltD-like domain of DPD at the bottom, suggesting that the *A. aeolicus* and *Pyrococcus* putative proteins may be GltD-like FAD-containing NAD(P)H oxidoreductases unrelated to glutamate synthase.


Ss-GltD
Pb-GltD
Ms-GlsN
Bs-GltD
Sc-GlsN
Ce-GlsN
Mt-GltD
Ab-GltD
Pa-GltD
Ec-GltD
Tb-GltD
Ec-AegA
Ph-GltD
Ps-GltA
Ae-GltD
Rc-GltX
Bt-DPD
FAD

Fig. 4.


301 350
Ss-GltD NTEIGKD..L PPETLLKD.Y DAVVLCTGAT KPRDLAIAGR E..LEGIHFA
Pb-GltD NTEIGKD..L PAEDLLRD.F DAVVLCTGAT KPRDLPIEGR Q..LKGIHFA
Ms-GlsN NANIGLDPLY SLERLREE.N DAIVLAVGAT KPRDLPVPGR E..LSGVHFA
Bs-GltD NTEIGVD..I TADELKEQ.F DAVILCTGAQ KQRDLLIEGR D..SKGVHYA
Sc-GltD NTEIGKT..I SMDELKNK.H NAVVYAIGST IPRDLPIKGR E..LKNIDFA
Ce-GlsN NTEIGKH..V PADFLLKE.N DAIIVCTGST TARDLNVEGR D..AKGVCFA
Mt-GltD GVNVGVD..I SAEKLRAD.F DAVVLAGGAT AWRELPIPGR E..LEGVHQA
Ab-GltD NFEVGRD..A SLPELRRK.H VAVLVATGVY KARDIKAPGS G..LGNIVAA
Pa-GltD NTEIGKD..V TMEQLLDE.Y DAVFMGMGTY TYMKGGFPGE D..LPGVHDA
Ec-GltD NTEVGRD..V QLDDLLSD.Y DAVFLGVGTY QSMRGGLENE D..ADGVYAA
Tb-GltD DTEVGRD..I SLGKLLED.F DAVFLGMGTY TAKTGDFPGE K..LPGVLKA
Ec-AegA NCEVGKD..V SLDSLLEQ.Y DAVFVGVGTY RSMKAGLPNE D..APGVYDA
Ph-GltD DHIVGRT..V TIPELLKE.Y DAIFIGTGAG TPKLLKIPGI N..LNGIYSA
Ps-GltA DHVVGRT..V TIEELLQE.Y DAVFIGSGAG TPRLINAPGI N..LNGIYTA
Ae-GltD GYLIGKN..I TLQELREK.Y DAVFIAVGAG RGKL.GLPGD H..LKGVYSA
Rc-GltX NWKLGRN..G SVADLLAE.H DAVFLGMGLA GVNALTIPGA EHATPATEFI
Bt-DPD GKSLSVND.I TLSTLKEEGY KAAFIGIGLP EPKKDHIFQG LTQDQGFYTS
351 400
Ss-GltD MEFLTANTKA ILD.KT...P GPNFISAKDK DVVIIGGGDT GTDCVGTSLR
Pb-GltD MDFLTANTQA VLD.QT...E PA..ITAQGK DVVIIGGGDT GTDCVGTSIR
Ms-GlsN MEFLHANTKS LLDSNL...Q DGNYISAKGK KVVVIGGGDT GTDCIGTSIR
Bc-GltD MDYLTLATKS YLDSNF...K DKQFIDAKGK DVIVIGGGDT GADCVATALR
Sc-GlsN MQLLESNTKA LLNKDL...E IIR.EKIQGK KVIVVGGGDT GNDCLGTSVR
Ce-GlsN MEYLEKSQRR RAGDDV...S WEGLDPA.NK KVIILGGGDT ATDCIATSNR
Mt-GltD MEFLPWANRV QEGDDVLDED GQPPITAKGK KVVIIGGGDT GADCLGTVHR
Ab-GltD LDYLTTSNKV SLG.DTVEAY ENGSLNAAGK HVVVLGGGDT AMDCVRTAIR
Pa-GltD LDFLIANVNR NLG..F.EKS AEDFVDMKGK RVVVLGGGDT AMDCNRTSIR
Ec-GltD LPFLIANTKQ LMG..FGETR DEPFVSMEGK RVVVLGGGDT AMDCVRTSVR
Tb-GltD LSYLIANTRH LMQ..LPD.P TLPYISLSGK RVVVLGGGDT AMDCLRTAIR
Ec-AegA LPFLIANTKQ VMG..LEELP EEPFINTAGL NVVVLGGGDT AMDCVRTARR
Ph-GltD NEFL...TRI NLM.KAYKFP EYDTPIIVGK RVIVIGAGNT AMDAARSALR
Ps-GltA NEFL...TRV NLM.KAYLFP EYDTPVYVGK KVIVIGAGNT AMDAARSARR
Ae-GltD IDFL...MRV NLY.KANEFP KKGVPVELGK RTVIIGGGFT AVDCAITANR
Rc-GltX AHLRQAGDLA TV..... .....PVGR RVVVIGGGMT AVDAAVQAKL
Bt-DPD KDFLPLVAK. ..SSKAGMCA CHSPLLSIRG TVIVLGAGDT AFDCATSALR
++ + + + + + +
========== NADPH ==
401 450
Ss-GltD HGCRSLVQLE IMPKPPEERA ANNPWPEWPK VYKMDYGQEE AAAVYGHDPR
Pb-GltD HGCNGLVQLE ILPQPPEERA PNNPWPEYPK VYKMDYGQEE AAAKFGGDPR
Ms-GlsN HGCTAVVNLE LLPQPPPTRA PGNPWPQWPR IFRVDYGHQE AETKFGKDPR
Bs-GltD QKAKSVHQFG KHPKLPPART NDNMWPEQPH VFTLEYAYEE REAKFGRDPR
Sc-GlsN HGAASVLNFE LLPEPPVERA KDNPWPQWPR VMRVDYGHAE VKEHYGRDPR
Ce-GlsN LGCKTVGAFE ILPQPGPNRK PENPWPEWPL IFRVDYGHEE AKEMTGSDPR
Mt-GltD QGAIAVHQFE IMPRPPDARA ESTPWPTYPL MYRVSAAHEE GGE.....R
Ab-GltD QGATSVKCLY RRDRKNMPGS ..... QREVAHAEEE GVEFI.....
Pa-GltD QGAKSVTCAY RRDEENMPGS ..... RKEVKNAKEE GVKFL.....
Ec-GltD QGAKHVTCAY RRDEENMPGS ..... RREVKNAREE GVEFK.....
Tb-GltD QGARQVTCVY RRDEENMPGS ..... RREVANAREE GVHFL.....
Ec-AegA HGASNVTCAY RRDEANMPGS ..... KKEAKNAREE GANFE.....
Ph-GltD LGA.EVIIAY RRGEEDMTAR ..... IEEVQHAKEE GVKFM.....
Ps-GltA FGA.EVTIAY RRGEEDVSAR ..... EEEVEHAKEE GIKFV.....
Ae-GltD LGV.ETHVVY RRTRETSSAR ..... QEEWDHLMEE GVKIH.....
Rc-GltX LGAETASIVY RRSQAEMPAS ..... AHEQEHAQKN GVRIVP....
Bt-DPD CGARRVFIVF RKGFVNIRAV ..... PEEVELAREE KCEFLP....
+ + #
==========
451 500
Ss-GltD SYLTTATKIE GDDQG....R VKAVHTVDVR W.EKDGQGRF IPQQVAGTEQ
Pb-GltD AYLRTATHFE GDEQG....N VKAVHTVEVQ W.ERNEQGQF IPKQIPGTEK
Ms-GlsN TYEVLTKRFV GDENG....V VKGLEVVRVC W.EKDETGKF QFKEIEGSEE
Bs-GltD EYSIQTTKMV ADKNG....K LKELHTIQME K.VKNEHGKY EFRELPGTEK
Sc-GlsN EYCILSKEFI GNDEG....E VTAIRTVRVE W.KKSQSGVW QMVEIPNSEE
Ce-GlsN TYSVSTKRFL TTTNAAGITV LTGLEIVDVE W.EKDDKGAW KLIEKNETIR
Mt-GltD VFSVNTEAFV GTDGRVSALR AHEVTMLDGK F.VK..... ....VEGSDF
Ab-GltD .WQAAPEGFT GDT......V VTGVRAVRIH LGVADATGRQ TPQVIEGSEF
Pa-GltD .FNRQPIAIV GED......R VEGVKVVETR LGEPDARGRR SPEPIPGSEE
Ec-GltD .FNVQPLGIE VNGNG....K VSGVKMVRTE MGEPDAKGRR RAEIVAGSEH
Tb-GltD .FHRQPVEVV GDAAS....G SPGRASGGDP SGEPDARGRR HPEIIPGSEQ
Ec-AegA .FNVQPVALE LNEQG....H VCGIRFLRTR LGEPDAQGRR RPVPVEGSEF
Ph-GltD .FFVNPVEFI GDENG....R VKAVKFEKMK PLEERDSRGK RKIIGTGEYV
Ps-GltA .YFVNPVEFI GDENG....R VKAVKFEKMK ALDERDARGK RKIVGTGEYV
Ae-GltD .WLTQPVEVI GNEKG....E VVGLKCIKME LGEPDESGRP RPVPVPNSEH
Rc-GltX ..NAAPVAIR KDDQGY.... ....EVE FARTETQGGK LVQL..DDTL
Bt-DPD .FLSPRKVI. .... VKGGRIVAMQ FVRTEQDETG KWNEDGDQIA
501 550
Ss-GltD VRPAQLVLLA MGFVGPE.SQ LLDAMKLDKD ARGNIKA.EY ..GQYETSIP
Pb-GltD IIPAQLVLLA MGFLGPE.QP LLDAMGLDRD ARSNIKS.EY ..GRYTTSIP
Ms-GlsN IIEADLVLLA MGFLGPE.AT IAEKLGVERD NRSNFKA.DY ..GRFSTSVD
Bs-GltD VWPAQLVFIA IGFEGTE.QP LLKQFGVNSV N.NKISA.AY ..GDYQTNID
Sc-GlsN IFEADIILLS MGFVGPE.LI NGNDNEVKKT RRGTIATLDD ..SSYSIDGG
Ce-GlsN TIECDLCILA MGFVGPE.KS VIEQLNLKTD PRSNILTPK. ..DKYDSDVA
Mt-GltD ELEADLVLLA MGFVGPERAG LLTDLGVKFT ERGNVARGD. ..D.FDTSVP
Ab-GltD TVQADLVIKA LGFE.PEDLP NAFDEPELKV TRWGTLLVDH RTK..MTNMD
Pa-GltD IIPAEAVLIA FGFR.PSPAP WFERFEIATD SQGRVVAPAQ AQFKHQTSNP
Ec-GltD IVPADAVIMA FGFR.PHNME WLAKHSVELD SQGRIIAPEG SDNAFQTSNP
Tb-GltD VLDADVVIIA FGFD.PSPAP WFAEHHITTD SRGRISTNAQ ....FQTSNP
Ec-AegA VMPADAVIMA FGFN.PHGMP W.LESHGVTV DKWGASSRMW .K.ASTVTRP
Ph-GltD TVETDTVVIA IGQT.PNRII WKTTPGLKVR ENGTIVVD.. ..ENLMTSIP
Ps-GltA TLEADTVIIA IGKH.PNRLI .INTPGLKV. ERGKIVVD.. ..ENLMTSIP
Ae-GltD IIECDSVIFA IGQRANPIAY .EGSGIKTTK W.GTIEVN.. ..ERYETNLE
Rc-GltX RIKADQIFTA IGQTLGPVPE GLTITGGKIE IDAQGR.... ......TMGA
Bt-DPD CLKADVVISA FGSVLSDPKV KEALSPIKFN RWDLPEVD.. .PETMQTSEP
551 600
Ss-GltD GVFAAGD.CR RGQSLVVWA. FNEGRGAAKA CDLYLMGETD LP*.......
Pb-GltD KVFAAGD.CR RGQSLVVWA. FNEGRGAARE CDLYLMGETN LP*.......
Ms-GlsN GVFAAGD.CR RGQSLVVWA. ISEGRQAAAQ VDSYLTNEDH GIDGNQDEFV
Bs-GltD GVFAAGD.AR RGQSLIVWA. INEGREVARE VDRYLMGSSV LP*.......
Sc-GlsN KTFACGD.CR RGQSLIVWA. IQEGRKCAAS VDKFLMDGTT YLPSNGGIVQ
Ce-GlsN KVFAAGD.CR RGQSLVVWA. IHEGRQAARQ VDEYLMGKTT LAGPGGIVTA
Mt-GltD GVFVAGD.MG RGQSLIVWA. IAEGRAAAAA VDRYLMGSSA LPAPVKPTAA
Ab-GltD GVFAAGD.IV RGASLVVWA. IRDGRDAGRG HPRLRQGEGR GTGCRGG*..
Pa-GltD KIFAGGD.MV RGSDLVVTA. IFEGRTAAEG ILDYLGV*.. ....
Ec-GltD KIFAGGD.IV RGSDLVVTA. IAEGRKAADG IMNWLEV*.. ....
Tb-GltD RIFAGGD.MR RGSDLVVTA. VDEGRRAAQG ILDYLGV*.. ....
Ec-AegA PIRKSSI.VV TPCVVRIWWL PQWQKDVMRH RGLLTGWG*. ....
Ph-GltD GVFAGGD.AI RGEATVILA. MGDGRKAAKA IHEYLSKKSA *.........
Ps-GltA GVFAGGD.AI RGEATVILAM GDGRKAAKAI HEYLTKKRQE KANA*.....
Ae-GltD GVFAGGD.VI NGGDRVVVAL AHGKEAAKYI HEYLMSNKEV RK*.......
Rc-GltX HVWAGGDCAT GGEDLTVTAV AQGRDAAEDI HAFLMGA*.. ....
Bt-DPD WVFAGGD.VV GIANTTVEAV NDGKQASWYI HRYIQSQYGA SVSAKPELPL
FAD-II

Fig. 4.

In the case of *A. aeolicus*, the *glt*D-like gene is located far from a putative gene that encodes a protein similar to GltB [63]. This gene could encode either an Fd-GltS or the α subunit of a NAD(P)H-GltS. If the putative GltB were the large subunit of an NAD(P)H-GltS and the GltD-like protein were indeed the small subunit of GltS, it would be difficult to explain how the bacterium could coordinate the simultaneous production of the two subunits, which appears to take place in eubacteria and, for the enzyme of *Azospirillum*, to be required for formation of the fully active GltS holoenzyme. In the absence of actual biochemical evidence, the hypothesis may be formulated that the *A. aeolicus glt*B-like gene encodes an Fd-GltS, as indicated by Deckert et al. [63], and that the GltD-like protein is part of the novel group of FAD- and Fe/S clusters-containing NAD(P)H oxidoreductases.

With respect to the *glt*D-like genes found in *Pyrococcus* species, it should be noted that the *Pyrococcus* proteins are similar to each other and that the protein encoded by the *glt*D-like gene of *Pyrococcus* sp. KOD1 has been overproduced in *E. coli* and purified [65]. It was reported to be a fully functional GltS on the basis of its ability to oxidise NADPH in the presence of L-glutamine (or ammonia) and 2-oxoglutarate. As a result it was designated GltA and proposed to be an ancestral form of GltS. Unfortunately, the identification of reaction products and the determination of the reaction stoichiometry were not reported. In particular, no attempt was made to explain how this protein can be a fully functional glutamate synthase in light of the known properties of eubacterial and eukaryotic GltS. A minimal functional GltS should be similar to the GltS α subunit or to Fd-GltS, which contain the glutamine and 2-OG sites, rather than to the β subunit.

Do archaeal GltS differ from other GltS?

The analysis of the sequence of the genome of two Archaea, *Archaeoglobus fulgidus* [66] and *Methanococcus jannaschii* [67], revealed the presence of two putative genes encoding proteins of approximately 500 residues (fig. 5) related to the GltS α subunit (here indicated as GltB being the *glt*B gene product). The putative proteins are similar to each other and were identified as GltB in both *A. fulgidus* (Af-GltB) and *M. jannaschii* (Mj-GltB). Although biochemical evidence for the function of the Archaea GltB-like proteins is still missing, it has been proposed that Mj-GltB and Af-GltB are ancestral fully functional GltS comprising only the minimal structural features needed to carry out glutamate synthesis from glutamine and 2-oxoglutarate [8]. The alignment of Af- and Mj-GltB with the Ab-GltS α subunit shows that there is a striking similarity between the archeal sequences and the 851–1170 region of the Ab-GltS α subunit (fig. 5). This region contains the FMN binding region, the Cys-rich region that in GltS should form the 3Fe-4S center along with the conserved regions CR4, CR5 and CR6, which appear to be typical of GltS. Close inspection of the archaeal GltB-like proteins also reveals that the Cys-rich region corresponding to position 1102 through 1113 of Ab-GltB contains four rather than three Cys residues. Their spacing is of the type $CX_2CX_2CX_4C$, which would suggest the presence of a 4Fe-4S cluster rather than a 3Fe-4S cluster. If the hypothesis is made that the Mj- and Af-GltB-like proteins are indeed ancestral GltS, it could be proposed that (i) the entire region corresponding to residues 851 through 1170 of Ab-GltS α subunit forms Site 2 of GltS and that (ii) in eubacteria the 3Fe-4S cluster derives from the loss of the second Cys residue in the 1102–1113 cluster of the ancestral GltS form. However, the ability of the proposed archaeal glutamate synthase to use glutamine in the absence of a glutamine amidotransferase domain should be explained. It could be proposed that in Archaea (i) GltS uses free ammonia instead of glutamine as the nitrogen source for the reaction or (ii) GltS is a heterodimer-formed by the GltB-like protein and an unidentified subunit carrying the glutamine binding site. In this

Figure 4. Comparison of GltD and GltD-like proteins or protein domains. The sequences of the β subunit of NADPH-GltS (GltD), of the C-terminal part of eukaryotic NADH-GltS (GlsN) and of GltD-like proteins or protein domains were aligned. The residues conserved only in GltD are in blue. The Cys residues that are proposed to participate in the formation of GltS 4Fe-4S clusters are in green and marked with their position in the Ab-GltD sequence. The regions that match the consensus sequence for the formation of ADP-binding fold for FAD (in red) and NADPH binding (in purple) are marked with +. In the ADP-binding region for NAD(P)H # indicates the proposed acidic residue at the C-terminus of the consensus sequence, which is absent in NADPH-dependent GltS. The arrow-head indicates the proposed basic residue at the C-terminus of the ADP-binding region, which may favor NADPH binding. The region and the residues that match the second FAD consensus sequence of Eggink et al. [60] are in red, underlined and marked (FAD-II). The sequences used can be retrieved from the GenBank/EMBL, SwissProt and PIR databases with the accession numbers indicated in parentheses: Ss-GltD, *Synechocystis* sp. PCC6803 (D09900); Pb-GltD, *P. boryanum* (D85230); Ms-GlsN, *M. sativa* (residues 1646–2177, L01660); Bs-GltD, *B. subtilis* (Z99114); Sc-GlsN, *S. cerevisiae* (residues 1605–2131, X89221); Ce-GlsN, *C. elegans* (residues 1702–2232, Z49889); Mt-GltD, *M. tuberculosis* (residues 1–485, MTCY1A6); Ab-GltD, *A. brasilense* (L04300); Pa-GltD, *P. aeruginosa* (PAU81261); Ec-GltD, *E. coli* (GltD_Ecoli); Tb-GltD, *T. ferrooxidans* (U36427); Ec-AegA, *E. coli aeg*A gene product (residues 149–652, YFFG_ECOLI); Ph-GltD, *Pyrococcus horikhoshii* OT3 (AP000007); Ps-GltA, *Pyrococcus* sp. KOD1 (D86223); Ae-GltD, *A. aeolicus* (AE000770); Rc-GltX, *Rhodobacter capsulatus* (AF031406); Bt-DPD, bovine dihydropyrimidine dehydrogenase (residues 1–523, U20981).

```
Af-GltB     .......... .......... .......... ........MM LEGEFHTHML
Mj-GltB     .......... .......... .......... .......... ...MIPSYVP
Ab-GltB     653-HYFAVL IGVGATTVNA YLAQEAIAER HRRGLFGSMP LEKGMANYKK
                 ___________CR2___________                        _

Af-GltB     PEFMIERRQD RCIRCRVCER QCGFNVHWYD EEMDMMREDE MKCVGCQRCA
Mj-GltB     PKYKVEVDPN RCMLCERCTI ECSWGVYR.. REGDRIISYS NRCGACHRCV
Ab-GltB     A...IDDGLL KIMSKMGISV ISSYRG.... .......GGN FEAIGLSRAL
            ______CR3_________________

Af-GltB     VMCPTNALVV KPHPGNYKPN ANWTRERLQD LKKQAETGGV LLTGSG....
Mj-GltB     VMCPRDAITI KENAISWRSH PLWDVDARVD IYNQAKTGCI LLSGMG....
Ab-GltB     VAEHFPAMVS RISGIGLNGI QKKVLEQHAT AYNE...EVV ALPVGGFYRF

Af-GltB     NDKPYRIYWD HIVLNASQ.. VTNPSIDPL. ........RE PMELRTFLGR
Mj-GltB     NAKEHPIYFD KIVLDACQ.. VTNPSIDPL. ........RE PMELRTYIGK
Ab-GltB     RKSGDRHGWE GGVIHTLQQA VTNDSYTTFK KYSEQVNKRP PMQLRDLLEL

Af-GltB     KQDRLEFKYS ED......FE DIEITTELYP NVQIETPIVF SAMSYGAISY
Mj-GltB     KPKQLEFEFV EEEIDGKKIK KAKLKTKIAP NLKLDTPIMI AHMSYGALSL
Ab-GltB     RSTKAPVPVD E......... .......VES ITAIRKRFIT PGMSMGALSP
                                                 _________CR4____

Af-GltB     QAFKSLAMAA SEFGTLFNTG EGGLPKELRK YGKN...... AIVQCASGRF
Mj-GltB     NAHLSFAKAV KECGTFMGTG EGGLPKALYP YADH...... IITQVASGRF
Ab-GltB     EAHGTLNVAM NRIGAKSDSG EGGEDPARFR PDKNGDNWNS AIKQVASGRF
            _____________________________________     ___________

Af-GltB     GVDPEYLNVA AVVEIKIGQG AKPGIGGHLP GEKVTLPISV TRMIPEGTDA
Mj-GltB     GVNEEYLMKG SAIEIKIGQG AKPGIGGHLP GEKVTAEISA TRMIPEGSDA
Ab-GltB     GVTAEYLNQC RELEIKVAQG AKPGEGGQLP GFKVTEMIAR LRHSTPGVML
            ____________________________________________________

Af-GltB     LSPAPQHDIY SIEDLSMLIY ALKEATNYEK PVSVKIAAVH NVAAIASGMV
Mj-GltB     ISPAPHHDIY SIEDLAQLVR SLKEATRWKK PVFVKIAAVH NAPAIAVGIA
Ab-GltB     ISPPPHHDIY SIEDLAQLIY DLKQINPDAK .VTVKLVSRS GIGTIAAGVA
            _____________________CR5_____________________________

Af-GltB     RAGADIIAID GLRGGTGAAP KMIRDNVGIP VELALAAVDQ RLRDEGIRNK
Mj-GltB     TSDADAVVID GYKGGTGAAP KVFRDHVGIP IEMAIAAVDQ RLREEGLRNE
Ab-GltB     KANADIILIS GNSGGTGASP QTSIKFAGLP WEMGLSEVHQ VLTLNRLRHR
            ____________________________________     ==========

Af-GltB     ASILVAGGFR CSADVVKAIA LGADAVYIGT PALVAMGCTL CQKCHTGICN
Mj-GltB     ISIIASGGIR CSADVFKAIA LGADAVYIGT AAMVALGCRV CGRCYTGLCA
Ab-GltB     VRLRTDGGLK TGRDIVIAAM LGAEEFGIGT ASLIAMGCIM VRQCHSNTCP
            =====FMN=============================     Fe/S-I

Af-GltB     WGICTQDPYL AKRLNPEITA KRLVNLLRAW SHEIKEMLGG MGINAIESLR
Mj-GltB     WGIATQRPEL VKRLDPEVGA RRVANLIKAW THEIKELLGA AGINSIESLR
Ab-GltB     VGVCVQDDKL RQKFVG..TP EKVVNLFTFL AEEVREILAG LGFRSLNEVI
                                     _________________CR6__________

Af-GltB     GNREQLRGVG LEDWELEVLG IK...GAGE*
Mj-GltB     GNRDRLRGVG LNEKELEVLG IK...AAGE*
Ab-GltB     GRTDLLHQVS RGAEHLDDLD LNPRLAQVDP GENARYCTLQ GRNEV-1207
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Figure 5. Comparison of *A. brasilense* GltB and GltB-like proteins from *Archaeoglobus fulgidus* and *Methanococcus jannaschii*. The sequence of the Ab-GltS α subunit (Ab-GltB) was aligned with the sequences of the GltB-like proteins of *A. fulgidus* (Af-GltB) and *M. jannaschii* (Mj-GltB). The Af-GltB was obtained by translating nt 3007 through 4542 of AE001038, whereas that of Mj-GltB is the translation of nt 1042 through 2574 (reverse) of U67575. The Ab-GltB sequence is annotated as in figure 1. The clusters of Cys residues are in bold.

respect, none of the putative proteins encoded by open reading frames adjacent to Mj- and Af-GltB exhibits any similarity to Ab-GltB or GltD. Another interesting aspect of the archeal GltB-like protein is the fact that the N-terminal region of the two GltB-like proteins exhibits low similarity with Ab-GltB and no similarity with GltD, but contains two Cys clusters with $CX_2CX_2CX_3C$ (residues 24–34 of Af-GltB) and $CX_2CX_2CX_3CP$ (residues 55–66 of Af-GltB) spacings that would predict two 4Fe-4S centers in addition to that predicted by the region following the putative FMN binding site. Thus, such a hypothetical alternative, and perhaps ancestral, form of GltS would also contain an N-terminal ferredoxin module. This domain would have been lost by the Fd-GltS and eubacterial GltB. However, it would be functionally replaced by free ferredoxin in Fd-GltS and by the N-terminal portion of GltD in the NAD(P)H-GltS of eubacteria and eukaryotes. Whether the GltB-like proteins of Archaea are indeed functional GltS remains to be established by studying the proteins purified from the bacteria in order to determine their catalytic activity, and the type and number of subunits and cofactors. An alternative approach would be the study of the catalytic activity of the fragment of Fd-GltS or GltS α subunit spanning from residues 850 through 1170 to test the hypothesis of the localisation of Site 2 of GltS within this part of the polypeptide. This and all other hypotheses that may be formulated at this stage on the structure and catalytic mechanism of GltS clearly need to be verified by further work on GltS from various sources. The finding, in our laboratory, of conditions that permit the preparation of fully active recombinant Ab-GltS holoenzyme and of its subunits from overproducing *E. coli* strains, should make some of these studies possible.

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