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# The unexpected structural role of glutamate synthase [4Fe–4S]$^{+1,+2}$ clusters as demonstrated by site-directed mutagenesis of conserved C residues at the N-terminus of the enzyme β subunit

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**Abstract**

*Azospirillum brasilense* glutamate synthase (GltS) is a complex iron–sulfur flavoprotein whose catalytically active αβ protomer (α subunit, 162 kDa; β subunit, 52.3 kDa) contains one FAD, one FMN, one [3Fe–4S]$^{0,+1}$, and two [4Fe–4S]$^{+1,+2}$ clusters. The structure of the α subunit has been determined providing information on the mechanism of ammonia transfer from L-glutamine to 2-oxoglutarate through a 30 Å-long intramolecular tunnel. On the contrary, details of the electron transfer pathway from NADPH to the postulated 2-iminoglutarate intermediate through the enzyme flavin co-factors and [Fe–S] clusters are largely indirect. To identify the location and role of each one of the GltS [4Fe–4S] clusters, we individually substituted the four cysteinyl residues forming the first of two conserved C-rich regions at the N-terminus of GltS β subunit with alanyl residues. The engineered genes encoding the β subunit variants (and derivatives carrying C-terminal $His_6$-tags) were co-expressed with the wild-type α subunit gene. In all cases the C/A substitutions prevented α and β subunits association to yield the GltS αβ protomer. This result is consistent with the fact that these residues are responsible for the formation of glutamate synthase [4Fe–4S]$^{+1,+2}$ clusters within the N-terminal region of the β subunit, and that these clusters are implicated not only in electron transfer between the GltS flavins, but also in αβ heterodimer formation by structuring an N-terminal [Fe–S] β subunit interface subdomain, as suggested by the three-dimensional structure of dihydropyrimidine dehydrogenase, an enzyme containing an N-terminal β subunit-like domain.


*Keywords:* Glutamate synthase; Flavoprotein; Iron–sulfur clusters; Site-directed mutagenesis; Dihydropyrimidine dehydrogenase; Ammonia assimilation; Protein engineering; Amidotransferase; Assembly of iron–sulfur clusters

Glutamate synthase (GltS)[1] catalyses the reductive synthesis of L-glutamate (L-Glu) from L-glutamine (L-Gln) and 2-oxoglutarate (2-OG), thus participating with glutamine synthetase to ammonia assimilation processes in microorganisms and plants. GltS from various sources fall in one of three classes and differ, mainly, for

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[1] *Abbreviations used:* 2-IG, 2-iminoglutarate; 2-ME, 2-mercaptoethanol; 2-OG, 2-oxoglutarate; β-His, C-terminally $His_6$-tagged β subunit of glutamate synthase; DPD, dihydropyrimidine dehydrogenase; FAD, flavin adenine dinucleotide; Fd, ferredoxin; Fd-GltS, Fd-dependent glutamate synthase; FMN, flavin monucleotide; GAT, glutamine amidotransferase; GltS, glutamate synthase; GltS-His, GltS variant formed by the wild-type α subunit and β-His; INT, iodonitrotetrazolium; IPTG, isopropyl thio β-galactoside; LB-Amp, Luria–Bertani broth supplemented with ampicillin (0.1 mg/ml); NTA, nitrilo acetic acid; NAD(P)H, reduced nicotinamide-adenine dinucleotide (phosphate); NAD(P)H-GltS, NAD(P)H-dependent glutamate synthase, Ntn-type amidotransferase, $NH_2$-terminal nucleophile amidotransferase; SDS, sodium dodecyl sulfate; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of SDS.

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subunit and co-factor composition, and physiological electron donors (see [1–4] for recent reviews).

*Azospirillum brasilense* GltS (NADPH-GltS) is the model of the bacterial enzyme form, which is NADPH-dependent and is catalytically active as an αβ protomer. According to current evidence, during the reaction, NADPH binds at site 1 (the NADPH-binding site) within the 52.3 kDa β subunit and is oxidized with parallel reduction of FAD, also at site 1 (Fig. 1). Reducing equivalents are transferred to FMN (on the 162 kDa α subunit) through the $[3Fe–4S]^{0,+1}$ cluster of GltS (within the α subunit) and at least one of the two $[4Fe–4S]^{+1,+2}$ centers of the enzyme. At site 2, on the α subunit, reductive glutamate synthesis takes place. At this site (also indicated as the synthase site) 2-OG binds, is converted to the postulated 2-iminoglutarate (2-IG) intermediate on addition of ammonia to its C(2) position, and is reduced by reduced FMN to L-glutamate. Ammonia derives from hydrolysis of glutamine at the Type II (or Ntn—from N-terminal nucleophile) glutamine amidotransferase (GAT) site (also referred to as the glutaminase site) of GltS α subunit from which it diffuses to the synthase site through the approximately 30 Å-long intramolecular tunnel identified by X-ray crystallography [5]. The ferredoxin-dependent glutamate synthase (Fd-GltS) is similar to the α subunit of bacterial NADPH-GltS. In Fd-GltS reducing equivalents derive from reversible association of the enzyme with reduced ferredoxin (Fd). Yeast, non-photosynthetic tissues of plants, and lower animals produce the third type of GltS (NADH-GltS), which appears to be NADH-dependent and to result from the fusion of the bacterial GltS α and β subunits. Based on sequence similarity with the NADPH-GltS α and β subunits, all eukaryotic NADH-GltS are proposed to be similar to the bacterial enzyme with respect to cofactor content and reaction mechanism.

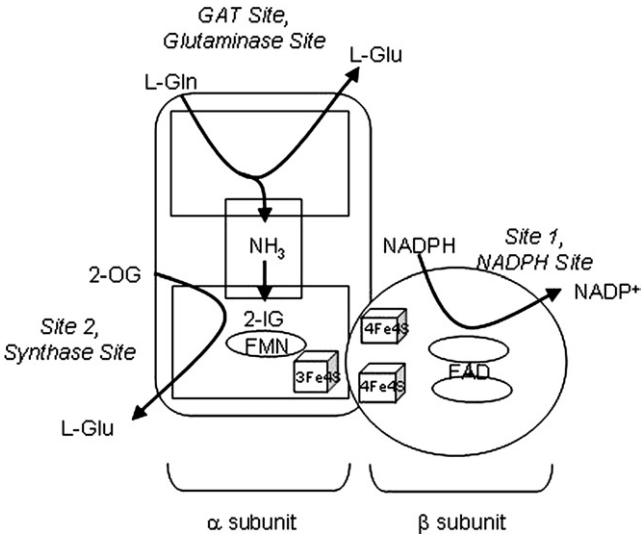


Fig. 1. Distribution of co-factors and catalytic sub-sites in NADPH-GltS. FMN (on the α subunit) and FAD (on the β subunit) are represented as an oval or two linked ovals, respectively. The [3Fe–4S] cluster (on the α subunit) and the [4Fe–4S] clusters (on the β subunit) are represented as cubes.

The three-dimensional structure of *A. brasilense* GltS α subunit in complex with the L-methionine sulfone (an enzyme inhibitor competitive with L-glutamine) and the 2-OG substrate [5] and those of *Synechocystis* Fd-GltS in the free form, in complex with 2-OG and that of the enzyme-oxo-L-norleucine analog in complex with 2-OG have been solved by X-ray crystallography [6,7], providing insights on the mechanism of L-Gln-dependent glutamate synthesis taking place within the GltS α subunit and the homologous Fd-GltS form. On the contrary, attempts to obtain crystals of the *A. brasilense* GltS αβ holoenzyme have not been successful, yet. Therefore, the location and role of the enzyme $[4Fe–4S]^{+1,+2}$ clusters only rely on sequence analyses and solution studies of the NADPH-GltS αβ holoenzyme and of its isolated α and β subunits. Electron paramagnetic resonance spectroscopy experiments on oxidized and reduced forms of GltS [8] and measurements of the mid-point potential values of the flavin co-factors and of the $[3Fe–4S]^{0,+1}$ center of the enzyme [9] led to the conclusion that the [3Fe–4S] cluster and at least one of the [4Fe–4S] centers of GltS are part of the electron transfer path from FAD to FMN. Modelling of the NADPH-GltS αβ protomer within the framework of studies aimed to obtain a low resolution structure of the enzyme are consistent with such hypothesis [10]. Analyses of the primary structures of NAD(P)H-GltS α and β subunits highlighted the presence of eight invariant C residues at the N-terminus of the β subunit, with no other conserved C residues except for C1 in the N-terminal GAT domain and those implicated in the formation of the [3Fe–4S] cluster in the α subunit [1]. In spite of the presence of the two C-rich regions, no [Fe–S] clusters are found on the β subunit when it is produced separately from the α subunit [11]. This observation led to the proposal that the α subunit is required for [4Fe–4S] clusters formation either because it provides a stabilizing environment for the clusters or because it provides ligands for cluster(s) formation. Interestingly, the spacing of the conserved C residues at the N-terminus of NADPH-GltS β subunits and in the corresponding part of NADH-GltS differs from the typical $CX_2CX_2CX_4CP$ fingerprint of ferredoxin-type [4Fe–4S] clusters, but it is conserved in a number of β subunit-like proteins or protein domains, which were found through databank searches [1]. Among these proteins, only *Pyrococcus furiosus* sulfide dehydrogenase [12], bovine dihydropyrimidine dehydrogenase (DPD, [13,14]), and GltS [8,9] have been characterized to any extent with respect to the properties of their iron–sulfur clusters, which have in common low to very low mid-point potential values.

To identify the role of the C residues conserved at the N-terminus of GltS β subunit we mutated *glt*D, the gene