The Subnanometer Resolution Structure of the Glutamate Synthase 1.2-MDa Hexamer by Cryoelectron Microscopy and Its Oligomerization Behavior in Solution

FUNCTIONAL IMPLICATIONS

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Glutamate synthases (GltS) are complex iron-sulfur flavoproteins that catalyze the reductive transfer of the L-Glu amide group to the C2 carbon of 2-oxoglutarate (2-OG), yielding two molecules of L-glutamate (L-Glu). GltS are found in bacteria, yeast, and plants, where they form with glutamine synthetase an essential pathway for ammonia assimilation (1–3). Bacterial NADPH-dependent GltS (NADPH-GltS) is formed by one α subunit (αGltS) and one β subunit (βGltS) of 162 and 52 kDa, respectively, for the Azospirillum brasilense GltS. The α protomer contains one FAD (on βGltS), one FMN (on αGltS), and three different iron-sulfur clusters (one [3Fe-4S]1/0+1/2+ cluster on αGltS and two [4Fe-4S]1/2+ centers on βGltS; Fig. 1A). On the basis of sequence, structural, and mechanistic similarities, the NADPH-GltS serves as a model for the other two main forms of GltS, namely (i) the ferredoxin-dependent GltS found in cyanobacteria and photosynthetic tissues of plants, which is similar to αGltS and (ii) the eukaryotic type of GltS, which is NADH-dependent and is found in yeast, nonphotosynthetic tissues of plants, and lower eukaryotes; this GltS species is formed by a single polypeptide chain derived from the fusion of bacterial α and β subunits.

Several lines of evidence support an essential role of GltS in all of these cell types, making it a potential target of novel drugs. For example, the NADPH-GltS has been found to be essential in Mycobacterium tuberculosis, where it has been shown that <1 μM azaserine, a GltS inhibitor, is sufficient to inhibit growth of greater than 90% cells (4). In Caenorhabditis elegans, inactivation of the GltS-encoding gene (W07E11) leads to a lethal phenotype (see the WormBase data base on the World Wide Web). Finally, in plants, inactivation of the genes encoding the NADH- or the ferredoxin-dependent GltS forms leads to mutants impaired in growth due to the involvement of the

The three-dimensional structure of the hexameric (αβ)6 1.2-MDa complex formed by glutamate synthase has been determined at subnanometric resolution by combining cryoelectron microscopy, small angle x-ray scattering, and molecular modeling, providing for the first time a molecular model of this complex iron-sulfur flavoprotein. In the hexameric species, interprotomeric α-α and α-β contacts are mediated by the C-terminal domain of the α subunit, which is based on a β helical fold so far unique to glutamate synthases. The αβ protomer extracted from the hexameric model is fully consistent with it being the minimal catalytically active form of the enzyme. The structure clarifies the electron transfer pathway from the FAD cofactor on the β subunit, to the FMN on the α subunit, through the low potential [4Fe-4S]1+/2+ centers on the β subunit and the [3Fe-4S]0/1 cluster on the α subunit. The (αβ)6 hexamer exhibits a concentration-dependent equilibrium with αβ monomers and (αβ)6 dimers, in solution, the hexamer being destabilized by high ionic strength and, to a lower extent, by the reaction product NADP+. Hexamerization seems to decrease the catalytic efficiency of the αβ protomer only 3-fold by increasing the Kₚ values measured for L-Gln and 2-OG. However, it cannot be ruled out that the (αβ)6 hexamer acts as a scaffold for the assembly of multienzymatic complexes of nitrogen metabolism or that it provides a means to regulate the activity of the enzyme through an as yet unknown ligand.

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†† The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1–5, Figs. 1–8, and Movie 1.

* We are deeply saddened that we must report the loss of our friend and colleague Dr. Nicolas Boisset who passed away on January 4, 2008.

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2 The abbreviations used are: GltS, glutamate synthase; αGltS, α subunit of glutamate synthase; βGltS, β subunit of glutamate synthase; 2-OG, 2-oxoglutarate; 2-I2, 2-iminoglutarate; EM, electron microscopy; DPD, dihydroxy-pyrimidine dehydrogenase; DTT, 1,4-dithiothreitol; Eₚₐₚ, midpoint potential; GltSHis, glutamate synthase formed by the wild-type α subunit and a β subunit form carrying a C-terminal hexahistidinyl extension; GltSHis37 or GltSHis440, GltSHis variant resulting from deletion of the C-terminal 7 or 40 residues, respectively, of the α subunit; MetS, l-methionine sulfone; MM, molecular mass; NAD(P)H-GltS, NAD(P)H-dependent glutamate synthase; SAXS, small angle x-ray scattering.
The Hexameric NADPH-GltS Structure

enzyme in primary nitrogen assimilation and in photosrespiration (5, 6). On the other hand, enhancing the GltS activity through protein and metabolic engineering is a viable option for modulating the NAD(P)⁺/NAD(P)H ratio (or the 2-OG level) in bioconversions (e.g. see Ref. 7), the nitrogen assimilation pathway in nitrogen-fixing bacteria used as biofertilizers, or the response to osmotic or acid stress (8). NADPH-GltS from A. brasilense, a root-growth promoting nitrogen-fixing bacterium, is the best studied GltS. The three-dimensional structure of αGltS in complex with l-methionine sulfoxide (MetS, an l-Gln analog) and the 2-OG substrate has been solved by x-ray crystallography (9), providing insights into the glutaminase and synthase catalytic subsites within the isolated enzyme (10). Furthermore, the location and the role of the [4Fe-4S] clusters of GltS within the 9.5 Å resolution cryo-EM-derived electron density shows how the C-terminal β helical domain of αGltS acts as a structural spacer, which establishes interprotomeric α-α and α-β contacts, playing a key role in the oligomerization process.

In the present work, we used three-dimensional cryoelectron microscopy (cryo-EM), small angle x-ray scattering (SAXS), and modeling to investigate the stoichiometry and the structure of the active oligomeric NADPH-GltS species in solution. The combined use of cryo-EM and SAXS allowed us to obtain an electron density map of the GltS (αβ)₆ hexamer at a subnanometer resolution, which has been reached only recently, and only in a few cases, for cryo-EM-based structure determinations (17–22). Moreover, we propose a homology model of βGltS, and, more importantly, that of the αβ protomer. The latter sheds light on the intramolecular electron transfer process from FAD to FMN along the enzyme iron-sulfur clusters. Finally, modeling of the (αβ)₆ oligomer of NADPH-GltS into the 9.5 Å resolution cryo-EM-derived electron density shows how the C-terminal β helical domain of αGltS acts as a structural spacer, which establishes interprotomeric α-α and α-β contacts, playing a key role in the oligomerization process.

EXPERIMENTAL PROCEDURES

Enzymes—GltS or the species formed by the wild-type α subunit and a C-terminally His₆-tagged variant of the β subunit (GltS-His) were overproduced in Escherichia coli BL21 (DE3) cells and purified, quantified, and assayed as described previously (15, 23). αGltS was prepared and characterized as described in Ref. 16. Steady-state kinetic analyses were carried out as in Ref. 15 and references therein. Construction of GltS deletion mutants and other techniques are described in the supplemental material.

Prior to each experiment, the protein solutions were transferred in 25 mM Hepes/KOH buffer, pH 7.5, containing 1 mM EDTA and 1 mM dithiothreitol by gel filtration through Sephadex G25 (medium) columns (PD10, prepacked disposable columns; GE Healthcare) equilibrated and eluted with the same buffer. If needed, the enzyme solution was concentrated to 10–30 mg/ml and stored frozen at −80 °C after flash freezing in liquid nitrogen. The same buffer was used for all dilutions.

Small Angle X-ray Diffraction Data Collection—Synchrotron x-ray scattering data from solutions of GltS in the presence and absence of ligands and/or substrates were collected at the X33 beamline (DESY, Hamburg) (24) at protein concentrations (c) ranging from 0.75 to 10.0 mg/ml. At a sample-detector distance of 2.7 m, the range of momentum transfer 0.1 < s < 5 nm⁻¹ was covered (s = 4π sin(θ)/λ, where 2θ is the scattering angle and λ = 0.15 nm is the x-ray wavelength). The data were processed using standard procedures by the program package PRIMUS (25). The forward scattering I(0) and the radii of gyration (Rg) were evaluated by the program AUTORG (26) using the Guinier approximation (27), assuming that at very small angles (s < 1.3/Rg), the intensity is represented as I(s) = I(0) exp[−(sRg)²/3]. The effective molecular mass of the solute (MM) was estimated by comparison of the forward scattering I(0) with that from reference solutions of bovine serum albumin (MM = 66 kDa). For the NaCl-containing solutions, the MM estimates were appropriately corrected to account for contrast reduction (factors 1.5 and 2.4 for 1 and 2 M NaCl, respectively).

The scattering intensities for the monomeric, dimeric, and hexameric GltS computed by CRYSO (28) from the atomic coordinates taken from the cryo-EM model were employed to

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analyze the oligomeric composition of all of the samples. The program OLIGOMER (25) was used to find the volume fractions of components minimizing the discrepancy $\chi^2$ (normalized sum of the reduced S.D. values) between the linear superposition of the weighted intensities of the components and the experimental data from the mixture.

Specimen Preparation and Cryo-EM Data Collection—Grids with holey carbon films were prepared and vitrified by flash freezing in liquid ethane (29). For the initial three-dimensional reconstruction, a GltS sample (6.2 mg/ml) was observed in a Philips CM12 electron microscope using an acceleration voltage of 120 kV and a magnification of $\times 45,000$. Each field was recorded with subsequent tilt angles of 45 and 0° and with defocus values of $-1.6$ and $-1.2 \mu m$, respectively. Additional untilted specimen images of the wild-type GltS complex and of the isolated $\alpha$GltS were recorded on the same instrument, with sample concentrations of 9.25 mg/ml and 7 mg/ml, respectively. For high resolution studies, the sample was observed in a JEOL JEM 2100F, using an acceleration voltage of 200 kV, a spherical aberration of 0.5 mm, and a magnification of $\times 50,000$. Images were recorded under low dose conditions (10 electrons/Å$^2$) on Kodak SO 163 film.

Three-dimensional Reconstruction—For the initial three-dimensional reconstruction, 437 pairs of particles were extracted from 12 pairs of tilted-untitled images, interactively, using the program WEB (30). A preliminary three-dimensional model was reconstructed with the software package SPIDER (30) using the random conical tilt series method (31). This model was refined using the projection matching method (32) with 1344 particle images extracted from five additional untilted specimen images. A few final refinement iterations were performed with imposed D3 symmetry until the resolution stabilized at 26 Å, as estimated with the Fourier shell correlation criterion (33). For the high resolution reconstruction, micrographs with anisotropic or no diffraction rings were removed using the enhanced power spectra sorting method (34). 60,000 particle images were selected automatically from 84 remaining micrographs using Roseman’s particle selection algorithm (35). However, only 13000 particle images were kept after visual inspection and splitting in defocus groups. The parameters of the contrast transfer function of the electron microscope were computed for each particle with the program CTFTILT (36). An iterative refinement of the low resolution three-dimensional model was performed with SPIDER, using the projection matching method coupled with the method of correction of the contrast transfer function by Wiener filtering of volumes from focal series (37). D3 symmetry was imposed, and different filters were applied on the reference volume at different stages of the refinement until the resolution improved and stabilized at 9.5 Å (33). The Fourier amplitudes of the final, nonfiltered volume were enhanced using the GltS SAXS data as described in Ref. 38.

Atomic Structure Fitting and Modeling—Sequences of $\beta$GltS (SwissProt accession number Q05756) and DPD (Protein Data Bank code 1h7w (14)) were first roughly aligned using ClustalW (39, 40). Then the secondary structure prediction methods SOPMA, PHD, and PREDATOR, integrated in the metaserver NPS@ (41), and hydrophobic cluster analysis (42, 43) were applied to $\beta$GltS. The secondary structures predicted for $\beta$GltS were compared with the corresponding ones of DPD calculated by DSSP (44), allowing us to optimize the alignment. This alignment was used with MODELLER 7V7 (45) to generate homology models by satisfaction of spatial restraints derived from the alignment. The $\alpha_2$GltS dimer (Protein Data Bank code 1ea0 (9)) was submitted to the algorithm “loop” of MODELLER to model the unresolved regions 305–307, 1172–1179, and 1194–1202 of chains A and B. Only the seven C-terminal residues (residues 1473–1479) of $\alpha$GltS, absent in the Protein Data Bank file, remain missing in the final pseudoatomic model of GltS.

To perform the fitting of atomic data into the cryo-EM map, the following programs were compared: (i) rigid body fitting in Fourier space with Colores and Colacor algorithms of SITUS (46, 47); (ii) real space fitting with the FoldHunterP algorithm of EMAN (48, 49); (iii) real space fitting using the operation “Fit Model in Map” of Chimera (50, 51). A first $\alpha_2$ dimer was fitted, and the 3-fold symmetry was used to calculate the atomic coordinates of the other two. Due to the unambiguous dispositions of the subunits, these different programs yielded equivalent results. Then a $\beta$GltS model was fitted independently, and D3 symmetry was used to calculate the positions of the other ones. An automatic check with Chimera software showed no significant steric hindrance between the fitted subunits. This fitting was compared with another procedure, consisting of fitting independently the $\alpha$- and $\beta$GltS monomers. However, this approach did not improve the model.

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by National Institutes of Health Grant P41 RR-01081). The experimental density map (accession code EMD-1440) and the fitted atomic coordinates of GltS (Protein Data Bank code 2vdc) have been deposited in the Macromolecular Structure Database.

RESULTS

Three-dimensional Reconstruction Volume of GltS by Cryo-EM at 9.5 Å Resolution

To obtain a preliminary three-dimensional reconstruction volume, preparations of GltS were observed by cryo-EM with the specimen grid tilted at 45 and 0°, respectively (Fig. 1B). Digitized images were subjected to the random conical tilt series technique, a three-dimensional reconstruction method inspired by tomographic approaches (31, 52). The volume obtained at this stage was solved at a resolution of 26 Å, which was sufficient to clarify two important points concerning the structure of the oligomeric complex. First, although no symmetry was enforced during the three-dimensional reconstruction process, the particles produced well defined rectangular side views (Fig. 1C) and triangular top views (Fig. 1D), specific for a 322 point group symmetry. Second, using the rigid body fitting techniques of SITUS (46, 47) and EMAN (48, 49) software, it was easy to fit the atomic structure of the crystallographic $\alpha_2$ dimers (Protein Data Bank code 1ea0 (9)) forming three large “pillars” within the complex (Fig. 1, C and D). Hence, the stoichiometry of the whole complex was unambiguously deter-
mined with six αGltS, forming the three pillars of the complex, and six smaller masses, connecting these pillars, most likely corresponding to βGltS. Interestingly, the crystallographic asymmetric unit of αGltS contains α₂ dimers, but the analysis of the geometry of adjacent dimers in the crystals revealed that they interact to form tetramers, with a structure essentially corresponding to that of two adjacent pillars of the cryo-EM-derived hexameric model of GltS (supplemental Fig. 1).

To improve the resolution of the three-dimensional reconstruction, as needed to propose a complete structural model of the (αβ)_6 oligomeric GltS complex, additional cryo-EM images were collected with the specimen grid untilted, using a JEOL 2100F electron microscope under low dose conditions. Micrographs of the highest quality were selected as described in Ref. 34, and an automatic identification of particles was performed on the selected micrographs using the procedure described in Ref. 35. After additional sorting of the particle images by visual inspection, the kept images were split into defocus groups and used for the high resolution three-dimensional reconstruction. Using the preliminary volume (Fig. 1, C and D) as the starting model, the new image set was subjected to an iterative process of three-dimensional projection alignment and contrast correction, until the resolution of the three-dimensional reconstruction volume stabilized at 9.5 Å. This better resolved structure has lost its smooth appearance but clearly shows finer details on the isosurface representations of the rectangular side view (Fig. 1E) and the triangular top view (Fig. 1F). This new structure was the template required for the complete modeling of the GltS complex.

**Homology Modeling of the GltS β Subunit**

In order to generate a model of the (αβ)_6 GltS hexamer from the cryo-EM-derived structure at 9.5 Å resolution, a structural model of βGltS was required. It has been previously shown (1, 12, 14) that βGltS and the N-terminal region of DPD are similar to each other. Thus, the DPD atomic coordinates (Protein Data Bank code 1h7w, chain A, residues 31–520) were used as a structural template to produce a homology model of βGltS (Fig. 2A). Overall, the DPD and βGltS sequences are very similar to each other (26% identity and 46% similarity on the
whole sequence calculated with BLOSUM62 substitution matrix) taking into account in βGltS the deletion of a small 8-residue α helix (residues 177–184; supplemental Fig. 2) and an insertion of 4 residues (residues 346–349) between strands β9 and β10 of DPD (supplemental Fig. 2). Only the region spanning residues 1–25 of βGltS (corresponding to residues 31–55 of DPD, supplemental Fig. 2, stars) could not be reliably modeled due to the low similarity and to the fact that the corresponding region of DPD is an α helix projected from subunit A toward subunit B, with which it interacts. Thus, the first 25-residue peptide of βGltS was omitted.

As previously suggested (12, 14, 15), the [4Fe-4S] clusters of βGltS are well modeled, assuming that one of these centers is formed by Cys47, Cys50, Cys55, and Cys108 of βGltS, corresponding to Cys79, Cys82, Cys87, and Cys130, forming the first N-terminal cluster of DPD (nFeS1 according to DPD nomenclature (14)), and the other (nFeS2 in DPD) is formed by Cys59, Cys98, Cys104, and Glu124. These Cys residues correspond to Cys91, Cys130, and Cys136 of DPD. Glu124 of βGltS substitutes Gln156 of DPD, in part justifying the different redox properties of the GltS [4Fe-4S] clusters with respect to the corresponding ones in DPD. Indeed, one of the GltS [4Fe-4S] clusters can be reduced by NADPH, and both clusters are reduced photochemically (53). On the contrary, none of the corresponding DPD clusters could be reduced even under mild denaturing conditions (13).

**Rigid Body Modeling of the GltS (αβ)_6 Hexamer**

With the crystallographic structure of αGltS and a model of βGltS available, it was possible to fit them into the 9.5 Å cryo-EM model (Fig. 1, E and F), using a rigid body approach. Three copies of the crystallographic αα dimer were positioned in the electron density of the three pillars of the oligomer to form the core of the complex. One copy of the βGltS model was positioned in each one of the triangular shapes extending at the periphery of the αGltS hexameric core. The best fit resulted in the model shown in Fig. 2, B and C (see also movie1.mov in the supplemental materials), which was fully supported by the SAXS data, as detailed below.

In the hexameric model of GltS, four kinds of contacts, which are relevant for its stabilization, can be described. The intradimeric contacts between α subunits in the (αβ)_6 dimers, forming the pillars of the complex, correspond to the α-α interactions found in the crystallographic αα dimer (9). The
Interprotomeric contacts deal with α-α and α-β interactions between subunits belonging to adjacent \((\alpha\beta)_2\) dimers (i.e. adjacent pillars). The α-α interprotomeric contacts involve residues 1433–1436, 1310–1313, 1228–1234, 1264–1269, and 1224–1228 of the C-terminal β helical domain of one αGltS, which are facing, respectively, residues 804–805, 806–808, 840–848, 876–879, and 896–902 of the FMN domain of the neighboring αGltS, belonging to the adjacent pillar (Fig. 3B). The interprotomeric α-β contacts involve residues 370–385 of one βGltS and residues 1438–1449 of the αGltS belonging to the αβ protomer of the adjacent pillar (Fig. 3C). The intraprotomeric α-β contacts correspond to the interface between the subunits in the αβ protomer. Here, residues 781–785, 791–798, and 1102–1118 of the FMN domain of αGltS face residues 54–59 and 108–113 of βGltS (Fig. 2D). The iron-sulfur clusters of GltS are close to the α-β interface.
and seem to structure loops 47–55 of βGltS and 1102–1118 of αGltS (Fig. 2E).

This observation agrees well with the results of site-directed mutagenesis of Cys residues of βGltS, which indicated a role of the βGltS [4Fe-4S] clusters in structuring the N-terminal region of this subunit allowing its association with αGltS to form the αβ protomer (15).

SAXS and Stoichiometry of the NADPH-GltS Complex in Solution

Overall Parameters—The x-ray scattering patterns from GltS solutions are given in Fig. 4. The effective \( R_g \) and MM computed from the SAXS data are presented in Table 1. Control measurements with GltSHis solutions (15) yielded, within the errors, the same scattering curves as the wild-type GltS at the given conditions. Thus, it was confirmed that the C-terminal His\(_6\) tag engineered in βGltS yields an enzyme form indistinguishable from the native GltS. The MM values obtained for solutions of unliganded GltS at low ionic strength (Table 1, lines 1–4) indicate that >90% of the protein is present as an (αβ)\(_6\) hexamer. GltS solutions that had been preincubated for up to 16 h with the l-Gln analog MetS (1 mM) and the GltS substrate 2-OG (1 mM), alone (not shown) or in combination, yielded similar \( R_g \) and MM values (Table 1, lines 17–20). On the contrary, preincubation with NADPH\(^+\) either alone or in combination with MetS and 2-OG (Table 1, lines 21–24 and 13–16) resulted in a decrease of both \( R_g \) and MM, suggesting a shift in the oligomeric equilibrium toward smaller particles. For all of the above solutions, the \( R_g \) and MM values at the highest protein concentrations (~8–10 mg/ml) were somewhat smaller than those obtained at the medium ones (~2–4 mg/ml), indicating a concentration effect (namely repulsion between particles) at the higher protein concentrations.

In order to identify the smaller particles found in the GltS solutions, the effect of the ionic strength of the solvent on the oligomerization state of the enzyme was studied by SAXS. Preliminary analytical dynamic light scattering and gel filtration experiments showed that increasing ionic strength above 0.7 M brought about dissociation of GltS or GltSHis into a smaller species (i.e. the αβ protomer; see below). The process was slow, reaching completion after 16–20 h of incubation at 4–15 °C with greater than 85% retention of activity (supplemental Fig. 3, D–C). Removal of NaCl by either dialysis or centrifugal gel filtration established that the dissociation of GltS αβ oligomer into αβ protomers was reversible and fast (supplemental Table 1 and supplemental Fig. 3D), except for a small fraction of protein that had irreversibly dissociated into the free α and β subunits, as revealed by SDS-PAGE of individual fractions obtained by gel filtration chromatography (not shown).

The SAXS data from GltS solutions that had been preincubated with 1 or 2 M NaCl reveal a dramatic decrease of \( R_g \) and MM, yielding a low mass species (Table 1, lines 5–12), presumably, the αβ protomer (see below). These parameters show a clear correlation with protein concentration, with 2 M NaCl exhibiting an effect stronger than that of 1 M NaCl on protein dissociation (compare lines 5–8 with lines 9–12 of Table 1).

Analysis of the Oligomeric Composition—The computed scattering from the cryo-EM model of GltS (Fig. 2, B and C) agrees well with the measured SAXS profile of salt- and ligand-free GltS at high protein concentrations. However, it displays some systematic deviations (Fig. 4, upper fit), which become very significant with the addition of salt and NADPH\(^+\) (Fig. 4). To determine the nature of the deviations, quantitative analysis of

FIGURE 4. Effect of protein concentration, ionic strength, and ligands on the aggregation state of NADPH-GltS. SAXS plots of NADPH-GltS samples described in Table 1, Plots 1–4, 10-0.75 mg/ml GltS; plots 5–8, 8.6-1 mg/ml GltS in the presence of 2 mM NaCl showing an almost complete dissociation of the complex; plots 9–12, 8.1-0.9 mg/ml GltS preincubated for 16 h with MetS (a l-Gln analog), 2-OG, and NADPH\(^+\) (1 mM each); plots 13–16, 8.9-1 mg/ml GltS preincubated for 16 h with MetS and 2-OG only; plots 21–24, 7.8-0.9 mg/ml GltS preincubated for 16 h with NADPH\(^+\) (1 mM). Experimental data are denoted by dots, and fits from OLIGOMER are presented as solid lines. In the top curve, the computed scattering from the cryo-EM model (Fig. 2) is denoted by triangles.
the oligomeric state of GltS as a function of concentration and ligands was performed. It was possible to fit the entire set of data (Fig. 4) by the scattering from mixtures of GltS αβ protomers, (αβ)_2 dimers (i.e. the pillars forming the GltS oligomer) and (αβ)_6 hexamers extracted from the cryo-EM model (Fig. 2, B and C). The volume fractions of these species were computed by OLIGOMER (25), and are presented in Table 1. Even without salt or ligands and at the highest protein concentration, GltS is not fully hexameric. Rather, the solution contains 7–10% of GltS αβ protomers. The same observations hold in the presence of MetS and 2-OG. The somewhat higher χ values obtained at the highest protein concentrations might be explained by the repulsive interactions between the particles already discussed above. The presence of salt significantly shifts the protomer-hexamer equilibrium toward the GltS protomer, possibly weakening the interprotomer α-α and α-β interactions as well as the α-α interactions within each (αβ)_2 dimer. OLIGOMER results confirm that GltS in 1 M NaCl completely dissociates into αβ protomers at concentrations below 2 mg/ml (Table 1, lines 11 and 12). Interestingly, only upon the addition of NADP⁺ (independently from the presence of the other two ligands), a small fraction of (αβ)_2 dimeric particles appeared (Table 1, lines 13–16 and 21–24). In the corresponding three-component mixtures, the volume fraction of αβ protomers remained almost unchanged with respect to free GltS (Table 1, lines 1–4), but the amount of the dimeric particles systematically grew with the dilution of the GltS solution. This finding suggests that NADP⁺ acts on the interface between (αβ)_2 dimers of adjacent pillars by disrupting to some extent the (αβ)_6 hexamers (Figs. 2 and 3). This is at variance with the effect of ionic strength, where only intraprotomeric α-β interactions are maintained. The possibility of dissociation of GltS into the isolated α and β subunits was also considered in OLIGOMER (not shown). In some cases, slightly better fits were obtained. However, the improvement was not systematic, and the volume fractions of the individual subunits were in all cases below 10%, in agreement with the <15% loss of activity observed with samples of GltS upon extended incubation with NaCl (supplemental Fig. 3 and Table 1).

Earlier SAXS results suggested a tetrameric (αβ)_4 assembly of GltS in solution (54), assuming monodispersity of the GltS samples. Given the new evidence for the co-existence of different GltS oligomers, the previous data were reanalyzed, leading us to the conclusion that the samples studied at that time were actually mixtures of about 20% αβ protomers and 80% (αβ)_6 hexamers (supplemental Fig. 4) as opposed to more than 90% hexamers from the current measurements.

The oligomeric state of αGltS in solution was also reanalyzed (54) by taking into account the possibility of sample heterogeneity. At variance with the GltS holoenzyme, the salt-induced dissociation of αGltS was completed in ~10–20 min (supplemental Table 2 and supplemental Fig. 5). This result indicated a lower stability of the high mass species of αGltS than that formed by the GltS holoenzyme. The SAXS patterns from solutions of αGltS were also measured, although not as titration series (supplemental Fig. 5). Primary analysis of the SAXS data (supplemental Table 3 and supplemental Fig. 5) suggested the presence of (αβ)_6 hexamers in solution, but their percentage was systematically lower than that found with the GltS αβ holoenzyme. The oligomeric composition of αGltS solutions was characterized by OLIGOMER (25) using mixtures of α monomers, (crystallographic) αβ dimers, and (αβ)_6 hexamers (extracted from the cryo-EM-derived GltS model, Fig. 2, B and C). The maximum content of (αβ)_6 hexamers did not exceed 84% (obtained for a 5 mg/ml solution). This finding indicates a higher stability of the GltS (αβ)_6 hexamer compared with αGltS, presumably due to the additional interprotomeric α-β interaction.
interactions. Furthermore, the results are in full agreement with the size distribution of particles observed by cryo-EM using αGltS preparations (supplemental Fig. 6). The addition of salt led to dissociation of α subunits similar to that observed with GltS, but at 2 mg/ml and in the presence of 1 M NaCl. α hexamers were almost fully dissociated into α monomers (supplemental Table 3).

The heterogeneity of the previous GltS preparation led to underestimating the oligomer stoichiometry (54). Both gel filtration and SAXS data pointed to a tetrameric assembly (the former due to the nonlinearity of calibration at high MM, the latter due to partial dissociation). Interestingly, even under incorrect stoichiometry and symmetry assumptions, the ab initio shape of GltS by (54) displays an overall hollow appearance similar to that of the present cryo-EM model (supplemental Fig. 7). Furthermore, assuming hexameric species and imposing D3 symmetry, ab initio shape reconstruction and rigid body refinement based on the present SAXS data yielded shapes of α6 and (αβ)6 hexamers similar to those derived from the cryo-EM model.

**Effect of the Oligomerization State on the Catalytic Activity of GltS**

In order to establish if the oligomerization state of GltS affects its catalytic activity, we first evaluated if we could generate variants of the α or β subunits capable of forming the αβ protomer but unable to establish interprotomeric contacts yielding the αβ protomer species at all protein concentrations and at low ionic strength. Inspection of the GltS (αβ)6 model showed that interprotomeric α-β and α-α contacts (Fig. 3) involve several residues without, however, well defined couples of strongly interacting residues (e.g. facing positively/negatively charged residues or hydrophobic patches). Therefore, in order to attempt to produce a monomeric form of GltS for further studies, we generated GltS species formed by the βGltS variant carrying a C-terminal His6 tag (15) and truncated variants of αGltS. Deletion of the C-terminal 40 residues of αGltS removed the 1438–1453 α helices resting on the β helical core, which are engaged in the interprotomeric α-β contacts (Fig. 3, A and B). However, this deletion was too drastic, leading to no production of the truncated αGltS protein. On the contrary, removal of the C-terminal 1473–1479 heptapeptide, which is disordered in the αGltS crystal structure (9), led to a GltS form (GltSHisΔ7), which was essentially indistinguishable from the full-length GltSHis with respect to protein production levels, behavior, and yields during purification (supplemental Table 4), activity, cofactor content, aggregation state, and its dependence on ionic strength (supplemental Fig. 3).

In light of these results, we turned our attention to the fact that incubation of GltS in the presence of 1 M NaCl leads to full dissociation into αβ protomers. Since dissociation is a slow process (supplemental Fig. 3), but reassociation upon removal of NaCl is fast (supplemental Table 1, supplemental Fig. 3), we had to carry out the determination of the apparent steady-state kinetic parameters (maximum velocity, $V_{max}$, and $K_m$ values for the three substrates) in assay mixtures containing 1 M NaCl using GltS solutions (1 mg/ml) that had been preincubated for 16–20 h in the absence or presence of 1 M NaCl. In the absence of NaCl, the (αβ)6 hexamer prevails, whereas preincubation with NaCl yields the monomeric αβ species. The inclusion of 1 M NaCl in the steady-state kinetic measurements done with the hexameric enzyme form led to a 3–6-fold decrease of the $V_{max}$ value with respect to that measured in the absence of NaCl (15, 23, 55). The $K_m$ values for NADPH and 2-OG increased by 2 orders of magnitude, and that for L-Gln increased 5–10 fold. The αβ species exhibited the same $V_{max}$ values as the hexameric one but 3-fold lower $K_m$ values for 2-OG and L-Gln, which are fully reflected in the catalytic efficiency ($V_{max}/K_m$) with these two substrates (Table 2). That dissociation brought about an increase in the catalytic efficiency of the enzyme was confirmed by the correlation of the decrease of the GltS radius (as monitored by dynamic light scattering) and the increase of initial velocity of reactions containing subsaturating concentrations of the substrates and 1 M NaCl when GltS was incubated with 1 M NaCl (supplemental Fig. 8). The modest effect of the oligomerization state on the kinetic properties of the enzyme makes it unlikely that the monomer/hexamer equilibrium significantly affects the biological function of GltS.

**DISCUSSION**

By combining cryo-EM, SAXS, and molecular modeling, it was possible to propose a structural model of the (αβ)6 hexam-

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**TABLE 2**

steady-state kinetic parameters of the glutamate synthase reaction catalyzed by the hexameric and monomeric forms of GltS.

| Substrate | $V_{max}$ | $K_m$ | $K_{cat}$ | $K_{m}$ | $V_{max}/K_m$ |
|-----------|-----------|-------|-----------|--------|--------------|
| NADPH     | 10.8 ± 0.9| 750 ± 90| 2.10 ± 0.5| 5.1    | 2.10 ± 0.5   |
| 2-OG      | 19.6 ± 1.0| 245 ± 30| 0.69 ± 0.09| 15.4  | 0.69 ± 0.09  |
| L-Gln     | 8.5 ± 0.2 | 230 ± 20| 3.0 ± 0.05 | 12.0  | 3.0 ± 0.05   |

*For comparison, the $V_{max}$ and $K_m$ values obtained for GltSHis under similar experimental conditions in the absence of NaCl in the assays are shown (15). Similar results are obtained with the native GltS (23, 55).*
The Hexameric NADPH-GltS Structure

The effective complex of the NADPH-dependent bacterial GltS, the essential enzyme of ammonia assimilation (Figs. 2 and 3). The combined approach allowed us to reconstruct the GltS particles and to solve any ambiguity that might have arisen in the reconstruction process. The high resolution reached (9.5 Å), which led to the observation of secondary structure elements, allowed us to fit the electron density with the atomic models of the GltS α and β subunits. Thus, we obtained information on the interprotomeric and intraprotomeric interface regions and, more importantly, on the structure of the αβ protomer, which is here confirmed to be the minimal catalytically active unit.

Our work exploited the complementarity of the two structural methods for the analysis of oligomeric assemblies, which is particularly important for dynamic situations where dissociation cannot be excluded. In cryo-EM analyses, images of individual particles can be selected and processed, allowing one to pick the species of interest. In SAXS, the scattering from the entire ensemble is recorded, and monodispersity has to be assumed for the ab initio or rigid body analysis. The particle symmetry cannot be directly deduced from the scattering data, and additional information is required. In Ref. 54, both gel filtration and SAXS data pointed to a tetrameric assembly, and a P222 symmetry was adopted for the reconstruction. The differences between the “old” and “new” SAXS models in supplemental Fig. 7 are not only due to a stronger dissociation of the old preparation but, mainly, to the different symmetry assumptions. The models generated without the symmetry from the previous and new data were rather similar to each other, albeit having lower resolution. Under the assumption of D3 symmetry, the independently generated ab initio SAXS model agrees well with the cryo-EM data. This example stresses the necessity of verification of sample monodispersity for the structural studies. Indeed, for a monodisperse system, SAXS is able to assess the oligomeric composition (thus, to provide a good estimate of the possible symmetry) simply by evaluating the MM of the solute. For mixtures, the experimental MM value from SAXS is an average over the ensemble, and, if dissociation is present, it underestimates the mass of the highest oligomer. At the same time, our work underlines the strength of SAXS in providing direct quantitative characterization of mixtures of different species in solutions as a function of concentration and of the presence of various factors.

In the GltS αβ hexamer, extended contacts are made within the α subunits forming the body of each one of the pillars (Figs. 1, E and F, and 2), which correspond to the crystallographic asymmetric unit. The interpre otomeric contacts between α-α and α-β subunits are weaker. The model is consistent with several observations. In the crystals of αGltS, α2 dimers form the asymmetric unit. However, adjacent dimers interact with each other forming tetramers resembling the cryo-EM determined oligomer, missing one of the pillars (supplemental Fig. 1). The presence of interprotomeric α-β contacts explains why the αGltS oligomer, which also forms hexamers at high protein concentration and low ionic strength (supplemental Table 3; supplemental Figs. 4 and 5), dissociates more readily than the αβ hexamer. The same interpreotomeric α-β contacts may rationalize the effect of NADP⁺ on the oligomerization state of the enzyme. NADP⁺, although at a saturating concentration, induced partial dissociation of the (αβ)₆ hexamer (with no activity loss; not shown) and led to the appearance of a species that could be identified with the (αβ)₂ dimer forming the hexamer pillar. Indeed, NADP⁺ binding to βGltS might induce a conformational change sufficient to weaken the interaction with the α subunit of the adjacent protomer. In support of NADP⁺-induced conformational changes is the fact that it was found to modify the kinetics of proteolytic cleavage of both α and β subunits with trypsin and chymotrypsin in the native GltS (56). Finally, the minimal stable unit of GltS is the αβ protomer, which is obtained at high ionic strength.

The interpreotomeric α-α and α-β contacts both involve residues of the C-terminal domain of the α subunit (residues 1203–1479; Fig. 3A). The core of this domain is formed by a right-handed β helical structure (residues 1226–1395), which is followed by three α helices (residues 1420–1433, 1438–1445, and 1447–1453) resting on the surface of the β helix. The rest of the crystallographically detectable polypeptide leads to a heptapeptide (residues 1473–1479), which was unresolved (i.e. disordered) in the crystallographic αGltS structure. More specifically, the interpreotomeric α-β interactions involve residues of the 1438–1454 α helices resting on the β helix core, which face residues 370–385 of βGltS of the neighboring αβ protomer (Fig. 3C). The interpreotomeric α-α interactions instead involve mainly residues 1214–1237 belonging to the N-terminal region of the β helical domain of one αGltS and residues of the FMN domain (i.e. residues 838–848, 870–880, and 894–902) of the other (Fig. 3B).

Thus, it appears that the C-terminal domain of αGltS, whose core is formed by a so far unique right-handed β helical fold, serves as a structural spacer in three ways: (i) its β helical core region participates in building the α subunit keeping the amidotransferase domain and the synthase domain in place and allowing them to form the intramolecular ammonia tunnel (Fig. 3A) (9); (ii) the α helices protruding from it and resting on its side mediate interprotomeric α-β contacts while (iii) its N-terminal part is implicated in the interpreotomeric α-α contacts.

To our knowledge, no protein of known structure, other than GltS, has been found to contain this β helical structure, so that it remains to be established if this fold plays a similar role in other proteins. Data bank searches revealed that the only proteins showing regions of significant sequence similarity with the C-terminal region of αGltS are the C subunits of tungsten- or molybdenum-dependent formyl methanofuran dehydrogenases (see Interpro classification: IPR002489). However, to our knowledge, no data are available on the specific role of this fold in these enzymes.

Because of the relatively low resolution of the current density map in terms of atomic interpretation and the absence of obvious residues that might be specifically responsible for the interpreotomeric α-α and α-β interactions, efforts to induce monomerization of GltS by site-directed mutagenesis were done by constructing deletion mutants. The deletion of the C-terminal 7 residues of αGltS (residues 1473–1479), which are unresolved in the crystal structure of αGltS,
led to a fully active enzyme, similar to the wild-type species with respect to stability and aggregation state. On the contrary, the deletion of the C-terminal 40 residues of GltS led to no protein production. This result indicates that the deletion prevents protein folding, in agreement with the fact that the deleted protein fragment forms the 1438–1454 α helices that cover a highly hydrophobic surface of the β helix and completes the β helical core contacting the seventh and final turn (Fig. 3A).

The structure of the αβ protomer extracted from the (αβ)6 hexamer is consistent with the hypothesis that the αβ protomer is the minimal catalytically active unit of GltS. In both the (αβ)6 hexamer and in the αβ protomer extracted from it, the three catalytic subunits are accessible to substrates, and no interprotomer electron transfer pathways can be observed, confirming previous spectroscopic observations (57). The spacial arrangement of the five cofactors is linear (Fig. 2E), implying that electron transfer from FAD to FMN occurs in two subsequent one-electron transfer events, with the two electrons following the same path. The \( E_m \) values of the oxidized and the hydroquinone forms of the FAD and FMN cofactors and of the oxidized and reduced [3Fe-4S] cluster have been determined (58). Estimates of the \( E_m \) values of the oxidized/semiquinone and semiquinone/hydroquinone forms of the flavins have also been calculated (58) (supplemental Table 5).

With those values and assuming that the two [4Fe-4S] clusters are equipotential with an \( E_m \) value as low as that of the NADP+/NADPH couple (−340 mV) (53, 58), the scheme shown in Fig. 5 can be built. This pathway differs from the previously proposed bifurcated electron transfer pathway (58) for a greater number of thermodynamically unfavored steps. The latter are, however, possible within an overall thermodynamically favored reaction with precedents found in, for example, the well characterized fumarate reductase (59).

Interestingly, Phe54 of βGltS, a residue conserved as a Phe or a Tyr in essentially all βGltS, seems to be suitably positioned to mediate the electron transfer from the βGltS [4Fe-4S] cluster closest to the interface to the [3Fe-4S] center in αGltS (Fig. 2E).

The question of the physiological meaning of the protomer/hexamer equilibrium in GltS remains open. We have measured the apparent steady-state kinetic parameters \( V_{max} \) and \( K_m \) for the three GltS substrates in the presence of 1 M NaCl using

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**FIGURE 5.** Electron transfer pathway in NADPH-GltS. The geometry of the five redox centers of GltS αβ protomer extracted from the cryo-EM-determined hexameric complex (Fig. 2) and the estimates of their midpoint potential \( (E_m) \) values (supplemental Table 5) (53, 58) allow us to propose that electron transfer from FAD to FMN takes place with two one-electron transfer processes that follow the same pathway. However, the energetics of transfer of the first (empty circle, continuous arrows, steps 2–4) and the second electron (full circle, dashed arrows, steps 5–7) differ due to the different \( E_m \) values of oxidized/semiquinone and semiquinone/hydroquinone forms of both flavin cofactors. In these schemes, the two low potential [4Fe-4S] clusters of GltS (53) are assumed to be equipotential with \( E_m \) values in the range of that of the NADP+/NADPH couple (−340 mV). The two-electron transfer process from NADPH to FAD (step 1), and from FMN hydroquinone to the 2-iminoglutamate intermediate resulting from the addition of ammonia and 2-OG (step 8) are shown with the broad arrows containing the electron pair. In the top panel, the electron transfer process is depicted using the \( E_m \) values determined for unliganded GltS, whereas in the lower panel, those determined for GltS in complex with 2-OG are used. It is not known if 2-OG binding precedes reduction of GltS cofactors, although it binds to oxidized GltS, and which is the effect of the 2-iminoglutamate intermediate on the redox properties of the cofactors.
envelope solutions that had been preincubated in the absence or presence of NaCl (1 M, 20 h). NaCl in reaction mixtures containing the hexameric species brought about 10–100-fold increase of the $K_m$ values for α-Gln, 2-OG, and NADPH (Table 2). This was accompanied by a 3–6-fold decrease of the $V_{\text{max}}$ value. The same experiments carried out with enzyme that had been preincubated with 1 M NaCl to ensure dissociation into αβ protomers showed $V_{\text{max}}$ values similar to those measured with the hexameric form. However, the $K_m$ values for 2-OG and α-Gln were ~3-fold lower than those measured for the hexameric form. The resulting modest (3-fold) increase of the catalytic efficiency with 2-OG and α-Gln of the αβ protomer, as compared with the hexameric form, leads to the conclusion that the oligomerization behavior of GltS may be relevant for its effect on its functionality in vivo. Rather, it may be suggested that the oligomerization state of GltS may be relevant for its interaction with (so far unknown) regulatory ligands or other proteins.

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