Supplementary Information for:

The subnanometer resolution structure of the glutamate synthase 1.2 MDa hexamer by cryo-electron microscopy and its oligomerization behavior in solution: functional implications.

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Supplementary Methods.

Construction of pETGltSHisΔ7 and pETGltSHisΔ40. The 3302 bp *Sma*I-*BamH*I fragment of the *glt*B gene encoding the α subunit of GltS was subcloned into pUC18 digested with the same enzymes (Agnelli, P., Mulazzi, S. and Vanoni M.A., unpublished). This plasmid (pPS) was used as the template for the introduction of stop codons followed by *BamH*I restriction sites at positions 4524 (Δ7) and 4437 (Δ40) of *glt*B (the numbering is that obtained by assuming the first nucleotide of the start codon of *glt*B as nucleotide 1). Polymerase chain reactions (PCR) were set up by mixing pPS (40 ng), Oligo 1 and Oligo 2 (or Oligo 3) (10 pmol each), the 4 dNTPs (10 nmol each) and Pfu DNA polymerase (1.5 U, Stratagene) in a final volume of 50 µl. The final buffer composition was 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 0.1 mg/ml nuclease-free bovine serum albumin.

Oligo 1: 5'-ggataacaatttcacacaggaaacagctatgacc-3'

Oligo 2: 5'-ggccttcgg***ggatcc***<u>**ttatta**</u>cagatggaccggaacttccaggcggttgagc-3'

Oligo 3: 5'-cggcggc***ggatcc***<u>**ttatta**</u>gaagcggctctgcgtctccgtcacatgctcttcg-3'

Oligo 1 is the sequence between nucleotides 464-497 of pUC18 and is upstream of the *Sma*I site. Oligo 2 and Oligo 3 are complementary to *glt*B coding strand in the 4494-4533 and 4392-4432 regions, respectively, except for the presence of the stop codons (underlined) and the *BamH*I site (italic). In bold are the mismatching bases.

PCR conditions were as follows: cycle 1: 1 min at 94 °C; cycle 2-34: 10 s at 98°C, 30 s at 55°C and 7 min at 68°C; cycle 35: 15 min at 68°C. Five parallel reactions were carried out and pooled at the end of the PCR cycles. The amplified 3339 and 3238 bp fragments were purified using the Wizard SV Gel and PCR Clean-up system kit (Promega), precipitated and digested with *Sma*I and *Bam*HI (GE Healthcare). After agarose gel electrophoresis, the extracted fragments were ligated with the 6411 bp fragment of pETGltSHis digested with the same restriction enzymes and purified. pETGltSHis is a pET23b derivative for the coproduction of the C-terminally His_6-tagged βGltS and the wild-type αGltS (1) each one under the control of the T7 promoter. The ligation was carried out using the T4 ligase (GE Healthcare) according to the manufacturer's instructions. After 2.5 h at 25°C, the ligation mixture was used to transform *E. coli* DH5α cells made competent with $CaCl_2$ using standard protocols (2,3). The inserts of

plasmids extracted from the transformants were sequenced (PRIMM srl, Milano), and, for each type of mutant, one of the plasmids that was found to carry the desired mutations was used in further studies. pETGltSHisΔ7 and pETGltSHisΔ40 were used to transform *E.coli* BL21(DE3) cells. Standard protocols were used for DNA and cell manipulations (2,3).

Production of the GltSHisΔ7 variant was carried out as described for GltSHis (1) except that IPTG was omitted. Namely, main cultures were maintained at 30°C until an absorbance value of approximately 2.0 was reached. The temperature was then lowered to 25°C and cells were harvested after 15-17 h. In experiments aimed to produce the GltSHisΔ40 variant, the cells were also grown at 25°C for 6-7 h. Alternatively, when an absorbance of approximately 2.0 was reached, the temperature was lowered to 15°C, IPTG was added to a final concentration of 0.1 mM, and cells were harvested after 17 h. Under no condition the production of the polypeptide corresponding to the truncated α subunit was observed.

The GltSHisΔ7 variant of GltS was purified as done for the native species (1) with similar chromatographic behavior, yields, specific activities at all stages.

Miscellaneous techniques. Absorbance spectra were recorded with a Hewlett Packard 8452 diode-array spectrophotometer (Agilent Technologies). Dynamic light scattering measurements were carried out using a DynaPro Instrument (Protein Solutions, Charlottesville, USA) in a 50 µl quartz cuvette at 15°C (average of twenty 30 s acquisitions, sensitivity 70-100%). Data were analyzed using the DynaPro software (version V5 or V6). Analytical gel filtration chromatography was carried out on a Superose 6 column (GE Healthcare).

Supplementary Table 1. Reversibility and time-frame of the salt-induced dissociation of GltS.

	Treatment	Radius nm	Polydispersity %	Specific Activity $U \cdot mg^{-1}$
Experiment 1[a]	None	10.43	21.2	20.3
	Dialysis against buffer with 1 M NaCl	5.43	14.5	18.0
	Dialysis against buffer without NaCl	10.08	11.8	19.4
Experiment 2[b]	None	10.19	11.0	22.3
	Incubation with buffer with 1 M NaCl	6.62	28.9	20.2
	Centrifugal gel filtration into buffer without NaCl	9.24	14.1	19.2

[a] A 800 µl aliquot of a GltS solution (1.7 mg/ml) in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 1 mM DTT and 1 M NaCl was dialyzed overnight against the same buffer containing 1 M NaCl (1 l) at 5°C in the dark. After 16 h a 200 µl aliquot was withdrawn and analyzed, while the dialysis tubing containg the residual sample was transferred to buffer without NaCl and dialyzed overnight. On the aliquots withdrawn before dialysis and after each one of the two equilibrations steps activity, protein and dynamic light scattering (DLS) measurements were carried out. Aliquots (100 µl) were also analyzed on a Superose 6 analytical gel filtration column equilibrated with dialysis buffer (not shown). The shift in the radius measured by DLS was mirrored by changes in the elution volume of the protein from the analytical gel filtration column. The small decrease of specific activity after dialysis against 1 M NaCl is explained by the irreversible dissociation of a small amount of GltS into free α and β subunits as detected by analytical gel filtration and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) of individual fractions from the column (not shown).

[b] A 200 µl aliquot of GltS (1 mg/ml) was incubated overnight on ice with buffer containing 1 M NaCl. A 70 µl aliquot was rapidly transferred into buffer without NaCl by centrifugal gel filtration on Bio-Spin columns (BioRad) in buffer without NaCl, which were eluted with 50 µl aliquots of the same buffer. Essentially all the undiluted protein was found in the first fraction. Aliquots of the protein solution immediately after dilution with 1 M NaCl, after overnight incubation and immediately after centrifugal gel filtration were used for activity, protein and DLS measurements.

Supplementary Table 2. Effect of solvent ionic strength on the size of αGltS as determined by dynamic light scattering.

Solutions (1 mg/ml) of αGltS were incubated in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 1 mM DTT, in the presence of different NaCl concentrations at 15°C. At different times aliquots (100 µl) were withdrawn, centrifuged for 15 min at 13000 rpm in a microfuge in the cold and the supernatant was subjected to dynamic light scattering measurements. The calculated radius in nm is reported.

Time, min	NaCl, M			
	0	0.15	0.5	1
0	9.6	—	—	—
15	—	8.7	6.0	5.5
60	—	8.6	5.7	—
120	—	9.0	5.6	—
240	—	9.0	5.4	—
300	9.4	—	—	—
420	—	8.1	5.3	—
1200	8.8	—	—	5.0

Supplementary Table 3. Overall parameters and volume fractions of the components in αGltS solutions obtained by SAXS.

The corresponding plots are shown in Supplementary Fig. 5. R_g and MM are the radius of gyration and the molecular mass calculated from the scattering data, respectively. The observed polydispersity of the samples is expressed by relative percentages of αGltS present as α monomers, α_2 dimers and α_6 hexamers in solution. The discrepancy between the computed curve and the experimental data is denoted as χ.

Additions	Plot # in Supplementary Fig. 5	α, mg/ml	R_g, nm	MM, kDa	α, %	α_6, %	χ
None	01	5.0	6.44±0.05	766±100	16	84	12.05
None	02	2.0	7.08±0.10	754±100	18	82	5.60
2 M NaCl	03	2.0	3.98±0.10	140±20	95	5	1.37
1 M NaCl	04	2.0	4.00±0.10	156±20	95	5	1.59

Supplementary Table 4. Purification of GltSHisΔ7.

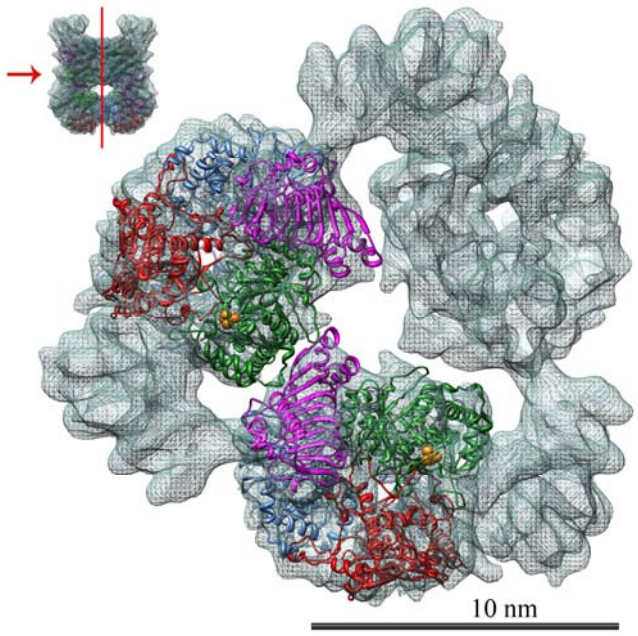
GltSHisΔ7 was purified from 33 g of *E. coli* BL21 (DE3) cells transformed with pETGltSHisΔ7 and grown as described in the Supplementary Methods section. Purification was carried out at 0-5°C as described for the corresponding wild-type form (1). Activity is expressed as μmol NADPH oxidised per minute per ml of enzyme solution at 25°C in 50 mM Hepes/KOH buffer, pH 7.5, in the presence of 0.1 mM NADPH, 2.5 mM 2-OG and 5 mM L-Gln.

	Activity U	Protein mg	Specific activity $U \cdot mg^{-1}$	Purification -fold	Yield %
Crude Extract	5457	3213	1.7	1	100
NiNTA-Sepharose	4337	229	19	11	79
Sephacryl S 300	2448	114	21.5	13	45

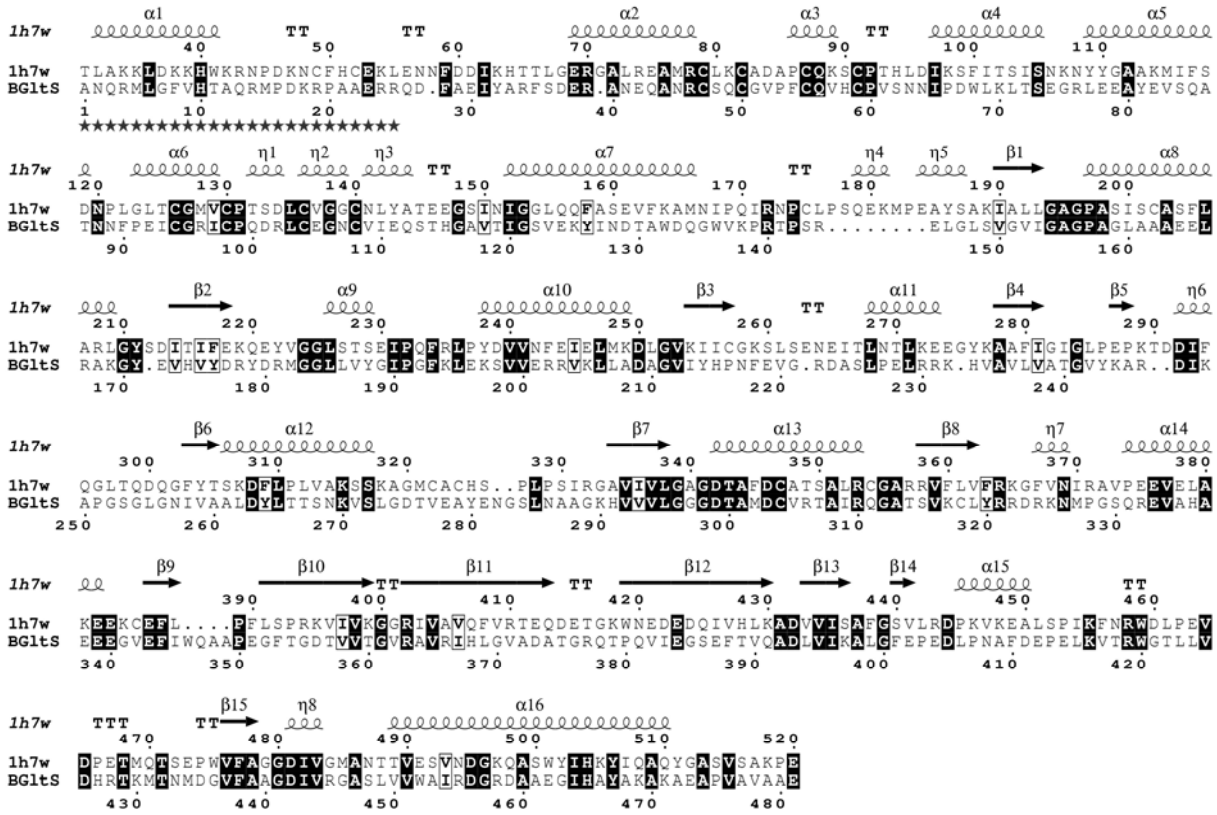
Supplementary Table 5. Summary of mid-point potential values estimated for the GltS cofactors.

Mid-point potential (E_m) values of the oxidized (ox)/hydroquinone (hq) couples for FAD and FMN and of the oxidized and reduced form of the [3Fe-4S] cluster have been calculated for the free and 2-OG bound GltS at pH 7.5 and 20°C in (4). 2-OG was found to affect only the E_m values of the [3Fe-4S] cluster and of the FMN cofactor. Estimates of the E_m values of oxidized/semiquinone (sq) and sq/hq couples of FAD and FMN have been obtained on the basis of the lack of accumulation of the semiquinone form of the flavins, which implies that, in both cases, the E_m values of the ox/sq couple is at least 120 mV less negative than that of the corresponding sq/hq couple.

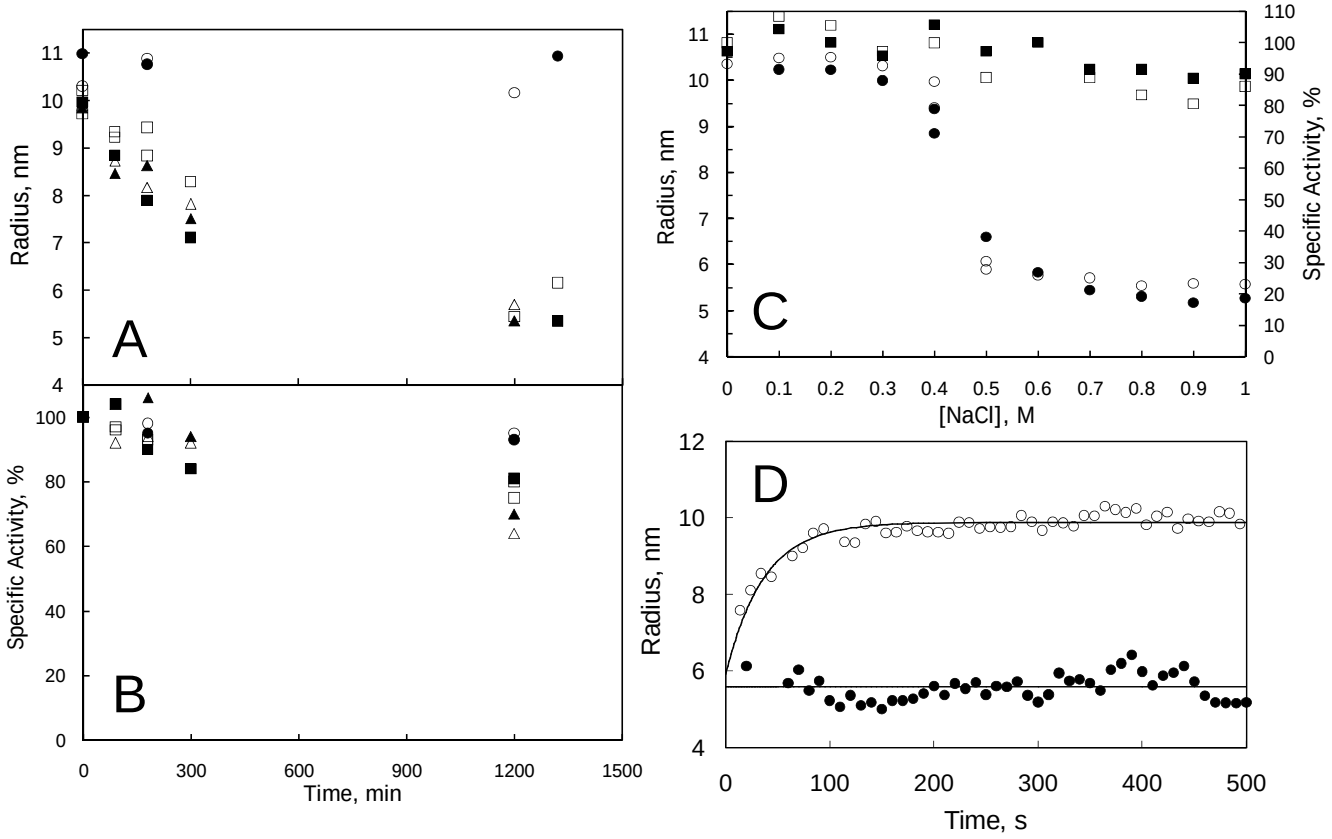
Enzyme	Redox Couple	E_m (mV)
GltS	FAD_{ox}/FAD_{hq}	-300
	FAD_{ox}/FAD_{sq}	-360
	FAD_{sq}/FAD_{hq}	-240
	$[3Fe\text{-}4S]^{+1}/[3Fe\text{-}4S]^{0}$	-280
	FMN_{ox}/FMN_{hq}	-240
	FMN_{ox}/FMN_{sq}	-300
	FMN_{sq}/FMN_{hq}	-180
GltS + 2-OG	FAD_{ox}/FAD_{hq}	-300
	FAD_{ox}/FAD_{sq}	-360
	FAD_{sq}/FAD_{hq}	-240
	$[3Fe\text{-}4S]^{+1}/[3Fe\text{-}4S]^{0}$	-190
	FMN_{ox}/FMN_{hq}	-270
	FMN_{ox}/FMN_{sq}	-339
	FMN_{sq}/FMN_{hq}	-210
	$NADP^{+}/NADPH + H^{+}$	-340
	$(2\text{-}OG + NH_3)/L\text{-}Glu$	-126



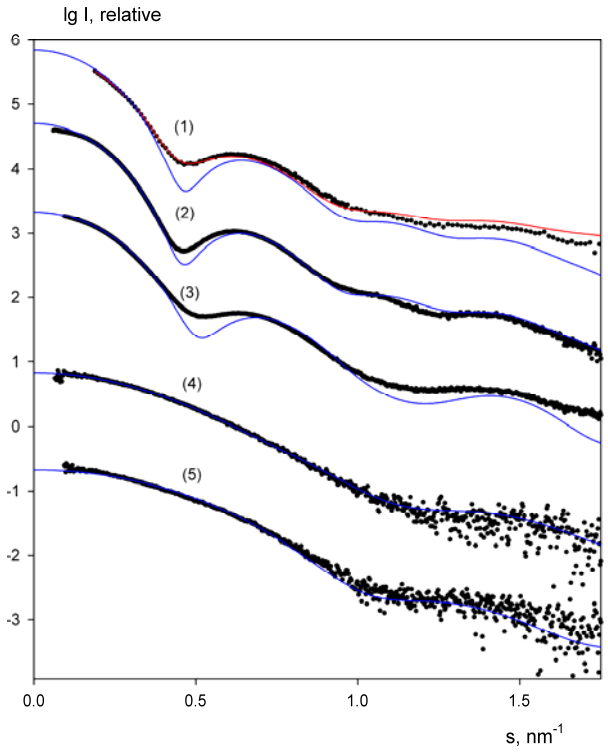
Supplementary Figure 1. Comparison of the 9.5 Å cryoEM volume of the GltS $(\alpha\beta)_6$ hexamer and of two adjacent α_2 dimers observed in the αGltS crystals. Fitting of two α_2 dimers found in two adjacent asymmetric units in crystals of αGltS within the cryoEM density map of GltS shows how the α_2-α_2 contacts in adjacent *pillars* of the $(\alpha\beta)_6$ hexamer are similar to those found in the αGltS crystals. The clipping plane and the direction of view are indicated in the image on the left. The color code is as in Fig. 1 C,D, main text.



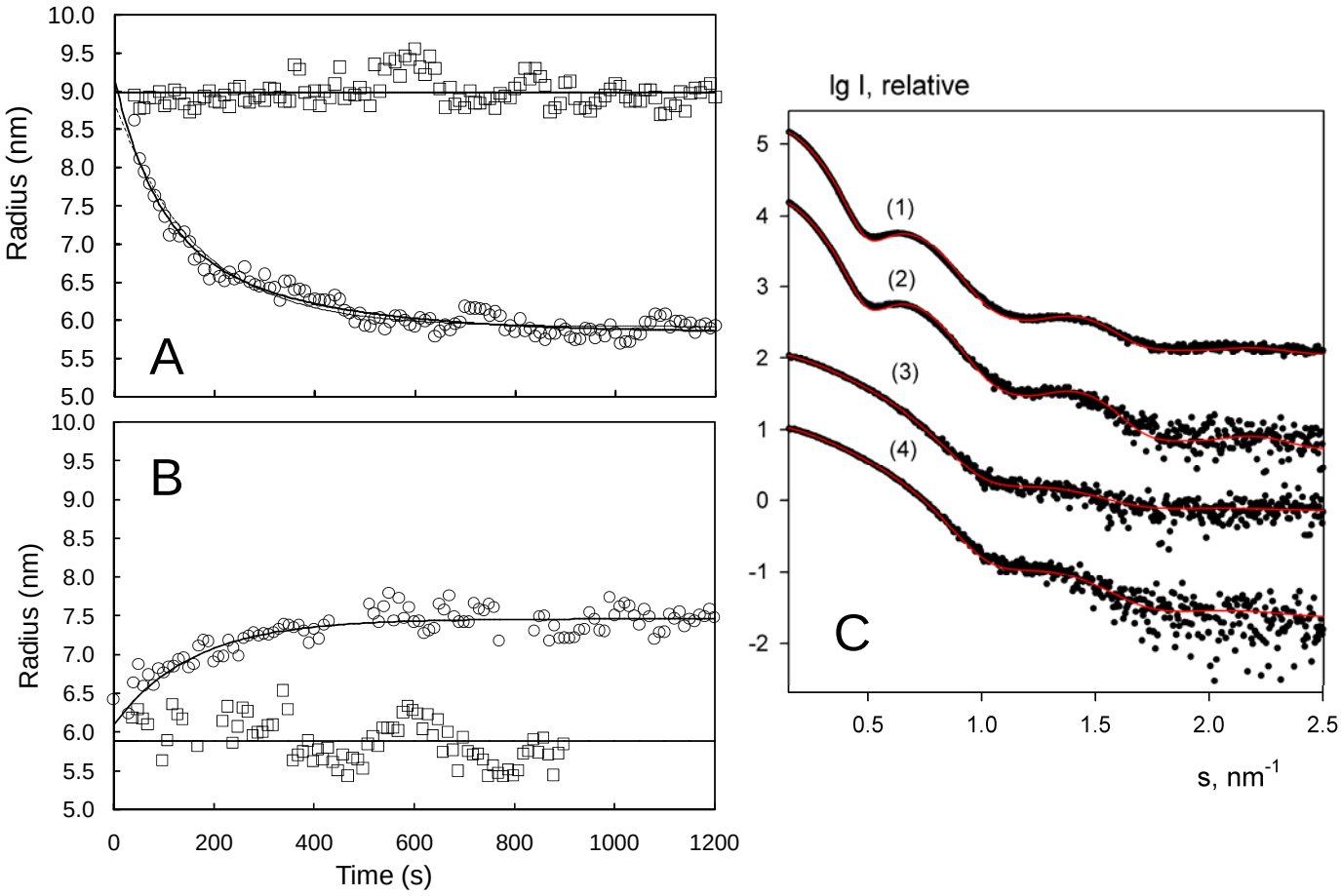
Supplementary Figure 2. Sequence alignment used to generate the homology model of βGltS. Sequence alignment of βGltS (residues 1-480) and of the N-terminal domain of DPD (PDB ID 1h7w, chain A, residues 31-520, (5)). Identical residues (in white against black background) and conservative substitutions (bold and boxed) have been calculated according to the substitution matrix BLOSUM62. The secondary structures of 1h7w are indicated above the alignment, and have been calculated by DSSP (6). The stars indicate the first 25 residues of βGltS, which were not modeled.



Supplementary Figure 3. Effect of solvent ionic strength on the size of GltS as determined by dynamic light scattering. Panels A-C: solutions (1 mg/ml) of GltSHis (empty symbols), GltSHisΔ7 (full symbols) were incubated in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 1 mM DTT, in the presence of different NaCl concentrations at 15°C. Panels A and B: at different times aliquots (100 µl) were withdrawn, centrifuged for 15 min at 13000 rpm in a microfuge in the cold and the supernatant was subjected to activity, protein and dynamic light scattering measurements. The calculated radius (A) and specific activities (B) as a function of time are shown. NaCl concentrations were: 0 M (circles), 1 M (squares) and 2 M (triangles). Panel C: activity, protein and dynamic light scattering measurements were done on samples incubated with 0-1 M NaCl for 17 h. The calculated radius (circles) and specific activities (squares) of GltSHis (empty symbols) and GltSHisΔ7 (full symbols) are shown. Similar experiments done with GltS samples yielded similar results. Panel D: kinetics of re-association of GltSHis (1.2 mg/ml) that had been incubated for 17 h in the presence of 1 M NaCl upon 10-fold dilution in buffer devoid of NaCl. The figure shows the radius calculated by DLS during 10 s acquisitions (open circles). The continuous line shows the fit of the calculated radius at the different times to a single exponential process (Radius = 3.95(1-exp(-0.027*t) + 5.93). A control sample was diluted 10-fold in buffer containing 1 M NaCl (closed circles). The line shows the average of the calculated radius, 5.59 nm.



Supplementary Figure 4. Experimental SAXS data from different GltS preparations and the scattering from the models. Comparison of the scattering curves obtained with GltS and αGltS solutions at low and high ionic strength. (1) Previous preparations of GltS (MM ~ 900 kDa, (7)); (2) newly acquired scattering curves of GltS at low ionic strength and high protein concentration (10 mg/ml in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 1 mM DTT, MM ~ 1200 kDa); (3) 5 mg/ml αGltS at low ionic strength (MM ~ 760 kDa); (4) 1.6 mg/ml GltS solution in the presence of 1 M NaCl (MM ~ 260 kDa); (5) 2 mg/ml αGltS in the presence of 2 M NaCl (MM ~ 140 kDa). In (1) the red line is OLIGOMER fit with 80% $(\alpha\beta)_6$ hexamers and 20 % αβ protomer. Blue solid lines indicate the scattering curves computed with CRYSOL (8) using the cryoEM-derived structure of the $(\alpha\beta)_6$ hexamer (curves 1,2), the α_6 hexamer (curve 3) and the αβ protomer (curve 4) extracted from it, and the crystallographic coordinates of αGltS (curve 5). The deviation from the experimental curve may be attributed to the polydispersity of the solutions.

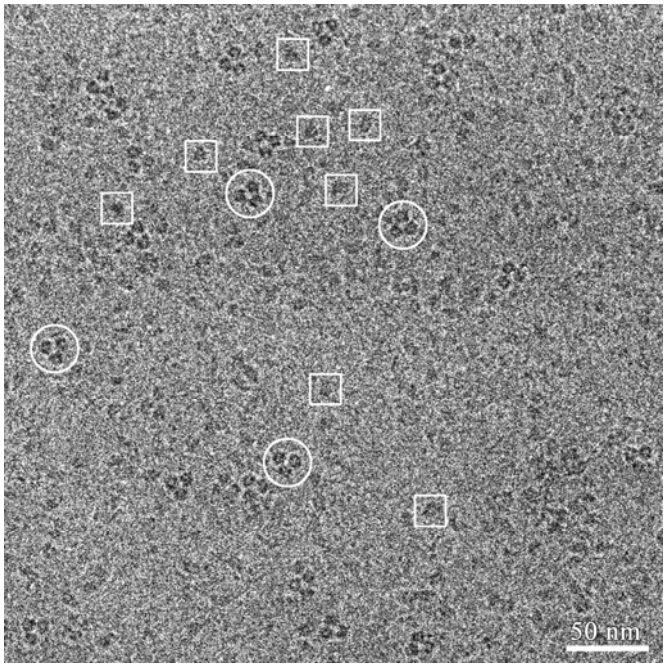


Supplementary Figure 5. Dissociation behaviour of αGltS.

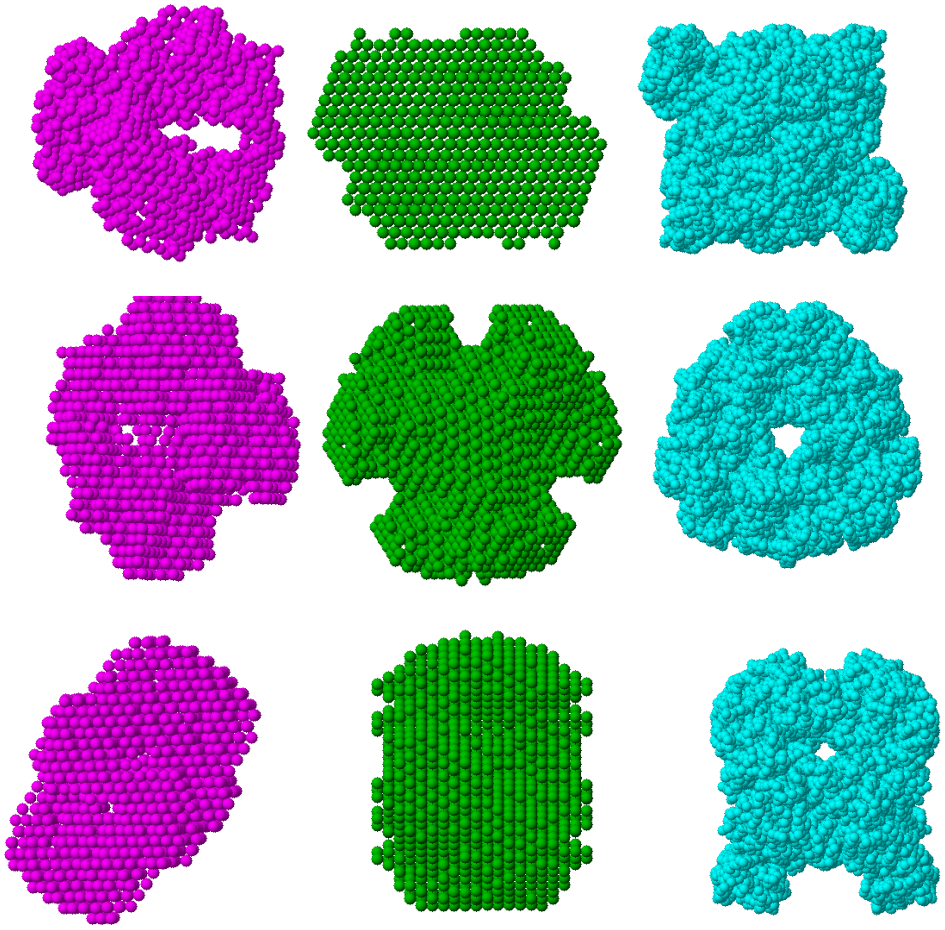
Panel A: The kinetics of dissociation of αGltS were determined by diluting a protein sample into 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 1 mM DTT and 1 M NaCl (final concentration) to obtain a 1.5 mg/ml αGltS solution. The solution was immediately transferred in the DLS cuvette and 10 s signals were acquired for 20 min (open circles). The calculated radius is shown as a function of time (in s). The continuous line shows the fit of the calculated radius to a double exponential decay curve (Radius = 1.89exp(-0.012*t) + 1.42exp(-0.0034*t) + 5.84); the thin dotted line is the fit to a single exponential decay curve (Radius = 2.93exp(-0.0062*t) + 5.92). The data were better fitted with the former equation suggesting that an intermediate species (perhaps the α_2 dimer) is formed as an intermediate. The corresponding values obtained in a control experiment in which the protein was diluted in the absence of NaCl are shown (open squares). The line shows the average of the calculated radius throughout the experiment (8.98 nm).

Panel B: The kinetics of re-association of αGltS were determined by incubating a 2 mg/ml enzyme sample in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA and 1 mM DTT in the presence of 1 M NaCl for 1 h. The sample was diluted 10-fold into buffer containing no NaCl, and the radius was measured by DLS as described for the experiment shown in Panel A (open circles). The curve shows the fit of the data to a single exponential process (Radius = 1.37(1-exp(-0.006*t) + 6.09). A control sample was diluted into buffer containing 1 M NaCl (open squares). The average of the calculated radius was 5.9 nm. The lack of recovery of the α_6 hexameric form is attributed to the combination of low protein concentration (0.1 mg/ml) and the presence of 0.1 M NaClin the solution, confirming the lower stability of the α_6 hexamer than that of the GltS oligomer (see Supplementary Figure 3D).

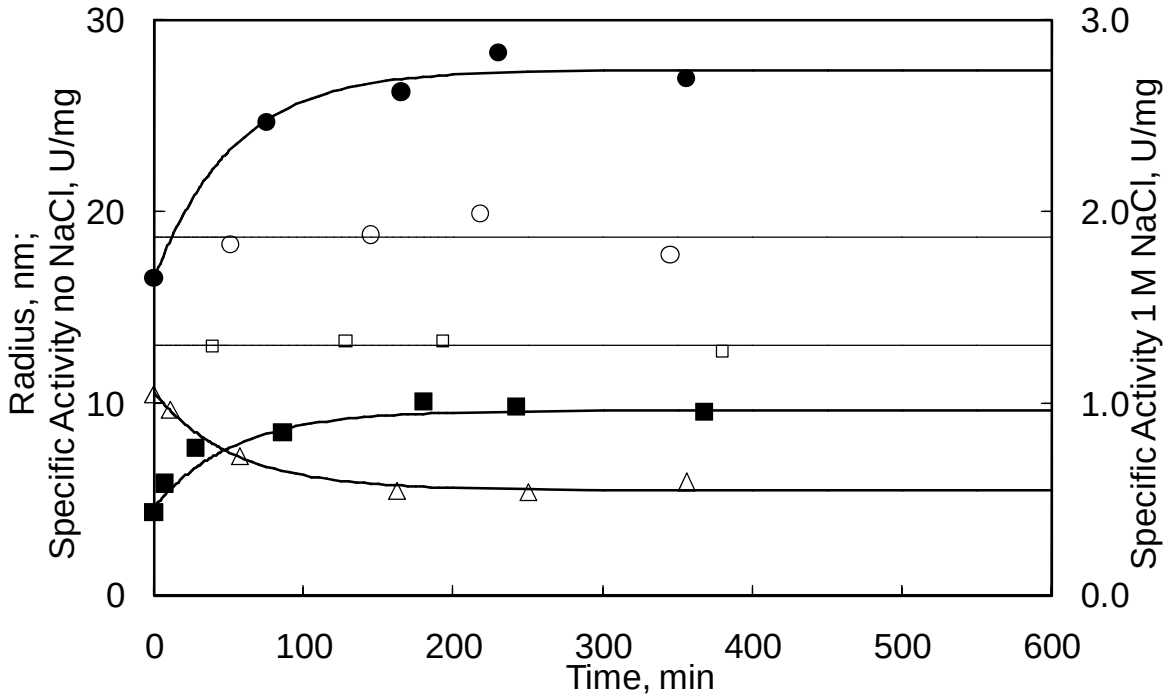
Panel C: SAXS plots measured on the αGltS samples described in Supplementary Table 3. Plots [1-2]: 5-2 mg/ml αGltS. Plots [3-4]: 2 mg/ml αGltS in the presence of 2 M and 1 M NaCl showing an almost complete dissociation of the protein into α monomers. Experimental data are denoted by dots, fits from OLIGOMER are presented as red solid lines.



Supplementary Figure 6. Cryoelectron micrograph of αGltS. Aliquots of a 7 mg/ml αGltS solution were analysed by cryoEM as described for GltS holoenzyme. Several types of oligomeric forms are visible from α monomers and α_2 dimers (rectangles) up to hexameric α_6 (circles) complexes, in full agreement with the SAXS data of Supplementary Figure 5 and Table 3.



Supplementary Figure 7. Comparison of GltS ab initio SAXS models reconstructed from the old (magenta) and the new (green) data with the cryo-EM model (blue). The models are shown using space filling representation. The middle view is rotated by 90 degrees about the horizontal axis with respect to the top line orientation. The bottom view results from further rotation by 90 degrees about the vertical axis.



Supplementary Figure 8. Correlation between the oligomerization state of GltS and the activity at sub-saturating concentrations of the enzyme substrates. A GltSHis sample (1.2 mg/ml) was incubated in 25 mM Hepes/KOH buffer, pH 7.5, 1 mM EDTA, 1 mM DTT and 1 M NaCl on ice. At different times aliquots were withdrawn for the measurement of the particles' radius by DLS (30 s acquisitions for 15 min, open triangles) and of the GltS activity in the presence of 5 mM L-Gln, 2.5 mM 2-OG, 50 mM Hepes/KOH buffer, pH 7.5 in the absence (open circles) or presence (closed circles) of 1 M NaCl or 0.5 mM L-Gln, 0.25 mM 2-OG in the absence (open squares) or presence (closed squares) of 1 M NaCl. Due to the effect of NaCl on the kinetic parameters of GltS, the assays containing 1 M NaCl were carried out at sub-saturating concentrations of substrates (Table 2). As expected from a decrease of the K_M values for 2-OG and L-Gln upon dissociation of GltS, the measured activity of the sample incubated in the presence of 1 M NaCl increased as the radius decreased at both substrates concentrations. Due to the rapid protein re-association at low ionic strength, no effect of incubation with 1 M NaCl was observed in assays carried out in the absence of 1 M NaCl. A control experiment in which GltS was incubated in the absence of NaCl showed no change of the radius (10.4 nm) and of the activity measured under the four different conditions after up to 20 hours of incubation (not shown). The curves show the fit of the measured radius and activity values in the NaCl-treated sample assayed in the presence of NaCl to functions describing processes that occur at the same rate of 0.019 min^{-1}.

Movie: file movie1.mov

Structure of the NADPH-dependent glutamate synthase hexamer: cryoEM density map and fitting of atomic data. The cryoEM volume, represented here by an isosurface, undergoes rotations about its D3 symmetry axes. The fitted αGltS and βGltS polypeptides are shown in ribbons. βGltS is yellow; αGltS is color coded as follows: glutamine amidotransferase domain, residues 1-422, blue; central domain, residues 423-780, red; FMN/synthase domain, residues 781-1202, green; C-terminal β helical domain, residues 1203-1472, purple. The cofactors are in spacefill and orange.

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