Combined use of small-angle X-ray scattering and functional studies: the case of glutamate synthase

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Glutamate synthase (GltS)

- The reaction
- Why studying GltS?
- (Some of) the known properties
- Structural information from SAXS
Glutamate synthase (GltS): the reaction

\[
\text{L-glutamine + 2-oxoglutarate} \quad \xrightarrow{\text{A}_{\text{red}} \xrightarrow{\text{A}_{\text{ox}}} \ x} \quad 2(\text{L-glutamate})
\]
Why studying glutamate synthase?

- GltS plays an essential role in ammonia assimilation in microorganisms and plants, thus:
  - target for drug design (in pathogens)
  - target of metabolic engineering for improved biofertilizers
  - target of metabolic engineering for controlling NAD(P)⁺/NAD(P)H levels and that of 2-OG in cells used for bioconversions
GltS plays a role in ammonia assimilation

Nitrogen is the second most abundant element in living organisms after Carbon

<table>
<thead>
<tr>
<th>Element</th>
<th>Dry Weight (%)</th>
<th>Elements Present in Trace Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>61.7</td>
<td>B</td>
</tr>
<tr>
<td>N</td>
<td>11.0</td>
<td>F</td>
</tr>
<tr>
<td>O</td>
<td>9.3</td>
<td>Si</td>
</tr>
<tr>
<td>H</td>
<td>5.7</td>
<td>V</td>
</tr>
<tr>
<td>Ca</td>
<td>5.0</td>
<td>Cr</td>
</tr>
<tr>
<td>P</td>
<td>3.3</td>
<td>Mn</td>
</tr>
<tr>
<td>K</td>
<td>1.3</td>
<td>Fe</td>
</tr>
<tr>
<td>S</td>
<td>1.0</td>
<td>Co</td>
</tr>
<tr>
<td>Cl</td>
<td>0.7</td>
<td>Cu</td>
</tr>
<tr>
<td>Na</td>
<td>0.7</td>
<td>Zn</td>
</tr>
<tr>
<td>Mg</td>
<td>0.3</td>
<td>Se, Mo, Sn, I</td>
</tr>
</tbody>
</table>


### Table 3–5: Molecular Components of an *E. coli* Cell

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage of total weight of cell</th>
<th>Approximate number of different molecular species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>70</td>
<td>1</td>
</tr>
<tr>
<td>Proteins</td>
<td>15</td>
<td>3,000</td>
</tr>
<tr>
<td>Nucleic acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RNA</td>
<td>6</td>
<td>&gt;3,000</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Lipids</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Monomeric subunits and intermediates</td>
<td>2</td>
<td>500</td>
</tr>
<tr>
<td>Inorganic ions</td>
<td>1</td>
<td>20</td>
</tr>
</tbody>
</table>
The nitrogen cycle

Nitrate $\rightarrow$ N$_2$ $\rightarrow$ Ammonia $\rightarrow$ Nitrite $\rightarrow$ Nitrate

Reduction by some anaerobic bacteria, most plants

N$_2$ fixation by some bacteria (e.g., Klebsiella, Azotobacter, Rhizobium)

Synthesis in plants and microorganisms

Degradation by animals and microorganisms

Amino acids and other reduced nitrogen-carbon compounds

Ammonia assimilation pathway

$\text{NH}_3$ $\rightarrow$ 2-OG $\rightarrow$ L-Gln

Glutamate dehydrogenase (GDH)

NADPH $\rightarrow$ NADP$^+$

GS: Glutamine synthetase

ATP

L-Glu $\rightarrow$ L-Gln

ADP + Pi

L-Glu $\rightarrow$ L-Glu
Glutamine and Glutamate are key amino acids


Glutamine-dependent amidotransferases
The amide N of glutamine can be viewed as a non-toxic form of NH₃ and is made available for biosyntheses by L-glutamine-dependent amidotransferases, an expanding class of enzymes.

A conserved glutaminase site within the conserved glutamine amidotransferase domain

An unrelated synthase site for the formation of the aminated or amidated product

2 main classes of amidotransferase domains

Common problem: Control and coordination of catalysis at a distance
Three main classes of glutamate synthases

Bacterial NADPH-GltS
- 1 x 150 kDa; 1 x 50 kDa
- 1 x FAD, 1 x FMN
- 1 x [3Fe/4S], 2 x [4Fe/4S]

Fd-GltS
- 1 x 160 kDa;
- 1 x FMN
- 1 x [3Fe/4S]

Eukaryotic NAD(P)H-GltS
- 1 x 200 kDa;
- 1 x FAD?, 1 x FMN?
- 1 x [3Fe/4S]?, 2 x [4Fe/4S]?
The (poorly characterized) archeal form of glutamate synthase derives from the assembly of individual domains of the bacterial GltS.
Why studying glutamate synthase?

It is a complex iron-sulfur flavoprotein:
- A multi-domain/multi-subunit protein
- With multiple redox centers

Evolutionary history of nowadays proteins
Protein-protein interaction

Novel redox centers or variations on old themes
Model to study the assembly of Fe/S clusters
Model to study the structural determinants/modulation of electron transfer (ET) among redox centers
Our approach:

gene cloning, engineering & expression

• protein (over)production & purification
  • structure-function studies
    – steady-state & pre-steady-state kinetics
    – absorbance & fluorescence spectroscopies

– EPR, NMR (D. Edmondson, Atlanta; W.R. Hagen, Delft)
  – X-ray crystallography (Andrea Mattevi, Pavia)
– Small-angle X-ray scattering (Dmitri Svergun, EMBL-Hamburg)
  – Cryoelectron microscopy (Nicolas Boisset, Paris VI)
  – Molecular dynamics (V. Coiro, A. Di Nola, Rome)
Model of GltS reaction
NADPH-GltS catalyzes and must coordinate 3 reactions that take place at separate sites.

Glutaminase reaction of the PurF (Ntn) type amidotransferase domain:

1. L-Gln → L-Gln
2. L-Gln + NH₃ → L-Glu + 1NH₂
3. L-Glu + H₂O → L-Glu + H₂O
4. L-Glu + H₂O → L-Glu + H₂O
5. L-Glu + H₂O → L-Glu + H₂O
6. L-Glu + H₂O → L-Glu + H₂O

Synthase site:

2-OG + H⁺ + 2 e⁻ → 2-IG + L-Glu

NADPH oxidizing site:

NADPH-GltS catalyzes and must coordinate 3 reactions that take place at separate sites.
Low Temperature EPR studies of Ab-GltS

\[ 1 \times [3\text{Fe-4S}]^0, +1 \]

\[ 2 \times [4\text{Fe-4S}]^{+1,+2} \]

Magnetic Field (Gauss)
In NADPH-GltS the glutaminase and synthase sites are tightly coupled so that 1 L-Glu is formed from 2-OG with 1 L-Gln being hydrolyzed and 1 NADPH being reduced.

No glutamine hydrolysis in the absence of NADPH and 2-OG
$^{15}$N-NMR spectroscopy to monitor NADPH-GltS reaction

**Figure 4:** Attempt to detect exchange of ammonia during GltS reaction with exogenous $^{15}$N-enriched ammonium ion. A solution containing 10 mM each L-glutamine, 2-oxoglutarate, NADPH, and $^{15}$N-enriched ammonium nitrate in 100 mM Hepes/K$^+$, pH 7.5/10 mM EDTA was made anaerobic. NMR spectra were recorded before and 18 h after the addition of GltS (0.15 unit).
In the isolated $\alpha$ subunit of NADPH-GltS the tight coupling of the glutaminase and synthase activities is partially lost:

The $\beta$ subunit not only serves to transfer reducing equivalents to the synthase site, but also to ensure the tight control of the glutaminase and synthase reaction in the $\alpha$ subunit through protein-protein interaction and conformational changes.
Lack of equilibration between FMN and 3Fe/4S in the isolated GltS $\alpha$ subunit
The β subunit is required to establish redox communication between the 3Fe/4S cluster and FMN on the α subunit.
The formation of the [4Fe-4S] clusters of NADPH-GltS requires the co-production of the α and β subunits.
Identification of the ligands of the 4Fe/4S centers of GltS:
effect of the C->A substitution in the Cysteine-rich regions of the GltS β subunit

Expected results:
no effect on isolated β subunit

Inactive αβ GltS protomer with some of the mutants?
The C/A mutant forms of the β subunit no longer associate with α subunit

The [4Fe-4S] clusters of NADPH-GltS are not only required to establish redox communication among centers, but also to structure the interface domain of the protomer.
Search for structural information on NADPH-GltS $\alpha\beta$ holoenzyme

1. Use X-ray crystallography
Crystallization experiments of NADPH-GltS in the presence of 2-OG and L-methionine sulfone (MetS, a L-Gln analog) yielded crystals of the $\alpha_2$ dimer

Mattevi et al. (Pavia)
The structure of the GltS $\alpha$ subunit dimer in complex with L-methionine sulfone (L-MetS) and 2-oxoglutarate (2-OG) confirmed the domain structure of $\alpha$GltS, the type of coenzymes present, the location of the substrate binding sites.

Binda et al., 2000
The structure of GltS α subunit shows 30 Å-long intramolecular tunnel for the transfer of ammonia released from L-glutamine at the glutaminase site to the synthase site.
The intramolecular «Ammonia Tunnel» that connects the conserved amidotransferase domain to the unrelated synthase domain is a common feature of amidotransferases.

The «ammonia tunnel» is a case of convergent evolution because it is formed by the unrelated synthase domain instead of the related amidotransferase domain.

The current scheme of amidotransferases

The textbook scheme of an amidotransferase
The amidotransferase and the synthase sites of amidotransferases communicate through (small?) conformational changes of residues of the tunnel.

There are differences among different amidotransferases with respect to:

- presence/shape of the tunnel in the absence of one of the substrates
- The degree of coupling between the glutaminase and the synthase site
Crystallization has trapped a catalytically inactive conformation of αGltS but indicates how the reaction takes place.

Loop 210-225 is open: ammonia would escape from the active site.

2-OG C(2) is well positioned to receive ammonia from the tunnel, but too far from the FMN N(5) position to be reduced.

Cys1 thiol points away from L-Gln C(5).

Obstruction of the tunnel.
NADPH-GltS catalyzes and must coordinate 3 reactions that take place at separate sites.

**Glutaminase reaction of the PurF (Ntn) type amidotransferase domain**

1. L-Glu $\rightarrow$ L-Gln + H$_2$O
2. L-Glu $\rightarrow$ L-Gln + H$_2$N$^+$
3. L-Gln + H$_2$O $\rightarrow$ L-Glu + NH$_3$
4. L-Glu + H$_2$N$^+$ $\rightarrow$ L-Glu + H$_2$NH$^+$
5. L-Glu + NH$_3$ $\rightarrow$ L-Glu + H$_2$N$^+$
6. L-Glu $\rightarrow$ L-Gln + H$_2$O

**Synthase site**

- $\text{COO}^- \text{NH}_3 \rightarrow \text{COO}^- \text{C} = \text{NH}_2 \rightarrow +\text{H}_3\text{N} \text{C} = \text{H}$
- 2-OG $\rightarrow$ 2-IG $\rightarrow$ L-Glu

**NADPH oxidizing site**
Study the ferredoxin-dependent GltS (FdGltS) from Synechococcus to look for alternative conformations.
The Structure of Fd-GltS is overall similar to that of αGltS except for:

- The presence of a loop at the surface of the synthase domain (“Fd loop”), which might serve for the docking of ferredoxin (Fd)
- The conformation of Cys1 is «catalytically competent»

Van den Heuvel et al. 2002-2004
The “Ferredoxin loop” may participate in the control of the glutamate synthase activity of the enzyme.
Reduced Fd activates Fd-GltS reaction and establishes redox communication between FMN and the 3Fe/4S cluster

Ravasio, S., Dossena, L., 2002-2003
Fd/Fd-GltS Stoichiometry?
Where does Fd bind?
Which is the catalytically relevant species?
Methods to determine binding stoichiometries:

- Gel filtration (needs tight complexes or lots of protein)
- Analytical ultracentrifugation (tight complexes (?))
- Biacore (surface plasmon resonance) (needs large change of mass on binding)
- Fluorescence-monitored titrations in the UV or Vis region
- Absorbance-monitored titrations in the Vis region
- Equilibrium dialysis (large amount of protein)
- Centricon
- Chemical Cross-linking
Small Angle X-ray Scattering Studies on Fd/FdGltS to study complex stoichiometry

Size, Mass

Compare scattering curve with known 3D structure

Size, Mass

Compare scattering curve with those obtained with individual proteins

Compare scattering curves with models of Fd/Fd-GltS complexes.
Small Angle X-ray Scattering Studies on Fd/FdGltS show that FdGltS is a monomer in solution and that it forms a 1:1 complex with Fd.

<table>
<thead>
<tr>
<th></th>
<th>Rg, nm</th>
<th>M, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Fd + Fd/FdGltS complex</td>
<td>1.43</td>
<td>12</td>
</tr>
<tr>
<td>FdGltS+Fd (1:1)</td>
<td>3.58</td>
<td>175</td>
</tr>
<tr>
<td>FdGltS+Fd (1:2)</td>
<td>3.63</td>
<td>191</td>
</tr>
<tr>
<td>Free Fd + Fd/FdGltS complex</td>
<td>3.38</td>
<td>146</td>
</tr>
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</table>
The proposed catalytically active FdGltS form

Catalytically competent species

L-Glu

L-Gln + 2-OG

Priming steps
(Some) potential targets of site-directed mutagenesis from analysis of the structure
E978 (E1013 in FdGltS) is the only residue of the synthase domain making contacts with residues of the glutaminase site: this residue is well positioned to signal the presence of 2-OG to the glutaminase and to the tunnel entrance.
Production and characterization of the E1013D, E1013N and E1013A mutants of Fd-GltS:

E1013D: Glu 1013 is substituted by another acidic aminoacid (Asp) but it is moved away by one methylene group;
E1013N: the negative charge is neutralised but the residue can still form hydrogen bonds (Asn);
E1013A: the side chain is removed (Ala)
Activities of FdGltS with $^{14}$C-labelled substrates

Glutamine-dependent GltS activity (overall reaction):
$L$-Gln + 2-OG + dithionite + Fd $\rightarrow$ $^*L$-Glu + L-Glu
$L$-Gln + *2-OG + dithionite + Fd $\rightarrow$ L-Glu + *L-Glu

Ammonia-dependent GltS activity (synthase site only)
$\text{NH}_3$ + *2-OG + dithionite + Fd $\rightarrow$ *L-Glu

Glu:INT oxidoreductase activity (synthase site only)
L-Glu + INT $\rightarrow$ 2-OG + $\text{NH}_3$ + INT$_{\text{red}}$

Glutaminase activity (glutaminase site only)
*L-Gln + $\rightarrow$ *L-Glu + $\text{NH}_3$

Separation of L-Gln, L-Glu and 2-OG on Dowex 1X8

Ferredoxin

Ammonia tunnel

GAT site
The E1013D, N and A variants of FdGltS

The effect of E1013 D, N and A substitutions is confined to the activities that involve the glutaminase site.

E1013 D, N and A substitutions significantly affect \( V \) but \( K_m \) for Gln and 2-OG only to a limited extent.

The negative charge of the 1013 residue is important for catalysis.

<table>
<thead>
<tr>
<th>Relative activity</th>
<th>( V ) (min(^{-1}))</th>
<th>( K_{L\text{-Gln}} ) (mM)</th>
<th>( K_{2\text{-OG}} ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>7500</td>
<td>2.4</td>
<td>0.98</td>
</tr>
<tr>
<td>E1013D</td>
<td>84</td>
<td>( \sim 10 )</td>
<td>0.52</td>
</tr>
<tr>
<td>E1013N</td>
<td>1.23</td>
<td>1.5</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Robert van den Heuvel, Laura Dossena, 2007
The E1013D, N and A variants of FdGltS

The E1013D/N/A mutants are not trapped as the Glutamyl-thioester intermediate. E1013 does not seem to be important for acid/base catalysis.

Conclusions:

The E1013D/N/A mutants are affected in the early steps of the glutaminase reaction, perhaps because E1013 is important for the correct formation of the oxyanion hole by interacting with N227.
Compare the initial velocities of formation of L-Glu from *L-Gln (○) or from *2-OG (●) to determine the degree of coupling between the sites.

\[
\text{L-Gln} + 2\text{-OG} \xleftrightarrow{\text{Fd}_{\text{red}}} \text{Fd} \xrightarrow{\text{L-Glu} + \text{L-Glu}} \text{L-Glu}
\]
E1013N/FdGltS:

The redox state and the presence of Fd activate the glutaminase activity, but the coupling is partial.
Sigmoid kinetics cannot be explained with a “classical” allosteric effect because SAXS told us that FdGltS is monomeric.

E1013D/FdGltS:
Coupled but sigmoid kinetics when L-Gln is varied at fixed (high) 2-OG.
Small Angle X-ray Scattering Studies on Fd/FdGltS show that FdGltS is a monomer in solution and that it forms a 1:1 complex with Fd.

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</tr>
</tbody>
</table>
Schemes leading to sigmoid kinetics in monomeric enzymes
(Segel, Enzyme kinetics, 1978)
The E1013D/FdGltS variant reveals a two-step activation process of the glutaminase site completed by L-Gln.
Search for structural information on NADPH-GltS $\alpha\beta$ holoenzyme

1. More crystallization trials: Use GltSHis

Obtain crystals containing both subunits, but very thin and do not diffract

2. Use SAXS to build a (low resolution) model of NADPH-GltS and study its behaviour in solution
The First series of SAXS measurements gave nice curves and reasonable models....

.....but:

- Some prep to prep variability
- The calculated mass was Not fully consistent with the tetrameric oligomerization state (Mr, 800000) expected from gel filtration
- Inconsistent correlation between effect of NaCl on dissociation of the holoenzyme into αβ protomers......
Combined use of Small Angle X-ray Scattering (SAXS) and low-temperature electron microscopy (cryoEM) to build a model of NADPH-GltS $\alpha\beta$ holoenzyme

Magali Cottevieille, Slavica Jonic, Eric Larquet, Nicolas Boisset (Paris VI, cryoEM)
Maxim Pethoukov, Dmitri Svergun (EMBL-Hamburg, SAXS)
Gianluca Caprini, Stefano Paravisi (MI)
Low-temperature electron microscopy (cryoEM)
Nicolas Boisset, Magali Cottevieille, Eric Larquet, Paris VI

60000 particles analyzed; 13000 particles used for reconstruction

Multivariate statistical analysis and 2D averages
From the initial CryoEM model at 20 Å resolution, GltS is a 1.2MDa \((\alpha\beta)_6\) hexamer:

The \(\alpha_2\) crystallographic dimer forms the body of each one of the three pillars, with \(\beta\text{GltS}\) at the periphery, attached to the corresponding \(\alpha\) subunit.
At the same time: New SAXS Measurements on several different GltS preparations and different conditions

GltS forms \((\alpha \beta)_6\) hexamers, arranged as a core of \(\alpha_6\) with 6 \(\beta\) subunits at the periphery, but even at high protein concentration and low ionic strength there are some lower mass species.
Rigid-body Fitting of the X-ray structures into the SAXS-corrected cryoEM model at 9.5 Å resolution.

A βGltS model based on the N-terminal domain of dihydropyrimidine dehydrogenase for which the structure is known.

The X-ray αGltS dimer.
How does the cryoEM/SAXS oligomeric model correlate with the crystal structure of the GltS α subunit?

Adjacent αGltS dimers in the crystals

The X-ray αGltS dimer in the asymmetric unit

9.5 Å resolution
Extraction of information from the GltS model:

- The interface regions
- The structure of the $\alpha\beta$ protomer
- The ET pathway
- Effect of oligomerization on the catalytic activity of GltS
The Interprotomeric $\alpha/\alpha$ and $\alpha/\beta$ contacts are mediated by the C-terminal $\beta$ helical domain of GltS $\alpha$ subunit.
The C-terminal domain of GltS α subunit is based on a novel right-handed β helix that acts as a “spacer”:

(1) to hold the GAT and synthase domains in place (with the central domain) and

(2) to assemble the oligomer.
The $\alpha\beta$ protomer model extracted from $(\alpha\beta)_6$ hexamer indicates a linear electron transfer path between the flavins, which is consistent with the redox potential values and behavior of the Flavin s and Fe/S centers.
SAXS Measurements:

GltS forms \((\alpha\beta)_6\) hexamers, arranged as a core of \(\alpha_6\) with 6 \(\beta\) subunits at the periphery, but even at high protein concentration and low ionic strength there are some lower mass species.

\[\ln I(s), \text{ relative}\]

\begin{align*}
\text{low ionic strength} & : \alpha\text{-GltS} \\
\text{high ionic strength} & : \text{GltS} \\
\end{align*}

\[s, \text{ nm}^{-1}\]

80% + 20%

5% + 95%

>80% + < 20%

5% + 95%

\((\alpha\beta)_6\)

\(\alpha\)

\(\alpha_6\)

\(\alpha_2\)

\(\alpha\beta\)
Effect of ionic strength and ligands on the GltS aggregation state from SAXS
**Effect of ionic strength and ligands on the GltS aggregation state from SAXS**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>none</th>
<th>MetS, 2-OG, MetS+2-OG</th>
<th>NADP⁺ or AADP (3-aminopyrimidine dinucleotide phosphate)</th>
<th>NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>GltS or GltSHis</td>
<td>&gt;80% $(\alpha\beta)_6$</td>
<td>~ 80% $(\alpha\beta)_6$</td>
<td>&lt;60% $(\alpha\beta)_6$</td>
<td>&lt;20% $(\alpha\beta)_6$</td>
</tr>
<tr>
<td></td>
<td>&lt;20% else</td>
<td>~ 20% else</td>
<td>&lt; 20% $(\alpha\beta)_2$</td>
<td>&gt;20% else</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;20% else</td>
<td>&gt;80% $(\alpha\beta)$</td>
</tr>
</tbody>
</table>

NaCl weakens both $\alpha/\alpha$ and $\alpha/\beta$ interprotomeric contacts

NADP⁺ weakens only the $\alpha/\beta$ interprotomeric contacts

20 nm
NADP$^+$ weakens the $\alpha/\beta$ interprotomeric contacts

The NADP effect is consistent with conformational changes induced by NADP binding to $\beta$-GltS, as previously detected by limited proteolysis.

<table>
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<tr>
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<th>MetS, 2-OG, MetS+2-OG</th>
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<td>&lt;20% else</td>
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</tr>
<tr>
<td></td>
<td>~ 80% ($\alpha\beta)_6$</td>
<td>&gt; 20% else</td>
<td>&gt;20% else</td>
</tr>
</tbody>
</table>

$(\alpha\beta)_6$ top view
Is the equilibrium between \((\alpha \beta)_6\) hexamers and \(\alpha \beta\) protomers physiologically relevant?

Our first plan:
- Try to Stabilize the \(\alpha \beta\) protomer by site directed mutagenesis
- Study the kinetic properties of the \(\alpha \beta\) protomer

GltSHisΔ7
Same as wt

GltSHisΔ40
No protein expressed
Our second (back-up) plan:

- Use NaCl to dissociate the \((\alpha \beta)_6\) hexamer into \(\alpha \beta\) protomers to Study the kinetic properties of the \(\alpha \beta\) protomer
Study of the dissociation behavior of the GltS \((\alpha\beta)_6\) hexamer (and of the GltS \(\alpha\) subunit) as a function of \([\text{NaCl}]\) by using:

- dynamic light scattering,
- gel filtration chromatographies,
- activity measurements

(Use the information to design SAXS measurements).
Dissociation behavior of the GltS (αβ)₆ hexamer as a function of [NaCl] by using dynamic light scattering, gel filtration, activity measurements.

Dissociation is slow ($k_{diss} = 0.019 \text{ min}^{-1} = 0.0003 \text{ s}^{-1}$ @ 6 μM GltS (as αβ protomers))

Dissociation leads to a small activity loss due to irreversible dissociation of some GltS into free α and β.

Full dissociation into αβ protomers is observed only at > 1 M NaCl with overnight incubation.
Dissociation behaviour of GltS ($\alpha\beta)_6$ hexamer caused by NaCl.

Dissociation is reversible.

Dialysis + Gel-chromatography

0 M NaCl

1 M NaCl, ON

0 M NaCl

Dynamic light scattering

Dilute 10-fold/no NaCl

Reassociation is fast

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radius</th>
<th>Polydispersity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10.43</td>
<td>21.2</td>
<td>20.3</td>
</tr>
<tr>
<td>Dialysis against buffer with 1 M NaCl</td>
<td>5.43</td>
<td>14.5</td>
<td>18.0</td>
</tr>
<tr>
<td>Dialysis against buffer without NaCl</td>
<td>10.08</td>
<td>11.8</td>
<td>19.4</td>
</tr>
<tr>
<td>None</td>
<td>10.19</td>
<td>11.0</td>
<td>22.3</td>
</tr>
<tr>
<td>Incubation with buffer with 1 M NaCl</td>
<td>6.62</td>
<td>28.9</td>
<td>20.2</td>
</tr>
<tr>
<td>Centrifugal gel filtration into buffer without NaCl</td>
<td>9.24</td>
<td>14.1</td>
<td>19.2</td>
</tr>
</tbody>
</table>

$k$, 0.027/s
The GltS $\alpha_6$ hexamer is less stable than the $(\alpha\beta)_6$ form.

Dissociation is (relatively) fast, and is reversible

$\alpha_2$ dimers and $\alpha$ monomers are detected by SAXS

$\alpha_2$ dimers are detected by cryoEM
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 α2 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).

Best fit with:
Radius = 1.89e^{-0.012*t} + 1.42e^{-0.0034*t} + 5.84
Suggesting: \( \alpha_6 \rightarrow \alpha_2 \rightarrow \alpha \)

Alternative fit:
Radius = 2.93e^{-0.0062*t} + 5.92
Implying: \( \alpha_6 \rightarrow \alpha \)

Radius = 1.37(1-e^{-0.006*t} + 6.09)
Is the equilibrium between \((\alpha\beta)_6\) hexamers and \(\alpha\beta\) protomers physiologically relevant? Is it a regulatory mechanism?
- Use NaCl to dissociate the \((\alpha\beta)_6\) hexamer into \(\alpha\beta\) protomers

- Compare the kinetic properties of the \((\alpha\beta)_6\) hexamer with those of the \(\alpha\beta\) protomer under the same ionic strength conditions:

- \((\alpha\beta)_6\) hexamer: when concentrated enzyme is diluted into a assay with 1M NaCl the dissociation is slow: during the time of the assay, the species in solution is the \((\alpha\beta)_6\) hexamer

- \(\alpha\beta\) protomer: when enzyme that had been incubated overnight with 1 M NaCl is diluted into a solution with 1M NaCl, during the time of the assay the species in solution is the \(\alpha\beta\) protomer
Steady-state kinetics in 1 M NaCl of GltS preincubated in low and high salt:

- High NaCl inhibits GltS and causes an increase of the $K_m$ values for the substrates, especially $K_{\text{NADPH}}$ and $K_{2-\text{OG}}$.

- The catalytic efficiency of the $\alpha\beta$ protomer with L-Gln and 2-OG is 2-3-fold higher than that of the $(\alpha\beta)_6$ hexamer.

<table>
<thead>
<tr>
<th></th>
<th>NADPH</th>
<th>2-OG</th>
<th>L-Gln</th>
<th>$V_{\max}$</th>
<th>$K_{\text{NADPH}}$</th>
<th>$K_{2-\text{OG}}$</th>
<th>$K_{\text{L-Gln}}$</th>
<th>$V_{\max}/K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(αβ)$_6$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2500</td>
<td>0.5 - 10</td>
<td>10.8 ± 0.9</td>
<td>—</td>
<td>—</td>
<td>2.10 ± 0.5</td>
<td>—</td>
<td>5.1</td>
</tr>
<tr>
<td>100</td>
<td>30 - 5000</td>
<td>10</td>
<td>9.1 ± 0.4</td>
<td>—</td>
<td>750 ± 90</td>
<td>—</td>
<td>—</td>
<td>12.1</td>
</tr>
<tr>
<td>20 - 700</td>
<td>5000</td>
<td>10</td>
<td>19.6 ± 1.0</td>
<td>245 ± 30</td>
<td>—</td>
<td>—</td>
<td>80.0</td>
<td></td>
</tr>
</tbody>
</table>

| αβ     |        |        |         |             |                   |                  |                   |                |
| 100     | 2500   | 0.5 - 15 | 10.6 ± 0.3 | —             | —                 | 0.69 ± 0.09      | —                 | 15.4           |
| 100     | 500 - 5000 | 10 | 8.5 ± 0.2 | —             | 230 ± 20          | —                 | —                 | 36.0           |
| 20 - 700 | 5000   | 10  | 19.5 ± 0.9 | 175 ± 25      | —                 | —                 | 112.0          |

| (αβ)$_6^a$ |        |        |         |             |                   |                  |                   |                |
| 100     | 2500   | 0.1-2  | 63.0 ± 0.6 | —             | —                 | 0.17 ± 0.005     | 370             |
| 100     | 15-100 | 5      | 63.6 ± 0.8 | —             | 5.1 ± 0.4         | —                 | 12470         |
| 15-100  | 2500   | 5      | 70.1 ± 1.3 | 3.0 ± 0.5     | —                 | —                 | 23366         |

$^a$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).
Monitoring the increase of activity of GltS during dissociation with 1 M NaCl due to decrease of $K_m$ for 2-OG and L-Gln.

\[
L\text{-Gln} + 2\text{-OG} + \text{NADPH} + H^+ \rightarrow 2 \times L\text{-Glu} + \text{NADP}^+
\]

$k_{\text{obs}} = 0.019 \text{ min}^{-1}$

Note: at low (subsaturating) $[S]$, a decrease of $K_m$ will result in an increase of $v$. 

\[
v = \frac{V_{\text{max}}[S]}{K_m + [S]}
\]
Both species are catalytically active, but we observed a 3-fold increase of catalytic efficiency with 2-OG and L-Gln on dissociation, entirely due to a decrease of the $K_m$ for 2-OG and L-Gln.
Biological meaning of \((\alpha\beta)_6 / \alpha\beta\) equilibrium?

Possible other roles of the hexamer/protomer equilibrium:

Regulation by unknown ligand that will differentially bind to hexamer or protomer?

The hexamer may act as a scaffold for organizing other proteins?