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Neutron crystallographic study of heterotrimeric glutamine amidotransferase CAB

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Heterotrimeric glutamine amidotransferase CAB (GatCAB) possesses an ammonia-self-sufficient mechanism in which ammonia is produced and used in the inner complex by GatA and GatB, respectively. The X-ray structure of GatCAB revealed that the two identified active sites of GatA and GatB are markedly distant, but are connected in the complex by a channel of 30 Å in length. In order to clarify whether ammonia is transferred through this channel in GatCAB by visualizing ammonia, neutron diffraction studies are indispensable. Here, GatCAB crystals were grown to approximate dimensions of 2.8 × 0.8 × 0.8 mm (a volume of 1.8 mm^3) with the aid of a polymer using microseeding and macroseeding processes. Monochromatic neutron diffraction data were collected using the neutron single-crystal diffractometer BIODIFF at the Heinz Maier-Leibnitz Zentrum, Germany. The GatCAB crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 74.6, b = 94.5, c = 182.5 Å and with one GatCAB complex (molecular mass 119 kDa) in the asymmetric unit. This study represented a challenge in current neutron diffraction technology.

1. Introduction

The correct pairing of an amino acid with its cognate tRNA is an essential step in maintaining accurate translation (Carter, 1993). In the majority of bacteria and all archaea, the formation of Gln-$tRNA^{Gln}$ involves a noncanonical indirect pathway via a two-step process (Raushel *et al.*, 2003; Nakamura *et al.*, 2010; Massière & Badet-Denisot, 1998). In the first step, $tRNA^{Gln}$ is mischarged with glutamic acid by a nondiscriminating glutamyl-tRNA synthetase (GluRS). Next, glutamine amidotransferase CAB (GatCAB), which is a heterotrimeric complex composed of A, B and C subunits with a molecular weight of 119 kDa, converts mischarged Glu-$tRNA^{Gln}$ to Gln-$tRNA^{Gln}$ by three reactions (Nakamura *et al.*, 2006): (i) mischarged Glu-$tRNA^{Gln}$ is activated to γ-phosphoryl-Glu-$tRNA^{Gln}$ by GatB (a kinase), (ii) the glutaminase GatA catalyzes the hydrolysis of glutamine and produces ammonia for the subsequent reaction, and (iii) γ-phosphoryl-Glu-$tRNA^{Gln}$ is transamidated to Gln-$tRNA^{Gln}$ by GatB (a transamidase) using the ammonia produced by GatA.

The X-ray crystal structures of *Staphylococcus aureus* GatCAB–ADP–AlF_4 and GatCAB–Gln showed that the two identified catalytic centers, the glutaminase reaction center in GatA and the transamidase reaction center in GatB, are markedly distant (Nakamura *et al.*, 2006, 2010). An intramolecular channel of about 30 Å in length connecting the two

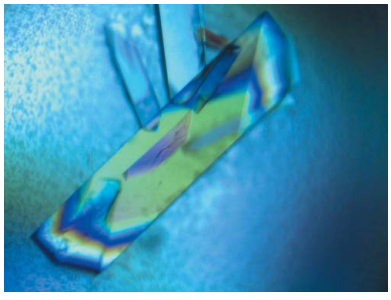

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Table 1
Macromolecule-production information (Nakamura *et al.*, 2006, 2010).

Source organism	*S. aureus* Mu50
Cloning site	NcoI/XhoI
Expression vector	pET-28b(+) vector (Novagen)
Expression host	*E. coli* B834 strain
Complete amino-acid sequence of the construct produced	
GatA	MSIRYESVENLLTLIKDKKIKPSDVVKDIYDAIEETDPTI KSFLALDKENAIKKAQELDELQAKDQMDGKLFGIPMGI KDNIITNGLETTCASKMLEGFVPIYESTVMEKLHKENA VLIGKLNMDEFAMGGSTETSYFKKTVNPFDHKAVPGGS SGGSAAAVAAGLVPLSLGSDTGGSIRQPAAYCGVVGMK PTYGRVSRFGLVAFASSLDQIGPLTRNVKDNAIVLEAI SGADVNDSTSAPVDDVDFTSEIGKDIKGLKVALPKEYL GEGVADDVKEAVQNAVETLKSLGAVVEEVSLPNTKFGI PSYYVIASSEASSNLSRFDGIRYGYHSKEAHSLEELYK MSRSEGFGKEVKRRIFLGTFALSSGYYDAYYKKSQKVR TLIKNDFDKVFENYDVVVGPTAPTTAFNLGEEIDDPLT MYANDLLTTPVNLAGLPGISVPCGQSNGRPIGLQFIGK PFDEKTLYRVAYQYETQYNLHDVYEKL
GatB	MHFETVIGLEVHVELKTDSKMFSPSPAHFGAEPNSNTNVI DLAYPGVLPVVNKRAVDWAMRAAMALNMEIATESKFDR KNYFYPDNPKAYQISQFDQPIGENGYIDIEVDGETKRI GITRLHMEEDAGKSTHKGEYSLVDLNRQGTPLIEIVSE PDIRSPKEAYAYLEKLRSIIQYTGVSDVKMEEGSLRCD ANISLRPYGQEKFGTKAELKNLNSFNYVRKGLEYEEKR QEEELLNGGEIGQETRRFDESTGKTILMRVKEGSDDYR YFPEPDIVPLYIDDAWKERVRQTIPELPDERKAKYVNE LGLPAYDAHVLTLTKEMSDFFESTIEHGADVKLTSNWL MGGVNEYLNKNQVELLDTKLTPENLAGMIKLIEDGTMS SKIAKKVFPELAAKGGNAKQIMEDNGLVQISDEATLLK FVNEALDNNEQSVEDYKNGKGKAMGFLVGQIMKASKGQ ANPQLVNQLLKQELDKRLEHHHHHH
GatC	MTKVTREEVEHIANLARLQISPEETEEMANTLESILDFAK QNDSADTEGVEPTYHVLDLQNVLREDKAIKGIPQELAL KNAKETEDGQFKVPTIMNEEDA

catalytic centers of GatCAB has been identified and proposed to be an ammonia channel. This ammonia channel couples the glutaminase and transamidase reactions, as the ammonia generated by GatA is transferred through the channel to the transamidase reaction center of GatB (Nakamura *et al.*, 2006; Horiuchi *et al.*, 2001). To elucidate the precise mechanism of ammonia transportation in GatCAB, the positions of hydrogens and the discrimination of water from ammonia molecules inside the channel are needed. However, this is challenging owing to the current limitations in detecting hydrogen positions, especially using X-ray crystallography, even if ultrahigh-resolution ($\leq$1.5 Å) data are collected (Blakeley *et al.*, 2015).

The neutron diffraction method can be used to directly determine the positions of H atoms (and isotopes) at moderate resolutions of 2.5 Å. This is because the neutron scattering length b_n of deuterium is $+0.667 \times 10^{-12}$ cm, which is larger than that of hydrogen ($b_n = -0.374 \times 10^{-12}$ cm) and is similar to those of carbon and oxygen (Sears, 1992). Furthermore, the neutron scattering length of nitrogen is 1.6 times that of oxygen. Comparison of the neutron structures of GatCAB and GatCAB–Gln will enable us to understand the ammonia-self-sufficient mechanism using the channel in GatCAB. Although neutron crystallography is a promising technique for elucidating the precise mechanism of ammonia transportation in GatCAB, two significant problems must be overcome. The first is the growth of large GatCAB crystals (with a volume of >1 mm^3) since the crystallization of *S. aureus* GatCAB shows low repeatability and the crystals usually have a narrow needle-like shape. The second is to obtain neutron diffraction data for GatCAB without overlap of Bragg peaks, because the unit cell of GatCAB is relatively large for neutron single-crystal diffractometers (Blakeley, 2009). Recently, Azadmenesh and coworkers reported the successful collection of time-of-flight (TOF) neutron diffraction data from a crystal with the largest unit-cell edge to date (240 Å) using the MaNDi instrument at the Spallation Neutron Source (Azadmanesh *et al.*, 2017, 2018).

Using the batch method with a polymer through microseeding and macroseeding processes, we successfully obtained large crystals suitable for neutron diffraction experiments. The neutron data were collected at room temperature and the neutron structure was successfully determined at 3.0 Å resolution. These results present a challenge to current neutron diffraction technology.

2. Materials and methods

2.1. Macromolecule production

The cloning, expression and purification of *S. aureus* GatCAB were carried out using previously described protocols (Nakamura *et al.*, 2006, 2010). For the neutron diffraction experiments, purified GatCAB was exchanged with deuterated buffer [20 m*M* Tris–DCl pD 7.5, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, 10%(*v*/*v*) glycerol-d_8; all components were dissolved in 99.9% D_2O] by repeated centrifugal ultrafiltration and dilution with deuterated buffer. For crystallization, GatCAB was concentrated to a final concentration of 15 mg ml^{-1} by ultrafiltration using Amicon Ultra centrifugal filter units. Macromolecule-production information is given in Table 1.

2.2. Crystallization

The crystallization solution (sample mixture), including GatCAB and crystallization buffer, was prepared by mixing 15 mg ml^{-1} GatCAB with deuterated crystallization buffer *A* [50 m*M* HEPES–NaOD pD 7.2, 5 m*M* $MgCl_2$, 3%(*v*/*v*) MPD, 26%(*w*/*v*) PEG 600, 4%(*w*/*v*) *n*-isopropylacrylamide-copolymerized PEG] in a ratio of 11:8. All of the components of the crystallization buffer were dissolved in 99.9% D_2O, and the sample mixture was centrifugated at 10 000 rev min^{-1} for 15 min at 4°C. The sample mixture was then used as the drop in both microseeding and macroseeding experiments. To overcome the problem of the low repeatability of GatCAB crystal formation, microseeding was first performed by the sitting-drop vapor-diffusion method in 96-well plates. A seed stock was prepared using deuterated buffer *B* [50 m*M* HEPES–NaOD pD 7.2, 5 m*M* $MgCl_2$, 3%(*v*/*v*) MPD, 15%–20%(*w*/*v*) PEG 600] while crushing the crystal with a pestle and was then centrifugated at 10 000*g* for 10 s. A 1 µl drop sample mixture with 0.1 µl seed stock was vapor-diffused with reservoir deuterated buffer *C* [50 m*M* HEPES–NaOD pD 7.2, 5 m*M* $MgCl_2$, 3%(*v*/*v*) MPD, 26%(*w*/*v*) PEG 600]. After incubation for two days, crystals of GatCAB appeared. Crystals of good quality were chosen for the subsequent

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Table 2
Crystallization.

Method	Batch method
Plate type	24-well sitting-drop plates (Hampton Research)
Temperature (K)	293
Protein concentration (mg ml^{-1})	15
Buffer composition of protein solution	50 m*M* HEPES–NaOD pD 7.2, 5 m*M* $MgCl_2$, 3%(*v*/*v*) MPD, 26%(*w*/*v*) PEG 600, 4%(*w*/*v*) *n*-isopropylacrylamide-copolymerized PEG; all components of the buffer were dissolved in 99.9% D_2O
Volume of drop (µl)	50

macroseeding experiment. In macroseeding, the volume of each drop was set to 50 µl sample mixture using 24-well sitting-drop crystallization plates (Hampton Research). No reservoir solution was added during crystal growth, as in the batch method. After a small seed crystal had been transferred to the drop, the crystal continued to grow to a volume of ~1.8 mm^3 (2.8 × 0.8 × 0.8 mm; Fig. 1) in one month. All crystallization experiments were carried out at room temperature. Crystallization information is given in Table 2.

2.3. Data collection and processing

The neutron diffraction experiment was performed using the single-crystal diffractometer BIODIFF at FRM-II at the Heinz Maier-Leibnitz Zentrum (MLZ), Germany. BIODIFF is a monochromatic diffractometer equipped with a cylindrical neutron image-plate detector (Niimura *et al.*, 1994). The cylindrical area detector provides high coverage of reciprocal space, thereby allowing a large number of Bragg reflections to be simultaneously recorded (Fig. 2). The sample-to-detector distance of the image-plate scanner is 199 mm. For data collection at room temperature, the largest crystal was mounted in a quartz capillary tube (VitroCom) with reservoir solution to avoid dryness and the tube was then sealed with vaseline, beeswax (Hampton Research) and nail polish. Because a pre-diffraction experiment using in-house X-ray diffractometry showed that the unit cell of the large crystals of GatCAB was similar to that of a previous X-ray crystal structure (Nakamura *et al.*, 2006, 2010), the data set was collected using a wavelength of 4.77 Å with a pyrolytic graphite monochromator (PG002). A total of 146 images were recorded with an oscillation range of 0.3° and an exposure time of 2 h per image. The diffraction data were indexed, integrated, scaled and merged using the *HKL*-2000 package (Otwinowski & Minor, 1997). Crystallization information is given in Table 3.

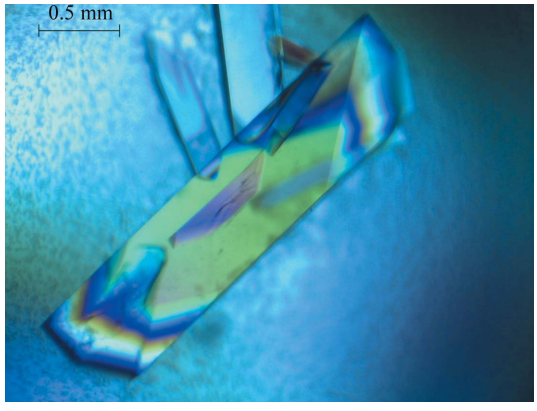

Figure 1
An H/D-exchanged crystal of GatCAB for neutron diffraction data collection. The crystal volume is approximately 1.8 mm^3.

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	FRM-II nuclear research reactor
Wavelength (Å)	4.77
Temperature (K)	293
Detector	Cylindrical neutron image plate
Crystal-to-detector distance (mm)	199
Rotation range per image (°)	0.3
Total rotation range (°)	43.5
Exposure time per image (h)	2
Space group	$P2_12_12_1$
a, *b*, *c* (Å)	74.6, 94.5, 182.5
Resolution range (Å)	50–3.0 (3.11–3.00)
Total No. of reflections	24626 (1741)
No. of unique reflections	15105 (1249)
Completeness (%)	56.3 (47.5)
Multiplicity	1.6 (1.4)
$\langle I/\sigma(I)\rangle$	2.7 (1.1)
R_{meas}	0.322 (0.799)
$R_{p.i.m.}$	0.209 (0.537)

3. Results and discussion

GatCAB is a difficult sample for obtaining crystals without microseeding and favors the growth of narrow needle-shaped crystals. Therefore, to obtain crystals that were large enough for neutron diffraction experiments, optimization of crystal growth was conducted. This process included optimization of the crystallization conditions, the crystallization containers, the volume of the drops, the crystal seed preparation, the crystallization method and additives. During the preparation of the seed stock in the microseeding stage, treatments using lower precipitant concentrations and centrifugation of the seed stock were found to improve the quality of the seed crystal used in the macroseeding stage. The lower precipitant concentration of buffer *B* can slightly dissolve the surface of the crystal seed, resulting in fresh crystal seed surface being exposed to the solution, which contributes to crystal growth. Centrifugation removed the large crystal seeds and made it

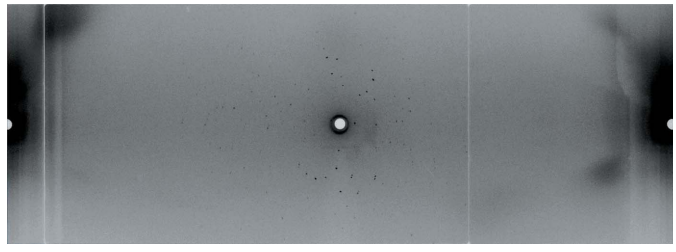

Figure 2
A representative monochromatic neutron diffraction image. The diffraction image was collected using the single-crystal diffractometer BIODIFF at the FRM-II research reactor at MLZ.

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easy to control the concentration of microseeds. The results of trial and error in the macroseeding stage indicated that the thickest and shortest seed crystals harvested from the microseeding experiment were good seeds for achieving large-volume crystals. For data collection, cooling crystals is a widespread and useful method. However, it is not a good choice for the large crystals of GatCAB because both direct cooling and flash-cooling may damage the crystal, although a cryoprotectant with 12.5% glycerol was successfully used to cool the smaller GatCAB crystals in the X-ray diffraction experiments. The large GatCAB crystal was sealed in a capillary for data collection at room temperature to avoid damage owing to freezing. The crystal quality was checked by in-house X-ray diffractometry before sending the crystal to the neutron beamline.

To avoid the spatial overlap of neutron reflections, the wavelength of the neutron beam was set to 4.77 Å. It took 292 h to obtain a data set at 3.0 Å resolution with an overall completeness of 56.3%. Table 3 shows the data-collection statistics. The crystals of GatCAB belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 74.6$, $b = 94.5$, $c = 182.5$ Å. One GatCAB molecule with a molecular weight of 119 kDa is present in the asymmetric unit. The neutron structure was determined by rigid-body refinement with *phenix.refine* (Adams *et al.*, 2010) using the X-ray crystal structure with PDB code 3ip4 (Nakamura *et al.*, 2006) as the model. After several cycles of refinement, H/D exchange (H/D at exchangeable sites, H elsewhere) was performed, and the values of R_{work} and R_{free} (5% data set) for neutron refinement were 23.7% and 28.7%, respectively.

This study demonstrated that it is possible to grow large crystals of a large protein complex (molecular mass of >110 kDa) with a large unit cell (one dimension of >180 Å) and analyze its neutron structure. In order to understand the ammonia-self-sufficient mechanism using the channel in GatCAB, we obtained large crystals of GatCAB complexed with the substrate glutamine (GatCAB–Gln). Techniques for improving the crystal quality to collect data to higher than 3.0 Å resolution are under development. Furthermore, we will use sufficient beam time to collect monochromatic or time-of-flight neutron diffraction data.

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