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Crystal structure of human gamma-butyrobetaine hydroxylase

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ABSTRACT

Gamma-butyrobetaine hydroxylase (GBBH) is a 2-ketoglutarate-dependent dioxygenase that catalyzes the biosynthesis of L-carnitine by hydroxylation of gamma-butyrobetaine (GBB). L-carnitine is required for the transport of long-chain fatty acids into mitochondria for generating metabolic energy. The only known synthetic inhibitor of GBBH is mildronate (3-(2,2,2-trimethylhydrazinium) propionate dihydrate), which is a non-hydroxylatable analog of GBB.

To aid in the discovery of novel GBBH inhibitors by rational drug design, we have solved the three-dimensional structure of recombinant human GBBH at 2.0 Å resolution. The GBBH monomer consists of a catalytic double-stranded β-helix (DBSH) domain, which is found in all 2KG oxygenases, and a smaller N-terminal domain. Extensive interactions between two monomers confirm earlier observations that GBBH is dimeric in its biological state. Although many 2KG oxygenases are multimeric, the dimerization interface of GBBH is very different from that of related enzymes.

The N-terminal domain of GBBH has a similar fold to the DUF971 superfamily, which consists of several short bacterial proteins with unknown function. The N-terminal domain has a bound Zn ion, which is coordinated by three cysteines and one histidine. Although several other 2KG oxygenases with known structures have more than one domain, none of them resemble the N-terminal domain of GBBH. The N-terminal domain may facilitate dimer formation, but its precise biological role remains to be discovered.

The active site of the catalytic domain of GBBH is similar to that of other 2KG oxygenases, and Fe(II)-binding residues form a conserved His–X–Asp–X_n–His triad, which is found in all related enzymes.

1. Introduction

Gamma-butyrobetaine hydroxylase (GBBH, EC1.14.11.1) is an enzyme that catalyzes the last step in the biosynthesis of L-carnitine, which is required for the transport of long-chain fatty acids into the mitochondrial matrix for subsequent β-oxidation and cellular energy production [1–3]. GBBH belongs to a class of enzymes called 2-ketoglutarate (2KG) oxygenases, which catalyze a broad range of oxidation reactions in virtually all living organisms. Hydroxylation is the most common reaction catalyzed by 2KG oxygenases and remains the only activity reported for these enzymes in animals [4]. All 2KG oxygenases utilize 2KG and O_2 as substrates and Fe(II) ion as a cofactor and, in addition to their specific biochemical product, release succinate and CO_2. Ascorbate and catalase are also important for the enzymatic activity of 2KG oxygenases. Ascorbate acts as a reducing agent, maintaining the Fe(II) ion in its reduced state, whereas catalase neutralizes hydrogen peroxide, a toxic by-product of oxidation reactions. Structurally, the catalytic domains of 2KG oxygenases share a common double-stranded β-helix fold (DBSH) and a conserved Fe(II) binding triad His–X–Asp/Glu–X_n–His. Apart from the DBSH core domain, several 2KG oxygenases and related enzymes have additional N- or C-terminal domains with various functions. For example, the N-terminal α-helical domain of hydroxypropylphosphonic acid epoxidase is involved in subunit oligomerization [5], and the C-terminal α-helical bundle domain of bacterial proline-3-hydroxylase has been suggested to play a role in catalysis [6].

Many 2KG oxygenases are monomeric in solution, whereas some exist as dimers, tetramers or hexamers [4]. GBBH has been shown to form stable dimers [7]. In general, 2KG oxygenases are structurally well characterized with more than 50 representatives in the Protein Data Bank (for review see [4]). Nevertheless, none of the available structures displays significant sequence identity to GBBH.

Abbreviations: GBBH, gamma-butyrobetaine hydroxylase; GBB, gamma-butyrobetaine; 2KG, 2-ketoglutarate; DBSH, double-stranded β-helix.

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In human tissues, GBBH is expressed only in liver, kidney and to a lesser extent in the brain [8,9]. The enzymatic activity of GBBH has been used to increase the bioavailability of L-carnitine by administering gamma-butyrobetaine (GBB), the substrate for GBBH and a bio-precursor of L-carnitine [1,10–12]. The only known synthetic inhibitor of GBBH is mildronate (3-(2,2,2-trimethylhydrazinium) propionate dihydrate), a structural non-hydroxylatable analog of GBB. Mildronate is clinically used as an anti-ischemic drug and a regulator of cellular energy metabolism [13,14]. By inhibiting GBBH and, subsequently, the availability of L-carnitine, mildronate stimulates glucose metabolism, which can be beneficial under certain physiological or pathological conditions, such as ischemia-related pathological states, metabolic syndrome and diabetes [13–15].

To find novel pharmacological agents for the regulation of L-carnitine pathways and to facilitate rational structure-based drug design, we have solved the crystal structure of GBBH. The structure has revealed several unknown features of 2KG oxygenases, such as an N-terminal zinc-binding domain and a novel dimerization interface.

2. Materials and methods

2.1. Cloning, expression and purification

The coding sequence of GBBH with *Escherichia coli*-optimized codons was provided by ASLA Ltd. (Riga). The gene was cloned in a modified pColdI vector (Takara Bio Inc.). As a result, the protein had the following amino acid sequence before the start codon of GBBH: MNHKV (pColdI leader sequence) – HHHHHH (His-tag) – IEGR (Factor Xa cleavage site)-S.

E. coli strain WK6 [16] was co-transformed with two plasmids encoding (a) the human recombinant gamma-butyrobetaine hydroxylase (GBBH) and (b) the bacterial chaperones GroES and GroEL under the control of an IPTG-inducible lac promoter. The transformed cells were grown in LB medium supplemented with 0.1% glucose, 50 mg/l ampicillin and 50 mg/l kanamycin at 37 °C until OD_{600} = 0.6. IPTG was added to a final concentration of 1 mM, and the culture was incubated at 37 °C for 30 min followed by 16 h at 15 °C. The cells were harvested by centrifugation, resuspended in PBS buffer, pH 7.4, lysed by sonication, and the insoluble fraction was removed by centrifugation. About 10% of the GBBH was in the soluble fraction. The lysate was applied to a Ni–NTA agarose column (Qiagen) followed by gel filtration on a Superdex 200 column (GE Healthcare) and ion exchange chromatography on a Mono Q column (GE Healthcare).

2.2. Production, purification and verification of SeMet-labeled GBBH

SeMet-labeled protein was produced and purified as above except that cells were grown in Overnight Express™ Autoinduction System 2 media (Novagen).

Initial attempts to obtain SeMet-labeled protein in a Met auxotrophic *E. coli* strain failed due to severely reduced protein expression levels.

Therefore, SeMet labeling was performed in WK6 cells, which are not Met auxotrophs. Incorporation of SeMet was verified by MALDI-TOF mass spectrometry of tryptic peptides as described previously [17], which indicated close to 100% incorporation of SeMet.

2.3. Activity determination

Activity was determined by measuring the formation of carnitine from GBB according to a slightly modified method from Lindstedt and Lindstedt [18]. The complete reaction mixture, in a final volume of 0.2 ml, contained the following: 20 mM potassium phosphate pH 7.0, 20 mM potassium chloride, 3 mM 2-ketoglutarate, 0.25 mM ferrous ammonium sulfate, 10 mM sodium ascorbate, 0.16 mg catalase, 3–500 μM GBB and an appropriate amount of GBBH. The reaction was initiated by addition of GBBH, and the mixture was incubated at 37 °C for 120 min. The reaction was stopped by addition of 0.8 ml of cold acetonitrile:methanol (1:3). The mixture was spun down at 20,000 g for 10 min at 4 °C; the supernatant was decanted and used to measure the amount of carnitine. The IC_{50} for mildronate was determined under the same conditions but with the addition of mildronate (3 μM to 300 μM). The concentration of L-carnitine was measured by ultra performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) in positive ion electrospray mode, as described previously [19].

2.4. Crystallization and data collection

GBBH was crystallized by a sitting drop vapor technique by mixing 1 μl protein (7 mg/ml) in 20 mM tris–HCl pH 8.0 with 1 μl of reservoir solution (1.2 M ammonium sulfate, 100 mM sodium acetate, pH 4.5). In co-crystallization trials with ligands, each ligand was added to the reservoir solution to a final concentration of 5 mM. Needle-shaped crystals appeared after 3–4 h and reached a maximum size of 0.2–0.4 mm in 24 h. SeMet-labeled protein was crystallized under the same conditions as above. The crystals were soaked in the mother liquor with 30% glycerol for approximately one minute and flash frozen in liquid nitrogen. Data were collected at ESRF beamlines ID23–1 and BM14u. The best diffracting crystals were those with mildronate, which diffracted to 2.0 Å resolution. SeMet-labeled crystals diffracted to 3.5 Å resolution, and crystals with other ligands diffracted to 3.0–3.2 Å resolution. From the SeMet-labeled crystal, two datasets were collected at 0.97856 Å (peak) and 0.91840 Å (remote) wavelengths at 3.5 Å resolution.

2.5. Structure determination

Indexing by MOSFLM [20] indicated a trigonal lattice, and subsequent scaling by SCALA [21] confirmed space group $P6_122$ or $P6_522$ as judged by scaling statistics and systematic absences. Determination of Se atom positions and initial phasing was performed in SHELX C/D/E [22] in both $P6_122$ and $P6_522$ space groups. The resulting electron density maps were manually inspected, and protein-like features, such as right-handed α helices and β strands, were detected for the $P6_522$ space group. Heavy atom refinement and phasing was done by SHARP [23], and the resulting map was improved by solvent flattening with SOLOMON [24]. The initial model was built automatically in BUCANEER [25]. The resulting partial model, which covered about 50% of the sequence, was manually inspected and improved in COOT [26]. The coordinates were used as a molecular replacement model for the 2.0 Å dataset, and the improved electron density map was used for an additional BUCANEER round. The remaining parts of the model were built manually in O [27] and COOT. Structure refinement was done by REFMAC [28]. Water molecules, zinc and sulfate ions were added in COOT. All automatically added water molecules were manually inspected, and some were removed due to bad density, bad contacts or very high B factors.

Data scaling, refinement and validation statistics are shown in Table 1.

2.6. Determination of Zn presence

Quantitative determination of Zn was done by electrothermal atomic absorption spectrometry (ETAAS). Measurements were performed on a Perkin–Elmer Model AAnalyst 600 with AC Zeeman-effect background correction using a modulated 0.8 Tesla magnetic