Structure of glycine N-acyltransferase clarifies its catalytic mechanism

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Abstract

Glycine N-acyltransferase (GLYAT; EC 2.3.1.13) is a key enzyme in mammalian homeostasis that has been linked to several pathologies in humans, including cancer. Here we report the first crystal structure of a member of the GLYAT family, and a detailed interpretation of its structure and enzymatic mechanism. This work will aid further studies on GLYAT and its involvement in metabolic diseases and cancer.

Background

GLYAT participates in phase II detoxification through conjugating benzoyl-CoA to glycine to form hippurate, one of the four most abundant metabolites in urine. This makes GLYAT essential to the homeostasis of hepatic and renal coenzyme A (CoASH), which would otherwise be sequestered as benzoyl-CoA^{1,2}. Recently, glycine conjugation has gained more awareness due to the excessive use of some products such as benzoate (as a preservative) and common drugs like aspirin. The metabolism of these everyday products exacerbates the dietary deficiency of glycine, a conditionally essential amino acid in humans, influencing the metabolism of not only glycine but also ATP and free CoASH, which participate in many metabolic pathways. Glycine deficiency is associated with pathologies like CASTOR (Coenzyme A Sequestration, Toxicity, and Redistribution) disorders² and recently its metabolic product, hippurate, has been identified as a biomarker for toluene exposure³. In addition, polymorphism of the *GLYAT* gene has been linked to variation in musculoskeletal growth, and its expression is downregulated in liver and breast cancer cells^{2,4}.

To gain insight into the catalytic mechanism, GLYAT from *Bos taurus* (bGLYAT) was recombinantly expressed and purified (Supplementary Fig. 1). The protein was crystallized both alone and in the presence of either glycine or benzoyl-CoA. The structures, to resolutions of 1.25 – 1.65 Å, were solved in parallel by both molecular replacement (MR) and single-wavelength anomalous diffraction phasing based on the intrinsic sulfur atoms (S-SAD) (Supplementary Table 1). Although we failed to obtain bGLYAT crystals in complex with glycine, electron density for an acetate molecule was detected and interpreted in the putative glycine binding site (Fig. 1 and Supplementary Fig. 2).

GLYAT belongs to the Gcn5-related *N*-acetyltransferase (GNAT) superfamily¹. Despite the low sequence identity amongst members, the catalytic domain's topology is conserved. However, members of the family have diverged to adopt additional structural elements (Fig. 2a). These structural differences significantly alter the substrate binding site's architecture and the mechanisms for deprotonation of the primary amine to promote nucleophilic attack⁵.

The bGLYAT structure comprises two distinct domains with almost identical topology: a mixed open central β -sheet with helical crossovers, typical of the GNAT fold⁵ (Fig. 2). The domains are linked by a disordered loop that showed poor to no density for residues 144-157. The N-terminal domain and C-terminal catalytic domain are related to each other by a pseudo-two-fold axis that is not altered by the binding of benzoyl-CoA (Fig. 1). The C-terminal strand β 12 extends and forms part of the N-terminal

central β -sheet, while the N-terminal strand $\beta 5$ extends to the C-terminal catalytic domain and positions antiparallel to $\beta 10$ and $\beta 11$, generating backbone contacts that stabilize the structure. A similar type of fold has been described for the human *N*-myristoyl transferase, although the catalytic site is found in the N-terminal domain⁶ (Supplementary Fig. 3).

In the catalytic domain, the topological switch point is located between strands $\beta 9$ and $\beta 10$, forming a V-shaped cleft that accommodates the benzoyl-CoA. The acyl donor adopts a C-shaped orientation and interacts with the enzyme via hydrophobic contacts and direct and water-mediated hydrogen bonds (Supplementary Fig. 4), similar to what has been reported for other GNAT-CoA structures⁵.

The benzoyl moiety is buried in a hydrophobic pocket formed by $\beta 9$ -10, $\alpha 8$, and the loop $\alpha 6$ -7, where it is stabilized by hydrophobic and van der Waals interactions with Met227, Met271, Ile246, and Ser262. In the same pocket, the acetate anion is found with its methyl group (where the amino group of glycine would be attached) pointing towards the thioester bond of the acyl donor (Fig. 1). A glycine molecule was modeled in the acetate position in 2 different C – Ca torsion angles showing the close proximity to the catalytic dyad and acyl donor (Supplementary Fig. 2). The carboxyl group of the acetate interacts with NH2 and NE of Arg228. In the apoenzyme, this residue coordinates a sulfate or malonate ion from the crystallization solution. Interestingly, a putative malonate adduct was observed in the active site of the apo enzyme.

Two characteristic GNAT features are present in the bGLYAT structure: the β -bulge, creating the oxyanion hole that stabilizes the tetrahedral intermediate during the reaction, and the 'P-loop', interacting with the pyrophosphate moiety of the CoA⁵. The structural fold resembles a $\beta\alpha\beta$ Rossmann fold, usually found in nucleotide diphosphate binding proteins. However, the characteristic glycine motif (Gly-X-Gly), which allows the hairpin turn and interacts with the diphosphate group, is replaced by Ala-X-Gly in the bGLYAT structure.

His263 is positioned near the substrates and forms a low-barrier hydrogen bond (LBHB) with Glu226 (Glu226 OE2 - His263 ND1 distance is ~ 2.5 Å). This interaction stabilizes the active site and positions His263 and is expected to facilitate catalysis by increasing the nucleophilicity of His263 NE2, driving the abstraction of the glycine proton. The mutation of this residue in bovine⁷ (E226Q) and human⁸ (E227Q) GLYAT decreased the enzymatic activity, supporting the key role of the LBHB for catalysis.

Based on analysis of the structure, we reevaluated the mechanism proposed previously⁷. The enzyme follows a general base-catalyzed, ternary complex mechanism coordinated by the catalytic dyad Glu226-His263 (Fig. 3). The structure also shows that His263 coordinates a water molecule; thus, the deprotonation of glycine could occur directly, or it is water mediated, as it has been observed for some GNAT⁵. The conserved Arg228 coordinates the carboxyl group of the acyl donor via hydrogen bonding and electrostatic contact. This interaction favors the activation of the substrate by polarizing the glycine molecule and therefore facilitating the removal of the proton and preventing the re-protonation before the nucleophilic attack occurs. Interestingly, the enzyme can use other amino acids (including Ala, Asn, Gln,

and Glu) as acyl acceptors, but with lower efficiency¹. Although these alternative acceptors are larger amino acids, we hypothesized that the effect on the reduced activity is probably due to a loss of the salt bridge polarization effect, and not due to steric hindrance.

After deprotonation, nucleophilic attack by the amino group of the glycine on the thioester carbonyl group of the acyl-CoA takes place. Arg228 was found in different conformations in the apo structure, depicting a certain degree of mobility (Fig. 1d and Supplementary Fig. 2b). We hypothesize that the residue could also play a crucial role in positioning the substrate to the His263 for deprotonation and subsequently re-positioning the activated glycine for nucleophilic attack.

Met229-Ala230 form the β -bulge in β 9; the amide groups of these residues are directed towards the carbonyl oxygen of the benzoyl-CoA. The oxyanion formed polarizes the thioester carbonyl group and stabilizes the zwitterionic tetrahedral intermediate.

Finally, the intermediate collapses into free CoASH and acyl-glycine products. With the exception of the now protonated His263, no obvious residue is present as a general acid candidate to protonate the thiolate anion. The protonation could, however, occur during molecular rearrangement of the intermediate through an intramolecular proton transfer from the amide group. As electron density for a number of water molecules was also observed within the active site, protonation of the leaving group from the solvent cannot be excluded (Fig 3).

Due to its physiological relevance, human GLYAT has recently received considerable attention. Many groups have reported genetic polymorphisms in the *GLYAT* gene, and *in vitro* studies of the prevalent variants showed some of the mutations affect enzymatic activity⁸⁻¹¹ (Supplementary Table 2). Without a representative GLYAT structure, previous analyses were based on homology models generated using low identity templates (<12 %)⁸, which could introduce severe bias. GLYAT is, however, highly conserved in mammals (> 70% pairwise identity), and the bovine and human enzymes share similar catalytic efficiencies and substrate specificities⁶. Not surprisingly, these two enzymes share 83% sequence similarity (76% pairwise identity) and the residues involved in catalysis are conserved (Supplementary Fig. 5). The bGLYAT structure can therefore contribute to the rational design of experiments to understand how these changes impact the efficiency of GLYAT and contribute to its pharmacogenetic consequences.

The overall structure is novel to the superfamily, with an N-terminal GNAT domain that does not have a clear function. In other members of the family, such as GLYAT-like2, it has been implicated in allosteric regulation of the enzyme's activity via acetylation¹². The GLYAT sequence presents several putative posttranslational modification sites for acetylation and phosphorylation (Supplementary Fig. 5). Interestingly, this enzyme has been related to kinase pathways, such as PI3K/AKT in human breast cancer cells⁵. All these require a more in-depth study of GLYAT and its multiple roles in human metabolism.

For many years, the study of glycine *N*-acyltransferase has been neglected and its importance underestimated. However, there is increasing evidence of the importance of this enzyme for the proper functioning of the organism. Recently, downregulation of GLYAT has been proposed as a biomarker and possible target for breast cancer therapy⁵. The crucial value of this protein requires an in-depth understanding of its structure and enzymatic mechanism. The crystal structure of the first member of the family is a step forward towards this goal.

Methods

Cloning and expression. The open reading frame (ORF) coding for *Bos taurus* glycine *N*-acyltransferase (bGLYAT, NP_803479) was fused to the small ubiquitin modifying protein (SUMO) tag¹³ (N-terminal), separated by a TEV protease cleavage site to facilitate cleavage of the tag (SUMO-TEV-bGLYAT). The construct was synthesized and cloned into pET-28a(+), using the NdeI and XhoI sites, by GenScript. *Escherichia coli* BL21-Gold(DE3) (Stratagene) was transformed with the plasmid and plated onto LB-agar supplemented with 30 µg mL⁻¹ kanamycin. Heterologous expression of the fusion protein was performed in the auto-induction medium ZYM-5052¹³ (30 °C for 48 h).

Constructs for the expression of TEV protease and Benzonase were generously donated by Prof. F. von Delft (Oxford University). Enzymes were expressed using *E. coli* BL21(DE3) cells. Cultures were grown in the auto-induction medium ZYM-5052 for 48 h at 25 °C.

Protein purification. For purification of bGLYAT, Benzonase, and TEV protease, cells were harvested by centrifugation (5000 x *g*, 10 min, 4 °C). The pellets were resuspended (0.2 g_{wet weight} mL⁻¹) in BugBuster protein extraction reagent (Novagen) containing 30 U mL⁻¹ lysozyme (Fluka) and 3 µg mL⁻¹ Benzonase and incubated at room temperature for 15 min. The insoluble material was removed by centrifugation (20 min, 20,000 x *g*, 4 °C) and the clarified lysate was loaded onto a 5 mL HisTrap FF column (GE Healthcare), previously equilibrated with buffer A (25 mM Tris-HCl, 40 mM imidazole, and 500 mM NaCl, pH 8.0). The column was washed with 10 column volumes of buffer A, and the protein was eluted with a linear gradient of imidazole (40 – 500 mM) using an ÄKTA pure automated system (GE Healthcare). Fractions containing the target protein were pooled, concentrated through ultrafiltration (Amicon Ultra-10 kDa MWCO (Merck)), and desalted by size exclusion chromatography (SEC) using PD-10 columns (GE Healthcare) pre-equilibrated with buffer B (25 mM Tris-HCl and 50 mM NaCl, pH 8). Cleavage of the SUMO tag from the GLYAT fusion protein were performed by the addition of TEV protease (1 µg TEV/100 µg fusion protein) to the desalted fraction and overnight incubation at 4 °C. The cleaved protein was passed through a 5 mL HisTrap FF column, equilibrated with buffer A, and the flow-through, containing the bGLYAT, was collected and concentrated as described above. The concentrated pool was used to perform SEC using Sephacryl S200HR (XK 26/100 column, GE Healthcare) equilibrated with buffer C (10 mM Tris-HCl, pH 8.0). The eluted bGLYAT was pooled and concentrated.

Benzonase and TEV protease were kept at -20 °C, after the addition of glycerol to a final concentration of 50%.

Protein concentration and activity determination. The protein concentration was determined by the BCA assay (Pierce) using bovine serum albumin as standard. The activity of bGLYAT was analyzed by following the reduction of DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] (Merck) at 412 nm using a Spectramax M2 spectrophotometer. Reactions were performed as described elsewhere⁸. Protein purity was analyzed by SDS-PAGE.

Crystallization, data collection, and structure determination. The purified bGLYAT was concentrated to 6 and 8 mg mL⁻¹. Crystals were grown by the sitting drop vapor diffusion method, by mixing 1 µL of protein and 1 µL of the reservoir solution (0.2 M sodium malonate, 20% w/v polyethylene glycol 3,350, pH 5.0, or 1.5 M ammonium sulfate, 0.1 M sodium acetate, pH 4.6). For crystals with benzoyl-CoA, the enzyme was crystallized in a 5-fold molar excess of the substrate (1 M sodium acetate, 30% v/v polyethylene glycol 300, pH 4.5). Plates were incubated at 16 °C. Crystals were soaked in a reservoir solution containing 20% v/v polyethylene glycol 400 before cryocooling. X-ray diffraction data were collected at Diamond Synchrotron (UK) on beamline I03 (0.9762 Å) at 93 K. Data were processed using XDS¹⁵.

The apoenzyme crystallized in either space group $P2_12_12_1$ or $P6_5$ with two molecules in the asymmetric unit (ASU) that are almost identical in each crystal form (Ca RMSD of 0.372 and 0.479 Å, respectively). The bGLYAT-benzoyl-CoA complex crystallized in space group $P4_32_12$ with one molecule in the ASU and a related packing to the orthorhombic crystal form.

De novo phasing through single-wavelength anomalous dispersion of S atoms (S-SAD) were performed on crystals without substrate. Data were collected on beamline I23 (Diamond Synchrotron) at 4.5 keV (2.755 Å) and processed using XDS through xia2i¹⁶. Four datasets were merged using XSCALE¹⁷. A good anomalous signal was detected at 2.2 Å. SHELXD¹⁸ and CRANK2¹⁹ were used for structure solution and initial model building.

Several attempts to use very distant homologs for molecular replacement (MR) failed. Instead, five bGLYAT models were generated *ab initio* with the recently developed method RosettaFold²⁰, a deep learning-based modeling tool for protein structure prediction. The models were superimposed into an ensemble to use as a search model for MR. A trimmed ensemble of the models was used for molecular replacement using PHASER²¹. Iterative cycles of model building in COOT²² and refinement using Refmac²³ were performed. Figures were prepared using PyMOL.

Data availability

The 3D structures of bGLYAT reported here have been deposited at the PDB under codes 7PK0 (in complex with benzoyl-CoA), 7PK1 (apoenzyme complexed with SO₄), 7PK2 (apoenzyme complexed with malonate).

Declarations

Acknowledgments

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Author contributions

D.J.O. and A.A.v.D. conceived the project and supervised the experiments. A.C.E. performed the experiments (cloning, purification and crystallization). C.P.S.B. participated in bGLAT biochemical studies and analysis of the results. A.C.E., D.J.O. and R.J.R. solved and analyzed the structures. All the authors contributed to the writing of the manuscript.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

Structure of bGLYAT in complex with benzoyl-CoA. a Comparison of the overall GLYAT structures with (blue) and without (grey) benzoyl-CoA (cyan). Changes in the structure upon substrate binding are highlighted in magenta. Residues missing from the flexible loop that links the N- and C-terminal domains (L147 – G157) are shown as broken lines. Structures aligned correspond to PDB ID 7PK0 and 7PK2. b

Binding of the acetate molecule (pink) in the putative binding site of glycine. Ionic and hydrogen-bonding interactions are shown as dashed lines and distances (Å) are displayed. c Electron density of benzoyl-CoA (cyan), acetate (pink), and the putative catalytic water (red sphere) are shown as mesh from the omit map 2Fo-Fc, contoured at $\sigma = 1.0$ Å. The catalytic dyad (Glu226-His263) is displayed, and hydrogen bonds are shown as dashed lines. d Movement of the residues in the catalytic site after binding of benzoyl-CoA. The structure of the apoenzyme is colored grey and the enzyme in complex with the substrate is colored green. Structures aligned correspond to PDB ID 7PK0 and 7PK2 (chain B).

Figure 2

Comparison of bGLYAT structure to the GNAT fold. a General GNAT topology. Conserved secondary structure elements are depicted in green, yellow, pink, and blue, whereas non-conserved secondary elements are colored grey. The positions of important catalytic residues (general base and acid) are indicated with arrows in the diagram. The β -bulge that forms the oxyanion hole is shown in green and the P-loop in orange. b Diagram of the bGLYAT topology showing structural GNAT features. Color codes are the same as in a. The arrows point to the position of the catalytic dyad (E226-H263). c Structural

alignment (Ca RMSD = 1.5 Å) of GLYAT (blue, N-terminal, and green, C-terminal catalytic domain) and the closest mammalian structural homolog with a known structure, human N- α -acetyltransferase 20 (grey, PDB ID 6VP9, chain A). d Overall view of the bGLYAT fold, labeled with secondary structure elements. The β -bulge (green) and P-loop (orange) are highlighted in the structure.

Figure 3

Proposed catalytic mechanism of bGLYAT. The scheme depicts the important residues involved in catalysis. The substrates are shown in purple (glycine) and blue (benzoyl-CoA), interactions are shown as dashed lines (black, hydrogen bond; blue, LBHB; red, salt bridge), and alternative pathways are shown as dashed arrows. a The catalytic dyad deprotonates the acyl acceptor directly or through a hydrogen-bonded water. b The activated nucleophilic glycine attacks the thioester carbonyl group of the benzoyl-CoA. c The oxyanion hole formed stabilizes the tetrahedral transition state and the intermediate receives an H⁺ from the bulk solvent, the protonated His263, or by intramolecular transfer during the molecule rearrangement. d The intermediate collapses, forming the products hippurate and free CoASH.

Supplementary Files

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