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Expression, Purification, and Characterization of Mouse Glycine *N*-acyltransferase in *Escherichia coli*

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Abstract

Glycine *N*-acyltransferase (GLYAT) is a phase II metabolic detoxification enzyme for exogenous (xenobiotic) and endogenous carboxylic acids; consisting of fatty acids, benzoic acid, and salicylic acid. GLYAT catalyzes the formation of hippurate (*N*-benzoylglycine) from the corresponding glycine and benzoyl-CoA. Herein, we report the successful expression, purification, and characterization of recombinant mouse GLYAT (mGLYAT). A 34 kDa mGLYAT protein was expressed in *Escherichia coli* and purified to homogeneity by nickel affinity chromatography to a final yield of 2.5 mg/L culture. Characterization for both amino donors and amino acceptors were completed, with glycine serving as the best amino donor substrate, $(k_{cat}/K_m)_{app}$ = (5.2 ± 0.20) × $10^2 M^{-1}s^{-1}$, and benzoyl-CoA serving as the best the amino acceptor substrate, $(k_{cat}/K_m)_{app}$ = (4.5 ± 0.27) × $10^5 M^{-1}s^{-1}$. Our data demonstrate that mGLYAT will catalyzed the chain length specific (C2-C6) formation of *N*-acylglycines. The steady-state kinetic constants determined for recombinant mGLYAT for the substrates benzoyl-CoA and glycine, were shown to be consistent with other reported species (rat, human, bovine, ovine, and rhesus monkey). The successful recombinant expression and purification of mGLYAT can lead to solve unanswered questions associated with this enzyme, consisting of what is the chemical mechanism and what catalytic residues are essential for the how this phase II metabolic detoxification enzyme conjugates glycine to xenobiotic and endogenous carboxylic acids.

Keywords

Glycine *N*-acyltransferase; *Escherichia coli*; hippurate; steady-state kinetics; *N*-acylglycine; benzoyl-CoA

Introduction

The detoxification of xenobiotics is generally biphasic, comprising of Phase I metabolism and Phase II metabolism [1,2]. The enzymes of Phase I metabolism catalyze the formation of more polar and reactive forms of the xenobiotic while the enzymes of Phase II catalyze the conjugation of the xenobiotic derivative to a species that is readily transported to the urine or bile for excretion [1-3]. The first step in the detoxification of xenobiotic carboxylates is the ATP-dependent formation of the acyl-CoA thioester: ATP + CoA-SH +

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R-COOH → R-CO-S-CoA + AMP + PP_i [4-6], as catalyzed by acyl-CoA synthetase. A family of acyl-CoA synthetases is known, differentiated by their specificity for the acyl-CoA substrates [6]. The acyl-CoA thioesters serve as susbstrates for a number of Phase II conjugating enzymes to produce glutathione adducts, *O*-acylcarnitines, or *N*-acylamino acids [6]. One focus of our research has been a specific Phase II conjugating enzyme, glycine *N*-acyltransferase (GLYAT, E.C. 2.3.1.13)[1]. GLYAT, a member the Gcn5-related *N*-acetyltransferase (GNAT) superfamily of enzymes [7], catalyzes the formation of *N*-acylglycines and *N*-arylalkylglycines from the corresponding acyl-CoA or arylalkyl-CoA thioesters and glycine (Fig. 1) [8,9]. Knowledge of this chemistry dates back to the 1840's with the demonstration that urinary hippurate (*N*-benzoylglycine) was derived from benzoic acid [10].

GLYAT has been characterized from a number of mammals, including human [9,11-13], rhesus monkey [11], rat [15,16], rabbit [15], and bovine [14,17-19], and is found in the mitochondria of the liver and kidney [9,11-13,16-18]. GLYAT exhibits a strong preference for glycine as the amino donor substrate as non-glycine amino acids are poor substrates [13,14] and benzoyl-CoA and short-chain acyl-CoA thioesters as amino acceptor substrates. The V/K values for the acyl-CoA substrates decrease as the acyl-chain length from C2-C10 [9,13]. Lauroyl-CoA and longer-chain acyl-CoA thioesters are, most likely, not substrates for GLYAT [20]. GLYAT is relevant medically as glycine supplementation may be useful for the treatment of organic acidemias to promote the excretion of *N*-acylglycines [21,22], benzoate therapy is used to treat hyperammonemia by excretion of hippurate [23], and the quantification of blood and urinary *N*-acylglycines is useful in the diagnosis of metabolic disorders that lead to the accumulation of specific organic acids [24-27].

We report herein the successful expression, purification, and characterization of recombinant mouse GLYAT (mGLYAT) in *Escherichia coli*. mGLYAT is a 34 kDa protein that has a high amino acid sequence identity ranging from 60% to 89%, when compared to the other previously characterized mammalian orthologs. We determined the steady-state kinetic constants for different amino acceptors (benzoyl-CoA and acyl-CoA thioesters) and amino donors (amino acids, amino acid esters, decarboxylated amino acids, and peptides). The steady-state kinetic constants we have determined for recombinant mGLYAT are consistent with similar values reported for other mammalian GLYATs.

Material and methods

Materials

Mouse *GLYAT* TrueClone™ Full Length cDNA Clone was purchased from Origene (MC201077). Oligonucleotides were purchased from Integrated DNA Technologies. PfuUltra High-Fidelity DNA polymerase was purchased from Aglient. BL 21 (DE3) *E.coli* cells, *Ni-NTA* His-Bind® *resin*, and *pET-21a(+)* vector were purchased from Novagen. *BamHI*, *HindIII*, Antarctic Phosphatase, and T4 DNA ligase were purchased from New England Biolabs. Mouse anti-6x-His epitope tag was purchased from Fisher Scientific. Immun-Blot goat anti-mouse IgG AP assay kit and AP conjugate substrate kit were purchased from Bio-rad. Ampicillin sodium salt and IPTG were purchased from Gold Biotechnology. Benzoyl-CoA, acetyl-CoA, butyryl-CoA, and hexanoyl-CoA were purchased from Sigma-Aldrich. All other reagents were of the highest quality available from either Sigma-Aldrich or Fisher Scientific.

[1]**Abbreviation:** DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GNAT, Gcn5-related *N*-acetyltransferase superfamily; GLYAT, glycine *N*-acyltransferase; GLYATL1, glycine *N*-acyltransferase like 1; GLYATL2, glycine *N*-acyltransferase like 2; GLYATL3, glycine *N*-acyltransferase like 3; mGLYAT, mouse glycine *N*-acyltransferase; OD, optical density.

Molecular Cloning

The *mGLYAT* (NCBI reference sequence NM_145935.3) gene was amplified from Origene GLYAT TrueClone™ Full Length cDNA using (primers: forward 5′ GAC TGG ATC CAT GAT TGT TCC ATT ACA AGG TGC A 3′ and reverse 5′ GAC TAA GCT TCA TAG GCA TGC ACT TCC ATT G 3′) PCR (95°C for 30 s; 55°C for 30 s; 72°C for 1 min, 30 cycles) and then inserted into a *pET-21a (+)* vector using the *BamHI* and *HindIII* restriction sites. Note that the primers were designed to create *BamHI* and *HindIII* restriction sites to facilitate insertion of the *mGLYAT* gene into the *pET-21a (+)* vector. The *mGLYAT pET-21a (+)* vector was then transformed into *E.coli* BL21 (DE3) cells for protein expression.

Expression and Purification of mGLYAT

The mGLYAT *E. coli* BL21 (DE3) cells were cultured in LB media with 100 μg/mL ampicillin at 37°C and induced at an OD_{600} of 0.6 with 1 mM isopropyl thio-β-D-galactoside for 4 h at 37°C. The final culture was then harvested by centrifugation at 5,000 g for 10 min at 4°C and the pellet was collected.

The pellet was resuspended in 20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 7.9; the cells disrupted by sonication; and then centrifuged at 10,000 g for 15 min at 4°C. The supernatant was then loaded onto 3 mL of *Ni-NTA* His-Bind® *resin*. The column was washed first with 10 column volumes of 20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9, then washed with 10 column volumes of 20 mM Tris-HCl, 500 mM NaCl, 60 mM imidazole, pH 7.9, and then finally eluted in 1 mL fractions of 20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.9.

Expression and Purification Analysis

The purified fractions were analyzed for protein concentration by the Bradford assay and purity was accessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 10% gel. The SDS-PAGE gel was visualized by using Coomassie stain. Purification of mGLYAT was confirmed by Western blot analysis by using a primary anti-6x-His epitope tag and a goat secondary anti-mouse antibody with alkaline phosphatase conjugated.

mGLYAT Steady-State Kinetics and Analysis of the Kinetic Data

mGLYAT activity was measured by following the release the free CoA-SH spectrophotometrically using Ellman's reagent (DTNB, ε_{412} = 13,600 $M^{-1}cm^{-1}$) [28] in 300 mM Tris-HCl pH 8.0, 150 μM DTNB, and the desired concentrations of the acyl-CoA and amino-containing substrates. We refer to the acyl-CoA substrates as the amino acceptors and the free amino-containing compounds as the amino donors. Steady-state kinetic constants were determined for each amino donor by holding the initial concentration of benzoyl-CoA at 300 μM while varying concentration of the amino donor. Steady-state kinetic constants were determined for each amino acceptor by holding the initial concentration of glycine concentration at 100 mM while varying the amino acceptor concentration. Steady-state kinetic constants were determined by fitting the data from each experiment to the Michaelis-Menten equation (1) using Sigma Plot 12.0:

$$v = (V_{max,app}[A]) / (K_{max,app} + [A]) \quad (1)$$

where v is the experimentally determined initial rate, [A] is the initial concentration of the substrate A, $K_{m,app}$ is the apparent Michaelis constant for the variable substrate, A, at a constant fixed concentration of the other substrate, and $V_{max,app}$ is the apparent maximum velocity at saturating [A] under conditions of experiment.

Inhibition Studies and the Determination of IC_{50} Values

A number of potential amino donor substrates were evaluated for their binding to mGLYAT by determining their respective inhibition of the mGLYAT-catalyzed conversion of benzoyl-CoA plus glycine to hippurate. This was accomplished by measuring the release of CoA-SH in 300 mM Tris-HCl pH 8.0, 150 μM DTNB, 9.4 μM benzoyl-CoA, 6.1 mM glycine, and 100 mM amino donor. The initial concentrations of benzoyl-CoA and glycine were fixed at their $K_{m,app}$ values.

The concentration of inhibitor required to decrease the initial rate of CoA-SH release under specific conditions, the IC_{50} value, was determined for two compounds: glycolic acid and oleoyl-CoA. Fractional velocity, v/v_i, as a function of glycolic acid or oleoyl-CoA concentration was determined by following CoA-SH release in 300 mM Tris-HCl, 150 μM DTNB, 9.4 μM benzoyl-CoA, 6.1 mM glycine, and either glycolic acid (1.0 – 200 mM) or oleoyl-CoA (500 nM – 1.0 mM). The IC_{50} value was determined by fitting the data for glycolic acid or oleoyl-CoA to equation (2) using Sigma Plot 12.0 [29]:

$$\text{Fraction velocity} = v/v_i = 1 + ([I]/IC_{50}) \quad (2)$$

where v is the determined initial rate obtained in absence of the inhibitor, I, v_i is the initial rate obtained in the presence of I, and [I] is the desired concentration of I (either glycolic acid or oleoyl-CoA).

Results and Discussion

Cloning, Expression, and Purification of mGLYAT

The *mGLYAT* gene was successfully amplified (Fig. 2A) from the mouse *GLYAT* TrueClone™ Full Length cDNA and inserted into a *pET-21a(+)* vector, in a manner that will result in the production of the mGLYAT protein with a His_6-tag C-terminal extension. The *mGLYAT pET-21a(+)* vector was then transformed into *E.coli* BL21 (DE3) cells, cultured in LB media supplemented with 100 μg/mL ampicillin, yielding mGLYAT (with the C-terminal His_6-tag) at a final yield of 2.5 mg/L culture. Soluble protein after sonication was then loaded onto a *Ni-NTA* His-Bind® affinity column and mGLYAT was purified using increasing concentrations of imidazole. Purity of mGLYAT was analyzed by SDS-PAGE (Fig. 2B), showing a single band of the proper molecular weight, 34 kDa. Additional data indicating that the protein at 34 kDa was recombinant mGLYAT came from Western blot analysis using a mouse anti-6x-His antibody as the primary antibody followed by treatment with a secondary goat anti-mouse antibody conjugated to alkaline phosphatase (Fig. 2C).

mGLYAT Substrate Specificity for the Amino Acceptors

The benzoyl-CoA and the acyl-CoAs are defined as the amino acceptor substrates for mGLYAT. Substrate specificity for amino acceptors was evaluated by fixing the initial glycine concentration at 100 mM, varying the concentration of the amino acceptor substrate, and measuring the initial rate of CoA-SH release using DTNB [28]. Benzoyl-CoA and short-chain acyl-CoAs are mGLYAT substrates with respectable $(k_{cat}/K_m)_{app}$ values (Table 1). Amino acceptor substrate preference ranked in decreasing order is benzoyl-CoA > butyryl-CoA > hexanoyl-CoA > acetyl-CoA under these standard conditions of this study. These data are consistent with earlier reports for mammalian GLYATs purified from natural sources [8,9, 11-14,30-34]. The activity data of Table 1 combined with the data included in Fig. 2 demonstrate that we have successfully expressed and purified active, recombinant mGLYAT from *E. coli*.

The recent discoveries of long-chain *N*-acylglycines like *N*-arachidonoylglycine [35], *N*-oleoylglycine [36], and *N*-palmitoylglycine [37] have pointed towards an GLYAT-like enzyme responsible for their biosynthesis: fatty acyl-CoA + glycine *N*-fatty acylglcyine [38]. At 100 mM glycine, we tested 500 μM oleoyl-CoA as a substrate and activity was not observed (data not shown). This result is consistent those of others demonstrating that the V/K for glycine conjugation decreases as the length of the acyl chain increases for the acyl-CoA substrates. It is possible that activity might be observed at higher concentrations of oleoyl-CoA, but our data and those from others suggest that mGLYAT will not catalyze the formation of long chain *N*-acylglycines *in vivo*.

The lack of any measurable activity from oleoyl-CoA does not eliminate the possibility of binding to mGLYAT and oleoyl-CoA is a known inhibitor for other enzymes of the GNAT family, including ovine serotonin *N*-acetyltransferase [39,40] and *Drosophila melanogaster* arylalkylamine *N*-acetyltrasferase [41]. To test this hypothesis, we determined the IC_{50} value for oleoyl-CoA while holding the concentration of glycine (6.1 mM) and benzoyl-CoA (9.4 μM) constant at their respective $K_{m,app}$ values. Oleoyl-CoA was found to have an IC_{50} value of 21 ± 3.4 μM (Table 2). These data indicate that oleoyl-CoA does bind to mGLYAT even though the our data suggests that oleoyl-CoA is not a substrate for this enzyme. Nandi *et al*. [17] showed that the kinetic mechanism for bovine GLYAT was ordered sequential mechanism with benzoyl-CoA binding first followed by the binding of glycine. Assuming a similar kinetic mechanism for mGLYAT, the binding of oleoyl-CoA could alter the active site conformation that precludes the optimum positioning oleoyl-CoA and glycine for effective conjugation chemistry. Alternatively, the long-chain acyl group of oleoyl-CoA could extend into the glycine binding pocket within the mGLYAT active site to either prevent or dramatically decrease the affinity of the enzyme for glycine. The resolution of these possibilities awaits the availability of mGYLAT structure. The binding of oleoyl-CoA points to a GLYAT-like enzyme that would accept long-chain acyl-CoA thioesters as substrates, enzymes that have been identified by Waluk *et al*. [38]. In addition, we have recently described a GNAT family enzyme that will utilize long-chain acyl-CoA thioesters and serotonin as substrates to produce long-chain *N*-acylserotonins [42].

mGLYAT Substrate Specificity for Amino Donors

The amino donor substrates for mGLYAT are defined as those compounds that contribute the nitrogen atom in the final amide product. The amino donor specificity was assessed by fixing the initial benzoyl-CoA concentration at 300 μM while varying the concentration of the putative amino donor substrates. Of the amino donors that we tested, only glycine, glycine methyl ester, and glycine ethyl ester were mGLYAT substrates. All the other amino donors included in our study, including those structurally very similar to glycine (L-alanine, β-alanine, and ethanolamine) were not substrates (Fig. 3A). Our data for the amino donor specificity is in agreement with earlier work on other mammalian GLYATs demonstrating a strong preference for glycine. Our experiments demonstrating that glycine methyl ester and glycine ethyl ester exhibit $(k_{cat}/K_m)_{app}$ values that are 6-11% of the value for glycine, $(k_{cat}/K_m)_{app,\ glycine}$ of 520 ± 20 $M^{-1}s^{-1}$ (Table 3) are the first report of any other amino donor substrate for GLYAT that has respectable $(k_{cat}/K_m)_{app}$ value relative to that for glycine. These data indicate that the carboxylate moiety of glycine is important, but not critical in the binding of the amino donors to mGLYAT.

The lack of any measurable product formation from the amino donors shown in Fig. 3A does not mean that these compounds cannot bind to mGLYAT. As detailed above, we found that oleoyl-CoA inhibits mGLYAT, but apparently is not an mGLYAT substrate. The non-substrate amino donors were screened at 100 mM for the inhibition of the mGLYAT-catalyzed formation of hippurate with the initial concentrations of benzoyl-CoA and glycine

being fixed at their $K_{m,app}$ values. L-Alanine, β-alanine, L-serine, L-valine, and L-threonine showed very weak inhibition under these conditions, 15% of the initial rate of hippurate formation (Fig. 3B). The other amino donors tested, Gly-Gly-Gly, ethanolamine, and tyramine, were better inhibitors, with percent inhibition values of 35%-55% (Fig. B). Given that Gly-Gly-Gly, ethanolamine, and tyramine were evaluated as mGLYAT inhibitors at 100 mM, even these 3 amino-containing compounds are weak inhibitors, at best. The data shown in Fig. 3B highlight the strong preference of mGLYAT for glycine as the amino donor substrate.

Previous reports for other enzymes in the GNAT family show that compounds containing a hydroxyl group in place of the amino group are inhibitors of amide formation [41,43]. There seems to be no evidence that enzymes from the GNAT family will utilize hydroxyl-containing compounds as substrates to form esters. Replacement of the amine in glycine with a hydroxyl results in glycolic acid, HOOC-CH_2-OH. Glycolic acid is not an mGLYAT substrate even at 100 mM (Fig. 3A); however, glycolic acid does inhibit the mGLYAT-catalyzed formation of hippurate from benzoyl-CoA and glycine. These data suggest that mGLYAT will not catalyze ester formation, but that hydroxyl-containing compounds can inhibit this enzyme. The IC_{50} value for glycolic acid, 58 ± 4.9 mM (Table 2), is ~10-fold higher than the $K_{m,app}$ value for glycine, 6.1 ± 1.2 mM (Table 3), indicating the amino group may decrease the binding affinity to mGLYAT ~1 kcal/mol relative to an hydroxyl group.

Ethanolamine, another hydroxyl-containing compound (H_2N-CH_2-CH_2-OH), inhibits mGLYAT weakly (Fig 3B), but this compound lacks a carboxylate and might bind to mGLYAT in more than one orientation with either its H_2N-group or the HO-group docked into the position of the H_2N-group of glycine within the mGLYAT active site. Therefore, a direct comparison of the binding of glycolic acid to mGLYAT to the binding of ethanolamine to mGLYAT (both relative to the binding of glycine) is probably naïve.

Comparison of Steady-state Kinetic Constants for Recombinant mGLYAT to those for Other Mammalian GLYATs

Literature values for the kinetic constants from other mammalian GLYATs show significant variation, even in comparing results for GLYAT from the same source (Table 4). For example, the $K_{m,app}$ value for benzoyl-CoA from human GLYAT ranges from 13 μM to 5800 μM plus there is a wide range in the reported specific activity values, 170-fold from 0.24μmoles/min/mg for rat GLYAT to 41 μmoles/min/mg to ovine GLYAT. Some of the variation must result from experimental differences between the various research groups and, as suggested by van der Sluis *et al*. [34], some of the variation likely is an outcome from differences in the quality of the particular GLYAT preparations under study.

Despite a few outliers, a review of the data presented in Table 4 shows a reasonable agreement in the values for the $K_{m,app}^{glycine}$, the $K_{m,app}^{benzoyl-CoA}$, and a range of ~3-fold for most of the mammalian GLYATs (7-24 μmoles/min/mg). The values that we have measured for recombinant *E. coli*-expressed mGLYAT possessing a C-terminal His_6-tag, $K_{m,app}^{glycine}$, $K_{m,app}^{benzoyl-CoA}$, and a specific activity = 7.44 ± 0.44 μmoles/min/mg are consistent with most of data shown in Table 4, indicating the this enzyme is very similar to wildtype GLYAT purified from mouse tissue.

Conclusion

In this present study, we were successful in the recombinant expression, purification, and characterization of mGLYAT from *E. coli* with a final yield of 2.5 mg/L culture of pure

enzyme. The steady-state kinetic constants for recombinant mGLYAT were consistent with those values measured for mGLYAT purified from natural sources and other mammalian GLYATs, as well. Thus, the C-terminal His_6-tag fused to the C-terminus of wildtype mGLYAT has little to no effect on the catalytic efficiency of mGLYAT and the recombinant enzyme we have produced in *E. coli* is catalytically comparable to wildtype enzyme.

We defined the substrate specificity of recombinant mGLYAT with respect to both the acyl-CoA acceptor substrates and the amino donor substrates. Benzoyl-CoA is the acceptor substrate with the highest $(V/K)_{app}$. A number of straight-chain acyl-CoA thioesters (acetyl-CoA, butyryl-CoA, and hexanoyl-CoA) were also substrates, but with lower $(V/K)_{app}$ values than that measured for benzoyl-CoA. Oleoyl-CoA was not an mGLYAT substrate, but did inhibit the enzyme with an IC_{50} value of 21 μM. The acceptor specificity we have defined for recombinant mGLYAT is consistent with similar work on other mammalian GLYATs and suggests that the GLYAT is probably not the biosynthetic enzyme for the long-chain mammalian *N*-fatty acylglycines that have recently been identified [35-37].

Our studies of the amino donor specificity reveal a strong preference for glycine. Glycine analogs like L-Ala, L-Ser, ethanolamine, and β-alanine are not substrates, but can inhibit mGLYAT with low affinity. We found that the glycine methyl and glycine ethyl ester are substrates, with $(V/K)_{app}$ values of ~10% of that for glycine, the first report of any amino donor other than glycine serving as a respectable substrate for mGLYAT. Glycolic acid, an analog of glycine with an α-hydroxyl group instead of an α-amino group, is not a mGLYAT substrate, but is an inhibitor suggesting a lead towards the development of inhibitors to this enzyme and other members of the GNAT family of acyltransferases. A more complete understanding of the strong preference mGLYAT exhibits for glycine, a structurally simple molecule, requires a three-dimensional structure of the protein. The successful expression and purification of recombinant mGLYAT from *E. coli* provides a convenient source of enzyme for future mechanistic and structural studies of this protein.

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Highlights

- Mouse glycine *N*-acyltransferase was successfully overexpressed and purified from *Escherichia coli*.
- Benzoyl-CoA and glycine were the substrates with the highest $(k_{cat}/K_m)_{app}$ values.
- Glycine esters have respectable $(k_{cat}/K_m)_{app}$ values relative to glycine.
- Oleoyl-CoA is an inhibitor, but not a substrate for mouse glycine *N*-acyltransferase.

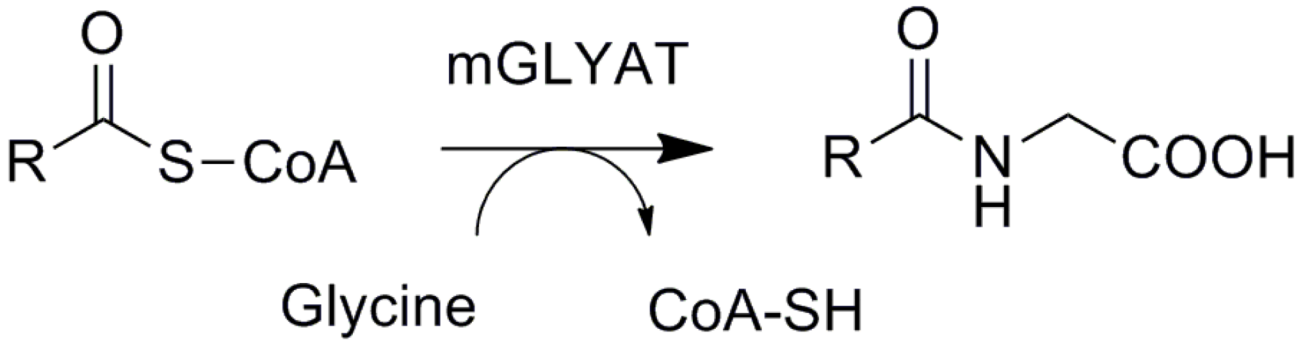

Figure 1.
Glycine *N*-acyltransferase reaction. Reaction catalyzed by mGLYAT where the corresponding amino acceptor and glycine is converted to *N*-acylglycine. R is different acyl chain lengths of the amino acceptor.

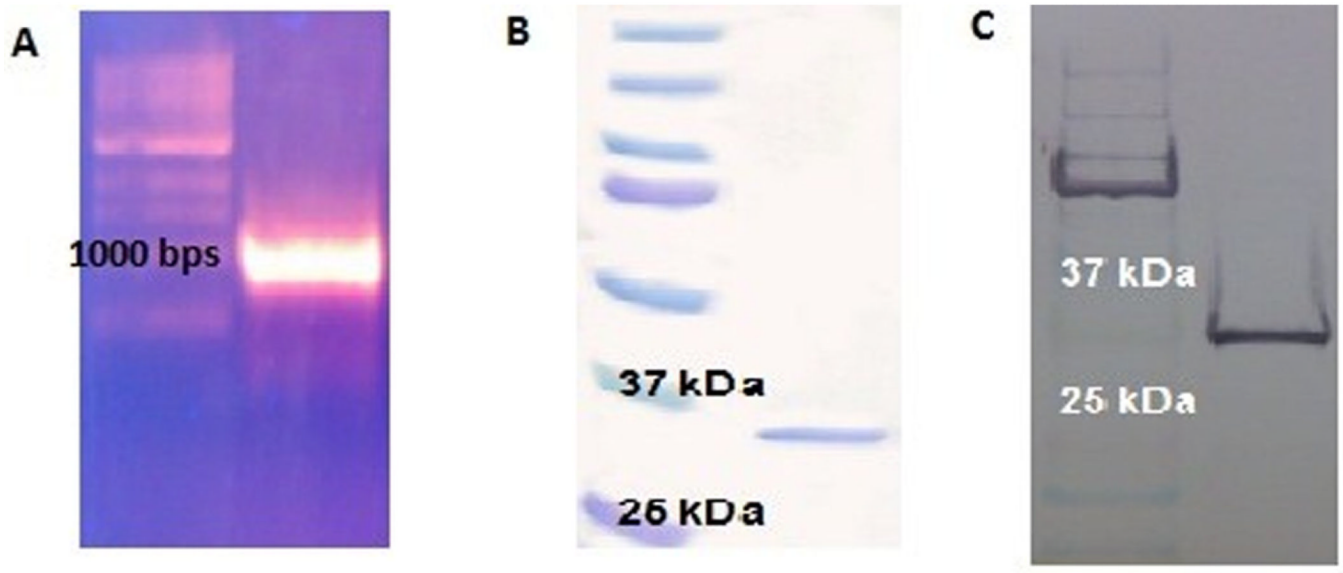

Figure 2.

Cloning and purification of mGLYAT. **A.** Cloning of *mGLYAT* from Origene (MC201077). Lane 1, 1 kb ladder; Lane 2, *mGLYAT* cloning product. **B.** SDS-PAGE of purified mGLYAT. Lane 1, Precision Plus Protein™ Kaleidoscope™ Standards; Lane 2, purified mGLYAT. **C.** Western blot analysis of mGLYAT. Lane 1, Precision Plus Protein™ Kaleidoscope™ Standards; Lane 2, purified mGLYAT after western blot analysis with conjugated alkaline phosphatase.

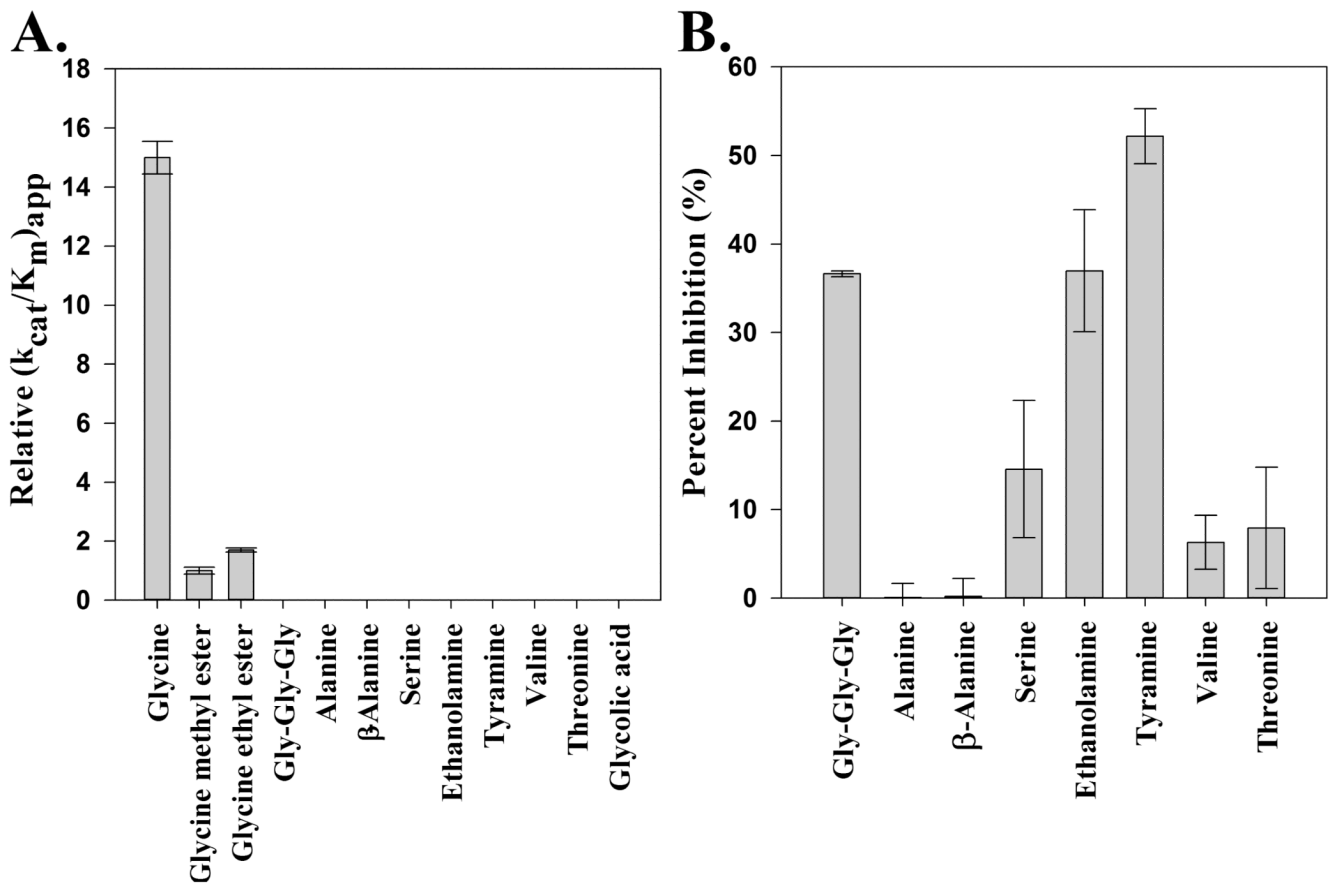

Figure 3.

Relative $(k_{cat}/K_m)_{app}$ and inhibition data for different amino donors. (A) Characterization of a panel of amino donors screened for activity with mGLYAT and 300 μM benzoyl-CoA. Data from screen is represented in the plot by the relative $(k_{cat}/K_m)_{app}$ indexed against the $(k_{cat}/K_m)_{app}$ value for glycine methyl ester, $3.5 \pm 0.41 \times 10^1 M^{-1}s^{-1}$. All amino donors were designated a relative $(k_{cat}/K_m)_{app}$ value of zero if activity was not observed at 300 μM benzoyl-CoA and 100 mM of the amino donor. (B) Percent inhibition of different amino donors screened for activity. Data was generated using the $K_{m,app}$ for both glycine (6.1 mM) and benzoyl-CoA (9.4 μM) and 100 mM of each amino donor.

Table 1

Steady-State Kinetic Constants for Different Amino Acceptors

Substrate[a]	$K_{m,app}$ μM	$k_{cat,app}$ s^{-1}	$(k_{cat}/K_m)_{app}$ $M^{-1}s^{-1}$	Relative $(k_{cat}/Km)_{app}$ [b]
Benzoyl-CoA	9.4 ± 14	4.2 ± 0.25	$(4.5 \pm 0.27) \times 10^5$	1 20 ± 7.0
Acetyl-CoA	209 ± 43	0.80 ± 0.074	$(3.8 \pm 0.35) \times 10^3$	1.0 ± 0.09
Butyryl-CoA	52 ± 7.9	15 ± 0.43	$(2.9 \pm 0.082) \times 10^5$	78 ± 2.2
Hexanoyl-CoA	45 ± 3.0	4.6 ± 0.085	$(1.0 \pm 0.019) \times 10^5$	27 ± 0.50

[a] For mGLYAT the apparent steady-state kinetic constants were determined by using a fixed initial concentration of glycine at 100 mM while varying the concentration of the amino acceptor substrate.

[b] Relative $(k_{cat}/K_m)_{app}$ values were indexed against acetyl-CoA.

Table 2

IC_{50} Values

Inhibitor	IC50 Value[a] (mM)
Oleoyl-CoA	0.021 ± 0.0034
Glycolic acid	58 ± 4.9

[a]The IC50 values were determined by using a fixed concentration of both substrates at their respective Km,app values (benzoyl-CoA - 9.4 μM and glycine - 6.1 mM) while varying the inhibitor concentration.

Table 3

Steady-State Kinetic Constants for Different Amino Donors

Substrate[a]	$K_{m,app}$ mM	$k_{cat,app}$ s^{-1}	$(k_{cat}/K_m)_{app}$ $M^{-1}s^{-1}$	Relative $(k_{cat}/K_m)_{app}$[b]
Glycine	6.1 ± 1.2	3.2 ± 0.12	520 ± 20	15 ± 0.55
Glycine methyl ester	29 ± 9.1	1.0 ± 0.12	35 ± 4.1	1 .0 ± 0.12
Glycine ethyl ester	9.7 ± 1.7	0.57 ± 0.023	59 ± 2.3	1.7 ± 0.07

[a] The apparent steady-state kinetic constants were determined by using a fixed concentration of benzoyl-CoA at 300 μM while varying the concentration of the amino donor substrate.

[b] Relative $(k_{cat}/K_m)_{app}$ values were indexed against glycine methyl ester.

Table 4

Steady-State Kinetic Constants for Different Mammalian GLYATs

Organism	Glycine	Benzoyl-CoA		Comment
	K_m mM	K_m μM	Specific Activity μmoles/min/mg	
Mouse	6.1 ± 1.2[a]	9.4 ± 14[b]	7.4 ± 0.44	This study
Rat	23 ± 4.0	31 ± 5.0	0.24 ± 0.044	[31]
Human	6.4[c] ND[d] 6.5 ± 1.0 27[c] ND[d]	13[c] 24 ± 3 67 ± 5.0[e] 209[c] 5800[c]	0.54 ± 21 0.73 ± 0.030 ND[d] 0.81[c] 17[c]	[14] [34] [18] [33] [12]
Bovine	2.0 ± 0.30 3.3[c,f] 6.2[c] 3.0[c] 6.0 ± 1.0	16 ± 1.0 9.0[c] 160[c] 20.0[c] 110 ± 31[e]	ND 10.4[c] 10 ± 0.095 24[c] 17 ± 2.0	[19] [30] [14] [17] [32]
Rhesus Monkey	20.0[c]	6.0[c]	9.0[c]	[11]
Ovine	ND[d]	2300[c]	41[c]	[12]

[a] For mGLYAT the apparent steady-state kinetic constants were determined by using a fixed concentration of benzoyl-CoA at 300 μM while varying the concentration of glycine.

[b] For mGLYAT, the apparent steady-state kinetic constants were determined by using a fixed concentration of glycine at 100 mM while varying the concentration of benzoyl-CoA.

[b] For mGLYAT the apparent steady-state kinetic constants were determined by using a fixed concentration of benzoyl-CoA at 300 μM while varying the concentration of glycine.

[c] Errors were not reported for these steady-state kinetic constants.

[d] Steady-state kinetic constant was not determined (ND) in these experiments.

[e] This value is the dissociation constant (K_d) for benzoyl-CoA.

[f] The K_m for glycine this study was determined by holding tiglyl-CoA at a fixed concentration.