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Enzymatic Characterization and Elucidation of the Catalytic Mechanism of a Recombinant Bovine Glycine *N*-Acyltransferase

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ABSTRACT:

Glycine conjugation, a phase II detoxification process, is catalyzed by glycine *N*-acyltransferase (GLYAT; E.C. 2.3.1.13). GLYAT detoxifies various xenobiotics, such as benzoic acid, and endogenous organic acids, such as isovaleric acid, which makes GLYAT important in the management of organic acidemias in humans. We cloned the open reading frame encoding the bovine ortholog of GLYAT from bovine liver mRNA into the bacterial expression vector pColdIII. The recombinant enzyme was expressed, partially purified, and enzymatically characterized. Protein modeling was used to predict Glu^{226} of bovine GLYAT to be catalytically important. This was assessed by constructing an E226Q mutant and comparing its enzyme kinetics to that of the wild-type recombinant bovine GLYAT. The Michaelis constants for benzoyl-CoA and glycine were determined and were similar for wild-type recombinant GLYAT, E226Q recombinant GLYAT, and GLYAT present in bovine liver. At pH 8.0, the E226Q mutant GLYAT had decreased activity, which could be compensated for by increasing the reaction pH. This suggested a catalytic mechanism in which Glu^{226} functions to deprotonate glycine, facilitating nucleophilic attack on the acyl-CoA. The recombinant bovine GLYAT enzyme, combined with this new understanding of its active site and reaction mechanism, could be a powerful tool to investigate the functional significance of GLYAT sequence variations. Eventually, this should facilitate investigations into the impact of known and novel sequence variations in the human GLYAT gene.

Introduction

Humans and other animals are constantly exposed to various toxic substances. Homeostasis is maintained by the detoxification and elimination of these toxins. Detoxification is commonly divided into phase I and phase II detoxification enzyme systems (Liska, 1998). Phase I detoxification enzymes convert toxic compounds to secondary metabolites that are frequently more chemically reactive and thus more toxic than the original compounds. Phase II detoxification enzymes decrease the toxicity of the secondary metabolites by means of conjugation to soluble carrier molecules such as glycine or glutathione. The resulting conjugates are usually less toxic, more water-soluble, and more readily excreted in urine than the secondary metabolites. In humans, impaired detoxification has been associated with diseases such as cancer and with adverse reactions to pharmaceutical drugs (Campbell et al., 1988; Liska, 1998; Wallig, 2004).

Glycine *N*-acyltransferase (GLYAT; E.C. 2.3.1.13) is a phase II detoxification enzyme found in the liver and kidney mitochondria of mammals (Schachter and Taggart, 1954). GLYAT is a member of the Gcn5-related *N*-acyltransferase (GNAT) superfamily, one of the largest and most functionally diverse superfamilies of enzymes known (Vetting et al., 2005). GLYAT uses glycine and an acyl-CoA as substrates, forming free CoA and an acylglycine as products (Dyda et al., 2000; van der Westhuizen et al., 2000; Vetting et al., 2005). The acylglycines are less toxic than the unconjugated organic acids and are more readily excreted into urine by the kidneys (Tanaka and Isselbacher, 1967; Duffy et al., 1995). Examples of xenobiotic metabolites that are detoxified by conjugation to glycine include salicylic acid, benzoic acid, and methylbenzoic acid, a metabolite of the industrial solvent xylene (Campbell et al., 1988; Duffy et al., 1995). Studies have shown that in humans, metabolism of aspirin to its glycine conjugate may be impaired by exposure to xylene, which is detoxified via the same pathway (Campbell et al., 1988). Organic acids of endogenous origin, including isovaleric acid, 3-methylcrotonic acid, tiglic acid, and hexanoic acid, are also detoxified by conjugation to glycine. For this reason, glycine conjugation is of key importance to the management of inherited organic acidemias in humans (Kølvraa and Gregersen, 1986; Ogier de Baulny and Saudubray, 2002). For example, isovaleric acidemia is treated by means of glycine supplementation, which enhances formation and excretion of isovalerylglycine (Tanaka et al., 1966; Tanaka and Isselbacher, 1967). Isovaleric acidemia is only one of a large group of human diseases known as CoA sequestration, toxicity, or redistribution (CASTOR) disorders (Mitchell et al., 2008), which represent a

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ABBREVIATIONS: GLYAT, glycine *N*-acyltransferase; GNAT, Gcn5-related *N*-acyltransferase; CASTOR, CoA sequestration, toxicity, or redistribution; PDB, Protein Data Bank; PAGE, polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BLAST, Basic Local Alignment Search Tool.

major segment of biochemical genetics. One of the primary mechanisms of pathogenesis in CASTOR disorders is depletion of free CoA, which derails cellular metabolism. GLYAT converts the accumulated acyl-CoA to acylglycines and free CoA, restoring levels of free CoA and carnitine (Sakuma, 1991). Significant interindividual variation in glycine conjugation capacity has been demonstrated using human liver samples (Temellini et al., 1993). The basis for this variability is not understood, but genetic variations in the coding sequence may be a factor. Six nonsynonymous single-nucleotide polymorphisms have been identified in the open reading frame of the human GLYAT gene. However, it is not yet understood whether, or how, these variations influence enzyme function (Yamamoto et al., 2009; Lino Cardenas et al., 2010).

Because of the large number of compounds metabolized by GLYAT, the human ortholog is of clinical interest. However, we have not yet been able to express an enzymatically active recombinant human GLYAT. Here, we report the bacterial expression and enzymatic investigation of a recombinant bovine GLYAT. Bovine GLYAT is an enzyme expressed in bovine liver and kidney mitochondria, with a molecular mass reported to be between 33 and 36 kDa (Nandi et al., 1979; van der Westhuizen et al., 2000). Investigations of human and bovine GLYAT have shown that these enzymes are similar in terms of molecular mass, reaction kinetics, and substrate specificity (Bartlett and Gompertz, 1974; Kelley and Vessey, 1993; van der Westhuizen et al., 2000). The recombinant bovine GLYAT, combined with molecular modeling and site-directed mutagenesis, was used to investigate the catalytic mechanism used by GLYAT. The data suggest that residue Glu^{226} of bovine GLYAT serves as a general base catalyst. The identification of this catalytic residue provides the first insights into the catalytic mechanism and active site location of the GLYAT enzymes.

Materials and Methods

Sequence Analysis and Molecular Modeling. The bovine GLYAT amino acid sequence (NP_803479) was submitted to the GenTHREADER server (Jones, 1999) for identification of potential structural homologs. An uncharacterized protein from *Drosophila melanogaster*, with Protein Data Bank (PDB) code 1SQH, was identified as the best homolog and was used for molecular modeling. Although this protein is only 13% identical to bovine GLYAT, it is structurally very similar to other GNAT enzymes and was used as template structure because of the exceptional conservation of structure in the GNAT superfamily of acyltransferases, despite there being virtually no sequence similarity between some members of the superfamily (Vetting et al., 2005). The alignment generated by GenTHREADER was used with the structure of 1SQH as input for model generation using MODELLER 9.3 (Eswar et al., 2008). For side-chain modeling, SCWRL 3.0 (Dunbrack Lab, Fox Chase Cancer Center, Philadelphia, PA) was used (Wang et al., 2008). The molecular model was superimposed with the structures of serotonin *N*-acetyltransferase (PDB code 1CJW), diamine *N*-acetyltransferase (PDB code 2Q4V), and Esa1 (PDB code 1GHE) using the matchmaker algorithm of University of California, San Francisco, Chimera (UCSF Chimera) (Pettersen et al., 2004). UCSF Chimera was used to generate images of the molecular model. The bovine GLYAT amino acid sequence was also submitted to a Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov) search, and the homologs were aligned using CLUSTALX 2.0.10.

Cloning of the Bovine GLYAT Open Reading Frame into the Bacterial Expression Vector pColdIII. Total RNA was isolated from bovine liver tissue using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Cloned avian myeloblastosis virus reverse transcriptase (Invitrogen, Carlsbad, CA) was used to generate cDNA from the bovine liver mRNA. The open reading frame of bovine GLYAT was amplified from the cDNA using oligonucleotide primers (5′-GCC GCA TAT GAT GTT CCT GCT GC-3′ and 5′-CAT CTC GAG TCA CAG AGG CTC AC-′3) that contained NdeI and XhoI restriction endonuclease recognition sites to facilitate cloning into pColdIII (Takara Bio USA, Madison, WI). Oligonucleotide primers were obtained from Inqaba Biotechnical Industries (Pretoria, South Africa). The pColdIII vector was modified to encode a C-terminal hexahistidine tag after the XhoI site. The recombinant plasmid was sequenced to confirm that bovine GLYAT had been cloned without any sequence aberrations.

Construction of the E226Q Mutant Recombinant Bovine GLYAT. Site-directed mutagenesis using a mega-primer method (Aiyar and Leis, 1993) was used to generate the E226Q mutant coding sequence. In a first polymerase chain reaction, the mutagenic oligonucleotide primer 5′-CCA GAC GGG ACA GAT GCG GAT GG-3′ was used with the reverse primer 5′-CTT CTC GAG AGG CTC ACA GTT CCA CTG G-3′ to generate a 240-base pair amplicon. This amplicon was gel-purified and was used in a second polymerase chain reaction with the forward primer 5′-GCC GCA TAT GAT GTT CCT GCT GC-3′ to generate a full-length mutated GLYAT coding sequence. The mutated amplicon was gel-purified, digested with NdeI and XhoI, and cloned into pColdIII.

Expression and Nickel-Affinity Purification of Wild-Type and E226Q Recombinant Bovine GLYAT. The pColdIII-bovine GLYAT plasmid was introduced into Origami cells (Novagen, Madison, WI) by electroporation. Expression of the recombinant GLYAT was performed as follows. The cells from 50 ml of overnight cultures in Luria Bertani medium, containing 100 μg/ml ampicillin, were harvested by centrifugation at 4000*g* for 5 min. The cells were resuspended in 200 ml of Luria broth medium containing 50 μg/ml ampicillin. The cultures were gently shaken at 15°C for 1 h before isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. The cultures were incubated at 15°C for 24 h with vigorous shaking. Cells were then harvested by centrifugation at 4000*g* for 20 min. The cell pellets were resuspended in 5 ml of BugBuster protein extraction reagent (Novagen) containing 30 U/ml lysozyme (Novagen) and 25 U/ml Benzonase nuclease (Novagen), followed by incubation at room temperature for 5 min. Insoluble material was removed by centrifugation at 12,000*g* for 25 min at 4°C. The cleared lysates were passed through Protino Ni-TED 2000 columns (Macherey-Nagel, Düren, Germany) equilibrated with buffer LEW (Macherey-Nagel). The columns were washed with 10 ml of buffer LEW containing 20 mM imidazole. The bound protein was eluted from the columns in 9 ml of buffer EB (Macherey-Nagel) and was added to Vivaspin 20 ultrafiltration devices (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The proteins were concentrated to approximately 500 μl by centrifugation in a fixed-angle centrifuge at 8000*g* for 15 min. The proteins were then washed by adding 10 ml of 50 mM Tris-HCl, pH 8.0, and repeating the centrifugation to again concentrate the solution to approximately 500 μl. Protein expression and purification were monitored by means of SDS-polyacrylamide gel electrophoresis (PAGE) analyses and Coomassie Brilliant Blue staining (Laemmli, 1970).

Preparation of an Extract Containing Bovine Liver Mitochondrial GLYAT. To prepare an extract containing bovine liver mitochondrial GLYAT, 100 g of liver tissue was homogenized in 400 ml of 0.13 M KCl. The homogenate was centrifuged for 10 min at 600*g*. The supernatant was centrifuged again at 9000*g* for 10 min to isolate mitochondria, which were then lysed by three cycles of freezing and thawing in 10 ml of 0.13 M KCl. The lysates were clarified by centrifugation at 35,000*g* at 4°C for 2 h. The GLYAT enzyme was further enriched from these lysates by collecting the fraction soluble between 40 and 60% ammonium sulfate. The precipitate was dissolved in 4 ml of 50 mM Tris-HCl, pH 8.0, and was dialyzed overnight against 1000 ml of 50 mM Tris-HCl, pH 8.0 (van der Westhuizen et al., 2000). This crude mitochondrial GLYAT preparation is referred to as "bovine liver GLYAT" in the remainder of this article.

Enzyme Assays for Determination of K_M Parameters. Reaction mixtures were 400 μl in volume and contained, in addition to enzyme, 25 mM Tris-HCl, pH 8.0, 0.1 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], and varying concentrations of substrates (Kølvraa and Gregersen, 1986). The glycine concentration was varied from 2.5 to 20 mM, and the benzoyl-CoA concentration was varied from 5 to 50 μM. The assays were performed at 30°C, and the change in absorbance at 412 nm over the first 4 min was measured using a Uvikon XS spectrophotometer (NorthStar Scientific, Bardsey, Leeds, UK) (van der Westhuizen et al., 2000). The absorbance change at 412 nm is the result of reduction of DTNB by the liberated thiol group of CoA, forming 2-nitro-5-thiobenzoate. 2-Nitro-5-thiobenzoate is a yellow species that absorbs at 412 nm with an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ (Ellman, 1959). To each reaction, 1 unit of GLYAT activity was added. A unit of GLYAT activity was defined as a change in A_{412} of 0.24 units, in 4 min, using cuvettes

with a 1-cm light path, 20 mM glycine, and 100 μM benzoyl-CoA. The amount of enzyme used per assay was defined in this way because the enzymes used were not purified to homogeneity. Although this precluded the determination of V_{max} values, K_M parameters could be determined using the partially purified enzyme preparations (van der Westhuizen et al., 2000; Palmer, 2001). All assays were performed in triplicate, and the data were analyzed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). To determine the apparent K_M values for isovaleryl-CoA, propionyl-CoA, benzoyl-CoA, and octanoyl-CoA (Sigma-Aldrich, St. Louis, MO), the same conditions were used, except that the glycine concentration was fixed at 200 mM and the acyl-CoA concentrations were varied from 10 to 600 μM (Nandi et al., 1979).

Determining the pH Dependence of the GLYAT Enzymes. Reaction mixtures were 100 μl in volume and consisted of 50 mM potassium phosphate buffer, 0.1 mM DTNB, 200 μM benzoyl-CoA, and 200 mM glycine, at various pH values (Fig. 4). Of the crude bovine liver GLYAT extract, 30 μg of protein was used per assay. An amount of protein that has activity comparable to the 30 μg of bovine liver GLYAT extract was used in each assay of the wild-type recombinant GLYAT. This was usually approximately 3 μg of protein. Because the proteins were not purified to homogeneity, SDS-PAGE analysis was generally used to determine the amount of E226Q recombinant GLYAT to use per assay. The mutant enzyme was diluted until the recombinant GLYAT band, as judged by SDS-PAGE, was of intensity equal to that in the wild-type recombinant GLYAT preparation. Because the same amount of enzyme is used in each assay in a particular experiment, pH is the only variable that influences activity. Reactions that contain all components except enzyme were also performed for control purposes. The reactions were monitored at 412 nm in 96-well plates on a BioTech plate reader and the accompanying Gen5 software (Bio-Tek Instruments, Winooski, VT). Measurements were made every 30 s for 6 min. Initial velocities were calculated and recorded as nanomoles per minute. Reactions were performed in triplicate, and the data were plotted as nanomoles per minute against pH, using a logarithmic scale for the *y*-axis. Data were plotted using GraphPad Prism 4.02 (GraphPad Software Inc., San Diego, CA).

Results

Prediction of the Catalytic Importance of Glu^{226} of Bovine GLYAT. To investigate the catalytic mechanism of bovine GLYAT, a candidate for the catalytic residue had to be identified. A BLAST search using the bovine GLYAT amino acid sequence as query was performed. After removing sequences of significantly different length to bovine GLYAT, a multiple sequence alignment was performed (Fig. 1A). The bovine GLYAT sequence was then used to construct a molecular model. By investigating the superposition of the GLYAT model with GNAT enzymes for which the catalytic residues are known, a putative catalytic residue was identified. The Glu^{226} residue of bovine GLYAT coincided spatially (Fig. 1B) with the catalytic residue, His^{120}, of serotonin *N*-acetyltransferase (Scheibner et al., 2002). Similar results were obtained when this superposition was done using other GNAT enzymes for which catalytic mechanisms are known (Fig. 1B). The Glu^{226} residue of bovine GLYAT was also conserved in the top 40 GLYAT homologs obtained by a BLAST search, suggesting the residue to be functionally significant (Fig. 1A shows part of the multiple alignment).

Expression and Purification of Recombinant Bovine GLYAT Enzymes. Both the wild-type and E226Q recombinant bovine GLYAT were expressed at high levels from the bacterial expression vector pColdIII. Most of the recombinant bovine GLYAT, both of the wild-type and the E226Q mutant, was insoluble (Fig. 2A; GLYAT is indicated by an arrow). Soluble wild-type and E226Q recombinant GLYAT was obtained by means of nickel-affinity chromatography and ultrafiltration (Fig. 2B). The lower bands in Fig. 2B (indicated by the arrow) represent soluble recombinant bovine GLYAT enzymes and some copurifying proteins. Expression of the recombinant bovine GLYAT enzymes with hexahistidine tags containing serine-glycine linkers of different lengths, to enhance the flexibility and accessibility of the tag, did not improve purification. Expression with the tags of different length did, however, result in size differences being observed for the lower bands in Fig. 2B, indicating that these bands represent the recombinant bovine GLYAT enzymes (results not shown). The recombinant bovine GLYAT proteins were not subjected to further purification, as the copurified proteins did not seem to interfere with any subsequent investigations.

Kinetic Properties of the Recombinant Bovine GLYAT Enzymes. To characterize the wild-type and E226Q recombinant bovine GLYAT enzymes, Michaelis constants were determined using glycine

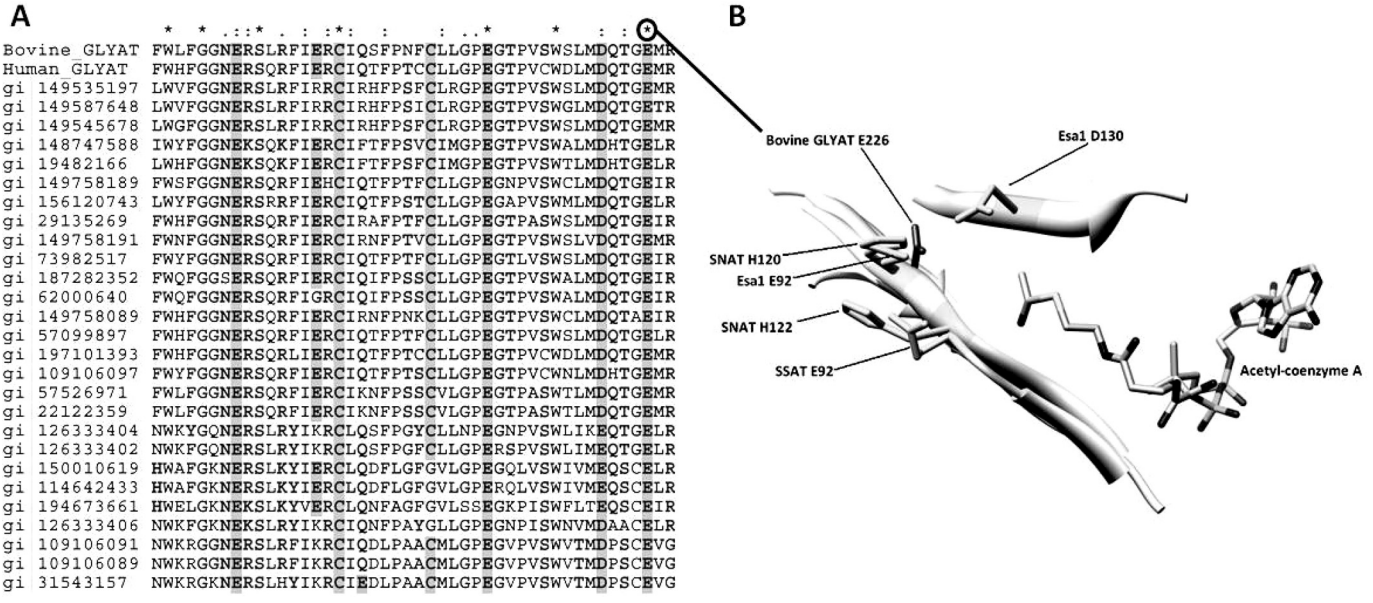

FIG. 1. Prediction of a putative catalytic glutamate residue for bovine GLYAT. A, part of a multiple alignment of sequences with significant similarity to bovine GLYAT, demonstrating that the Glu^{226} residue is conserved. Homologs are results of a BLAST search and GenBank accession numbers are shown to the left of the alignment. B, part of a structural superposition of the structures of SNAT, SSAT, and Esa1 with the bovine GLYAT model. The catalytic residue side chains of the GNAT enzymes are shown. The bovine GLYAT Glu^{226} residue is indicated on both the multiple alignment and the molecular model. The small molecule to the right is CoASAc, a substrate bound to SNAT. This image was generated using UCSF Chimera.

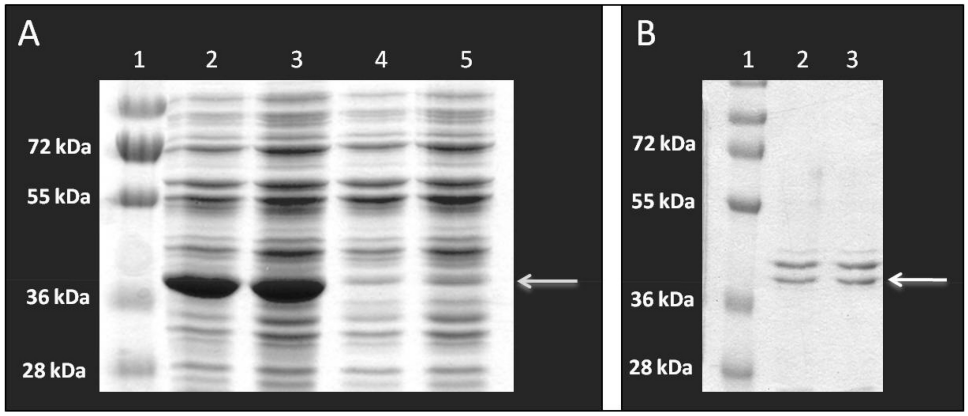

FIG. 2. Bacterial expression and partial purification of the recombinant bovine GLYAT enzymes. A, SDS-PAGE analyses of the recombinant bovine GLYAT expression. Lanes: 1, PageRuler protein size marker; 2, wild-type, total fraction; 3, E226Q, total fraction; 4, wild-type, soluble fraction; 5, E226Q, soluble fraction. The arrow indicates the position of recombinant bovine GLYAT on the gel. The molecular mass of the recombinant bovine GLYAT enzymes is approximately 36.7 kDa. B, SDS-PAGE analyses of recombinant bovine GLYAT partially purified by nickel-affinity chromatography. Lanes: 1, PageRuler protein size marker; 2, soluble, partially purified wild-type recombinant GLYAT; 3, soluble, partially purified E226Q mutant recombinant GLYAT. Recombinant bovine GLYAT is indicated by the arrow, as some unidentified copurifying proteins are also visible on the gel.

and benzoyl-CoA and were compared to those for GLYAT extracted from bovine liver mitochondria. Nonlinear regression was used to analyze the kinetic data, and Lineweaver-Burk plots were used to visually represent the data (Fig. 3). The K_M values for benzoyl-CoA were similar for the bovine liver GLYAT and the recombinant bovine enzymes, at approximately 16 μM (Table 1). The K_M values for glycine for bovine liver GLYAT and wild-type recombinant GLYAT were also similar, at approximately 2 mM. However, the K_M value for glycine of the E226Q mutant was higher, at approximately 7 mM (Table 1). The apparent K_M values (at 200 mM glycine) were also determined for bovine liver GLYAT and wild-type recombinant bovine GLYAT, using propionyl-CoA, isovaleryl-CoA, benzoyl-CoA, and octanoyl-CoA. These values were also comparable for the two enzymes (Table 2).

The pH Dependence of Bovine GLYAT Enzymes. The catalytic importance of the Glu^{226} residue of bovine GLYAT was investigated by determining the pH dependence of wild-type and E226Q mutant recombinant bovine GLYAT enzymes and comparing it to that of GLYAT extracted from bovine liver mitochondria. Increasing pH resulted in increased reaction rates for all three enzymes. Both the bovine liver GLYAT and wild-type recombinant bovine GLYAT enzymes had relatively low activity at pH 6.0, and activity increased with pH to a maximum at pH 7.5. As the pH increased further from 7.5 to 9.6, no significant increase in enzyme activity was observed (Fig. 4). The activity of the E226Q mutant GLYAT enzyme increased as the pH was increased from 6.0 to 9.6. In a representative experiment, the activity of the E226Q mutant bovine GLYAT was approximately 6% of the activity of the wild-type recombinant bovine GLYAT, at pH 8.0. At higher pH values, the activity of the mutant was increased significantly, with the E226Q mutant being approximately 111% as active as the wild-type recombinant GLYAT, at pH 9.6 (Fig. 4). This effect was consistently observed using different preparations of the enzymes. Because the recombinant bovine GLYAT enzymes were only partially purified, SDS-PAGE had to be used to compare the recombinant bovine GLYAT content of the wild-type and E226Q recombinant GLYAT preparations. This method worked well but is not completely accurate, which explains the difference between the activities of the wild-type and E226Q mutant recombinant GLYAT at pH 9.6.

Discussion

The purpose of this study was to generate and enzymatically characterize a recombinant bovine GLYAT and to initiate investigations of the catalytic mechanism of the enzyme. It is important to understand the molecular and biochemical characteristics of the GLYAT family of enzymes, because these enzymes metabolize a

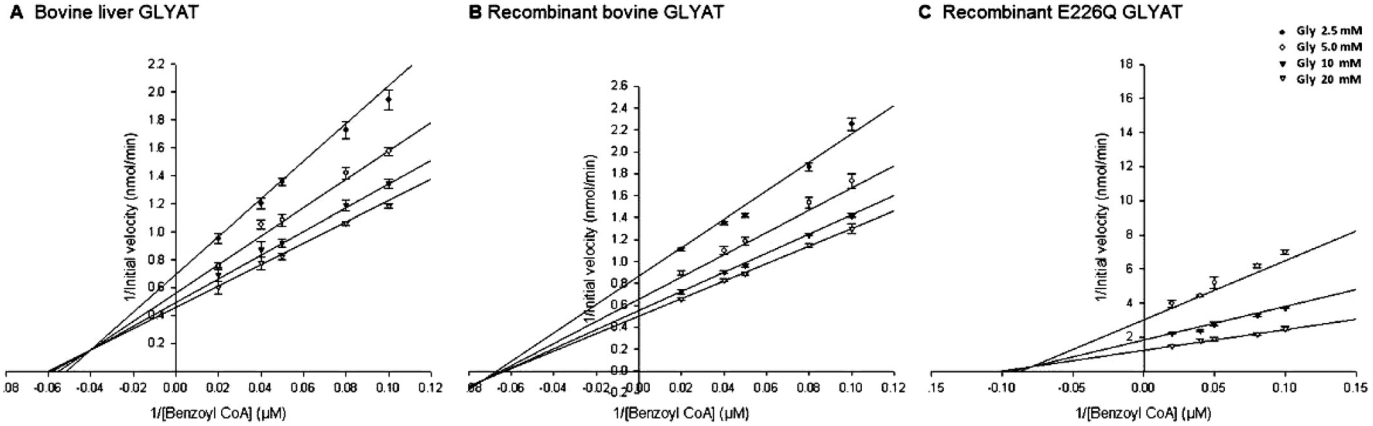

FIG. 3. Lineweaver-Burk plots used to visualize the kinetic parameters of the GLYAT enzymes. Benzoyl-CoA and glycine were used as substrates. Benzoyl-CoA concentrations were 10, 15, 25, 40, and 50 μM. The data points indicate average values ± S.D. of triplicate assays. A, bovine liver GLYAT; B, recombinant bovine GLYAT; C, recombinant E226Q bovine GLYAT. Plots were generated using SigmaPlot 11.0.

TABLE 1

K_M values for wild-type and E226Q mutant recombinant bovine GLYAT and GLYAT from bovine liver (n = 3)

Data are presented as means ± S.D.

GLYAT Enzyme	K_M Benzoyl-CoA	K_M Glycine
	μM	*mM*
Recombinant bovine GLYAT	16 ± 1	2 ± 0.3
E226Q recombinant GLYAT	18 ± 4	7 ± 4
GLYAT from bovine liver	16 ± 3	1.6 ± 0.5

wide range of endogenous and xenobiotic compounds and may present as yet unknown targets for the therapeutic manipulation of inherited metabolic disorders or exposure to toxins. A recombinant bovine GLYAT was used in this study because it could be expressed in an enzymatically active form in *Escherichia coli*. Our attempts to express enzymatically active human GLYAT in bacteria have thus far been unsuccessful. This perhaps correlates with the observation that GLYAT isolated from human liver is less active and less stable than GLYAT isolated from bovine liver (Nandi et al., 1979; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000). However, the reaction kinetics and substrate specificity of the human and bovine GLYAT enzymes are similar, and we started investigations of the GLYAT domain architecture, catalytic mechanism, and functional residues, using the bovine ortholog (Schachter and Taggart, 1954; Bartlett and Gompertz, 1974; Nandi et al., 1979; Kølvraa and Gregersen, 1986; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000).

Our results show that recombinant bovine GLYAT, expressed in *E. coli*, has enzymatic properties similar to those of GLYAT present in an extract of bovine liver mitochondria. The K_M values for several acyl-CoA substrates and glycine were determined and found to be similar for these two enzymes (Tables 1 and 2). In the literature, there is great variation in the K_M values reported for bovine liver GLYAT. The K_M values for benzoyl-CoA range from 9 to 310 μM, and those for glycine range from 2 to 15 mM (Schachter and Taggart, 1954; Bartlett and Gompertz, 1974; Nandi et al., 1979; Gregersen et al., 1986; Kelley and Vessey, 1986, 1993, 1994; Kølvraa and Gregersen, 1986; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000). The K_M values we determined for the bovine liver GLYAT and recombinant bovine GLYAT (approximately 16 μM for benzoyl-CoA and 2 mM for glycine) fall within the range reported in the literature. The similarity of recombinant bovine GLYAT to bovine liver GLYAT, in terms of K_M parameters, suggested that the recombinant enzyme could be a valuable tool for investigation of the catalytic residues of bovine GLYAT. When glycine was omitted from the enzyme assays, no activity could be observed, confirming that the enzyme preparations were not contaminated with any proteins that nonspecifically hydrolyze benzoyl-CoA.

TABLE 2

Apparent K_M values for acyl-CoA substrates of bovine liver and recombinant GLYAT enzymes (n = 3)

Data are presented as means ± S.D.

Acyl-CoA	K_M for Acyl-CoA	
	Bovine Liver GLYAT	Recombinant GLYAT
	μM	
Benzoyl-CoA	18 ± 5	19 ± 2
Octanoyl-CoA	70 ± 10	66 ± 8
3-Methylcrotonyl-CoA	140 ± 24	123 ± 19
Propionyl-CoA	184 ± 30	143 ± 23
Isovaleryl-CoA	195 ± 32	127 ± 20

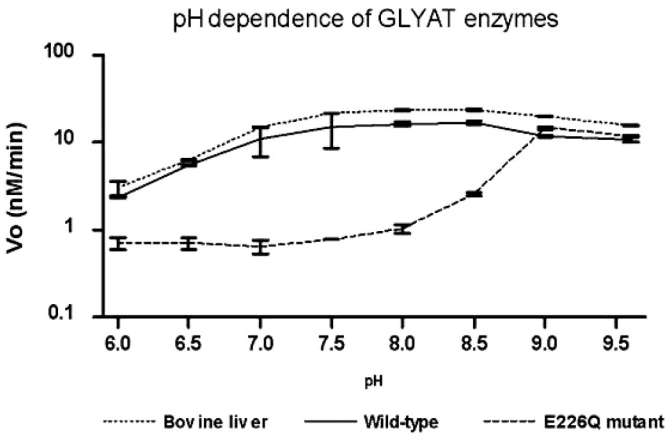

FIG. 4. The pH dependence of the bovine liver, wild-type and E226Q recombinant GLYAT enzymes. Representative initial velocity data (nanomole product formed per minute) is plotted against pH on a logarithmic scale. For each reaction, 30 μg of bovine liver GLYAT, or 3 μg of either recombinant enzyme, was used. Error bars indicate the mean ± S.D. of triplicate assays. Data were plotted using GraphPad Prism 4.02.

Based on the pH dependence of the human and bovine GLYAT enzymes, as reported in the literature, we anticipated that a general base catalyst is involved in the reaction (Schachter and Taggart, 1954; Nandi et al., 1979; Mawal and Qureshi, 1994). Our hypothesis, based on molecular modeling and the similarity of GLYAT to the GNAT superfamily of acyltransferases, was that the residue Glu^{226} of bovine GLYAT served as a general base catalyst in the GLYAT reaction. To investigate this hypothesis, an E226Q mutant was expressed and enzymatically characterized. When similar amounts of wild-type and E226Q recombinant bovine GLYAT were assayed at pH 8.0, there was a difference of approximately 20-fold in activity between the two enzymes. Similarity between the mutant and wild-type enzymes in terms of the K_M values for glycine (approximately 7 and 2 mM, respectively) and benzoyl-CoA (approximately 18 and 16 μM, respectively) suggested that loss of substrate-binding ability of the mutant could not solely account for the lower activity of the mutant enzyme. This is because the assay mixture contained 200 μM benzoyl-CoA and 200 mM glycine, concentrations much higher than the K_M values of both wild-type and E226Q recombinant GLYAT, meaning that both substrates were saturating.

The loss of catalytic activity displayed by the E226Q mutant GLYAT is not sufficient evidence to conclude that our bioinformatic analyses and prediction of the catalytic importance of Glu^{226} of bovine GLYAT are valid. This is because the mutation may simply have altered some structural component of the enzyme, lowering the catalytic rate. Because our objective was to demonstrate that Glu^{226} serves as a general base catalyst, the pH dependence of the mutant and wild-type enzymes was investigated. The wild-type recombinant bovine GLYAT and bovine liver GLYAT reached maximal activity at pH 7.5, and further increases in pH did not have a significant effect. However, the activity of the E226Q mutant increased significantly as pH increased from 6.0 to 9.6. At pH 9.6, the E226Q mutant had activity comparable to that of the wild-type recombinant bovine GLYAT and bovine liver GLYAT. When interpreted in light of the similarity of the kinetic parameters of the wild-type and mutant enzymes (suggesting that the mutant is structurally intact), it was concluded that the Glu^{226} residue is catalytically important in a pH-dependent fashion and is probably the catalytic base residue. This interpretation does not exclude the possibility that the Glu^{226} residue acts in concert with another as yet unidentified residue to catalyze the deprotonation of glycine. What is clear is that the deprotonation of the

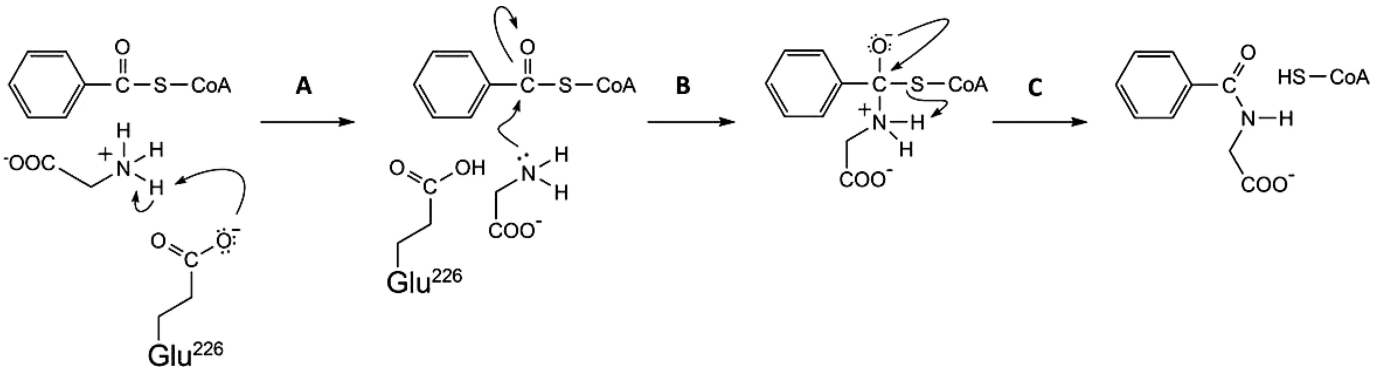

FIG. 5. A schematic representation of the general base-catalyzed, ternary-complex mechanism proposed for bovine GLYAT. A, for nucleophilic attack to occur, the glycine amino group must be deprotonated by Glu^{226}. B, a tetrahedral intermediate is formed after nucleophilic attack by the amino group of glycine on the thioester carbonyl group. C, finally, the tetrahedral intermediate collapses, forming the peptide product and CoA. ChemDraw 10.0 (CambridgeSoft, Cambridge, MA) was used to produce this schematic.

amino group of glycine is important, because it is chemically impossible for the protonated amine to act as a nucleophile, and that Glu^{226} seems to be involved in the process.

For acyl-transfer reactions, there is an alternative mechanism to the ternary-complex, direct transfer mechanism, commonly known as the ping-pong mechanism (Dyda et al., 2000; Berndsen and Denu, 2005). The literature supports our analyses that bovine GLYAT employs a general base-catalyzed, ternary-complex mechanism. First, the reaction kinetics support a ternary-complex mechanism but not a ping-pong mechanism (Nandi et al., 1979; van der Westhuizen et al., 2000). Second, GLYAT is insensitive to thiol-modifying reagents, such as iodoacetamide and *N*-ethylmaleimide, which would inactivate the active site cysteine residue of a ping-pong enzyme (Nandi et al., 1979). Finally, GLYAT is homologous to the GNAT superfamily of acyltransferases, of which members studied to date all employ direct transfer mechanisms.

Based on our analyses, we propose the ternary-complex, base-catalyzed reaction mechanism, as depicted in Fig. 5, for bovine GLYAT. In brief, the glycine amino group is deprotonated by the Glu^{226} residue to increase its nucleophilic character. The nucleophilic amine then attacks the thioester, forming a tetrahedral intermediate that collapses to form the CoA and acylglycine products. We speculate that this mechanism should be conserved among the GLYAT enzymes of different species, based on the conservation of the Glu^{226} residue in the homologs of bovine GLYAT (Fig. 1). If our interpretation is valid, this may provide the first insight into the active site of the human ortholog of bovine GLYAT, an enzyme of increasing clinical relevance. Conclusive evidence for the ternary-complex, base-catalyzed mechanism we propose for bovine GLYAT awaits the determination of a crystal- or NMR structure of a GLYAT enzyme, preferably with bound substrates or an inhibitor. Repeating the photoaffinity labeling of GLYAT, performed by Lau et al. (1977), combined with mass spectrometric identification of the labeled residues, would be another means of investigating the GLYAT active site.

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Authorship Contributions

Participated in research design: Badenhorst, Van Dijk, and Jooste.

Conducted experiments: Badenhorst and Jooste.

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References

Aiyar A and Leis J (1993) Modification of the megaprimer method of PCR mutagenesis: improved amplification of the final product. *Biotechniques* **14:**366–369.
Bartlett K and Gompertz D (1974) The specificity of glycine-N-acylase and acylglycine excretion in the organicacidaemias. *Biochem Med* **10:**15–23.
Berndsen CE and Denu JM (2005) Assays for mechanistic investigations of protein/histone acetyltransferases. *Methods* **36:**321–331.
Campbell L, Wilson HK, Samuel AM, and Gompertz D (1988) Interactions of m-xylene and aspirin metabolism in man. *Br J Ind Med* **45:**127–132.
Duffy LF, Kerzner B, Seeff L, Barr SB, and Soldin SJ (1995) Preliminary assessment of glycine conjugation of para-aminobenzoic acid as a quantitative test of liver function. *Clin Biochem* **28:**527–530.
Dyda F, Klein DC, and Hickman AB (2000) GCN5-related N-acetyltransferases: a structural overview. *Annu Rev Biophys Biomol Struct* **29:**81–103.
Ellman GL (1959) Tissue sulfhydryl groups. *Arch Biochem Biophys* **82:**70–77.
Eswar N, Eramian D, Webb B, Shen MY, and Sali A (2008) Protein structure modeling with MODELLER. *Methods Mol Biol* **426:**145–159.
Gregersen N, Kølvraa S, and Mortensen PB (1986) Acyl-CoA: glycine N-acyltransferase: in vitro studies on the glycine conjugation of straight- and branched-chained acyl-CoA esters in human liver. *Biochem Med Metab Biol* **35:**210–218.
Jones DT (1999) GenTHREADER: an efficient and reliable protein fold recognition method for genomic sequences. *J Mol Biol* **287:**797–815.
Kelley M and Vessey DA (1986) Interaction of 2,4-dichlorophenoxyacetate (2,4-D) and 2,4,5-trichlorophenoxyacetate (2,4,5-T) with the acyl-CoA: amino acid N-acyltransferase enzymes of bovine liver mitochondria. *Biochem Pharmacol* **35:**289–295.
Kelley M and Vessey DA (1993) Isolation and characterization of mitochondrial acyl-CoA: glycine N-acyltransferases from kidney. *J Biochem Toxicol* **8:**63–69.
Kelley M and Vessey DA (1994) Characterization of the acyl-CoA:amino acid N-acyltransferases from primate liver mitochondria. *J Biochem Toxicol* **9:**153–158.
Kølvraa S and Gregersen N (1986) Acyl-CoA:glycine N-acyltransferase: organelle localization and affinity toward straight- and branched-chained acyl-CoA esters in rat liver. *Biochem Med Metab Biol* **36:**98–105.
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:**680–685.
Lau EP, Haley BE, and Barden RE (1977) Photoaffinity labeling of acyl-coenzyme A:glycine N-acyltransferase with p-azidobenzoyl-coenzyme A. *Biochemistry* **16:**2581–2585.
Lino Cardenas CL, Bourgine J, Cauffiez C, Allorge D, Lo-Guidice JM, Broly F, and Chevalier D (2010) Genetic polymorphisms of glycine N-acyltransferase (GLYAT) in a French Caucasian population. *Xenobiotica* **40:**853–861.
Liska DJ (1998) The detoxification enzyme systems. *Altern Med Rev* **3:**187–198.
Mawal YR and Qureshi IA (1994) Purification to homogeneity of mitochondrial acyl coa:glycine n-acyltransferase from human liver. *Biochem Biophys Res Commun* **205:**1373–1379.
Mitchell GA, Gauthier N, Lesimple A, Wang SP, Mamer O, and Qureshi I (2008) Hereditary and acquired diseases of acyl-coenzyme A metabolism. *Mol Genet Metab* **94:**4–15.
Nandi DL, Lucas SV, and Webster LT Jr (1979) Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. Purification and characterization. *J Biol Chem* **254:**7230–7237.
Ogier de Baulny H and Saudubray JM (2002) Branched-chain organic acidurias. *Semin Neonatol* **7:**65–74.
Palmer T (2001) *Enzymes: Biochemistry, Biotechnology, Clinical Chemistry. Horwood Series in Chemical Science*, 5th ed, Horwood Publishing Limited, Chichester.
Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, and Ferrin TE (2004) UCSF Chimera–a visualization system for exploratory research and analysis. *J Comput Chem* **25:**1605–1612.
Sakuma T (1991) Alteration of urinary carnitine profile induced by benzoate administration. *Arch Dis Child* **66:**873–875.
Schachter D and Taggart JV (1954) Glycine N-acylase: purification and properties. *J Biol Chem* **208:**263–275.

Scheibner KA, De Angelis J, Burley SK, and Cole PA (2002) Investigation of the roles of catalytic residues in serotonin N-acetyltransferase. *J Biol Chem* **277:**18118–18126.
Tanaka K, Budd MA, Efron ML, and Isselbacher KJ (1966) Isovaleric acidemia: a new genetic defect of leucine metabolism. *Proc Natl Acad Sci USA* **56:**236–242.
Tanaka K and Isselbacher KJ (1967) The isolation and identification of N-isovalerylglycine from urine of patients with isovaleric acidemia. *J Biol Chem* **242:**2966–2972.
Temellini A, Mogavero S, Giulianotti PC, Pietrabissa A, Mosca F, and Pacifici GM (1993) Conjugation of benzoic acid with glycine in human liver and kidney: a study on the interindividual variability. *Xenobiotica* **23:**1427–1433.
van der Westhuizen FH, Pretorius PJ, and Erasmus E (2000) The utilization of alanine, glutamic acid, and serine as amino acid substrates for glycine N-acyltransferase. *J Biochem Mol Toxicol* **14:**102–109.
Vetting MW, S de Carvalho LP, Yu M, Hegde SS, Magnet S, Roderick SL, and Blanchard JS (2005) Structure and functions of the GNAT superfamily of acetyltransferases. *Arch Biochem Biophys* **433:**212–226.
Wallig MA (2004) Glucuronidation and susceptibility to chemical carcinogenesis. *Toxicol Sci* **78:**1–2.
Wang Q, Canutescu AA, and Dunbrack RL Jr (2008) SCWRL and MolIDE: computer programs for side-chain conformation prediction and homology modeling. *Nat Protoc* **3:**1832–1847.
Yamamoto A, Nonen S, Fukuda T, Yamazaki H, and Azuma J (2009) Genetic polymorphisms of glycine N-acyltransferase in Japanese individuals. *Drug Metab Pharmacokinet* **24:**114–117.

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