Chapter 4: The influence of genetic variation on the enzyme activity of a recombinant human GLYAT

Paper IV

Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase

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Short Communication

Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase

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ABSTRACT

Human glycine N-acyltransferase (human GLYAT) detoxifies a wide range of endogenous and xenobiotic metabolites, including benzoate and salicylate. Significant inter-individual variation exists in glycine conjugation capacity. The molecular basis for this variability is not known. To investigate the influence of single nucleotide polymorphisms (SNPs) in the GLYAT coding sequence on enzyme activity, we expressed and characterised a recombinant human GLYAT. Site-directed mutagenesis was used to generate six non-synonymous SNP variants of the enzyme (K16N; S17T; R131H; N156S; F168L; R199C). The variants were expressed, purified, and enzymatically characterised. The enzyme activities of the K16N, S17T and R131H variants were similar to that of the wild-type, whereas the N156S variant was more active, the F168L variant less active, and the R199C variant was inactive. We also generated an E227Q mutant, which lacks the catalytic residue proposed by Badenhorst et al. (2012). This mutant was inactive compared to the wild-type recombinant human GLYAT. A molecular model of human GLYAT containing coenzyme A (CoA) was generated which revealed that the inactivity of the R199C variant could be due to the substitution of the highly conserved Arg^{199} and destabilisation of an α-loop-α motif which is important for substrate binding in the GNAT superfamily. The finding that SNP variations in the human GLYAT gene influence the kinetic properties of the enzyme may explain some of the inter-individual variation in glycine conjugation capacity, which is relevant to the metabolism of xenobiotics such as aspirin and the industrial solvent xylene, and to the treatment of some metabolic disorders.

1. Introduction

Detoxification is an essential physiological process as it serves to decrease the toxicity of compounds that are not catabolised (Liska, 1998). These compounds include several endogenous metabolites such as steroid hormones and exogenous toxins such as compounds in food or industrial chemicals (Campbell et al., 1988). Detoxification is generally divided into two phases of which phase I detoxification activates metabolites by adding functional groups to them. The activated compounds are often more toxic than the original compounds and are rapidly acted on by phase II detoxification systems (Kinzig-Schippers et al., 2005). These reactions include methylation and conjugation to sulfate, glucorunide and glycine. The purpose of these conjugation reactions is to make the compounds less toxic and more soluble for excretion in the urine and bile (Jakoby and Ziegler, 1990; Liska, 1998; Swinney et al., 2006).

Impaired phase II detoxification has been associated with adverse reactions to pharmaceutical drugs and may be involved in the pathogenesis of complex multifactorial diseases like cancer (Nebert et al., 1996; Wallig, 2004). Glycine N-acyltransferase (EC 2.3.1.13, GLYAT) is a phase II detoxification enzyme found in mitochondria of mammalian liver and kidney (Nandi et al., 1979; Schachter and Taggart, 1954). GLYAT is a member of the Gcn5-related N-acetyltransferase (GNAT) superfamily of N-acyltransferases which uses an acyl-CoA and glycine as substrates (Schachter and Taggart, 1954; Vetting et al., 2005). The products of the reaction are free CoA and an acylglycine that is less toxic and more readily excreted by the kidneys than the original compound (Bartlett and Gompertz, 1974). Little is currently understood about the physiological significance of glycine conjugation and the implications of its impairment (Bartlett and Gompertz, 1974; Campbell et al., 1988; Gregersen et al., 1986; Kolvraa and Gregersen, 1986; Tanaka and Isselbacher, 1967).

A large number of metabolic disorders, collectively named CASTOR disorders (coenzyme A sequestration, toxicity, and redistribution), influence the metabolism of CoA (Mitchell et al., 2008; Ogier and Saudubray, 2002). One of the primary mechanisms of pathogenesis in CASTOR disorders is depletion of free CoA. This depletion is caused by the accumulation of acyl-CoAs, which in turn leads to a depletion of free carnitine, as acylcarnitines are excreted in the urine. Conjugation of accumulating acyl-CoAs to glycine normalises cellular metabolism as levels of free CoA and carnitine are restored (Bartlett and Gompertz, 1974; Mitchell et al.,

Abbreviations: CASTOR, coenzyme A sequestration, toxicity, and redistribution; CoA, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); human GLYAT, human glycine N-acyltransferase; GNAT, Gcn5-related N-acetyltransferase; SNP, single nucleotide polymorphism.

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Table 1
Parameters for GLYAT isolated from human liver.

Parameters	Values	Reference
Protein size (kDa)	27	van der Westhuizen et al. (2000)
	30	Mawal and Qureshi (1994)
	30.5	Kelley and Vessey (1994)
	33.9	Based on sequence NM_201648.2
K_M (benzoyl-CoA) (μM)	13	van der Westhuizen et al. (2000)
	67 ± 5	Kelley and Vessey (1994)
	209	Matsuo et al. (2012)
	57900	Mawal and Qureshi (1994)
V_{max} (nmol/min/mg)	543 ± 21	van der Westhuizen et al. (2000)
	807	Matsuo et al. (2012)
	17100	Mawal and Qureshi (1994)

2008; Sakuma, 1991; Tanaka and Isselbacher, 1967). For example, in isovaleric acidemia, isovaleryl-CoA accumulates to toxic levels. Since isovaleryl-CoA is a good substrate for GLYAT, glycine supplementation enhances excretion of isovalerylglycine, making it a very effective therapy (Dercksen et al., 2012; Tanaka and Isselbacher, 1967; Tanaka et al., 1966).

Xenobiotics which are detoxified by conjugation to glycine include benzoate, salicylate, and methyl-benzoate, a metabolite of the industrial solvent xylene (Levy, 1965; Tremblay and Quereshi, 1993). Competition between different substrates for glycine conjugation is demonstrated by the reduced excretion of salicylurate and methyl-hippurate when aspirin and xylene are co-administered to healthy individuals (Campbell et al., 1988). Significant inter-individual variability in glycine conjugation capacity has been demonstrated using human liver samples (Temellini et al., 1993). There is also large variation in the amount of glycine conjugates excreted after administering benzoate to different individuals (Campbell et al., 1988). No work to date has explained this inter-individual variation in glycine conjugation capacity, but substrate availability and levels of GLYAT expression may be important factors (Matsuo et al., 2012; Tanaka and Isselbacher, 1967). It is not known at present whether known non-synonymous variations in the GLYAT gene may account for some of this variability (Lino Cardenas et al., 2010; Yamamoto et al., 2009).

The enzymatic characteristics of GLYAT have been studied using enzymes isolated from the liver and kidney of several mammals (Bartlett and Gompertz, 1974; Gregersen et al., 1986; Kelley and Vessey, 1993, 1994; Kolvraa and Gregersen, 1986; Mawal and Qureshi, 1994). The values reported for the molecular mass, apparent Michaelis constant (K_{Mapp}), and maximum velocity of human liver GLYAT, are highly variable (Table 1). This variation in values is unexplained, but factors like substrate quality and/or concentration, experimental technique, and genetic heterogeneity of the GLYAT gene may be responsible (Kolvraa and Gregersen, 1986; Lino Cardenas et al., 2010; Yamamoto et al., 2009).

Recently, there have been several reports on the expression, purification, and partial enzymatic characterisation of recombinant GLYATs. A recombinant human GLYATL2 (GLYAT-like 2) was shown to be an acyltransferase that produces long-chain acylglycines, precursors to cannabinoid-like signalling hormones. It was also demonstrated that human GLYATL2 is regulated by reversible acetylation of Lys^{19} (Waluk et al., 2010, 2012). Site-directed mutagenesis revealed that Glu^{226} is the catalytically important residue of a recombinant bovine GLYAT (Badenhorst et al., 2012). Most recently, a recombinant human GLYAT was expressed, purified, and partially characterised (Matsuo et al., 2012). In the work we present here, we used site-directed mutagenesis to investigate the influence of SNPs in the human GLYAT gene on the enzymatic properties of a bacterially expressed recombinant human GLYAT.

2. Methods

2.1. Generation of human GLYAT variants

The human GLYAT reference sequence (GenBank ID: NM_201648.2), cloned into a pET32a(+) expression vector, was purchased from GeneArt (Piscataway, NJ). Mutations of the GLYAT coding sequence, to generate the desired GLYAT variants (Table 2), were introduced using the Phusion Site-Directed Mutagenesis kit (Finnzymes, Vantaa, Finland). The following mutagenic oligonucleotides were employed: **K16N**, CTG CAG ATG CTG GAG AAT TCC TTG AGG AAG; **S17T**, CAG ATG CTG GAG AAA ACC TTG AGG AAG AGC; **K20R**, TCC TTG AGG CGG AGC CTC CCA; **K20Q**, TCC TTG AGG CAG AGC CTC CCA; **R131H**, GTC AAA CAA ACA CAA CAC ATT CTC TAT ATG GCA; **N156S**, AAG ATT TTA TCT CCC AGT GGT GGC AAA CCC AAG; **F168L**, ATC AAC CAA GAG ATG TTA AAA CTC TCA TCC ATG; **R199C**, CAG AGA TTC ATT GAG TGC TGC ATT CAG ACC TTT; and **E227Q**, ATG GAC CAG ACT GGA CAG ATG AGA ATG GCA. All oligonucleotides are in the standard 5′ → 3′ orientation, and were purchased from IDT (Coralville, Iowa). All constructs were Sanger sequenced to confirm introduction of the various mutations.

2.2. Expression and nickel-affinity purification of recombinant human GLYAT and selected variants

All proteins were expressed with an N-terminal His-tag to facilitate purification. Expression of the recombinant human GLYAT variants was carried out as follows. Purified plasmid DNA was used to transform Origami™ cells (Novagen, Madison, WI) already containing the pGro7 chaperone expression plasmid (Takara, Madison, WI). An expression medium containing 2% bacto-tryptone, 1.25% yeast extract, 0.625% NaCl, 0.5% $Na_2HPO_4 \cdot 12H_2O$, 0.1% KH_2PO_4, and 0.2% glucose was used. Colonies of co-transformed Origami™ cells were used to inoculate 10 ml cultures in expression medium containing 50 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 2 mg/ml L-arabinose. The cultures were incubated overnight at 37 °C. Cells were harvested by centrifugation at 4000 ×*g* for 5 min and resuspended in 100 ml of the same antibiotic containing medium. All centrifugation steps were performed at 4 °C. The cultures were further incubated at 37 °C until they reached an optical density of 0.4 at 600 nm. The cultures were then transferred to 28 °C for 24 h. Cells were harvested by centrifugation at 4000 ×*g* for 15 min. Cells were resuspended in 10 ml of a buffer, pH 8.0, containing 50 mM NaH_2PO_4, 300 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, and

Table 2
Variants of human GLYAT investigated in this study.

Recombinant human GLYAT variants		Allele frequencies (%)	
Amino acid change from wild-type	Accession number or RS number	95 Japanese subjects (Yamamoto et al., 2009)	55 Caucasian subjects (Lino Cardenas et al., 2010)
Wild-type	NM_201648.2		
K16N	rs17850556	Not detected	Not detected
S17T	rs10896818	27.4	16.4
R131H	rs117149346	0.5	Not detected
N156S	rs675815	85.3	97.3
F168L	rs1045359	Not detected	Not detected
R199C	rs138125182	Not detected	0.9
E227Q	Novel variant	n/a	n/a

10 μl of Lysonase Bioprocessing Reagent (Novagen, Madison, WI). The resuspended cells were incubated at room temperature for 10 min before being disrupted by five passes through a 22 G needle. The lysates were cleared by centrifugation at 14 000 ×g for 25 min and passed through Protino Ni-TED 2000 columns equilibrated with buffer LEW (Macherey-Nagel Inc., Bethlehem, PA). Columns were washed with 10 ml of buffer LEW before eluting the bound proteins with 3 ml of buffer EB (Macherey-Nagel Inc., Bethlehem, PA). Eluates were immediately desalted on 10 ml columns of Sephadex G25 (Sigma, St. Louis, MO), pre-equilibrated with 25 mM Tris–HCl, pH 8.0. Glycerol was added to 12.5% (v/v), followed by concentration to approximately 1 ml using Vivaspin 6 ultra-filtration devices (GE Healthcare, Björkgatan, Uppsala, Sweden). Expression and purification procedures were monitored by means of SDS-PAGE and Coomassie brilliant blue staining (Laemmli, 1970). Protein concentrations were determined based on the bicinchoninic acid method (Smith et al., 1985) using a kit from Sigma-Aldrich (Sigma, St. Louis, MO). To confirm that the purified proteins contained equal amounts of human GLYAT, western blot analyses were performed using an anti-GLYAT polyclonal antibody (Abcam, Cambridge, MA).

2.3. Enzyme assays for determination of relative activities and K_{Mapp} values

The relative activities of the recombinant human GLYAT variants were determined using a colourimetric assay that measures glycine dependent release of CoA at 412 nm, in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Kolvraa and Gregersen, 1986). Enzyme assays were generally 100 μl in volume, and contained 50 mM Tris-acetate, pH 8.0, 200 mM glycine, 100 μM DTNB, 200 μM benzoyl-CoA, and 0.5 μg of a particular recombinant human GLYAT variant. Reactions were carried out at 37 °C in 96-well plates and monitored for 10 min using a BioTek plate reader and accompanying Gen5 software (BioTek, Winooski, VT). Activities from triplicate assays were calculated from the linear change at 412 nm over 10 min, and expressed as nmol/min/mg. For estimation of V_{max} and apparent K_M (benzoyl-CoA) values, similar conditions were used, except that the concentration of benzoyl-CoA was varied from 10 μM to 100 μM in a 200 μl reaction, containing 1.5 μg of a particular recombinant human GLYAT variant. Initial velocities were calculated over the first 2.5 min, where the reaction was linear even for the assays where a low concentration of benzoyl-CoA was used. Data of six replicate assays were used to estimate kinetic parameters by nonlinear regression, using SigmaPlot 12.0 (Systat software, Inc., San Jose, CA).

2.4. Molecular modelling and sequence analyses

To investigate the possible structural significance of the amino acid variations investigated in this study, molecular modelling was used. A molecular model of human GLYAT, containing a molecule of CoA, and a multiple alignment of sequences with high homology to human GLYAT, were generated as described previously (Badenhorst et al., 2012). The unusually high degree of structural homology in the GNAT superfamily of enzymes (Dyda et al., 2000; Vetting et al., 2005) allowed an approximate model of human GLYAT to be generated, using template structure 1SQH. The use of this model is discussed by Badenhorst (Badenhorst et al., 2012). UCSF Chimera (Pettersen et al., 2004) was used to generate molecular graphics and to investigate positions on the model of residues significant to this study.

3. Results and discussion

3.1. Wild-type human GLYAT and selected variants: constructs and expression

In this study the catalytic effects of selected amino acid substitutions on the activity of a recombinant human GLYAT were investigated. The variants studied included six known polymorphisms K16N, S17T, R131H, N156S, F168L, and R199C. An E227Q mutant lacking the proposed GLYAT catalytic residue (Badenhorst et al., 2012), was also investigated. Wild-type recombinant human GLYAT and the selected variants (Table 2) were expressed from pET32a(+) in a soluble form as Trx–His-fusions of approximately 56 kDa. The thioredoxin (Trx) fusion protein is a highly soluble polypeptide (Sachdev and Chirgwin, 1998) used to enhance the production of soluble, enzymatically active human GLYAT. The expression of soluble, enzymatically active recombinant human GLYAT could be improved by lowering the temperature of expression to 28 °C and by not using isopropyl-1-thio-β-D-galactopyranoside to induce expression. The recombinant enzymes were highly purified by means of nickel-affinity chromatography (Fig. 1) and could be stored at 4 °C for at least one week without loss of enzyme activity.

3.2. Relative activities

To investigate the relative activities of the wild-type human GLYAT and selected variants, 0.5 μg of each enzyme was assayed using substrate concentrations where V_{max} could be measured (200 μM benzoyl-CoA and 200 mM glycine) (Nandi et al., 1979). Relative activities of the variants, in nmol/min/mg, were calculated from the 10 min period where the reaction proceeded linearly (Fig. 2). The wild-type recombinant human GLYAT had activity comparable to the K16N, S17T, and R131H variants. The N156S variant showed an increase in enzyme activity when compared to the wild-type GLYAT, while the F168L variant showed a decreased enzyme activity. Both the R199C and E227Q variants of recombinant human GLYAT were virtually inactive, having less than 5% of the activity of the wild-type. To verify that each variant was assayed using the same amount of recombinant GLYAT protein, SDS-PAGE and western blot analyses were performed on samples diluted to equal protein concentration (Fig. 1). The relative enzyme activity shown in Fig. 2 was consistently observed using independently expressed and purified batches of enzymes.

3.3. Kinetic parameters

The recombinant human GLYAT variants were further compared by determining V_{max} values and K_{Mapp} (benzoyl-CoA) values. Lineweaver–Burk plots are shown in Fig. 3, and the results are summarised in Table 3. The V_{max} values range from approximately 500 nmol/min/mg to 1200 nmol/min/mg, and the K_{Mapp} (benzoyl-CoA) values range from approximately 20 μM to 70 μM (Table 3).

The kinetic parameters reported in the literature for human GLYAT vary substantially. The K_{Mapp} (benzoyl-CoA) is reported to be between 13 μM and 57.9 mM, and the V_{max} value between 543 nmol/min/mg and 17100 nmol/min/mg (Table 1) (Bartlett and Gompertz, 1974; Gregersen et al., 1986; Kelley and Vessey, 1993, 1994; Kolvraa and Gregersen, 1986; Mawal and Qureshi, 1994; van der Westhuizen et al., 2000). The V_{max} values we determined for the recombinant human GLYAT variants fall within the range reported in the literature. The K_{Mapp} (benzoyl-CoA) values we determined for the recombinant human GLYAT variants fall on the lower end of the range in the literature, being comparable to the values of 13 μM (van der Westhuizen et al., 2000) and 67 μM (Kelley and Vessey, 1994). It is difficult to explain the large variation in literature values, but differences in the quality of enzyme preparations studied may be partly responsible. The exact experimental conditions used in previous reports are unclear, making interpretation difficult.

The recombinant enzyme characterised by Matsuo et al. (2012) is the N156S variant. They report a K_M (benzoyl-CoA) of 209 μM (Matsuo et al., 2012). This value is much higher than the value of 38 μM we determined for the N156S variant (Table 3). This difference in K_{Mapp} value obtained for the N156S variant could be due to significant differences in the reaction composition, substrate concentrations used, quality of purified protein preparations used, and data analysis.

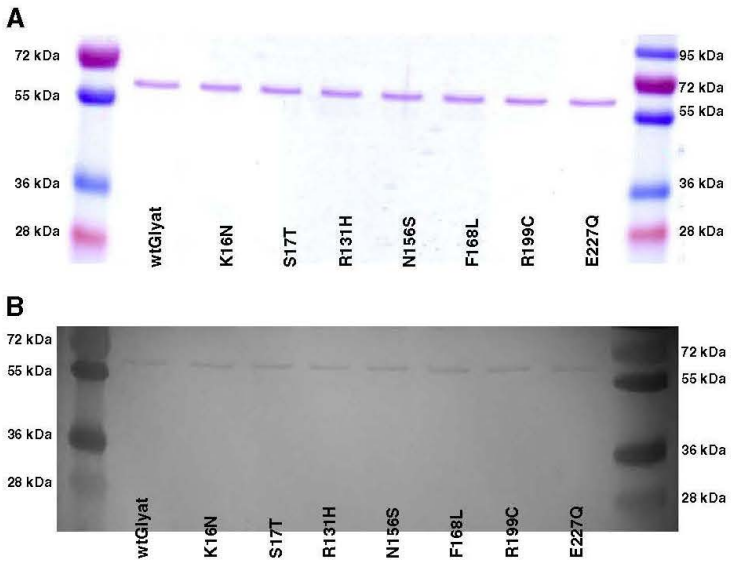

Fig. 1. 10% SDS-PAGE (A) and western blot analyses (B) of the purified protein samples of the wild-type human GLYAT and the selected variants. Lane 1: protein ladder – 28 kDa, 36 kDa, 55 kDa, and 72 kDa; Lane 2: wild type human GLYAT; Lane 3: K16N; Lane 4: S17T; Lane 5: R131H; Lane 6: N156S; Lane 7: F168L; Lane 8: R199C; Lane 9: E227Q and Lane 10: protein ladder – 28 kDa, 36 kDa, 55 kDa, 72 kDa, and 95 kDa. The purified recombinant proteins are visible as a band of approximately 56 kDa.

When comparing the kinetic parameters of the wild-type to that of the variants, the wild-type, K16N, and S17T variants had comparable K_{Mapp} (benzoyl-CoA) values (24 μM, 21 μM, and 28 μM, respectively). The R131H variant had an increased K_{Mapp} (benzoyl-CoA) value of about 71 μM. Interestingly, the N156S variant had a V_{max} value (1230 nmol/min/mg) greater than that of the wild-type enzyme (730 nmol/min/mg), despite having a higher K_{Mapp} (benzoyl-CoA) value of 38 μM. The F168L variant also had an increased K_{Mapp} value of about 53 μM, but a decreased V_{max} value of 500 nmol/min/mg. The R199C substitution resulted in less than 5% of wild-type activity, and its K_M (benzoyl-CoA) could not be determined.

The differences in V_{max} and K_{Mapp} (benzoyl-CoA) values reported in this study, for variants of recombinant human GLYAT, may partly explain the significant variation in glycine conjugation capacity that has been observed between human liver samples (Temellini et al., 1993). However, since Temellini and co-workers investigated the formation of hippurate from benzoate, ATP, and glycine, using hepatocyte homogenates, there may also have been other factors, such as variation in the rate of benzoyl-CoA formation, that influenced the rates of glycine conjugation. The inter-individual variation in urinary acylglycine excretion may similarly be accounted for, in part, by the different properties we report here for human GLYAT variants (Campbell et al., 1988; Sakuma, 1991).

The clinical variability of certain inborn errors of organic acid metabolism may be influenced by inter-individual differences in glycine conjugation capacity. This is demonstrated by the variation in clinical presentation of a large group of South African isovaleric acidemia patients, all having the same disease-causing mutation (Dercksen et al., 2012). Because of its critical importance in the management of

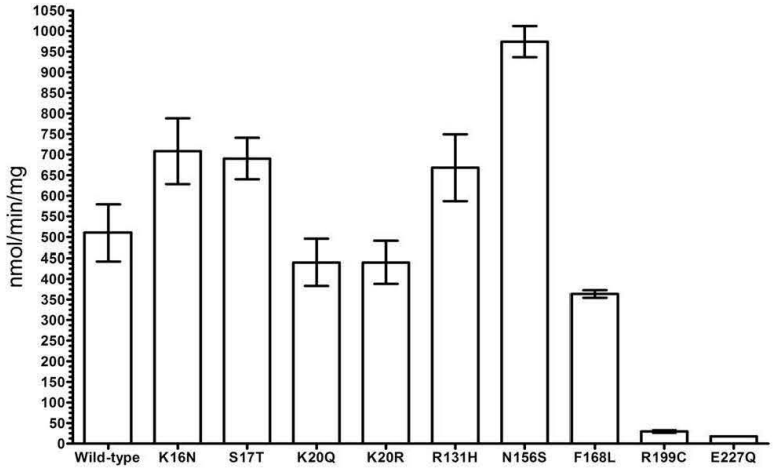

Fig. 2. Relative enzyme activities of recombinant human GLYAT and selected variants. Enzymes (0.5 μg) were assayed at 37 °C using 200 μM benzoyl-CoA and 200 mM glycine. Error bars indicate the mean ± standard deviation for triplicate assays.

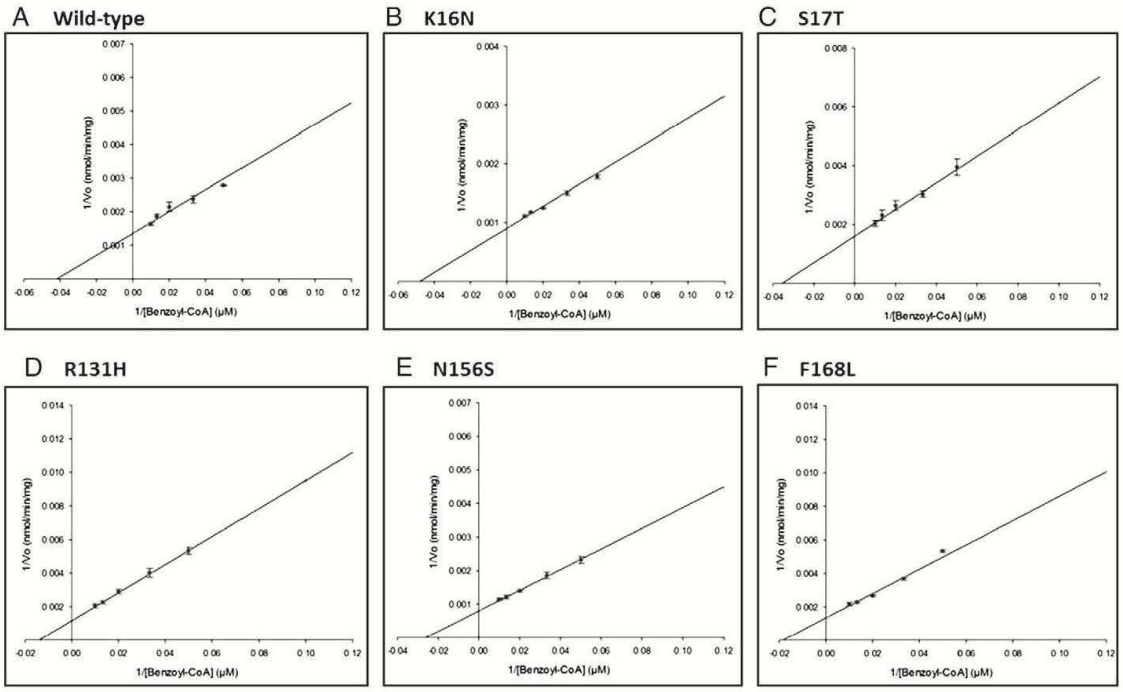

Fig. 3. Lineweaver–Burk plots for determination of K_{Mapp} (benzoyl-CoA) and V_{max} values at 200 mM glycine. Error bars indicate the mean ± standard deviation for six replicate assays. Benzoyl-CoA concentrations used were 20 uM, 30 uM, 50 uM, 75 uM, and 100 uM. A) Wild-type recombinant human GLYAT; B–F) K16N, S17T, R131H, N156S, and F168L recombinant human GLYAT variants.

isovaleric acidemia, variation in glycine conjugation capacity is thought to impact on clinical presentation of the disease, and this needs to be further investigated (Bartlett and Gompertz, 1974; Dercksen et al., 2012; Tanaka and Isselbacher, 1967). Another question that needs to be addressed is whether variants of human GLYAT have different substrate specificities, which would also help explain why some patients with isovaleric acidemia excrete more glycine conjugates than others (Dercksen et al., 2012).

It has been proposed that the fraction of a dose of para-aminobenzoate, excreted as glycine conjugates (hippurates), be used as a convenient test of liver function. It was demonstrated that there exists a correlation between this hippurate ratio and the extent of liver damage, with a lower hippurate ratio indicating more severe damage (Duffy et al., 1995). Based on the known inter-individual variation in glycine conjugation capacity, and the different kinetic parameters of the GLYAT variants reported in this study, we suggest that tests of liver function based on the hippurate ratio be interpreted with caution.

Table 3
Kinetic parameters obtained for recombinant human GLYAT variants.

Variant	K_{Mapp} (benzoyl-CoA) μM[a]	V_{max} (nmol/min/mg)[a]
Wild-type	24 ± 3	730 ± 30
K16N	21 ± 1	1030 ± 20
S17T	28 ± 5	665 ± 40
R131H	71 ± 11	1040 ± 85
N156S	38 ± 4	1230 ± 60
F168L	53 ± 6	500 ± 30

[a] Values are reported as mean ± standard deviation for six replicates.

3.4. Molecular model and sequence analyses

Previously, a molecular model of bovine GLYAT was generated, which suggested that the highly conserved Glu^{226} residue was catalytically important, and this was supported by investigation of an E226Q mutant (Badenhorst et al., 2012). We constructed, expressed, and purified the equivalent E227Q recombinant human GLYAT. This variant had no enzyme activity (Fig. 2). A molecular model of human GLYAT, based on the model reported for bovine GLYAT (Badenhorst et al., 2012), was constructed to help explain some of the differences between the human GLYAT variants investigated in this study. Locations of the Arg^{131}, Asn^{156}, Phe^{168}, and Arg^{199} residues of human GLYAT are indicated on the model (Fig. 4A). Residues in contact with these residues (Asp^{48}, Leu^{242} and Thr^{244}) are also indicated. Conservation of selected residues is also indicated on part of a multiple sequence alignment (Fig. 4B).

The R131H substitution removes a positively charged residue from a binding cavity on the model, perhaps explaining its increased K_{Mapp} value for the negatively charged benzoyl-CoA (Fig. 3 and Table 3). The F168L substitution alters the highly conserved Phe^{168}, which on the model is in contact with the well conserved Leu^{242}, found with Thr^{244} on the first turn of an alpha helix important for acyl-CoA binding in the GNAT superfamily. The Thr^{244} residue is in contact with the CoA pyrophosphate moiety, a very common feature of GNAT enzymes (Dyda et al., 2000; Vetting et al., 2005). This may explain the high K_{Mapp} (benzoyl-CoA) and low V_{max} values obtained for the F168L variant (Fig. 3 and Table 3). The inactivity of the R199C variant may be explained by the presence of the highly conserved Arg^{199} in an α-loop-α motif, where it is in contact with the highly conserved, negatively charged Asp^{48}. Substitution of Arg^{199} may destabilise this motif, which is important for substrate binding in the GNAT superfamily

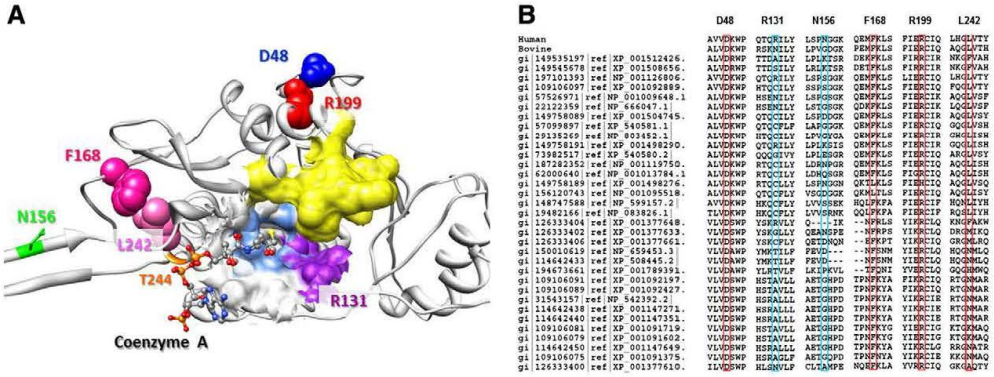

Fig. 4. A molecular model and multiple sequence alignment of human GLYAT to visualise selected residues mutated in this study. A) The molecular model of human GLYAT, with bound CoA. Arg^{131} (in purple) forms part of a putative substrate binding cavity (light blue). Asn^{156} (green) is located on a poorly modelled loop. Phe^{168} (dark pink) is in contact with Leu^{242} (lighter pink), on the same helix as Thr^{244} (orange), which is in contact with the pyrophosphate of CoA. Arg^{199} (red) is in contact with Asp^{48} (blue). Arg^{199} is part of an α-loop-α motif important for substrate binding (yellow loop). UCSF Chimera was used to generate this image. B) A multiple alignment of sequences homologous to human GLYAT. Accession numbers are shown to the left of the alignment. The Arg^{131} and Asn^{156} residues are not well conserved. The Phe^{168} residue and the Leu^{242} residue are well conserved. The Arg^{199} and Asp^{48} residues are also well conserved. ClustalX 2.0.12 was used to align sequences.

(Dyda et al., 2000; Vetting et al., 2005). On the model, Asn^{156} is on a poorly predicted loop from Lys^{159} to Met^{167}, making interpretation of its role in enzyme activity difficult (Fig. 4A). However, it has been suggested that residues 130 to 180 of human GLYAT may be important in substrate binding (Matsuo et al., 2012), which could perhaps explain why the N156S variant had altered K_{Mapp} (benzoyl-CoA) and V_{max} values.

3.5. Allele frequencies of selected variants

The allele frequencies of the SNPs for S17T, R131H, N156S, and R199C human GLYAT have been investigated in small Japanese and French Caucasian populations (Lino Cardenas et al., 2010; Yamamoto et al., 2009). There seems to be some interesting correlations between the allele frequencies reported for these variants and the kinetic parameters we determined. The altered kinetic parameters of R131H, F168L and R199C (Fig. 3 and Table 3) may explain the low frequencies of these alleles (Table 2). In contrast, the allele frequencies for the N156S variant were 85% and 97% (Table 2). Based on the higher activity (Fig. 2) and higher allele frequency of the N156S variant, it appears that choosing the GLYAT sequence from GenBank (NM_201648.2, Table 2) as a reference sequence for a typical wild-type human GLYAT, may be erroneous. It was also suggested by Lino Cardenas et al. (2010) that the N156S variant is the wild-type allele in the French Caucasian population.

3.6. Regulation of human GLYAT activity

Amino acid substitutions in the GLYAT sequence can influence mitochondrial import and perhaps regulation of enzyme activity (Stadtler et al., 2005; Waluk et al., 2012). The activity of human GLYATL2, a paralogue of human GLYAT, is regulated by reversible acetylation on Lys^{19}. Enzyme activities of K19Q and K19R mutants of GLYATL2 were decreased by about 80%, demonstrating the importance of this residue (Waluk et al., 2012). To investigate whether human GLYAT activity is regulated similarly, the corresponding K20Q and K20R mutants of GLYAT were expressed, purified, and assayed as for the other variants in this study. These mutants had activity comparable to the wild-type GLYAT, indicating that human GLYAT is not similarly subject to regulation by modification of this residue (Fig. 2).

3.7. Conclusion

By using a recombinant human GLYAT we demonstrated that SNP variations found in the human GLYAT gene may result in altered kinetic properties of the enzyme. These differences may help explain some of the observed inter-individual variation in glycine conjugation capacity, which is relevant to the treatment of some metabolic disorders and to the metabolism of xenobiotics such as aspirin and the industrial solvent xylene.

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