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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