EXPERT OPINION

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Glycine conjugation: importance in metabolism, the role of glycine *N*-acyltransferase, and factors that influence interindividual variation

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Introduction: Glycine conjugation of mitochondrial acyl-CoAs, catalyzed by glycine *N*-acyltransferase (GLYAT, E.C. 2.3.1.13), is an important metabolic pathway responsible for maintaining adequate levels of free coenzyme A (CoASH). However, because of the small number of pharmaceutical drugs that are conjugated to glycine, the pathway has not yet been characterized in detail. Here, we review the causes and possible consequences of interindividual variation in the glycine conjugation pathway.

Areas covered: The authors review the importance of CoASH in metabolism, formation and toxicity of xenobiotic acyl-CoAs, and mechanisms for restoring levels of CoASH. They focus on GLYAT, glycine conjugation, how genetic variation in the GLYAT gene could influence glycine conjugation, and the emerging roles of glycine metabolism in cancer and musculoskeletal development.

Expert opinion: The substrate selectivity of GLYAT and its variants needs to be further characterized, as organic acids can be toxic if the corresponding acyl-CoA is not a substrate for glycine conjugation. GLYAT activity affects mitochondrial ATP production, glycine availability, CoASH availability, and the toxicity of various organic acids. Therefore, variation in the glycine conjugation pathway could influence liver cancer, musculoskeletal development, and mitochondrial energy metabolism.

Keywords: acyl-coenzyme A, benzoate, CASTOR disorder, coenzyme A, coenzyme A sequestration, GLYAT, glycine conjugation, glycine *N*-acyltransferase, hepatocellular carcinoma, xenobiotics

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1. Introduction

The study of drug metabolism started with the discovery of glycine conjugation. The excretion of hippuric acid after ingestion of benzoic acid was discovered in 1841 by Alexander Ure [1]. This was later confirmed by Wilhelm Keller in 1842 who ingested 32 grains of benzoic acid and isolated hippuric acid from his urine the next morning [2]. Later, in 1845, it was demonstrated by Dessaignes that hippuric acid was in fact an amide conjugate between glycine and benzoic acid, making this the first conjugation reaction to be discovered [3]. Since this epic discovery, interest in glycine conjugation has faded significantly, and only sporadically has anything on the subject been published in the last 168 years. In this review, we wish to re-emphasize the importance of glycine conjugation and clarify its influence on the metabolism of CoASH and glycine. We also point out some

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Article highlights.

- GLYAT is the enzyme responsible for glycine conjugation of the acyl-CoA esters of several xenobiotic organic acids.
- Glycine conjugation is important for the detoxification of benzoate and hydroxybenzoates that are conjugated to coenzyme A in the liver and kidneys.
- Interindividual variation in glycine conjugate excretion has been observed but the mechanisms underlying this variation are not understood.
- SNPs in human GLYAT have been shown to influence the enzymatic activity, but it is not clear how this influences variation in the glycine conjugation pathway.
- The high exposure to xenobiotics in modern times may exacerbate dietary glycine deficiency.
- Because of its influence on glycine availability, GLYAT may play a role in the development of hepatocellular carcinoma and may be involved in musculoskeletal development.

This box summarizes key points contained in the article.

serious deficiencies, of paramount importance, in our understanding of the glycine conjugation pathway.

Humans have several biotransformation systems, including conjugation to sulfate, glucuronate, and glycine, that convert various endogenous and xenobiotic metabolites to more hydrophilic conjugates that can be excreted in the urine [3-8]. The resulting conjugates are often less toxic than the parent compound, with some exceptions such as reactive acyl-glucuronides [9]. Glycine *N*-acyltransferase (GLYAT) is responsible for the glycine conjugation of xenobiotics such as benzoic acid (**Figure 1**). Although the small range of substrates for glycine conjugation, when compared with glucuronidation, may have contributed to the relatively little research that has been done on GLYAT [3], we will argue that the enzyme plays a central role in maintaining CoASH homeostasis in the liver. We briefly review and discuss acyl-CoA metabolism, glycine conjugation, interindividual variations, and some factors that may influence glycine conjugation. Finally, we review the literature on the GLYAT gene and enzyme, and what is known about genetic variation in the GLYAT gene and its consequences.

2. Acyl-CoA metabolism and toxicity

2.1 The importance of coenzyme A in metabolism

Coenzyme A is an extremely important molecule that can be seen as a central hub around which much of metabolism revolves [10-13]. Acyl-CoA esters are important intermediates in many anabolic and catabolic reactions. Almost all catabolic reactions result in the formation of acetyl-CoA, the fuel for both oxidative phosphorylation and lipogenesis (**Figure 2**) [10,14]. It is thus clear that disturbances of coenzyme A metabolism, and changes in the relationships between CoASH and acyl-CoAs, can have severe and far-reaching consequences for metabolism as a whole [12,13]. Therefore, coenzyme A metabolism is tightly regulated, and even under ischemic conditions, levels of free and acylated CoASH in the liver stay the same, despite a doubling in acetyl-CoA levels [12].

An interesting study in which rats were fed with the pantothenate analog hopantenate demonstrates the tight regulation of hepatic coenzyme A metabolism. Hopantenate inhibits CoASH biosynthesis, and the rats died of hypoglycemia within 2 weeks with fatty liver and mitochondrial dysmorphology [13]. It was shown that hopantenate initiates a transcriptional reprogramming of the liver, which leads to an increase in expression of acyl-CoA thioesterases, and pyruvate dehydrogenase kinase isoform 1, which decreases pyruvate dehydrogenase activity. The result is increased liberation and decreased consumption of CoASH [11-13]. These observations emphasize the importance of tight regulation of hepatic CoASH metabolism and the consequences of disruption of CoASH homeostasis.

2.2 Formation of xenobiotic acyl-CoAs

Several fatty acids and xenobiotic carboxylic acids that are conjugated to amino acids must first be activated to acyl-CoAs by ATP-dependent acid:CoA ligases [3,4,15,16]. These ligase enzymes exhibit selectivity for short-, medium-, long-, or very long-chain fatty acids [15]. Several long-chain forms have been identified, which have different activities and tissue localization, and enable site-specific activation of fatty acids for specific metabolic requirements [12,17]. Most xenobiotics that undergo glycine conjugation are activated by the mitochondrial medium-chain ligases, which also activate C4–C12 acids for β-oxidation [15,18-20]. This dual role of the medium-chain ligases for fatty acid oxidation and xenobiotic activation is one of the reasons why mitochondrial accumulation of xenobiotic acyl-CoA esters may interfere with β-oxidation and disturb mitochondrial metabolism [3,21-23].

Four distinct medium-chain ligases, XL-I, XL-II, XL-III, and XL-J, have been identified in bovine liver, and have overlapping substrate specificities [18,19,22,24,25]. XL-I, XL-II, and XL-III all activate C3–C10 fatty acids and a range of arylacetic and aromatic carboxylic acids, including benzoate, 4-aminobenzoate, 4-chlorobenzoate, 4-nitrobenzoate, napthylacetate, and salicylate [15,18,20]. In humans, there are five medium-chain xenobiotic-activating enzymes. These are ACSM1, ACSM2A and ACSM2B, ACSM3, and ACSM5 [26]. Vessey *et al.* characterized two human liver medium-chain ligases, HXM-A and HXM-B, with activity toward a range of xenobiotics [3,18,27,28]. HXM-A is encoded by the ACSM2A gene [26,28]. These enzymes are less well characterized than the corresponding bovine enzymes, but have been shown to activate benzoate, hexanoate, octanoate, and decanoate. There is evidence of activation of valproate by HXM-A and salicylate by HXM-B [3,27,28]. Xenobiotics that involve activation to an acyl-CoA ester include pivalate,

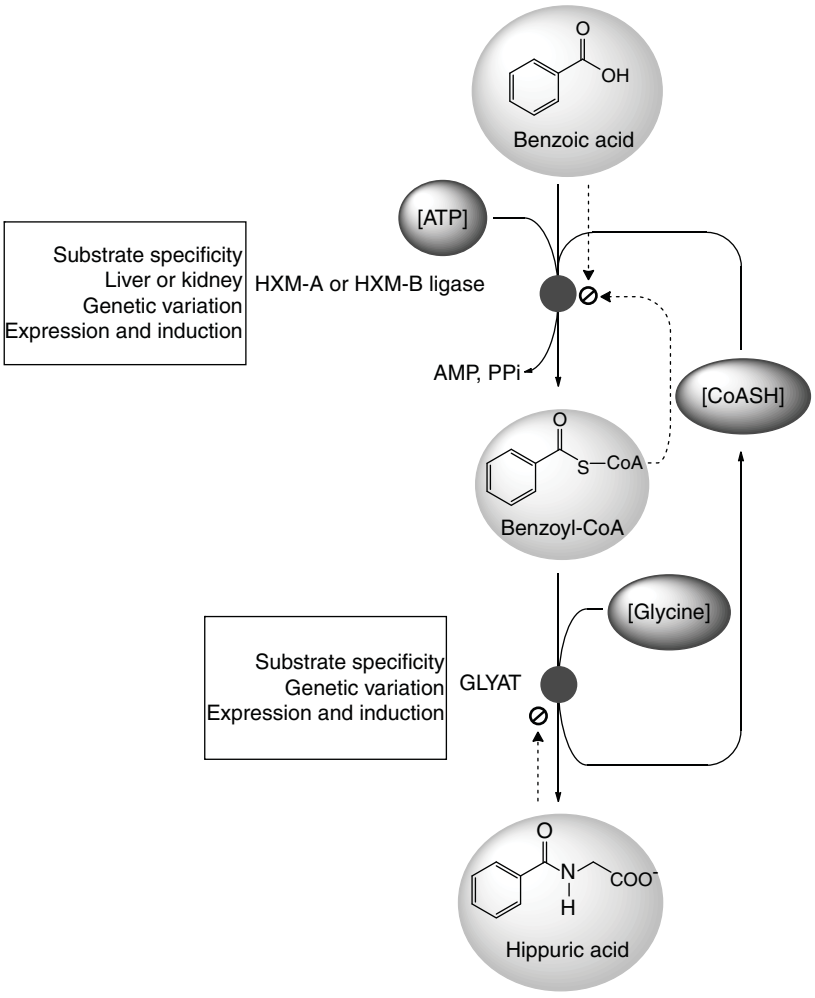

Figure 1. Glycine conjugation of benzoic acid. The glycine conjugation pathway consists of two steps. First benzoate is ligated to CoASH to form the high-energy benzoyl-CoA thioester. This reaction is catalyzed by the HXM-A and HXM-B medium-chain acid:CoA ligases and requires energy in the form of ATP. Some acyl-CoA esters can competitively inhibit the ligase enzymes. The benzoyl-CoA is then conjugated to glycine by GLYAT to form hippuric acid, releasing CoASH. In addition to the factors listed in the boxes, the levels of ATP, CoASH, and glycine may influence the overall rate of the glycine conjugation pathway. The black circles indicate the ligase and GLYAT enzymes.

AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; CoASH: Coenzyme A; GLYAT: Glycine *N*-acyltransferase; PPi: Pyrophosphate.

valproate, benzoate, salicylate, phenylbutyrate, and several others, summarized in **Table 1**. Depending on the xenobiotic, glycine conjugation may occur primarily in either the liver or kidney, reflecting differences between hepatic and renal acyl-CoA formation, but this is not discussed here [29]. If a xenobiotic acyl-CoA is formed that cannot be metabolized further, it will accumulate, resulting in toxicity [4,23,30]. The mechanisms of acyl-CoA toxicity are briefly described in the following section, before looking at pathways that can restore CoASH levels and homeostasis.

2.3 Mechanisms of acyl-CoA toxicity and pathogenesis

All disorders, acquired or inherited, that involve coenzyme A sequestration, toxicity, or redistribution were conceptually united into a group called CASTOR disorders by Mitchell *et al.* [12]. In CASTOR disorders, the degradation of acyl-CoA esters is impaired [10,14]. Grouping of the CASTOR disorders enables a clearer grasp of the underlying pathophysiology and enables better understanding of potential therapeutic strategies. The mechanisms of pathogenesis can be divided broadly into effects caused by depletion of CoASH, and effects caused by the accumulated acyl-CoA itself [4,12,30].

2.3.1 Depletion of CoASH

Depletion of CoASH is often one of the most severe consequences of acyl-CoA accumulation [4,12,13,31,32]. As described in Section 2.1, coenzyme A is a central metabolic hub and depletion can, indirectly, have far-reaching implications for both intermediary and energy metabolism. When CoASH

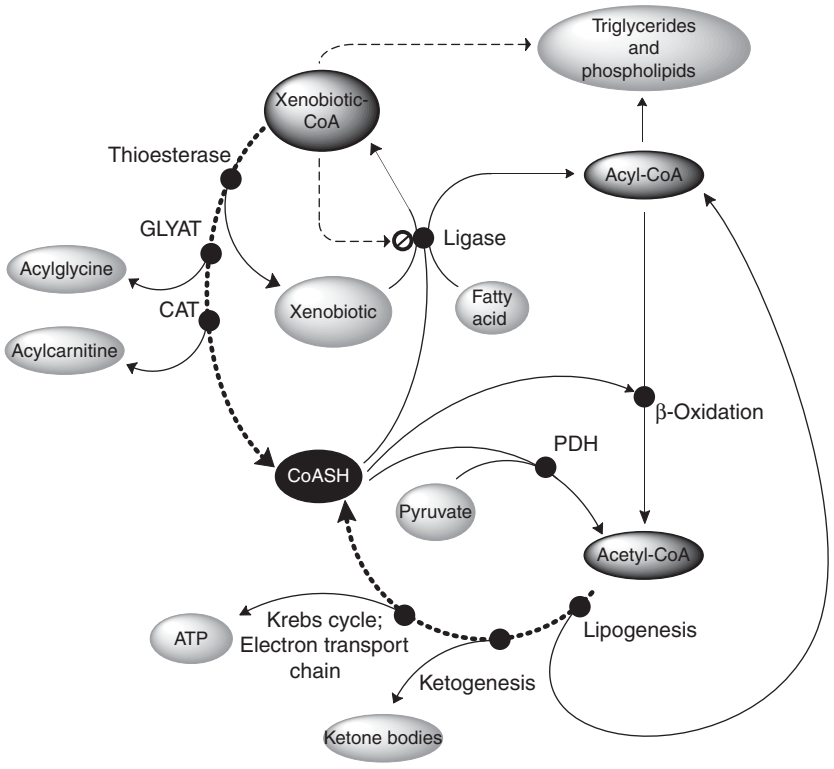

Figure 2. An overview of coenzyme A and acyl-CoA metabolism. The main pathways that produce and consume CoASH are demonstrated. It is extremely important that CoASH always be available in the cell because of its role in β-oxidation and the conversion of pyruvate to acetyl-CoA. Acetyl-CoA is the product of most catabolic reactions and provides the fuel for ketogenesis and mitochondrial ATP production. When xenobiotics are converted to xenobiotic-CoA esters, CoASH can be sequestered, disrupting the ATP synthesis from pyruvate and fatty acids. As demonstrated by the fine broken arrows, accumulating xenobiotic-CoAs can inhibit the acid:CoA ligases or be incorporated into unnatural triglycerides and membrane phospholipids. The bold broken arrows indicate pathways that release bound CoASH. The black circles indicate important processes involved in the formation and degradation of acyl-CoAs.

CAT: Carnitine acyltransferase; CoASH: Coenzyme A; GLYAT: Glycine *N*-acyltransferase; PDH: Pyruvate dehydrogenase.

becomes limiting, energy metabolism is impacted on several levels [13,14,33]. The consumption of glucose, a primary metabolic fuel, results in the formation of pyruvate, which requires CoASH in order to be converted to acetyl-CoA by pyruvate dehydrogenase [13]. CoASH is also needed for β-oxidation of fatty acids, which are broken down to two-carbon units in the form of acetyl-CoA [10,13,14,31,33]. Thus, if CoASH is depleted, glucose and lipids cannot be efficiently utilized for the production of energy by oxidative phosphorylation. The result is diminished capacity for mitochondrial ATP production, increased dependence on glycolysis, and altered ratios of cellular NAD^+ and NADH [12,13,31,32]. NAD^+ is required for activity of the sirtuins, a family of NAD^+-dependent deacetylases and ADP-ribosyltransferases [34]. These proteins play important roles in energy metabolism by regulating the activities of enzymes involved in gluconeogenesis, β-oxidation, and the electron transport chain [34]. Disturbances of NAD^+ levels can thus negatively impact the regulation of energy metabolism, but this falls outside the scope of this review.

The effects of CoASH sequestration are demonstrated by the metabolism of valproate, an anti-epileptic drug that is metabolized to valproyl-CoA. As valproyl-CoA is not a substrate for glycine conjugation, it can accumulate in the liver, deplete CoASH, and may eventually cause hepatic steatosis [3,30,31,33]. This effect is not caused by an α-fluorinated derivative of valproate, which is either not a good substrate for ligation to CoASH, or because of its increased acidity forms a less stable thioester that spontaneously hydrolyses. As a result, this α-fluorinated derivative does not cause CoASH sequestration and hepatic steatosis [3].

2.3.2 *Toxic effects of accumulating acyl-CoAs*

Accumulation of acyl-CoAs can also negatively influence energy metabolism by causing a depletion of carnitine, which is the transporter of fatty acids over mitochondrial membranes [10,14]. When an acyl-CoA accumulates to high enough amounts, it may become a substrate for carnitine acyltransferases, resulting in the formation of an acyl-carnitine that can be

Table 1. Xenobiotics that are metabolized to acyl-CoA and glycine conjugates.

Xenobiotic	Glycine conjugate formed in humans	CoA sequestration or toxicity	Notes
2,4,5-Trichlorophenoxyacetate	No	Unknown	Glycine conjugate formed by bovine GLYAT [3]
2,4-Dichlorophenoxyacetate	No	Unknown	Glycine conjugate formed by bovine GLYAT [3]
3-Hydroxybenzoate	Yes	No	Product of dietary polyphenol fermentation by gut microorganisms [44]
4-Aminobenzoate (PABA)	Yes	No	Seems to be well tolerated; slow glycine conjugation of PABA has been correlated to liver failure and hepatitis probably because of decreased formation of aminobenzoyl-CoA [3,47,65,66]
4-Hydroxybenzoate	Yes	No	Product of dietary polyphenol fermentation by gut microorganisms [44]
Astemizole	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Benzoate	Yes	No	Good substrate for glycine conjugation; large doses of benzoate are tolerated; we believe that benzoate would cause severe CoASH sequestration in the absence of GLYAT activity [2,27,45,59,78]
Brompheniramine	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Ferulic acid	Yes	No	From metabolism of ferulate-containing plant material by gut microorganisms [100]
Hypoglycine	Yes	Sequestration and toxicity	Glycine conjugation is not fast enough to detoxify the acyl-CoA metabolite, which is an irreversible inhibitor of dehydrogenase enzymes [3,4]
Ibuprofen	No	Sequestration	Taurine conjugate is formed; interaction between salicylate and ibuprofen was observed for the bovine medium-chain ligase enzymes; causes CoASH sequestration in rat liver [3,4,22]
Indoleacetic acid	Yes	No	Usually conjugated to glutamine; associated with gut microbe dysbiosis; not activated to acyl-CoA by HXM-A or HXM-B [27,81,100]
Naphthylacetic acid	No	Unknown	Weak activation by HXM-A and HXM-B; unlikely to cause CoASH sequestration [27]
Nicotinic acid	Unknown	No	Very weak activation by HXM-B [27]
Permethrin	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Phenylacetic acid	Yes	Unknown	Usually conjugated to glutamine; associated with gut microbe dysbiosis [81,100]
Pivalic acid	No	Sequestration	Not a substrate for human GLYAT [3,32]
Salicylate	Yes	Uncertain, toxic	Activation of salicylate to salicylyl-CoA is slow, making CoASH sequestration unlikely; toxicity and associated Rye-like syndrome are possibly caused by inhibition of carnitine acyltransferases by salicylyl-CoA; salicylic acid at therapeutic doses can also inhibit bovine ligase enzymes, suggesting another mechanism of toxicity [3,22,27,39,78]
Toluene	Yes	No	Metabolized to hippuric acid; no interaction was observed between toluene and the xylenes at the doses used in the investigation [83]
Triflusal	Yes	Unknown	As a glycine conjugate is detected, formation of an acyl-CoA is assumed [3]
Valproate	No	Sequestration and toxicity	Can cause hepatic steatosis because of CoASH sequestration [3,18,67]
Xylenes	Yes	No	These solvents are metabolized to methylhippurates; *m*-xylene has been shown to interact with salicylate conjugation [63,83]

PABA: *Para*-aminobenzoic acid.

excreted in the urine [21,32,35,36]. For example, benzoic acid administration results in benzoyl-carnitine excretion and a decrease in plasma-free carnitine levels [35].

Xenobiotic acyl-CoAs can substitute for acetyl-CoA in lipogenesis, resulting in odd-chain, branched-chain, aromatic, and other unnatural fatty acids, which cannot be properly catabolized and may be incorporated into cell membranes [3,30,31]. For example, propionyl-CoA, which accumulates in propionic acidemia, can be the substrate for synthesis of odd- and branched-chain fatty acids [37]. It has also been shown that 2-arylpropionyl-CoA esters, metabolites of the nonsteroidal anti-inflammatory drugs (NSAIDS), can be incorporated into adipocyte triglycerides [15,31]. Enzymes may be competitively or allosterically inhibited by acyl-CoAs, with effects that are difficult to predict [12,21-23,30,38,39]. For example, protein kinase C activity, important in signal transduction, is perturbed by ciprofibroyl-CoA, a metabolite of the hypolipidaemic drug ciprofibrate [15]. Propionyl-CoA, at high concentrations, inhibits formation of N-acetylglutamate by N-acetylglutamate synthetase, resulting in urea cycle dysfunction and hyperammonemia [37].

2.4 Restoration of CoASH levels

There are a few basic mechanisms that can restore depleted CoASH reserves, including conjugation to amino acids or to carnitine, and hydrolysis of acyl-CoAs by thioesterases (**Figure 2**) [3,12,40]. Acyl-CoA thioesterases hydrolyze acyl-CoA esters to free organic acids and CoASH. This is an indispensable metabolic necessity, because CoASH must always be available to maintain a proper metabolic milieu [10,14]. Thioesterases may have selectivity for short-, medium-, long-, and very long-chain acyl-CoAs, and are found in almost every compartment of the cell, including the cytoplasm, peroxisomes, microsomes, and mitochondria [3,12,41]. There is a direct relationship between cellular levels of CoASH, long-chain acyl-CoAs, peroxisomal β-oxidation, and cellular thioesterase activity, with thioesterases playing a role in regulation of peroxisomal and intracellular lipid metabolism [10,41]. It has been suggested that the accumulation of a particular xenobiotic acyl-CoA will, in part, reflect the relative activity and substrate selectivity of the various thioesterases [15]. In the following section, the main focus is on glycine conjugation, a primary mechanism for the restoration of CoASH levels.

3. Glycine conjugation and interindividual variation

3.1 The metabolic role of glycine conjugation

Although GLYAT can conjugate a variety of endogenous and xenobiotic acyl-CoAs to glycine, the normal metabolic role of GLYAT seems to be the detoxification of dietary benzoates [4,42-45]. On a daily basis, humans consume varying quantities of benzoate, a metabolite found in plant material [45]. In addition, plant material contains complex polyphenols, which are fermented by the colonic flora to benzoate, 3- and 4-hydroxybenzoates, and the corresponding hydroxyphenyl-propionates [44]. After intestinal absorption, these compounds are transported to the liver, where they are conjugated to CoASH by the medium-chain xenobiotic acid:CoA ligases [3,15,18,45]. Because these benzoates are ubiquitous in plant-containing diets, it is clear that hepatic CoASH would be rapidly sequestered if the xenobiotic acyl-CoAs could not be further metabolized. GLYAT plays a major role in restoring CoASH levels by conjugating these xenobiotic acyl-CoAs to glycine. Therefore, excretion as the corresponding glycine conjugates is the major metabolic fate of ingested polyphenols [44,45]. Other natural substrates for conjugation to glycine include salicylate, a common plant metabolite, and 4-aminobenzoate [39,46-48]. About 83 – 90% of ingested benzoate and about 75 – 84% of ingested salicylate are excreted as glycine conjugates [49]. Decreased benzoate production by the gut microorganisms in patients with Crohn's disease is correlated with decreased hippurate excretion in the urine [45].

3.2 Glycine conjugation in metabolic diseases

In several organic acidemias, an acyl-CoA accumulates to toxic levels because of a defect of the enzyme acting on it [14,43,50]. This results not only in CoASH sequestration, but because of thioesterase activity, free organic acids are released, causing potentially deadly acidoses [51-53]. Because some of the acyl-CoAs that accumulate in organic acidemias are substrates for GLYAT, glycine conjugation impacts on the biochemical profiles and clinical outcomes of some of these metabolic defects [42,43,53-55]. Glycine conjugation under these abnormal conditions sheds light on the important role GLYAT plays in maintaining CoASH levels.

In some cases, the accumulating acyl-CoA can be conjugated to glycine by GLYAT, decreasing the severity of CoASH sequestration and avoiding acidosis, as a less toxic acylglycine is formed and excreted [42,43,50,56]. It was demonstrated that a relationship exists between the kinetics and substrate selectivity of a bovine liver GLYAT, and the acylglycines excreted in the urine of patients with organic acidemias [42]. For example, in isovaleric acidemia, where isovaleryl-CoA accumulates, large amounts of isovalerylglycine are excreted in the urine because isovaleryl-CoA is a good substrate for GLYAT [50,55,57]. However, in propionic acidemia, only relatively small amounts of propionate are excreted as propionylglycine [37]. This is because, despite having similar K_M values for isovaleryl-CoA and propionyl-CoA, bovine GLYAT conjugates propionyl-CoA at a much lower rate [42,58]. Unfortunately, a similar comparison cannot yet be made for the human enzyme, as its kinetic parameters are not as well characterized. Glycine conjugates are also excreted in several other organic acidemias, and include 3-methylcrotonylglycine, hexanoylglycine, butyrylglycine, and tiglylglycine [14,43,52,53]. There is no simple relationship between GLYAT substrate selectivity, in terms of K_M and V_{max} parameters, and acyl

group structure. This makes it difficult to predict the extent to which glycine conjugation will influence the outcome of any particular organic acidemia [4,42].

3.3 Glycine availability can be a limiting factor in glycine conjugation

The presence of isovalerylglycine in the urine of patients with isovaleric acidemia suggested that glycine supplementation may help to detoxify the accumulating isovaleryl-CoA [50,55]. In fact, glycine supplementation has been shown to increase the levels of isovalerylglycine excreted, and usually decreases the severity of the disease to allow normal physical and mental development [50,55,57]. This suggests that availability of glycine may be a limiting factor in glycine conjugation under some conditions [50,59,60]. When glycine conjugation is maximal, glycine may be depleted, and other amino acids may be used, as suggested by the detection of isovaleryl-conjugates of 19 other amino acids in the urine of isovaleric acidemia patients [61]. It was proposed, although not experimentally verified, that these conjugates are also formed by GLYAT. Glycine availability also influences benzoate conjugation, as demonstrated by the dose-dependent increase in hippurate formation after administration of glycine [59]. Cysteamine, which decreases the activity of the glycine cleavage system and doubles hepatic glycine content, increases the benzoate clearance by 50% in rats [59].

3.4 Interindividual variation in glycine conjugation

Glycine conjugation is also manipulated for the treatment of hyperammonemia in urea cycle disorders, by administration of benzoate, which is conjugated to form hippurate, allowing excess nitrogen to be excreted from the body [36,60]. Interindividual variation in responsiveness to administration of glycine and benzoate, respectively, was observed in both isovaleric acidemia and hyperammonemia [56,57,62]. For example, variation in clinical outcome and responsiveness to glycine supplementation was observed in a group of South African isovaleric acidemia patients, all homozygous for the same isovaleryl-CoA dehydrogenase mutation [56]. It was suggested that interindividual variation in GLYAT activity may partly account for this, but further investigation is needed.

It has also been shown that there is significant interindividual variation in the rate of glycine conjugation of xenobiotics [35,48,49,62,63]. Greater similarity between identical twins than between fraternal twins in the glycine conjugation of salicylate suggested that there is a genetic component to this variation [48]. Using human liver samples, it was demonstrated that there is interindividual variation in the capacity for hippurate synthesis from benzoate, and that the elderly seem to have a slightly decreased capacity [64]. In a large group of subjects, a coefficient of variation of approximately 15 – 24% was found for the formation of hippurate and salicylurate from benzoate and salicylate, respectively [49]. However, no significant difference was found between the mean values for children, adults, the elderly, or patients with liver disease. There was, however, higher variation between the individuals with liver disease, suggesting that the rate of glycine conjugation is influenced by liver disease (Section 4.1) [47,49,65,66]. Glycine conjugation is a saturable process, and this has consequences for co-administration of different substrates for this pathway [3,4,39,45,48,49,64]. For example, although there is interindividual variation in the total amount of glycine conjugates excreted by healthy adults, the total amount of glycine conjugates excreted is the same for each individual whether aspirin and *m*-xylene (excreted as 3-methylhippurate) are administered separately or simultaneously [63].

It is important to note that all these studies on glycine conjugation of xenobiotics report variation for the whole pathway, including ligation to CoASH and conjugation to glycine [3,35,48,49,62-64]. Several factors can influence the overall rate of glycine conjugation, including the availability of ATP, CoASH, and glycine, variation in acid:CoA ligase activity, and GLYAT enzyme activity (**Figure 1**) [3,4,55,59,67]. The limiting step is substrate dependent and can be either ligation to CoASH, as with salicylate, or conjugation to glycine, as with benzoate [3,29,64].

4. GLYAT, liver cancer, hepatitis, and musculoskeletal development

4.1 GLYAT, liver cancer, and hepatitis

Recently, a complete downregulation of transcription of the GLYAT gene was observed in 32 of 41 hepatocellular carcinoma specimens investigated, with significant downregulation in the other nine specimens [68]. This was confirmed by immunohistochemistry using a GLYAT-specific antibody, which revealed that GLYAT is not expressed in cancerous cells, but is expressed in neighboring healthy hepatocytes. Interestingly, GLYAT expression was found to be significant and similar in all noncancerous liver specimens studied, including 60 samples from patients with chronic hepatitis of various etiologies [68]. This observation may be explained by the expression of GLYAT in differentiated hepatocytes, but not in dedifferentiated cancerous cells. On the basis of these findings, Matsuo *et al.* proposed that suppression of GLYAT transcription may be a novel marker of hepatocellular carcinoma and is a key event in the development of liver cancer. There could also be a relationship between GLYAT activity, glycine availability, and cancer cell proliferation [69,70]. This is further elaborated on in Section 7.

It has been reported that the fraction of 4-aminobenzoate excreted as glycine conjugates correlates well to functional hepatic reserves in patients with hepatitis. Therefore, the measurement of glycine conjugation of 4-aminobenzoate has been proposed as a liver function test [47,65,66]. GLYAT expression is normal in hepatitis specimens and it has been suggested that the lower glycine conjugation observed for hepatitis patients could be explained by impaired hepatic β-oxidation and lower availability of ATP for ligation of benzoate to CoASH [3,68].

4.2 GLYAT, glycine, and musculoskeletal development

It was recently proposed that the GLYAT gene may be involved in determining lean muscle mass and bone size in humans [71]. About 690 000 single nucleotide polymorphisms (SNPs) were analyzed in large groups of unrelated Han Chinese (1627) and American Caucasian (2286) individuals to search for variations in the genome that correlate to variation in lean muscle mass and bone size. Fourteen SNPs with significant correlation were identified, three of which are located in or near the GLYAT gene (rs2507838, rs7116722, and rs11826261). Guo *et al.* explained this correlation by stating that GLYAT is important in the metabolism of glucose, but we are not aware of a direct relationship between GLYAT and glucose metabolism. The correlation they report is significant, however, suggesting that GLYAT may play an as yet unknown role in musculoskeletal development [71]. The significance of this observation is further elaborated on in Section 7.

5. Glycine *N*-acyltransferase

5.1 Biochemical and enzymatic characteristics of GLYAT

GLYAT is a monomeric detoxification enzyme found in the mitochondrial matrix of mammalian liver and kidney [58,60,68,72-80]. GLYAT was first identified in bovine liver mitochondria in 1953 and subsequently isolated and characterized from human liver mitochondria in 1976 [16,81]. GLYAT catalyses the transfer of an acyl group from an acyl-CoA to the amino group of glycine, forming an acylglycine and CoASH. Both products of the reaction are powerful inhibitors, and product inhibition is readily observed in enzyme assays [73,82]. Human GLYAT can use several endogenous and xenobiotic acyl-CoAs as substrates, as is evidenced by excretion of corresponding acylglycines in urine (**Table 1**) [4,35,42,43,50,54,63,83]. However, very little information is available on the kinetic parameters of human GLYAT [60,72,74,78,81]. The apparent K_M (benzoyl-CoA) value is reported to range from 13 μM to 57.9 mM, and the V_{max} value using benzoyl-CoA and glycine is reported as 700 nmol/min/mg and 17.1 μmol/min/mg (**Table 2**). This large variation in reported values is difficult to explain, but differences in the method of kinetic analysis, substrate quality, enzyme quality, experimental technique, and perhaps genetic heterogeneity of the GLYAT gene may be responsible [81,84]. The molecular mass of human GLYAT has been reported as 24, 27, 30, and 30.5 kDa [60,74,78,81]. This variation in reported values may be partly explained by the different techniques used in the different studies. For example, Kelley and Vessey [73] found that bovine GLYAT bound to their gel filtration matrix, resulting in erroneous molecular mass estimates.

No structure has been reported for GLYAT; thus, little is known about structure–function relationships. However, GLYAT is a member of the GNAT (Gcn5-related N-acetyltransferase) superfamily. Because of the remarkable structural conservation in the GNAT superfamily, a molecular model of bovine GLYAT could be generated by homology modeling [85]. The model was used to propose that Glu^{226}, a highly conserved residue, is catalytically important. Kinetic characterization and pH profiling of an E226Q mutant demonstrated that Glu^{226} acts as a general base that deprotonates glycine before nucleophilic attack on the carbonyl of the acyl-CoA thioester (**Figure 3**) [85].

5.2 The GLYAT gene and genetic variation

The human GLYAT gene is located on chromosome 11 at position 11q12, spans over 23 000 base pairs, and contains six exons [40]. Two splice variants of human GLYAT mRNA exist, coding for isoforms a (296 residues) and b (162 residues). The transcript for isoform b does not contain exon 6, and there is no protein level evidence for the existence of isoform b [86]. Within the GLYAT gene, there are approximately 668 known SNPs (www.ensembl.org, February 2013), of which 12 are synonymous and 39 are nonsynonymous. Only two studies on relatively small groups of Japanese and French Caucasian individuals have reported novel genetic polymorphisms and allele frequencies of SNPs in human GLYAT [86,87]. The N156S variant had allele frequencies of 97 and 85% in the French Caucasian and Japanese populations, respectively. Because of this high frequency, it was suggested that the N156S allele, rather than the reference sequence (NM_201648.2), should be considered as the wild-type allele [86,87].

In a recent study, the relative enzyme activities of six known polymorphisms (K16N, S17T, R131H, N156S, F168L, and R199C) of a recombinant human GLYAT were compared to the enzyme encoded by the reference sequence (NM_201648.2) [84]. The N156S variant had a greater relative activity than the reference sequence, and this might further support the suggestion that the N156S allele represents the wild-type enzyme. It is interesting to note that the variants with low allelic frequencies (R131H, F168L and R199C) had higher apparent K_M (benzoyl-CoA) values or lower relative enzyme activity when compared to the reference sequence [84,86,87]. The V_{max} values of the variants investigated range from approximately 500 to 1200 nmol/min/mg, and the apparent K_M (benzoyl-CoA) values range from approximately 20 to 70 μM (**Table 3**). Compared to the reference sequence, the K16N, S17T, and R131H variants had similar activities, the F168L variant had decreased activity and an increased K_M (benzoyl-CoA) value, while the R199C variant had < 5% activity. These results indicate that SNP variations found in the human GLYAT gene may result in altered properties of the enzyme, and could perhaps explain some of the differences in kinetic parameters reported in the literature [84]. A molecular model of human GLYAT was used to help explain the altered kinetic properties of the R131H, F168L, and R199C variants of human GLYAT [84].

5.3 Paralogs of the human GLYAT gene

GLYAT is one of four putative glycine-conjugating enzymes. Two GLYAT-like genes, GLYAT-L1 and GLYAT-L2, are

Table 2. Kinetic parameters of human GLYAT.

Parameters	Values
K_M (benzoyl-CoA) (μM)	13 [60] 67 ± 5 [74] 57900 [78]
V_{max} (nmol/min/mg)	700 [60] 17100 [78]

located with the GLYAT gene on chromosome 11q12.1, while the GLYAT-L3 gene is located on chromosome 6p12.3 [40,68,88]. In addition to GLYAT, primates have another transferase that conjugates arylacetyl-CoAs to glutamine, forming phenylacetylglutamine and indoleacetylglutamine [74,81]. The GLYAT-L1 gene codes for the glutamine-conjugating enzyme in humans [68]. Both mitochondrial and cytoplasmic localizations of GLYAT-L1 have been reported, and this could be explained by the two alternative splice variants of GLYAT-L1 mRNA, which code for two isoforms (333 and 302 residues) with distinct N-termini and possibly different subcellular localization [40,81]. The 302-residue isoform of GLYAT-L1 is located in the cytoplasm and transcriptionally activates the heat shock factor pathway in HEK293T cells [40]. The two isoforms might thus have different functions in the mitochondria and cytoplasm [40,68,81]. It has not been investigated why primates, unlike other mammals, conjugate arylacetates to glutamine instead of glycine.

GLYAT-L2 mRNA is expressed in salivary gland, trachea, spinal cord, and skin fibroblasts. The enzyme is localized to the endoplasmic reticulum, and a recombinant GLYAT-L2 catalyses the formation of long-chain acylglycines such as N-arachidonoylglycine and N-oleoylglycine [88,89]. These are members of a class of cannabinoid-like signaling hormones that activate G-protein-coupled receptors and have antinociceptive, anti-inflammatory, and antiproliferative effects [90]. GLYAT-L2 activity is regulated by acetylation on Lys^{19} and mutation of Lys^{19} of a recombinant GLYAT-L2 to arginine or glutamine resulted in a 70 – 80% decrease in enzyme activity [89]. Mutation of the equivalent Lys^{20} residue of a recombinant human GLYAT to arginine or glutamine did not cause a similar reduction in enzyme activity, suggesting that acetylation of this lysine residue is not important in regulation of GLYAT activity [84,89]. No enzyme activity has been reported for GLYAT-L3, which does not seem to have the catalytic glutamate residue proposed for the GLYAT reaction mechanism, but the significance of this is unclear [85,88].

6. Summary

Compared to the cytochrome P450 and UDP-glucuronosyltransferase superfamilies of biotransformation enzymes, GLYAT is not very well characterized [3,4]. This may be because of the small number of pharmaceutical drugs that are metabolized to glycine conjugates and the difficulty in obtaining human material and xenobiotic acyl-CoA substrates for research [3]. In this review, we have demonstrated that glycine conjugation is an important metabolic pathway that plays a role in the metabolism of CoASH and glycine and can influence mitochondrial energy production (**Figures 2** and **4**) [4,84]. Recent studies suggest that GLYAT may also be an important factor in the development of hepatocellular carcinoma and could influence musculoskeletal development and growth [68,71].

A range of xenobiotic acylglycines are excreted in urine, indicating that either the parent xenobiotic or a carboxylate metabolite is a substrate for ligation to CoASH, and that the acyl-CoA is a substrate for glycine conjugation (**Table 1**) [4,29,35,44,50,63,65,83]. The toxicity of xenobiotic carboxylates is partially determined by the extent to which an acyl-CoA, that cannot be conjugated to glycine or some other acceptor, is formed [3,4,31,33]. This leads to accumulation of the acyl-CoA, which can have several toxic effects in addition to disrupting mitochondrial energy production [23,33]. In severe cases, this can lead to hepatic steatosis and death [3,12]. As the glycine conjugation pathway is saturable, variation in the rate of glycine conjugation influences the clearance of xenobiotics and thus toxicity [3,64]. If the rate of conjugation by GLYAT is low, glycine conjugation may not prevent the toxicity of an acyl-CoA, even if it is a substrate for the enzyme (**Table 1**) [3,37,42,57].

It is often unclear whether variation in the rate of glycine conjugation results from differences in acid:CoA ligase activity or GLYAT activity (**Figure 1**) [4,55,59,67]. This is complicated by the observation that the limiting step depends on the xenobiotic, making it difficult to compare the results of different studies [29,64]. The recent expression and characterization of recombinant human GLYAT enzymes made an important contribution to our understanding of variation in glycine conjugation by demonstrating that genetic variation in the GLYAT gene can influence GLYAT enzyme activity [68,84,88,89].

7. Expert opinion

7.1 GLYAT and its relationship to liver cancer and musculoskeletal development

It was recently proposed that the GLYAT gene may play important roles in development of both hepatocellular carcinoma and the musculoskeletal system [68,71]. We suggest that these relationships may be explained by the role of GLYAT in glycine metabolism. Glycine is commonly considered as a nonessential amino acid because it can be synthesized from serine (**Figure 4**) [91,92]. However, studies over the past two decades have shown that glycine is in fact a semi-essential amino acid, that humans may have a daily shortage of about 10 g of glycine and that this may impact on collagen turnover and the synthesis of bile acids, creatine, glutathione, and heme [8,91-93].

Under certain conditions, GLYAT can conjugate sufficient amounts of glycine to limit its availability for other metabolic

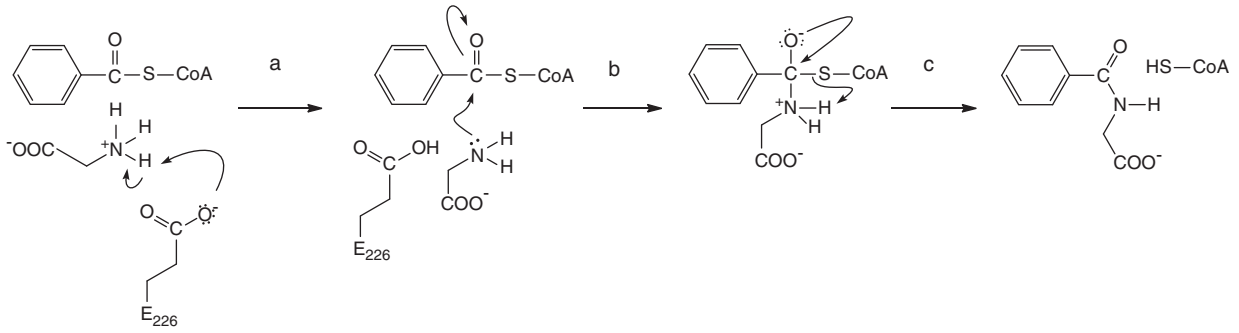

Figure 3. The catalytic mechanism proposed for bovine GLYAT. Bovine GLYAT employs a ternary complex mechanism, where Glu^{226} serves as a general base catalyst. (a) For nucleophilic attack to take place, the glycine amino group must be deprotonated by Glu^{226}; (b) A tetrahedral intermediate is formed, following the nucleophilic attack by the amino group of glycine on the thioester carbonyl group. (c) Finally, the tetrahedral intermediate collapses, forming benzoylglycine and CoASH. ChemDraw 10.0 (CambridgeSoft, Cambridge, MA) was used to produce this schematic.
Reproduced with permission [85] from the American Society for Pharmacology and Experimental Therapeutics.

Table 3. Kinetic parameters of recombinant human GLYAT enzymes.

Recombinant GLYAT	K_M (benzoyl-CoA) (μM)	V_{max} (nmol/min/mg)
Flag-His_6-hGLYAT (N156S)	209	807 [68]
Trx-His_6-hGLYAT (NM_201648.2)	24 ± 3	730 ± 30 [84]
Trx-His_6-hGLYAT (K16N)	21 ± 1	1030 ± 20 [84]
Trx-His_6-hGLYAT (S17T)	28 ± 5	665 ± 40 [84]
Trx-His_6-hGLYAT (R131H)	71 ± 11	1040 ± 85 [84]
Trx-His_6-hGLYAT (N156S)	38 ± 4	1230 ± 60 [84]
Trx-His_6-hGLYAT (F168L)	53 ± 6	500 ± 30 [84]
Trx-His_6-hGLYAT (R199C)	Not determined	Not determined [84]

processes (**Figure 4**) [61,94,95]. For example, it has been shown that administration of benzoate to rats can reverse chemically induced porphyria by diverting glycine away from heme biosynthesis. This results in normalization of urinary δ-aminolevulinate, porphobilinogen, and porphyrin levels, an effect cancelled out by co-administration of glycine [8,94]. We suggest that the recently reported correlation of SNPs in and near the GLYAT gene to variation in lean muscle mass and bone size could be explained, in part, by the impact of GLYAT on the availability of glycine for the synthesis of creatine, collagen, and elastin [71,91,92]. It is interesting that apart from the normal expression of GLYAT in liver and kidney, low levels of GLYAT expression have also been observed in skeletal muscle, but the significance of this observation is unclear [68].

It was recently demonstrated that glycine is a metabolite crucial for rapid division of cancer cells and that inhibition of glycine uptake or biosynthesis impaired the cancer cell growth, probably by slowing the synthesis of nucleic acids (**Figure 4**) [69]. We suggest that this helps to explain why GLYAT is not expressed in hepatocellular carcinoma, as depletion of hepatic glycine by GLYAT would inhibit rapid proliferation of cancer cells [68-70,96,97]. This could have significant implications for both the diagnosis and treatment of liver cancer.

7.2 The increased demand for glycine conjugation in modern life

In modern times increasing exposure to benzoate, salicylate, solvents, and drugs that are metabolized to acyl-CoA intermediates places more pressure on the glycine conjugation pathway, possibly exacerbating metabolic glycine shortage discussed previously [3,4,63,83,84,91,93]. Therefore, the consequences of interindividual variation in the glycine conjugation pathway may become more significant as more xenobiotic organic acids are encountered in the future [3].

In addition to xenobiotics, SCFAs produced by intestinal microbes are another potential source of substrates for glycine conjugation and may contribute to glycine depletion under some conditions [98]. Gut microbes produce large amounts of SCFAs that account for 5 – 10% of the total dietary energy intake in humans [98]. Indeed, the gut contains an active SCFA ligase for metabolizing these organic acids [99]. Gut dysbiosis, caused by antibiotic use, for example, can result in increased SCFA production and this has been associated with obesity and diabetes [100,101]. SCFAs are not usually

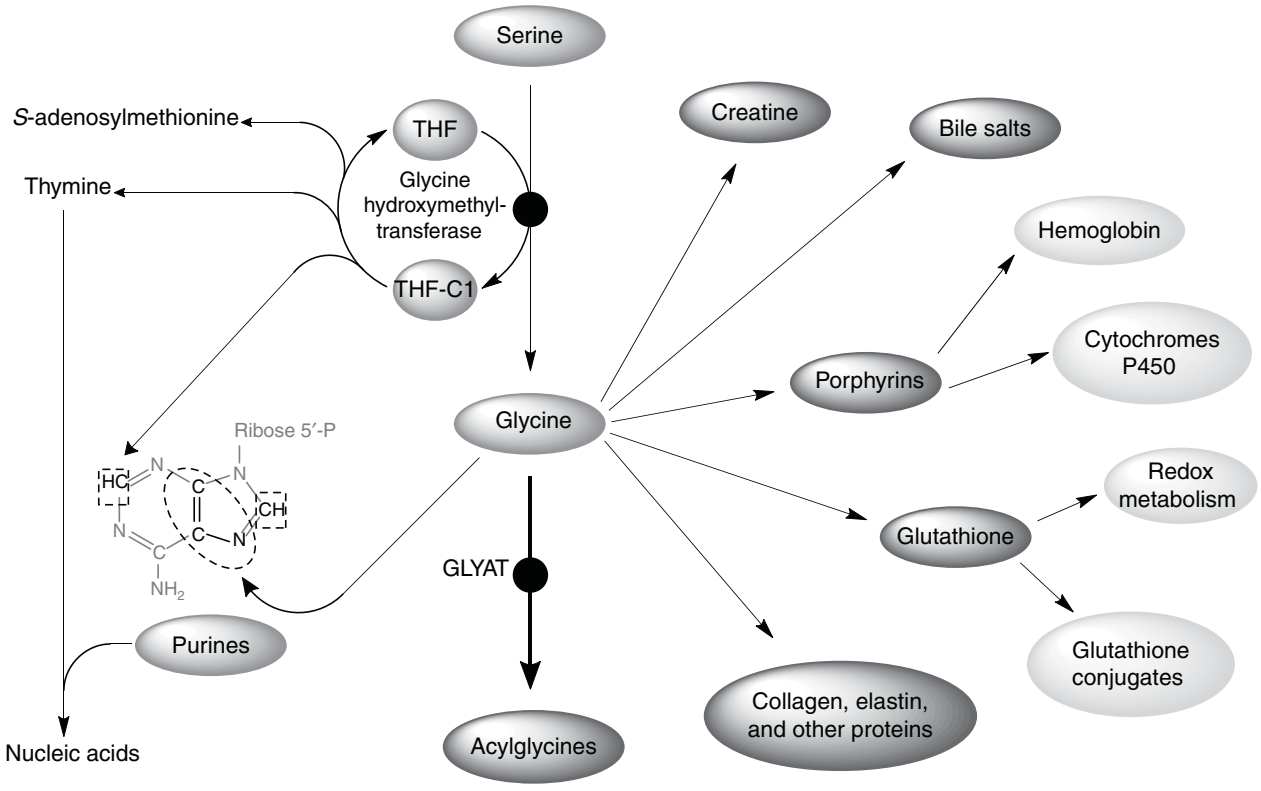

Figure 4. Biosynthesis and metabolic consumption of glycine. Glycine is biosynthesized from serine by glycine hydroxymethyltransferase. The reaction converts tetrahydrofolate (THF) to tetrahydrofolate-C1 (THF-C1) for each molecule of glycine produced. The total amount of glycine synthesized can thus not exceed the amount of THF-C1 consumed through the production of *S*-adenosylmethionine, thymine, and purines. Glycine is used in the production of glutathione, creatinine, bile salts, porphyrins, collagen, elastin, and other proteins. The bold arrow indicates the formation of xenobiotic acylglycines by GLYAT. The parts of purine rings derived from glycine and THF-C1 are indicated by the dashed ellipse and squares, respectively. The black circles indicate the glycine hydroxymethyltransferase and GLYAT enzymes.

conjugated to glycine, as this would be energetically wasteful [14,42,43]. However, it is our opinion that if sufficiently large amounts of SCFAs are produced, hepatic metabolism of CoASH and glycine will be affected. A recent observation in our laboratory seems to support this idea. A patient with unusually high levels of urinary butyrate complained of bad body odor and was referred to our laboratory by a physician. The increased butyrate excretion was not the result of a short-chain acyl-CoA dehydrogenase defect, and gut dysbiosis was suspected. Glycine supplementation was recommended and this resulted in significantly increased butyrylglycine excretion, decreased butyrate excretion, and disappearance of the body odor (unpublished results).

7.3 Future investigations of interindividual variation in glycine conjugation

In conclusion, we believe that it is important to study the relationships between genetic variation in the GLYAT gene, GLYAT enzyme activity, the *in vivo* rate of glycine conjugation, and physiological consequences of variation in the glycine conjugation pathway.

Existing publications on interindividual variation in the glycine conjugation pathway do not discriminate between variation in acyl-CoA formation and variation in glycine conjugation. It is important to remember that glycine conjugation is a two-step process and that the overall rate of glycine conjugation can be influenced by several factors (**Figure 1**) [3,4,55,59,67]. Most importantly, the limiting step in the glycine conjugation pathway depends on the xenobiotic used [29,64]. We suggest that future studies employ at least salicylate and benzoate as probe compounds, on separate occasions, to enable differentiation between variation in acid:CoA ligase and GLYAT activities, respectively.

The use of benzoate as a probe compound is, however, complicated by the metabolism of gut microorganisms. It was mentioned in Section 3.1 that gut dysbiosis in Crohn's disease results in decreased microbial benzoate production and lower levels of hippurate in urine [45]. Gut metabolism will thus influence relative increases in urinary hippurate levels after benzoate ingestion without necessarily affecting the rate of hepatic glycine conjugation [44,45]. Therefore, it is important to determine increases in hippurate excretion rather

than ratios to baseline levels [45]. An alternative probe compound is 4-aminobenzoate [65]. However, substituted benzoates are generally activated to acyl-CoAs more slowly than benzoate [24,27,29]. This suggests that glycine conjugation of 4-aminobenzoate, as with salicylate, is limited at the acid: CoA ligase step. This is consistent with the suggestion that the decreased glycine conjugation of 4-aminobenzoate by hepatitis patients is because of decreased mitochondrial ATP production [3]. Determination of hippurate formed from an oral dose of stable isotope labelled benzoate is one suggestion to simplify the interpretation of benzoate conjugation data, which we believe cannot be substituted for by salicylate or 4-aminobenzoate.

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