18 Amino Acid Conjugation: A Novel Route of Xenobiotic Carboxylic Acid Metabolism in Man

KATHLEEN M. KNIGHTS and JOHN O. MINERS

Department of Clinical Pharmacology, Flinders University, Bedford Park, Australia

18.1 SUMMARY

Compared to most xenobiotic-metabolizing enzymes, knowledge of amino acid conjugation in terms of enzyme multiplicity, protein structure, and xenobiotic substrate selectivity is less advanced. Amino acid conjugation is a novel biotransformation pathway in man for a number of xenobiotic carboxylic acids, including arylacetic, aryloxyacetic, aromatic, and heteroaromatic acids. A notable example is salicylurate formation, which accounts for up to 85% of total salicylic acid clearance in humans.

Amino acid conjugation of xenobiotics involves two enzymatic processes that are localized in mitochondria. First, formation of a reactive xenobiotic–CoA thioester intermediate, catalyzed by an ATP-dependent acid:CoA synthetase (ACSM), and second, linkage of the activated acyl group via an acyl-CoA:amino acid *N*-acyltransferase (GLYAT) to the amino group of the acceptor amino acid. In humans, the principal amino acid utilized is glycine. Formation of a xenobiotic acyl-CoA thioester is an obligatory step in the overall process of amino acid conjugation. However, it is apparent that there are many more xenobiotics that form acyl-CoA thioesters than there are xenobiotics that are substrates for sequential metabolism by ACSM and GLYAT.

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.

Although the active sites of the ACSMs and GLYATs clearly differ, detailed understanding of the metabolism of xenobiotic carboxylic acids via amino acid conjugation awaits the in-depth structural characterization of both proteins.

18.2 INTRODUCTION

The early pioneering work of Alexander Ure in 1841 laid the foundation for the field of amino acid conjugation by demonstrating that exogenously administered benzoic acid was metabolized to hippuric acid [1]. It was not until Dessaignes in 1845 determined that hippuric acid was a benzoic acid linked to glycine (benzoylglycine) that the first evidence of both a conjugation reaction per se and an amino acid conjugation reaction in humans was provided [2]. The future of the field seemed assured following the recognition that amino acid conjugation is the predominant route of metabolism of salicylic acid, with salicylurate (the glycine conjugate) formation accounting for 68–85% of the excretion of salicylic acid in urine. Although knowledge of conjugation reactions involving glucuronic acid, sulfate, and glutathione has advanced significantly, in relative terms, amino acid conjugation remains poorly understood and is limited to a number of arylacetic, aryloxyacetic, aromatic, and heteroaromatic acids.

For the majority of xenobiotic carboxylic acids, amino acid conjugation is a novel but minor metabolic pathway. However, rather than assignation in terms of importance to the bottom of the drug metabolism, enzymology ladder amino acid conjugation (or lack there of) could be viewed as an evolutionary piece de resistance. Instead of exhibiting the substrate promiscuity of the cytochrome P450s (CYPs) or UDP-glucuronosyltransferases (UGTs), the relative substrate selectivity of the enzymes of amino acid conjugation has been very stable. This stability implies that the enzymes have a central role in the connectivity of biotransformation reactions, and hence, any disruption may have the potential to influence multiple pathways crucial to cellular integrity.

18.3 MECHANISM OF AMINO ACID CONJUGATION

A limited number of carboxylic acids are conjugated with an amino acid (principally glycine) before excretion. These include endogenous molecules (e.g., bile acids, branched chain fatty acids), environmental chemicals (e.g., herbicides), food preservatives (e.g., substituted benzoates), and drugs (e.g., salicylic acid and valproic acid (VPA)). In general, bile acid conjugation forming glycine and taurine conjugates is catalyzed by extramitochondrial (microsomal and peroxisomal) enzymes, while xenobiotic amino acid conjugation is mediated by mitochondrial enzymes. The latter is the focus of this chapter, and while much of our knowledge of amino acid conjugation both *in vivo* and *in vitro* is based on glycine as the acceptor amino acid, other amino acids are utilized in both man and other species. These include glutamine, glutamate, and taurine in man [3–5]; ornithine in birds and reptiles; and glutamine in ferrets, rabbits, and rats [6]. Formation of amino acid conjugates increases solubility and facilitates xenobiotic excretion in bile or urine.

In contrast to other conjugation reactions, amino acid conjugation is a coupled two-enzyme system. The general consensus is that amino acid conjugation proceeds through

- initial activation of the carboxylic acid moiety with ATP, generating an acyl adenylate and pyrophosphate;
- the reaction of the bound acyl adenylate with coenzyme A (CoASH) catalyzed by an ATP-dependent acid:CoA synthetase (AMP forming) to yield a "high energy" xenobiotic–CoA thioester intermediate;
- linkage of the activated acyl group via the action of an acyl-CoA:amino acid *N*-acyltransferase to the amino group of the acceptor amino acid with regeneration of CoASH (Fig. 18.1).

The formation of an acyl adenylate has not been proven unequivocally. However, multiple studies using proteins from liver and kidney of multiple species with an array of substrates indicate that the reaction follows a bi uni uni bi ping-pong mechanism. For example, using benzoate as the substrate, ATP binds first to the enzyme, followed in order by benzoate binding, pyrophosphate release, CoA binding, benzoyl-CoA release, and AMP release [7].

It is important to recognize at this stage that as a coupled enzyme reaction, a xenobiotic is first a substrate for the acid:CoA synthetase and then the xenobiotic–CoA conjugate is a substrate for the acyl-CoA:amino acid *N*-acyltransferase. Thus, specificity or selectivity of this metabolic route can be conferred at the level of either one or both enzymes. Furthermore, lack of amino acid conjugation does not necessarily infer lack of xenobiotic–CoA conjugation.

18.4 MITOCHONDRIAL MEDIUM CHAIN ACYL-CoA SYNTHETASES (ACSM)

An understanding of amino acid conjugation is predicated on the knowledge of the process of fatty acid activation and the commonality of the enzymes involved. Short-, medium-, and long-chain and very-long-chain fatty acids are activated to their corresponding acyl-CoA thioesters by different families of acyl-CoA synthetases (ACS). Since the fatty acid carbon chain length defines the selectivity of the different enzymes, the ACS superfamily has been subdivided into multiple subfamilies on the basis of fatty acid substrate selectivity. In biochemical terms, these enzymes were previously referred

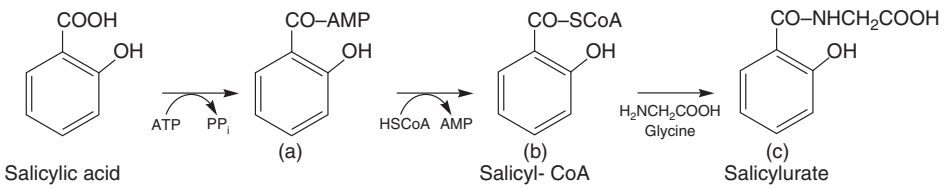

Figure 18.1 Salicylurate formation. (a) Formation of acyl adenylate, (b) formation of salicyl–CoA catalyzed by medium chain acyl-CoA synthetase, and (c) conjugation with glycine to form salicylurate catalyzed by acyl-CoA:glycine *N*-acyltransferase.

to as *ligases* because of their role in "ligating" a fatty acid to CoA. However, in view of the diversity of substrates utilized, both endogenous and exogenous, synthetase was adopted to describe the activity of the enzymes and acyl-CoA for the product [8]. The approved root symbol is ACS followed by a letter that specificies the carbon chain length of the fatty acid substrates, for example, S, short; M, medium; and L, long. The ACS nomenclature was introduced in 2004 for the medium chain ACS (ACSM) subfamily.

Short-chain fatty acids (C_2-C_4) are primarily activated by the cytosolic short-chain ACSs, and these enzymes are not considered to contribute to xenobiotic metabolism. Long-chain fatty acids ($C_{12}-C_{20}$) are activated by members of the ACSL family and are present in the outer mitochondrial membrane, endoplasmic reticulum (ER), and peroxisomes. The microsomal ACSL catalyze formation of the CoA thioesters of nafenopin, clofibric acid, and the R(-)enantiomers of the 2-arylpropionic acid non-steroidal anti-inflammatory drugs [9,10]. In general, the xenobiotic–CoA thioesters formed in the ER are not substrates for the mitochondrial acyl-CoA:amino acid *N*-acyltransferases. Likely explanations include the fact that xenobiotic–CoAs do not cross biological membranes readily, and they may also be poor substrates for the carnitine shuttle.

The most important enzymes in terms of amino acid conjugation are the medium chain ACSs (ACSM). The ACSMs are located in the mitochondrial matrix and activate C_4-C_{12} fatty acids. These medium chain fatty acyl-CoA esters are then utilized either for β-oxidation or in anabolic pathways for the formation of cellular lipids. The commonality of involvement of ACSM in both activation of fatty acids and xenobiotics suggests that intramitochondrial formation of a xenobiotic–CoA has the potential to perturb mitochondrial function.

A purified beef liver mitochondrial enzyme ("Mahler's enzyme") was first described to catalyze CoA conjugation of C_4-C_{13} fatty acids and their oxidized derivatives, benzoic acid, phenylacetic acid, and 2,4-dichlorophenoxyacetic acid (2,4-D) [11]. Subsequent studies, however, determined that salicylic acid and *p*-aminosalicylic acid were not substrates for "Mahler's enzyme," and mitochondrial "salicyl-CoA" and "propionyl-CoA" ligases were isolated from bovine and guinea pig liver, respectively [12–14]. For the next 20 years, progress was slow because of persistent methodological problems with lability of the mitochondrial ACS and hydrolysis of the xenobiotic–CoA conjugates from contaminating acyl-CoA hydrolases. In 1995, Vessey and Hu used column chromatography to purify three mitochondrial medium chain ACSs from bovine liver [15]. The three forms were designated XL-I, XL-II, and XL-III, and their apparent molecular masses were 54.7, 55.6, and 52.5 kDa, respectively. Partial sequencing of purified XL-I revealed regions of high homology with the essential hypertension SA protein, suggesting a role for the metabolism of endogenous carboxylic acids in the regulation of blood pressure [16]. Subsequent screening of a bovine cDNA library with oligonucleotide probes complementary to short sequences of the N-terminus of XL-I or XL-III resulted in the isolation of a 2065-bp cDNA encoding a protein of 577 amino acids (molecular mass, 64 kDa, XL-III) and an incomplete (75%) cDNA encoding XL-I. Comparison of the amino acid sequence of XL-III and the partial sequence of XL-I revealed an overall identity of 60%, suggesting a common ancestral gene [17]. A fourth bovine liver mitochondrial medium chain ACS (molecular mass, 65.5 kDa) was purified in 1996 by Kasuya *et al*. [18] and for convenience has been termed XL-IV in this chapter.

Additionally, ACSMs have been purified from mouse kidney [19] and mouse liver mitochondria [20]. In a comparative study, Kasuya *et al*. established that there was 48.0% amino acid identity among the mitochondrial ACSMs of mouse, rat, and bovine liver [20]. Furthermore, they reported that the mouse butyryl-CoA synthetase (SA protein) shared 74.2% amino acid identity with bovine XL-III. The latter is identical to a lipoate-activating enzyme that was purified from bovine liver mitochondria [21].

Two human liver mitochondrial xenobiotic/medium chain fatty acid ACSs kinetically distinct from the corresponding bovine liver XL forms were isolated in 1999: HXM-A (now designated ACSM2B) and HXM-B [22]. Subsequent cloning of human liver cDNAs determined that HXM-A was a 64-kDa protein exhibiting 56.2%, 58.7%, and 81.5% amino acid identity to MACS1, XL-III, and XL-I, respectively [23]. The recombinant protein (expressed in COS-7 cells) had greater activity toward benzoic acid than phenylacetic acid, which was consistent with the substrate selectivity of the purified HXM-A protein [22]. In 2001, Fujino *et al*. [24] characterized two medium chain ACSs (MACS1, now ACSM1; and SA, now ACSM3) located on human chromosome 16p. Both the proteins were localized to the mitochondrial matrix, and MACS1 appears to be the same as HXM-B.

18.4.1 Xenobiotic Substrates of ACSM

It is not surprising, given the high amino acid sequence identity between the mitochondrial ACSMs, that there is considerable overlap in terms of fatty acid and xenobiotic substrates. Tables 18.1 and 18.2 provide an overview of the *in vitro* substrate profiles of purified or recombinant bovine and murine (Table 18.1) and human (Table 18.2) ACSM proteins. It is difficult to compare both the substrate selectivity and relative enzyme activity of the ACSMs because the range of substrates varies between different studies, and different protein sources and preparations have been used. Although relative activities with each substrate vary enormously, common substrates for all bovine XL enzymes and murine ACSMs include hexanoic acid, octanoic acid, benzoic acid, 4-chlorobenzoic acid, and VPA. Salicylic acid is metabolized by XL-I, XL-II, and XL-III, with respective apparent K_m values of 2.4, 2.2, and 2.5 μM and respective V_{max} values of 1.2, 1.4, and 0.24 nmol salicyl-CoA formed per minute milligram of protein [25]. However, negligible activity was observed with XL-IV [26]. From the data detailed in Table 18.1, 1-napthylacetic acid and 3-phenoxybenzoic acid are not substrates for XL-I and XL-II but are substrates for XL-III, XL-IV, and the murine liver and kidney forms of ACSM [15,18–20,24–27].

In contrast to the bovine and murine proteins, the substrate profile of the human forms HXM-A and HXM-B is poorly characterized (Table 18.2). Of the limited number of fatty acids investigated, substrate selectivity (C_4, C_6, and C_{10}) is similar to the bovine and murine enzymes. The only xenobiotic investigated was salicylic acid; K_m^{app} values for salicyl-CoA formation by HXM-A and HXM-B were 7.4 and 25 μM, respectively [22]. Formation of salicyl-CoA in humans is thought to be the rate-limiting step in clearance of salicylic acid to salicylurate, the latter accounting for the majority of total salicylic acid clearance in humans [28]. Salicylic acid clearance is greater in males than females due largely to enhanced salicylurate formation, but oral contraceptive steroid (OCS) use negates the gender difference in salicylic acid disposition parameters between males and OCS users [29]. At therapeutic doses, salicylurate formation

TABLE 18.1 Substrate Profile of Bovine, MACS1 and Murine Medium Chain Acyl-CoA Synthetases

Substrate	Bovine Liver				MACS1	Murine	
	XL-I	XL-II	XL-III	XL-IV		Kidney	Liver
Fatty Acid							
C_3	√	√	Neg	√	NA	NA	NA
C_4	√	√	√	NA	Neg	NA	NA
C_6	√	√	√	√	√	√	√
C_8	√	√	√	√	√	√	√
C_{10}	√	√	√	NA	√	NA	√
C_{12}	Neg	Neg	√	√	√	NA	NA
C_{16}	×	×	×	NA	Neg	NA	NA
Xenobiotics							
Benzoic acid	√	√	√	√	√	√	√
4-Chlorobenzoic acid	√	√	√	√	√	√	√
DTA	×	×	√	NA	NA	NA	NA
Ibuprofen	×	×	Neg	NA	NA	NA	NA
3-Methoxybenzoic acid	NA	NA	NA	√	NA	√	√
1-Napthylacetic acid	×	×	√	√	NA	√	√
1-Napthoic acid	×	√	√	NA	NA	√	√
3-Phenoxybenzoic acid	×	×	√	√	NA	√	√
Salicylic acid	√	√	√	NA	NA	×	×
Valproic acid	√	√	Neg	NA	NA	Neg	Neg

√, activity reported in nmol/min mg; ×, activity not detected; Neg, negligible activity; NA, not assayed; DTA, 2,4,6,8-decatetraenoic acid.
XL-I to XL-IV, kidney and liver ACSM column-purified proteins; MACS1, recombinant protein [24].
For further comprehensive data, see Refs 15,18–20, and 24–27.

is a saturable process, and this contributes to the dose-dependent nonlinear pharmacokinetics of salicylic acid [30,31]. Although the V_{max} value of the purified HXM-A protein was 0.7 nmol/min.mg [22], the catalytic efficiency calculated as k_{cat}/K_m is 0.006 μL/min.pmol protein, suggesting that HXM-A is unlikely to be the sole enzyme responsible for salicyl-CoA conjugation *in vivo*.

In addition to the xenobiotics listed in Tables 18.1 and 18.2, mitochondrial ACSM catalyzes CoA conjugation of hypoglycin A [32], pivalate [33], 2-tetradecylglycidic acid (TDGA) [34]), 3-mercaptopropionic acid [35], zomepirac [36], and 3-oxapentanoic and 3,6-dioxaheptanoic acid, the carboxylic acid metabolites of the ethylene-glycol-based solvents 2-ethoxyethanol and diglyme, respectively [37].

18.4.2 Substrate Structure–Activity Relationships

As relatively few drugs and chemicals are activated to acyl-CoA thioesters, distinct physiochemical features of the xenobiotic presumably influence the initial conjugation reaction. Using a variety of aromatic and arylacetic acids and purified bovine liver ACSM, Kasuya *et al*. [18] observed high activity toward saturated straight medium chain fatty acids in contrast to aromatic acids. Investigation of benzoic acid derivatives showed that highest activity occurred with alkyl or alkoxyl substitutions in the para- or meta-position of the benzene ring, while ortho-substituted benzoic acids were not

TABLE 18.2 Substrate Profile and Kinetic Parameters for Acyl-CoA Conjugation by Human Medium Chain Acyl-CoA Synthetases HXM-A and HXM-B

Substrate	HXM-A	K_m (μM)	V_{max} (nmol/min.mg)	V_{max}/K_m (mL/min.mg)	HXM-B	K_m (μM)	V_{max} (nmol/min.mg)	V_{max}/K_m (mL/min.mg)
Fatty Acid								
C_3	√	—	—	—	—	√	—	—
C_4	√	800	27	0.03	√	570	24	0.04
C_6	√	74	88	1.19	√	34	118	3.47
C_8	√	—	—	—	√	—	—	—
C_{10}	√	110	15	0.14	√	20	8	0.4
C_{12}	Neg	60	0.2	0.003	Neg	24	0.15	0.006
Xenobiotics								
Benzoate	√	13	103	7.9	√	21	9	0.4
Naphthylacetate	Neg	—	—	—	Neg	—	—	—
Nicotinate	×	—	—	—	—	Neg	—	—
Phenylacetate	√	160	29	0.18	√	143	2	0.014
Salicylate	√	7.4	0.7	0.1	Neg	25	<0.1	<0.004
Valproate	√	—	—	—	—	√	—	—

√, activity reported in nmol/min.mg; ×, activity not detected; Neg, negligible activity.
For further details, see Ref. 22.

activated to their corresponding acyl-CoA esters. Arylacetic acids, such as 1-naphthylacetic acid, exhibited similar activity to benzoic acid, while VPA, a branched medium chain fatty acid, exhibited negligible activity [18]. High activity (relative to hexanoyl-CoA formation) has also been observed for benzoic acids with methyl, methoxy, ethoxy, phenoxy, and *n*-pentyl and *n*-heptyl substituents [38].

Studies investigating the mechanism of valproyl-CoA toxicity found that α-fluorination of Δ^4-VPA [39] and VPA [40] prevents the formation of the corresponding acyl-CoA derivative and, in the case of Δ^4-VPA, prevents hepatic steatosis [39]. Similarly, lack of formation of acyl-CoA thioesters has been shown for α-fluoropalmitic acid, perfluorooctanoic acid, and perfluorodecanoic acid [41,42]. These data suggest that fluorine, an electron-withdrawing substituent, alters the molecular recognition by ACSM. As noted above, substitution of fluorine at the α-carbon in VPA prevents formation of Δ^4-VPA acyl-CoA. This may be explained by the increased acidity of the carboxyl group due to the electronegativity of the fluorine atom (F-VPA, pK_a 3.55) in comparison to VPA (pK_a 4.80) [40]. Recently, a positive electron-deficient "patch" adjacent to the electron-withdrawing fluorine substituent has been identified on the electrostatic surface of Δ^4-VPA [43]. This electrostatic feature of the fluorinated analog may account for the loss of molecular recognition by ACSM.

Recently, a crystal structure (3EQ6) of human ACSM2A with butyric acid bound in the active site has been reported [44], although full details of the structure appears not to have been published. Fifteen putative substrate-determining residues (SDRs) were identified: Phe-234, Asp-235, Ala-236, Trp-239, Thr-278, Ile-299, Ala-301, Gly-302, Ala-322, Tyr-323, Gly-324, Pro-325, Ile-330, Cys-331, and Lys-517. In addition to the 15 SDRs common to the ACS family of enzymes, an additional conserved histidine at position 210 was identified to be important in determining the chain length of medium chain fatty acid substrates, while disfavoring the binding of long-chain fatty acids [44]. Further site-directed mutagenesis studies are required to elucidate the importance of these residues in the binding of xenobiotics.

18.4.3 Inhibitors of ACSM

The bovine forms XL-I and XL-III show substrate-inhibition kinetics with salicylic acid, and although ibuprofen is a specific substrate for XL-III, it inhibits all three XL forms with K_i values in the range of 70–125 μM [25]. Using a purified mouse liver ACSM and hexanoic acid as the substrate, Kasuya *et al*. demonstrated competitive inhibition by diflunisal (K_i0.6 μM), 2-hydroxynaphthoic acid (K_i13.4 μM), 2-hydroxydodecanoic acid (K_i15 μM), nalidixic acid (K_i12.4 μM), and salicylic acid (K_i19.6 μM) and mixed-type inhibition with enoxacin and ofloxacin with respective K_i values of 23.7 and 38.2 μM [45]. Recently, it was demonstrated that while triacsin C is a potent inhibitor of ACSL ($K_i < 1$ μM), this compound is a relatively weak inhibitor of HXM-A and HXM-B ($K_i > 100$ μM) [46].

18.5 MITOCHONDRIAL ACYL-CoA:GLYCINE *N*-ACYLTRANSFERASE (GLYAT)

The second step in the overall process of amino acid conjugation in mammals most commonly involves transfer of the acyl group to the amino group of glycine. This

reaction is catalyzed by acyl-CoA:glycine *N*-acyltransferases (GLYAT, EC 2.3.1.13) located in the mitochondrial matrix. Kinetic studies with bovine GLYAT are consistent with a sequential bi–bi mechanism where the acyl-CoA substrate binds first, followed by addition of the amino acid, before dissociation of CoA, and the release of the peptide product as the final step [47]. GLYATs, which are principally found in the mitochondria of liver and kidney, differ from the bile acid:CoA amino acid *N*-acyltransferase (EC 2.3.1.65) that conjugates bile acids with either taurine or glycine. Following identification in beef liver mitochondria in 1953 [48], two glycine *N*-acyltransferases were purified from human liver mitochondria in 1976 [49] and 1994 [50]. One was specific for glycine and utilized either butyryl-CoA or benzoyl-CoA as acyl donors, while the second enzyme conjugated phenylacetyl-CoA or indoleacetyl-CoA with glutamine [49].

18.5.1 Xenobiotic Substrates of GLYAT

Specificity of the bovine liver GLYATs has been demonstrated. The "phenylacetyltransferase" protein exhibits activity toward phenylacetyl-CoA, phenoxyacetyl-CoA, and 2,4-D but not toward 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), while the "benzoyltransferase" protein conjugates both 2,4-D and 2,4,5-T [51]. However, both GLYATs exhibited very high K_m values for glycine (100 and 1000 mM) and slow catalytic rate constants with the phenoxyherbicide-CoAs [51].

Detailed *in vitro* studies of the substrate profile of human GLYAT are lacking. Mawal and Qureshi [50] reported K_m values of 58 and 84 mM for benzoyl-CoA and salicyl-CoA, respectively. In general, evidence of amino acid conjugation principally results from identification of conjugates in human urine (Table 18.3). Glycine conjugation has been observed for the histamine H_1 receptor antagonists astemizole [52] and brompheniramine [53], the insecticide permethrin [54], the antiplatelet agent triflusal [55], and the solvent *m*-xylene [56]. In contrast, a taurine conjugate of ibuprofen was identified in human urine [5].

18.5.2 Substrate Structure–Activity Relationships

Current data indicates that CoA thioesters, which are substrates for amino acid conjugation, exhibit varying affinities for GLYAT [57]. From studies of the reactivity of acyl-CoA thioesters with glutathione, a simple structure–activity relationship based on substitution of the carbon α to the acyl carbon and on the presence of an oxygen atom β to the acyl carbon has been proposed [58]. Clearly, structural aspects of the xenobiotic–CoAs influence reactivity, and this is most likely associated with the functionality adjacent to the carboxylic acid group. Although this is an interesting observation, it does not account for the selectivity of the GLYATs, and no studies to date have characterized the factors that determine the recognition of xenobiotic acyl-CoAs by GLYAT.

18.5.3 Glycine Conjugation *In Vivo*

Unlike the majority of metabolic pathways that follow first-order kinetics *in vivo*, glycine conjugation is readily saturable, and hence, metabolism of some carboxylic acids is dose dependent. Depending on the substrate, the rate-limiting step can be either formation of the acyl-CoA intermediate or acyl transfer of the amino acid. In

TABLE 18.3 Xenobiotics Metabolized via Amino Acid Conjugation as Either the Parent Acid or Carboxylic Acid Metabolite in Humans

Parent Compound	Carboxylic Acid Metabolite	Amino Acid Conjugate	References
Astemizole	4-Fluorobenzoic acid	2-[4-Fluorobenzoyl)amino]acetic acid[a]	50
	2-(4-Methoxyphenyl)acetic acid	2-[[2-(4-Methoxyphenyl)acetyl]amino]acetic acid	50
	2-(4-Hydroxyphenyl)acetic acid	2-[[2-(4-Hydroxyphenyl)acetyl]amino]acetic acid	50
Brompheniramine	3-(4-Bromophenyl)-3-pyridin-2-yl-propanoic acid	2-[[3-(4-Bromophenyl)-3-pyridin-2-yl-propanoyl]amino]acetic acid	51
Ibuprofen	NA	2-(2-[4-(2-Methylpropy)phenyl]propanoylamino)ethanesulfonic acid	5
Permethrin	3-(2,2-Dichloroethenyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid	2-[[3-(2,2-Dichloroethenyl)-2,2-dimethyl-cyclopropane carbonyl]amino]acetic acid	52
Triflusal	2-Hydroxy-4-(trifluoromethyl)benzoic acid	2-[[2-Hydroxy-4-(trifluoromethyl)benzoyl]amino]acetic acid	53
m-Xylene	NA	2-[3-Methylbenzoyl)amino]acetic acid	54

Formation of the respective acyl-CoA thioester conjugate has not been demonstrated.
Abbreviation: NA, not applicable.
[a]Benzoylaminoacetic acid is commonly referred to as *hippuric acid*.
Source: Adapted from Knights *et al*. (2007) [43].

addition to the kinetic characterization of ACSM and GLYAT, the two other factors that could result in saturation are the availabilities of the cosubstrates, coenzyme A, and glycine. Indeed, conjugation of benzoic acid and salicylic acid *in vivo* is concentration dependent but the mechanism differs. Formation of hippuric acid from benzoic acid is limited by the availability of glycine [6], while in humans, formation of salicyl-CoA is the rate-limiting step in the amino acid conjugation of salicylic acid [6,29].

Administration of exogenous glycine to rats confirmed that hippuric acid formation was limited by the availability of glycine [59]. Increasing the availability of the hepatic pool of CoA following dietary feeding with fenofibrate or bezafibrate led to an increase (8- to 10-fold) in hepatic CoA concentration and no change in benzoyl-CoA synthetase activity but a decrease in GLYAT activity [60]. Despite an increase in the available pool of CoA, the effect of fibrates was so complex that no conclusive evidence could be provided that the availability of CoA influenced glycine-conjugating capacity.

In humans, glycine conjugation of salicylic acid is capacity limited at therapeutic doses; however, oral administration of glycine does not "unsaturate" salicylic acid conjugation, suggesting that glycine availability is not a limiting factor [31]. Further studies conducted in 1969 in humans identified that formation of hippuric acid following oral administration of benzoic acid was also capacity limited. Concomitant administration of glycine markedly increased the rate of formation of hippuric acid. In contrast, prior administration of salicylic acid (1–3 g) had no measurable effect on hippuric acid formation [61]. The same study confirmed that salicylurate formation was capacity limited and that glycine availability had no measurable effect. However, administration of benzoic acid inhibited salicylurate formation, an effect not ameliorated by coadministration of glycine [61]. These data support the proposition that the availability of glycine is rate limiting for hippuric acid formation but not for salicylurate formation, while acyl-CoA conjugation is the rate-limiting step for salicylic acid but not for benzoic acid.

18.5.4 Inhibitors of Amino Acid Conjugation

As indicated, benzoic acid inhibits the elimination of salicylurate in man [61]. Since the activation step is rate limiting for salicylic acid and both are common substrates for the same ACSM, it is reasonable to assume that benzoic acid will inhibit salicyl-CoA formation *in vivo*. Indeed, benzoic acid does inhibit salicylic acid activation by bovine ACSMs *in vitro* [25]. However, salicylic acid also inhibits benzoic acid conjugation *in vitro* [25], and yet, in man, it does not appear to affect the formation of hippuric acid *in vivo* [62]. Ethanol is another proposed inhibitor of amino acid conjugation. Although ethanol was found to have no effect on the elimination of hippuric acid, it inhibited benzoyl-CoA conjugation [62]. In contrast, ethanol had no effect on either salicyl-CoA formation or the excretion of salicylurate [62].

18.6 LIFE SPAN ASPECTS OF AMINO ACID CONJUGATION

Human newborns have limited capacity to excrete *p*-aminobenzoic acid as a glycine conjugate [63]. Consistent with this *in vivo* finding, human liver and kidney homogenates from newborns have limited capacity to form glycine conjugates of benzoic and *p*-aminobenzoic acid [64,65]. Not surprisingly, wide variability in

hippuric acid excretion is observed in neonates and infants administered benzoic acid for the treatment of hyperammonemia [66]. This may be explained in part by immaturity of GLYAT, which appears to attain peak activity at 18 months of age [67]. However, salicylurate formation is detectable in fetal human liver slices [64]. To date, developmental maturity of the human ACSMs has not been investigated.

The capacity for glycine conjugation appears to decline with advancing age, and *in vivo* formation of hippuric acid from benzoic acid is known to decrease in the elderly [68]. Using human tissue homogenates from individuals aged 23–86 years, it was demonstrated that the capacity of the liver (but not the kidney) to form hippuric acid decreased slightly with age [69].

18.7 CONCLUSIONS

With the exception of salicylic acid, amino acid conjugation is a relatively minor pathway of metabolism of carboxylic acid xenobiotics. The limited number of xenobiotics that have been identified to form amino acid conjugates may reflect poor sensitivity of metabolite assays and also limited recognition of this metabolic route in humans. However, manipulation of this pathway is used clinically in the treatment of nonketotic hyperglycinemia and hyperammonemia in neonates and infants [66,70,71]. Additionally, the serum concentration of *p*-aminohippuric acid has been investigated as a prognostic test in hepatic failure [72,73], and 2-[[3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carbonyl]amino]acetic acid, the glycine conjugate of the pyrethroid insecticide permethrin, has been proposed as a urinary biomarker of human exposure [52]. These studies provide little in the way of mechanistic insight as availability of the cosubstrates, CoASH and glycine, and the substrate selectivity of ACSM and GLYAT can all impact the overall process of amino acid conjugation.

It is evident, however, that there are many more xenobiotics that form acyl-CoA thioesters, which are not substrates for amino acid conjugation, than there are xenobiotics that are substrates for both the ACSM and GLYAT. Limited data from structure–activity studies have alluded to the subtle nature of the active site of human ACSMs, but these studies have provided no information on factors influencing recognition of the xenobiotic acyl-CoA by GLYAT. Further elucidation of the critical structural aspects of substrates that confer amino acid conjugation and knowledge of the active sites of the enzymes involved, namely, ACSM and GLYAT, are essential for understanding the broader potential contribution of amino acid conjugation to xenobiotic disposition.

REFERENCES

1. Ure A. On gouty concretions; with a new method of treatment. Pharm J Trans 1841;1:24.
2. Dessaignes V. Nouvelles recherches sur l'acide hippurique, l'acide benzoique et le sucre de gelatine. Compte Rendu de l'Academie des Sciences (Paris) 1845;21:1224–1227.
3. Webb E, Kim YT. The urinary excretion of amino acid conjugates in free living adult males. Toxicol Ind Health 1991;7:119–124.
4. Gopaul VS, Tang W, Farrell K, *et al*. Amino acid conjugates: metabolites of 2-propylpentanoic acid (valproic acid) in epileptic patients. Drug Metab Dispos 2003;31:114–121.

5. Shirley MA, Guan X, Kaiser DG, *et al*. Taurine conjugation of ibuprofen in humans and in rat liver *in vitro*. Relationship to metabolic chiral inversion. J Pharmacol Exp Ther 1994;269: 1166–1175.
6. Hutt AJ, Caldwell, J. Amino acid conjugation. In: Mulder GJ, editor. Conjugation reactions in drug metabolism. London: Taylor Francis; 1990. pp. 273–305.
7. Vessey DA, Kelley M. Characterization of the reaction mechanism for the XL-1 form of bovine liver xenobiotic/medium-chain fatty acid:CoA ligase. Biochem J 2001;357:283–288.
8. Mashek DG, Bornfeldt KE, Coleman RA, *et al*. Revised nomenclature for the mammalian long-chain acyl-CoA synthetase gene family. J Lipid Res 2004;45:1958–1961.
9. Knights KM, Drogemuller CJ. Xenobiotic-CoA ligases: kinetic and molecular characterization. Curr Drug Metab 2000;1:49–66.
10. Knights KM. Long-chain-fatty-acid CoA ligases: the key to fatty acid activation, formation of xenobiotic acyl-CoA thioesters and lipophilic xenobiotic conjugates. Curr Med Chem Immunol Endocrinol Metab Agents 2003;3:235–244.
11. Mahler HR, Wakil SJ, Bock RM. Studies on fatty acid oxidation 1. Enzymatic activation of fatty acids. J Biol Chem 1953;204:453–468.
12. Killenberg PG, Davidson ED, Webster LT. Evidence for a medium-chain fatty acid: coenzyme A ligase (adenosine monophosphate) that activates salicylate. Mol Pharmacol 1971;7:260–268.
13. Groot PHE, Scholte HR, Hulsmann WC. Fatty acid activation: specificity, localization, and function. Adv Lipid Res 1976;14:75–126.
14. Groot PH. Acyl-CoA synthetases in guinea-pig liver mitochondria: purification and characterization of a distinct propionyl-CoA synthetase. Biochim Biophys Acta 1976;441:260–267.
15. Vessey DA, Hu J. Isolation from bovine liver mitochondria and characterization of three distinct carboxylic acid:CoA ligases with activity toward xenobiotics. J Biochem Toxicol 1995;10:329–337.
16. Vessey DA, Kelley M. Purification and partial sequencing of the XL-I form of xenobiotic-metabolizing medium chain fatty acid:CoA ligase from bovine liver mitochondria, and its homology with the essential hypertension protein. Biochim Biophys Acta 1997;1346:231–236.
17. Vessey DA, Lau E, Kelley M. Isolation and sequencing of cDNAs for the XL-1 and XL-III forms of bovine liver xenobiotic-metabolizing medium-chain fatty acid:CoA ligase. J Biochem Mol Toxicol 2000;14:11–19.
18. Kasuya F, Igarashi K, Fukui M, *et al*. Purification and characterization of a medium chain acyl-coenzyme A synthetase. Drug Metab Dispos 1996;24:879–883.
19. Kasuya F, Igarashi M, Fukui M. Characterization of a renal medium chain acyl-CoA synthetase responsible for glycine conjugation in mouse kidney mitochondria. Chem Biol Interact 1999;118:233–246.
20. Kasuya F, Tatsuki T, Ohta M, *et al*. Purification, characterization, and mass spectrometric sequencing of a medium chain acyl-CoA synthetase from mouse liver mitochondria and comparisons with homologues of rat and bovine. Protein Expr Purif 2006;47:405–414.
21. Fujiwara K, Takeuchi S, Okamura-Ikeda K, *et al*. Purification, characterization, and cDNA cloning of lipoate-activating enzyme from bovine liver. J Biol Chem 2001; 276:28819–28823.
22. Vessey DA, Kelley M, Warren RS. Characterization of the CoA ligases of human liver mitochondria catalyzing the activation of short- and medium-chain fatty acids and xenobiotic carboxylic acids. Biochim Biophys Acta 1999;1428:455–462.
23. Vessey DA, Lau E, Kelley M, *et al*. Isolation, sequencing, and expression of a cDNA for the HXM-A form of xenobiotic/medium-chain fatty acid:CoA ligase from human liver mitochondria. J Biochem Mol Toxicol 2003;17:1–6.

24. Fujino T, Takei YA, Sone H, *et al*. Molecular identification and characterization of two medium-chain acyl-CoA synthetases, MACS1 and the Sa gene product. J Biol Chem 2001;276:35961–35966.
25. Vessey DA, Hu J, Kelley M. Interaction of salicylate and ibuprofen with the carboxylic acid:CoA ligases from bovine liver mitochondria. J Biochem Toxicol 1996;11:73–78.
26. Kasuya F, Igarashi K, Fukui M. Participation of a medium chain acyl-CoA synthetase in glycine conjugation of the benzoic acid derivatives with the electron-donating groups. Biochem Pharmacol 1996;51:805–809.
27. Vessey DA, Kelley M, Lau E. Development of a radiolabeled ATP assay for carboxylic acid:CoA ligases and its use in the characterization of the xenobiotic carboxylic acid:CoA ligases of bovine liver mitochondria. J Biochem Mol Toxicol 1998;12:151–155.
28. Von Lehmann B, Wan SH, Riegelman S, *et al*. Renal contribution to overall metabolism of drugs IV: biotransformation of salicylic acid to salicyluric acid in man. J Pharm Sci 1973;62:1483–1486.
29. Miners JO, Grgurinovich N, Whitehead AG, *et al*. Influence of gender and oral contraceptive steroids on the metabolism of salicylic acid and acetylsalicylic acid. Br J Clin Pharmacol 1986;22:135–142.
30. Levy G. Salicylurate formation—demonstration of Michaelis–Menten kinetics in man. J Pharm Sci 1965;54:496.
31. Levy G. Pharmacokinetics of salicylate elimination in man. J Pharm Sci 1965;54:959–967.
32. Sherratt HAS. Hypoglycin, the famous toxin of the unripe Jamaican ackee fruit. Trends Pharmacol Sci 1986;7:186–191.
33. Ruff LJ, Brass EP. Metabolic effects of pivalate in isolated rat hepatocytes. Toxicol Appl Pharmacol 1991;110:295–302.
34. Kiorpes TC, Hoerr D, Ho W, *et al*. Identification of 2-tetradecylglycidyl coenzyme A as the active form of methyl 2-tetradecylglycidate (methyl palmoxirate) and its characterization as an irreversible, active site-directed inhibitor of carnitine palmitoyltransferase A in isolated rat liver mitochondria. J Biol Chem 1984;259:9750–9755.
35. Cuebas D, Beckmann JD, Frerman FE, *et al*. Mitochondrial metabolism of 3-mercaptopropionic acid: chemical synthesis of 3-mercaptopropionyl coenzyme A and some of its S-acyl derivatives. J Biol Chem 1985;260:7330–7336.
36. Olsen J, Li C, Bjornsdottir I, *et al*. *In vitro* and *in vivo* studies on acyl-coenzyme A-dependent bioactivation of zomepirac in rats. Chem Res Toxicol 2005;18:1729–1736.
37. Panuganti SD, Penn JM, Moore KH. Hepatic enzymatic synthesis and hydrolysis of CoA esters of solvent-derived oxa acids. J Biochem Mol Toxicol 2003;17:76–85.
38. Kasuya F, Igarashi K, Fukui M. Participation of a medium chain acyl-CoA synthetase in glycine conjugation of the benzoic acid derivatives with the electron-donating groups. Biochem Pharmacol 1996;5:805–809.
39. Tang W, Abbott FS. A comparative investigation of 2-propyl-4-pentenoic acid (4-ene VPA) and it's α-fluorinated analogue: phase II metabolism and pharmacokinetics. Drug Metab Dispos 1997;25:219–227.
40. Grillo MP, Chiellini G, Tonelli M, *et al*. Effect of α-fluorination of valproic acid on valproyl-S-acyl-CoA formation *in vivo* in rats. Drug Metab Dispos 2001;29:1210–1215.
41. Soltysiak RM, Matsuura F, Bloomer D, *et al*. D, L-alpha-fluoropalmitic acid inhibits sphingosine base formation and accumulates in membrane lipids of cultured mammalian cells. Biochim Biophys Acta 1984;792:214–226.
42. Kuslikis BI, Vanden Heuvel JP, Peterson RE. Lack of evidence for perfluorodecanoyl or perfluorooctanoyl-coenzyme A formation in male and female rats. J Biochem Toxicol 1992;7:25–29.
43. Knights KM, Sykes MJ, Miners JO. Amino acid conjugation: contribution to the metabolism and toxicity of xenobiotic carboxylic acids. Expert Opin Drug Metab Toxicol 2007;3:159–168.

44. Khurana P, Gokhale RS, Mohanty D. Genome scale prediction of substrate specificity for acyl adenylate superfamily of enzymes based on active site residue profiles. BMC Bioinformatics 2010;11:57.
45. Kasuya F, Hiasa M, Kawai Y, *et al*. Inhibitory effect of quinolone antimicrobial and nonsteroidal anti-inflammatory drugs on a medium chain acyl-CoA synthetase. Biochem Pharmacol 2001;62:363–367.
46. Vessey DA, Kelley M, Warren RS. Characterization of triacsin C inhibition of short-, medium-, and long-chain fatty acid:CoA ligases of human liver. J Biochem Mol Toxicol 2004;18:100–106.
47. Nandi DL, Lucas SV, Webster LT. Benzoyl-coenzyme A:glycine N-acyltransferase and phenylacetyl-coenzyme A:glycine N-acyltransferase from bovine liver mitochondria. J Biol Chem 1979;254:7230–7237.
48. Schachter D, Taggart JV. Benzoyl coenzyme A and hippurate synthesis. J Biol Chem 1953;203:925–934.
49. Webster LT, Siddiqui UA, Lucas SV, *et al*. Identification of separate acyl-CoA:glycine and acyl-CoA:L-glutamine N-acyltransferase activities in mitochondrial fractions from liver of rhesus monkey and man. J Biol Chem 1976;251:3352–3358.
50. Mawal YR, Qureshi IA. Purification to homogeneity of mitochondrial acyl CoA:glycine N-acyltransferase from human liver. Biochem Biophys Res Commun 1994;205:1373–1379.
51. Kelley M, Vessey DA. Interaction of 2,4-dichlorophenoxyacetate (2,4-D) and 2,4,5-trichlorophenoxyacetate (2,4,5-T) with the acyl-CoA:amino acid N-acyltransferase enzymes of bovine liver mitochondria. Biochem Pharmacol 1986;35:289–295.
52. Meuldermans W, Hendrickx J, Lauwers W, *et al*. Excretion and biotransformation of astemizole in rats, guinea-pigs, dogs, and man. Drug Dev Res 1986;8:37–51.
53. Bruce RB, Turnbull LB, Newman JH, *et al*. Metabolism of brompheniramine. J Med Chem 1986;11:1031–1034.
54. Ahn KC, Ma S-J, Tsai H-J, *et al*. An immunoassay for a urinary metabolite as a biomarker of human exposure to the pyrethroid insecticide permethrin. Anal Bioanal Chem 2006;384:713–722.
55. Murdoch D, Plosker GL. Triflusal a review of its use in cerebral infarction and myocardial infarction, and as thromboprophylaxis in atrial fibrillation. Drugs 2006;66:671–692.
56. Campbell L, Wilson HK, Samuel AM, *et al*. Interactions of m-xylene and aspirin metabolism in man. Br J Ind Med 1988;45:127–132.
57. Boelsterli UA. Xenobiotic acyl glucuronides and acyl CoA thioesters as protein-reactive metabolites with the potential to cause idiosyncratic drug reactions. Curr Drug Metab 2002;3:439–450.
58. Sidenius U, Skonberg C, Olsen J, *et al*. *In vitro* reactivity of carboxylic acid-CoA thioesters with glutathione. Chem Res Toxicol 2004;17:75–81.
59. Gregus Z, Fekete T, Varga F, *et al*. Dependence of glycine conjugation on the availability of glycine: the role of the glycine cleavage system. Xenobiotica 1993;23:141–154.
60. Gregus Z, Fekete T, Halaszi E, *et al*. Effects of fibrates on the glycine conjugation of benzoic acid in rats. Drug Metab Dispos 1998;26:1082–1088.
61. Amsel LP, Levy G. Drug biotransformation interactions in man II: A pharmacokinetic study of the simultaneous conjugation of benzoic and salicylic acids with glycine. J Pharm Sci 1969;58:321–326.
62. Levy G. Pharmacokinetics of salicylate in man. Drug Metab Rev 1979;9:3–19.
63. Vest MF, Salzberg R. Conjugation reactions in the newborn infant; the metabolism of p-aminobenzoic acid. Arch Dis Child 1965;40:97–105.
64. Irjala K. Synthesis of p-aminohippuric, hippuric, and salicyluric acids in experimental animals and man. Ann Acad Sci Fenn A (Helsinki) 1972;154:1–40.
65. Pacifici GM, Mogavero S, Giuliani L, *et al*. Conjugation of benzoic acid with glycine in the human fetal and adult liver and kidney. Dev Pharmacol Ther 1991;17:52–62.

66. Batshaw ML, MacArthur RB, Tuchman M. Alternative pathway therapy for urea cycle disorders: twenty years later. J Pediatr 2001;138:S46–S55.
67. Mawal Y, Paradis K, Qureshi IA. Developmental profile of mitochondrial glycine N-acyltransferase in human liver. J Pediatr 1997;130:1003–1007.
68. Stern K, Hinds EG, Askonas BA. Studies in hippuric acid synthesis during psychoses of the involutional and old age group. Am J Psychiatry 1945;102:325–329.
69. Temellini A, Mogaavero S, Giulianotti PC, *et al*. Conjugation of benzoic acid with glycine in human liver and kidney: a study on the interindividual variability. Xenobiotica 1993;23:1427–1433.
70. Hamosh A, Maher J, Bellus G, *et al*. Long-term use of high-dose benzoate and dextromethorphan for the treatment of nonketotic hyperglycinemia. J Pediatr 1998;132: 709–713.
71. Van Hove JLK, Vande Kerckhove K, Hennermann JB, *et al*. Benzoate treatment and the glycine index in nonketotic hyperglycinaemia. J Inherit Metab Dis 2005;28:651–663.
72. Furuya KN, Durie PR, Roberts EA, *et al*. Glycine conjugation of para-aminobenzoic acid (PABA): a quantitative test of liver function. Clin Biochem 1995;28:531–540.
73. Lebel S, Nakamachi Y, Hemming A, *et al*. Glycine conjugation of para-aminobenzoic acid (PABA): a pilot study of a novel prognostic test in acute liver failure in children. J Pediatr Gastroenterol Nutr 2003;36:62–71.