Chapter 6: Summary and Conclusions

6.1 Investigating the importance of GLYAT and glycine conjugation in metabolism

Despite being the first biotransformation reaction to be discovered, glycine conjugation is still a poorly understood metabolic pathway (Badenhorst et al., 2013, Knights and Miners, 2012, Knights et al., 2007). As discussed in Chapter 2, the importance of glycine conjugation in normal metabolism seems to have been neglected for two main reasons. First, compared to the sulfonation, glucuronidation, oxidation, and hydrolysis pathways, relatively few pharmaceutical compounds are metabolised to glycine conjugates (Knights et al., 2007). Second, investigations of glycine conjugation have often led to the identification of other seemingly more rewarding research topics, such as the importance of glycine conjugation in the treatment of inborn errors of metabolism (Barshop et al., 1989, Bartlett and Gompertz, 1974, Batshaw and Brusilow, 1981, Batshaw et al., 1988, Kolvraa and Gregersen, 1986, Schachter and Taggart, 1954, Tanaka and Isselbacher, 1967). Therefore, the main objectives of this study were to investigate the importance of glycine conjugation in normal metabolism and to understand the role of GLYAT in this process. The aims of this study were fulfilled by writing the four scientific papers included in this thesis. As described in Chapter 1.3, the aims of this study were:

I) To develop an understanding of the role of glycine conjugation in metabolism by reviewing the available literature **(Paper I and Paper II, submitted manuscript)**.

II) To elucidate the catalytic mechanism employed by GLYAT and to identify one or more amino acid residues located in the GLYAT active site **(Paper III)**.

III) To investigate the effects of genetic variation in the human GLYAT gene on the catalytic properties of a recombinant human GLYAT **(Paper IV)**.

In the two review articles (Papers I and II) it was demonstrated that the role of glycine conjugation, under normal conditions, is to detoxify benzoate and related aromatic acids that are encountered in the diet. The catalytic residue of bovine GLYAT (Paper III) and a residue that may be located in the binding site of bovine GLYAT (Chapter 5) were identified by means of homology modelling and site-directed mutagenesis. Finally, site-directed mutagenesis was used to demonstrate that known polymorphisms in the human GLYAT gene influence the catalytic properties of a recombinant human GLYAT (Paper IV).

6.2 The purpose of the glycine conjugation pathway is to detoxify benzoate

The most important aim of this study was to develop an understanding of the role of glycine conjugation in metabolism. This was achieved by conducting a literature review that culminated in the writing of two review articles (Papers I and II). Paper I discussed the metabolism and toxicity of acyl-CoAs and the role of GLYAT in the detoxification of these reactive metabolites. The factors that influence interindividual variation in the rate of glycine conjugation were also reviewed, with a focus on the significance of genetic variation in the human GLYAT gene. It was concluded that the purpose of glycine conjugation is to detoxify benzoyl-CoA and other acyl-CoA metabolites that are derived from dietary polyphenols and aromatic acids (Badenhorst et al., 2013).

If the purpose of glycine conjugation was only to detoxify the benzoyl-CoA derived from benzoate, it should be asked why benzoate is metabolised to benzoyl-CoA in the first place. This question is important because an alternative to the detoxification theory of amino acid conjugation, the glycine deportation hypothesis, was recently proposed (Beyoglu and Idle, 2012, Beyoglu et al., 2012). This hypothesis states that glycine conjugation should be viewed as a neuroregulatory process, important for the regulation of CNS glycine levels, rather than as a detoxification mechanism (Table 1 of Paper II). This issue was addressed by writing a review (Paper II) that carefully analysed the glycine deportation hypothesis and reassessed the assumption

that glycine conjugation is a detoxification mechanism. Paper II demonstrated that benzoate and other aromatic acids are toxic to mitochondria, and argued that glycine conjugation decreases this toxicity by forming more hydrophilic conjugates that are more readily excreted into the urine. It was further argued that, because human diets seem to be moderately deficient in glycine, it is unreasonable to view glycine conjugation as a means of removing excess glycine from the body (Badenhorst et al., 2013, Melendez-Hevia et al., 2009, Wu, 2013, Wu et al., 2013). However, humans are nowadays exposed to large amounts of benzoate, which is a commonly used preservative (Lees et al., 2013, Piper et al., 2001, Tfouni and Toledo, 2002). It may therefore be said that human glycine deficiency is not relevant under natural conditions, making it irrelevant to the discussion of the purpose of glycine conjugation. However, the growth of other animals such as birds and pigs is also known to be limited by glycine availability (Wang et al., 2013, Wu, 2010, Wu, 2013). Furthermore, herbivorous animals synthesise very large amounts of hippurate, which in some cases uses up to 20% of their available nitrogen (Au et al., 2013, Marsh et al., 2006, Lowry et al., 1993). Since nitrogen availability is often a limiting factor in the growth and development of animals, it seems unreasonable to think that so much nitrogen is used, simply to remove glycine from the body.

6.3 Identification of Glu^{227} as the catalytic residue of bovine GLYAT

In order to understand the functional significance of certain amino acid substitutions on the catalytic properties of GLYAT, it is important to have some knowledge of the enzyme's structure. To date, however, no crystal structure has been reported for any GLYAT ortholog. Therefore, the structure of bovine GLYAT was predicted by means of homology modelling. Paper III reported the use of the molecular model of bovine GLYAT to identify Glu^{227} as a potential catalytic residue (Badenhorst et al., 2012). To experimentally verify this prediction, a system for the bacterial expression of a recombinant bovine GLYAT was developed. The open reading frame of bovine GLYAT, amplified from bovine liver mRNA by means of reverse transcription and PCR, was cloned into a pColdIII expression vector with a C-terminal hexahistidine tag to facilitate

purification. The recombinant bovine GLYAT was expressed and partially purified by means of nickel-affinity chromatography. The K_M values for glycine and some acyl-CoA substrates were determined and shown to be similar to those of a crude preparation of bovine liver GLYAT. Site-directed mutagenesis was then used to generate an E227Q mutant recombinant bovine GLYAT, lacking the proposed catalytic residue. This enzyme was significantly less active than the wild-type enzyme at pH 7.5, but had activity comparable to the wild-type at pH 9.6. Based on these findings a catalytic mechanism for bovine GLYAT was proposed (Figure 5 of Paper III). In this catalytic mechanism Glu^{227} acts as a general base catalyst to deprotonate glycine, which makes glycine more nucleophilic and therefore capable of reacting with benzoyl-CoA. Although elucidation of the GLYAT catalytic mechanism is valuable in understanding the function of the enzyme, it would be valuable to have more information on the residues that form the binding site of the enzyme.

6.4 Identification of Asn^{131} of bovine GLYAT as a potential active site residue

In Chapter 5 the identification of Asn^{131} of bovine GLYAT as a potential active site residue was discussed. In addition to GLYAT, bovine liver contains a phenylacetyltransferase that conjugates phenylacetyl-CoA to glycine. As discussed in Chapter 5, the bovine liver phenylacetyltransferase is sensitive to inhibition by the sulfhydryl reagent DTNB, while bovine liver GLYAT is not inhibited by DTNB. The observation that phenylacetyl-CoA can partially protect the phenylacetyltransferase from inhibition by DTNB suggested that a cysteine residue may be situated in the active site of the phenylacetyltransferase (Nandi et al., 1979, Kelley and Vessey, 1993, Vessey and Lau, 1998, Kelley and Vessey, 1992). Both bovine GLYAT and bovine phenylacetyltransferase contain five cysteine residues, four of which are aligned between the two proteins. One cysteine residue, Cys^{130} of the phenylacetyltransferase, is not aligned with a cysteine residue in bovine GLYAT (Figure 5.1A), suggesting that Cys^{130} of the phenylacetyltransferase could be the residue responsible for inhibition by DTNB (Vessey and Lau, 1998). Inspection of the molecular model of bovine GLYAT described in Paper III revealed that the corresponding Asn^{131} residue of bovine GLYAT is situated in the cavity that

forms the acyl-CoA binding site (Figure 5.1B). This further suggested that the Asn^{131} residue is located in the active site of bovine GLYAT. To experimentally investigate this possibility, site-directed mutagenesis was used to generate an N131C mutant recombinant bovine GLYAT. An N131Q mutant was also generated to serve as a control. It was demonstrated that while the wild-type and N131Q variants were insensitive to inhibition by DTNB, the N131C variant was significantly inhibited (Figure 5.3).

Another technique that could be used to identify GLYAT active site residues is photoaffinity labelling. The use of p-azidobenzoyl-CoA as an active site-directed photoaffinity labelling reagent for bovine GLYAT has been reported (Lau et al., 1977). Although p-azidobenzoyl-CoA is not an inhibitor of bovine GLYAT, ultraviolet irradiation of a complex of p-azidobenzoyl-CoA and bovine GLYAT results in partial, but irreversible, inhibition of GLYAT activity (Lau et al., 1977). This is because the photosensitive azido group decomposes upon exposure to ultraviolet light, forming a reactive nitrene radical that can covalently attach to nearby amino acid side chains in the enzyme's active site. This covalent modification blocks the GLYAT active site, irreversibly inhibiting enzyme activity. This photoaffinity labelling experiment should be repeated, followed by separation of the labelled and unlabelled forms of bovine GLYAT by means of blue dextran affinity chromatography. The purified labelled bovine GLYAT should then be trypsinised to generate peptide fragments, which can then be analysed by means of mass spectrometry. In so doing, it should be possible to identify the GLYAT active site residues that are covalently labelled by p-azidobenzoyl-CoA. The results of an experiment of this kind can then be used to evaluate the accuracy of the molecular model of bovine GLYAT described in Paper III.

6.5 Genetic variation in the human GLYAT gene may influence the catalytic properties of human GLYAT

Interindividual variation in the rate of glycine conjugation has been an important theme throughout this thesis. It seems as though the availability of CoASH, ATP, and glycine are the most important factors

determining the rate of glycine conjugation (Gregus et al., 1996a, Gregus et al., 1992, Gregus et al., 1993, Knights and Miners, 2012, Knights et al., 2007). However, as discussed in Paper IV, variation in the ability of human liver and kidney homogenates to synthesise hippurate, from large and identical amounts of benzoate, CoASH, ATP, and glycine, has been reported. This suggests that the enzyme activities of ACSM2A and/or GLYAT in these homogenates were not identical, but these activities were not individually determined (Temellini et al., 1993). Genetic variation in the human GLYAT gene is one of the factors that may have contributed to the variation in GLYAT activity between these liver samples (van der Sluis et al., 2013). To investigate this possibility, the open reading frame of human GLYAT was purchased and cloned into a pET32a expression vector. Site-directed mutagenesis was then used to generate variants of the recombinant human GLYAT corresponding to six known SNPs in the human GLYAT gene. These variants were expressed and partially purified by means of nickel-affinity chromatography. The relative activities, using benzoyl-CoA and glycine as substrates, and the K_M (benzoyl-CoA) parameters were then determined for each variant. Paper IV reported three very important results. First, known SNP variations in the human GLYAT gene influenced the enzyme activity and K_M (benzoyl-CoA) parameter of a recombinant human GLYAT. Second, a molecular model of human GLYAT, similar to the bovine GLYAT model described in Paper III, could be used to explain the effects of some of the SNP variations on the enzyme activity or K_M (benzoyl-CoA) parameter of the recombinant human GLYAT. Finally, and most importantly, there seemed to be a correlation between the kinetic parameters of the recombinant human GLYAT variants and the allele frequencies of these variants. For example, the SNPs that negatively influenced the enzyme activity of the recombinant human GLYAT had very low allele frequencies. Since the reliability of these results is limited by the small number of SNPs that were investigated, it would be valuable to compare the allele frequencies of several other human GLYAT SNPs to the influence these variations have on the catalytic properties of a recombinant human GLYAT (van der Sluis et al., 2013).

6.6 In some individuals low GLYAT activity may negatively impact hepatic CoASH metabolism

It is possible that the high allele frequencies, of human GLYAT variants with high enzyme activity, reflects some selective advantage to having high GLYAT activity. However, it is not yet clear whether genetic variation, which seems to influence human GLYAT activity, also influences the *in vivo* rate of glycine conjugation. The consequences of variation in GLYAT activity on the metabolism of CoASH in the liver are also not clearly understood at present. This is because it is not currently known if, or under which conditions, GLYAT activity is the limiting factor in the glycine conjugation pathway (Badenhorst et al., 2013). It does seem reasonable, however, to think that the amount of CoASH available in the liver will be directly related to the level of GLYAT activity if a few simplifying assumptions are made. First, that a dose of benzoate is consumed that is not large enough to deplete hepatic glycine. Second, that the concentration of ATP in the liver remains approximately constant. Third, that the activity of ACSM2 is approximately constant during the course of a single exposure to benzoate. Finally, that the pool of total coenzyme A (CoASH plus acyl-CoAs) is constant. Under these conditions the rate of conversion of benzoyl-CoA to CoASH, and therefore the availability of CoASH, should be directly related to GLYAT activity. This suggests that, in order to prevent CoASH sequestration, the rate of glycine conjugation by GLYAT must exceed the rate of benzoyl-CoA formation. That high GLYAT activity is needed to prevent CoASH sequestration is supported by the observation that the rate of glycine conjugation in liver homogenates is 10 to 300 times higher than the rate of benzoyl-CoA formation (Forman et al., 1971, Huckle et al., 1981).

If the foregoing argument is correct, genetic variations that influence human GLYAT enzyme activity may have significant consequences for the metabolism and availability of CoASH in the liver. Therefore, it is possible that some individuals may have insufficient GLYAT activity, which would negatively influence all metabolic processes dependent on free CoASH, including ATP production. This in turn suggests that at least some individuals may benefit from strategies to increase the rate of hepatic glycine conjugation, such as glycine supplementation. Another strategy to increase the rate of hepatic glycine conjugation could be to

target a recombinant human GLYAT enzyme for delivery to the hepatic mitochondria. A patent application for the therapeutic use of a recombinant GLYAT, which we call "GLYAT augmentation therapy", has been filed and is included in this thesis as Appendix I.

6.7 Conclusion and suggestions for future research

In conclusion, the glycine conjugation pathway seems to be very important for maintaining normal mitochondrial function in the liver and kidneys. This is because aromatic acids such as benzoate are capable of accumulating in the mitochondrial matrix (Beyoglu and Idle, 2012, Gatley and Sherratt, 1977, Gatley and Sherratt, 1976). This accumulation of benzoate in the mitochondrial matrix can result in uncoupling of oxidative phosphorylation, generation of reactive oxygen species, inhibition of mitochondrial enzymes, and possibly inhibition of the organic anion transporters in the inner mitochondrial membrane. In Paper II it was argued that glycine conjugation prevents this accumulation of benzoate in the matrix by forming the more hydrophilic hippurate which can be excreted more readily (Figure 4 of Paper II).

It is becoming clear, for two major reasons, that it is important to investigate the glycine conjugation pathway in more detail. First, as discussed in Papers I and II, the metabolism of glycine, ATP, and CoASH, versatile metabolites that participate in several metabolic pathways, can be influenced by the glycine conjugation pathway. Second, as discussed in Paper II, urinary hippurate excretion could be a valuable diagnostic parameter, especially if interpreted in relation to the levels of other urinary metabolites (Lees et al., 2013). For example, altered hippurate synthesis has been correlated with several disease states such as hepatitis, schizophrenia, and diabetes. At present, however, it is very difficult to accurately interpret the relationship between disease and urinary hippurate excretion, since the glycine conjugation pathway is not understood well enough.

In order to gain a more complete understanding of the glycine conjugation pathway, several studies still need to be performed. Suggestions for future studies were made in the concluding sections of Papers I and II. One of the most important unanswered questions is whether, or under which conditions, GLYAT is the limiting step in the glycine conjugation pathway. Until this situation is understood more clearly, the effects of genetic variation in the human GLYAT gene on the *in vivo* rate of glycine conjugation will remain unclear. In this regard, it is important to keep in mind that the limiting step of the glycine conjugation pathway depends on the substrate used (Section 7 of Paper I and Sections 2 and 7 of Paper II). For example, using salicylate as a probe compound is unlikely to reveal anything on variation in hepatic GLYAT activity, since the formation of salicylyl-CoA is the limiting step in salicylurate synthesis. Furthermore, because phenylpropionate seems to be the major precursor for hippurate synthesis, it could be valuable to compare the rates of hippurate synthesis from benzoate and phenylpropionate. As discussed in Paper II, phenylpropionate differs from benzoate as a precursor for hippurate synthesis by generating 15 molecules of ATP, instead of consuming two, and by requiring two molecules of coenzyme A while benzoate requires only one (Figure 1 of Paper II). These differences could mean that interindividual variation in the rate of glycine conjugation, determined using benzoate as a probe compound, could differ from the variation observed when using the more natural phenylpropionate as a probe compound.