Chapter 1: Introduction, Aims, and Methods

1.1 Introduction

Glycine N-acyltransferase (GLYAT, E.C. 2.3.1.13) is the enzyme responsible for conjugating a range of endogenous and xenobiotic organic acids to glycine (Nandi et al., 1979, Schachter and Taggart, 1954). The unusual range of xenobiotic and endogenous glycine conjugates formed by GLYAT makes the true metabolic function of glycine conjugation unclear. The purpose of this thesis is to clarify the role of GLYAT and glycine conjugation in metabolism, and to investigate the possible consequences of genetic variation in the human GLYAT gene.

Glycine conjugation is commonly assumed to be a mechanism for the detoxification of aromatic acids such as benzoic acid (Badenhorst et al., 2013, Nandi et al., 1979). Glycine conjugation is also important in the management of some inherited disorders of organic acid catabolism, such as isovaleric acidemia, an inborn error of leucine catabolism that occurs relatively frequently in South Africa (Dercksen et al., 2012, Tanaka and Isselbacher, 1967, Tanaka et al., 1966). Isovaleric acidemia is treated by means of glycine supplementation, which stimulates the conversion of the toxic isovalerate that accumulates in this disease to isovalerylglycine, which is less toxic and more readily excreted into urine (Tanaka and Isselbacher, 1967, Sweetman and Williams, 2013). It is well known that different isovaleric acidemia patients do not respond equally well to glycine supplementation therapy, even in South Africa, where all the currently known patients are homozygous for the same disease-causing mutation (Dercksen et al., 2012, Itoh et al., 1996). One possible explanation that has been proposed is that variation in the human GLYAT gene determines how effectively isovalerate can be detoxified to isovalerylglycine (Badenhorst et al., 2013, Dercksen et al., 2012). This, together with the interindividual variation in the glycine conjugation of benzoate observed in humans,

suggests that it is important to develop an understanding of the consequences of genetic variation in the human GLYAT gene.

1.2 Hypotheses investigated in this study

Since the initial report of alkaptonuria by Garrod in 1902, several inborn errors of metabolism have been discovered and studied (Garrod, 1902). Inborn errors of metabolism are caused by genetic mutations or other genomic aberrations which result in a deficiency of one or more crucial metabolic enzymes, and therefore disease (Childs et al., 2001). An organism is a whole, composed of its parts, with each part playing an important role in homeostasis. Homeostasis is the process by which organisms maintain internal stability in order to survive in a complex world. By means of its metabolism, an organism transforms the external world into an environment that is favourable to its survival (Smuts, 1926). In inborn errors of metabolism, the ability of an organism to maintain a favourable internal environment is compromised, resulting in what is called “disease” (Childs, 2001). It is commonly understood that there is an inverse relationship between the importance of a metabolic pathway and the incidence of genetic defects of that pathway (Childs, 2001, Childs et al., 2001). Interestingly, no defect of the glycine conjugation pathway has ever been reported, suggesting that this pathway is fundamentally important to metabolic homeostasis. Despite this, however, the role of glycine conjugation in metabolism remains very poorly understood. Therefore, the following three hypotheses were investigated in this study:

I) Glycine conjugation is a fundamentally important metabolic pathway.

II) The function of the glycine conjugation pathway is to detoxify dietary benzoates.

III) Genetic variation in the human GLYAT gene is partly responsible for interindividual variation in the rate of glycine conjugation.

1.3 Aims of this study

To explore the hypotheses stated in Section 1.2, the aims of this study were:

I) To develop an understanding of the role and importance of glycine conjugation in metabolism by conducting an extensive literature review **(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)**.

1.4 The structure of this thesis

Chapter 2: Literature review

In this chapter the literature on GLYAT and glycine conjugation is reviewed. The literature review consists of a historical introduction to glycine conjugation and two review articles:

- **Paper I:** CPS Badenhorst, R van der Sluis, E Erasmus, and AA van Dijk. Glycine conjugation: Importance in metabolism, the role of glycine N-acyltransferase, and the factors that influence interindividual variation. Expert Opinion on Drug Metabolism and Toxicology 2013; 9: 1139-1153.

- **Paper II:** CPS Badenhorst, E Erasmus, R van der Sluis, C Nortje, and AA van Dijk. A new perspective on the importance of glycine conjugation for the detoxification of benzoic acid (Manuscript submitted to *Drug Metabolism Reviews*).

Chapter 3: The catalytic mechanism of a recombinant bovine GLYAT

This chapter consists of a paper describing the use of a bacterially expressed recombinant bovine GLYAT to investigate the GLYAT catalytic mechanism:

- **Paper III:** CPS Badenhorst, M Jooste, and AA van Dijk. Enzymatic characterisation and elucidation of the catalytic mechanism of a recombinant bovine glycine N-acyltransferase. Drug Metabolism & Disposition 2012; 40(2): 346-352.

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

This chapter consists of a paper describing the consequences of genetic variation in the human GLYAT gene on the enzyme activity of a recombinant human GLYAT:

- **Paper IV:** R van der Sluis, CPS Badenhorst, FH van der Westhuizen, and AA van Dijk. Characterisation of the influence of genetic variations on the enzyme activity of a recombinant human glycine N-acyltransferase. Gene 2013; 515: 447-453.

Chapter 5: Identification of a potential active site residue of bovine GLYAT

This chapter describes the use of molecular modelling and site-directed mutagenesis to identify a residue that might be located in the active site of bovine GLYAT. These results have not been published yet.

Chapter 6: Summary and conclusion

In this chapter the most important conclusions derived from the work in this thesis are discussed. Finally, a novel concept called *"GLYAT augmentation therapy"* is introduced.

Appendices

The recombinant therapeutic GLYAT patent application, annotations for some important references, a patent application for a method to synthesise acyl-CoA thioesters, a list of publications, a list of scientific posters, a list of figures, and a list of tables, are included as appendices at the end of the thesis.

1.5 Materials and methods used in this study

The materials and methods used in this study are described in the *Materials and Methods* sections of the published papers in Chapters 3 and 4. The methods are summarised in Table 1 below.

Table 1.1: Summary of the methods used in this study

Methods	**Chapters where method is described and used**
Multiple sequence alignments	3, 4, 5
Molecular homology modelling	3, 4, 5
Visualisation and comparison of molecular models	3, 4, 5
RNA isolation and cDNA synthesis	3
Polymerase chain reactions	3, 4, 5
Analysis of DNA by agarose gel electrophoresis	3, 4, 5
Restriction digestion, ligation, and transformation	3, 4, 5
Site-directed mutagenesis	3, 4, 5
Sanger sequencing of plasmid DNA	3, 4, 5
Expression of recombinant bovine GLYAT cloned into pColdIII	3, 4
Expression of recombinant human GLYAT cloned into pET32a	4
Nickel-affinity purification of His_6-tagged recombinant proteins	3, 4, 5
Crude fractionation of bovine liver GLYAT	3
Analysis of protein expression and purification by SDS-PAGE	3, 4, 5
Western blots for detection of human GLYAT	4
GLYAT enzyme assays using DTNB	3, 4
GLYAT enzyme assays without DTNB	5
Nonlinear regression analysis of enzyme kinetics data	3, 4