

Chapter 5: Concluding summary and future prospects

5.1 Concluding summary

The background for this study was the importance of glycine N-acyltransferase (EC 2.3.1.13) in detoxification of organic acids that accumulate in the blood and tissues of patients with defects of organic acid metabolism. It is our long term aim to develop a recombinant therapeutic GLYAT enzyme for the treatment of propionic acidemia and perhaps other organic acidemias. This idea was inspired by a unique individual with propionic acidemia. This person is relatively healthy for a sufferer of propionic acidemia. This is thought to be because of exceptional detoxification of propionic acid, which is demonstrated by the observation that the levels of propionylglycine excreted in the urine of this patient is three orders of magnitude higher than normal (Prof LJ Mienie, personal communication).

In order to rationally design novel variants of GLYAT with altered substrate specificities, a good understanding of the relationship between the amino acid sequence of the GLYAT enzyme and its substrate specificity is needed. For this purpose a valuable tool is a recombinant enzyme expression system. Such a system can be used to study variants of the enzyme by using site-directed mutagenesis. At present, we are not aware of a system for the expression of soluble and enzymatically active human GLYAT. This is a potential difficulty for our long term plans, but we will experiment with eukaryotic expression systems in the future. In a recent study in our laboratory, it was found that bovine GLYAT could be expressed in a partially soluble and enzymatically active form (M Snyders, unpublished work). Although the human and bovine GLYAT enzymes are not identical, they have similar properties. We argue that studies of the substrate binding mechanics of the GLYAT enzyme may be undertaken using the bovine GLYAT enzyme as model and that the knowledge so gained could be used to aid design of novel human GLYAT variants. In this

way the research can continue without having to wait for the development of a system for the expression of enzymatically active human GLYAT.

In this study the main aim was to further investigate the bovine GLYAT expression system. The work of Maritza Snyders showed that bovine GLYAT has enzyme activity if expressed at 15 °C from pColdIII, with co-expression of the GroES-GroEL-TF chaperone team. The recombinant bovine GLYAT did not have any fusion tags to facilitate purification. The first objective was to confirm that the expression system works, and to optimise expression conditions. The vast majority of GLYAT expressed using this system was insoluble. Optimisation of IPTG inducer concentration and time of induction did not significantly increase the yields of soluble and enzymatically active recombinant bovine GLYAT. It was also found that chaperone co-expression did not contribute to the yield of enzymatically active recombinant GLYAT.

The second main objective was to purify the expressed recombinant bovine GLYAT. For this purpose a recombinant bovine GLYAT with a C-terminal histidine tag was constructed. The histidine tagged bovine GLYAT had enzyme activity, and was purified using nickel affinity chromatography. Complete purification could not be achieved, and two major proteins were co-purified (Figure 2.12). Whether the two proteins represent different forms of the recombinant GLYAT (different translation initiation or termination sites, or perhaps partial proteolytic degradation) could not be determined. An argument was put forward that the smaller of the two proteins may represent the enzymatically active recombinant GLYAT, but the identity of the other protein is not yet known.

The third main aim of the study was to kinetically characterise the recombinant bovine GLYAT. For this purpose, the recombinant enzyme was expressed and purified. The GLYAT enzyme from bovine liver was extracted and enriched for purposes of comparison. It was found that the K_M values for various substrates corresponded reasonably well between the bovine liver GLYAT enzyme and the recombinant GLYAT enzyme. Furthermore, the kinetic parameters determined corresponded well to those reported in the literature. This suggests that the recombinant bovine GLYAT may be used as a tool to study the relationship between enzyme sequence and substrate specificity. This work is currently being written up in the form of a peer-reviewed scientific paper for publication. It is

also the basis for a patent application which we are currently writing. The patent will cover the use of recombinant GLYAT enzymes for therapeutic purposes.

As a demonstration of the power of the recombinant expression system described, it was used to elucidate the catalytic mechanism of the bovine GLYAT enzyme. A catalytic mechanism for the enzyme was predicted based mainly on the conservation of the catalytic mechanisms in the GNAT superfamily. It was argued that bovine GLYAT must have a general base residue as part of its catalytic mechanism. This residue would remove a proton from glycine, making it a strong nucleophile and enabling direct acyl transfer by means of nucleophilic attack. A putative catalytic glutamate residue was proposed based on bioinformatic analyses. This prediction was experimentally analysed using site-directed mutagenesis. The E226 residue was mutated to a glutamine residue. It was shown that the activity of this E226Q mutant was dependent on pH in the way predicted by the catalytic model proposed (Figure 3.18). At pH 7.0 there was almost two orders of magnitude difference between the enzyme activity of the wild type recombinant enzyme and the E226Q mutant. At pH 8.0, the activity of the E226Q mutant was approximately a third of that of the wild type recombinant GLYAT and the GLYAT isolated from bovine liver. This demonstrated that the function of the E226 residue is probably to remove a proton from glycine in the catalytic mechanism, as predicted. Kinetic analysis of the mutant showed that it has a decreased K_M value for benzoyl-coenzyme A and an increased K_M value for glycine. The K_M value obtained for glycine was about 3X of that for the wild type GLYAT enzyme. Since the glycine concentration used for the standard GLYAT assay is 200 mM, a concentration 30X the K_M value of the E226Q mutant, it was argued that the altered K_M value alone would not account for the decreased enzyme activity of the mutant protein. Together, these experiments provided strong support for the catalytic mechanism drawn in Figure 3.21. To our knowledge this is the first mechanism to be worked out for a GLYAT enzyme. We are currently writing up an article on the catalytic mechanism of bovine GLYAT for publication in a peer reviewed scientific journal.

A final objective of the study was to implement a system for the low cost in-house synthesis of coenzyme A derivatives in our laboratory. Using methods adapted from the literature, a novel approach to the synthesis of benzoyl-coenzyme A was developed (Al Arif & Blecher, 1969, Lapidot et al., 1967, Nazi et al., 2004). Benzoyl-pantethine was synthesised from

pantetheine and benzoic acid via the N-hydroxy succinimide ester of benzoic acid. The method was developed such that all steps are performed in a single test tube, and that no purification steps were necessary. This benzoyl-pantetheine was then used as the precursor for the synthesis of benzoyl-coenzyme A, using three recombinant enzymes prepared from *Escherichia coli*, as catalysts. The product was usually obtained with a yield of 75% or higher, based on the amount of pantetheine used. The methods for the use of the NHS ester of benzoic acid to synthesise benzoyl-pantetheine, and the enzymatic synthesis of coenzyme A analogues were adapted from the literature (Al Arif & Blecher, 1969, Lapidot et al., 1967, Nazi et al., 2004). However, the synthesis of benzoyl-pantetheine from pantetheine and benzoic acid, and the use of benzoyl-pantetheine to synthesise benzoyl-coenzyme A, has not been reported in the literature. We are currently writing up an article on this very convenient and efficient synthesis of benzoyl-coenzyme A for publication in a peer reviewed scientific journal.

5.2 Future prospects

One of our most important future objectives is to obtain a sample of blood from the unique propionic acidemia patient described in Chapter 1. The aim is to determine the coding sequence of the GLYAT gene of this individual. The extremely elevated levels of propionylglycine excreted in the urine of this individual cannot be easily explained by simply increased levels of GLYAT activity, such as increased expression of the enzyme. The metabolite profile more likely points to a novel variant of glycine N-acyltransferase with altered substrate specificity. If this is the case, the sequence for the recombinant therapeutic enzyme we aim to develop may be based on this design from nature. Other researchers in our laboratory will sequence the entire 23 kb GLYAT gene of this individual by means of long PCR and pyrosequencing as part of our effort to understand the unique metabolic profile of the patient.

Another approach to understanding the nature of the active site and substrate specificity of the GLYAT enzyme is photoaffinity labelling. The synthesis of an aryl-azide photoaffinity labelling reagent, p-azidobenzoyl-coenzyme A, and its use to covalently label the active site of the GLYAT enzyme has been reported (Lau et al., 1977). We plan on repeating this

experiment and using tandem mass spectrometry to determine which amino acid residues are labelled in the process. These residues should be in or very near the binding site for the acyl group on the enzyme. Identification of one or more of these residues should point out the mapping of the active site to the enzyme primary structure. More importantly, it will provide a rational starting point for site-directed mutagenesis oriented investigations of the function of specific residues in the enzyme active site. The experiment will also give some new information about the exact molecular weight and post-translational modifications of the bovine GLYAT enzyme, which could clear up some of the uncertainties on the topic (Section 1.4.6).

Finally, we intend to develop an affinity binding column for purification of GLYAT enzymes from various sources, such as liver tissue. One approach would be to use the system described in Chapter 4 to synthesise benzyl-coenzyme A, an analogue of benzoyl-coenzyme A without the carbonyl group of the thioester. This compound should bind tightly to the GLYAT enzyme, but it cannot serve as a substrate in the reaction. If this compound is linked, by means of diaminoethane for example, to a resin such as Sepharose, it may be used to selectively bind GLYAT from protein preparations, while most non-specific proteins can be easily washed off. This system, if developed, should facilitate the simple purification of large amounts of the GLYAT enzyme. This could provide the option of attempting crystallisation of a GLYAT enzyme for structure determination.