

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

## 5.1 Introduction

Biological organisms are very complex structures that are composed of an immense number of chemical compounds. These compounds participate in an even greater number of chemical reactions that make up the metabolism of the organism. At physiological temperatures, however, virtually all of these chemical reactions would usually take place at rates that are too slow to maintain homeostasis. Furthermore, the metabolic reactions compose only a small subset of the possible chemical reactions. Therefore, the chemical reactions of life are catalysed by enzymes, complex protein molecules that significantly increase the rates of biochemical reactions (Buchner, 1897, Sumner, 1926, Sumner, 1933). Enzymes also ensure that the necessary reactions are favoured over alternative side reactions, catalysing the formation of specific isomers, for example. Enzymes are capable of catalysing such highly selective reactions because of the complex three-dimensional structures of these biomolecules (Kendrew et al., 1958, Fischer, 1894). Enzymes are linear chains of amino acids known as polypeptides. The amino acid sequence, or primary structure, of an enzyme determines how parts of the polypeptide chain fold to give rise to its secondary structure. The secondary structure elements, mainly  $\alpha$ -helices,  $\beta$ -sheets, and random coils, are further organised to define the tertiary structure of the enzyme (Pauling and Niemann, 1939). The unique three-dimensional structure of an enzyme is therefore essentially determined by its primary structure or amino acid sequence (Kendrew et al., 1958, Pauling and Niemann, 1939). The three-dimensional structure in turn determines the catalytic properties of the enzyme, such as the reaction mechanism, substrate selectivity, and the rate of catalysis (Fischer, 1894).

In enzyme engineering the catalytic properties of an enzyme are changed by manipulating its amino acid sequence, and therefore its three-dimensional structure (Rastetter, 1983). Enzyme engineering has benefited greatly from the development of recombinant DNA technology, especially the polymerase chain reaction, site-directed mutagenesis, and recombinant protein expression (Mullis and Faloona, 1987, Rastetter, 1983, Saiki et al., 1988, Wetzel, 1980). Using enzyme engineering technology, several novel enzymes with important industrial applications have been developed (Cherry and Fidantsef, 2003, Estell, 1993, Rastetter, 1983). For example, the engineering of proteases and lipases, to achieve greater thermal stability and resistance to bleach, is important in developing laundry detergents with improved cleaning properties (Chauhan et al., 2013, Wells et al., 1987). Enzyme engineering is also becoming important in the field of organic chemistry (Nair et al., 2010, Turner, 2003). For example, lipases with dramatically enhanced enantioselectivity have been engineered. Racemic mixtures of esters, which are easily synthesised, can be treated with these enantioselective esterases to obtain high yields of the desired enantiomer of the ester. This approach is much more efficient than trying to selectively synthesise the desired enantiomer or separating racemic mixtures by standard purification techniques (Bottcher and Bornscheuer, 2006).

As discussed in Appendix I, there are potential applications for GLYAT enzymes engineered for altered substrate selectivity and increased activity. For example, a GLYAT engineered to use isovaleryl-CoA more efficiently could possibly have value for the treatment of isovaleric academia. However, because enzymes, including GLYAT, are made up of large numbers of amino acid residues, it can be very difficult to decide which residues to alter. Therefore, some knowledge of the enzyme's structure is very valuable. This is because it is much more likely that the desired results will be achieved by mutating residues in and around the enzyme's active site than by mutating residues in distant, catalytically unimportant parts of the enzyme (Wells et al., 1987).

### *5.1.1 Identification of an active site residue of bovine GLYAT by means of sequence analysis*

Two bovine liver amino acid N-acyltransferases have been isolated and characterised. These are GLYAT (E.C. 2.3.1.13) and the phenylacetyl-CoA:glycine N-acyltransferase (E.C. 2.3.1.192) (Webster et al., 1976). As discussed in Chapter 2, GLYAT catalyses the formation of hippurate (benzoylglycine) from benzoyl-CoA, and the phenylacetyltransferase catalyses the formation of phenylacetylglycine from phenylacetyl-CoA. It has been demonstrated that incubation at 30 °C for two minutes with 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) inhibits the activity of the bovine liver phenylacetyltransferase. In contrast, bovine liver GLYAT is not inhibited by this reagent under similar conditions (Nandi et al., 1979). The phenylacetyltransferase could be partially protected from inhibition by DTNB by 100 µM of its substrate phenylacetyl-CoA (30% instead of 90% inhibition). These results suggest that a cysteine residue, sensitive to modification by DTNB, is present in the active site of the phenylacetyltransferase, but not in the active site of GLYAT (Nandi et al., 1979). Interestingly, both the phenylacetyltransferase and GLYAT enzymes contain five cysteine residues, suggesting that the sulfhydryl reagent-sensitive cysteine of the phenylacetyltransferase could be identified by comparing the amino acid sequences of GLYAT and the phenylacetyltransferase. It has thus been proposed that the Cys<sup>130</sup> residue of the phenylacetyltransferase could be located in the enzyme's active site (Vessey and Lau, 1998). It seemed reasonable to expect that introduction of a cysteine residue at the corresponding position in bovine GLYAT would result in a DTNB-sensitive mutant of bovine GLYAT. In this chapter an experiment, where the bovine GLYAT molecular model (described in Paper III) was used to identify a residue that is potentially situated in the active site of bovine GLYAT, is described. Although this work is still in progress, it suggests that the bovine GLYAT molecular model could be useful in identifying other residues that make up the GLYAT active site, which in turn is valuable for the future goal of engineering a variant of GLYAT with altered substrate selectivity.

## 5.2 Materials and methods

The materials and methods used in this study are similar to those described in Paper III (Chapter 3) and Paper IV (Chapter 4).

### 5.2.1 Generation of N131Q and N131C recombinant bovine GLYAT mutants

The Phusion Site-Directed Mutagenesis Kit (Finnzymes, Vantaa, Finland) was used to generate N131Q and N131C mutants of recombinant bovine GLYAT, as described in Paper IV. The primers used for site-directed mutagenesis were 5'-CAA GCG ATC AAA ACA GAT TCT CTA CAT GGC-3' and 5'-ACT TTG AAG GAT TTC GTG GCT GC-3' for the N131Q mutant, and 5'-CAA GCG ATC AAA ATG CAT TCT CTA CAT GGC-3' and 5'-ACT TTG AAG GAT TTC GTG GCT GC-3' for the N131C mutant. The primers were purchased from IDT (Coralville, Iowa, USA). The pColdIII-bovineGLYAT plasmid described in Paper III was used as the template for site-directed mutagenesis.

### 5.2.2 Expression, purification, assay, and inhibition of GLYAT variants by DTNB

The wild-type, N131Q, and N131C recombinant bovine GLYAT variants were expressed, purified, and assayed using methods similar to those described in Paper III, with a minor modification of the purification protocol. Instead of using pre-packed Ni-TED 2000 columns, columns of 1 cm by 10 cm were packed with the Ni-TED resin (Macherey-Nagel, Düren, Germany). Equilibration, loading, and washing were done as described in Paper III. The columns were then eluted with 15 ml of buffer EB, and fractions of approximately 500 µl were collected. Fractions were assayed for GLYAT activity and the fractions with the highest GLYAT activity were pooled and analysed by means of SDS-PAGE. Since DTNB is used to inhibit the N131C recombinant bovine

GLYAT mutant, an additional protocol was used to assay the GLYAT variants. The modified assay was the same as that described in Paper III, except that DTNB was not used in the assay. Because DTNB was excluded from the reactions, the change in absorbance at 412 nm ( $\Delta A_{412}$ ) could not be used to determine enzyme activity. Instead, the disappearance of the substrate benzoyl-CoA was monitored at 280 nm (Schachter and Taggart, 1954) in UV-transparent 96-well plates (Sigma, St. Louis, MO, USA). An extinction coefficient of 0.0049 AU/ $\mu$ M at 280 nm, for a 200  $\mu$ l reaction, was used for the calculation of initial velocities. For each assay 0.2  $\mu$ g of protein was used, and the change in absorbance over the first four minutes was used to calculate initial velocities, which were expressed as nmol/min/mg of protein. For enzyme inhibition experiments, each recombinant bovine GLYAT variant was incubated at room temperature for two minutes in the presence of 0.1 mM DTNB, before determining residual activity with the  $\Delta A_{280}$  GLYAT assay.

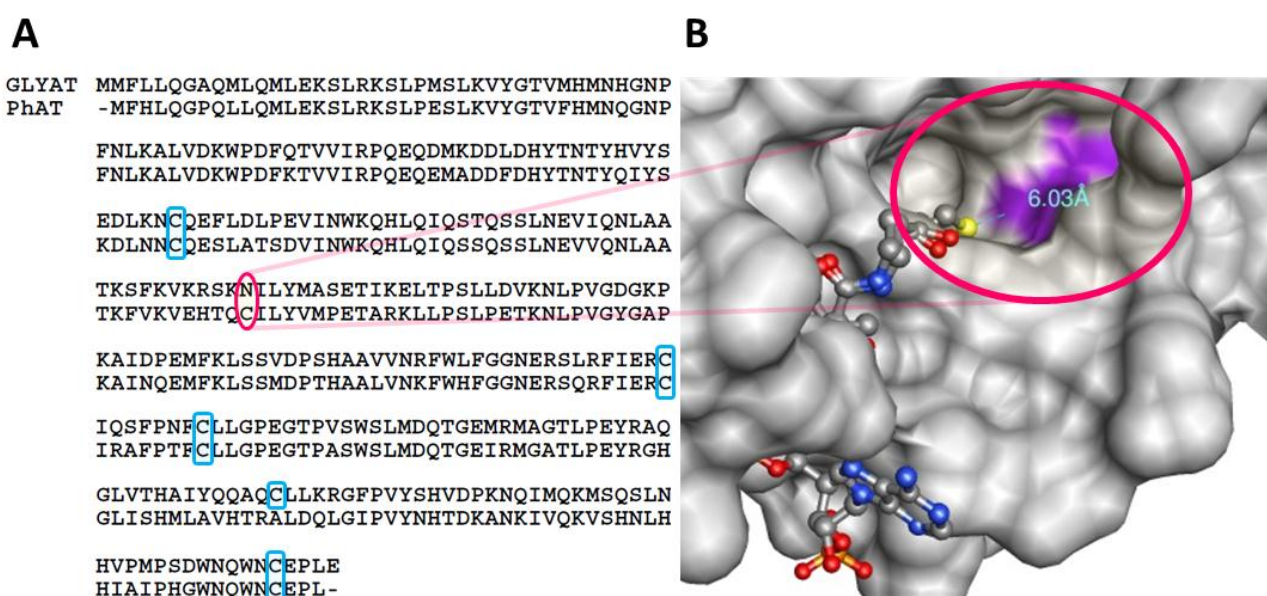
## 5.3 Results and discussion

### 5.3.1 Identification of Asn<sup>131</sup> of bovine GLYAT as a potential active site residue

An alignment of the amino acid sequences of bovine GLYAT (NP\_803479) and phenylacetyltransferase (NP\_803452.1) was generated using CLUSTALX 2.0.10 (Figure 5.1A). The alignment demonstrated that four of the five cysteine residues of each protein aligned. The Cys<sup>130</sup> residue of the phenylacetyltransferase, however, did not correspond to a cysteine residue in the bovine GLYAT sequence and was aligned with the Asn<sup>131</sup> residue of bovine GLYAT.

This suggested that Cys<sup>130</sup> of the phenylacetyltransferase was the DTNB-sensitive cysteine residue. To further investigate this possibility, the bovine GLYAT model described in Paper III was inspected. The Asn<sup>131</sup> residue of the bovine GLYAT model was situated in a cavity on the surface of the enzyme, approximately 6 Å from the thiol group of the CoASH molecule on the model (Figure 5.1B). This observation supported the

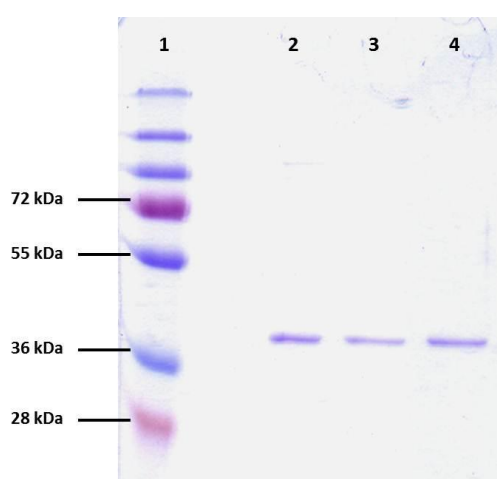
suggestion, based on the alignment of the bovine GLYAT and phenylacetyltransferase amino acid sequences, that Asn<sup>131</sup> is situated in the bovine GLYAT active site. Site-directed mutagenesis was used to generate an N131C recombinant bovine GLYAT, containing a cysteine residue in the same position as the Cys<sup>130</sup> residue of the bovine phenylacetyltransferase. An N131Q recombinant bovine GLYAT was also generated to serve as a control. It was predicted that the N131C recombinant bovine GLYAT would be sensitive to inhibition by DTNB, while the wild-type and N131Q mutant would be insensitive to inhibition by DTNB.



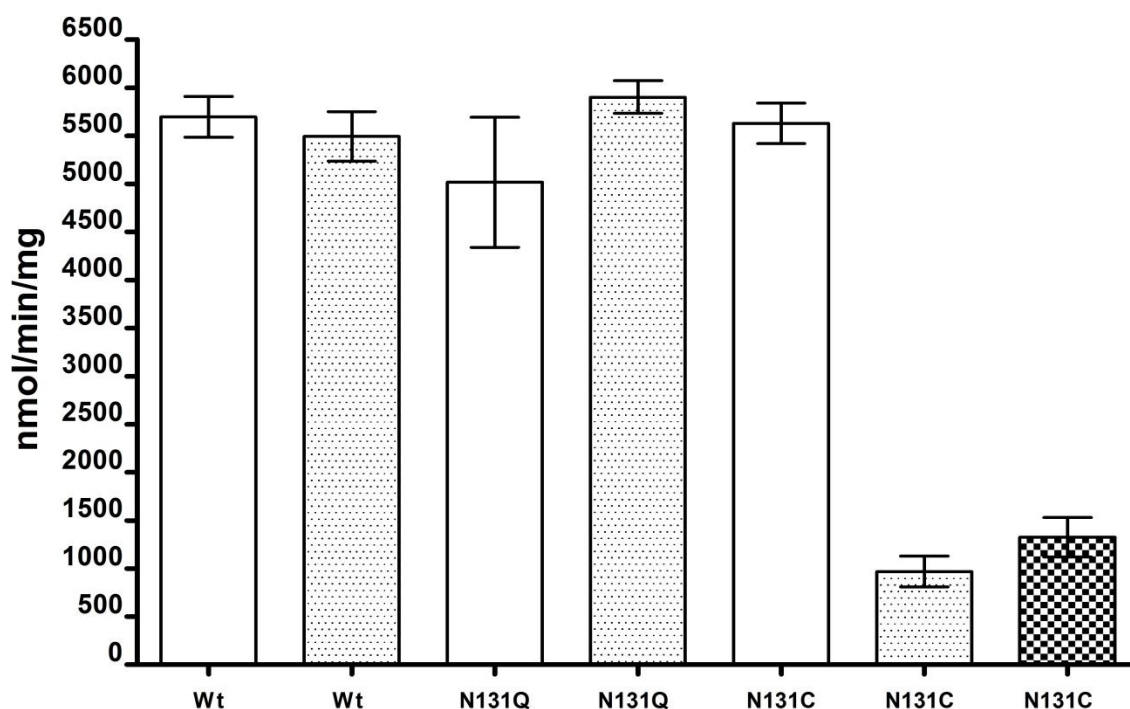
**Figure 5.1 Identification of a potential active site residue of bovine GLYAT.** **A)** A pairwise alignment of the amino acid sequences of bovine GLYAT and bovine phenylacetyltransferase (PhAT). Both sequences contain five cysteine residues. The residue of bovine phenylacetyltransferase thought to be responsible for its sensitivity to inhibition by DTNB, Cys<sup>130</sup>, is indicated by the pink oval. Aligned with this residue is Asn<sup>131</sup> of bovine GLYAT, which is also indicated by the pink oval. The alignment was generated using ClustalX 2.0.10. **B)** A view of the bovine GLYAT molecular model showing a molecule of CoASH situated in a binding cavity on the model. The purple surface represents the Asn<sup>131</sup> residue, which is situated about 6 Å away from the sulfur atom of the CoASH molecule. It appears as though the Asn<sup>131</sup> residue forms part of the benzoyl-CoA binding site of bovine GLYAT. UCSF Chimera was used to generate this image.

### 5.3.2 Expression, purification and inhibition of recombinant bovine GLYAT variants

The wild-type, N131Q, and N131C variants of recombinant bovine GLYAT were expressed and partially purified by means of nickel-affinity chromatography, as described in Paper III (Figure 5.2). The relative activities of the recombinant bovine GLYAT variants were determined using the  $\Delta A_{280}$  assay, in the absence of DTNB. As shown in Figure 5.3, the wild-type, N131Q, and N131C variants had comparable enzyme activity. The recombinant bovine GLYAT variants were also assayed after incubation for two minutes with 0.1 mM of DTNB, at room temperature. As demonstrated in Figure 5.3, the wild-type and N131Q variants of recombinant bovine GLYAT were not inhibited by DTNB. However, DTNB inhibited the activity of the N131C mutant by approximately 80%. This result was consistent with the prediction that the N131C mutant, like the bovine liver phenylacetyltransferase, would be sensitive to inhibition by DTNB. Although Nandi and co-workers reported partial protection of the bovine liver phenylacetyltransferase from DTNB by pre-incubation with 100  $\mu$ M of phenylacetyl-CoA, pre-incubation of the N131C recombinant bovine GLYAT with 100  $\mu$ M of benzoyl-CoA did not significantly protect the enzyme from inhibition by DTNB (Figure 5.3).



**Figure 5.2 SDS-PAGE analysis of the partially purified recombinant bovine GLYAT variants.** Lanes: 1) PageRuler protein size marker; 2) Wild-type recombinant bovine GLYAT; 3) N131Q recombinant bovine GLYAT; 4) N131C recombinant bovine GLYAT. The molecular mass of the recombinant bovine GLYAT is approximately 36.7 kDa.



**Figure 5.3 Relative activities of the recombinant bovine GLYAT variants, and the effects of the sulfhydryl reagent inhibitor DTNB.** The initial velocities of the wild-type, N131Q, and N131C recombinant bovine GLYAT variants are plotted as nmol/min/mg, represented by the white bars. Experiments where the inhibitor DTNB were added are represented by the dotted grey bars. The experiment where 100  $\mu$ M benzoyl-CoA was used to protect the N131C recombinant bovine GLYAT from inhibition by DTNB is represented by the checked bar. Error bars indicate the mean  $\pm$  standard deviation for four replicate assays.

#### 5.4 Conclusions and future work

Comparison of the bovine GLYAT and phenylacetyltransferase amino acid sequences (Figure 5.1A) led to the identification of Asn<sup>131</sup> of bovine GLYAT as a potential active site residue (Vessey and Lau, 1998). Inspection of the molecular model of bovine GLYAT described in Paper III further suggested that Asn<sup>131</sup> was situated in the active site of bovine GLYAT (Figure 5.1B). As expected, the activity of the N131C recombinant bovine GLYAT could be inhibited by incubation with DTNB, which also inhibits the bovine phenylacetyltransferase, which has a cysteine residue (Cys<sup>130</sup>) corresponding to the Asn<sup>131</sup> residue of bovine GLYAT. Although 100  $\mu$ M of benzoyl-CoA did not protect the N131C recombinant bovine GLYAT from inhibition by DTNB, it is possible that this protection could be achieved by further optimization of the experimental conditions. It is



interesting to note that this is not the first experiment to suggest that the Asn<sup>131</sup> residue might be situated in the bovine GLYAT active site. The corresponding residue in human GLYAT is Arg<sup>131</sup>, which is changed to a histidine residue by the R131H SNP in the human GLYAT gene. As discussed in Paper IV, the  $K_M$  (benzoyl-CoA) value for the R131H recombinant human GLYAT was significantly higher than for the wild-type recombinant human GLYAT. It was argued that loss of the positive charge of the arginine residue from the binding site of the R131H mutant resulted in a decreased affinity for the negatively charged benzoyl-CoA substrate.

The identification of Asn<sup>131</sup> as a potential bovine GLYAT active site residue is an important step towards a more complete understanding of the GLYAT active site. What makes this finding important is that it seems to provide further confirmation that the bovine GLYAT molecular model may be used to identify GLYAT residues that are of functional significance. However, it is important to optimise the experimental conditions described in this chapter in order to determine whether benzoyl-CoA, or some other acyl-CoA, can protect the N131C recombinant bovine GLYAT from inhibition by DTNB. This is important, because without this result it cannot be said with certainty that the Asn<sup>131</sup> residue is situated in the bovine GLYAT active site. Chapter 6.4 describes the possibility of using photoaffinity labelling to identify other amino acid residues located in the active site of bovine GLYAT.