AN INVESTIGATION INTO THE ORIGIN OF ACYL-AMINO ACID CONJUGATION IN THE HUMAN AND BOVINE METABOLISM

Francois Hendrikus van der Westhuizen
M.Sc.

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Supervisor: E. Erasmus
Co-supervisor: Prof. P.J. Pretorius
Co-supervisor: Dr. K.M. Gibson

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O LORD, how manifold are thy works!
In wisdom hast thou made them all;
the earth is full of thy creatures.

(Psalm 104:24)

I dedicate this work to my daughter, Helena.

CONTENTS

ABSTRACT

Glycine conjugation and subsequent excretion of carboxylic acids of endogenous and exogenous origin is a normal detoxication process. A wide variety of short- and medium-chain, mono and dicarboxyl, acylglycine conjugates are also excreted in the urine of patients suffering from various metabolic disorders. The formation of these conjugates is catalyzed by glycine *N*-acyltransferase (EC 2.3.1.13). During the last two decades acylamino acid conjugates with amino acid moieties other than glycine have also been detected in the urine of patients with metabolic disorders. These include propionylalanine, propionylglutamic acid, benzoylalanine, isovalerylalanine, isovalerylglutamic acid, and 3-methylcrotonylglutamic acid. This study investigated the conjugation of benzoyl-CoA with the aliphatic and acidic amino acids as well as their amides by glycine *N*-acyltransferase. Bovine and human glycine *N*-acyltransferase was purified from liver mitochondria in a five-step strategy and conjugation was investigated using these preparations in addition to mitochondrial lysates. A new electrospray ionization tandem mass spectrometry-based method was developed to characterize as well as quantify benzoylamino acid conjugation. Bovine glycine *N*-acyltransferase conjugated benzoyl-CoA with glycine (Km_{Gly} = 6.2 mM), asparagine (Km_{Asn} = 129 mM), glutamine (Km_{Gln} = 353 mM), alanine (Km_{Ala} = 1573 mM), and glutamic acid (Km_{Glu} = 1148 mM) in a sequential mechanism. Benzoyl conjugation with serine was also detected. Human glycine *N*-acyltransferase, on the other hand, showed a less diverse amino acid substrate utilization and conjugated benzoyl-CoA with glycine (Km_{Gly} = 6.4 mM), alanine (Km_{Ala} = 998 mM), as well as glutamic acid. The results indicated that, compared to glycine, all other amino acids conjugated with benzoyl-CoA at much lower rates, which explains the relative low levels of the newly detected conjugates in urine. Inhibition of human and bovine glycine *N*-acyltransferase by benzoylamino acid conjugates other than glycine were much lower compared to benzoylglycine and it is unlikely to have an inhibitory effect on glycine *N*-acyltransferase *in vivo*.

A human liver cDNA clone was obtained using bovine N-terminal sequence data which shared a strong homology (82 %) with bovine GNAT cDNA as well as bovine glutamine *N*-phenylacetyltransferase cDNA (77 %). Attempts to express the cDNA in E.*coli* were not successful and the identity of this cDNA could therefore not be confirmed.

OPSOMMING

Glisien konjugering en ekskresie van karboksielsure van endogene en eksogene oorsprong is 'n normale detoksifiserings proses. 'n Wye verskeidenheid kort- en mediumketting, mono- en dikarboksiel, asiel-glisien konjugate word ook gevorm en uitgeskei in die urine van pasiënte wat ly aan verskeie metaboliese defekte. Die vorming van hierdie konjugate word deur die ensiem, glisien *N*-asieltransferase (EC 2.3.1.13), gekataliseer. Gedurende die afgelope twee dekades is asiel-aminosuur konjugate, wat aminosuur-komponente anders as glisien bevat en waarvan die oorsprong onbevestig is, in die urine van pasiënte met metabolies defekte geïdentifiseer. Hierdie konjugate sluit propionielalanien, propionielglutamiensuur, benzoïelalanien, isovalerielalanien, isovalerielglutamiensuur en 3-metielkrotonielglutamiensuur in. Hierdie studie het die konjugering van benzoïel-KoA met die alifatiese aminosure, serien asook die suur aminosure en hul amiede, deur glisien *N*-asieltransferase, ondersoek. Bees en mens glisien *N*-asieltransferase is deur middel van 'n vyf-stap strategie vanuit lewer mitochondria geïsoleer en konjugering is ondersoek deur van hierdie asook mitochondriale lisate gebruik te maak. 'n Nuwe elektrosproei-ionisasie tandem-massaspektrometrie-gebasseerde metode is ontwikkel vir die karakterisering asook kwantifisering van benzoïel-aminosuur konjugate. Bees glisien *N*-asieltransferase het benzoïel-KoA met glisien (Km_{Gly} = 6.2 mM), asparagien (Km_{Asn} = 129), glutamien (Km_{Gln} = 1148), alanien (Km_{Ala} = 1573 mM) en glutamiensuur (Km_{Glu} = 1148 mM) in 'n opeenvolgende reaksiemeganisme gekonjugeer. Benzoïel konjugering met serien is ook waargeneem. Mens glisien *N*-asieltransferase het egter 'n minder omvattende aminosuur substraat-gebruik getoon en het benzoyl-CoA met glisien (Km_{Gly} = 6.4 mM), alanien (Km_{Ala} = 998 mM) asook glutamiensuur gekonjugeer. Die resultate het getoon dat, in vergelyking met glisien, die ander aminosure met benzoïel-KoA teen 'n baie laer tempo gekonjugeer het, wat die relatiewe lae konsentrasie van die nuut-geïdentifiseerde konjugate in urine verklaar. Inhibisie van mens en bees glisien *N*-asieltransferase deur benzoïel-aminosuur konjugate anders as glisien was heelwat laer in vergelyking met benzoïelglisien en dit is onwaarskynlik dat hierdie konjugate 'n inhiberende effek op glisien *N*-asieltransferase *in vivo* sal hê.

'n Mens lewer cDNA kloon is verkry deur bees GNAT N-terminale aminosuurvolgordes te gebruik. Hierdie cDNA toon sterk homologie (82%) met bees GNAT cDNA asook bees glutamien N-fenielasetieltransferase cDNA (77%). Pogings om hierdie cDNA in E.*coli* uit te druk was onsuksesvol en dus kan die identiteit van hierdie cDNA nie bevestig word nie.

CHAPTER 1

INTRODUCTION

Conjugation of compounds foreign or harmful to the human body is one of a wide variety of important detoxication mechanisms. It is also a normal and ongoing process as intermediary metabolism performs this function daily. Hereto the human body possesses a wide variety of different conjugation mechanisms to counter the wide range of compounds that need to be detoxicated. The importance of conjugation for detoxication purposes can also clearly be seen in patients suffering from metabolic disorders where metabolites, such as organic acids, accumulate as a result from an enzymatic defect which are then conjugated with compounds such as glycine and carnitine and eventually excreted in the urine. The detection of conjugates in plasma and urine are also useful in the diagnosis of various metabolic disease.

This study originated from the screening program for metabolic disorders at the Department of Biochemistry and Microbiology at the Potchefstroom University for Christian Higher Education (Reinecke *et al.*, 1983). The program initially focused on the occurrance of metabolic disorders in mentally handicapped people, but the focus has since been directed at metabolic disorders in neonates. A major research interest of this program has been the characterization of previously unidentified metabolites in the plasma and urine of patients suffering from various metabolic disorders. During the last decade, a number of previously uncharacterized metabolites have been identified which have sometimes led to the identification of alternative metabolic processes (Mienie, 1994). Some of the compounds have recently been identified, here and elsewhere, as acyl-amino-acid conjugates of unknown (albeit speculated) origin, which are excreted in low amounts in metabolic disorders such as propionic acidemia (Erasmus *et al.*, 1998; Erasmus and Mienie, 1998) and isovaleric acidemia (Lehnert, 1981, 1983). Their detection bacame possible by the development of much more sensitive analytical techniques, particularly gas- and liquid chromatography-mass spectrometry.

As the title suggests, the formation (origin) of these acylamino acid conjugates has been the primary focus of this study. A concise review of conjugation mechanisms with the focus on acylamino acid conjugation in general, as well as more specific aspects of acylamino acid conjugation and the hypothesis of this study, is presented in the following chapter.

CHAPTER 2

LITERATURE REVIEW AND HYPOTHESIS

2.1 The discovery and nature of conjugation mechanisms.

Conjugates are enzymatically formed products of the metabolism of a wide variety of endogenous or exogenous (xenobiotic) molecules. The discovery of conjugates dates as far back as biochemistry itself. In fact, even before Friedrich Wöhler synthesized urea in 1828, Rouelle in 1784 reported what he believed to be benzoic acid in the urine of cows and camels (Smith and Williams, 1970; Hutt and Caldwell, 1990). It was, however, not until 1829 when Liebig isolated a compound from horse urine, which he called hippuric acid (Gk: acid from horse urine), and 1842 when Keller, using himself as "guinea pig", proved that conjugation of ingested benzoic acid with glycine occurs to form hippuric acid which is then excreted in the urine (Smith and Williams, 1970; Hutt and Caldwell, 1990). A comprehensive discussion of the early history of the discovery of the metabolism of foreign compounds can be found in two works by Williams (1947, 1959).

The terms "conjugated", "conjugate", and "conjugation" most likely originated from the German word "gepaart" and "gepaarte Schwefelsäure" used by Baumann in 1876 to describe the indigo-forming substances in urine, which consist of carbon containing complexes (giving rise to indigo) and sulfuric acid (Smith and Williams, 1970). The majority of conjugation reactions, that is the conjugation of glycine, sulfate glucuronic acid, ornithine, mercapto uric acid, methylation, acetylation, and cyanide detoxication, were discovered during the nineteenth century. For many years during the late nineteenth and early twentieth centuries, after the discovery that organic compounds were excreted in the conjugated form in the urine, the process was viewed as *detoxication*. The reason for this was that the theory that the body uses these conjugation mechanisms to reduce the toxicity of administered compounds [only],

was widely accepted (Fishman, 1970). This view was not shared by A.J. Quick in 1927, who stated that "*the idea is no longer tenable that these conjugations are more or less unimportant mechanisms concerned solely with the detoxication powers of the organism. If these synthetic processes are looked upon as normal and common chemical reactions made manifest because the body is applying them to a foreign substance, it is possible to perceive how the study of conjugation of benzoic acid may help to solve various problems of metabolism which at present seem quite unrelated to hippuric acid and glucuronic acid*" (Fishman, 1970). It is now well known that certain functional metabolites, such as conjugated steroids, are conjugates of endogenous origin and can not merely be regarded as useless excretory products.

Conjugation, which is therefore not exclusively a detoxication mechanism, is essentially a transferase reaction and can be regarded as one of the mechanisms by which detoxication occurs. As Jakoby (1981) puts it: "The enzymes of detoxication catalyze a large variety of reactions in which xenobiotics, i.e. foreign compounds, are oxidized, reduced, hydrolyzed, and conjugated". Therefore, when referring to *detoxication* a wide variety of catalytic mechanisms are included, one of which is conjugation (Jakoby, 1980; Jakoby, 1981).

Conjugation in mammals includes an extremely wide variety of enzymatic reactions. Detoxication is usually attributed to the addition of a molecule which makes the target substrate more soluble for excretion in the urine or, in some cases, bile. Table 2.1 gives an overview of the major conjugating reactions which contributes to metabolic detoxication and where the enzymes, their substrates and metabolic role have been delineated. It is generally accepted that, for detoxication, there are six major mammalian conjugation reactions (Caldwell, 1986; Mulder, 1990), which include peptide bond conjugation, glucuronidation, methylation, acetylation, glutathione conjugation and sulfation. A complete review of each can be found in a number of literature references (Fishman, 1970; Jakoby, 1980; Jakoby, 1981; Paulson *et al*, 1986; and Mulder, 1990). Since this study focuses on mammalian peptide bond conjugation, it will be discussed in more detail.

Table 2.1 Major mammalian conjugation (detoxication) mechanisms and their properties.

Mechanism	Enzyme/s (Official name)	Tissue location and subcellular distribution	Reaction catalyzed	Physiological role
Peptide bond formation	Glycine *N*-acyltransferase (EC 2.3.1.13)	Mitochondrial matrix of liver and kidney.	Acyl-CoA + glycine → CoA + *N*-acylglycine Comment: The CoA derivatives of a number of aliphatic and aromatic acids, but not phenylacetyl-CoA or indole-3-acetyl-CoA, can act as donor.	Detoxication. Some conjugates, e.g. benzoylglycine, may have functional role. Possible role in fatty acid amide biosynthesis.
	Glutamine *N*-phenylacetyltransferase (EC 2.3.1.14)	Mitochondrial matrix of liver and kidney.	phenylacetyl-CoA + L-glutamine → CoA + alpha-*N*-phenylacetyl-L-glutamine	Detoxication.
	Glycine *N*-choloyltransferase (EC 2.3.1.65)	Microsomes of liver.	Choloyl-CoA + glycine → CoA + glycocholate Comment: taurine can act, more slowly, as acceptor. Acts on CoA derivatives of other bile acids.	Detoxication.
Glucuronidation	UDP-glucuronosyltransferases (EC 2.4.1.17) Some of the activities catalysed were previously listed separately as EC 2.4.1.42, EC 2.4.1.59, EC 2.4.1.61, EC 2.4.1.76, EC 2.4.1.77, EC 2.4.1.84, EC 2.4.1.107 and EC 2.4.1.108. A temporary nomenclature for the various forms whose delineation is in a state of flux.	Integrated with endoplasmic reticulum and nuclear envelope of liver, kidney, gastrointestinal tract and skin.	UDP-glucuronate + acceptor → UDP + acceptor beta-D-glucuronoside Comments: Family of enzymes accepting a wide range of substrates,including phenols, alcohols, amines and fatty acids.	Detoxication. Some compounds, e.g. glucuronides of retinoic acid, may have functional role.
	Bilirubin-glucuronoside glucuronosyltransferase (EC 2.4.1.95)	Microsomes of liver.	2-Bilirubin-glucuronoside → bilirubin + bilirubin-bisglucuronoside	Excretion of bilirubin diglucuronide in bile.
N-Methylation	*N*-methyltransferases e.g. Phenylethanolamine *N*-methyltransferase (EC 2.1.1.28) Histamine *N*-methyltransferase (EC 2.1.1.8) Amine *N*-methyltransferase (Indolethylamine *N*-methyltransferase) (EC 2.1.1.49)	Wide distribution and in cytosol.	norepinephrine + AdoMet → epinephrine + AdoHcy histamine + AdoMet → 3-*N*-methyl histamine + AdoHcy RCH_2NH_2 + AdoMet → RCH_2NHCH_3 + AdoHcy AdoMet = S-adenosyl-L-methionine AdoHcy = S-adenosyl-L-homocysteine	Detoxication of xenobiotics. Inactivation of number of normal amine metabolites such as the neurotransmitters.
S-Methylation	Thiol S-methyltransferases (EC 2.1.1.9)	Cytosol and microsomal form in the liver and kidney	AdoMet + thiol → AdoHcy + thioesther Comment: H_2S and a variety of alkyl, aryl and heterocyclic thiols and hydroxy thiols can act as acceptors.	Detoxication of xenobiotic thiols.

(Table 2.1 continues)

O-Methylation	Catechol O-methyltransferase (EC 2.1.1.6) Acetylserotonine O-methyltransferase (EC 2.1.1.4)	Wide distribution in cytosol.	AdoMet + catechol (wide selectivity) → AdoHcy + *meta/para* methylated catechol AdoMet + *N*-acetylserotonin → AdoHcy + *N*-acetyl-5-methoxytryptamine	Inactivation of catechol xenobiotics, circulating catecholamines and catechol neurotransmitters. Control of catechol-mediated functions. Synthesis of melatonin. Regulation and function of specialized proteins (protein carboxy O-methyltransferase).
Glutathione Conjugation	Glutathione S-transferase (EC 2.5.1.18) (Collective name) γ-Glutamyltransferase (EC 2.3.2.2)	Cytosol of a wide variety of tissues. Wide distribution although highest in kidney. Plasma membrane bound.	RX + glutathione → HX + R-S-G Comment: - R may be an aliphatic, aromatic or heterocyclic group. - X may be a sulfate, nitrite or halide group. - Also catalyzes the addition of aliphatic epoxides and arene oxides to glutathione; the reduction of polyol nitrate by glutathione to polyol and nitrite; certain isomerization reactions and disulfide interchange. (5-L-glutamyl)-peptide + amino acid → peptide + 5-L-glutamyl-amino acid	Very versatile enzymes with the conjugation of glutathione with a great variety of hydrophobic compounds for detoxication. Also noted for their role as binding proteins with a storage function for toxic compounds. Transpeptidase activity towards various S-derivatives of GSH indicates detoxication role.
Acetylation	Arylamine *N*-acetyltransferase (EC 2.3.1.5)	Cytosol of a wide variety of tissues.	acetyl-CoA + arylamine → CoA + *N*-acetylarylamine Comment: - Wide specificity for aromatic amines, including serotonin. - Also catalyzes acetyl-transfer between arylamines without CoA.	Acetylation is the major route of arylamine and hydrazine metabolism. Modulatory role in metabolic activation of mutagenic and carcinogenic aromatic amines and various food pyrolysates classified as carcinogens.
Sulfation	Aryl sulfotransferase (EC 2.8.2.1) Alhoholsulfotransferase (EC 2.8.2.2) Arylaminesulfotransferase (EC 2.8.2.3) Estrone sulfotransferase (EC 2.8.2.4) Tyrosine-ester sulfotransferase (EC 2.8.2.9) Bile-salt sulfotransferase (EC 2.8.2.14) Thiosulfate sulfurtransferase (EC 2.8.1.1)	Cytosol of a wide variety of tissues. Predominately in the matrix of mitochondria the liver. Wide distribution.	R-OH + PAPS → R-OSO_3H + PAP R-OSO_3H + R^1-OH → R-OH + R^1-OSO_3H PAP = Adenosine 3′,5′-biphosphate thiiosulphate + cyanide → sulphite + thiocyanate	Biotransformation of xenobiotic and endogenous compounds such as neurotransmitters and steroid hormones. May play regulatory role of thyroid and steroid hormone biosynthesis. Protein sulfation indicates possible change in function. Detoxication of cyanide and inorganic sulfide.

Compiled from Fishman (1970); Jakoby (1980); Jakoby (1981); Paulson *et al.* (1986); Mulder (1990); Merkler *et al.* (1996), and ExPASy Molecular Biological Server (1998).

2.2 Peptide bond conjugation and detoxication of carboxylic acids.

A wide variety of chemical compounds contain carboxylic acid moieties in their structures. These carboxylic acids can be metabolized in various ways such as glucuronic acid conjugation, amino acid conjugation, β-oxidation and conjugation with carnitine (Kanazu and Yamaguchi, 1997). Under normal metabolic conditions the role of carnitine as a buffer for excess organic acids probably is minor. However, under abnormal conditions, such as diabetes, anoxia, or several organic acidurias, carnitine is of major importance in maintaining mitochondrial function and viability (Rebouche, 1986).

Glycine conjugation is a very common route for xenobiotic carboxylic acids, such as salicylate (from aspirin) to form salicyluric acid (Levy, 1965) and with benzoate to form benzoylglycine (hippuric acid). It is also the case with endogenously formed carboxylic acids such as propionic acid and isovaleric acid which accumulates in propionic- and isovaleric acidemias respectively, in which case propionylglycine and isovalerylglycine are formed (Bartlett and Gompertz, 1974). The type and amount of conjugation varies according to the type of acid and the animal species. Larger and complex cyclic acids tend to be excreted as glycosides whereas simpler acids, such as benzoic, aliphatic (short and medium-chain) as well as arylacetic acids, are more commonly excreted in the form of amino acid (glycine) conjugates. Bile acids, which are large heterocyclic endogenously synthesized acids, are also conjugated with mainly taurine in a peptide bond forming reaction (Killenberg and Webster, 1980).

The amino acid involved in a conjugation reaction depends on the species as well as the bioavailability from endogenous and dietary sources. Glycine conjugation is the most common amino acid conjugation in mammals and the comparative rate of conjugation occurs in the order : herbivores > omnivores > carnivores (Killenberg and Webster, 1980).

In mammals conjugation of carboxylic acids with amino acids takes place in the matrix of the mitochondria of liver and kidney (Gatley and Sherratt, 1977; Vessey and

Hu, 1995). This effectively results in the joining of the carboxylic group of the acid to the amino nitrogen of the amino acid (eq. 1.3). This reaction is preceded by an ATP-dependent activation of the carboxylic acid with Coenzyme A (CoA) by carboxylic acid:CoA ligase to form an intermediate acyl-CoA product (eq. 1.1 and 1.2) (Schachter and Taggart, 1953; Gatley and Sherratt, 1977; Vessey and Hu, 1995).

$$RCOOH + ATP \rightarrow RCO\text{-}AMP + PPi + H_2O \quad (1.1)$$

$$RCO\text{-}AMP + HSCoA \rightarrow RCO\text{-}SCoA + AMP \quad (1.2)$$

$$RCO\text{-}SCoA + NH_2CH_2COOH \rightarrow RCONHCH_2COOH + CoASH \quad (1.3)$$

Carboxylic acid:CoA ligases which are specific for long-chain fatty acids have been characterized in microsomes, peroxisomes as well as mitochondria, whereas a carboxylic acid:CoA ligase (EC 6.2.1.3), with an affinity towards very long chain fatty acids, have been characterized in rat peroxisomes and microsomes (Singh and Poulos, 1988; and Vessey and Hu, 1995). Mitochondria also may contain several short- (C2, C3, and C4) and medium-chain fatty acid ligase (EC 6.2.1.2) and it is the latter enzyme that has been the catalyst for the activation of xenobiotic carboxylic acids (Mahler *et al*, 1953; Groot *et al*., 1976). Two forms of this xenobiotic/medium-chain acid ligases have initially been found in mitochondria. The first is the so-called Mahler form (Mahler *et al.*, 1953), which activates C4 - C12 fatty acids as well as benzoate, substituted benzoates, cinnamate, 2,4-D (dichlorophenoxyacetate), nicotate, phenylacetate, phenylpropionate and branched-chain fatty acids (Mahler *et al*, 1953, Vessey and Hu, 1995). The second is the so-called salicylate form (Groot *et al.*, 1976), which has an affinity towards salicylate, benzoate, *p*-aminosalicylate, *o*-methoxybenzoate and hexanoate (Vessey and Hu, 1995). Recently it was noted that these two forms of mitochondrial medium-chain fatty acid ligases are likely to be mixtures of three distinct forms (in bovine at least): a XL-I form which preferentially activates nicotinic acid and benzoate, a XL-II form with affinity towards salicylate, and a XL-III form which predominately activates medium-chain fatty acids and 2,4,6,8-decatetranoic acid (Vessey and Hu, 1995).

Activation of carboxylic acids takes place at rates at least 100-fold slower than the subsequent conjugation of the thioester with an amino acid, which indicates that this activating step is likely to be the rate-limiting step *in vivo* (Vessey and Hu, 1995). The transfer of the acyl group from a Coenzyme A thioester to an amino acid is subsequently catalyzed by an acyl-CoA:amino acid *N*-acyltransferase. As discussed in this chapter, the aim of this study was to determine whether the new conjugates are formed by glycine *N*-acyltransferase, and therefore the properties of acyl-CoA:amino acid N-acyltransferases will be reviewed in more detail.

2.3 Mammalian acyl-CoA:amino acid N-acyltransferases.

2.3.1 Introduction.

During the early 1950's, Schachter and Taggart (1953) described an enzyme partially purified from bovine liver mitochondria with an absolute specificity towards glycine with the ability to catalyze conjugation with both aliphatic and aromatic acyl groups. Some years later, Moldave and Meister (1957) also found that hippurate-forming fractions from bovine liver mitochondria synthesized phenylacetylglycine, but not phenylacetylglutamine. The latter authors also observed that partially purified fractions from human liver and kidney catalyze the formation of phenylacetylglutamine, phenylacetylglycine, and hippurate but not indoleacetylglutamine. These two reports raised a number of questions regarding the conjugation profile that was observed at that time (Webster *et al.*, 1976). For example, the absence of phenylacetylglycine in human urine after ingestion of phenylacetic acid was incompatible with the phenylacetyl-CoA:glycine N-acyltransferase activity previously reported by Moldave and Meister (1957). Webster *et al.* (1976) subsequently identified separate acyl-CoA:amino acid *N*-acyltransferases in the liver mitochondria of rhesus monkeys and humans. The first, which was named *acyl-CoA:glycine N-acyltransferase,* utilizes benzoyl-CoA or butyryl-CoA as acyl donors and glycine as acyl acceptor, and the second, *acyl-CoA:glutamine N-acyltransferase,* utilizes either phenylacetyl-CoA or indoleacetyl-CoA as acyl donors

and glutamine as acyl acceptor. Nandi *et al.* (1979), some time later, made similar observations using bovine liver mitochondria. The two enzymes were then referred to as *benzoyl-CoA:glycine N-acyltransferase*, with an affinity towards benzoyl-CoA, salicyl-CoA and certain short and medium chain (straight and branched) acyl-CoA substrates and glycine as acyl acceptor, and *phenylacetyl-CoA:glycine N-acyltransferase,* which also shares glycine as preferred acyl (phenylacetyl-CoA or indoleacetyl-CoA) acceptor. Both bovine enzyme forms showed some low activity towards glutamine[1] and asparagine (Nandi *et al.*, 1979).

This indicates differences in glutamine and glycine conjugation between primates and non-primates. Further studies by Kelley and Vessey during the last decade confirmed these reports. These authors, who incidentally also have a different view regarding nomenclature of the acyl-CoA:amino acid *N*-acyltransferases, concluded that primate glutamine *N*-phenylacetyltransferase (which utilizes glutamine) is not more efficient at glutamination than the bovine form (utilizing both glutamine and glycine), but rather that the primate enzyme are defective in that it has "lost" the glycine conjugation ability, and not gained glutamine conjugation ability (Kelley and Vessey, 1994a).

Differences in substrate affinities between the two acyl-CoA:amino acid *N*-acyltransferases are accompanied by structural differences which indicates that these enzymes are similar with regard to function, but are clearly distinct proteins. Except for reports of difference in molecular weight between the liver purified primate (30.5 kDa and 32.5 kDa) (Kelley and Vessey, 1994a) and the bovine (33.7 kDa and 33.5 kDa) (Kelley and Vessey, 1992) glycine *N*-acyltransferase and glutamine *N*-acyltransferase respectively, N-terminal analysis on bovine glutamine *N*-phenylacetyltransferase indicated a blocked N-terminus, whereas it was not the case with bovine glycine *N*-acyltransferase (Kelley and Vessey, 1992). The bovine cDNA's of the two enzymes have recently been characterized. According to these data both enzymes consists of 295 amino acids with some small differences in the sequence to give molecular weights of 31690 for glycine *N*-acyltransferase (Vessey

[1] Amino acids referred to in this study are all in the L-isomeric configuration.

and Lau, 1996) and 31909 for glutamine *N*-acyltransferase (EMBL accession number: embAJ001396), respectively.

Although both acyl-CoA:amino acid *N*-acyltransferases are found in the mitochondrial matrix of both liver and kidney (Gatley and Sherratt, 1977; James and Bend, 1978), there are differences in the expression of the two enzymes in liver and kidney (Kelley and Vessey, 1993). The relative activities of acyl-CoA:amino acid *N*-acyltransferases in kidney and liver varies within species and with different substrates. For example, benzoic acid conjugation in rat and guinea pig is greater in liver than kidney, whereas the situation is reversed in the cat and dog (Hutt and Caldwell, 1990). Furthermore, phenylacetic acid conjugation (compared to other acyl donors) in the kidney was found to be much higher than in the liver (Hutt and Caldwell, 1990).

2.3.2 Non-conformity in the nomenclature of acyl-CoA:amino acid *N*-acyltransferases.

The lack of conformity that exists with regard to the nomenclature of these enzymes in the literature is a troublesome aspect that, in some way, should be addressed by the researchers involved in studying these enzymes. As indicated in the previous section, most authors have named the two enzymes differently. This aspect is of particular interest and might have originated from the observation that these enzymes have not been studied in such detail as related enzymes such as glycine *N*-choloyltransferase. In the most recent papers on the acyl-CoA:amino acid *N*-acyltransferases, Kelley and Vessey (1986, 1990, 1992, 1993, 1994a, and 1994b), have named the enzymes in a number of related but different ways. This non-conformity in nomenclature is very much apparent from the information compiled in Table 2.2. As indicated in this table, the official names given to the acyl-CoA:amino acid *N*-acyltransferases according to the recommendations of the NC-IUBMB *are glycine N-acyltransferase* and *glutamine N-acyltransferase,* respectively (IUBMB, 1993). The respective systematic names are *acyl-CoA:glycine N-acyltransferase* and *phenylacetyl-CoA:L-glutamine α-N-phenylacetyltransferase.* In view of the currently documented substrate profile for human acyl-CoA:amino acid *N*-acyltransferases (Webster *et al.*, 1976; Kelley and Vessey, 1994a), this nomenclature fits well.

Table 2.2 Nomenclature used for acyl-CoA:amino acid *N*-acyltransferases since the 1970's.

Reference	Glycine *N*-acyltransferase	Glutamine *N*-acyltransferase	Species
Bartlett and Gompertz (1974) Grøn *et al.* (1978)	Glycine *N*-acylase	-	bovine
Webster *et al.* (1976)	Acyl-CoA:glycine *N*-acyltransferase	Acyl-CoA:L-glutamine *N*-acyltransferase	monkey human
Gatley and Sherratt (1976) Kølvraa and Gregersen (1986) Gregersen *et al.* (1986) Mawal and Quereshi (1994:1,2) Merkler *et al.* (1996)	Acyl-CoA:glycine *N*-acyltransferase	-	rat human mammals
Nandi *et al.* (1979)	Benzoyl CoA:glycine *N*-acyltransferase	Phenylacetyl CoA:glycine *N*-acyltransferase	bovine
Kelley and Vessey (1986)	Benzoyltransferase	Phenylacetyltransferase	bovine
Kelley and Vessey (1990) Kelley and Vessey (1992)	Aralkyl-CoA:*N*-acyltransferase	Arylacetyl-CoA:*N*-acyltransferase	bovine
Asaoka (1991)	Benzoyl-CoA:amino acid *N*-acyltransferase	Phenylacetyl-CoA:amino acid *N*-acyltransferase	monkey
Kelley and Vessey (1993)	Aralkyltransferase	Arylacetyltransferase	bovine
Kelley and Vessey (1994a)	Aralkyl *N*-acyltransferase	Arylacetyl *N*-acyltransferase	human
Kelley and Vessey (1996)	Aralkyl acyl-CoA:*N*-acyltransferase	-	bovine

However, as indicated in the previous section, it has been shown that both enzyme forms in bovine can utilize glutamine as well as asparagine (Nandi *et al.* 1979; Kelley and Vessey, 1990). In fact, Nandi *et al.* (1979) have shown that bovine glutamine *N*-acyltransferase utilizes glycine much better than glutamine or asparagine. Thus, especially with regard to the use of the name *glutamine N-acyltransferase* in the bovine system there has to be some doubt of its validity.

The non-conformity with regard to nomenclature may be attributed to the difference in substrate utilization of the various mammalian forms of acyl-CoA:amino acid *N*-acyltransferases. As the reader will note during the course of this work, the amino acid utilization of bovine as well as human glycine *N*-acyltransferase are much

broader than was previously accepted, which may cast some more doubt on the validity of the official nomenclature of this particular enzyme. As this study addresses some key issues of glycine *N*-acyltransferase substrate use, the nomenclature issue are again discussed in Chapter 5 (Section 5.6). For the purpose of this study, the official NC-IUBMB nomenclature of the acyl-CoA:amino acid *N*-acyltransferases will be used. Although this institution do not recommend the use of abbreviations for enzymes, the abbreviation, "GNAT", for glycine *N*-acyltransferase will be used in this thesis to allow for the fluency of text.

2.3.3 Mammalian glycine *N*-acyltransferase (GNAT) and conjugation of acyl-amino acid conjugates.

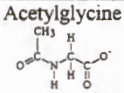
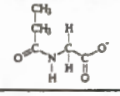
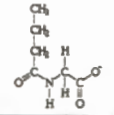
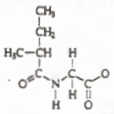
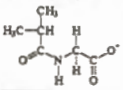
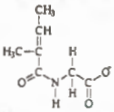
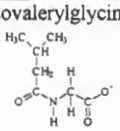
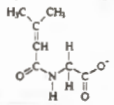
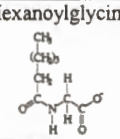
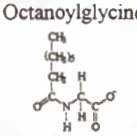
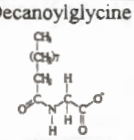
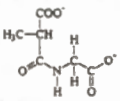
This study has focused on the amino acid substrate profile of GNAT and the formation of previously undetected amino acid conjugates. This enzyme catalyzes glycine conjugation via a sequential mechanism with the acyl-CoA binding first, followed by glycine to form a ternary complex with the subsequent release of the peptide conjugate (Nandi *et al.,* 1976). As indicated previously, GNAT is specific for glycine as amino acid substrate with some minor affinity for glutamine and asparagine in the case of bovine GNAT. The primary role of GNAT has been regarded as one of detoxication of a variety of acyl-CoA esters (Mulder, 1990). Recently, however, it was reported that acylglycines may also play a role in the biosynthesis of fatty acid amides, where it was postulated that they could serve as substrates for peptidylglycine α-amidating enzyme (Merkler *et al.*, 1996).

As far as could be determined, it appears that there has not yet been a major effort to investigate the structural aspects and catalytic mechanism of GNAT - aspects that would be very interesting in light of the wide substrate utilization of GNAT. Sequence data of bovine GNAT have been published, as mentioned in Section 2.3.1, but this is not the case for human GNAT. A number of reports on the molecular mass for liver purified human GNAT, however, exist which ranges between 24 kDa (Webster *et al.* 1976), 30 kDa (Mawal and Quereshi, 1994) and 30.5 kDa (Kelley and Vessey, 1994a).

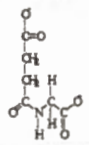
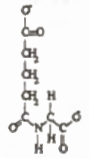
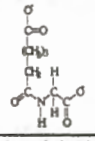
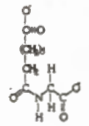
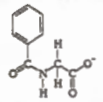
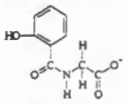
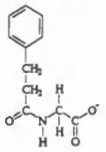
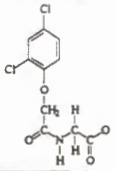
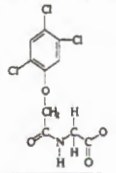
An notable feature of GNAT is that it can accommodate a wide variety of short- and medium-chain, mono- and dicarboxyl, acyl-CoA substrates. Benzoyl-CoA has been the acyl-CoA substrate most commonly used to describe the activity of this enzyme and has even been used in the nomenclature of the enzyme (see Table 2.2). However, compared to benzoyl-CoA at 100 %, and at optimal conditions for the respective acyl-CoA esters, bovine GNAT has showed activity towards butyryl-CoA (223 %), tiglyl-CoA (115 %), isovaleryl-CoA (103 %), propionyl-CoA (53 %), salicyl-CoA (35 %), acetyl- and 3-methylcrotonyl-CoA (32 %), isobutyryl-CoA (25 %) and methylmalonyl-CoA (3 %) but no activity towards malonyl-, phenylacetyl- and indoleacetyl-CoA (Nandi *et al.* (1979). No similar data is available for human GNAT. Table 2.3 reviews the documented acylglycines that are formed and the Km-values of the corresponding acyl-CoA substrates in human and bovine GNAT. Although the values have been determined by a number of investigators, which does not allow for a good comparison of the values, it can be seen that in the case of bovine GNAT benzoyl-CoA have the lowest Km-value and along with salicyl-CoA also the lowest for human GNAT. No correlation is apparent from a comparison of the Km-values and carbon length of the acids or with methyl substitution in the carbon chain. This is an interesting aspect of the enzymes catalytic function and a clarification of the structure and function of the active site of GNAT is eagerly awaited to understand its mechanism of action with different substrates.

It is not clear to what extent the intramitochondrial acyl-CoA concentrations relates to the reported Km-values since very little information is available on the *in vivo* concentrations of acyl-CoA esters. Intramitochondrial benzoyl-CoA concentration, which is reported to be in the region of 20 μM under normal physiological conditions (Kelley and Vessey, 1990), is also affected by benzoyl-CoA hydrolase. This enzyme has a relative high Km. This high value is thought to protect against free CoA depletion when insufficient amino acid acceptor is available for conjugation (Gatley and Sherratt, 1977). Conjugation of benzoate is, however, more dependent on the availability of glycine, where normal plasma concentrations are in the range of 100 - 400 μM (Shih, 1996), rather than the acyl-CoA (Hutt and Caldwell, 1990).

Table 2.3 Acylglycine formation and acyl-CoA utilization of human and bovine GNAT.

Acylglycine	Bovine GNAT $Km_{acyl\text{-}CoA}$* (μM)	Ref.**	Human GNAT $Km_{acyl\text{-}CoA}$* (μM)	Ref.**
Acetylglycine 	210	*2*	not known	
Propionylglycine 	180	*2*	nd	*10*
Butyrylglycine 	130	*1*	~ 500 2400	*5* *8*
2-Methylbutyrylglycine 	not known		nd	*14*
Isobutyrylglycine 	nd	*1*	5580	*8*
Tiglylglycine 	110	*2*	nd	*11*
Isovalerylglycine 	180	*2*	124 672	*6* *8*
3-Methylcrotonylglycine 	14	*2*	nd	*12*
Hexanoylglycine 	not known		2680	*8*
Octanoylglycine 	not known		198 322	*6* *8*
Decanoylglycine 	not known		2408	*8*
Methylmalonylglycine 	360	*1*	not known	

(Table 2.3 continues)

Succinylglycine 	nd	*9*	not known	
Glutarylglycine 	nd	*9*	not known	
Adipylglycine 	nd	*9*	not known	
Suberylglycine 	nd	*9*	nd	*13*
Benzoylglycine 	20 9 110 60	*1* *2* *3* *4*	6 57.9 67	*5* *6* *7*
Salicylglycine 	74	*3*	3 83.7	*5* *6*
Phenylpropionylglycine 	not known		nd	*15*
2,4-dichlorophenoxyacetylglycine 	95	*4*	not known	
2,4,5-trichlorophenoxyacetylglycine 	60	*4*	not known	

nd = not determined, but detected.; * With glycine as acyl acceptor.

** 1. Nandi *et al.* (1979); 2. Bartlett and Gompertz (1974); 3. Kelley and Vessey (1993); 4. Kelley and Vessey (1986); 5. Webster *et al.* (1976); 6. Mawal and Quereshi (1994); 7. Kelley and Vessey (1994a); 8. Gregersen *et al.* (1986); 9. Grøn *et al.* (1978); 10. Rasmussen *et al.* (1972); 11. Gompertz and Draffman (1972); 12. Gitzelmann *et al.* (1987); 13. Gregersen *et al.* (1976); 14. Sweetman and Williams (1995); 15. Bennet *et al.* (1990).

Supporting data for the limited conjugation of benzoate, being due to the restricted availability of glycine rather than benzoyl-CoA, arises from the higher Km-values for the amino acid substrate (in the millimolar range) than for the acyl-CoA substrates (in the micromolar range).

Benzoate, which is the main acyl-CoA substrate for GNAT, is ingested as a natural constituent of plants, with high amounts present in various fruits and berries, as well as in the form of sodium benzoate, a food preservative in many domestic products (Tremblay and Quereshi, 1993). Human metabolism has a substantial capacity to convert benzoate to benzoylglycine. For example, up to 10 g administered to adult males can quantitatively be recovered in the urine in as little as six hours (Tremblay and Quereshi, 1993). Although the clinical use of benzoate as a treatment for hyperammonemias (Tremblay and Quereshi, 1993) and non-ketotic hyperglycemia (Barshop *et al.*, 1989) is associated with an improved prognosis, the search for biochemical evidence in support of the rationale of this treatment has produced some conflicting results. The mechanism by which benzoate lowers blood ammonia is generally thought to be the result of diversion of waste nitrogen via glycine to benzoylglycine. There are, however, indications that plasma glycine is typically normal in patients with urea cycle defects and unaffected by benzoate therapy (Tremblay and Quereshi, 1993). Studies with isolated perfused rat liver indicated that benzoate inhibits the utilization of ammonia for the synthesis of glutamine as well as urea and that it stimulates glutamate export (Hausinger *et al.*, 1989). Although hepatic consumption of ammonia was found to be reduced by benzoate, glutamate export for synthesis of glutamine could aid the disposal of waste nitrogen during benzoate therapy (Tremblay and Quereshi, 1993).

2.4 Amino acid conjugates in patients with metabolic disorders.

The excretion of monocarboxylic acids in patients with organic acidurias are often accompanied by the excretion of the corresponding N-acylglycine (Bartlett and Gompertz, 1974; Grøn *et al.*, 1978; Gregersen, 1986). As indicated in Table 2.4,

isovalerylglycine can be found in the urine of patients suffering from isovaleryl-CoA dehydrogenase deficiency (Tanaka and Isselbacher, 1967) which can be attributed to the conjugation of accumulated isovaleryl-CoA with glycine. The accumulated material can be as much as 2000 - 15 000 μmol/day compared to normal excretions of less than 15 μmol/day (Sweetman and Williams, 1995). Isovalerylglycine appears to be nontoxic and is readily excreted. GNAT has the capacity to conjugate nearly all of the accumulated isovaleryl-CoA, except during acute episodes of the disease when the free acid becomes more elevated (Sweetman and Williams, 1995). In the case of isovaleric acidemia other minor metabolites include isovalerylglutamic acid (Lehnert, 1981) and isovalerylalanine and isovalerylsarcosine (Lehnert, 1983). It has not been proven that these compounds are formed by the action of GNAT although Lehnert (1983) speculated that this is likely to be the case.

Other branched chain organic acidurias in which urinary glycine conjugates are found include 3-methylcrotonyl-CoA carboxylase deficiency, in which case 3-methylcrotonylglycine is excreted (Beemer *et al.*, 1982) (Table 2.4). This conjugate is also found at elevated levels in holocarboxylase synthetase or biotinidase deficiency as well as 3-hydroxy-3-methylglutaric aciduria (Hoffmann, 1996). In mitochondrial branched chain 3-oxothiolase deficiency often tiglylglycine can be detected in the urine (Daum *et al.*, 1973). In the case of 2-methylbranched chain acyl-CoA dehydrogenase deficiency, isobutyrylglycine was detected in urine samples of patients suffering from this disorder (Burlina *et al.*, 1991).

The formation of propionylglycine has been reported in propionyl-CoA carboxylase deficiency (Rasmussen *et al.*, 1972). The same conjugate was detected in the urine of methylmalonic acidemia patients (Hoffmann, 1996). Even though this conjugate appears to account only for a small fraction of the propionate pool that accumulates in these diseases, it may be important in alleviating the toxic effects of propionate excess (Fenton and Rosenberg, 1995).

Table 2.4 Urinary acylglycine excretion in metabolic disorders.

Acylglycine	Normal range (mmol.mol^{-1} creatinine)		Classic abnormal range (mmol.mol^{-1} creatinine)	Enzyme deficiency
	Newborn (30 wk to 5 yr)	Adult		
Benzoylglycine (hippurate)	2 - 120	170 - 390	-	not known
Isovalerylglycine	nd	nd	2000 - 9000 0 - 1000	Isovaleryl-CoA dehydrogenase Multiple acyl-CoA dehydrogenase
3-Methylcrotonylglycine	0.2 - 2.5	nd	400 - 1000 30 -260 0 - 400	3-Methylcrotonyl-CoA carboxylase Holocarboxylase synthetase 3-Hydroxy-3-methylglutaryl-CoA lyase
Tiglylglycine	nd	nd	0 - 1000	Mitochondrial branched chain 3-oxothiolase
Hexanoylglycine	nd	nd	2 - 730	MCAD
Phenylpropionylglycine	nd	nd	1 - 90	MCAD
Suberylglycine	nd	nd	6 - 2200	MCAD
Butyrylglycine	nd	nd	not known	SCAD
Isobutyrylglycine	nd	nd	0 - 200	Multiple acyl-CoA dehydrogenase 3-Hydroxyisobutyryl dehydrogenase Methylmalonic semialdehyde dehydrogenase Methylbranched chain acyl-CoA dehydrogenase
2-Methylbutyrylglycine	nd	nd	0 - 200	Multiple acyl-CoA dehydrogenase
Propionylglycine	nd	nd	0 - 450	Propionyl-CoA and methylmalonyl-CoA carboxylase

nd = none detected
compiled from Sweetman (1991) and Hoffmann (1996).

In mitochondrial acyl-CoA dehydrogenase disorders the acylglycine conjugation profile are very prominent and useful to distinguish between the variants of these disorders. The development of the stable isotope dilution gas chromatography-mass spectrometry (GC-MS) assay for acylglycines has substantially aided the diagnoses of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (Roe and Coates, 1995). Organic acids excreted in this disorder include hexanoylglycine and suberylglycine, resulting from glycine conjugation to the corresponding elevated acyl-CoA esters, both substrates for GNAT (Roe and Coates, 1995). These two conjugates are also excreted in the multiple acyl-CoA dehydrogenase disorder, although butyrylglycine is additionally excreted in the latter disorder. A specific marker for MCAD is phenylpropionylglycine, originating from the conjugation of phenylpropionate, a metabolic product of intestinal bacteria (Bennet *et al.*, 1990), and glycine. In short-chain acyl-CoA dehydrogenase (SCAD) deficiency butyrylglycine has been found in the urine of some of the patients (Roe and Coates, 1995).

It is therefore clear that glycine conjugation, which is a natural occuring detoxicating process, may also be regarded as a detoxicating mechanism in a number of metabolic disorders in which accumulating acyl-CoA esters of various chain-lengths are conjugated to glycine in the mitochondria of liver and kidney. Notably important is the wide range of acyl-CoA substrate utilization of GNAT, which makes it serve a useful purpose in a number of metabolic disorders. It is not yet clear whether these conjugates may also have some damaging effect on metabolism.

2.5 Acyl conjugation with amino acids other than glycine: newly detected minor acylamino acid conjugates in the urine of patients with metabolic disease.

Conjugates between carboxylic acids and amino acids other than glycine have been reported during the last three decades. These conjugates are summarized in Table 2.5. Only conjugates with glutamine and asparagine can be regarded as products of glycine-*N*-acyltransferase because these amino acids were proven to alternative substrates (Nandi *et al.*, 1979).

Table 2.5 Mammalian amino acid conjugation of limited occurrence.

L-Amino Acid	Carboxylic Acid	Species	Reference
alanine	4,4′-dichlorophenylacetic acid	mouse	Wallcave *et al.* (1974)
	piperonylic acid	hamster	Gingell (1976)
	isovaleric acid	human*	Lehnert (1983)
	benzoic acid	human*	Shinka (1985)
	propionic acid	human*	Erasmus and Mienie (1998)
arginine	benzoic acid	scorpion	Hitchcock and Smith (1966)
asparagine	benzoic acid, salicylic acid phenylacetic acid, indole acetylic acid	bovine	Nandi *et al.* (1979)
aspartic acid	2,4′-dichlorodiphenylacetic acid	rat	Reif and Sinsheimer (1975)
glutamic acid	benzoic acid	Indian fruit bat	Idle *et al.* (1975)
		African bat	Collins *et al.* (1977)
	trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid	cow	Elliot (1977)
	3-phenoxybenzoic acid	cow	Elliot (1977)
	isovaleric acid	human*	Lehnert (1981)
	3-methylcrotonic acid	human*	Rolland *et al.* (1991)
	propionic acid	human*	Erasmus *et al.* (1998)
glutamine	benzoic acid, salicylic acid, phenylacetic acid, indole acetic acid	bovine	Nandi *et al.* (1979), Kelley and Vessey (1990), Moldave and Meister (1957)
	phenylacetic acid, indole acetic acid	human*	Webster *et al.* (1976)
	benzoic acid, phenylacetic acid	monkey	Asaoka (1991), Webster *et al.* (1976)
histidine	benzoic acid	peripatus	Jordan et al (1970)
serine	2,4′-dichlorodiphenylacetic acid	rat	Reif and Sinsheimer (1975)
	4,8-dihydroxyquinaldic acid	rat	Rothstein and Greenberg (195
	4,4′-dichlorodiphenylacetic acid	mouse	Gingell (1976)

* Humans with inborn errors of metabolism.

There are four reports in the literature that were of particular interest to this study. In the first report, Lehnert (1981) described the excretion of isovalerylglutamic acid in patients with isovaleric acdemia. He initially suggested that *N*-acetylglutamate synthetase (EC 2.3.1.1) is the catalyst for the formation of this conjugate. As was noted by this author, isovaleryl-CoA is a very poor substrate for this enzyme relative to acetyl-CoA (100 times slower turnover) (Coude *et al.*, 1979), and he expressed some doubt as to the origin of this conjugate. In the second report by Lehnert (1983),

in which he described the excretion of isovalerylalanine and -sarcosine, he speculated that these compounds are formed by GNAT-catalyzed conjugation of isovaleryl-CoA and the two respective amino acids. Since isovaleryl-CoA is one of the better substrates of GNAT in humans, and alanine and sarcosine resembles the structure of glycine closely, this route of conjugation seemed to us to be a very likely possibility.

In the third report, Shinka *et al.* (1985) described the excretion of benzoylalanine at levels ranging from 164 to 392 $mmol.mol^{-1}$ creatinine in the urine of five hyperammonemic patients which were treated with sodium benzoate. It was observed that these levels increased in parallel with blood ammonia levels. Comparatively, benzoylglycine concentrations were between 2116 to 4551 $mmol.mol^{-1}$ creatinine in the urine of the same patients. It was also noted in this report that this conjugate could be formed by GNAT. In the fourth report it was briefly mentioned that 3-methylcrotonylglutamic acid was detected in the urine of a patient suffering from 3-methylcrotonyl CoA carboxylase deficiency (Rolland *et al.*, 1991).

During the past few years a number of acyl conjugates with amino acids other than glycine have been detected in the urine of patients with propionic acidemia in our own screening program for metabolic disorders. These include propionyl conjugates with alanine (Erasmus and Mienie, 1998) and glutamic acid (Erasmus *et al.*, 1998). In one patient (WB), who was treated with alanine, the levels of these conjugates was 16.3 $mmol.mol^{-1}$ creatinine (propionylalanine) and 3.5 $mmol.mol^{-1}$ creatinine (propionylglutamic acid), respectively, whereas propionylglycine was 430.0 $mmol.mol^{-1}$ creatinine. Preliminary results have been presented at International symposia (van der Westhuizen *et al.*, 1994; Erasmus *et al.*, 1995).

2.6 Hypothesis and objectives.

The question that this study addressed, and which this literature review has put forward, is whether GNAT can accommodate amino acid substrates other than glycine. As discussed in Section 2.3.1, Schachter and Taggart (1954) initially reported

that GNAT is absolutely specific for glycine, which was later confirmed by Webster *et al.* (1976). Nandi *et al.* (1979) subsequently determined that bovine GNAT could additionally utilize asparagine and glutamine. More recently, Asaoka (1991) examined Japanese monkey GNAT and the conjugation of twenty-eight different amino acids with benzoyl-CoA as acyl donor. He reported, and thus confirmed what is commonly believed, that primate GNAT is absolutely specific for glycine. However, as indicated in the previous section, acylamino acids which share acyl moieties of known products of GNAT, but which also have amino acid moieties which are not known to originate from GNAT substrates, have been detected. These include acyl conjugates of alanine, serine, and glutamic acid. The catalytic origin in all of these conjugates are, although some speculations have been made, as yet unconfirmed and we believe this warrants a new investigation into this matter.

It is unfortunate that their are no data available on the structure of the active site of this enzyme. It was therefore not possible to investigate acyl-CoA and amino acid substrate binding additionally by using molecular models. However, in view of the structures of the newly described conjugates (Section 2.5), the hypothesis formulated for this study was that these conjugates are enzymatically formed by an acyltransferase reaction of an acyl-CoA to the acyl acceptor, the latter being the amino acids alanine, serine, and glutamic acid and that GNAT is the catalyst of these conjugation reactions.

To test this hypothesis, the objective was to focus the investigation on the formation of benzoylamino acid conjugation with respectively bovine and human GNAT. The motivation for initially focusing on benzoyl-CoA, even though most of the newly identified conjugates have other acyl moieties, is that it is the best characterized as well the acyl-CoA with the lowest Km-value and highest Vmax-value for GNAT. We therefore believe it to be a logical starting point to focus on when studying acyl-conjugation of amino acids other than glycine. The amino acids that were investigated were the aliphatic amino acids, serine, as well as the acidic amino acids and their respective amides.

Although our main interest was the conjugation of benzoylamino acids in humans, bovine GNAT was focussed on initially for the following reasons: firstly, practical experience in the purification, handling properties, and analysis of this enzyme would prove extremely useful when subsequently working with human material. The availibilty of human material is very limited, especially at our location as facilities that could provide such material are not close by. It was also clear from the onset of this study that there is more information available on the structural and catalytic properties of bovine GNAT than of any other mammalian species. Secondly, although there are clear differences in the catalytic, substrate and structural properties between human and bovine GNAT, they also share properties with regard to acyl-CoA and amino acid substrate utilization. A comparison of the results would therefore be interesting and useful. Bovine GNAT thus seemed a logical target to initially focus on. Futhermore, preliminary and subsequent analysis indicated it to be not only more stable, but also much more active compared to human GNAT preparations.

The following basic protocol was followed during this study: Firstly, benzoylamino acid conjugation in crude liver mitochondrial preparations was identified and compared to that of purified GNAT. Chemical characterization of conjugates were performed to validate the identity of conjugates. Secondly, the kinetic properties of the observed conjugation reactions were investigated. This same basic protocol was applied using bovine (Chapter 3) as well as human (Chapter 4) material.

CHAPTER 3

BENZOYLAMINO ACID CONJUGATION BY BOVINE GLYCINE *N*-ACYLTRANSFERASE

3.1 Introduction.

Conjugation of carboxylic acids with amino acids catalyzed by GNAT have been investigated in a number of mammalian species. These include ovine (Schachter and Taggart, 1953), human and monkey (Webster *et al.*, 1976), rat (Kølvraa and Gregersen, 1986) and bovine (Nandi *et al.*, 1979). It has been clear from the onset of this study that, even though the major interest relates primarily to conjugation of acylamino acids in humans, investigating conjugation in an animal model prior to using human material would provide important advantages (see Section 2.6). A similar investigation on bovine GNAT almost two decades ago (Nandi *et al.*, 1979) was a major stimulus for the current study and a major part of the work described builds on these investigations. Their findings, which were supported by an earlier publication on human GNAT by Webster *et al.* (1977) and later by Asaoka (1991), indicated that Gly[2], Asn and Gln are the only amino acid substrates for bovine GNAT. We and other investigators made some observations which suggested additional low-level conjugation of acylamino acids by GNAT (Section 2.5). In addition, we applied more advanced and sensitive technology to investigate the substrate specificity of GNAT.

3.2 Purification of bovine GNAT.

The protocol for the purification of bovine GNAT was essentially that described for the purification of bovine GNAT described by Nandi *et al.* (1979) and in more detail

[2]The three-letter abbreviation for amino acids will subsequently be used.

by Webster (1981). Mawal and Qureshi (1994a) made some modifications to this basic protocol for the purification of human GNAT. We used this latter protocol with some modifications as a basis for the purification of bovine GNAT. After some preliminary experimentation with the order of the various purification steps as well as the parameters for the low-pressure chromatography steps, we concluded that the method described here was the most appropriate in our hands. As the purification of GNAT was an integral part of this study and since purification of GNAT to near homogeneity was only achieved in a few published cases (Kelley and Vessey, 1992; Mawal and Qureshi, 1994), it will be described here in more detail.

A five-step strategy was followed and all purification steps where carried out as near to 4 °C as possible. GNAT activity in the various fractions was monitored using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) method (Ellman, 1959; Webster, *et al.*, 1976) as described by Nandi *et al.* (1979). Materials used in this study were of the highest purity that were available and were, unless otherwise specified, all obtained from Sigma Chemical Company.

The reaction mixture consisted of 25 mM Tris.HCl (pH 8.0), 0.1 mM benzoyl-CoA, 0.1 mM DTNB, and 200 mM Gly (pH 8.0) (BDH) in a final volume of 100 µl. The reaction was initiated by the addition of a volume of the fractionated sample of which the protein content varied depending on the stage of purification. Gly-dependent CoA release (DTNB reduction) from benzoyl-CoA was measured at 412 nm and 37 °C over a period of 10 minutes using a Beckman DU7000 spectrophotometer. Gly-independent DTNB reduction was subtracted from the measured values by using a blank in which Gly was omitted. This made a significant impact when the more crude enzyme preparations were tested. The molar extinction coefficient used for quantification of DTNB reduction by Gly-dependent CoA release was E_{412} = 13.6 $mM^{-1}.cm^{-1}$ (Ellman, 1959). Elution of protein was continuously monitored at 280 nm and compared with the GNAT specific activity in the eluted fractions. For the determination of specific activities of crude samples and eluted fractions after chromatographic separation, protein content was determined using the bicinchoninic

acid (BCA) method (Smith *et al.*, 1985), and was expressed as μmole.min^{-1}.mg^{-1} The following purification steps were followed :

3.2.1 Step 1. Mitochondrial supernatant preparation.

Freshly obtained chilled bovine liver (180 g) was homogenized using a Potter-Elvehjem homogenizer in four volumes of homogenization buffer (0.25 M sucrose; 20 mM Tris.HCl, pH 8.0). An enriched mitochondrial fraction was obtained by differential centrifugation of the homogenate at 1000 x *g* for 10 minutes followed by centrifugation of the supernatant at 10 000 x *g* for 10 minutes using a Beckman JA14 rotor. The mitochondrial enriched pellet was suspended in one tenth of the original volume of homogenizing buffer and frozen at -75 °C until use, which was usually within a few days. The suspension was thawed and the freeze-thaw step repeated after which the mitochondrial lysate was obtained by centrifugation of the suspension at 30 000 x *g* for 2 hours using a SW28 rotor (Beckman).

3.2.2 Step 2. Ammonium sulfate precipitation.

The protein concentration of the mitochondrial supernatant was adjusted to 20 mg.ml^{-1} and fractionated with solid ammonium sulfate. The pellet of the 40 - 60 % fraction, which was obtained after centrifugation at 10 000 x *g*, was suspended in as small volume as possible of Buffer A (20 mM Tris.HCl, pH 8.0). This fraction was desalted by gelfiltration using PD-10 Sephadex G25M desalting columns (Supelco) as instructed by the manufacturer.

3.2.3 Step 3. Hydroxyapatite chromatography.

The ammonium sulfate fraction was applied to a column (2.5 x 14.5 cm) containing Bio-Gel HT hydroxyapatite (Bio-Rad). After loading of the sample and running five volumes of Buffer A through the column, a gradient of 0 - 150 mM K_3PO_4, 10 mM $MgCl_2$, in Buffer A, with a combined volume of 200 ml, was applied at a linear flow rate of 12 cm.hr^{-1}. Fractions were collected in 3 ml volumes and those having the highest specific activities were pooled.

3.2.4 Step 4. Gelfiltration.

The combined fractions of the previous step were concentrated to a volume of 2 ml using ammonium sulfate precipitation, desalted as described in Step 2 of the protocol and applied to a calibrated gelfiltration column. The gelfiltration matrix consisted of Toyopearl HW 55-F (TosoHaas) packed into a glass column (1 x 86 cm). A linear flow rate of 14 $cm.hr^{-1}$ was applied and Buffer A was used as elution buffer. Eluted fractions were collected in 0.5 ml volumes and fractions having the highest specific activity where pooled for the following purification step.

3.2.5 Step 5. Chromatofocusing.

The pooled gelfiltration sample was applied to a chromatophocusing column (1 x 7 cm) packed with Polybuffer Exchanger 94 (Pharmacia) and pre-equilibrated with 25 mM Tris.CH_3COOH, pH 8.3. A pH range of 8 - 6 was used to elute the sample. This was achieved using nine bed volumes of the eluting buffer, Polybuffer^{TM} 96 (diluted 1:13 in water), at a linear flow rate of 22 $cm.hr^{-1}$. GNAT activity was monitored and collected as described in the previous steps, after which it was dialyzed against 100 volumes of Buffer A with two changes of buffer. The sample was stored in small aliquots at -75 °C until use.

3.2.6 Results and discussion.

Figures 3.1 to 3.3 show a series of chromatograms that is typical from what was obtained in a number of purifications. The results are given in Table 3.1. The first two crude steps in the protocol limited the amount of sample used for the chromatography steps to 90 mg of total protein. The inclusion of a salt precipitation step increased the activity to almost four times, which was comparable to that found by Mawal and Qureshi (1994), but with a loss of half the GNAT activity. This was a relatively high loss, but nevertheless the increase in specific activity was larger than was previously reported (Webster *et al.*, 1976; Nandi *et al.*, 1979).

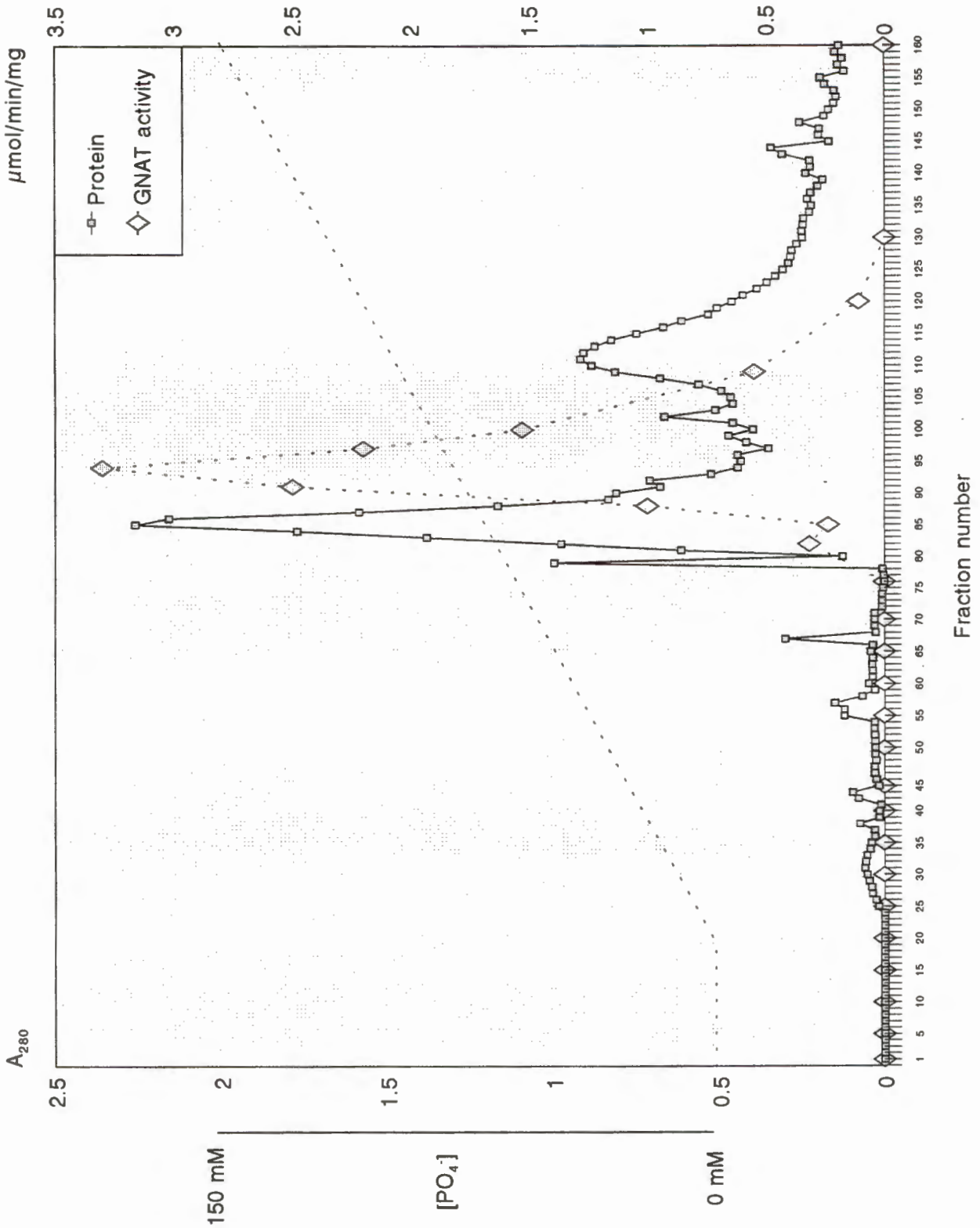


Figure 3.1 **Purification of bovine GNAT: Hydroxyapatite chromatography step** (see Section 3.2.3).

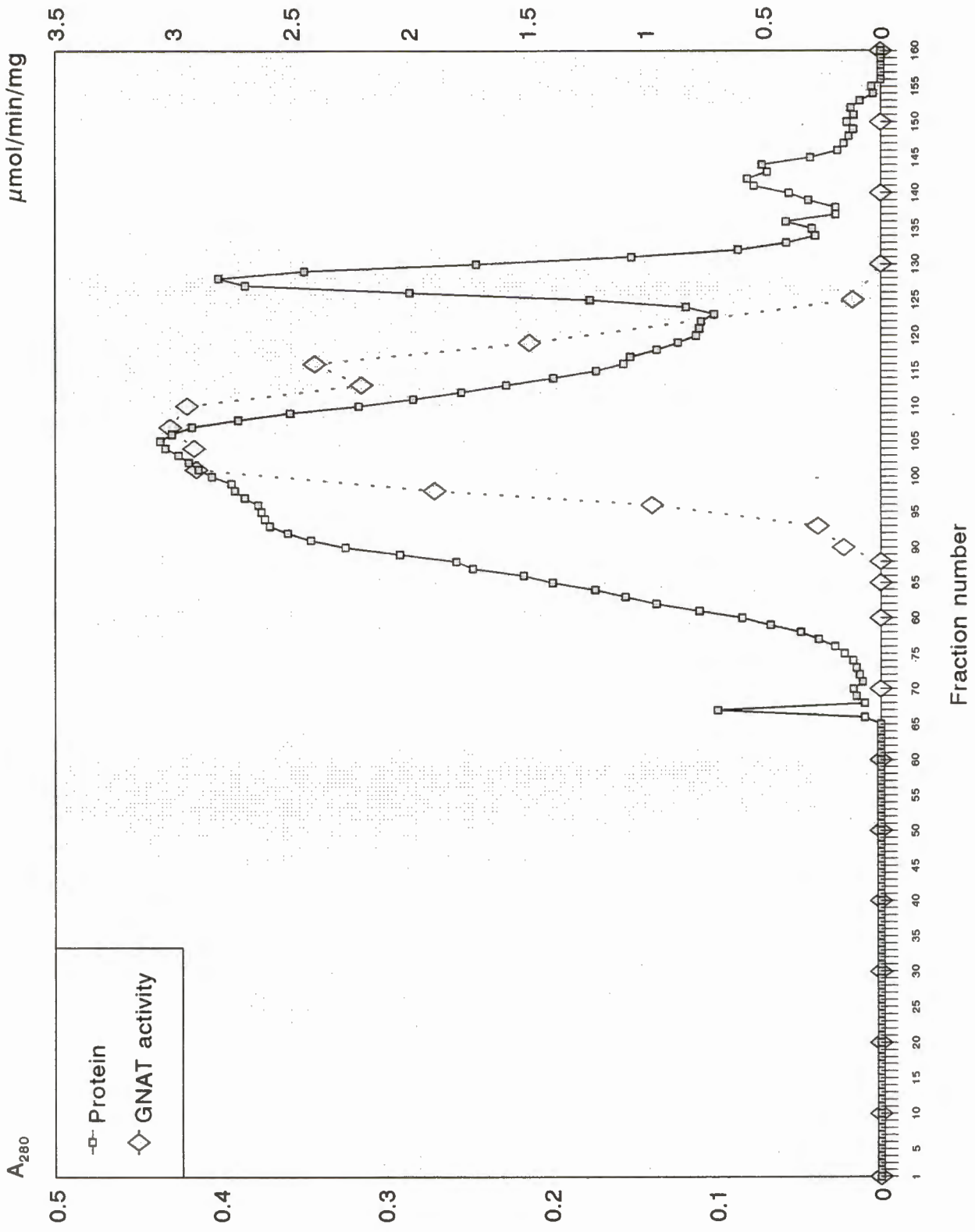


Figure 3.2 Purification of bovine GNAT: Gelfiltration step (see Section 3.2.4).

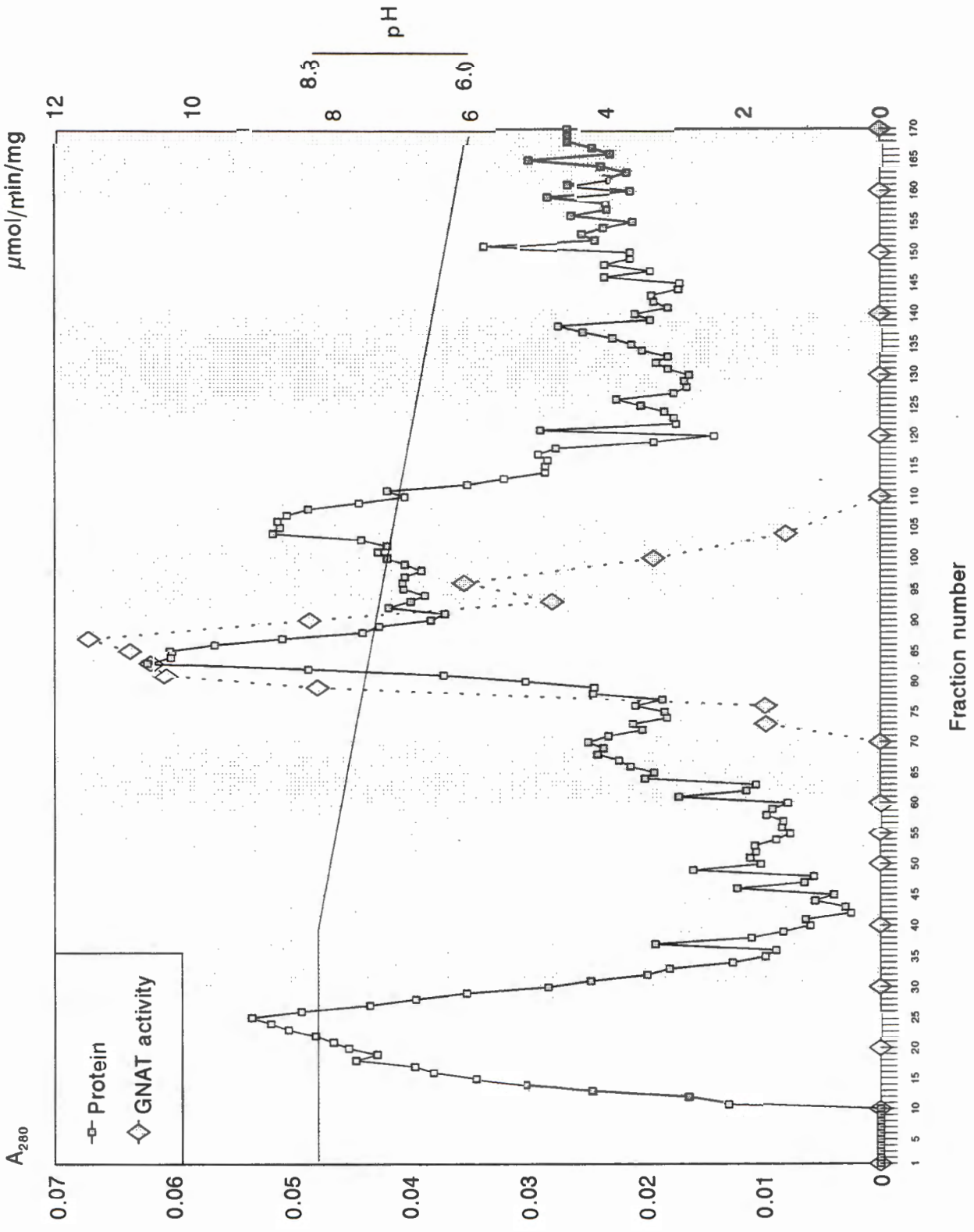


Figure 3.3 **Purification of bovine GNAT: Chromatophocusing chromatography step** (see Section 3.2.5).

Table 3.1 Purification of bovine glycine *N*-acyltransferase.

Step	Volume (ml)	Total protein (mg)	[Protein] ($mg.ml^{-1}$)	Total Activity* ($\mu mole.min^{-1}$)	Specific Activity* ($\mu mole.min^{-1}.mg^{-1}$)	Purification factor (*relative to previous step*)
1. Mitochondrial lysate	40	572	14.3	143	0.25	1
2. $(NH_4)_2SO_4$ precipitation (40 - 60 %)	15	90	6.0	85.5	0.95	3.8
3. Hydroxyapatite chromatography	25	7.5	0.3	21.3	2.84	3.0
4. Gelfiltration	8.5	1.96	0.23	11.5	5.86	2.1
5. Chromatofocusing	5.5	0.24	0.044	2.33	9.71	1.7

* Benzoylglycine formation.

Hydroxyapatite chromatography (Fig. 3.1) clearly indicates the elution of the majority of total protein (fractions 82 - 90) just prior to the elution of the fraction containing the highest GNAT activity (fractions 90 - 102). This chromatogram compares well with that shown by Nandi *et al.* (1979), who used this as the final step in their purification protocol. Another major total protein elution peak can be seen in the region of fraction 107 - 118, which according to Nandi *et al.* (1979), contains the majority of glutamine *N*-phenylacetyltransferase. This step thus separated the two acyl-CoA:amino acid *N*-acyltransferases. This was confirmed as the final GNAT preparation after the final step contained no activity towards glutamine *N*-phenylacetyltransferase, which was measured as described by Webster (1981). As indicated in Table 3.1, this first chromatography step resulted in an increase in specific activity of three times that of the previous step but with the loss of three quarters of total GNAT activity.

As can be seen in the chromatogram (Figure 3.2), the separation that was achieved during the gelfiltration step was not as successful as the previous step. An increase of twice the specific activity was achieved but a loss of half of the total activity. Two major elution peaks can be observed in the chromatogram of which the last twenty fractions of the first peak contained the highest GNAT specific activity. From a calibration graph obtained from running standard molecular weight markers (Boehringer Mannheim), it was determined that the elution of the GNAT activity was in the molecular mass region of 30 - 40 kDa.

The chromatographic separation of the sample obtained after gelfiltration, using chromatophocusing, can be seen in Figure 3.3. In this figure it can clearly be seen that the highest GNAT activity eluted together with the second major protein peak. It was also the first peak following the beginning of the pH gradient in the column. Elution mostly occurred in the region of pH 7.4 which was a surprising result since it was reported that bovine GNAT has a pI-value of 6.4 (Kelley and Vessey, 1990). With regard to this step results have been somewhat inconsistent as elution seldom occurred at the same pH, although it was always higher than 7.0. Combined fractions (80 - 90) collected from chromatofocusing had an increase in GNAT specific activity of just

below two times compared to the gelfiltration sample, with a loss of more than 80 % of the total activity. Assuming that GNAT activity remained reasonably stable during the purification procedure, an overall specific activity increase of almost fourty times resulted with a yield of 240 μg of bovine GNAT at a specific activity of 9.71μmole.min^{-1}.mg^{-1}. With this protocol reasonably consistent results were obtained, even though elution of GNAT was not always in identical fractions.

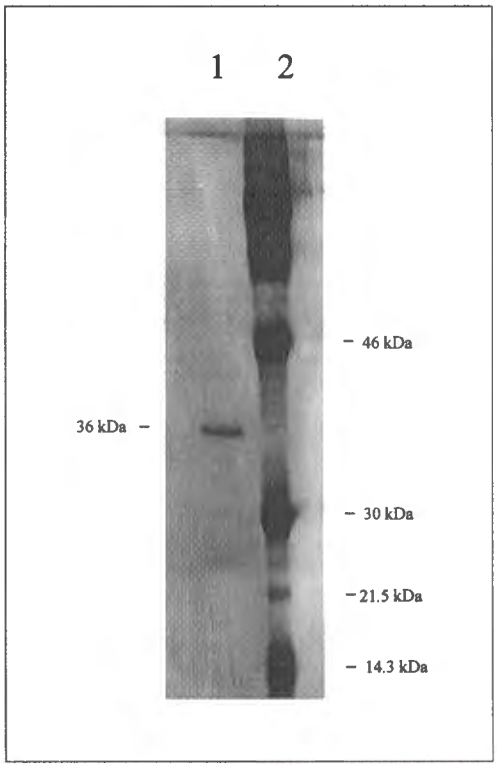


Figure 3.4 SDS-PAGE analysis of purified bovine GNAT.

The purified protein (lane 1) was electrophoresed on a 10 % SDS-PAGE gel in the presence of 0.1 % SDS. The gel was silver stained (Sasse and Gallagher, 1991) and compared with standard molecular weight markers (Amersham) as indicated in lane 2.

To determine the purity of the final GNAT preparation SDS-PAGE was used (Laemmli, 1970). As can be seen in Figure 3.4 one major protein band can be observed with a molecular mass of 36 kDa. This is higher than the 33.7 kDa which was previously reported to be the mass for bovine GNAT (Kelley and Vessey, 1992,

Nandi *et al.*, 1976). Two faint bands were visibly in the region of 28 and 40 kDa, respectively, when staining gels for extended periods using silver staining (Figure 3.4). No changes that were made to the purification protocol produced any better results than shown in this figure. The addition of blue-dextran affinity chromatography to the protocol, as described by Lau *et al.* (1977) and used by Mawal and Qureshi (1994) for the purification of GNAT, did not improve the results.

N-terminal sequencing of electroeluted bovine GNAT sample was performed by the laboratory of Dr. Clive Slaughter[3] using Edman-degradation chemistry on an automated sequencer. The N-terminal was not blocked and the following N-terminal sequence of nineteen amino acids was obtained:

M-F-L-L-Q-G-A-Q-M-L-Q-M-L-E-K-S-L-R-K

Protein database analysis identified a perfect match to a sequence of fifteen amino acids for the N-terminal of bovine *aralkyl-CoA N-acyltransferase* (Kelley and Vessey, 1992) which is, as discussed in Section 2.3.2, the same enzyme. At that time, relevant literature was obtained using, amongst other, the nomenclature for the enzyme as used in this study. This was, however, insufficient to locate the publications of Kelley and Vessey. This furthers illustrates the problem which non-conformity in nomenclature of this enzyme presents (discussed in Section 2.3.2).

The N-terminal sequence also matched to the recently published cDNA sequence for bovine *aralkyl acyl-CoA:amino acid N-acyltransferase* (Vessey and Lau, 1996). This confirmed the identity of the isolated protein although a substantial difference between the molecular mass of the purified enzyme (36 kDa) and the mass deduced from the cDNA (31.7 kDa). The reason for this discrepancy is not yet clear. From the results it was, however, concluded that the final enzyme preparation obtained was a very near-homogenous bovine GNAT sample and suitable to be used for the investigation of bovine GNAT kinetics.

[3] Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern MedicalSchool, Dallas, TX, USA.

3.3 Investigating GNAT conjugation of amino acids with benzoyl-CoA using the DTNB-based spectrophotometric assay.

The DTNB-based assay for measuring GNAT activity (Webster *et al.*, 1976) has been used by most researchers that have previously investigated and reported on the properties of this enzyme. We were, however, not sure whether this approach would be either sensitive or accurate enough for the detection of low-level conjugation for the following reasons: Firstly, we assumed that any possible conjugation of benzoyl-CoA with amino acids other than Gly, glutamine or asparagine would represent much lower enzyme activity, as none was previously detected using this same approach; therefore this approach might lack sensitivity. To illustrate this, consider the following hypothetical experimental setup: a molar extinction coefficient at 412 nm (E_{412}) of 13.6 $mM^{-1}.cm^{-1}$, a cuvet with a 1 cm light path and 0.2 ml reaction volume. If 1 µg of the enzyme is used with a specific activity of 9.7 $\mu mole.min^{-1}.mg^{-1}$ at optimal conditions, the change of absorbance of 0.66 units per minute would be measured. However, if the rate were 100 times (or more) lower, as it might be for other low affinity amino acid substrates, the absorbance change per minute would be 0.0066 or less. If less optimal conditions, such as lower substrate concentrations when doing kinetic experiments, are to be employed changes in absorbance would then become smaller and therefore less accurate. Secondly, non-substrate specific DTNB reduction was detected even though attempts were made to compensate for it by using blank reactions in which the either substrates was omitted.

To investigate conjugation of benzoyl-CoA with the aliphatic amino acids as well as serine, the acidic amino acids and their amides, purified GNAT as well as crude mitochondrial lysate were used in a standard assay reaction for GNAT. The basic reaction consisted of 20 mM Tris.HCl (pH8.0), 0.1 mM DTNB, 0.1 mM benzoyl-CoA, 100 mM amino acid (pH 8.0), and 1 - 100 µg of enzyme in in a final volume of 300 µl. In the case of Asp, due to low solubility, a concentration of 38 mM was used. These conditions were comparable to two previous reports where benzoylamino acid conjugation was investigated (Webster *et al.*, 1976; Asaoka, 1991). Although these conditions may not have been optimal for all the conjugation reactions that were subsequently detected, the objective in this case was to determine whether conjugation

could be detected under similar and relative high substrate concentrations which would then provide a basis on which further kinetic experiments could be performed. The reactions were initiated by the addition of amino acid and where followed at 412 nm for 30 minutes at 37 °C. The linear rate of increase was measured and blank values subtracted. The amount of conjugate formed in the various reactions, run in triplicate, was calculated and expressed as nmole.min^{-1}.mg^{-1} (Table 3.2).

Table 3.2 Specific activities of benzoylamino acid conjugation by purified bovine GNAT compared to a mitochondrial lysate.

Amino acid substrate	Benzoylamino acid conjugation* (nmole.min^{-1}.mg^{-1}) (n = 3)	
	Mitochondrial lysate	GNAT preparation
Gly	250 ± 3.4	9730 ± 54.0
Asn	4.79 ± 0.8	97.7 ± 3.6
Gln	0.36 ± 0.2	18.0 ± 1.3
Ala	0.65 ± 0.3	11.6 ± 1.4
Ser	0.54 ± 0.2	<D
Glu	0.26 ± 0.2	<D
Asp	1.49 ± 0.3	<D
Ile	0.38 ± 0.2	<D
Val	0.60 ± 0.1	<D

D = minimal activity that could be measured

*Benzoyl-CoA was used as acyl-donor at 0.1 mM and amino acid concentration was, with the exception of Asp (38 mM), set at 100 mM (pH 8.0).

These results indicate amino acid dependent release of CoA with all of the amino acid substrates tested when using mitochondrial lysate or purified GNAT as enzyme source. Where mitochondrial lysate was used the reaction rates were detected in all cases. Gly had the highest rate followed by Asn, which is one of the two known alternative substrates for bovine GNAT. The rate in this case was almost fifty times lower at the specific assay conditions used. The rest of the amino acids had similar conjugating rates with Asp being the highest, 170 times lower than Gly, followed by Ala, Val, Ser, Ile, Glu, and Glu in this order, all being approximately 500 times less active than Gly. Gln conjugation was at a notably low rate in view of the reports that it is a known substrate for bovine GNAT (Nandi *et al.*, 1979). A further observation that was made during the conjugation analysis using mitochondrial lysate was that non-amino acid dependent DTNB reduction was high and a very small net change in absorbancy was detected, as can be deduced from the results in Table 3.2.

With purified bovine GNAT as enzyme source a much lower non-amino acid dependent DTNB reduction was observed and thus made results more reliable. In this case DTNB reduction could only be detected when using Gly, Asn, Gln, and Ala as amino acid substrates (Table 3.2). The relative rates that were detected were in the following order: Gly >> Asn > Gln > Ala. It was also clear that the difference between the comparative rates between the latter three amino acids and Gly were much higher when using purified GNAT. For example, the activity with Ala compared to Gly was 0.26 % in the mitochondrial lysate experiment, as compared to 0.12 % in the case of purified GNAT. If assuming the conditions are not more optimal for Ala when using mitochondrial lysate, the reason for this observation could be that a part of the change in absorbance detected using the mitochondrial lysate are due to some background DTNB reduction that was not compensated. This may also explain the rates measured for some of the amino acids, such as the branched-chain amino acids, whereas none was detected when purified bovine GNAT was used.

These initial results also confirmed that for the purpose of doing the envisioned enzyme kinetic experiments, the DTNB reduction assay would not be sensitive enough for practical purposes. Supply of purified GNATwould not enable multiple analyses using the spectrophotometric assay. It was therefore clear that an alternative, more sensitive, quantification method was required to accurately measure the rate of the formation as well as to confirm the formation of the newly detected benzoyl conjugates.

3.4 Investigating GNAT conjugation of amino acids with benzoyl-CoA utilizing electrospray ionization tandem mass spectrometry (ESI-MS-MS).

The confirmation of newly detected GNAT-catalyzed conjugates were essential to this study. ESI-MS-MS was a logical choice as tool for the investigation of benzoyl conjugation for a number of reasons. Firstly, its diverse applications as well as its sensitivity for a wide range of compounds is well documented (Smith *et al.*, 1990; Voyksner, 1992; Cole, 1997). Secondly, the technology as well as the expertise to apply it was available to this study. Furthermore, a recent study on carnitine

palmitoyltransferase I has confirmed that ESI-MS-MS could be utilized as an extremely useful tool for *in vitro* enzyme activity studies (Nolte *et al.* 1998). Therefore, in addition to the chemical characterization of benzoyl conjugates, it also served as an alternative quantification tool to the spectrophotometric approach, whose limitations were already mentioned.

3.4.1 Characterization of benzoylamino acid conjugates.

Benzoyl conjugates of the various amino acids as listed in Table 3.2 (Section 3.3) were chemically synthesized essentially as described by Gregersen *et al.* (1976). Products obtained were dissolved in water and extracted twice with ethylacetate and dried under nitrogen. The purity of these conjugates were estimated by ESI-MS-MS by scanning for the positive ions of the reactants. In all cases none or negligible traces of the reactants were present. Conjugates were thus essentially pure with the exception of benzoylglutamine, in which case a very high level of contamination with benzoylglutamic acid was always detected. As the glutamic acid content of the glutamine used (Sigma) in the synthesis of benzoylglutamine is less than 0.1 % the only explanation for this observation is the deamination of benzoylglutamine to benzoylglutamic acid during the synthesis process.

Reactions used for determining benzoylamino acid formation were carried out as described in Section 3.3, with the exception that 0.15 µg of purified bovine GNAT protein or 6.0 µg mitochondrial lysate was used and DTNB was omitted. Reactions proceeded at 37 °C for 5 - 30 minutes after which they were terminated by the addition of 20 µl concentrated formic acid. Benzoyl conjugates in the reaction mixtures were extracted once with two volumes of ethylacetate and dried under vacuum or nitrogen, after which they were suspended in 50 µl of a 50 % (v/v) acetonitrile:water solution (containing 1% formic acid). In the case of benzoylglutamic acid, the product was butylated, as described by Millington *et al.* (1990), prior to analysis since detection of this conjugate was highly improved by this modification.

ESI-MS-MS analysis were carried out on a VG Quattro II triple quadropole instrument (Micromass, UK). For the identification and characterization of benzoyl conjugates the first MS was kept static, monitoring the positive ion mass of the conjugate, for example monitoring for a 194 mass/charge ratio (*m/z*) for benzoylalanine (Mw = 193). The second MS was set in the daughter ion scan mode of operation and was set to monitor daughter ions of the parent conjugate in the first MS over a range of 20 - 200 *m/z*. The detection of a characteristic fragment ion of *m/z* 105 (Shinka, 1985) in the second MS, which corresponds to the structure as indicated in Figure 3.5, along with the correct parent ion detected in the first MS, were the main objectives of the characterization of benzoyl conjugates. The collision energy was set at 20 eVolts and the following parameter settings were further applied to the apparatus:

		Source (ES^+)		
Capillary		3.5 kVolts		
HV lens		0.5 kVolts		
Cone		30 Volts		
Skimmer offset		3 Volts		
Skimmer		1.5 Volts		
RF lens		0.2 Volts		
Source Temperature		60 °C		
		MS1		MS2
Ion energy		0.8 Volts		2.5 Volts
Ion energy ramp		0.0 Volts		0.0 Volts
LM resolution		14.5		15.0
HM resolution		14.5		15.0
Lenses	(# 5)	100 Volts	(# 7)	250 Volts
	(# 6)	5 Volts	(# 8)	40 Volts
			(# 9)	0 Volts
Multiplier		650 Volts		650 Volts

Nitrogen was used as drying and nebulizing gas. The flow rates for drying and nebulizing were set at 350 and 20 $L.h^{-1}$, respectively.

Collision-induced dissociation (CID) mass spectra of all the benzoyl conjugates detected by ESI-MS-MS are given in Figures 3.5 - 3.10. In these figures the chemically synthesized conjugates were compared to conjugates formed by bovine GNAT, namely benzoylglycine (Fig. 3.5), benzoylglutamine (Fig. 3.6), benzoylasparagine (Fig. 3.7), benzoylalanine (Fig. 3.8), benzoylserine (Fig. 3.9), and

benzoylglutamic acid (butylated) (Fig. 3.10). The identity of some of the characteristic ion fragments that formed under the specific conditions are indicated on the mass spectra of the synthesized compound (Figures 3.5A - 3.10A). The comparison with the bovine GNAT products are also summarized in Table 3.3. Although Gly, Asn, and Gln are known substrates of bovine GNAT, they were included in these experiments to compare the full range of amino acid substrates. As can be seen in Figures 3.8 - 3.10 benzoyl conjugates with alanine, serine and glutamic acid could be detected as well. None of these conjugates were detected where enzyme was omitted from the reaction mixtures. Benzoyl conjugates of Asp, or any of the branched-chain amino acids were not detected using either mitochondrial or purified bovine GNAT preparations.

Table 3.3 CID profile of chemically synthesized and bovine GNAT-catalyzed formation of benzoylamino acid conjugates.

Benzoyl conjugate	Chemical synthesized conjugate fragmentation* (*m/z*)	Bovine GNAT-catalyzed conjugate fragmentation* (*m/z*)
benzoylglycine	105, 77, 95, 180	105, 77, 95, 180
benzoylasparagine	105, 87, 122, 77, 70, 192, 220	105, 87, 122, 77, 70, 192, 220
benzoylglutamine	122, 105, *79*, 129, 84	105, 122, *84*, 83, 129, 77, *130*
benzoylalanine	105, 77, 44, 148, 194, 95	105, 77, 44, 148, 194, 95
benzoylserine	105, 77, 88, 60, 70, 118, 210, 193, 118	105, 77, 88, *146*, 60, *195*, 193, 60, *69*, 210
benzoylglutamic acid	105, 84, 140, 186, 188, 262, 290, 130	105, 84, 140, 186, 262, 188, 290, 130

* Major fragments detected are listed in the order of decreasing intensity.

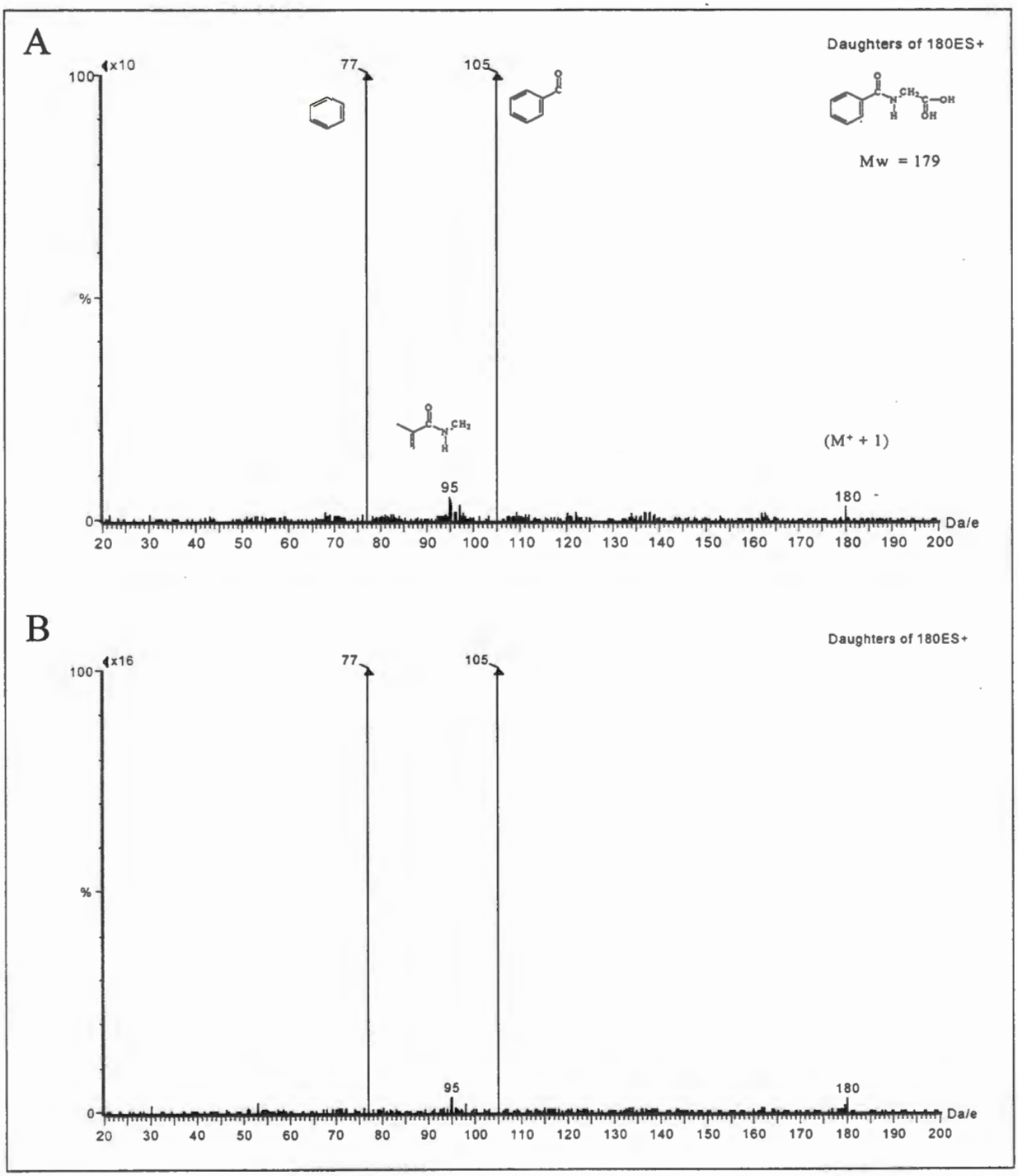


Figure 3.5 **CID mass spectra of chemically synthesized *benzoylglycine* (A) as compared to bovine GNAT-catalyzed benzoylglycine (B).**
Raw data was processed by using the *background subtraction* option (with the following settings: polynomal order: 1; below curve: 10 %; tolerance: 0.01) and by using the *smoothing* option (with the following settings: peak width (Da): 1.0; number of smoothing: 2; and using the *mean smooth* option and *center* option).

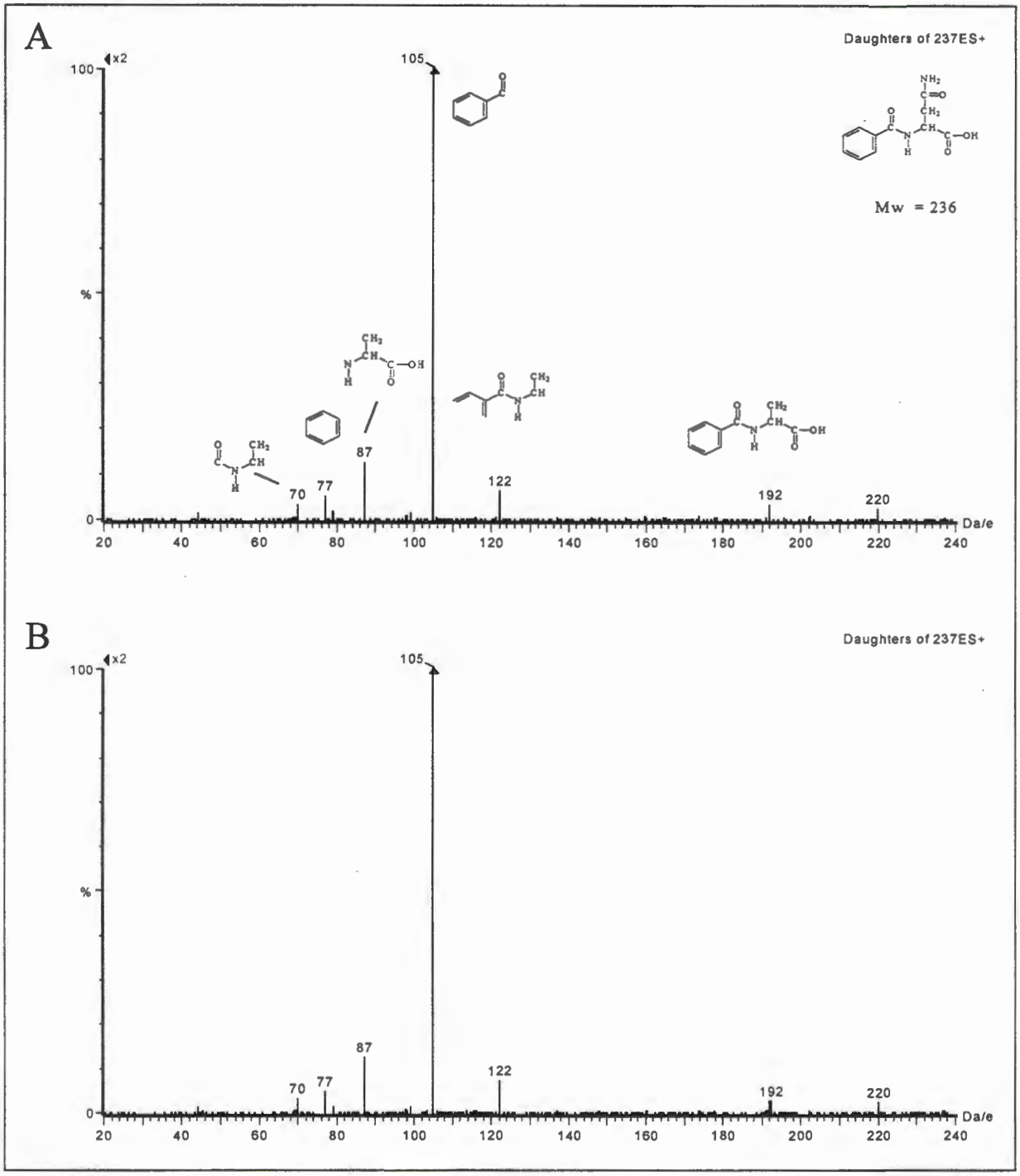


Figure 3.6 **CID mass spectra of chemically synthesized *benzoylasparagine* (A) as compared to bovine GNAT-catalyzed benzoylasparagine (B).**
Raw data was processed as described in Figure 3.5.

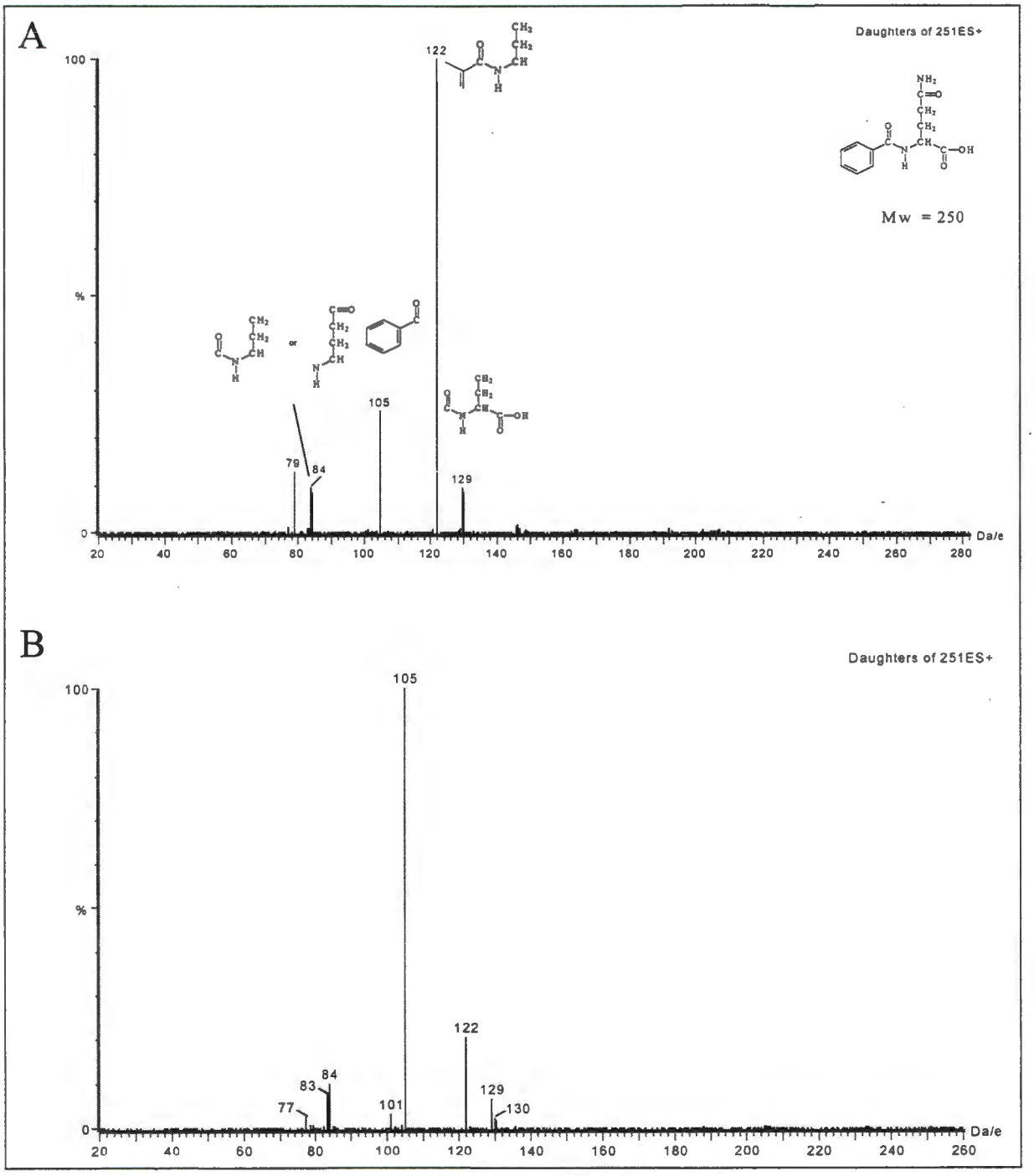


Figure 3.7 **CID mass spectra of chemically synthesized *benzoylglutamine* (A) as compared to bovine GNAT-catalyzed benzoylglutamine (B).**
Raw data was processed as described in Figure 3.5.

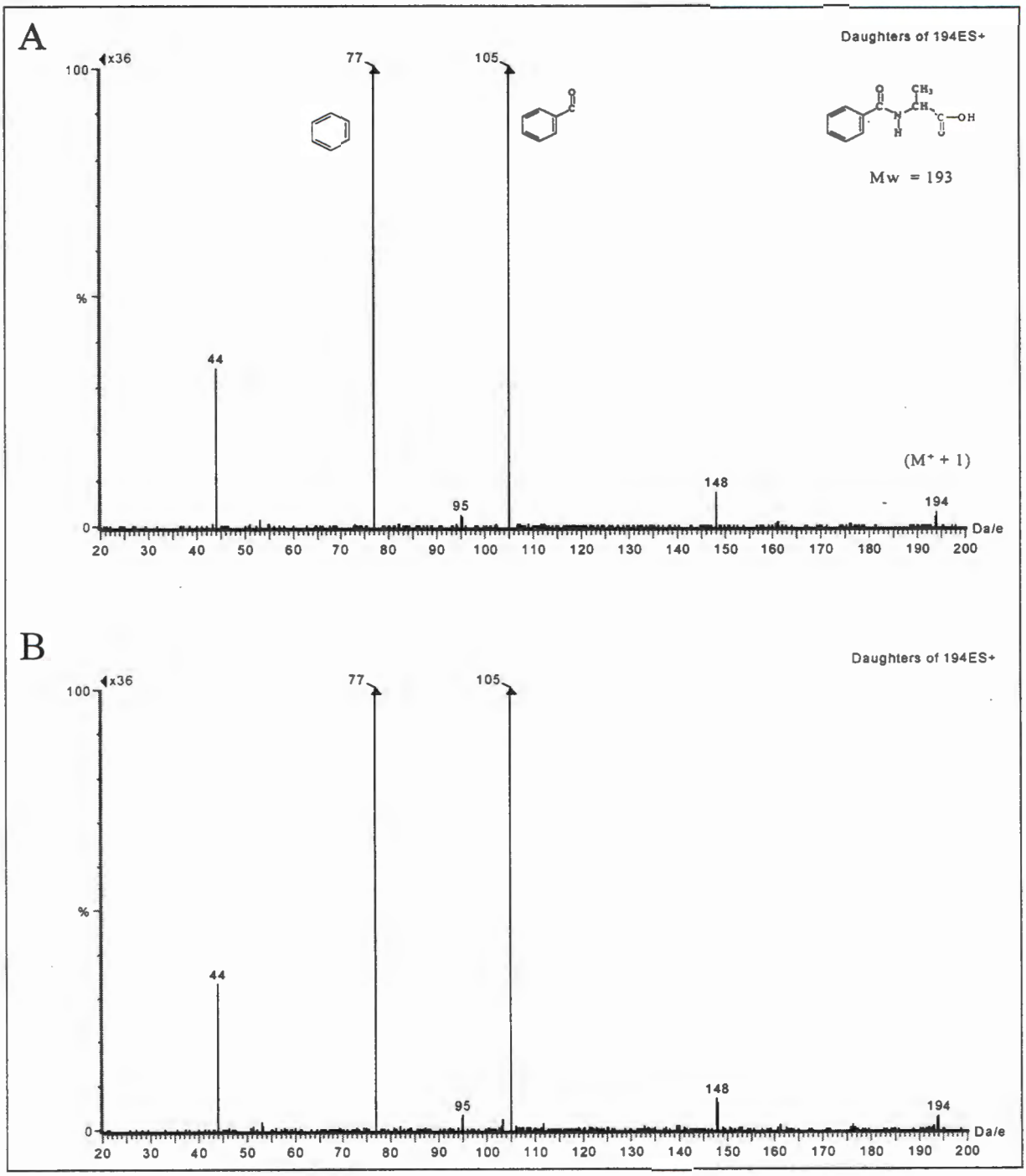


Figure 3.8 **CID mass spectra of chemically synthesized *benzoylalanine* (A) as compared to bovine GNAT-catalyzed benzoylalanine (B).**
Raw data was processed as described in Figure 3.5.

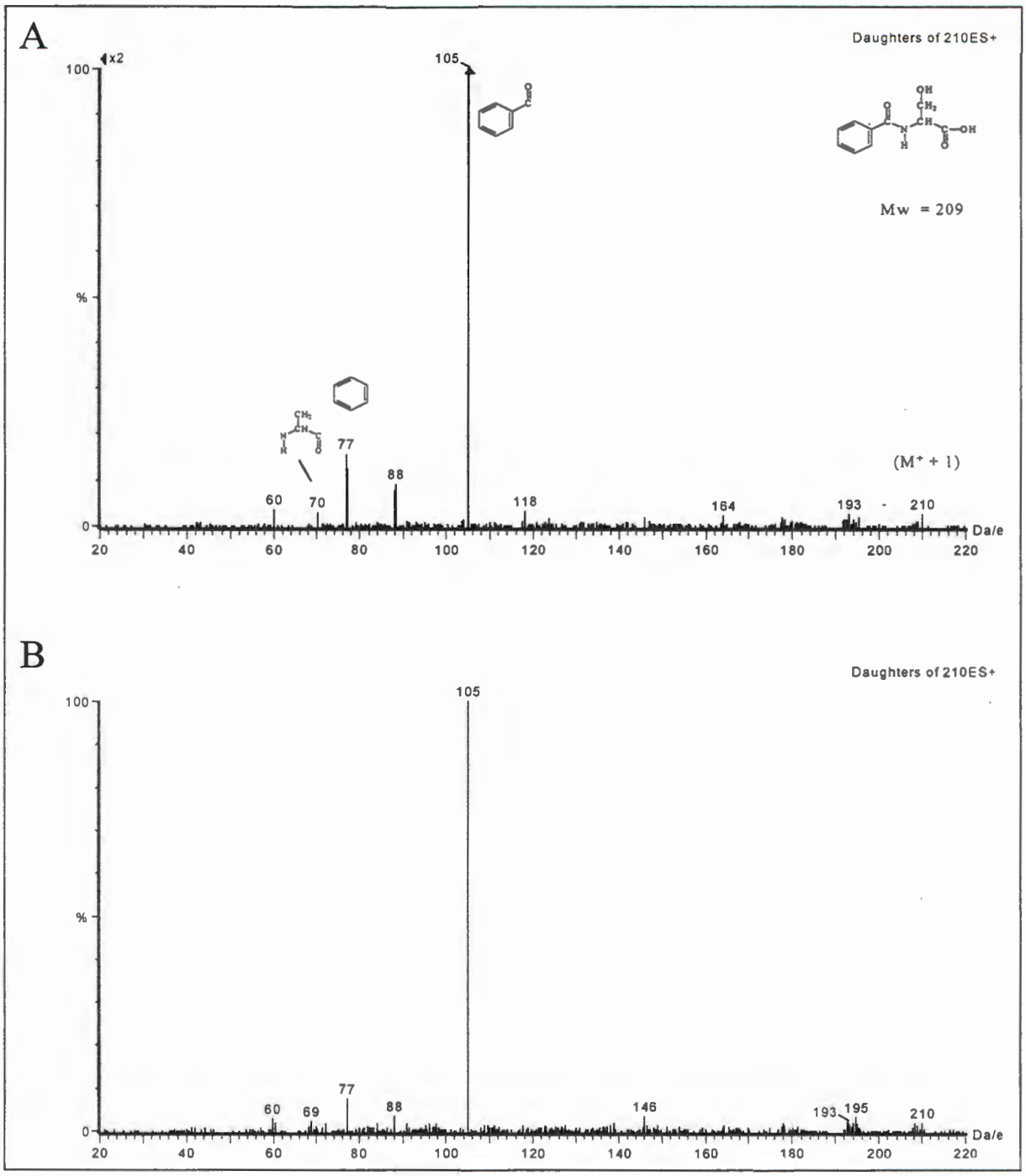


Figure 3.9 **CID mass spectra of chemically synthesized *benzoylserine* (A) as compared to bovine GNAT-catalyzed benzoylserine (B).**
Raw data was processed as described in Figure 3.5.

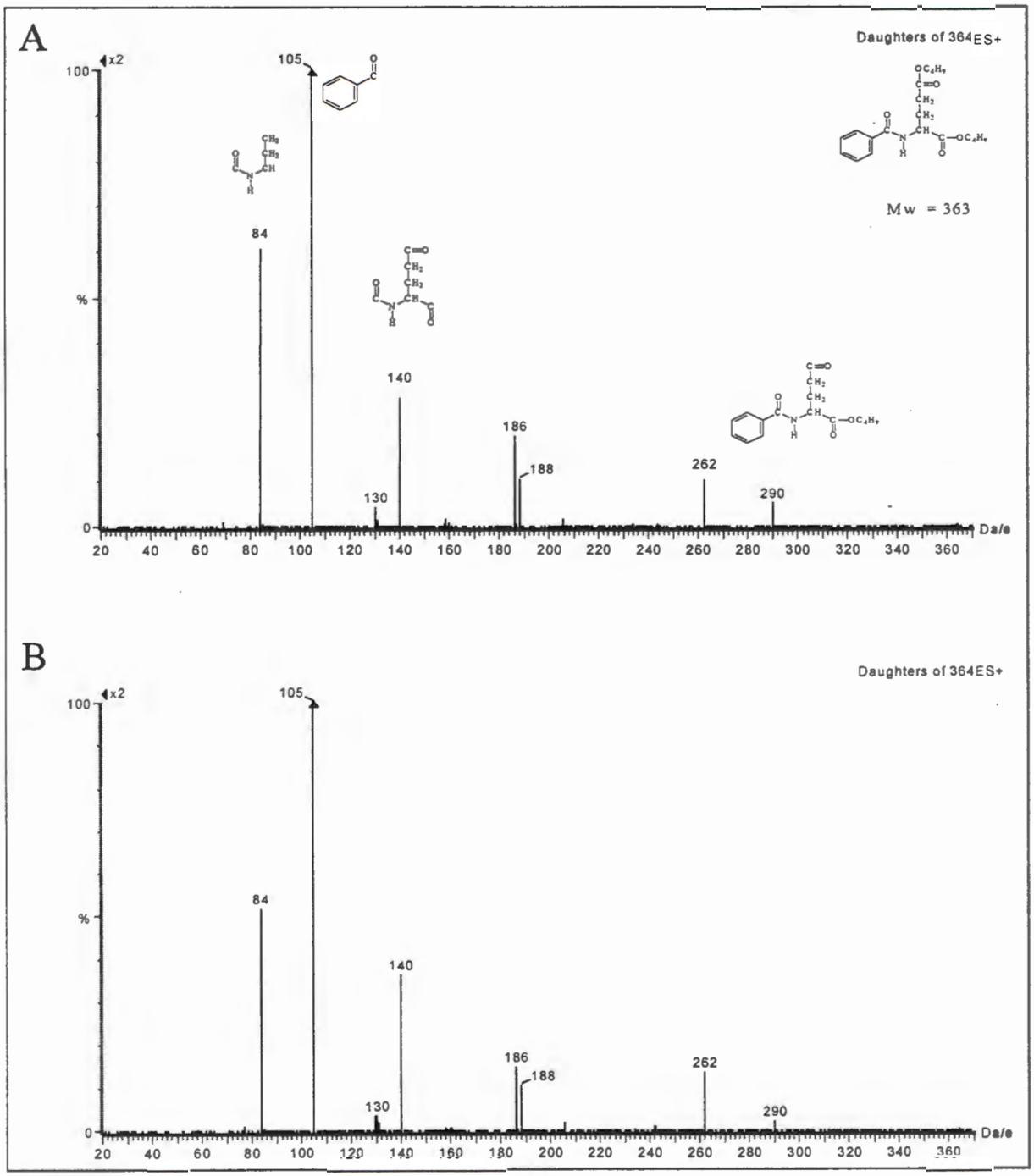


Figure 3.10 CID mass spectra of chemically synthesized *benzoylglutamic acid* (butylated) (A) as compared to bovine GNAT-catalyzed benzoylglutamic acid (butylated) (B).
Raw data was processed as described in Figure 3.5.

As can be seen in the various CID mass spectra of the synthesized conjugates as compared to the enzymatically formed conjugates, not only where the correct mass of each conjugate detected, but similar fragmentation patterns where detected in all cases as well. Some minor exceptions were observed which include fragments that was detected from the CID mass spectra of benzoylglutamine and benzoylserine (indicated in Italic font in Table 3.3). The identity of these fragments could not be determined.

Our findings indicate that benzoyl conjugtes of Gly, Asn, Gln as well as Ala, Glu, and Ser were indeed formed by bovine GNAT. How well and at what rate these newly detected benzoyl conjugates are formed is a major question. To determine this, we needed to quantitfy the formation of these compounds and determine the kinetic parameters. The preliminary data obtained from the spectrophotometric method, discussed in Section 3.3, indicated that the rates would be extremely low in comparison to Gly conjugation. A new, and more sensitive method was therefore required to answer this question.

3.4.2 Quantification of the formation of benzoylamino acid conjugates using ESI-MS-MS.

Benzoylamino acid conjugates were quantified using the same principle applied by Nolte *et al.* (1998). ESI-MS-MS was carried out using a slightly different approach to the one described in the previous section. In this case the parent ion (PAR) mode of operation was used where the second mass spectrometer was kept static, monitoring the CID daughter ion fragment at *m/z* 105 (which represents a characteristic moiety of benzoylamino acid conjugates (see Figures 3.5 - 3.10 and Table 3.3). The first MS was set to scan over a wide range for all parent ions of *m/z* 105. The positive ion mass of the corresponding benzoylamino acid conjugate, producing an ion of *m/z* of 105 in the second MS, would thus be detected in the first MS. The parameter settings were similar to those described for fragmentation except that the source cone voltage was set slightly lower, at 25 V. The settings for the ion energy and resolutions in MS1 were changed for those of MS2 and vice versa.

Benzoyl conjugates were quantified relative to an external standard, the stable isotope benzoyl-[2,2-D_2]glycine. This was synthesized essentially as described by Gregersen *et al.* (1976) using [2,2-D_2]glycine. For each of the benzoylamino acids investigated except benzoylglutamine, for which a pure synthesyzed product could not be obtained, the *ratio of ionization* (RI) of the conjugate relative to the stable isotope was predetermined using a calibration curve. In this curve the ratio of the relative intensities of ionization of the two compounds (peak area) and the concentration of the conjugate was compared at a fixed concentration of the stable isotope. The slope of this graph indicated the RI of the two compounds. The samples used for this calibration were prepared in the same way as those for the subsequent enzyme assay samples. (Section 3.4.1). As an example, Figure 3.11 shows the calibration curve for benzoylasparagine and the RI determined from the slope that was subsequently used for the quantification of this compound.

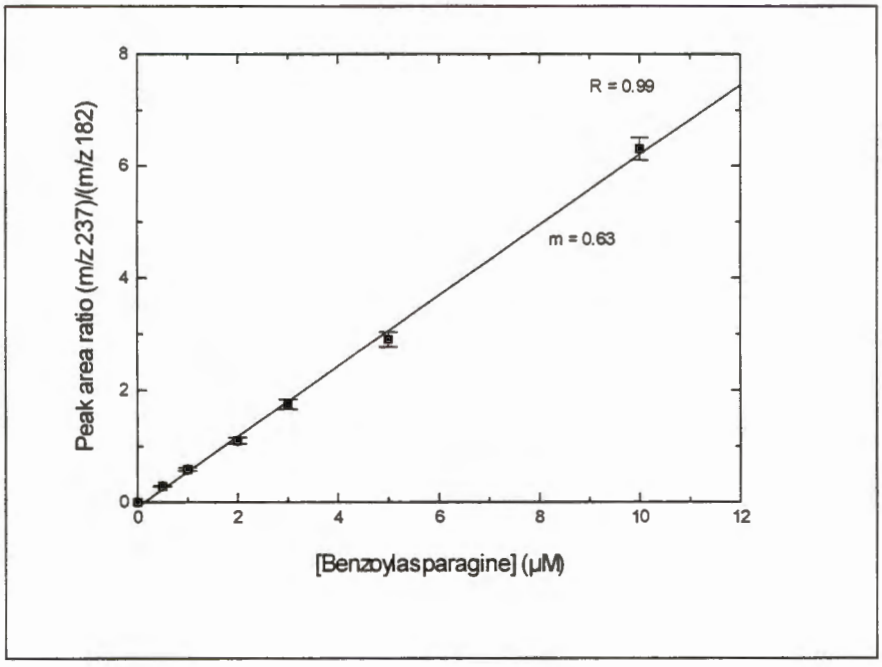


Figure 3.11 Calibration curve for determining the ratio of ionization (IR) of benzoylasparagine with the stable isotope, benzoyl-[2,2-D_2]glycine (1 µM). The slope (m) was subsequently used to determine the RI-value.

The RI-values obtained for the various conjugates under investigation were as follows: benzoylglycine (1.01), benzoylasparagine (0.63), benzoylalanine (0.48), benzoylserine (0.065), and benzoylglutamic acid (0.58).

In subsequent reactions, the isotope was added after the termination of each reaction in amounts such that the subsequent peak area ratio of the conjugate and the isotope would fall within the calibration curve. As an example, the time-dependent increase of benzoylalanine can be viewed in Figure 3.12. The amount of isotope added usually was in the region of 0.05 - 50 nmoles depending on the conjugate under investigation. The rate of benzoyl conjugation was thus measured in a end-point analysis and was expressed in nmole.min^{-1}.mg^{-1}, using equation 3.1 :

$$\mathbf{Vo} = \mathbf{PA} \cdot \mathbf{RI^{-1}} \cdot \mathbf{BG} \cdot \mathbf{mg^{-1}} \cdot \mathbf{min^{-1}} = \text{nmole.min}^{-1}\text{.mg}^{-1} \qquad \text{..... 3.1}$$

where PA : peak area ratio (conjugate/isotope)
RI : ratio of ionization (slope of calibration graph)
BG : amount of benzoyl-[2,2-D_2]glycine added (nmoles)

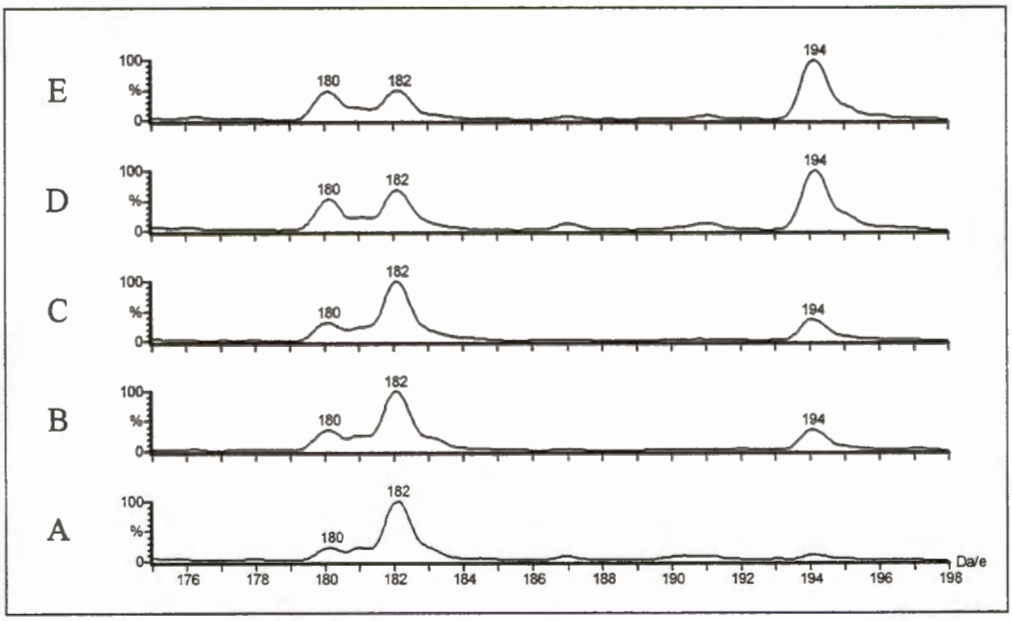


Figure 3.12 Time-dependent formation of benzoylalanine.
Mass spectra of a series of five separate bovine GNAT reactions are shown at the time intervals, 0 (A), 5 min (B), 10 min (C), 20 min (D), and 30 min (E). The peaks that are indicated are benzoylglycine (*m/z* 180), benzoyl-[2,2-D_2]glycine (*m/z* 182), and benzoylalanine (*m/z* 194).

3.4.3 Kinetic parameters of benzoylamino acid conjugation.

The methodology optimized for quantification of the benzoyl conjugates (as described in the previous section) was used for determination of rates of benzoyl conjugation. As was described in Section 3.3, the comparative rates of formation of conjugates using mitochondrial lysate and purified bovine GNAT were firstly compared at 100 mM amino acid (pH 8.0) and 0.1 mM benzoyl-CoA. GNAT conjugation of the various benzoylamino acid conjugates using mitochondrial lysate and GNAT preparations was quantified for the reasons discussed in Section 3.4.2., as well as to complement the detection of the conjugates already described in Section 3.4.2. The results are summarized in Table 3.4. The time- and protein-dependent formation of the benzoyl conjugates were investigated at the above mentioned substrate concentrations and the results are given in Figures 3.13 (benzoylalanine), 3.14 (benzoylserine), and 3.15 (benzoylglutamic acid).

Table 3.4 Formation of benzoylamino acid conjugates in bovine mitochondrial and purified bovine GNAT preparations using ESI-MS-MS.

Amino acid substrate	Benzoylamino acid conjugation* ($nmole.min^{-1}.mg^{-1}$)	
	Mitochondrial lysate	GNAT preparation
Gly	232	10266
Asn	1.0	193
Gln	nq	nq
Ala	0.2	24
Ser	0.08	6.4
Glu	0.15	3.5
Asp	<D	<D
Ile	<D	<D
Leu	<D	<D
Val	<D	<D

*Benzoyl-CoA was used as acyl-donor at 0.1 mM and amino acid concentration was, with the exception of Asp (38 mM), set at 100 mM (pH 8.0).
D = minimal activity that could be measured
nq = not quantifiable

As the results indicate, the specific activity (benzoylglycine formation) that was measured for purified GNAT using this ESI-MS-MS based method was 10266 $nmole.min^{-1}.mg^{-1}$. This was a fourty-fold increase from activity measured using mitochondrial lysate and is comparable, even though slightly higher, to the value obtained using the spectrophotometric approach (Table 3.2, Section 3.3). From this it was clear that ESI-MS-MS is an accurate alternative to the spectrophotometric method for the quantification of benzoylglycine.

Under the specific reactions conditions that was used, the rate of formation of benzoylglycine using both enzyme preparations was the highest followed by benzoylasparagine, benzoylalanine, benzoylserine, and benzoylglutamic acid. The relative rates of conjugation were 1 : 54 : 435 : 1604 : 2900, respectively, using purified GNAT as enzyme source. The rate of formation of the alternative benzoyl conjugates compared to benzoylglycine mainly increased from using the mitochondrial lysate to using purified bovine GNAT which could be attributed to the difference in composition of the two enzyme samples. From the data obtained in Section 3.3 (Table 3.2) it was observed that benzoylglutamine formed at a rate higher than benzoylalanine and lower than benzoylasparagine under the same reaction conditions. This conjugate could not be quantified accurately using ESI-MS-MS for the reason that an RI-value could not accurately be obtained from the chemically synthesized compound (see Section 3.1). A further important observation was the contaminating formation of benzoylglycine when using Ala as substrate (see Fig. 3.12). Although a high purity of Ala was used and a number of suppliers was tested, this was most likely due to contaminating Gly (less than 0.1 % according to one supplier) that was present. It may also account for the higher comparative benzoylalanine activity that was detected when the DTNB-based approach was used (Section 3.3), in which case approximately half of the activity monitored for benzoylalanine could have been due to the formation of benzoylglycine. Even though some trace amounts of Gly was present in the assay, Ala was by far the most abundant substrate and it was believed that the contaminating trace amounts of Gly would not substantially influence the results.

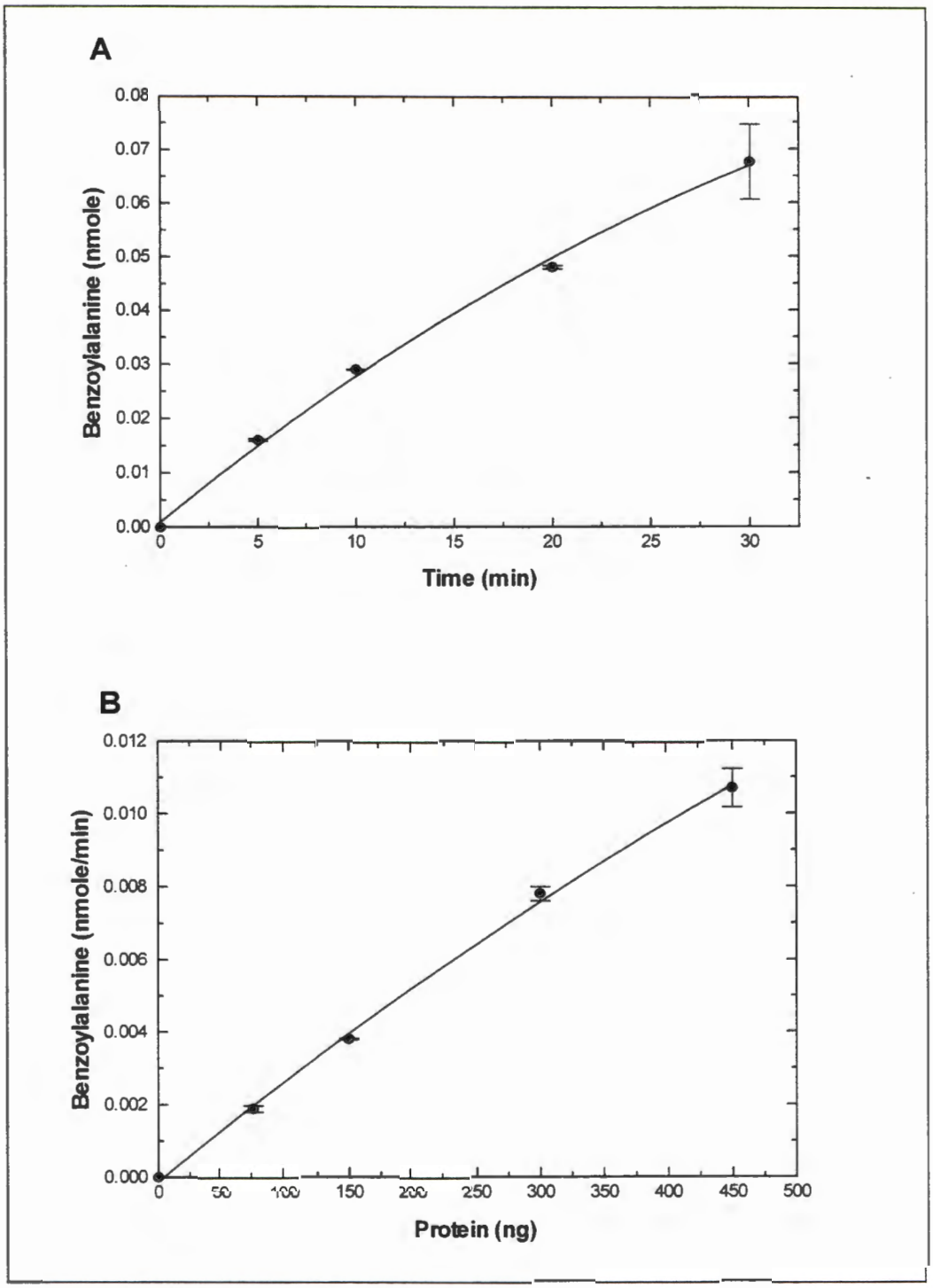


Figure 3.13 Time (A) and protein-dependent (B) formation of benzoylalanine by bovine GNAT. In B the assays were run for 10 minutes. The standard deviation of the mean ($n = 2$) are indicated on the graph.

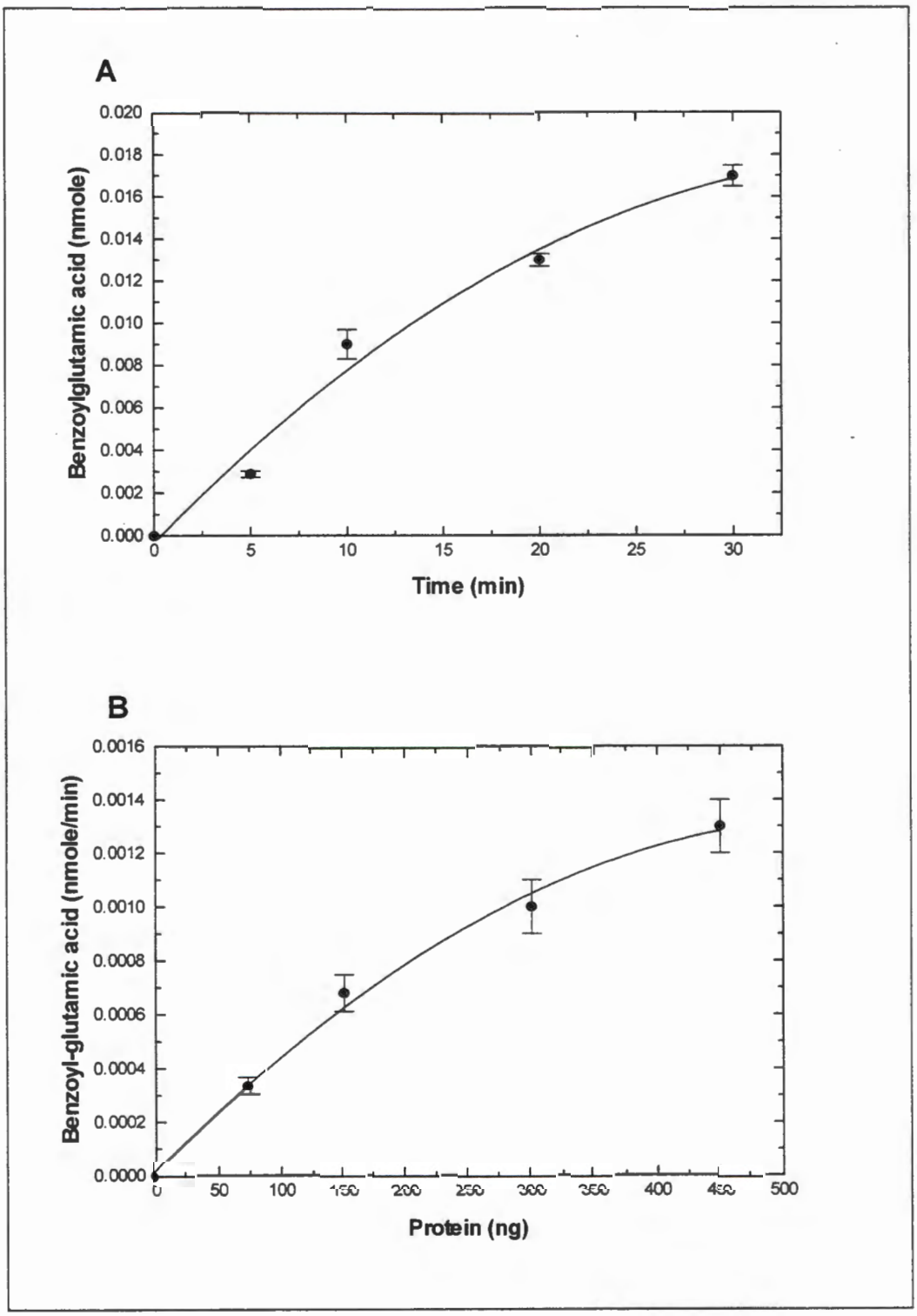


Figure 3.14 **Time (A) and protein-dependent (B) formation of benzoylglutamic acid by bovine GNAT. In B the assays were run for 10 minutes. The standard deviation of the mean (n = 2) are indicated on the graph.**

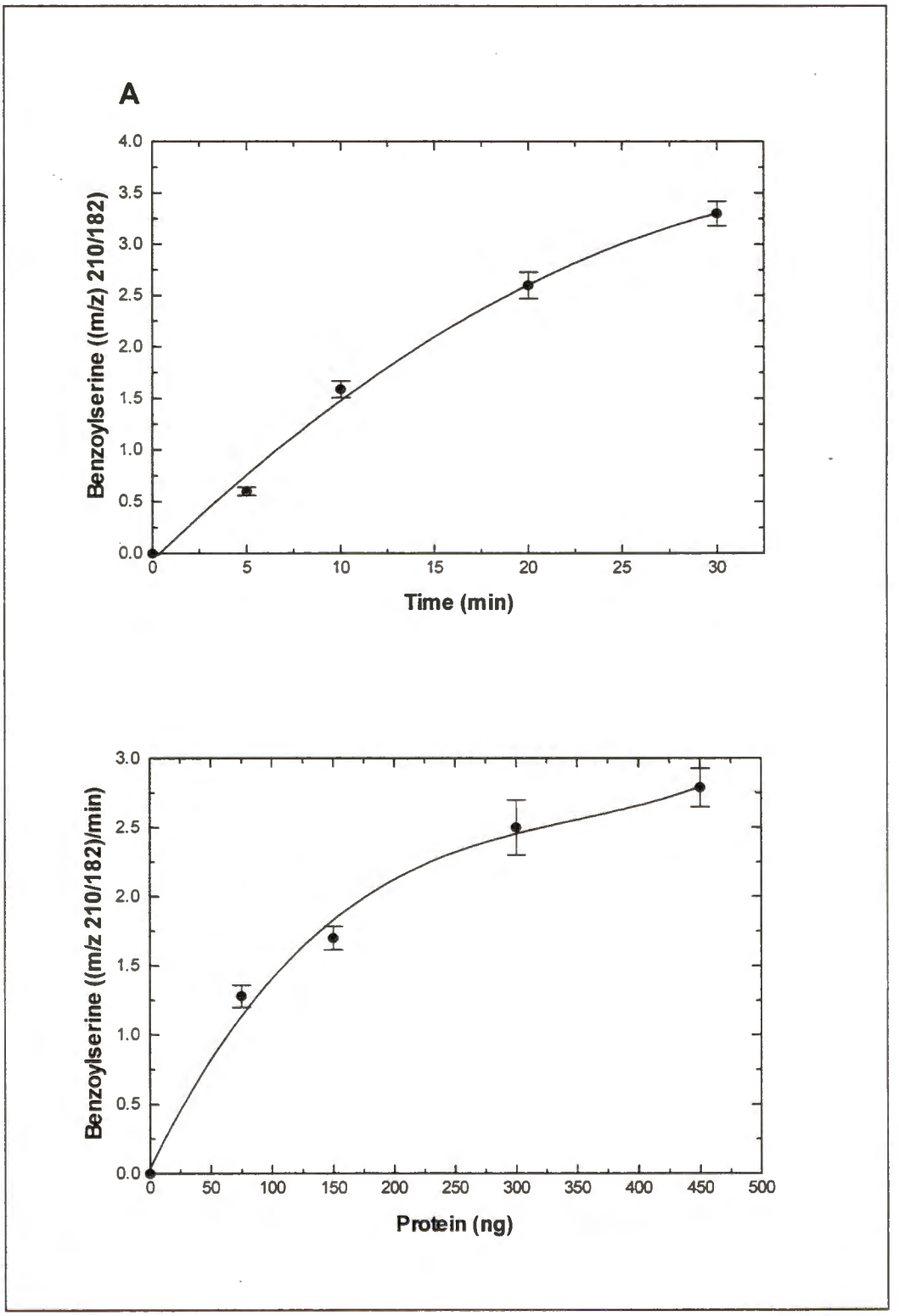


Figure 3.15 **Time (A) and protein-dependent (B) formation of benzoylserine by bovine GNAT. In B the assays were run for 10 minutes. The standard deviation of the mean (n = 2) are indicated on the graph.**

An important observation that was made from the protein-dependent benzoylamino acid formation experiments was that no conjugation could be detected where no enzyme was present, as can be deduced from Figure 3.13 - 3.15 (B). This clearly indicates that these compounds are not formed without the presence of GNAT as the catalyst. With benzoylalanine formation, a linear increase in conjugate was observed (Figure 3.13B) and in the case of the other two conjugates formation progressed in a more hyperbolic fashion (Figure 3.14 and 3.15B). Under the conditions used, a near linear increase of conjugate formation was observed for the first ten minutes with each of the three conjugates investigated (Figure 3.13 - 3.15A). This time frame was subsequently used in the kinetic experiments.

Reactions used for determining kinetic parameters for benzoylamino acid formation contained 20 mM Tris.HCl (pH 8.0), 0.15 µg protein, and varying substrate concentrations in a final volume of 200 µl. The substrate concentration range was from 2.5 to 400 mM, pH 8.0 (37 °C) for the amino acids and from 5 to 150 µM for benzoyl-CoA. Since monovalent cations have an effect on GNAT activity (Nandi *et al.* 1979; Kelly and Vessey, 1990), monovalent cation content was kept constant at 10 mM. Reactions used for kinetic analysis proceeded at 37 °C and not 30 °C as commonly used for kinetic analysis (Palmer, 1995), to allow for higher rates of low-level conjugation. The pH at which these and subsequent kinetic experiments for GNAT were performed were set at 8.0 since this was the pH that was used in all of the previous published kinetic investigations of GNAT. Reactions proceeded for 5 - 10 minutes after which they were terminated by the addition of 20 µl formic acid. After the addition of stable isotope, the samples were prepared as described in Section 3.4.1 and conjugates quantified as described in the Section 3.4.2. Conjugate formation was subsequently expressed as the initial reaction velocity (Vo) of the reaction.

Kinetic parameters were determined graphically using linear regression analysis of double reciprocal primary and secondary rate plots following established methods (Engel, 1977; Palmer, 1995) and expressed using the suggested terminology of the NC-IUB (NC-IUB, 1982). Figures 3.16 - 3.20 are the primary and secondary rate plots of benzoylglycine (Figure 3.16), benzoylglutamine (Fig 3.17), benzoylasparagine

(Fig 3.18), benzoylalanine (Fig 3.19), and benzoylglutamic acid (Fig. 3.20). Because of the extremely low detection capability of benzoylserine, no reliable data for kinetic parameters could be obtained. Vmax was derived from the intercept (1/Vmax) of the secondary rate plot with primary intercepts indicated as the ordinate, whereas $Km_{amino\ acid}$ was derived from the slope ($Km_{amino\ acid}$/Vmax) of the same plot. $Km_{benzoyl\text{-}CoA}$ was subsequently derived from the intercept ($Km_{benzoyl\text{-}CoA}$/Vmax) on the secondary rate plot with the primary slopes used as the ordinate (Engel, 1977; Palmer, 1995). The results of the kinetic analysis are summarized in Table 3.5.

Primary rate plots, in which linear extrapolated lines could be drawn on double reciprocal plots, indicated that the benzoylamino acid conjugations investigated obeyed Michealis-Menten kinetics. The varying slopes between the respective series, which were observed from the primary plots, were consistent with a compulsory order ternary complex reaction mechanism in each case, as parallel lines indicated a classical ping-pong (non-sequential) mechanism (Palmer, 1995). This observation is also consistent with the initial studies by Nandi *et al.* (1979).

Evaluation of Michaelis-Menten kinetics of two-substrate reactions revealed that the Michaelis constants determined in the current study could be expressed as $Km_{benzoyl\text{-}CoA}$ and $Km_{amino\ acid}$, and not apparent constants ($Km_{benzoyl\text{-}CoA}^{app}$ and $Km_{amino\ acid}^{app}$), since the concentration of the amino acid substrate, as indicated in the secondary rate plots, was extrapolated to an infinitely high value (Engel, 1977; IUB, 1982). It is important to note that the constants determined here are thus not "operational" Km-values that are sometimes determined at concentrations of metabolites *in vivo,* Km-values which may sometimes have more practical meaning for metabolic biochemists (Engel, 1977).

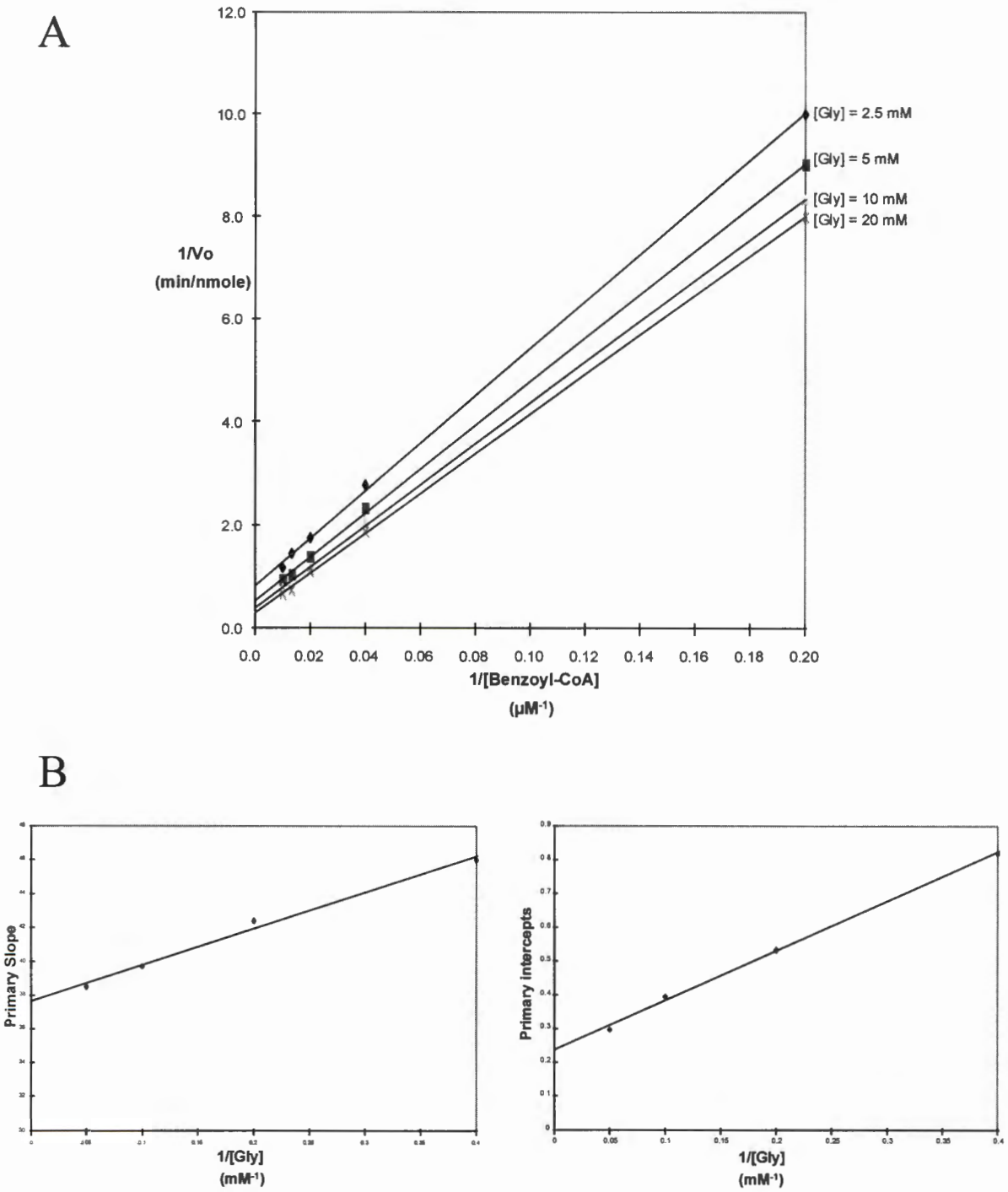


Figure 3.16 **Primary (A) double reciprocal, and secondary (B) rate plots used for the determination of kinetic parameters for *benzoylglycine* formation using bovine GNAT.**

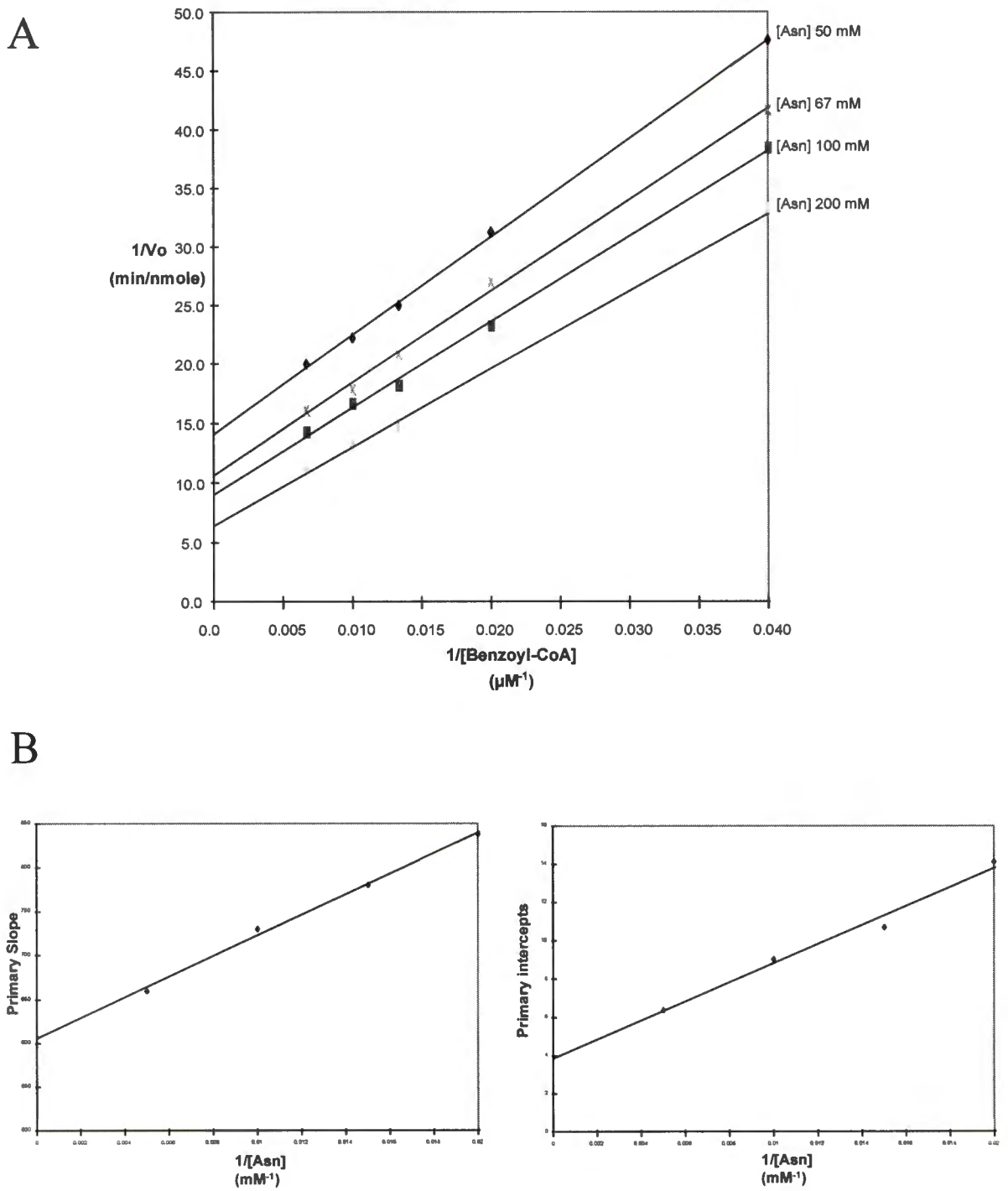


Figure 3.17 **Primary (A) double reciprocal and secondary (B) rate plots used for the determination of kinetic parameters for *benzoylasparagine* formation using bovine GNAT.**

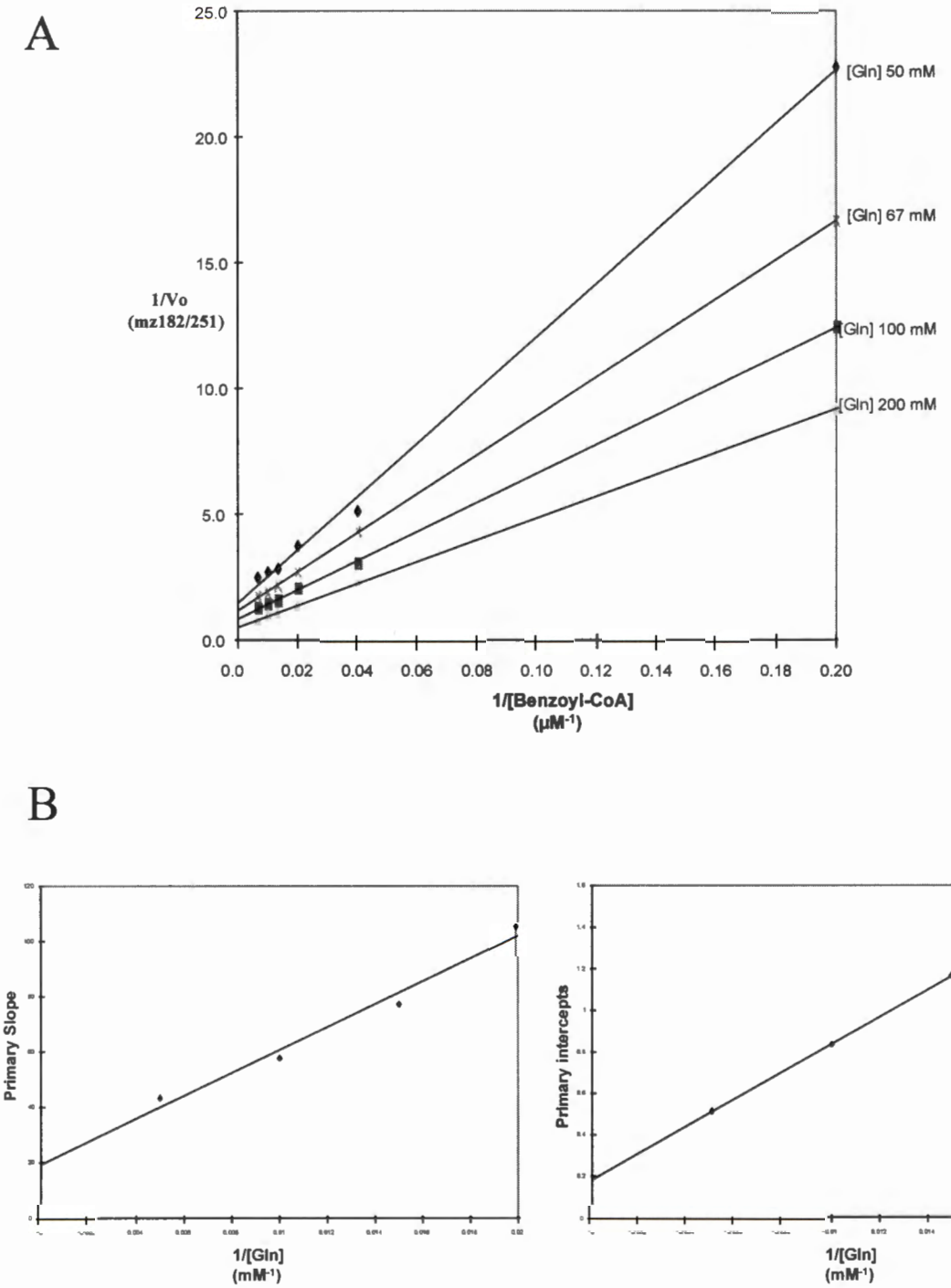


Figure 3.18 **Primary (A) double reciprocal and secondary (B) rate plots used for the determination of kinetic parameters for *benzoylglutamine* formation using bovine GNAT.**

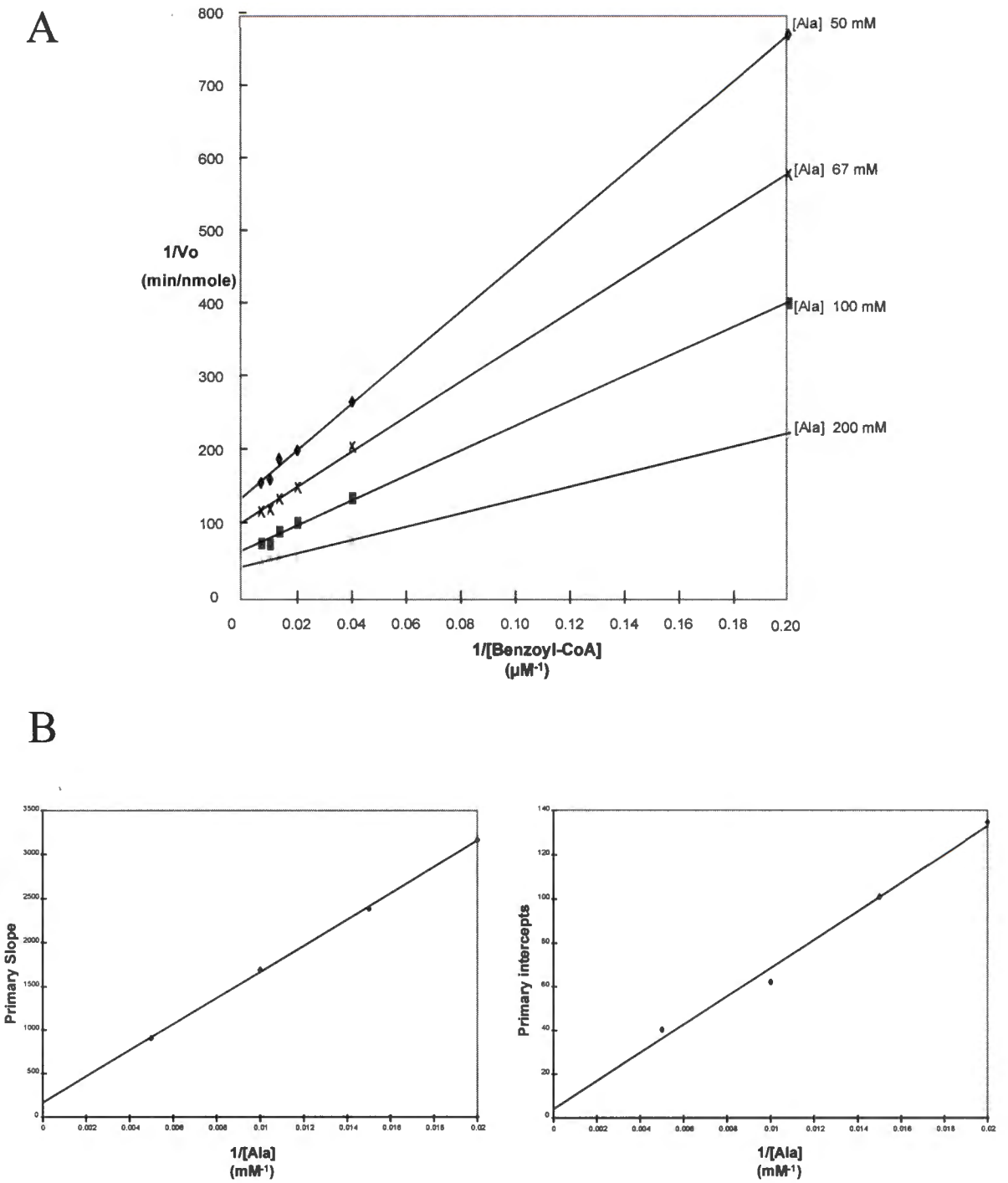


Figure 3.19 **Primary (A) double reciprocal and secondary (B) rate plots used for the determination of kinetic parameters for *benzoylalanine* formation using bovine GNAT.**

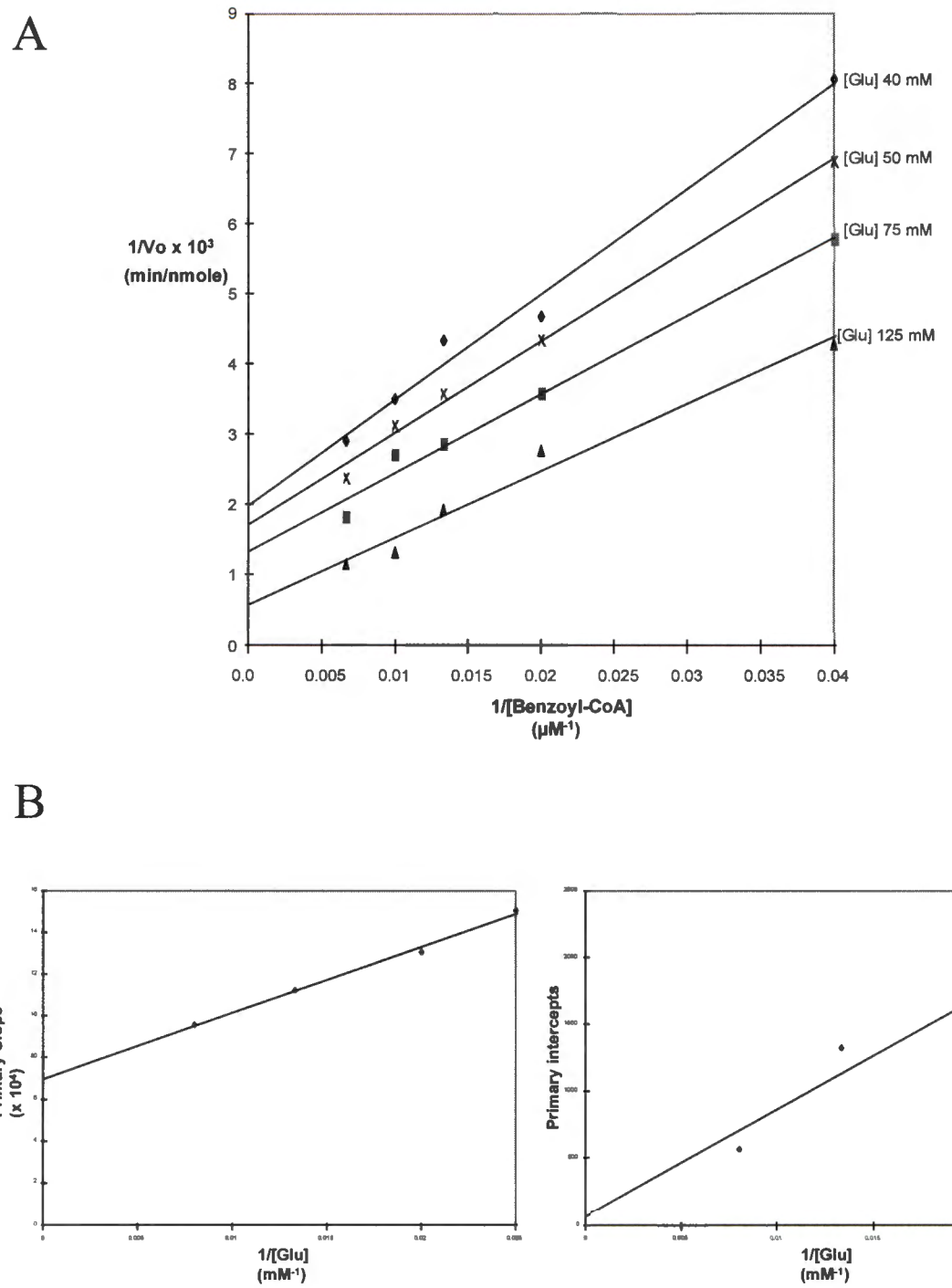


Figure 3.20 **Primary (A) double reciprocal and secondary (B) rate plots used for the determination of kinetic parameters for *benzoylglutamic acid* formation using bovine GNAT.**

Table 3.5 Kinetic parameters of bovine GNAT for the formation of benzoyl-amino acids.

Benzoylamino acid	$Km_{benzoyl\text{-}CoA}$ (μM)	$Km_{amino\ acid}$ (mM)	Vmax ($\mu mole.min^{-1}.mg^{-1}$)
benzoylglycine	160	6.2	28.2
benzoylglutamine	105	353	nq
benzoylasparagine	157	129	6.7
benzoylalanine	41	1573	1.6
benzoylglutamic acid	998	1148	0.1

nq: not quantifiable

The results (Table 3.5) confirmed that Gly is the best acyl acceptor for bovine GNAT, with the lowest $Km_{amino\ acid}$ (6.2 mM) compared to the other substrates. Even though our kinetic analysis was performed at 37 °C and not 30 °C, as was the case with most of the other studies on bovine GNAT, this compares well with previous reports of 3 mM (at 0.1 mM benzoyl-CoA, Nandi *et al.* (1979)), 15 mM (Kelley and Vessey, 1986), 2.0 mM (Kelley and Vessey, 1990), and 6 mM (bovine kidney GNAT, Kelley and Vessey, 1993). The higher utilization is also indicated in the much higher Vmax. Asn and Gln, the other two known substrates for bovine GNAT has twenty and fifty-seven times higher Km-values, at 129 mM and 353 mM respectively, with Asn having a similar lower Vmax (Vmax could not be determined for Gln for the reason explained in Section 3.4.2). The solubility of these two amino acids, however, does not allow rate determinations at even their respective Km-values, which is suboptimal for accurate determination of Km-values. Our data, including the comparative specific activities determined by spectrophotometry (Section 3.3, Table 3.2), clearly indicates that Asn is a much better substrate for bovine GNAT than Gln. Nandi *et al.* (1979), however, determined Km^{app}-values for Asn and Gln to be 170 and 130 mM respectively, at 0.1 mM benzoyl-CoA, which would suggest otherwise. The reason for this inconsistency is not clear. Gly and Asn shared similar $Km_{benzoyl\text{-}CoA}$-values (157 μM) with Gln somewhat lower at 105 μM. For Gly this value is rather high compared

to previous reports of 9, 20, 60, and 110 µM respectively (Bartlett and Gompertz, 1974; Nandi *et al.*, 1979; Kelley and Vessey, 1986; Kelley and Vessey, 1993). The $Km_{benzoyl\text{-}CoA}$-values for Asn and Gln are also much higher than the reported 45 µM for both amino acids, as reported by Nandi *et al.*(1979).

Kinetic parameters determined for Ala and Glu clearly indicate that these amino acids are very poor substrates for bovine GNAT, with $Km_{amino\ acid}$-values in the molar region (see Table 3.3). The same practical problem with respect to increasing the concentrations to levels higher than the Km-value also occurred with the kinetic analysis of these two amino acids. It was, however, possible to obtain straight lines in primary and secondary rate plots for these two amino acids (Figures 3.19 and 3.20). It was interesting to note that the Vmax for Ala is similar to that for Asn, which are the second best utilized amino acid substrate. This value is, off course, even less attainable than that of Asn when taking into consideration the high $Km_{amino\ acid}$ of Ala. Also interesting is the surprisingly low $Km_{benzoyl\text{-}CoA}$ (41.0 µM) obtained in the presence of Ala. Although no data on spectrophotometric kinetic analysis was presented in Section 3.3, early attempts to use this approach produced a similar low $Km_{benzoyl\text{-}CoA}^{app}$ (27 µM) when Ala was used as fixed substrate. It therefore appears possible that the adition of Ala decreases the dissociation of the GNAT-benzoyl-CoA complex and possibly increase the affinity of the enzyme for benzoyl-CoA (if Km would be equal to the true dissociation constant, Ks, of the GNAT-benzoyl-CoA complex).

3.5 Inhibition of benzoylglycine conjugation by formed benzoylamino acid conjugates.

Benzoylamino acid conjugates clearly share structural similarities. This have led us to investigate the inhibitory effect of benzoylamino acid conjugates on the benzoylglycine conjugation activity of bovine GNAT. The DTNB-based method was used in this case. GNAT activity was continuously monitored at 412 nm as described before (Kølvraa and Gregersen, 1986). Reactions proceeded at 37 °C in a final

volume of 400 µl, containing 20 mM Tris.HCl (pH 8.0), 0.1 mM DTNB, 0.5 - 1.0 µg enzyme with benzoyl-CoA (5 - 75 µM) and Gly (2 - 75 mM, pH 8.0) as the respective varying substrates. Gly concentration was fixed at 200 mM when benzoyl-CoA was varied and with varying Gly concentration, benzoyl-CoA was fixed at 0.1 mM (Nandi, 1979). Inhibitor concentrations were varied between 0.25 and 20 mM. As mentioned in Section 3.4.3, the effect of monovalent cations was taken into account by keeping it constant at 10 mM as far as possible. At this concentration it was found to have little effect on the GNAT activity. The linear increase in A_{412} over 5 minutes was used as initial velocity and kinetic constants of substrates and inhibitors were determined graphically using linear regression analysis of double reciprocal primary and secondary rate plots, following the steady state approach as described for general two substrate reactions (Engel, 1977; IUB, 1982).

Table 3.6 Inhibition of GNAT benzoylglycine formation by benzoylamino acids.

Benzoylamino acid	**Inhibition constants** (mM)				
	Variable [Benzoyl-CoA]		Variable [Gly]		
		K^{app}_{ic}		K^{app}_{iu}	K^{app}_{ic}
Benzoylglycine	Competitive	0.085	Mixed (Pure non-competitive)	0.23	0.23
Benzoylasparagine	Competitive	3	Mixed (Predominantly competitive)	8.6	3.9
Benzoylalanine	Competitive	7.9	Mixed (Predominantly competitive)	55.2	15
Benzoylserine	Competitive	4.1	Mixed (Predominantly competitive)	8.2	1.0
Benzoylglutamic acid	Competitive	8	Mixed (Predominantly uncompetitive)	11.8	23.8

K^{app}_{ic} : competitive inhibition constant for I (also known as *Ki*, (Palmer, 1995))
K^{app}_{iu} : uncompetitive inhibition constant for I (also known as *KI*, (Palmer, 1995))

A summary of data obtained from primary and secondary rate plots (not shown) of benzoyl conjugates of Gly, Asn, Ala, Ser, and Glu used as inhibitors is given in Table 3.6. The results show that, compared to benzoylglycine, all other conjugates had a substantially lower inhibitory effect with inhibition constants relatively high and in the mM region. Nevertheless, all conjugates exerted a competitive inhibitory effect with respect to varying benzoyl-CoA with K^{app}_{ic}-values increasing in the order benzoylglycine >> benzoylasparagine > benzoylserine > benzoylalanine ~ benzoylglutamic acid. Benzoylglycine at 500 μM showed an inhibitory effect on GNAT of nearly 50 % at the applied assay conditions, whereas the same inhibitory effect was only seen at concentrations higher than 20 mM with all of the other conjugates investigated. With respect to varying Gly concentrations, mixed inhibition patterns were observed with benzoylglycine giving a *pure non-competitive* inhibition pattern. With the exception of benzoylglutamic acid, which showed a *mixed, predominantly uncompetitive* inhibition (the terminology "non-competitive/uncompetitive inhibition" is also commonly used in this case (Palmer, 1995), a pattern of *mixed, predominantly competitive* inhibition (the terminology "competitive/non-competitive inhibition" is also commonly used) was observed with the rest of the benzoyl conjugates investigated with respect to varying Gly concentration.

Some investigation of inhibitors of GNAT have been presented (Webster *et al.*, 1976; Nandi *et al.*1979; Kelley and Vessey, 1990). Of the compounds investigated, phenylacetyl-CoA and indoleacetyl-CoA, the substrates for glutamine *N*-phenylacetyltransferase, have at this stage been identified to have the greatest inhibitory effect (competitive) with a Ki ~ 2 μM (Webster *et al.*, 1977; Nandi *et al.*, 1979). Gregus *et al.* (1996) identified lipoic acid, an important endogenous and also therapeutic compound, as an inhibitor of Gly conjugation of benzoic acid (IC_{50} = 0.3 mM) as well as renal excretion of benzoylglycine. Some divalent and monovalent cations, such as Mg^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, K^{+}, Na^{+}, Li^{+}, and Rb^{+} have some reversible inhibitory effects on GNAT activity at physiological substrate concentrations (Kelley and Vessey, 1990). Benzoylglycine, which theoretically should competitively inhibit GNAT with respect to benzoyl-CoA binding, as it binds to the same enzyme form (in

a compulsory order mechanism), and displays a mixed inhibition pattern with respect to Gly as varying substrate (Cleland, 1970), has been reported to have a Ki-value of 30 μM with respect to varying benzoyl-CoA concentration (Kelley and Vessey, 1990). This is somewhat lower than what was observed in this study (K^{app}_{ic} = 85 μM). Compared to the product inhibition of benzoylglycine, the other benzoylamino acids investigated in this study have much lower inhibitory effects on GNAT activity. The high inhibition constants of these compounds, in the mM range, are relatively high. Keeping also in mind that it is likely that these additional benzoylamino acids are formed at relatively low rates *in vivo* (See Section 2.5), and assuming transport of these compounds are simillar to that of benzoylglycine, our data would then suggest that they would have a negligible inhibitory effect on GNAT activity *in vivo*, if any at all.

3.6 Summary

I investigated the role of bovine glycine *N*-acyltransferase in order to determine the origin of amino acids to benzoic acid. Bovine GNAT was purified to near homogeneity and benzoylamino acid conjugation was investigated using the DTNB reduction based assay. Although Ala was detected as an additional, previously undetected, amino acid substrate for GNAT the detection limitations of the method were apparent. To solve this problem, ESI-MS-MS was developed into an quantification tool for benzoylamino acid conjugates in addition to the characterization capability that it was initially intended for. It was subsequently shown that bovine GNAT could not only utilize Gly, Asn and Glu, but also Ala, Glu and Ser as acyl acceptors for benzoyl-CoA. The Km-values obtained indicates that the order of reactivity was Gly >>> Asn > Gln > Ala > Glu. Although benzoylserine could be detected and quantified at high substrate levels, kinetic data could not be obtained as conjugation at lower substrate levels was not detectable. Inhibition studies using benzoylamino acid conjugates indicated very low levels of reversible inhibition, which suggests that these compounds would probably have little or no effect on GNAT function at the levels that these compounds are likely to exist *in vivo*.

CHAPTER 4

BENZOYLAMINO ACID CONJUGATION BY HUMAN GLYCINE *N*-ACYLTRANSFERASE

4.1 Introduction.

The study of GNAT began in the early 1950's using porcine and bovine liver (Schachter and Taggart, 1953, 1954) and it was not until the second half of the 1970's that the properties of human GNAT and the notable differences from bovine GNAT had been investigated (Webster *et al.*, 1976). As discussed in Section 2.3.1, these differences include not only protein structure, but also the amino acid substrates used by the two forms of GNAT. Unlike bovine GNAT, which can utilize Gly, Asn, and Gln as well as Ala, Glu, and Ser (as shown in the previous chapter), human GNAT is thought to be absolutely specific for Gly (Webster *et al.*, 1976; Gregersen *et al.*, 1986; Asaoka, 1991; Kelley and Vessey, 1994). New insights into the scope of benzoylamino acid conjugation for *bovine* GNAT (Chapter 3) derived from our investigation of GNAT amino acid substrate utilization, which was based upon new observations of low-level conjugates in the urine of patients with metabolic disorders. Our studies suggested that low-level benzoyl conjugation of amino acids other than Gly were also products for human GNAT.

4.2 Purification of human GNAT.

Permission was granted[4] to obtain liver tissue for a limited time and the use of it in this study was approved by the Ethical Committee of the Potchefstroom University for Christian Higher Education (Reference number BCH2M5-96). It was, for ethical

[4] Reference Number 16/7/4 in terms of Section 9 (3) of the Human Tissue Act, 1983 (Act 65 of 1983) as ammended by the Human Tissue Act, 1989 (Act 51 of 1989). Authorized by the Department of Health, Pretoria 0001, South Africa.

reasons, not possible to obtain fresh human liver in the same way and as often as bovine liver. Postmortem liver was obtained[5] from subjects younger than 40 years of age and within 12 hours after death. This material was suitable for this study since it has been shown that GNAT is stable postmortally at 4°C for as long as 72 hours (Caldwell *et al.*, 1976) and GNAT activity remains constant from ages 18 months to 40 years (Mawal *et al.*, 1997).

Human GNAT purification was based upon the purification of human GNAT described by Mawal and Qureshi (1994). As far as could be determined, this report is the only one in which human GNAT was purified to homogeneity. With the purification of human GNAT it was observed that the use of blue dextran Sepharose-4B affinity chromatography instead of chromatofocusing in the final step produced better results. This step was performed as described by Mawal and Qureshi (1994) with minor modifications. Blue dextran (Pharmacia) was immobilized onto Sepharose-4B (Pharmacia) as described by Ryan and Vestling (1974). The sample obtained from the gelfiltration step was applied to the column (1.5 x 3 cm), unbound protein washed out with five column volumes of Buffer A (Section 3.2.2), and the enzyme was eluted with a gradient of 50 - 100 mM KCl in Buffer A. The chromatograms of the last three steps of the purification protocol for human GNAT are given in Figures 4.1 - 4.3 and the results summarized in Table 4.1. In addition to Gly, Ala was also tested as substrate using the DTNB reduction assay.

Some differences between this purification results and that described for bovine GNAT (Section 3.2) were observed. From the onset a much lower yield in mitochondrial lysate was obtained from roughly the same amount of tissue. One reason for this is the longer time that it had taken before human tissue could be used. Salt precipitation led to an increase of almost three times in specific activity (benzoylglycine), which is a much lower increase for this step than that initially reported by Mawal and Qureshi (1994). As was the case with the bovine GNAT

[5] Department of Forensic Medicine, University of The Orange Free State, Bloemfontein 9300, South Africa.

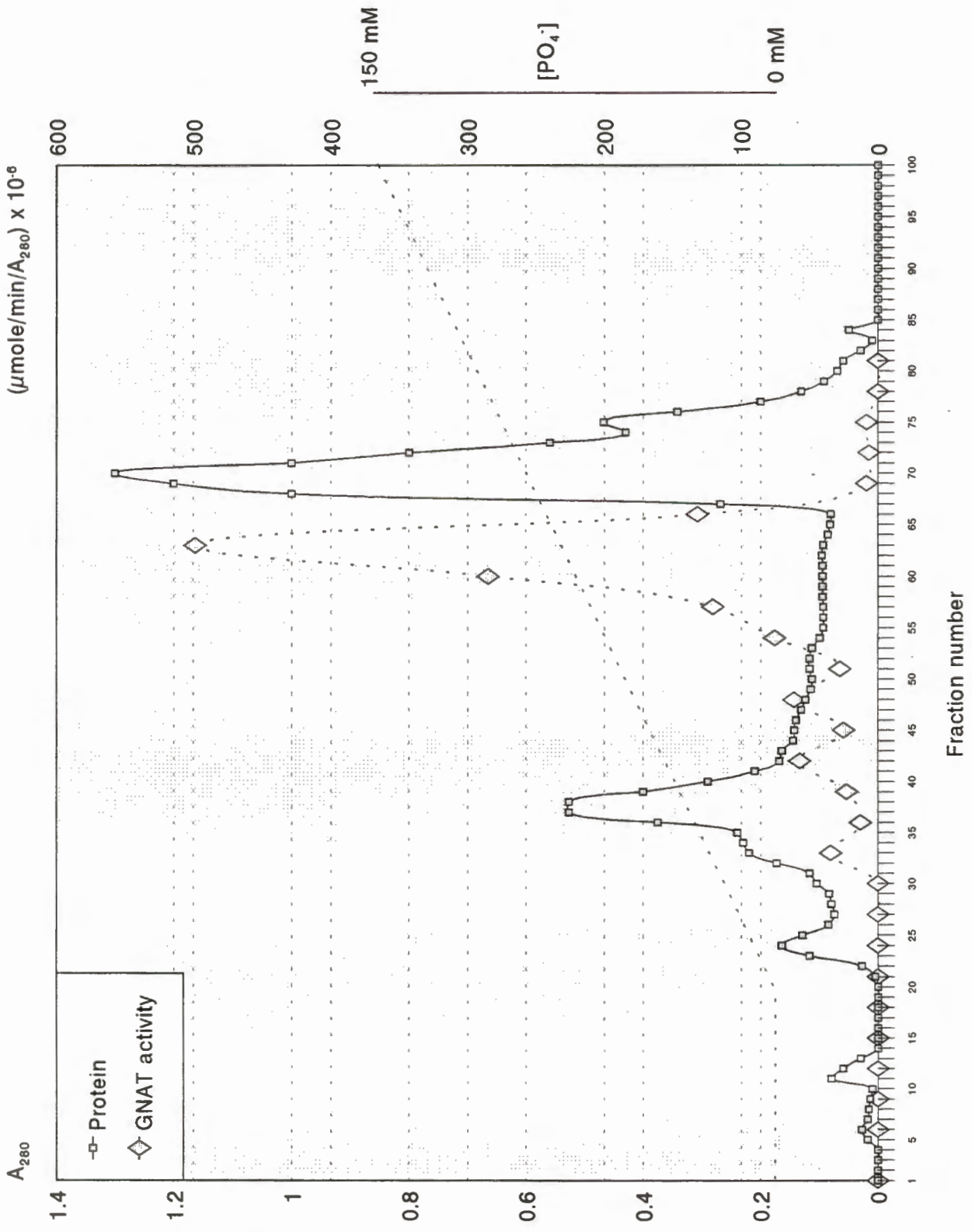


Figure 4.1 **Purification of human GNAT: Hydroxyapatite chromatography step** (see Section 3.2.3).

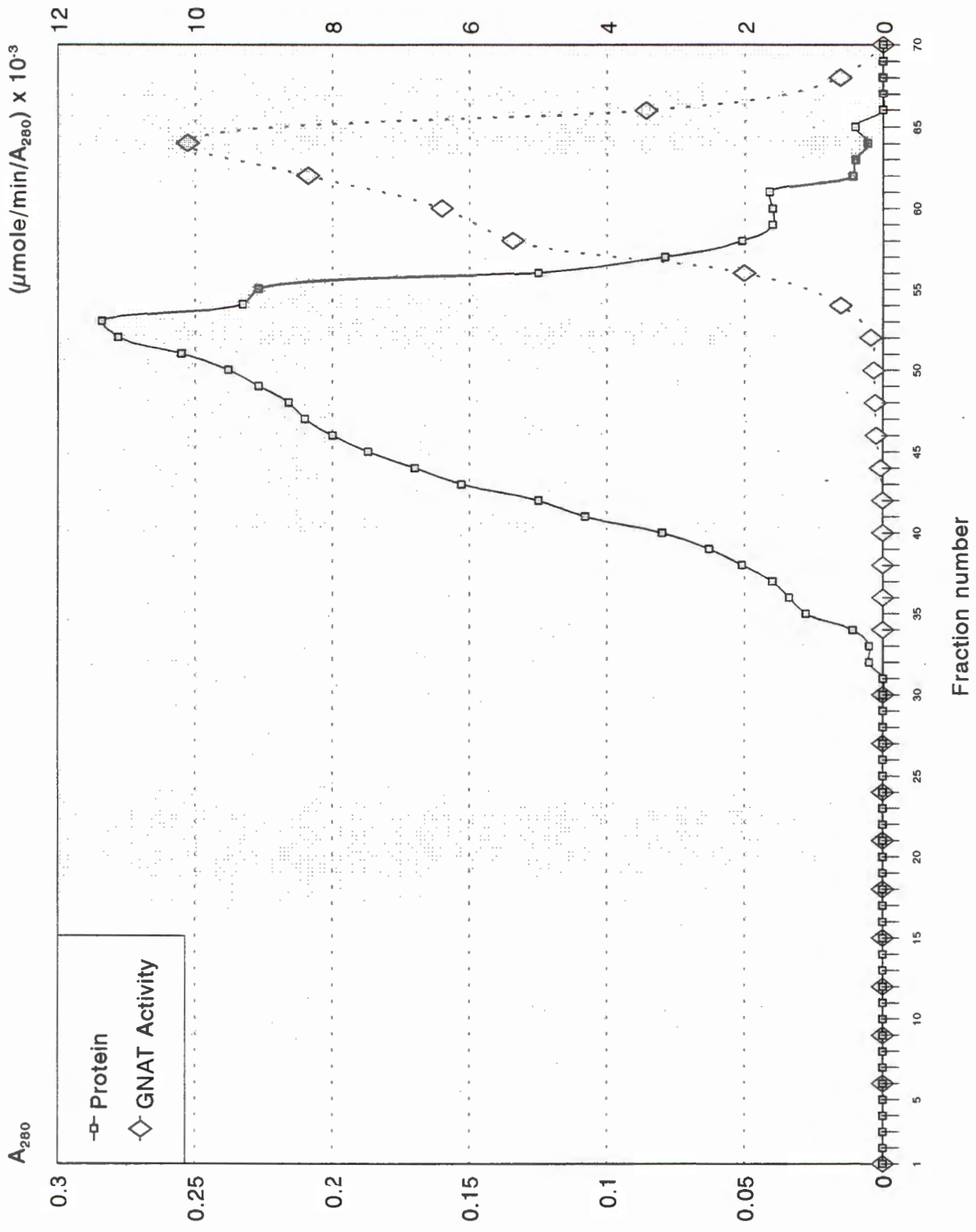


Figure 4.2 Purification of human GNAT: Gelfiltration step (see Section 3.2.4).

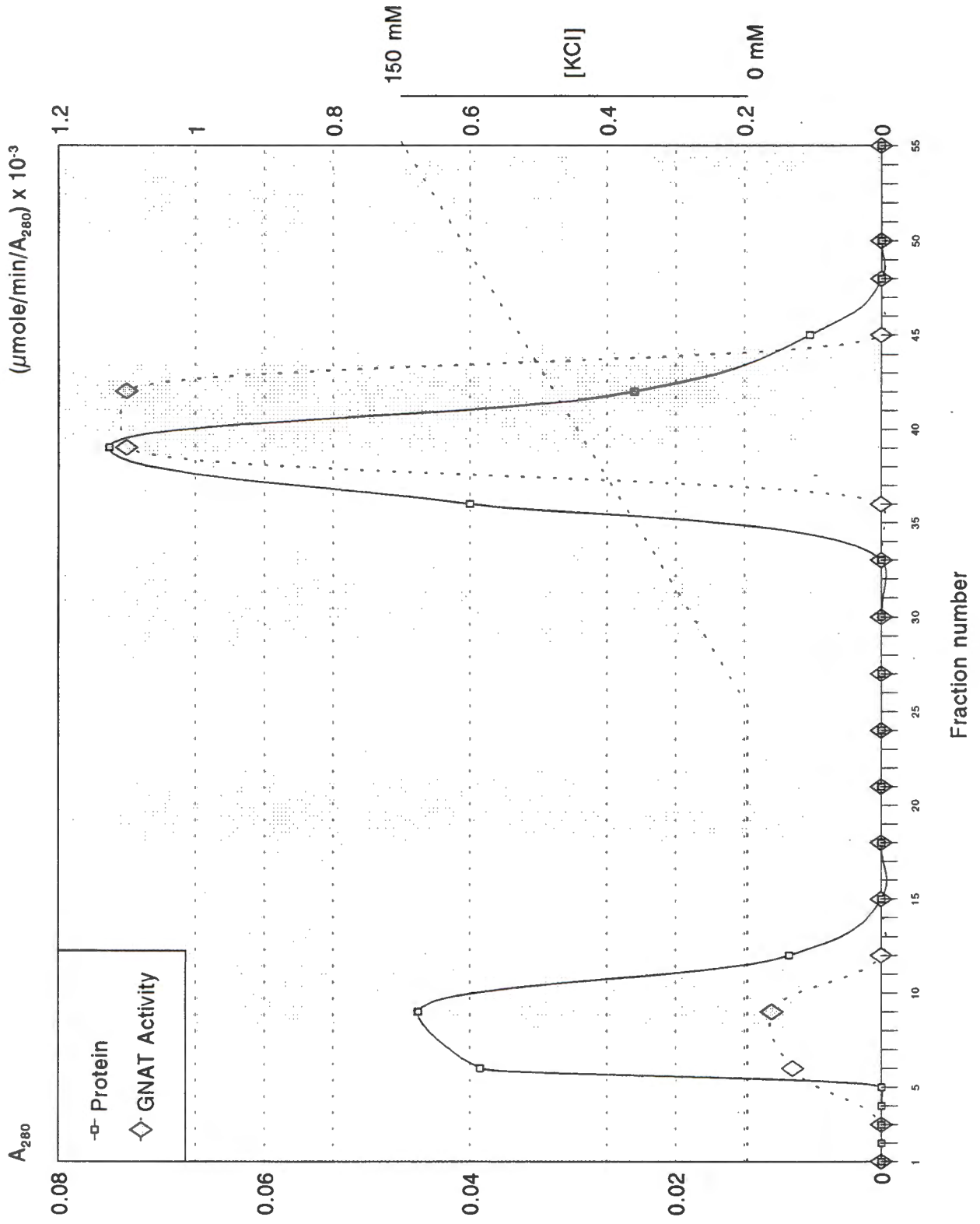


Figure 4.3 Purification of human GNAT: Blue Dextran Sepharose-4B chromatography step (see Section 4.2).

Table 4.1 Purification of human glycine *N*-acyltransferase.

Step	Volume (ml)	Total protein (mg)	[Protein] ($mg.ml^{-1}$)	Total Activity[1] ($nmole.min^{-1}$)	Benzoyl conjugation ($nmole.min^{-1}.mg^{-1}$)		Purification factor (relative to previous step)
					Gly[1]	*Ala*[2]	
1. Mitochondrial lysate	36	148	4.1	1672	11.3	*0.6*	1
2. $(NH_4)_2SO_4$ precipitation (40 - 60 %)	6	30	5.0	867	28.9	*1.4*	2.6
3. Hydroxyapatite chromatography	35	9.0	0.26	515	57.2	*3.4*	2.0
4. Gelfiltration	11	0.51	0.05	87	170.3	*10.0*	2.9
5. Blue Dextran Sepharose-4B	5	0.1	0.02	68	683.7	*33.0*	4.0

[1] Benzoylglycine formation.
[2] Benzoylalanine formation

purification, the loss in total activity was high compared to previous reports (Webster *et al.*, 1976; Nandi *et al.*, 1979). The hydroxyapatite chromatographic separation produced a different chromatogram (Fig. 4.1) from that for bovine (Fig 3.1, Section 3.2). As can be seen on the chromatogram, GNAT activity eluted in the region of fraction 60 - 65 and just before the second major protein elution peak. This elution profile was consistently obtained in a number of separations. Specific activity doubled after this step, which, even though it was higher than that achieved by Mawal and Qureshi (1994), seemed somewhat low if one considers the good separation that was achieved from this particular step.

Gelfiltration resulted into a much better separation of human GNAT activity (fractions 60 - 65, Fig 4.2) than was achieved with bovine GNAT purification. A three-fold increase in specific activity was observed, but with a five-fold loss in total GNAT activity. The subsequent use of an affinity resin, blue dextran, resulted in a four-fold increase in specific activity with a relative small loss in total activity. This also compares well to that reported by Mawal and Qureshi (1994). The amount of enzyme obtained after this step was 0.1 mg at 683.7 nmole.min^{-1}.mg^{-1}. Any further separations, for example the use of chromatophocusing before, after or instead of blue dextran affinity chromatography, resulted in a greater loss with little increase in specific activity.

The protocol used for human GNAT purification yielded a sixty-fold increase in specific activity using Gly as amino acid substrate (Table 4.1), which surpassed that for bovine purification (Table 3.1, Section 3.2). A similar increase, at activities more or less twenty times lower, was detected using Ala as substrate. As mentioned in Section 3.4.3, some of this activity could have been due to trace amounts of Gly contaminating the Ala preparation. However, subsequent analysis of the products using ESI-MS-MS (Section 4.4) confirmed that benzoylalanine were indeed formed by the enzyme preparations.

The overall increase in GNAT activity between the mitochondrial lysate preparation and the final GNAT preparation was almost six times higher than what was reported

by Mawal and Qureshi (1994), which produced a pure form of human GNAT at a specific activity of 13.9 μmole.min^{-1}.mg^{-1}. Even though the specific activity of our preparation was much lower, which could have been due to a number of reasons, such as the loss in activity, the overall increase seemed to be comparatively substantial.

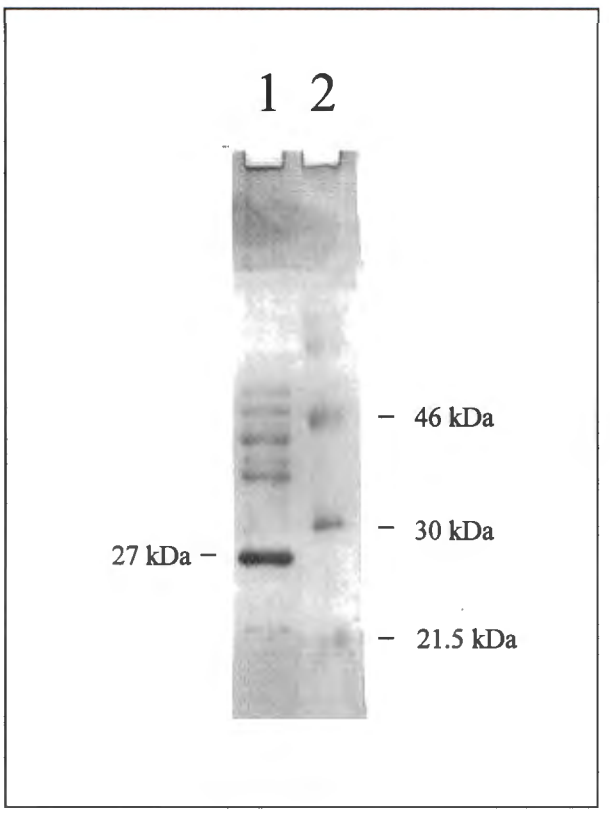


Figure 4.4 **SDS-PAGE analysis of purified human GNAT.**
A sample from the final enzyme preparation (lane 1) was electrophoresed on a 10 % SDS-PAGE gel in the presence of 0.1 % SDS. The gel was silver stained (Sasse and Gallagher, 1991) and compared with standard molecular weight markers (Amersham) as indicated in lane 2.

Evaluation of the homogeneity of the sample by SDS-PAGE (Figure 4.4), however, indicated that our final GNAT preparation was not homogenous, with at least nine contaminating minor protein bands when silver-staining the gel for a prolonged period. A major band with a molecular mass of 27 kDa was observed, which was

the band that became the prominent protein band during the purification procedure. This is smaller than the 30 kDa reported by Mawal and Qureshi. If the SDS-PAGE results shown in the publication of the latter authors are analyzed, and dissregarding the time that the sample and molecular weight markers were allowed to run, there may be some room for deviation from the reported mass of 30 kDa. As discussed in Section 2.3.3, there have been at least three different reports on the molecular mass of human GNAT, whith ranges between 24 and 30 kDa (Webster *et al.*, 1976; Mawal and Qureshi, 1994; Kelley and Vessey, 1994a). Thus, the major protein that eluted during our purification procedure is in the correct molecular mass region for human GNAT.

The homogeneity of the GNAT preparation was essential to us not both to determine whether GNAT is the catalyst of benzoyl-amino conjugation other than benzoylglycine, but also to assess kinetic parameters of GNAT without possible interfering factors. Additional changes to the purification protocol did not improve the homogeneity of the enzyme. The protocol was repeated with kind permission at the laboratory of Dr. K.M. Gibson[6], then at the Institute of Metabolic Disease, Baylor University Medical Center, Dallas, Texas. As was the case before, human GNAT could not be purified to homogeneity. Two options were considered for the further investigation of benzoylamino acid conjugation of human GNAT. The first was to use the final GNAT preparation in subsequent analysis and rely on the observation that the relative increase in benzoylalanine conjugation, which was similar to that of benzoylglycine (see Table 4.1), was an indication that GNAT indeed catalyzes the formation of both conjugates. The second option was to produce recombinant GNAT from an aquired putative GNAT cDNA, which will be discussed in the following section.

[6] Dr. K.M. Gibson. Biochemical Genetics Laboratory, Department of Molecular Genetics, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201-3098, USA.

4.3 Preliminary investigation into the production of recombinant human GNAT.

4.3.1 A putative cDNA for human GNAT.

The cDNA sequence of bovine GNAT has only recently been published (Vessey and Lau, 1996). It contains an open reading frame which encodes 295 amino acid residues in a 1199 bp cDNA clone and is consistent with previous data on the N-terminal sequence of the bovine liver and kidney enzyme (Kelley and Vessey, 1994). We acquired similar sequence data on the bovine GNAT N-terminal sequence as discussed in Section 3.2.6. While working at the laboratory of Dr. K.M. Gibson, protein and DNA database analyses of this sequence was performed with the kind assistance of Dr. K.L. Chambliss. During the analysis this amino acid sequence was matched to a 317 bp non-redundant expression sequence tag (Clone ID 124365) of a human fetal liver cDNA clone in the I.M.A.G.E. Consortium (LLNL) cDNA Clones Bank[7] (Lennon *et al.*, 1996). Subsequent sequencing of this clone, performed at the Jackson Laboratory[8], indicated that this cDNA clone consists of a novel 1083 bp sequence (Figure 4.5).

Analysis of the acquired cDNA (Figure 4.5) showed that it contains an open reading frame of 486 bp, encoding 162 amino acids, and an 86 and 511 bp 5'- and 3'-NTR, respectively. The bovine cDNA sequence contains a much larger open reading frame of 885 bp (295 amino acids) with a 126 and 203 bp 5'- and 3'-NTR, respectively (Vessey and Lau, 1996). Analysis of human and bovine translated sequences indicated no recognizable mitochondrial targeting signal, except for the motif, LRKSLP, at position 17. Mitochondrial targeting of GNAT may, however, occur through a nonconservative pathway via the cytoplasm. The unmodified translated product of this putative human GNAT cDNA clone should theoretically have a molecular mass of 18.3 kDa, which is much smaller than native purified human GNAT (24 - 30.5 kDa) as previously reported (Webster *et al.* 1976, Mawal and Qureshi, 1994, Kelley and Vessey, 1994a) or observed in this study (Section 4.2).

---

[7] Integrated Molecular Analysis of Genomes and their Expression.. Lawrence Livermore National Laboratory. The Regents of the University of California. Livermore, California 94550, USA.
[8] The Jackson Laboratory, 600 Main Street, Bar Harbour, Main 04605-1500, USA.

```
1     GCACGAGCTCCCAGAAGGGTGTTGCTCATCGTTTCTTCCCGGAAACATCTGCAGAGACTA
61    GCTTTTCAGGCTAAGGTATCCTCCATGATGTTACCATTGCAAGGTGCCCAGATGCTGCAG
                 human          MetLeuProLeuGlnGlyAlaGlnMetLeuGln
                 bovine         ...PheLeu.....................

121   ATGCTGGAGAAATCCTTGAGGAAGAGCCTCCCAGCATCCTTAAAGGTTTATGGAACTGTC
      MetLeuGluLysSerLeuArgLysSerLeuProAlaSerLeuLysValTyrGlyThrVal
      ..............................Met.......................

181   TTTCACATAAACCATGGAAATCCATTCAATCTGAAGGCTGTGGTGGACAAGTGGCCTGAT
      PheHisIleAsnHisGlyAsnProPheAsnLeuLysAlaValValAspLysTrpProAsp
      Met...Met............................Leu..................

241   TTTAATACAGTGGTTGTCTGCCCTCAGGAGCAGGATATGACAGATGACCTTGATCACTAT
      PheAsnThrValValValCysProGlnGluGlnAspMetThrAspAspLeuAspHisTyr
      ...Gln.........IleArg..................Lys..................

301   ACCAATACTTACCAAATCTACTCCAAAGATCCCCAAAACTGTCAGGAATTCCTTGGATCA
      ThrAsnThrTyrGlnIleTyrSerLysAspProGlnAsnCysGlnGluPheLeuGlySer
      ............HisVal......Glu...LeuLys.................AspLeu

361   CCAGAACTCATCAACTGGAAACAGCATTTACAGATTCAAAGTTCACAGCCTAGCCTGAAT
      ProGluLeuIleAsnTrpLysGlnHisLeuGlnIleGlnSerSerGlnProSerLeuAsn
      ......Val................................Thr...Ser.........

421   GAGGCTATACAAAATCTTGCAGCCATTAAGTCCTTCAAAGTCAAACAAACACAACGCATT
      GluAlaIleGlnAsnLeuAlaAlaIleLysSerPheLysValLysGlnThrGlnArgIle
      ...Val..................Thr..................ArgSerLysAsn...

481   CTCTATATGGCAGCTGAAACAGCCAAGGAACTGACTCCTTTCCTGCTGAAATCAAAGATT
      LeuTyrMetAlaAlaGluThrAlaLysGluLeuThrProPheLeuLeuLysSerLysIle
      ............Ser......Ile..............Ser......AspVal...Asn

541   TTATCTCCCAGTGGTGGCAAACCCAAGGCCATGTGAGTTTGATAAAATCCAGTCTGTACC
      LeuSerProSerGlyGlyLysProLysAlaMet*** 162
      ...ProValGlyAsp..............Ile... 295

601   ACTCACACTTCTCAAGTAACCTCCCAACTTCTCTCCCTGCATTCACTCTGGCTTTCCTAC
661   AATCAATGTTGTACAAAGAAATCAGAATTACATTTTTAAAAAATAACTCAGAACATGTTT
721   CTCTCCTGCTTAAAAGACTCCACCATCTCCCTTCTCATCTAGAATAAACCAGGCCTCTGG
781   CTACCACTTGGACCTTTATTGCCCTTCCTGCTTTCTTCCTCCAACAGCTCTGACCTTCAC
841   TCTGTTCCTCTTTCATCACAAGCTCATCTATATTCCAGGACTTCACATTTGTTATTTCTT
901   CTGTCTACTCTGTCTCTCAAGACAATAGAGGTCACAGCAAATACAAACCTTTATACTAAT
961   GGATTAAGTTTTCTTCTTGGACCCTCCATAGCAACCAAGAGATGTTAAAACTCTAATCCA
1021  TGGATGTTACCCATGCTCACTTGGTGAATAAATTCTGGCATTTTGGTGGTAAAAAAAAAA
1081  AAAA
```

Figure 4.5 **Nucleotide and deduced amino acid sequences of the putative human GNAT cDNA sequence.**

The stop codon is indicated with asterisks. A comparison with the deduced amino acid sequence of bovine GNAT cDNA (Vessey and Lau, 1996) is illustrated in the lower amino acid strand. Only differences in amino acids are indicated, with the amino acids displayed in the lower strand. Dots indicate identical residues.

```
Upper line: bovine GNAT, from 56 to 690
Lower line: human GNAT, from 16 to 1070

56    AGGGCTCCTCTTCAGGTGTTCCTGCAAAGCTTGGTGTGAAGAC.AGCTTTCCAGGCTTACGTGTCTTGCA
      ****     **    ** *     *  *      **   **** ****** ****** * ** ** * **
16    AGGGTGTTGCTCATCGTTTCTTCCCGGAAACATCTGCAGAGACTAGCTTTTCAGGCTAAGGTATCCTCCA

125   TGATGTTCCTGCTGCAAGGTGCCCAGATGCTGCAGATGCTGGAGAAATCCTTGAGGAAGAGCCTTCCTAT
      ******* *   ************************************************** **
86    TGATGTTACCATTGCAAGGTGCCCAGATGCTGCAGATGCTGGAGAAATCCTTGAGGAAGAGCCTCCCAGC

195   GTCCTTAAAGGTTTATGGGACCGTCATGCACATGAACCATGGAAACCCATTCAATCTAAAGGCCCTGGTG
       ***************** ** *** * ***** ********** ********** *****  *****
156   ATCCTTAAAGGTTTATGGAACTGTCTTTCACATAAACCATGGAAATCCATTCAATCTGAAGGCTGTGGTG

265   GACAAGTGGCCTGATTTTCAGACCGTGGTTATCCGCCCTCAGGAGCAGGACATGAAAGATGACCTTGATC
      ****************** * ** ****** ** *************** **** **************
226   GACAAGTGGCCTGATTTTAATACAGTGGTTGTCTGCCCTCAGGAGCAGGATATGACAGATGACCTTGATC

335   ACTACACTAATACTTACCATGTCTACTCTGAAGATCTTAAGAATTGTCAGGAATTCCTTGACTTACCAGA
      **** ** **********  *******  ******   * ** ***************  * ******
296   ACTATACCAATACTTACCAAATCTACTCCAAAGATCCCCAAAACTGTCAGGAATTCCTTGGATCACCAGA

405   AGTCATCAATTGGAAACAGCATCTGCAGATCCAAAGTACACAGTCCAGCCTGAATGAAGTAATACAAAAT
      * ******* ************ * ***** ****** ***** * ********** *  *********
366   ACTCATCAACTGGAAACAGCATTTACAGATTCAAAGTTCACAGCCTAGCCTGAATGAGGCTATACAAAAT

475   CTTGCAGCCACGAAATCCTTCAAAGTCAAGCGATCAAAAAACATTCTCTACATGGCATCTGAGACAATAA
      **********  ** ************** * * ** **  ********* ****** **** ***   *
436   CTTGCAGCCATTAAGTCCTTCAAAGTCAAACAAACACAACGCATTCTCTATATGGCAGCTGAAACAGCCA

545   AGGAACTGACTCCGTCCTTGCTGGATGTAAAGAACTTACCAGTTGGCGATGGCAAACCAAAGGCCATCGA
      ************* * * ***** *   *****  *** *     * * ********* ********
506   AGGAACTGACTCCTTTCCTGCTGAAATCAAAGATTTTATCTCCCAGTGGTGGCAAACCCAAGGCCATGTG

625   CCC.....
576   AGTTTGATAAAATCCAGTCTGTACCACTCACACTTCTCAAGTAACCTCCCAACTTCTCTCCCTGCATTCA

      ........................................................................
646   CTCTGGCTTTCCTACAATCAATGTTGTACAAAGAAATCAGAATTACATTTTTAAAAAATAACTCAGAACA

      ........................................................................
716   TGTTTCTCTCCTGCTTAAAAGACTCCACCATCTCCCTTCTCATCTAGAATAAACCAGGCCTCTGGCTACC

      ........................................................................
786   ACTTGGACCTTTATTGCCCTTCCTGCTTTCTTCCTCCAACAGCTCTGACCTTCACTCTGTTCCTCTTTCA

      ........................................................................
856   TCACAAGCTCATCTATATTCCAGGACTTCACATTTGTTATTTCTTCTGTCTACTCTGTCTCTCAAGACAA

      ........................................................................
926   TAGAGGTCACAGCAAATACAAACCTTTATACTAATGGATTAAGTTTTCTTCTTGGACCCTCCATAGCAAC

     ..AGAGATGTTTAAGCTCTCATCTGTGGATCCTAGCCACGCAGCTGTGGTGAACAGATTCTGGCTTTTCG
       ********* ** **** ***  *****  ** *** **     ******* * ******** *** *
996   CAAGAGATGTTAAAACTCTAATCCATGGATGTTACCCATGCTCACTTGGTGAATAAATTCTGGCATTTTG

686   GTGGC
      ****
1066  GTGGT
```

Figure 4.6 **Homology between putative human GNAT cDNA and bovine GNAT cDNA (Vessey and Lau, 1996).**
Alignment was performed using the DNAman sequence analysis program. *K-tuple* was set at 4 and the *gap penalty* at 7. Asterisks indicate the homology between the two sequences.

The sequence data has been submitted to the GenBank data base (GenBank AF023466). Data bank analysis was frequently performed (the last on the 25th of May 1998) using analysis facilities such as the BLAST (Basic Local Alignment Search Tool, Altschul *et al.*, 1990) facility at the Internet site[9] of the NCBI (National Center for Biotechnology Information, USA). As was expected, the nucleotide sequence were matched to the bovine GNAT cDNA sequence (Vessey and Lau, 1996; EMBL accession number embAJ223301), of which the alignment is illustrated in Figure 4.6. As shown in this figure these two sequences share 82 % homology with a BLAST score of 1764 (Karlin and Altschul, 1990). The results further indicate that the human cDNA sequence displays 77 % homology (BLAST score 1512) with the bovine glutamine *N*-phenylacetyltransferase cDNA sequence, which was recently submitted to the EMBL data bank (EMBL accession number embAJ001396). Apart from these two sequences, no other significant matches to this human cDNA were observed.

Comparison of the deduced *amino acid* sequence of the three cDNA's indicated that the homology shared between the putative human and bovine GNAT sequences is somewhat larger (77 %, as illustrated in Fig 4.5) in comparison to the homology shared between any of the two GNAT sequences and the bovine glutamine *N*-phenylacetyltransferase sequence (70 %). Thus, based on the nucleotide and amino acid sequence comparisons, there appears to be a higher probability that the acquired human cDNA may be the cDNA for human GNAT rather than glutamine *N*-phenylacetyltransferase. This, however, could only be established without doubt by either comparing with amino acid sequence of human GNAT, or by analysis of the properties of the translated protein of the acquired cDNA. Since no amino acid sequence data of human GNAT was (and still is) unavailable, our approach was to express the cDNA in a prokaryotic system and investigate the properties of the recombinant protein.

[9] http://www.ncbi.nlm.nih.gov/

4.3.2 Prokaryotic expression of the putative human GNAT cDNA.

The acquired cDNA clone was contained in the vector pT7TPac (Pharmacia), which was used to construct the cDNA library. For expression the prokaryotic expression vector, pGEX4T2 (Pharmacia) was employed as vector. With this vector system the insert DNA fragment is transcribed and translated into a fusion protein with glutathione S-transferase (GST), which can then be removed by thrombin.

The subcloning strategy used is schematically illustrated in Figure 4.7. As indicated in this figure, PCR was used to amplify the cDNA from the vector pT7TGNAT as well as to insert a 5' *Bam* HI-site to assist subcloning into pGEX4T2. The optimum $MgCl_2$ concentration and primer annealing temperature for PCR was 2 mM and 50 °C, respectively. To prevent mistakes during amplification, the Expand™ High Fidelity PCR System (Boehringer Mannheim) was used. Modified PCR fragments were subcloned into the base vector to form two vectors, pGG101 (with 3' NTR intact) and pGG102 (without 3'NTR). Standard DNA modification, cloning and analysis procedures were used as described before (van der Westhuizen, 1993). The plasmids were characterized by restriction endonuclease digestion using the enzymes indicated in Figure 4.7 (results not shown). DNA sequencing confirmed that the inserted cDNA was in the correct reading frame with respect to the glutathione transferase fusion protein. Plasmid DNA of both vector constructs were transformed into the E.*coli* host strains, BL21, Top10, MC1061, NM522, and TB1. Single colonies of transformed E.*coli* were cultured and expression was induced with 0.1 mM IPTG at 37 °C, as instructed by the supplier of the expression system (Pharmacia). The induction time (0 - 16 hours) as well as the IPTG concentration (0 - 10 mM) were varied to investigate the effect of these parameters on the expression of the fusion protein. Cell lysates (sonicates) were analyzed by SDS-PAGE, and the formation of a fusion protein with a molecular mass of 47 kDa was assessed.

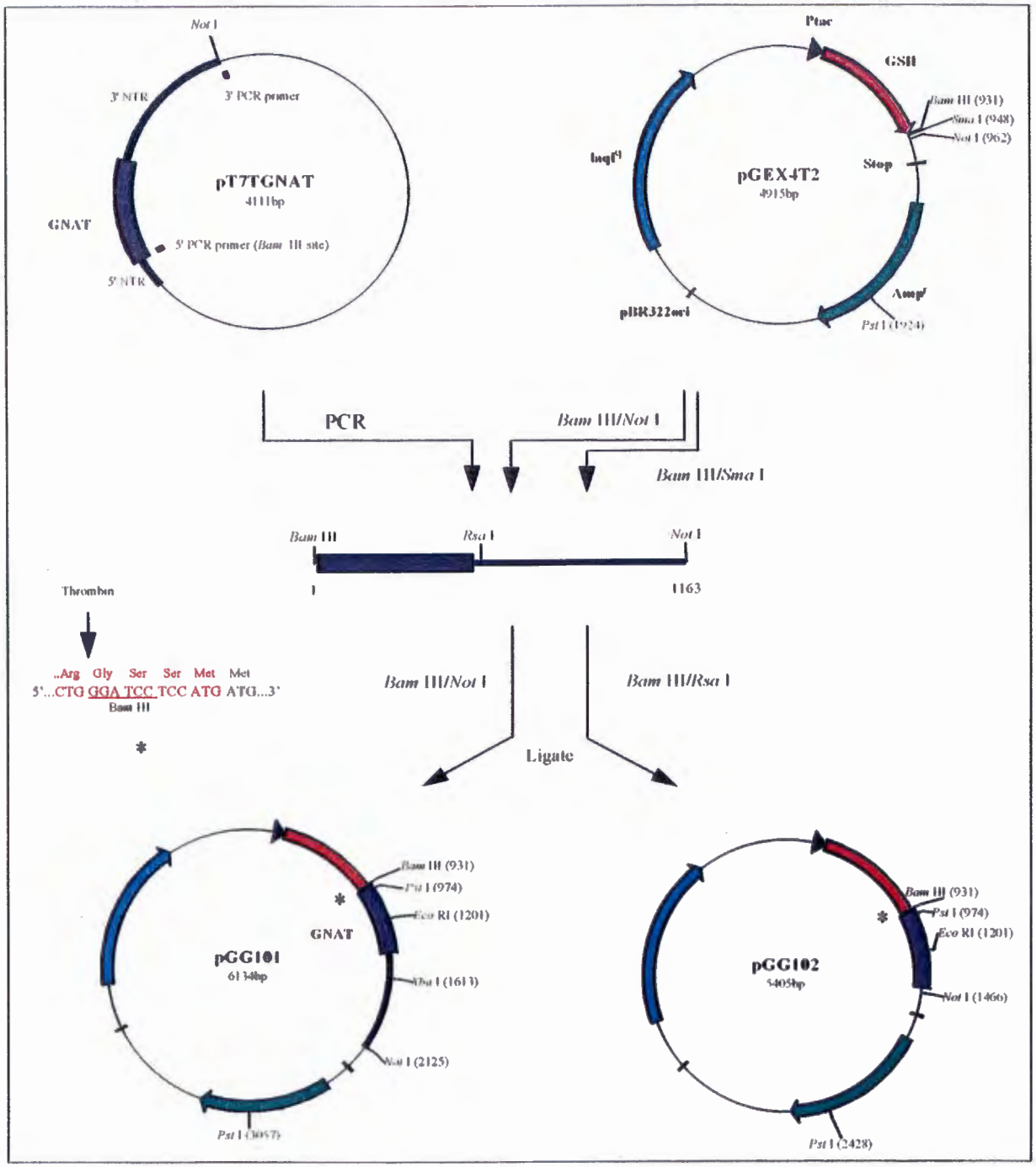


Figure 4.7 **Subcloning of putative human GNAT cDNA to form the expression vectors pGG101 and pGG102 (see Section 4.3.2).**
Only those restriction endonuclease that were used in subcloning and characterization of the constructs are shown. The asterisk indicates the nucleotide sequence and reading frame at the 5' end of the inserted DNA fragments, with the initiation codon indicated in blue.

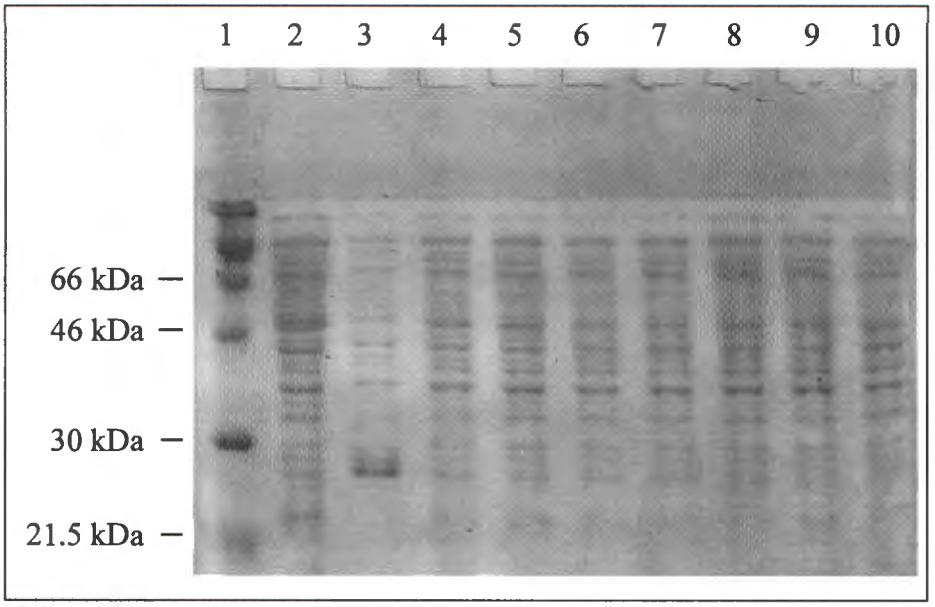


Figure 4.8 **SDS-PAGE analysis of putative human GNAT cDNA expression in E.*coli* BL21.**

Cells were incubated for 4 hours (37 °C) at various IPTG concentrations. 10 µl of the sonicate was loaded on an 10 % SDS-PAGE gel. The gel was stained with Coomasie Blue (Sasse and Gallagher, 1991).

Lane 1. Standard molecular weight markers (Amersham).
2. Untransformed E.*coli* BL21 (1 mM IPTG).
3. pGEX4T2-transformed E.*coli* BL21 (1 mM IPTG).
4 - 10. pGG101-transformed E.*coli* BL21 at 0, 0.05, 0.1, 0.5, 1, 5, and 10 mM IPTG.

Figure 4.8 shows the SDS-PAGE result that was obtained when the recommended E.*coli* host, BL21, containing pGG101 was analyzed after a 4 hr induction period at increasing IPTG concentration. As can be seen in this figure (lane 3), induction of a pGEX4T2 transformed colony induced expression of unfused GST (29 kDa). This protein could be isolated using Glutathion Sepharose 4B affinity matrix (Sigma). However, the induced expression of an expected 47 kDa fusion protein, or any other size, was not observed. This was the case with all E.*coli* strains and at any tested induction time or IPTG concentration (results not shown). No improvement of expression was observed with a lower incubation temperature either.

A number of reasons can be proposed to explain these results. The most likely explanations, including incorrect translation frame, poor induction conditions,

insufficient lysis, were unlikely the cause, since these factors were thoroughly tested. It is possible that inclusion bodies may have formed even though the use of less optimal induction conditions (lower temperature, shorter induction time, lower IPTG concentration) and the inclusion of Triton X-100 (1 %) in the cell lysates, as recommended by the supplier of the expression system (Pharmacia), did not improve the results. We intend to investigate the use of denaturants to improve solubilization of inclusion bodies that may have formed (Riggs, 1991). Expression may also have been too low for detection, but large scale preparations and subsequent attempts to isolate expressed fusion protein using the affinity matrix did not produce any protein. We are also considering the use of eukariotic expression systems to investigate the identity and function of this particular cDNA as possible post-translational modification of GNAT may be another aspect to investigate.

4.4 Investigation of human GNAT benzoylamino acid conjugation.

4.4.1 Introduction.

For the investigation of human GNAT conjugation our initial objective was to use liver GNAT purified to homogeneity. However, as discussed in Sections 4.2 and 4.3, neither GNAT (homogenous) nor recombinant GNAT could be obtained. The investigation of human GNAT benzoylamino acid conjugation using partially purified human GNAT preparations was continued for two reasons: Firstly, the observation that benzoylalanine formation monitored in the various pooled fractions during the purification process increased with approximately the same factor relative to benzoylglycine formation suggested that these two activities were associated with the same enzyme (Table 4.1). Secondly, the fact that *bovine* GNAT-catalyzed benzoylalanine conjugation was a further indication that the benzoylalanine conjugation detected using partially purified human GNAT could be attributed to human GNAT. For the characterization and kinetic analysis of benzoylamino acid conjugation the ESI-MS-MS-based assay was used, since the investigation of bovine

GNAT activities indicated that the DTNB-based method could be misleading (see Section 3.3).

4.4.2 Detection, characterization and kinetics of human GNAT-catalyzed benzoylamino acid conjugation.

Benzoylamino acid conjugation of human GNAT was investigated using the same approach, materials and methods as described for the study of bovine GNAT conjugation (Section 3.4). Conjugation of the amino acids listed in Table 4.2 with benzoyl-CoA was initially performed at standard conditions described for human GNAT, which was 20 mM Tris.HCl (pH 8.0, 37 °C), 100 μM benzoyl-CoA, and 100 mM amino acid (pH 8.0, 37 °C) (Mawal and Qureshi, 1994). For amino acids other than glycine the concentrations were also increased to the maximal level. Benzoyl conjugates were characterized as described in Section 3.4.1 and quantified as described in Section 3.4.2, using ESI-MS-MS.

Three different conjugates, benzoylglycine, benzoylalanine, and benzoylglutamic acid were detected using both mitochondrial lysate as well as the human GNAT preparation. The CID mass spectra for the latter two are given in Figure 4.9. We applied the same ESI-MS-MS conditions as employed for characterization of the conjugates investigated for bovine GNAT (Section 3.4.1). The CID mass spectra were compared to that of the corresponding synthesized conjugates illustrated in Figure 3.8A (benzoylalanine) and Figure 3.10A (benzoylglutamic acid) (Section 3.4.1). For enzymatically produced benzoylalanine the daughter ion fragments of *m/z* 105, 77, 44, 148, 194, 95 were detected (Figure 4.9A). This corresponded to that of the chemically synthesized conjugate. CID of benzoylglutamic acid formed daughter fragment ions of *m/z* 105, 84, 140, 186, 188, 262, 290, 130 (Figure 4.9B), which corresponds to that of the chemically synthesized conjugate. It was therefore no doubt that the benzoyl conjugates of Ala and Glu were enzymatically formed.

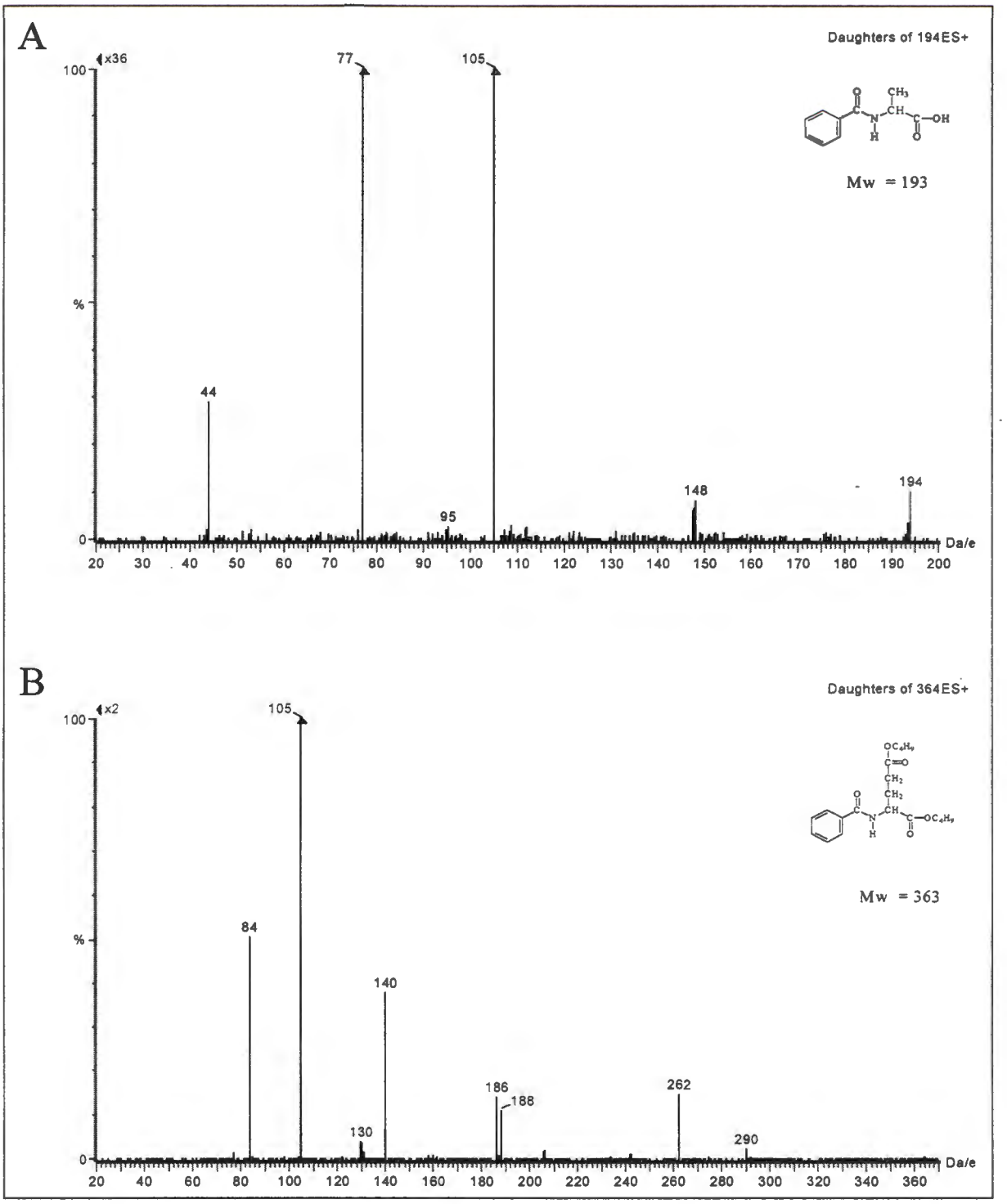


Figure 4.9 **CID mass spectra of partially purified human GNAT-catalyzed benzoylalanine (A) and benzoylglutamic acid (B).**
The settings for ESI-MS-MS as well as processing of data were as described in Section 3.4.1.

Table 4.2 Benzoylamino acid conjugates formed by human mitochondrial and partially purified human GNAT preparations (measured by ESI-MS-MS).

Amino acid substrate	Benzoylamino acid conjugation* (nmole.min^{-1}.mg^{-1}) (n = 2)	
	Mitochondrial lysate	GNAT preparation
Gly	9.2 ± 0.8	543 ± 21
Ala	0.4 ± 0.1	27 ± 4.5
Glu	0.07 ± 0.03	4.8 ± 0.7
Gln	<D	<D
Asn	<D	<D
Ser	<D	<D
Asp	<D	<D
Ile	<D	<D
Leu	<D	<D
Val	<D	<D

*Benzoyl-CoA was used as acyl-donor at 0.1 mM and the amino acid concentration was fixed at 100 mM (pH 8.0), except for Asp (38 mM).
D = minimal activity that could be measured

The specific activities of formation of the various benzoylamino acid conjugates formed by using mitochondrial lysate preparations and human GNAT preparation are presented in Table 4.2. This data summarizes the activities at the same reactant concentrations (100 mM amino acid) except for Asp which was set at 38 mM. Benzoylglycine, -alanine, and -glutamic acid were detected in both mitochondrial lysate and GNAT preparations. At these reaction conditions, and even at higher substrate concentrations, none of the other amino acids conjugated with benzoyl-CoA.

The specific activity of benzoylglycine formation was similar to that measured using the DTNB-based method. It was, however, much lower than previously reported by Mawal and Qureshi (1994) using human mitochondrial lysate and GNAT preparations (1.2 and 13.9 μmole.min^{-1}.mg^{-1} respectively). Benzoylalanine was detected under the same reaction conditions at a rate twenty times lower than benzoylglycine in both preparations. This is comparable to the results obtained during the purification process (Section 4.2, Table 4.1). Benzoylglutamic acid formation, on the other hand, was measured at rates 130 and 113 times lower compared to benzoylglycine using human mitochondrial lysate and GNAT preparations, respectively. It is interesting to note that benzoylalanine and benzoylglutamic acid were formed at nearly the same

rate by *bovine* GNAT, whereas benzoylglycine formation was almost twenty times lower in the human compared to the bovine system (see Table 3.4, Section 3.4.3).

The formation of the newly-detected conjugates were also investigated (in duplicate) at various time-intervals and protein concentrations using the reaction composition described earlier in this section. Benzoylalanine formation increased linearly over 30 minutes (Figures 4.10A) whereas benzoylglutamic acid formation increased hyperbolically over the same period (Figure 4.11A). This was also the case with *bovine* GNAT formed benzoylglutamic acid (Section 3.4.3). As illustrated in Figures 4.10B and 4.11B, both conjugates were formed linearly with respect to increased protein content.

For the determination of the kinetic parameters, Km and Vmax, the same reaction conditions were used as described for bovine GNAT (Section 3.4.3), where conjugation proceeded at pH 8.0 and 37 °C. In this case benzoylamino acid concentration was varied between 15 to 150 μM at a number of fixed amino acid concentrations. Gly was set between 1 and 20 mM, Ala between 20 and 400 mM, and Glu between 7.5 and 150 mM. Reactions were terminated after 10 minutes when Ala and Glu were substrates, or at 5 minutes when Gly was used. The limited amount of human material that was available prevented the reactions to be performed in duplicate. Benzoyl conjugates were quantified and kinetic parameters determined as described in Section 3.4.3. The primary and secondary rate plots are given in Figure 4.12 (benzoylglycine) and Figure 4.13 (benzoylalanine) and the Km^{app}- and Vmax-values are summarized in Table 4.3. As was the case with benzoylserine in the previous Chapter (Section 3.4.3), accurate kinetic data could not be obtained for benzoylglutamic acid in this case. The combination of the relatively low rates at which this conjugate forms and the limitations that solubility of Glu presents, made it extremely difficult to obtain data at low substrate concentrations, which is necessary for accurate kinetic analysis.

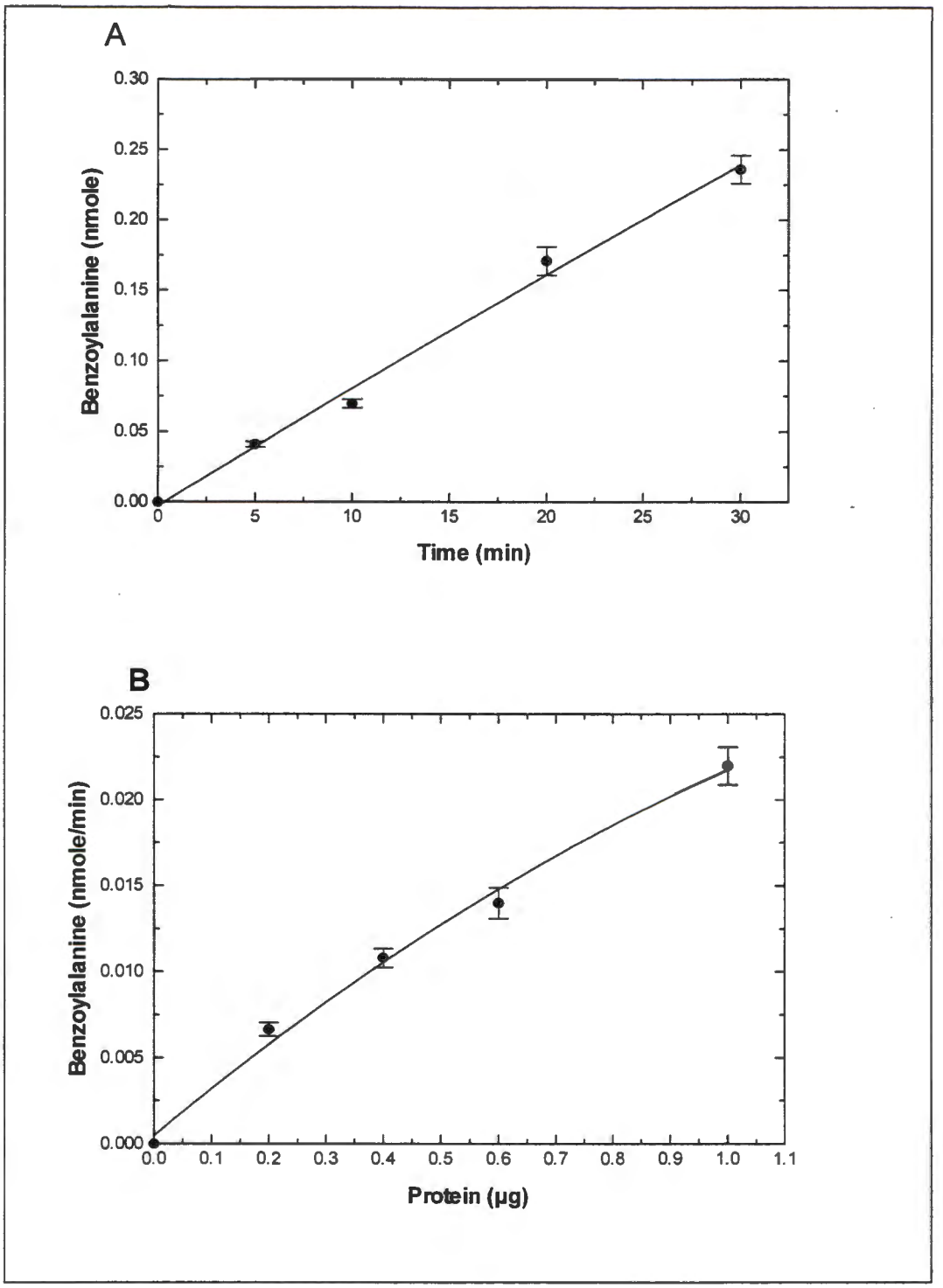


Figure 4.10 **Time (A) and protein-dependent (B) formation of benzoylalanine by human GNAT. In B the assays were run for 10 minutes. The standard deviation of the mean (n = 2) are indicated on the graph.**
Reaction conditions were as described in Section 4.4.2.

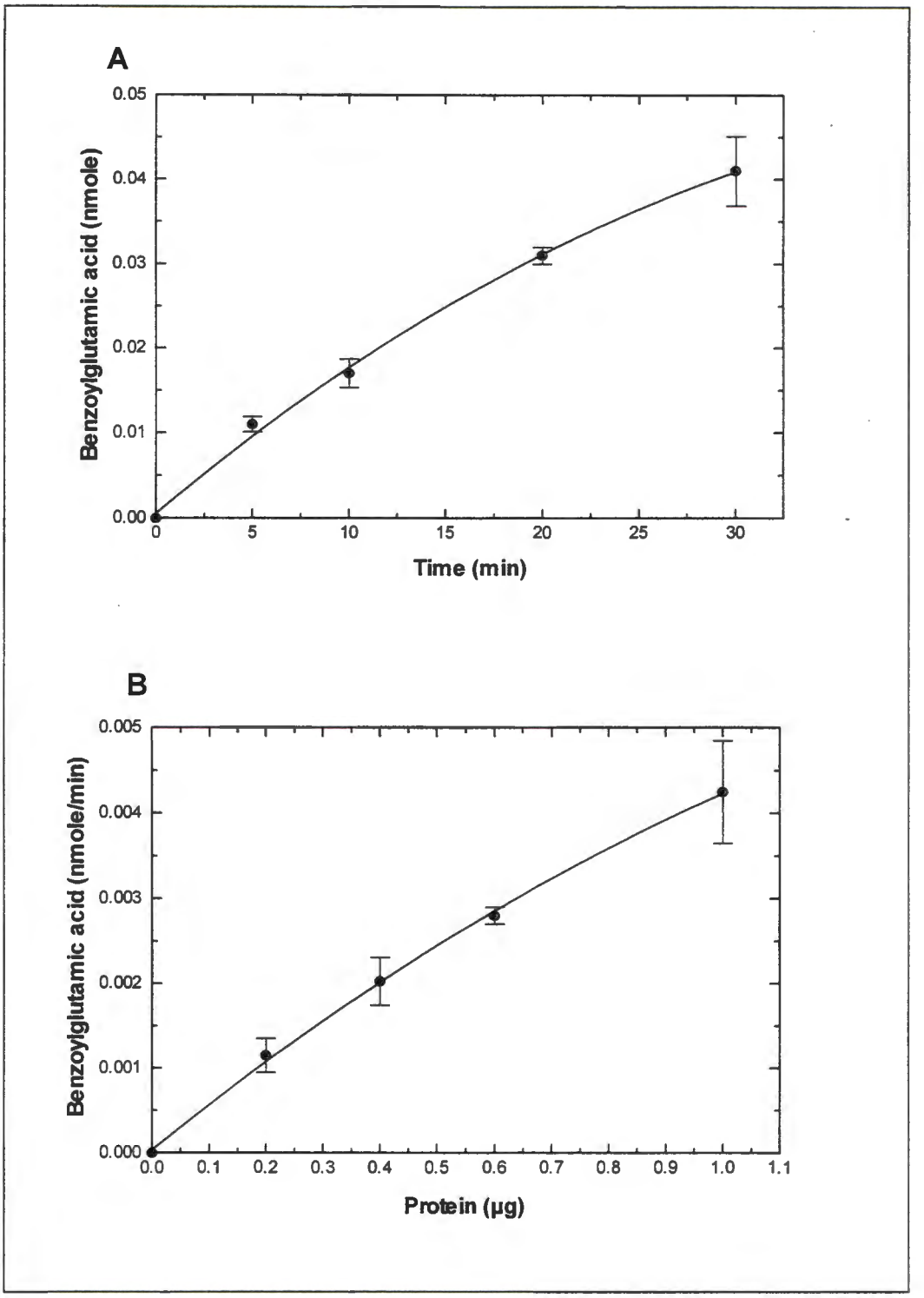


Figure 4.11 Time (A) and protein-dependent (B) formation of benzoylglutamic acid by human GNAT.
Reaction conditions were as described in Section 4.4.2.

For kinetic analysis of benzoylglycine and benzoylalanine formation, six sets of reaction velocity data were derived which could be plotted on primary rate plots (Figures 4.12A and 4.13A). Subsequent secondary rate plots (Figures 4.12B and 4.13B) were used for the determination of the kinetic parameters. As indicated in Table 4.3 the $Km^{app}_{benzoyl\text{-}CoA}$, when using Gly as fixed substrate, was 13.0 μM. Three previous studies reported different values for this parameter which ranged between 6 μM (Webster *et al.*, 1976), 57.9 μM (Mawal and Qureshi, 1994) and 67 μM (Kelley and Vessey, 1994a). These studies utilized 30 °C as temperature for the enzyme assays, which may explain the differences. Unlike bovine GNAT, where the $Km_{benzoyl\text{-}CoA}$ was decreased by the use of Ala as second substrate, a similar value (15.2 μM) was obtained in our study.

Table 4.3 Kinetic parameters of human GNAT for the formation of benzoylamino acids.

Benzoylamino acid	$Km^{app}_{benzoyl\text{-}CoA}$ (μM)	$Km^{app}_{amino\ acid}$ (mM)	Vmax ($nmole.min^{-1}.mg^{-1}$)
benzoylglycine	13.0	6.4	658
benzoylalanine	15.2	997	290

The Km^{app}_{Gly} for human GNAT was 6.4 mM, which compares well with the value (6.5 mM) reported by Kelley and Vessey (1994a). This is somewhat lower than the value (20 mM) reported by Webster *et al.* (1976). As was expected, Km^{app}_{Ala} was much higher at almost 1M (997 mM). Consequently, the Vmax-value for benzoylalanine formation is much lower than that of benzoylglycine (290 $nmole.min^{-1}.mg^{-1}$, compared to 658 $nmole.min^{-1}.mg^{-1}$) and is practically unachievable due to the high Km^{app}_{Ala}-value. As discussed in Section 3.4.3, the relatively high $Km^{app}_{amino\ acid}$-value that was observed for Ala, and which would most likely also be the case for Glu if it can accurately be determined, presents the problem that setting amino acid concentrations higher than the Km-values would be impossible due to the solubility of these amino acids. These results again underscores the fact that Gly is a much better substrate for

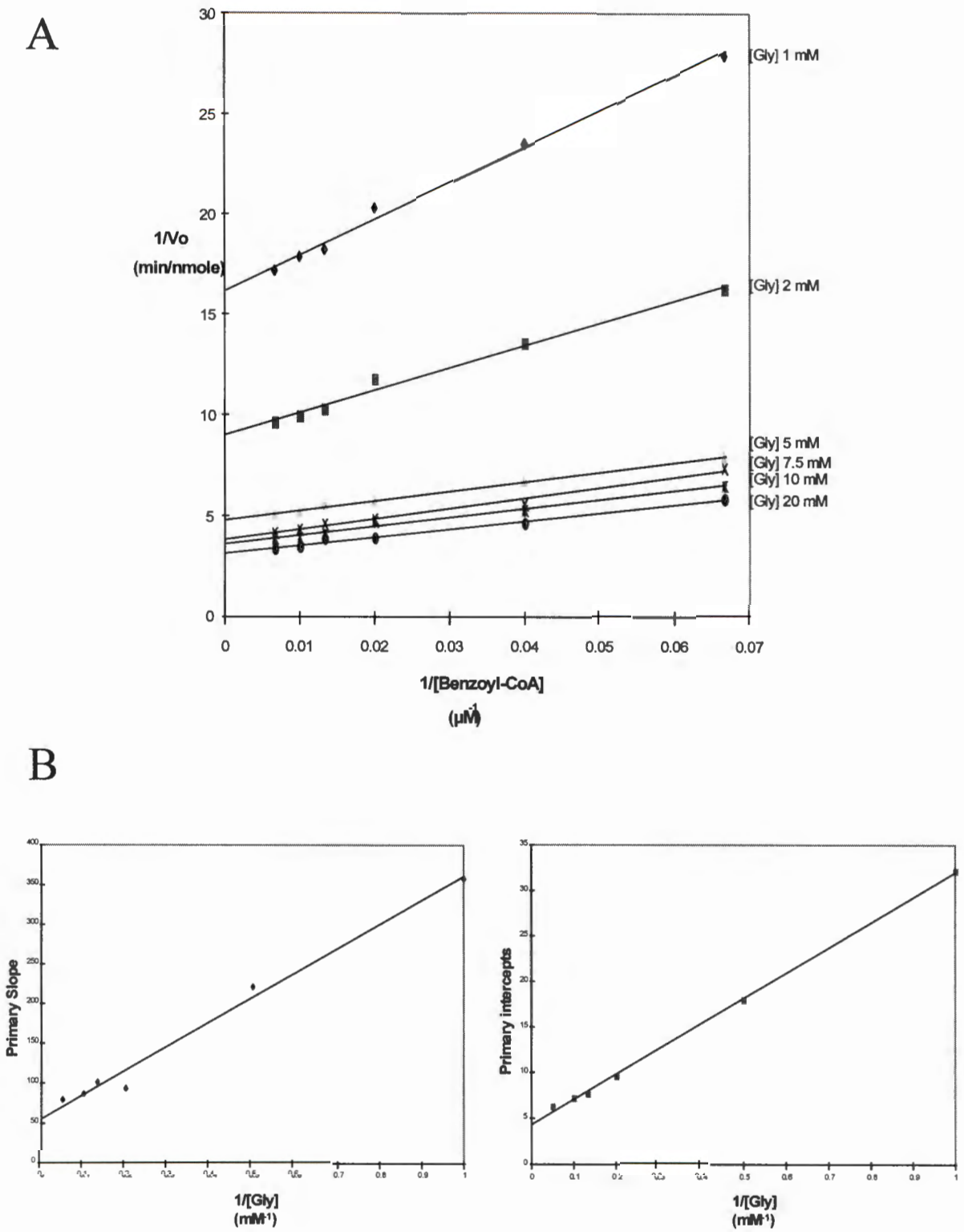


Figure 4.12 **Primary (A) double reciprocal, and secondary (B) rate plots employed for the determination of kinetic parameters for *benzoylglycine* formation using human GNAT.**
V_o was measured by ESI-MS-MS (Section 3.4.3).

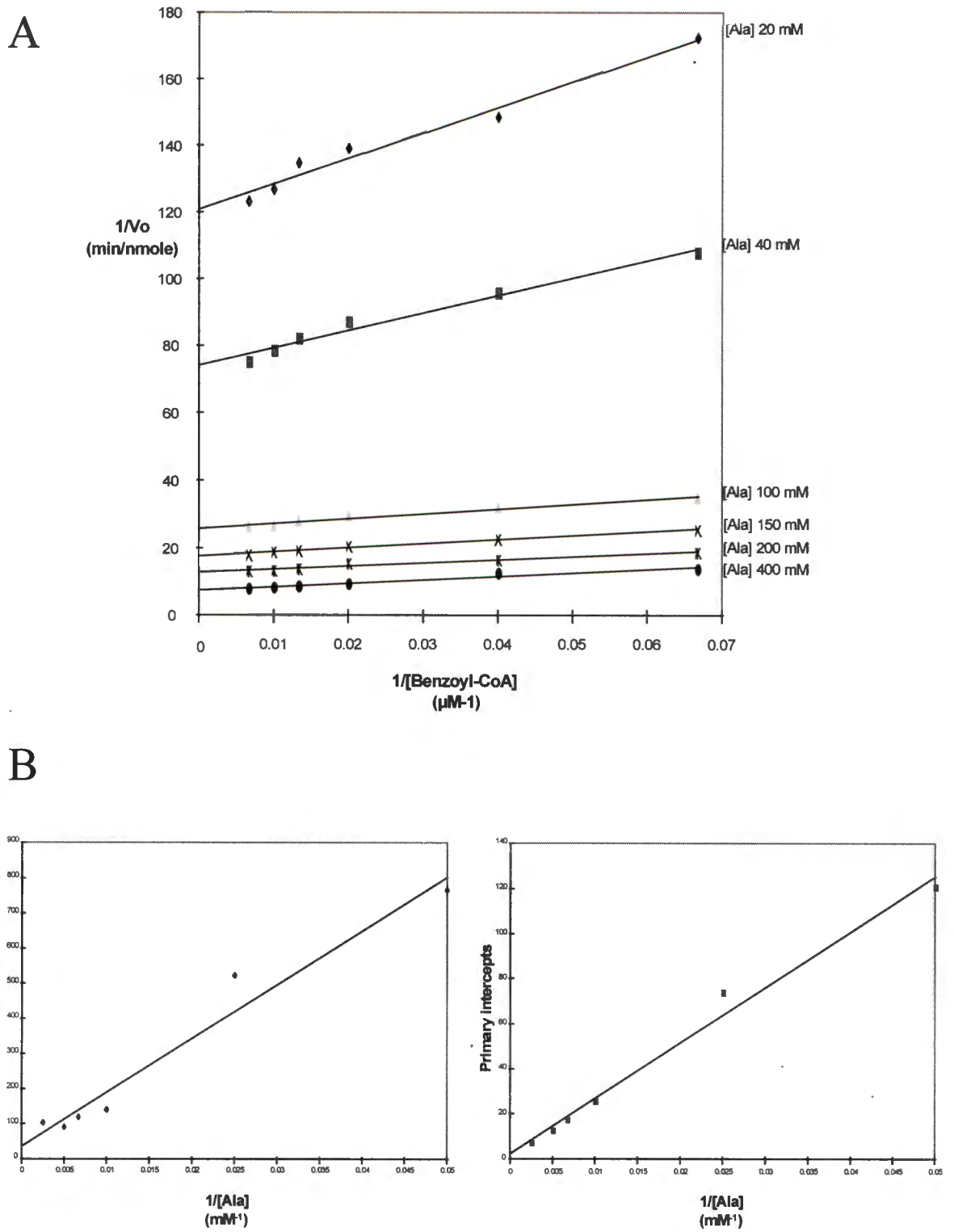


Figure 4.13 **Primary (A) double reciprocal, and secondary (B) rate plots employed for the determination of kinetic parameters for *benzoylalanine* formation using human GNAT.**
V_o was measured by ESI-MS-MS (Section 3.4.3).

human GNAT although the results presented here also identified Ala as well as Glu as additional, albeit very poor, substrates for human GNAT.

4.5 Inhibition of human GNAT by formed benzoylamino acid conjugates.

Due to the small amount of human material that was available it was not possible to perform intricate inhibition studies of the three benzoylamino acid conjugates formed by human GNAT, as was the case for bovine GNAT (Section 3.5). Preliminary inhibition studies of the three benzoyl conjugates were, however, performed using the DTNB-based assay for GNAT as described in Section 3.5. In this case, physiological substrate concentrations at 20 μM for benzoyl-CoA and 3.5 mM for Gly (Kelley and Vessey, 1990) were used in the assays. The inhibitory effect of benzoylglycine, -alanine, and -glutamic acid was determined at various concentrations and the concentration at which 50 % inhibition was observed (IC_{50}) under the specific substrate conditions was determined. These results are given in Table 4.4.

Table 4.4 Inhibition of human benzoylglycine formation* by benzoylamino acids.

Benzoylamino acid	IC_{50} (mM)
benzoylglycine	0.3
benzoylalanine	12.0
benzoylglutamic acid	10.0

*GNAT activity was monitored at 412 nm using physiological concentrations of benzoyl-CoA and glycine as described before (Kelley and Vessey, 1990).

As was the case with inhibition studies of bovine GNAT, the inhibitory effect of the "common" product of GNAT, benzoylglycine, is by far greater than any of the other two alternative products. This is clearly demonstrated in the IC_{50}-value of benzoylglycine (0.3 mM), which is more than thirty times lower than benzoylglutamic acid (IC_{50} = 10 mM) and benzoylalanine (IC_{50} = 12 mM). As was discussed in

Section 3.5, the relatively low inhibitory effect of the latter two conjugates on GNAT *in vitro* would, in view of the relatively low rates of formation of benzoylalanine and -glutamic acid, indicate that the inhibitory effect that they may have on human GNAT *in vivo* is likely to be negligible.

A similar inhibitory effect of benzoylglycine was observed with bovine GNAT measured at optimal substrate conditions (Section 3.5). Considering the normal plasma concentration of benzoylglycine, which is reported to be between 1.2 to 10.5 μM in healthy humans (Arends *et al.*, 1990; Hoffman *et al.*, 1993), benzoylglycine should not inhibit its own production under normal circumstances. However, elevated plasma benzoylglycine concentrations, reported in patients with renal failure (up to 904 μM, Igarashi *et al.*, 1987) as well as in cases of hyperammonemia (up to 400 μM, Simell *et al.*, 1986), may indeed have some product inhibitory effect on GNAT.

4.6 Summary.

The benzoylamino acid conjugation ability of human GNAT was the primary focus of this chapter. Human liver GNAT was partially purified in a five-step protocol. Analysis of the benzoylamino acid conjugation capabilities of this enzyme preparation, using ESI-MS-MS, resulted in the identification of human GNAT-catalyzed benzoyl conjugation with, apart from Gly, Ala as well as Glu. These conjugates, which could also be detected when using human mitochondrial lysate, formed at rates 20 (benzoylalanine) and 113 times (benzoylglutamic acid) lower compared to benzoylglycine under specified assay conditions. Similar $Km^{app}_{benzoyl\text{-}CoA}$-values were obtained using either Gly or Ala as amino acid substrate (13 and 15 μM, respectively), whereas the $Km^{app}_{amino\ acid}$-value for Gly (6.4 mM) was more than 150 times lower than the value determined for Ala (997 mM). This indicates that Gly is by far the better amino acid substrate for human GNAT when using benzoyl-CoA as acyl-donor. Preliminary inhibition studies of human GNAT indicated that benzoylglycine has a much (thirty times) higher inhibitory effect *in vitro* on the

enzyme than the other two conjugates and that the inhibitory effect of these alternatively slowly-formed products is likely to be negligible *in vivo*.

A human fetal liver cDNA clone in the I.M.A.G.E. Consortium (LLNL) cDNA Clones Bank was identified that matches bovine GNAT amino acid sequence data. Nucleotide sequence data showed that it consists of a novel 1083 bp sequence which can be translated into a 162 amino acid protein of Mr 18.3 kDa. The nucleotide sequence shared 82 % homology with bovine GNAT as well as a 77 % homology with bovine glutamine *N*-phenylacetyltransferase cDNA. Expression of this putative human GNAT cDNA in prokaryotic hosts is in progress.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Introduction.

The important metabolic role of peptide bond conjugation in the detoxication of carboxylic acids of exogenous and endogenous origin in mammals has been reviewed in Chapter 2 (Section 2.2). It is clear that this conjugation process not only serves a major detoxication role in normal metabolic processes, but in some organic acidemias this even more important to remove elevated carboxylic acids. It was surprising to us that this major conjugation mechanism did not receive much interest by researchers during the past few decades. The literature review indicated that less than thirty publications appeared in journals during the last two decades in which some aspect of GNAT was directly investigated (see Chapter 2), which is, compared to other conjugating enzymes such as glutathione S-transferase, a relatively small number.

What is known of this conjugation system ? The cellular location where it occurs, and the enzymes involved are known. The major substrates, products, their relative turnover in the body, as well as their respective metabolic purposes, have been established. Data on the molecular aspects of the three enzymes involved have been published during the past few years (Vessey and Lau, 1996; Falany *et al.*, 1994, 1997; EMBL accession number: embAJ001396).

Our interest in peptide bond conjugation, as discussed in Chapter 2 (Section 2.5), originated from the detection of unusual, previously undetected conjugates formed in patients with metabolic disorders. These include acyl conjugates with Ala, Glu, and Ser which were reported by a number of authors (Lehnert, 1981, 1983; Shinka, 1985; Rolland *et al.*, 1991; Erasmus and Mienie, 1998; Erasmus *et al.*, 1998). Structurally these latter conjugates relate to the products of GNAT. This was the basis of my

hypothesis that GNAT can utilize Ala, Ser and Glu in addition to Gly, Gln and Asn (the latter two only in the case of bovine GNAT) as amino acid substrates in a conjugation reaction with benzoyl-CoA. Although this aspect was previously investigated, with negative results (Webster *et al.*, 1976; Nandi *et al.*, 1979; Asaoka, 1991), I hoped to establish the origin of these conjugates, since technological advances in the detection of similar compounds make the detection of conjugates at much lower levels currently possible.

In this chapter the major aspects of this study are summarized to emphasize the contribution that was made to GNAT-catalyzed acylamino acid conjugation.

5.2 In vitro formation and detection of benzoylamino acid conjugates by bovine and human GNAT.

GNAT was purified from bovine and human liver samples in a five-step protocol which was similar for both organisms (Section 3.2 and 4.2). Bovine GNAT was purified to apparent homogeneity, whereas homogeneity was not as high in the case of the human enzyme. These two GNAT preparations, as well as mitochondrial lysates, were used to investigate the conjugation of the major acyl-CoA substrate for GNAT, benzoyl-CoA, with the aliphatic amino acids as well as serine, the acidic amino acids and their amides. Initially GNAT activity was monitored using the commonly used DTNB-based assay (Ellman, 1959; Webster *et al.*, 1976). Although conjugation of all of the amino acids was detected in this way when using bovine mitochondrial lysate, only Gly, Asn, Gln, and Ala conjugated with benzoyl-CoA when purified bovine GNAT was used. The lack of specificity as well as the detection limitations of this method, however, indicated that it was not the ideal method for the detection of conjugates that form at rates in the low nmole.min^{-1}.mg^{-1} range (Section 3.3). This was probably the reason why the previous investigations did not detect additional amino acid substrates for GNAT other than Gly, Gln and Asn for bovine GNAT (Nandi *et al.*, 1979) and Gly for primate GNAT (Webster *et al.*, 1976; Asaoka, 1991).

For the identification, characterization and quantification of benzoylamino acid conjugates, ES-MS-MS was used (Section 3.4). Conjugates that formed when *bovine* GNAT was used as catalyst were benzoyl conjugates of Gly, Asn, Gln as well as Ala, Ser and Glu (Section 3.4.1). The latter three conjugates were not previously identified to be products of bovine GNAT activity (Nandi *et al.*, 1979). In the case of *human* GNAT, benzoyl conjugates of Gly as well as Ala and Glu were detected (Section 4..4.2) of which the latter two are newly identified substrates for human GNAT. The same conjugates were detected using bovine and human mitochondrial lysates and no additional conjugates were detected in these lysates. Contrary to bovine GNAT, conjugates of Ser were not detected using human GNAT as catalyst. As this conjugate could not be formed using human mitochondrial lysate preparations either, I believe that acyl conjugates of Ser are either not formed in humans or its rate of formation is so low that it can not be detected by even sensitive techniques such as mass spectrometry. It may also be possible that benzoyl-CoA is not the best acyl donor where serine is used as acyl acceptor. At this stage, however, there no indication that enzymes other than GNAT catalyze the formation of the newly detected benzoyl conjugates. These conjugates are only formed in the presence of GNAT containing preparations. Comparative CID mass spectra of the GNAT-catalyzed conjugates and the corresponding synthesized conjugates were identical, which confirmed without any doubt the identity of the newly observed conjugates.

In a preliminary investigation of human GNAT-catalyzed conjugation of isovaleryl-CoA, propionyl-CoA, and 3-methylcrotonyl-CoA with Ala and Glu, respectively, the corresponding masses of the conjugates were indeed detected (results not shown). These results confirm that the previously detected conjugates, benzoylalanine (Shinka, 1985), isovalerylalanine and -glutamic acid (Lehnert, 1981, 1983), as well as 3-methylcrotonylglutamic acid and propionylalanine (Section 2.5) are catalyzed by human GNAT. Even though investigating the full scope of acyl-CoA substrates were beyond the focus of this study, we believe that GNAT may conjugate Ala and Glu, in addition to Gly, with a number of acyl-CoA substrates other than benzoyl-CoA, propionyl-CoA, isovaleryl-CoA or 3-methylcrotonyl-CoA (see Table 2.3, Section 2.3.3). Figure 5.1 illustrates the proposed substrate utilization of GNAT in view of the results obtained during this study. Conjugation of amino acids other than Gly,

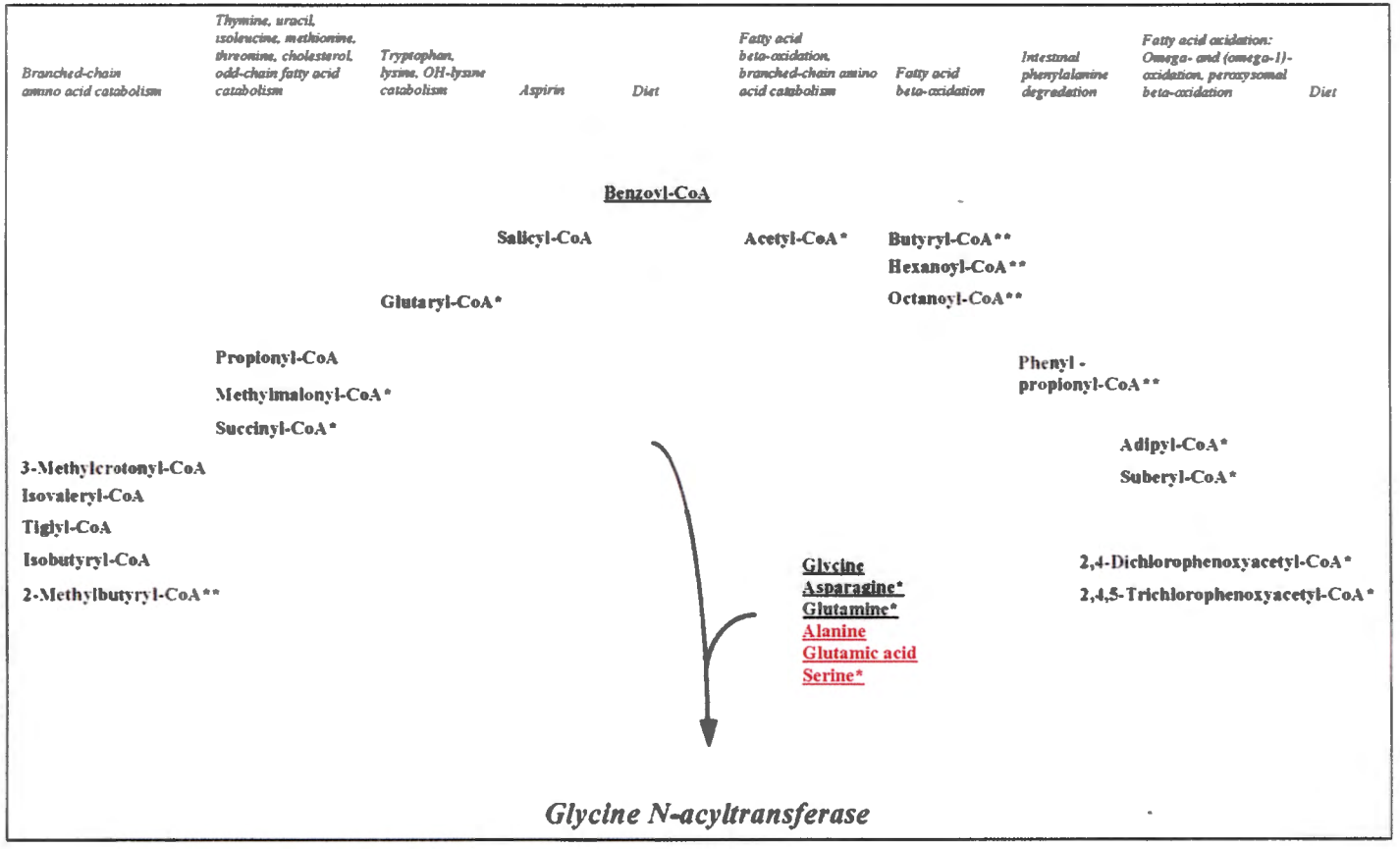


Figure 5.1 **Proposed substrate utilization by GNAT.**
Asterisks indicate where the substrates only apply to *bovine* GNAT whereas double asterisks indicate where substrates only apply to *human* GNAT. The underlined substrates indicate the substrates investigated in this study and newly identified substrates for GNAT are shown in red.

however, may be limited to certain acyl-CoA substrates. Further investigation of the conjugation of the various acyl-CoA substrates with the additional amino acid substrates needs to be performed to determine the full conjugation capacity of this enzyme. Although current kinetic and structural data suggest that the kidney enzyme is similar to the hepatic form, at least in bovine (Kelley and Vessey, 1993), very little is known of the kidney form of human GNAT, which also needs to be investigated further.

The additional use of mass spectrometry for the monitoring of GNAT activity is not a novel approach since Gregersen *et al.* (1986) used gas chromatography-mass spectrometry (GC-MS) for the detection of various GNAT-catalyzed acylglycine conjugates. They also emphasized the problem with non-specificity of the spectrophotometric based assay but further stated that "*with this* (GC-MS) *assay it was practically impossible to measure a sufficient number of assay mixtures to calculate the "true" kinetic constants (K_m, V_{max}) for this two-substrate reaction...*". However, with the novel *electrospray*-based mass spectrometry method used in this study, sample preparation and analysis took less time and were easier to perform, which enabled study of a number of assays required for the kinetic analysis of a two-substrate enzyme. The specific way in which benzoylamino acids were detected (see Section 3.4.2) made this approach, unlike the spectrophotometric method, highly specific for the detection of acylamino acids. As low as 5 nM of benzoylglycine could be detected, which is approximately 100 times more sensitive than the DTNB-based method.

5.3 Kinetic parameters of GNAT-catalyzed benzoylamino acid conjugation and its significance in human metabolism.

Kinetic data on benzoylamino acid formation was generated for bovine as well as human GNAT. These kinetic data followed Michaelis-Menten type kinetics for a sequential two-substrate mechanism (Palmer, 1995; see Sections 3.4.3 and 4.4.2). From these results it was concluded that for bovine, as well as human GNAT, Gly is

the preferred amino acid substrate. For *bovine* GNAT the Km_{Gly} at 6.2 mM was twenty times lower than Km_{Asn}, whereas Km_{Gln} was almost sixty times higher than Km_{Gly} (Table 3.5, Section 3.4.3). Both Ala and Glu were extremely poor substrates for bovine GNAT compared to Gly with Km-values which are in the molar range. The Km_{Ala} and Km_{Glu} were 250 and 185 times higher than Km_{Gly}, respectively. These trends were also observed in the Vmax-values as well as the relative rates of conjugate formation that were detected under specific substrate concentrations (Table 3.4 and 3.5, Section 3.4.3).

Benzoylglycine formation by *human* GNAT occurred at a much lower rate (approximately twenty times) compared to bovine under standard assay conditions, although the Km_{Gly}^{app} was similar at 6.4 mM (Section 4.4.2, Table 4.3). The kinetic data confirmed that Gly is by far the preferred substrate of *human* GNAT followed by Ala for which a very high Km_{Ala}^{app}-value of 997.4 mM was determined. When considering its extremely low rate of formation, Glu is also a relatively poor substrate for human GNAT although an accurate Km^{app}-value could not be determined. The high Km-values of the newly identified amino acid substrates for bovine and human GNAT did not allow development of reaction conditions for Vmax to be reached in these two cases.

To determine whether these conjugates have any significance in human metabolism, consider *in vivo* conjugation of Ala and Glu hypothetically. In humans acyl conjugates of these amino acids have only been detected in cases of certain abnormal metabolic states. Plasma Ala concentration under normal circumstances varies between 120 - 600 μM, which is more or less in the same region as Gly (100 - 400 μM), whereas Glu is in the region of 6 - 78 μM (Shih, 1996). As Ala and Glu are far less likely to conjugate with benzoyl-CoA than Gly, the formation of these conjugates in the liver and kidney could be expected to be extremely low under normal metabolic conditions when one considers the Km-values of these amino acids. This would explain why these conjugates are not detected under normal metabolic conditions, but rather under more specific metabolic conditions. In the cases where Ala and/or Glu conjugates with benzoic acid (Shinka, 1985), isovaleric acid (Lehnert, 1981, 1983), 3-

methylcrotonic acid (Rolland, 1991) and propionic acid (Erasmus and Mienie, 1998; Erasmus *et al.*, 1998) were detected, two specific metabolic conditions could have lead to their increased conjugation with an acyl donor.

Increased plasma Ala levels are associated with hyperammonemic syndromes along with lactic acidemia (Blom and Huijmans, 1992; Shih, 1996) as well as during histidine treatment of patients with histidinemia (Hamblin and Holton, 1972). Isovaleric acidemia and propionic acidemia, which are two disorders where alanine conjugates were detected (Section 2.5), are associated with hyperammonemia (Sweetman and Williams, 1995; Fenton and Rosenberg, 1995), whereas benzoylalanine was also detected in hyperammonemic patients (Shinka, 1985). In the case where the detection of propionylalanine was reported (Erasmus and Mienie, 1998), the patient were additionally treated with Ala, which would increase plasma levels of this amino acid. Elevation of Glu levels is only associated with two metabolic disorders, glutamic aciduria and dicarboxylic aminoaciduria (Shih, 1996).

Isovaleryl-CoA, propionyl-CoA, 3-methylcrotonyl-CoA and benzoyl-CoA, which are all substrates for human GNAT (Section 2.3.3, Table 2.3), are markedly elevated in cases where the corresponding conjugates were detected. This would make these substrates more available for the conjugation with amino acids. It was furthermore noted by Lehnert (1983) and Shinka (1985) that Gly conjugation could deplete Gly levels and provide a higher probability for Ala and Glu to conjugate with the acyl-CoA. However, the notion that benzoate treatment of hyperammonemic syndromes decreases Gly through benzoylglycine conjugation, has been investigated and was in fact found not to be the case (Tremblay and Qureshi, 1993; Section 2.3.3). This would suggest that the relative availability of Ala and Glu would not increase due to a decrease in Gly concentration, at least in case of sodium benzoate treatment of hyperammonemia.

An important question is to what extent Ala and Glu conjugates contribute to acylamino acid conjugation and detoxication of elevated acyl-CoA esters. In the case where benzoylalanine were detected it contributed 7.7 % to the total benzoylamino

acid pool (Shinka, 1985), wereas propionylalanine and propionylglutamic acid contributed 3.6 and 0.8 %, respectively, to the combined pool of propionylamino acid conjugates excreted in one specific propionic acidemia patient (Erasmus and Mienie, 1998). If one therefore considers the levels at which some of these conjugates were excreted in the urine of the patients in which they were detected (Section 2.5) as well as the kinetic behaviour of Ala and Glu in comparison to Gly in conjugating with benzoyl-CoA *in vitro* (Section 4.4.2, Table 4.3) it appears unlikely that these two amino acids would significantly contribute to the lowering of harmful, elevated levels of organic acids. Ala and Glu may conjugate better with acyl-CoA esters other than benzoyl-CoA and this, along with more data on the excretion of these conjugates, needs to be determined to derive the full contribution of acylamino acid conjugation to metabolic detoxication.

The inhibitory effect of the newly detected benzoylamino acid conjugates on GNAT were investigated to determine whether their presence may prove to be detrimental to normal GNAT function *in vivo*. In the case of bovine GNAT (Section 3.5) as well as human GNAT (Section 4.5) inhibition by all the benzoyl conjugates were detected, albeit relatively low. The results indicated that, relative to benzoylglycine, inhibition of all other benzoyl amino acid conjugates were thirty to forty times less effective. We believe these conjugates would have little, if any, inhibitory effect on GNAT *in vivo*, considering that the kinetic data clearly indicates that they are formed at extremely low levels compared to Gly conjugates.

5.4 Sequence analysis and expression of a putative human GNAT cDNA.

A human liver cDNA clone was obtained using bovine N-terminal sequence data which shared a strong homology (82 %) with bovine GNAT cDNA (Vessey and Lau, 1996) as well as bovine glutamine *N*-phenylacetyltransferase cDNA (77 %) (Section 4.3.1). Attempts to express the cDNA in E.*coli* were not successful (Section 4.3.2) and further investigations are planned. At this stage we can therefore only speculate on the identity of this cDNA. The strong homology that this cDNA shares with

bovine cDNA sequences is the only indication of its identity. The smaller size (18.3 kDa) of its translated sequence compared to purified, native human GNAT (24 - 30.5 kDa) is a concern and the expression of the recombinant protein needs to be performed successfully to establish whether this is indeed the cDNA for human GNAT. Another option is amino acid analysis of purified human GNAT or screening of a suitable cDNA library for additional clones.

5.5 Nomenclature of GNAT.

The lack of conformity in the nomenclature of GNAT has been discussed in Section 2.3.2. The NC-IUBMB recommended official name of this enzyme, *glycine N-acyltransferase*, has been used in this study. If the major amino acid substrate, Gly, is taken into account this name, as well as the systematic name (acyl-CoA:glycine *N*-acyltransferase) would accurately reflect the catalytic ability of this enzyme. However, if the total substrate profile of GNAT (Figure 5.1) is taken into account, these names, as well as some others previously used, seem to be insufficient with regard to accounting for its total amino acid substrate profile. This is the case for *bovine* GNAT, and in light of the results obtained in this study, as well as *human* GNAT (see Section 2.3.2). Alternative nomenclature has been used in the past (Table 2.2, Section 2.3.2) which in various ways altered the name for this enzyme. However, none of these proposed names seem to accurately reflect the enzyme's catalytic properties. As the enzyme utilizes different acyl-CoA esters as well as amino acid substrates it would be difficult to systematize an accurate and unique name for GNAT as there are two related enzymes, glutamine *N*-phenylacetyltransferase and glycine *N*-choloyltransferase, which share some properties with regard to substrate specificity (see Table 2.1, Section 2.3.2). The question should also be raised whether it would indeed serve a useful purpose to change the name from its current form, which does reflect its major catalytic role. The *IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (JCBN)* invites new suggestions for more accurate nomenclature of enzymes and we will communicate with other interested researchers to explore this possibility.

5.6 Conclusion.

The question that was put forward from the literature, as well as our own observations of acylamino acid conjugation of patients suffering from metabolic disorders, was whether the formation of a diverse group of newly discovered acylamino acid species in urine of patients is catalyzed by the same enzyme. Although previous investigations have suggested otherwise, I hypothesized that the acyl conjugates of Ala, Ser and Glu were catalyzed by GNAT. Benzoyl-CoA was used as acyl donor in this study. In the case of *bovine* GNAT the hypothesis was found to be true with all three benzoyl conjugates being catalyzed by the enzyme, whereas the hypothesis was partly true with respect to *human* GNAT where the benzoyl conjugates of Ala and Glu were catalyzed. Although Gly was again identified as the major amino acid substrate for GNAT, my work has proven that GNAT is not completely specific with regard to amino acid substrate utilization as previously reported.

ABBREVIATIONS*

Ala	Alanine
Asn	Asparagine
Asp	Aspartic acid
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary dioxyribonucleic acid
CID	Collision-induced dissociation
CoA	Coenzyme A
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EC	Enzyme Commission (of the IUBMB)
EMBL	European Molecular Biology Laboratory
ESI-MS-MS	Electrospray ionization tandem mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
GNAT	Glycine *N*-acyltransferase
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
IC_{50}	Inhibitor concentration at 50 % inhibition
IPTG	Isopropyl β-D-Thiogalactopyranoside
Ile	Isoleucine
IUBMB	International Union of Biochemistry and Molecular Biology
Km	Michaelis constant (substrate concentration at Vmax/2)
Leu	Leucine

MCAD	Medium-chain acyl-CoA dehydrogenase
MS	Mass spectrometry
NC-IUBMB	Nomenclature Commission of the IUBMB
NTR	Non-translatable region
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SCAD	Short-chain acyl-CoA dehydrogenase
Val	Valine
Vmax	Maximum reaction velocity
Vo	Initial reaction velocity

*Units of measurements and of physical and chemical quantities were used as recommended by The American Society for Biochemistry and Molecular Biology (1997).

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