

**ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF AN  
N-ACETYLTRANSFERASE FROM *Streptomyces akiyoshiensis* L-138**

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## ABSTRACT

A novel N-acetyltransferase (NAT) with high specificity for L-dopa was partially purified and characterized during this study. *Streptomyces akiyoshiensis* NAT was isolated from liquid cultures and the cell free extract was partially purified via two chromatographic approaches. The purest enzyme preparation (354.4-fold) was obtained through a combination of affinity (Affi-gel blue) and gel filtration chromatography. This preparation could not be stored due to poor stability. An alternate approach using affinity and anion-exchange column chromatography (macro-prep DEAE) provided a 125-fold purification. A second macro-prep DEAE showed the enrichment of a band at around 14.4 kDa. The enzyme activity was strongly inhibited by the presence of  $\text{Cu}^{2+}$  and  $\text{Hg}^{2+}$  salts suggesting the presence of critical histidine or cysteine residues. In addition, NaCl plays an important role as a stabilizing agent and/or slight activator of this enzyme. The  $K_m$  and  $V_{max}$  values for L-dopa determined at 30°C were  $5.39 \times 10^{-2}$  mM and  $2.19 \times 10^{-5}$  mM·s, respectively. At 37°C these kinetic constants were 0.11 mM and  $2.57 \times 10^{-4}$  mM·s, respectively. The  $K_m$  and  $V_{max}$  values for AcCoA determined at 30°C were 0.42 mM and  $4.34 \times 10^{-4}$  mM·s, respectively. At 37°C these values were 0.58 mM and  $1.15 \times 10^{-4}$  mM·s, respectively. The optimal temperature and pH were 43°C and 8.0 respectively. *S. akiyoshiensis* NAT acetylated arylalkylamine substrates but not arylamine substrates to any significant extent. These findings suggest that this enzyme may be closely related to arylalkylamine-N-acetyltransferases (AANATs), however the certification of this postulate requires the knowledge of the amino acid sequence and ultimately 3D structure of *S. akiyoshiensis* NAT.

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## LIST OF ABBREVIATIONS

AAT	aminoglycoside N-acetyltransferase
AcCoA	acetyl coenzyme A
2-AF	2-aminofluorene
ANAT	arylamine-N-acetyltransferase
AANAT	arylalkylamine-N-acetyltransferase
bis-tris propane	1,3-bis[tris(hydroxymethyl)methylamino]propane
bisubstrate analog	coenzyme-A-S-N-acetyltryptamine
CDDP	cisplatin
Da	dalton
kDa	kilodalton
DEAE	diethylaminoethyl
D-dopa	D-3,4-dihydroxyphenylalanine
Dopamine	3-hydroxytyramine
EDTA	ethylenediamino tetraacetic acid
×g	× gravitational force
GCN-5	general control nonrepressed factor-5
GNAT	GCN-5 related superfamily of enzymes
GSH	glutathione
h	hour
HAT	histone N-acetyltransferase
HIC	hydrophobic interaction chromatography

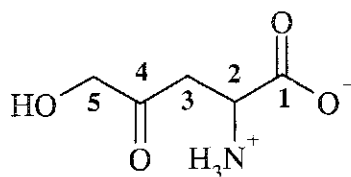
HON	5-hydroxy-4-oxo-L-norvaline
HON <sup>-</sup>	HON non producing phenotype
HPLC	high pressure liquid chromatography
INH	isoniazid
L-dopa	L-3,4-dihydroxyphenylalanine
MAO	monoamine oxidase
min	minute
Melatonin	N-acetyl-5-methoxytryptamine
Mr	apparent molecular weight
NAT	N-acetyltransferase
N-Acetyl-L-dopa	N-acetyl-L-3,4-dihydroxyphenylalanine
NMR	nuclear magnetic resonance
NTG	N-methyl-N'-nitro-nitrosoguanidine
OATs	O-acetyltransferases
PABA	p-aminobenzoic acid
PAC	puromycin AANAT
PAS	p-aminosalicylic acid
PMSF	phenylmethylsulfonyl fluoride
rpm	revolutions per minute
ScAANAT	<i>Saccharomyces cerevisiae</i> AANAT
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Serotonin	5-hydroxytryptamine
TBAH	tetrabutylammonium hydrogen sulfate
TFA	trifluoroacetic acid
TG	100 mM tris(hydroxymethyl)aminomethane, 10% glycerol
TGE	100 mM tris(hydroxymethyl)aminomethane, 10% glycerol, 2 mM EDTA
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume

## 1. INTRODUCTION

The order Actinomycetales includes a diverse array of bacteria, from the causative agents of tuberculosis and leprosy (*Mycobacterium tuberculosis* and *M. leprae*) to the harmless saprophytic relatives of the species *Streptomyces*, the most abundant and ubiquitous soil bacteria (Bentley *et al.*, 2002; Hopwood, 1999). Streptomycetes are gram-positive bacteria that constitute an important source of secondary metabolites (Hopwood, 1999). Although mostly antibiotics have been described, there is a vast repertoire of bioactive compounds generated by Streptomycetes. These include antiviral, antitumour, herbicidal, insecticidal and antiparasitic compounds (Sangler *et al.*, 1993; Vining, 1990). The biosynthesis of so many different compounds implies a plethora of enzymes involved in the catalysis of different reactions.

In 1956, a new strain (H-8998) of Streptomycete was reported to produce a new tuberculous antibiotic (Kanazawa and Tsuchiya, 1960). This new strain was designated *Streptomyces akiyoshiensis* nov. sp. The new antibiotic was found to be a homoserine-like amino acid identified as (*S*)-5-hydroxy-4-oxonorvaline (HON) and coded as RI-331 (Figure 1). Tatsuoka *et al.* (1961) reported the effectiveness *in vitro* of RI-331 against



**Figure 1.** Structure of 5-hydroxy-4-oxo-L-norvaline (HON, RI-331)

(Adapted from Yamaki *et al.*, 1992)

strains of human-type tubercle bacilli, sensitive and isoniazid-resistant as well as streptomycin-resistant. In 1988, HON was found to exhibit antifungal activity against several pathogenic fungi of medical importance including *Candida albicans* and *Cryptococcus neoformans* (Yamaki *et al.*, 1992; Yamaki *et al.*, 1990; Yamaguchi *et al.*, 1988, Yamaki *et al.*, 1988).

In 1999, HON was reported to deplete intracellular glutathione (GSH) and subsequently reverse resistance to cisplatin (CDDP) in human ovarian tumour cells (Tagashira *et al.*, 1999). This finding suggested a potential use of HON as a therapeutic agent for the chemotherapy of cancer.

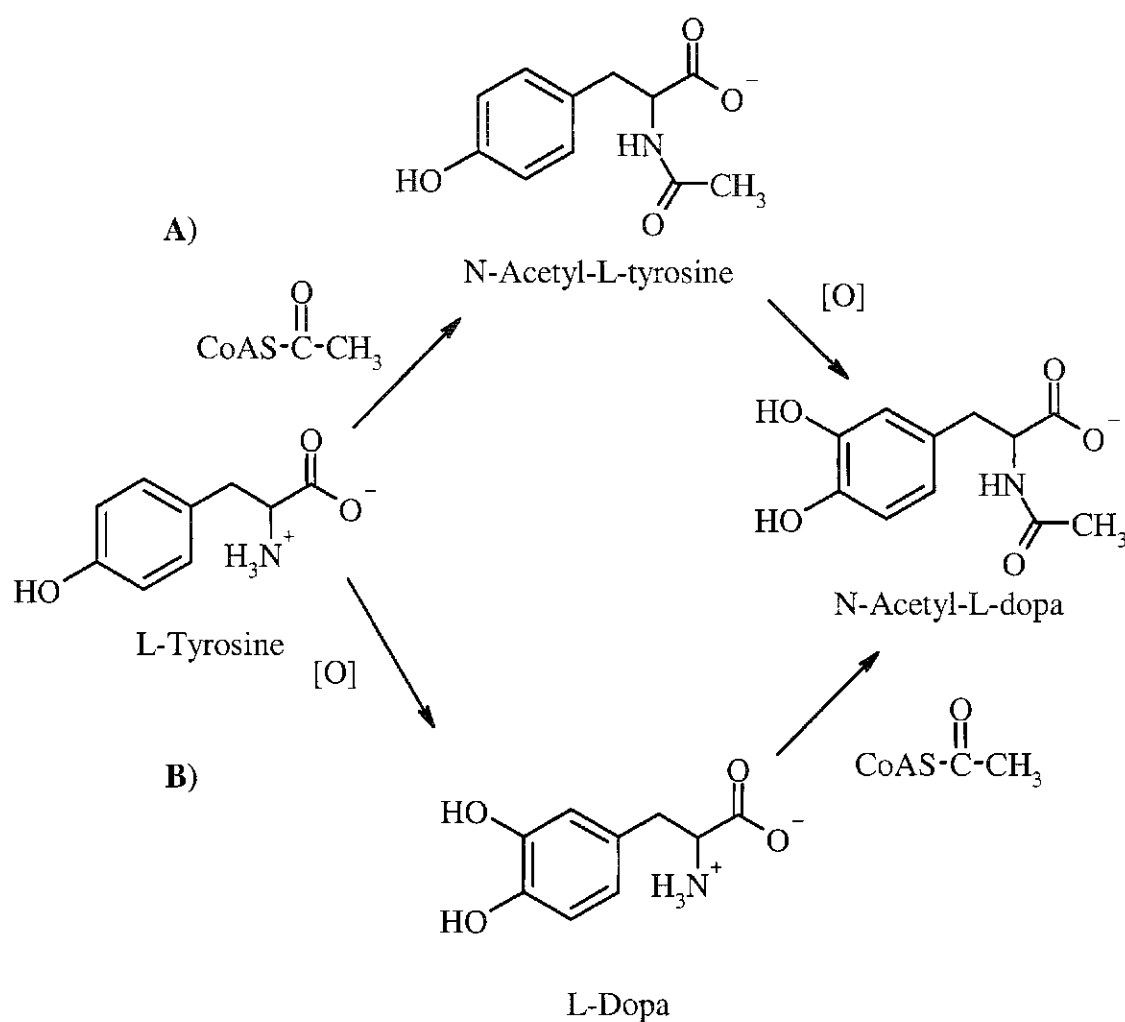
### **1.1. Mutants of *S. akiyoshiensis* blocked in HON production**

The first steps towards the elucidation of the biosynthetic pathway of HON were described in the early 1990s. In 1994, HON was reported to result from the precursors L-aspartic acid with C-5 coming from the methyl moiety of an acetate unit (White *et al.*, 1994). In an attempt to elucidate the identity of the intermediates of this pathway, a series of non-auxotrophic mutants created by chemical mutagenesis were isolated which were unable to produce HON. In addition, cross-feeding experiments suggested that these mutants were blocked in the biosynthetic pathway of HON. During attempts to isolate a potential intermediate from the mutant L-138, the metabolite N-acetyl-L-3,4-dihydroxyphenylalanine (N-acetyl-L-dopa) was isolated (Smith, 1995). Feeding studies showed however that N-acetyl-L-dopa was not related to HON biosynthesis. The production of N-acetyl-L-dopa, which interestingly was present not only in HON<sup>-</sup> mutants

of *S. akiyoshiensis* but also in the wild type, generated a question on the possible precursor(s) of this metabolite.

## 1.2. N-Acetyl-L-dopa and N-Acetyltransferase Activity

Given the structural resemblance, the most likely precursors for N-acetyl-L-dopa were thought to be either L-phenylalanine or L-tyrosine, which are derived from the shikimate pathway (Knaggs, 2003). Further investigations supported L-tyrosine as the precursor for this secondary metabolite (Smith and White, 1995). Two alternative pathways were proposed (Figure 2): A) the acetylation of L-tyrosine to N-acetyl-L-tyrosine and further hydroxylation to produce N-acetyl-L-dopa and, B) the initial hydroxylation of L-tyrosine to L-dopa followed by the acetylation of L-dopa to N-acetyl-L-dopa. Tyrosinase, an enzyme capable of catalysing hydroxylation of L-tyrosine and N-acetyl-L-tyrosine to L-dopa and N-acetyl-L-dopa, respectively, was shown to be active in *S. akiyoshiensis* (Smith, 1995). However, the required acetylation step was demonstrated to occur only with L-dopa. Finally, the existence of enzymatic activity capable of N-acetylation of L-dopa in cell-free extracts provided further evidence that L-dopa was the immediate precursor of this metabolite, thus, showing the existence of an N-acetyltransferase catalysing this nucleophilic acyl substitution. Therefore, this experimental evidence demonstrated that pathway B was operational in *S. akiyoshiensis* (Figure 2). In order to gain some understanding on the role of this enzyme in the biosynthetic machinery of *S. akiyoshiensis*, the isolation and characterization of this N-acetyltransferase was undertaken in the present study.



**Figure 2.** Biosynthetic Pathway for N-acetyl-L-dopa.

(Adapted from Smith 1995)

The present project focussed on *S. akiyoshiensis* N-acetyltransferase, which is a very distinctive enzyme, with a high specificity for an arylalkylamine (L-dopa). The intention of the work presented in this thesis is to corroborate the biosynthetic pathway

for N-acetyl-L-dopa and contribute to the understanding of the structural and functional roles of this bacterial N-acetyltransferase by 1) isolating the enzyme from cell-free extracts of *S. akiyoshiensis* L-138, 2) partially purifying enzyme activity from crude extract, and 3) studying its enzymatic properties with respect to pH and temperature optima, temperature effect, activators and inhibitors, substrate specificity as well as determine the kinetic parameters ( $K_m$  and  $V_{max}$ ). This study provides a useful background to improve purification strategies that will lead to a homogeneous protein. In addition, this work provides an insight into the classification of this enzyme as an arylalkylamine N-acetyltransferase (AANAT) or an arylamine N-acetyltransferase (ANAT or NAT). Finally, the information gathered in this thesis work describes an N-acetyltransferase that uses a different substrate from those characterized to date belonging to the eukaryotic AANATs and ANATs. This is particularly interesting since few members of the prokaryotic AANATs and ANATs have been described to date.

## 2. LITERATURE REVIEW

### 2.1. Acetyltransferases

Acetyltransferases are enzymes that transfer an acetyl group from acetyl Coenzyme A (AcCoA) to an acyl acceptor substrate (Delomenie *et al.*, 2001; Payton *et al.*, 1999; Weber and Hein, 1985). Acceptor substrates range from proteins and amino acids to aromatic amines and hydrazines (Delomenie *et al.*, 2001). Three main types of acetyltransferases have been described in the literature (Delomenie *et al.*, 2001; Hsieh *et al.* 1998; Watanabe *et al.*, 1992) that differ in the specificity of the functional groups to which the acetyl group can be transferred.

N-Acetyltransferases (NATs) are enzymes that catalyse the transfer of the acetyl group from AcCoA to the free amino group of arylamines (Sinclair *et al.*, 2000).

N-acetylation activity has recently been described in *Mycobacterium tuberculosis*, where this reaction is responsible for some instances of tubercular resistance to antibiotics such as isoniazid (INH, Kawamura *et al.*, 2002) (Figure 3).

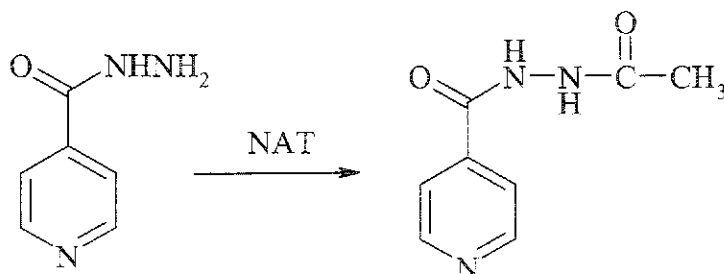
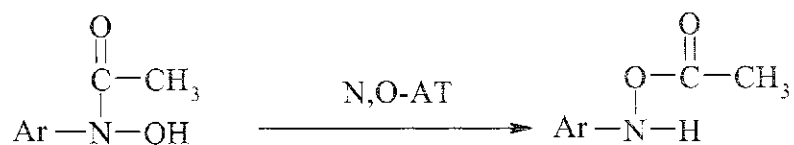


Figure 3: Metabolic Acetylation of Isoniazid.

(Adapted from Hanna, 1994)

O-Acetyltransferases (OATs) catalyse the transfer of the acetyl moiety from AcCoA to the oxygen atom of the hydroxyl group of N-hydroxyarylamines (Hanna, 1994). In mammals, heterocyclic arylamines and nitroaromatic compounds are metabolically converted to N-hydroxyarylamines (Figure 4). O-Acetylation activates the latter compounds to reactive and carcinogenic N-acetoxyarylamines that function as

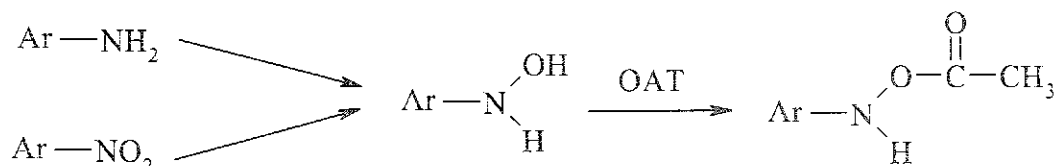


**Figure 4:** Bioactivation of Arylamines and Nitroaromatic Compounds.

(Adapted from Hanna, 1994)

DNA-binding reactants. Bacterial OAT activity has been reported in *Salmonella typhimurium* and is responsible for the bioactivation of nitroaromatic compounds and arylamines to mutagens (Delomenie *et al.*, 2001; Payton *et al.*, 1999). Interestingly, OAT activity has been associated with NAT activity. Purified mouse liver NAT exhibited OAT activity (Mattano *et al.*, 1989). In the same manner *S. typhimurium* OAT showed both OAT and NAT activity (Snyderwine *et al.*, 1988).

Finally, the third kind of acetyltransferase is called N-arylhydroxamic acid N,O-acetyltransferase (N,O-AT) and catalyses the transfer of the acetyl group from the nitrogen atom to the oxygen atom of the arylhydroxamic acid (Figure 5) (Hanna, 1994).



**Figure 5** : N-Arylhydroxamic Acid N,O-Acyltransfer

(Adapted from Hanna, 1994)

This process results in the formation of the same type of reactive compounds produced by OAT-catalysed reactions. N,O-AT activity has been reported in numerous mammalian tissues such as rat and rabbit liver (Delomenie *et al.*, 2001; Weber and Hein, 1985; Payton *et al.*, 1999). Interestingly, evidence has revealed that cytosolic NAT, OAT and N,O-AT activities are associated with single proteins (Hanna, 1994; Watanabe *et al.*, 1992). Nevertheless, the main focus of this study are N-acetyltransferases.

## 2.2. N-Acetyltransferases

N-acetyltransferases (NATs), also called transacetylases, are widely distributed in all organisms; these enzymes catalyse the transfer of an acetyl group from AcCoA (“the donor”) to diverse primary amines (“the acceptor”) (Lee *et al.*, 1988). The best characterized NATs belong to two unrelated groups of enzymes: the arylamine N-acetyltransferases (ANATs or NATs), and the GCN5-related (general control nonderepressible-5-protein) N-acetyltransferases (GNATs).

## 2.2.1. Arylamine N-acetyltransferases (ANATs or NATs)

### 2.2.1.1. Eukaryotic NATs

In humans, NATs are involved in the biotransformation of many arylamines and hydrazine drugs, as well as several known carcinogens present in the diet, cigarette smoke and the environment (Butcher *et al.*, 2002; Sim *et al.*, 1992; Sim *et al.*, 2000; Sinclair *et al.*, 2000). Arylamine N-acetyltransferase activity was first identified in humans as being responsible for the inactivation of INH, the major antitubercular agent (Sandy *et al.*, 2002; Upton *et al.*, 2001). There are two cytoplasmic N-acetyl-transferases (NATs) in humans, the N-acetyltransferase isoenzymes Type I (NAT1) and Type II (NAT2) (Payton *et al.*, 2001b; Sim *et al.*, 2000). In the mouse, there are three NATs designated NAT1, NAT2 and NAT3 (Martell *et al.*, 1992). Cats and other felids have only one NAT, while dogs lack NAT activity (Butcher *et al.*, 2002; Upton *et al.*, 2001). Although human NAT1 and NAT2 share 81% amino-acid sequence identity (Rodrigues-Lima *et al.*, 2001) their substrate selectivity for amine substrates differ markedly (Butcher *et al.*, 2002). In general, p-aminobenzoic acid (PABA), p-aminobenzoyl glutamate and p-aminosalicylic acid (PAS) are considered specific substrates for human NAT1 (or mouse NAT2). In contrast, sulfamethazine, procainamide, dapson and INH are acetylated primarily by human NAT2 (De Leon *et al.* 2000). Other compounds such as 2-aminofluorene (2-AF) are good substrates for both isoenzymes. NAT isoenzymes from other vertebrate species present a substrate specificity profile that appears to be similar to those of NAT1 and NAT2 (Payton *et al.*, 2001a).

NAT activity is associated with the metabolic activation and detoxification of arylamine and heterocyclic carcinogens (Dupret *et al.*, 1992; Upton *et al.*, 2001). Several studies have concentrated on the relationship between the NAT2 acetylator status and cancer risk. The consensus is that the slow acetylator phenotype represents a risk factor in bladder cancer while the rapid acetylator phenotype has been considered a strong risk factor in colorectal cancer (Butcher *et al.*, 2002). Recently, the NAT2 acetylator phenotype has also been linked to increased risk associated with neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease.

An endogenous role for NATs in human and mammalian development has been proposed based on its widespread tissue distribution (Sim *et al.*, 2000). Studies using the murine analog of human NAT1 have shown a putative role in folate metabolism. This NAT is expressed at the blastocyst stage and its distribution is not uniform, being more concentrated in the developing neural tube. This observation is important since folate prevents neural tube defects. Furthermore, other murine and human NAT genes are not expressed at this stage (Sim *et al.*, 2000; Upton *et al.*, 2001).

#### 2.2.1.2. Prokaryotic NATs

The first prokaryotic NAT was isolated from *Salmonella typhimurium* (Watanabe *et al.*, 1992). Within the last few years arylamine NATs have been reported in several bacterial species (Delomenic *et al.*, 2001; Sim *et al.*, 2000). Recently, the distribution of NATs in prokaryotes has been described based on activity studies (Delomenie *et al.*, 2001) and genome analysis (Payton *et al.*, 2001b). Payton *et al.* (1999) suggested the

presence of a single NAT gene in prokaryotes. However, more recent findings (Sim *et al.*, 2000) point to multiple NAT genes which code for proteins with a similar substrate specificity as human NAT2. Prokaryotic NATs acetylate INH and 2-AF but do not acetylate PABA. However, the prokaryotic NATs are also capable of acetylating 5-aminosalicylic acid, a human NAT1 specific substrate. Therefore, prokaryotic NATs should not be considered functionally equivalent to either NAT1 or NAT2 in humans (Butcher *et al.*, 2002).

The fact that prokaryotic NATs do not acetylate PABA is not surprising because bacteria use this compound to synthesize folate *de novo* from their environment. However the endogenous function of NATs in bacteria is still unknown (Mushtaq *et al.*, 2002; Sim *et al.*, 2000). Floss and Yu (1999) have described a prokaryotic member of the NAT family, rifamycin amide synthase (RifF), in *Mycolatosia mediterrani*. This enzyme presents an amino acid similarity of 45% to other prokaryotic NATs and catalyses an amide bond formation during the latter stages of rifamycin B synthesis. The relevance of this discovery is that there might be other NAT variants such as RifF. These NATs might become evident as more genomes are sequenced and it is possible that NATs have evolved to perform a range of specific roles in different organisms (Mushtaq *et al.*, 2002; Payton *et al.*, 2001a; Payton *et al.*, 2001b). Recently, an arylamine NAT was identified in *Mycobacterium*, which includes major human pathogens such as *M. tuberculosis*, *M. leprae* and *M. avium*, the major opportunistic pathogenic agent affecting HIV-infected people in the developed world (Payton *et al.*, 2001b; Sinclair *et al.*, 2000; Sim *et al.*, 2000). INH needs to be activated by oxidation to be effective against

*M. tuberculosis* (Sim *et al.*, 2000). N-Acetylation renders INH inactive in Mycobacteria (Payton *et al.*, 2001a). Payton and coworkers (1999) have shown that a *M. smegmatis nat* knockout mutant exhibited an increased sensitivity to INH while the heterologous expression of the gene for *M. tuberculosis* NAT resulted in *M. smegmatis* resistance to INH. Therefore, it is possible that increased expression of the gene or mutations resulting in the increase of NAT activity may contribute to resistance to INH (Payton *et al.*, 2001a).

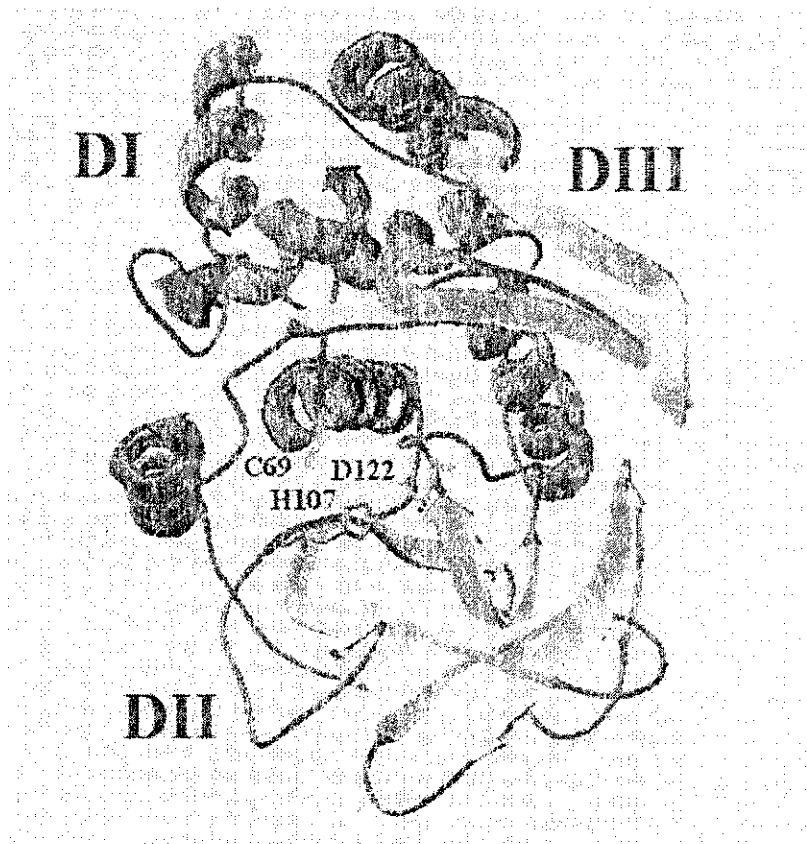
#### 2.2.1.3. Characteristics of NATs

Members of the arylamine NAT family are described as monomeric, dimeric or even tetrameric enzymes with a molecular weight range between 30-34 kDa (254-332 amino acids in length) (Butcher *et al.*, 2002; Mushtaq, 2002; Sinclair *et al.*, 2000). The optimum temperature for arylamine NATs range from 25°C to 37°C (Chen *et al.*, 1999; Chung, 1998; Chung *et al.*, 1998; Hsieh *et al.*, 1998; Lee *et al.*, 1988). The optimum pH for these enzymes varies from 7 to 8.5 (Chen *et al.*, 1999; Chung, 1998; Chung *et al.*, 1998; Hsieh *et al.*, 1998; Lee *et al.*, 1988). Divalent cations, such as Zn<sup>2+</sup>, Ca<sup>2+</sup>, Fe<sup>2+</sup>, Mg<sup>2+</sup>, Cu<sup>2+</sup> and Co<sup>2+</sup> act as potent inhibitors of NATs (Chen *et al.*, 1999; Chung, 1998; Chung *et al.*, 1998; Hsieh *et al.*, 1998; Lee *et al.*, 1988).

The structural features of arylamine NATs have been studied using homologous enzymes from *S. typhimurium* (Sim *et al.*, 2000; Upton *et al.*, 2001). The N-terminal region is the most highly conserved whereas the C-terminal region exhibits very little conservation between species. In *S. typhimurium* arylamine NAT (StNAT), conserved cysteine (Cys<sup>69</sup>), histidine (His<sup>107</sup>), and aspartate (Asp<sup>122</sup>) residues have been implicated as

a catalytic triad. In humans, Cys<sup>68</sup> is crucial for NAT activity as determined by functional analysis and site-directed mutagenesis (Rodrigues-Lima and Dupret, 2002). This Cys-His-Asp catalytic triad is highly conserved in all NATs identified to date (Sim *et al.*, 2000).

The first crystal structure of an arylamine N-acetyltransferase belonged to StNAT (Figure 6) (Sinclair *et al.*, 2000). More recently, Sandy *et al.* (2002) have solved the crystal structure for *M. smegmatis* NAT. The overall sequence identity for these NATs was 34%, with 52% identity in the first two domains. The overall fold of these two enzyme molecules was very similar with only slight differences observed in the relative disposition of the domains, potentially due to the low resolution of the StNAT structure. The information gathered from these crystal structures revealed a number of surprising features that provided novel structural and functional information about these enzymes. Particularly, the Cys-His-Asp catalytic triad was confirmed to be in the N-terminal region of the protein. The analysis of the structural motif revealed three domains of roughly equal length (Figures 6). Domain I consists of a helical bundle, which forms one side of a cleft in which the cysteine involved in acetyl transfer resides. All known NATs are highly homologous in this region (Sinclair *et al.*, 2000; Sandy *et al.*, 2002). Domain II is located on the other side of the cleft and consists mostly of a  $\beta$ -barrel. The last domain, domain III, at the carboxyl terminus is linked to the first two by an interdomain helix and is a combination of  $\beta$ -sheets and  $\alpha$ -helices ( $\alpha/\beta$  "lid") showing most of the diversity between species. The interface between the first and third domain including the interdomain region constitute the active site cleft (Sandy *et al.*, 2002). The fold for arylamine NATs shows no similarity to that of the GCN-5 related superfamily (GNATs) (Sandy *et al.*, 2002).



**Figure 6.** Three-Dimensional Structure of *S. typhimurium* NAT. Catalytic residues are highlighted: C69 = Cys<sup>69</sup>, H107 = His<sup>107</sup>, D122 = Asp<sup>122</sup>. Domains are: DI = domain I, DII = domain II, and DIII = domain III.

(Picture prepared using Swiss-Pdb Viewer, pdb code 1e2t)

However, there is a remarkable structural similarity to cysteine proteases, a superfamily of proteins that includes the transglutaminases, cathepsins and caspases. Cysteine proteases generally catalyse the hydrolysis of proteins and peptides. Nevertheless, a member of the cysteine protease family, transglutaminases, catalyse an acyl transfer that results in amide bond formation (Butcher *et al.*, 2002; Rodrigues-Lima *et al.*, 2001; Sandy *et al.*, 2002). Therefore, NATs and cysteine proteases might have a common protein ancestor (Sinclair *et al.*, 2000).

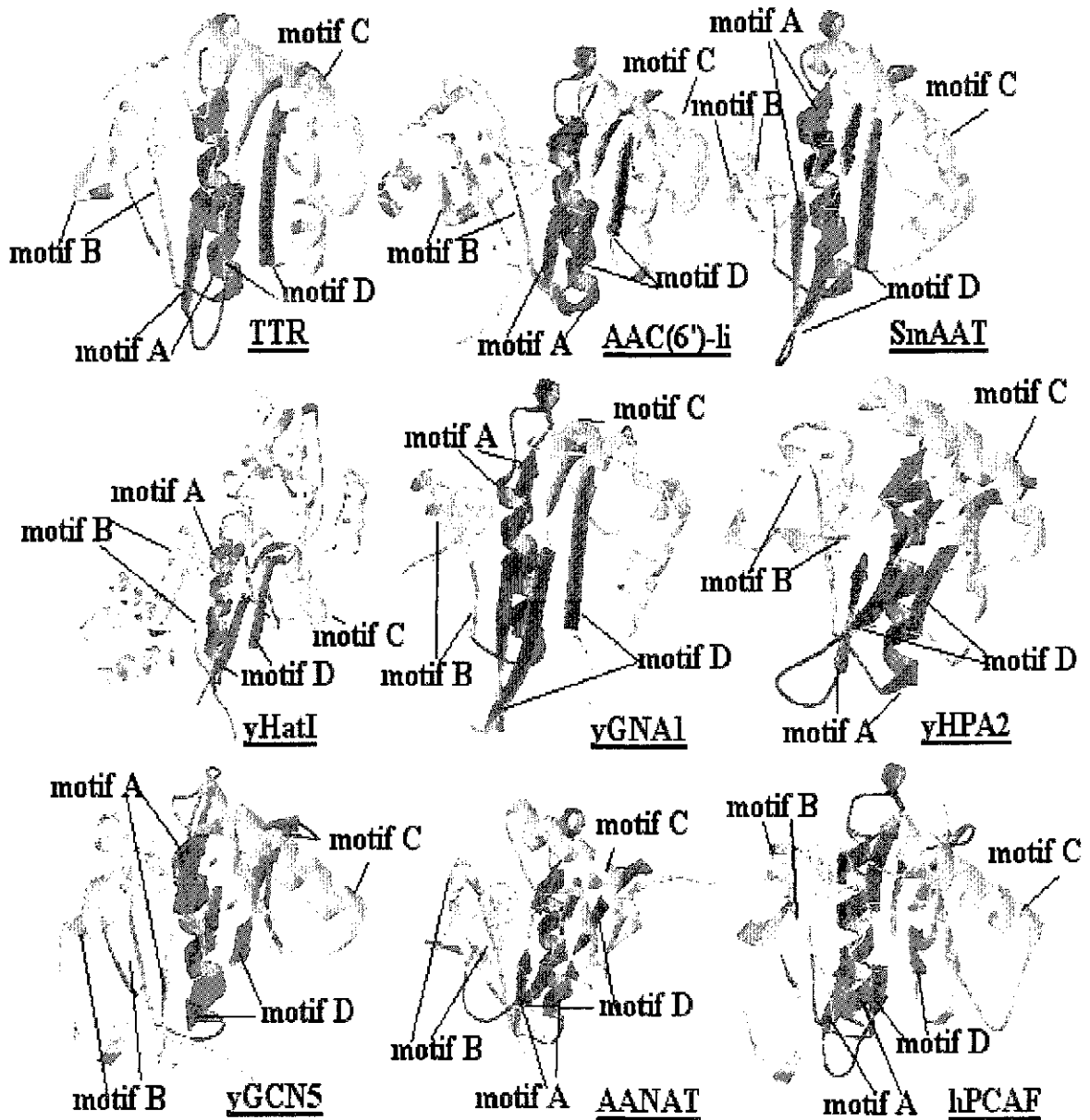
The accepted mechanism of the arylamine N-acetyltransferases is a ping-pong “Bi-Bi” reaction mechanism (Butcher *et al.*, 2002; Delgoda *et al.*, 2003). This reaction mechanism proposes two separate steps. Initially, the cysteine residue (Cys<sup>69</sup>) is activated after binding and transfer of the acetyl group from the cofactor (AcCoA), thereby releasing coenzyme A. The second step involves the binding of the arylamine substrate to the acetyl-enzyme intermediate followed by transfer of the acetyl moiety to the arylamine (Butcher *et al.*, 2002; Sim *et al.*, 2000; Sinclair *et al.*, 2000). However, some recent data are contradictory: (1) Mushtaq *et al.* (2002) have shown that NAT-catalysed AcCoA hydrolysis cannot be detected unless a hydrazine or arylamine substrate is present, and (2) the C-terminal region in StNAT has been shown to regulate the binding and hydrolysis of AcCoA in the absence of INH or any other substrate. Therefore, it has been suggested that once acetylated, the substrates do not remain bound to the protein. In summary, these findings suggested that the NAT protein must initiate a conformational change when binding INH, which promotes efficient acetyl CoA hydrolysis, and thus the likely transfer of the acetyl to the bound arylamine ligand (Mushtaq *et al.*, 2002; Sandy *et al.*, 2002).

Delgoda *et al.* (2003), using one-dimensional NMR studies have shown that INH and acetylated-INH do not compete for the same binding site in StNAT. This result provided more evidence for the binding of INH to StNAT prior to AcCoA. Whether the binding of AcCoA also leads to a conformational change that activates NATs constitutes a question that remains to be answered.

#### 2.2.2. GCN-5 (general control nonrepressed factor-5 related) N-acetyltransferases (GNATs)

GCN-5 related N-acetyltransferases (GNATs) constitute a superfamily of functionally diverse enzymes (more than 200 distinct enzymes) that catalyse the transfer of an acetyl group to a substrate (Dyda *et al.*, 2000; Modis and Wierenga, 1998; Stemglanz & Schindelin 1999; Wolf *et al.*, 1998). Most of the members of the GNAT superfamily share four conserved structural motifs C, D, A and B (in N-terminal to C-terminal order) (Figure 7). Interestingly, crystal structure analysis has determined that amino acid sequence homology between GNAT superfamily members may be undetectable in some instances (Butcher *et al.*, 2002; He *et al.*, 2003; Peneff *et al.*, 2001; Wybenga-Groot *et al.*, 1999). All members possess motif A, and nearly all have motifs B and D. However, motif C is not always evident based solely on amino acid sequence (Dutnall *et al.*, 1998). These observations emphasized the need to fully characterize more GNAT family members to conclude how this diverse group of enzymes performs their conserved tasks.

The GNAT superfamily includes proteins with such diverse functions as



**Figure 7.** Three-Dimensional Structures of GCN-5-Related N-acetyltransferases. The four conserved motifs of the GNAT superfamily - C, D, A, and B- are shown in green, blue, red and yellow respectively, and are labelled appropriately. The conserved tyrosine residue (phenylalanine in yHat1) is marked in yellow and the  $\beta$ -bulge is circled in green and cyan, respectively. (Adapted from He *et al.* 2003)

providing antibiotic resistance to regulating the circadian rhythms in mammals (Dyda *et al.*, 2000). The most intensively studied proteins of this superfamily include: histone N-acetyltransferases (HATs), aminoglycoside N-acetyltransferases (AATs), and arylalkylamine N-acetyltransferases (AANATs).

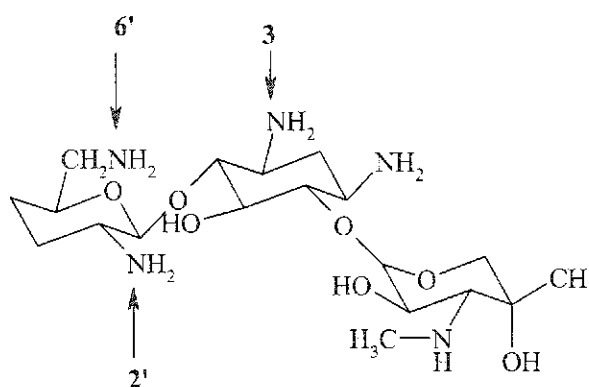
#### 2.2.2.1. Histone N-Acetyltransferases (HATs)

Histone N-acetyltransferases (HATs) acetylate histones thereby governing gene expression via its effect on chromatin structure and assembly. These enzymes exist in both prokaryotes and eukaryotes (He *et al.*, 2003). HATs are subdivided in two categories: Type A, located in the nucleus and Type B located in the cytoplasm (Martin *et al.*, 2001). The majority of HATs do not show motif C at the amino acid sequence level (Dutnall *et al.*, 1998). However, the crystal structures solved for these enzymes show this motif (Figure 7). The best characterized HATs are transcription factors such as: *Saccharomyces cerevisiae* GCN5 (yGCN5), *S. cerevisiae* HAT 1 (yHAT1), *S. cerevisiae* GNA1 (yGNA1), human p300/CREB-binding protein-associated factor (hPCAF), and the most recent *S. cerevisiae* HPA2 (hHPA2) (Angus-Hill *et al.*, 1999; Lau *et al.*, 2000; Martin *et al.*, 2001; Poux *et al.*, 2002; Sternglanz and Schindelin, 1999).

#### 2.2.2.2. Aminoglycoside N-Acetyltransferases (AATs)

Aminoglycoside N-acetyltransferases (AATs) catalyse the acetyl group addition to an amine group of aminoglycoside antibiotics reducing the affinity for their targets (Wolf *et al.*, 1998). AATs are of particular interest to clinical medicine due to the growing

problem of broad-spectrum aminoglycoside resistance. The presence of AATs generally provides resistance against most of the commonly used aminoglycosides, including gentamicin, tobramycin, and netilmicin. There are three main types of AATs which differ in the amine group that is acetylated: 3, 2', and 6' (Figure 8). Crystal structures for



**Figure 8.** Structure of Gentamicin C<sub>1α</sub>. Arrows are showing the three types of acetyl group addition (3, 2', and 6').

(Adapted from Wolf *et al.* 1998).

*Serratia marcescens* aminoglycoside 3-N-acetyltransferase (SmAAT, Figure 7) (Wolf *et al.*, 1998), and *Enterococcus faecium* aminoglycoside 6'-N-acetyltransferase, type li (AAC(6')-li) (Wolf *et al.*, 1998; Wybenga-Groot *et al.*, 1999) have reported agreement with the four conserved motifs described for the GNAT superfamily of enzymes. More recently, Vetting *et al.* (2002) have reported the structure of aminoglycoside 2'-N-acetyltransferase from *M. tuberculosis*. This enzyme not only acetylates aminoglycosides

containing 2'-amino substituents but also molecules with 2'-hydroxyl substituents such as kanamycin A. This finding indicates the capacity of the enzyme to perform N-acetyl and O-acetyl transfer (Hegde *et al.*, 2001; Vetting *et al.*, 2002). In addition, He *et al.* (2003) have demonstrated that *Pseudomonas syringae* tabtoxin resistant protein (TTR) belongs to the GNAT superfamily by solving its crystal structure (Figure 7). TTR not only acetylates the  $\beta$ -lactam phytotoxin but also has shown HAT activity. Structurally, TTR appears to be more closely related to yHAT1 and to the AAC(6')-li than to other members of the GNAT superfamily. Interestingly, AAC(6')-li was shown to acetylate histones and other small proteins indiscriminately (Eckermann *et al.*, 2003; He *et al.*, 2003; Wybenga-Groot *et al.*, 1999). Therefore, it seems likely that a common ancestral protein, maybe a HAT, has evolved to serve several functions and as more structures become available, more enzymes could be classified as belonging to the GNAT superfamily of proteins (He *et al.*, 2003).

The three-dimensional structures determined for some members of the GNAT superfamily have demonstrated a remarkable consistency in protein topology (Dyda *et al.*, 2000). The binding site for the donor substrate, AcCoA, is outstandingly conserved. The binding site for the acceptor substrate has only been characterized in two cases and showed that the same region of the proteins are involved. However, the conformations and length of analogous loops, which create a substrate binding cleft, differ drastically (Dyda *et al.*, 2000). This notion might explain the variability in substrate specificity (Ganguly *et al.*, 2001). Future investigations will provide more information on modes of acceptor substrate binding, particularly interesting for those members whose substrate

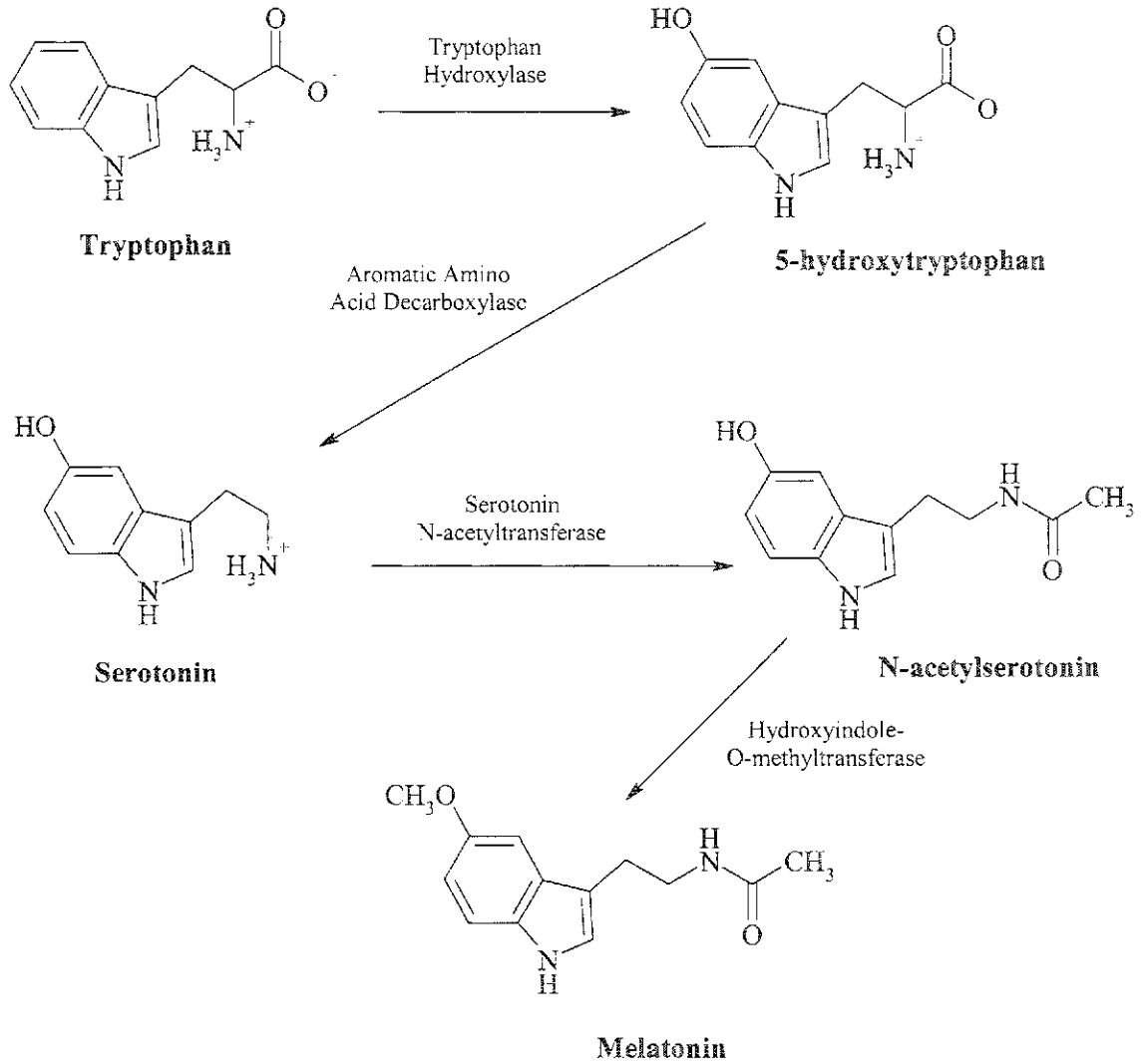
differ from those characterized to date (Dyda *et al.*, 2000).

#### 2.2.2.3. Arylalkylamine N-acetyltransferases (AANATs)

Arylalkylamine N-acetyltransferases (AANATs) are enzymes that exhibit selectivity for arylalkylamines substrates. The best known substrates for these enzymes include serotonin (5-hydroxytryptamine), tryptamine and phenylethylamine (De Angelis *et al.*, 1998; Ganguly *et al.*, 2001). However, AANATs generally do not acetylate arylamines such as aniline or 2-AF. This narrow specificity is not apparent with arylamine NATs, which acetylate both arylamines and arylalkylamines (Coon *et al.*, 1995; Sim *et al.*, 1992; Voisin *et al.*, 1984).

##### a. Eukaryotic AANATs

The most studied arylalkylamine N-acetyltransferase is serotonin AANAT (referred to as simply AANAT). This enzyme of 23 kDa catalyses the reaction of serotonin with AcCoA to form N-acetylserotonin and has a major function in the regulation of the melatonin circadian rhythm in vertebrates (Figure 9) (Ferry *et al.*, 2000). Melatonin plays an important role in regulating the sleep-wake cycle, seasonal breeding patterns, and adaptation to environmental changes in dark-light patterns. Other roles have been suggested related to aging, cardiovascular function, malignancy, and the immune system (Wolf *et al.*, 2002; Zheng and Cole, 2002). Serotonin AANAT is expressed primarily in two tissues, the pineal gland and the retina. Since serotonin AANAT levels follow a daily rhythm, with night-time levels being higher than in the daytime, this



**Figure 9.** Biosynthetic Pathway of Melatonin from Tryptophan.

(Adapted from Zheng *et al.* 2002)

enzyme is thought to be the key regulatory step in melatonin production (Ferry *et al.*, 2000). Hydroxyindole-O-methyltransferase (HIOMT), the other enzyme in the synthesis of melatonin, does not display such day-night changes (Ferry *et al.*, 2000; Zheng *et al.*, 2001).

This enzyme is involved in at least three different physiological functions in insects (Ichihara *et al.*, 2001; Muimo and Isaac, 1993). First, since monoamine oxidase (MAO, the enzyme that inactivates catecholamines) exhibits weak activity in insects, AANAT appears to be the major route of neurotransmitter catabolism producing N-acetylated monoamine such as N-acetyldopamine, N-acetylnorepinephrine, and N-acetyloctopamine; which are employed in sclerotization of the exoskeleton. Second, AANAT generates N-acetyldopamine that is oxidized to a quinone, which cross-links proteins and/or chitin contributing to the sclerotization of the insect cuticle. Finally, AANAT is involved in the synthesis of melatonin, although its function has not yet been linked with a circadian rhythm in insects. In *Periplacenta americana*, the activity of AANAT was shown to fluctuate in daily rhythm (Ichihara *et al.*, 2001). However, in *Drosophila melanogaster* AANAT activity was not rhythmic though melatonin fluctuated rhythmically (Amherd *et al.*, 2000). It is not yet clear if a single type of AANAT is responsible for all the metabolic reactions attributed to this enzyme, or if the existence of multiple forms of these enzyme allows each one to regulate a unique function. However, Ichihara *et al.* (2001) recently demonstrated the existence of two different forms of AANAT in *P. americana*. These two forms of AANAT presented a similar molecular weight (28 kDa) and neither of them acetylated arylamines, which is consistent with

AANATs. However these two AANATs differed in pH optimum, one being acidic (pH ~6.0) and the other basic (pH ~10.0). Furthermore, the substrate specificity of these two types of enzymes was different, acidic AANAT exhibited low  $K_m$  values (~0.02 mM) for tryptamine, serotonin, dopamine, octopamine and tyramine. On the other hand, basic AANAT had much higher  $K_m$  values for all of these substrates (0.32-1.52 mM) except for tyramine (0.02 mM). These findings suggest the feasibility of different AANATs in different regions/tissues and performing different roles (Ichihara *et al.*, 2001).

In helminths, biogenic amines, such as serotonin, dopamine and octopamine function as neurotransmitters and hormones (Aisien *et al.*, 1996; Muimo and Isaac, 1993). MAO activity in helminths has been found to be weak (Muimo and Isaac, 1993) while N-acetylation appears to be a major pathway for inactivation of these amines (Aisien *et al.*, 1996; Muimo and Isaac, 1993). In trematodes a single enzyme has been found responsible for the acetylation of arylalkylamines, diamines and polyamines. However, in nematodes the acetylation of diamines and arylalkylamines was found to be controlled by two independent enzymes, an arylalkylamine N-acetyltransferase (AANAT) and a diamino N-acetyltransferase (Aisien *et al.*, 1996). Aisien *et al.* (1996) have reported the characterization of an AANAT from *Onchocerca volvulus*, the causative organism of river blindness. However, no crystal structure has been solved for these enzymes yet.

According to database searches, AANATs occur in fungi, including *Saccharomyces cerevisiae*, and possess similar catalytic and kinetic characteristics to those found in vertebrates. Structural similarity is restricted to the catalytic core and motifs A/B that are distinctive of the GNAT superfamily (Dyda *et al.*, 2000; Ganguly *et al.*, 2001).

*S. cerevisiae* AANAT (ScAANAT) apparently functions in melatonin synthesis and in general detoxification of arylalkylamines. The molecular model of ScAANAT has revealed two main differences compared to mammalian AANAT (Ganguly *et al.*, 2001). One is the lack of similarity in regions flanking the catalytic core. These regions are likely to be involved in regulation (cAMP regulates mammalian AANAT) and are absent in the yeast AANAT. This observation indicates that through evolution AANAT became involved in photochemical transduction in multicellular eukaryotes. The second difference occurs in loop 1, which is involved in the formation of arylalkylamine binding site. The crystal structures for ovine AANAT (oAANAT) are consistent with loop 1 moving and undergoing a  $\alpha$ -helical reorganization during catalysis (Hickman *et al.*, 1999). The only conserved residues in ScAANAT are observed at the ends of this loop, while the length and the intervening sequence are very different (Ganguly *et al.*, 2001). These differences might account for the lower specificity of ScAANAT for serotonin and related substrates.

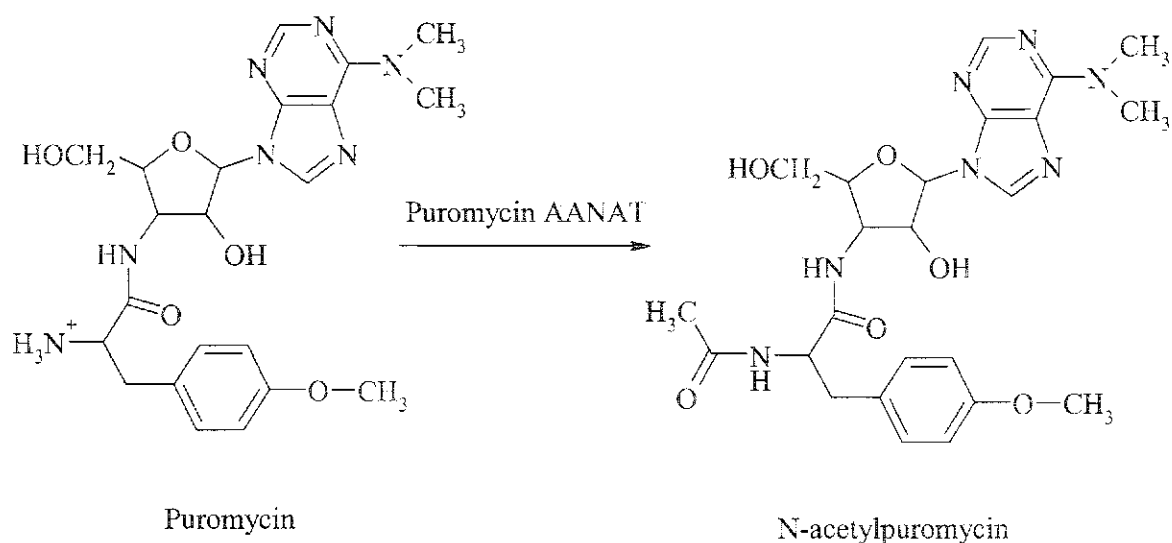
It is clear that in vertebrates, AANAT is an integral component of the melatonin synthesis and circadian rhythm and has no other apparent function. This role is reflected in the substrate specificity for serotonin and related substrates as well as the tissues in which this enzyme is expressed. The role of AANATs in simpler organisms is still not well understood, nevertheless a much broader function such as detoxification has been suggested (Ganguly *et al.*, 2001). This latter comment implies that substrate specificity for serotonin and related substrates (involved in the circadian rhythm machinery) might be low while the specificity for other arylalkylamines, more specific to their immediate

environment, might be high.

#### b. Prokaryotic AANATs

There is minimal information regarding bacterial AANATs in the literature. However, puromycin N-acetyltransferase (PAC, puromycin AANAT) isolated from *Streptomyces alboniger* (Vara *et al.*, 1985) is a protein of 24 kDa that catalyses the acetylation of the aminoacyl nucleoside antibiotic puromycin at the arylalkylamino group (Figure 10) and therefore has been considered an AANAT. Although this AANAT was partially purified and characterized in 1985 (Vara *et al.*, 1985), no crystal structure of this molecule has been generated to date. However, a multiple alignment and database search included PAC as part of the GNAT superfamily (Neuwald and Landsman, 1997). A three-dimensional structure of PAC would be necessary to confirm the conserved motifs of the members of the GNAT superfamily.

Antibiotic inactivation in aminocyclitol-producing *Streptomyces* proceeds through either acetylation or phosphorylation. These types of drug modification in certain antibiotic-producing organisms are connected with a self-protection mechanism against antibiotic products and toxic precursor molecules (Lacalle *et al.*, 1989; Vara *et al.*, 1985). Puromycin AANAT is a very specific enzyme that acetylates puromycin and other structurally related substrates such as O-demethylpuromycin, which is an intermediate in the biosynthesis of puromycin and presents antibiotic activity itself (Vara *et al.*, 1985). Interestingly, puromycin AANAT does not catalyse the acetylation of tyrosine nor puromycin aminonucleoside even when these constitute part of the whole puromycin



**Figure 10.** Reaction Catalysed by Puromycin AANAT.

(Adapted from Vara *et al.*, 1985)

molecule (Vara *et al.*, 1985), emphasizing the high specificity of this enzyme. However, there are no reports on the acetylating activity of puromycin AANAT on serotonin and related substrates. Therefore, in light of the above studies, further investigation of puromycin AANAT and other related proteins would most likely illuminate the role of these enzymes in the producing organism.

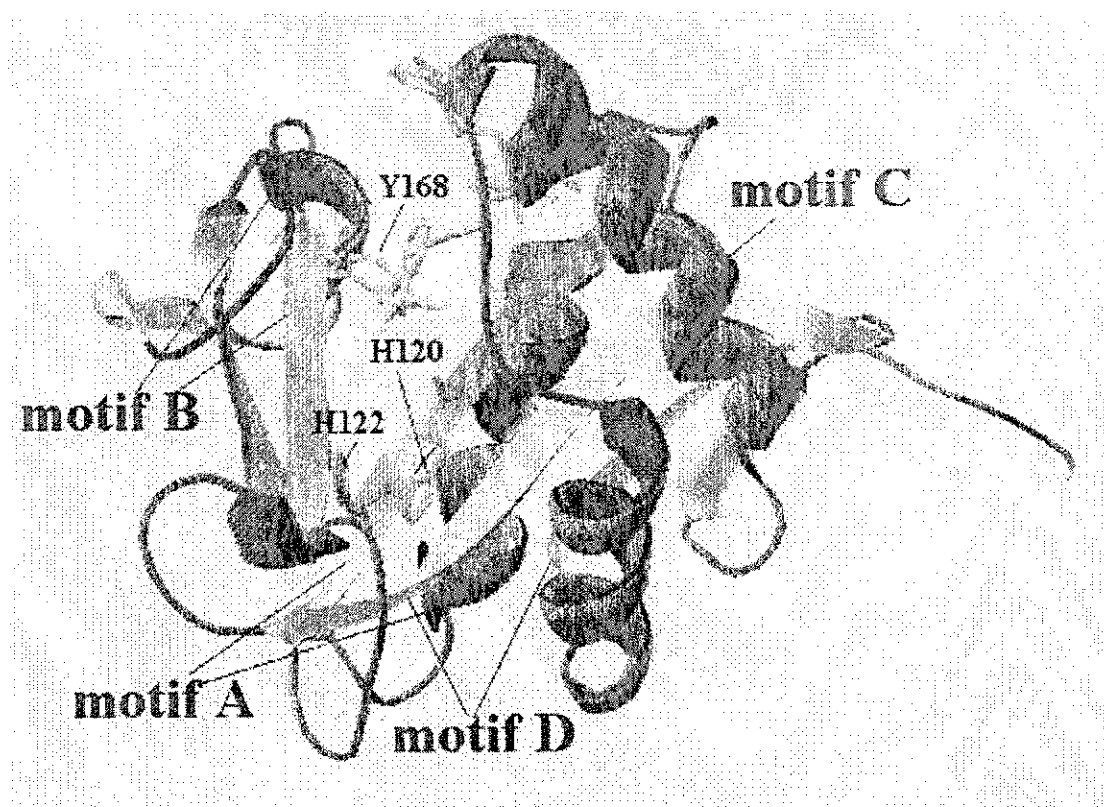
### c. Characteristics of AANATs

AANATs have been described as monomeric enzymes with a range in size between 20-30 kDa (Ichihara *et al.*, 2001; Wolf *et al.*, 2002). These enzymes contain the four conserved sequence motifs (A through D) of the GNAT superfamily. The optimal temperature for AANATs has been reported to be variable ranging from 37°C to 55°C

(Ganguly *et al.*, 2001; Ichihara *et al.*, 2001; Muimo and Isaac, 1993). The optimal pH has been shown to be highly variable with values ranging from 5.8 to 10 (Ganguly *et al.*, 2001; Ichihara *et al.*, 2001). These enzymes have shown inhibition with  $\text{Cu}^{2+}$  while effects with other divalent cations have been variable (Craft and Zhan-Poe, 2000; Falcon *et al.*, 2001; Morton, 1987; Morton, 1989).

The only three-dimensional structure of an AANAT, available to date, corresponds to a truncated form of ovine serotonin AANAT (oAANAT). This structure has been solved both in the unbound form (Hickman *et al.*, 1999a) and bound to a bisubstrate analog (coenzyme-A-S-N-acetyl tryptamine, Figure 11) (Hickman *et al.*, 1999b). This three-dimensional form of the enzyme provides a very comprehensive view of structural roles played by each of the four conserved motifs of this type of proteins. The crystal structure presents a single domain V-shaped molecule consisting of a seven-stranded mixed  $\beta$ -sheet flanked by five  $\alpha$ -helices. The  $\beta$ -strands run antiparallel, except for the two central ones that give rise to the V-shaped structure of this molecule. Despite the limited primary sequence homology among the members of the GNAT superfamily, the central fold of oAANAT is very similar to that of HAT1 (Dutnall *et al.*, 1998) and AAT (Wolf *et al.*, 1998).

The four most highly conserved motifs occur from the N-terminus in the order C, D, A, and B (Figure 11) (Dyda *et al.*, 2000). Motif C is characterized by a strand, a long loop and two  $\alpha$ -helices and constitutes the most mobile region of the structure. Motif D consists of two  $\beta$ -strands and turns into a  $\beta$ -strand where motif A starts. Motif A is the longest and most highly conserved motif. Motif A includes one of the strands involved in



**Figure 11 .** The Three-Dimensional Structure of Ovine Serotonin AANAT (oAANAT) Bound to the Bisubstrate Analog. Tyr<sup>168</sup>, His<sup>120</sup>, and His<sup>122</sup> are highlighted as important residues in catalysis.

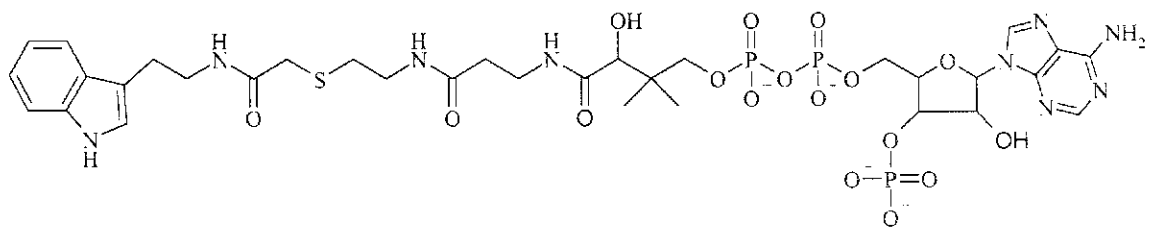
(Picture prepared using Swiss-Pdb Viewer, pdb code 1cju)

the V-shaped region of this molecule and one  $\alpha$ -helix. Finally, motif B presents a  $\beta$ -strand that defines the other side of the V-shaped region and an  $\alpha$ -helix. The AcCoA binding site has been identified as a cleft formed at the site where the two parallel strands come apart (inside the V-shaped region of the molecule) where potentially critical residues for AANAT have been identified: Tyr<sup>168</sup>, His<sup>120</sup>, and His<sup>122</sup> (Figure 11) and showed to be important in catalysis (Scheibner *et al.*, 2002).

The accepted catalytic mechanism of oAANAT to date is of an ordered “Bi-Bi” ternary complex kinetic mechanism (De Angelis *et al.*, 1998; Khalil *et al.*, 1999; Khalil and Cole, 1998; Zheng and Cole, 2002). According to this reaction mechanism AcCoA binds first followed by the amine substrate (usually serotonin or tryptamine for oAANAT). This mechanism involves the binding of both AcCoA and the amine substrate before the release of the products coenzyme A (CoASH) or N-acetylated amine (Khalil *et al.*, 1999; Zheng *et al.*, 2001). This notion is supported by the lack of plausible residues in the active site for intermediate formation (Hickman *et al.*, 1999a). Based on this catalytic mechanism, a bisubstrate analog, which links tryptamine and coenzyme A via an acetyl bridge (coenzyme-A-S-N-acetyltryptamine, Figure 12) was synthesized and shown to be a very potent and specific AANAT inhibitor *in vitro* (Hickman *et al.*, 1999b; Kim and Cole, 2001; Khalil *et al.*, 1999; Wolf *et al.*, 2002; Zheng *et al.*, 2001). This potent inhibitor closely approximates the apparent catalytic intermediate (Obsil *et al.*, 2001). This ordered binding of substrates has been thought to involve a conformational change in AANAT upon binding of the first substrate allowing the second substrate to bind more effectively (Hickman *et al.*, 1999b; Wolf *et al.*, 2002b; Zheng *et al.*, 2001). This

conformational change was observed during analysis of the crystal structures of free-enzyme and its form bound to the bisubstrate analog inhibitor (Hickman *et al.*, 1999b).

The only three-dimensional structure for an AANAT determined to date has shown consistency in protein topology with the GNAT superfamily. However, most of the research has focussed on eukaryotic AANATs as shown by the scarce information on prokaryotic AANATs. Future investigations on prokaryotic AANATs will provide new crystal structures that may help to elucidate the actual mode of catalysis for these enzymes.



**Figure 12.** Structure of Coenzyme-A-S-N-Acetyltryptamine (Bisubstrate Analog)

(Adapted from Khalil and Cole, 1998)

### 3. MATERIALS AND METHODS

#### 3.1. General

Water for microbiological media and most solutions were deionized (resistivity of 18 M $\Omega$ -cm) and filtered (0.20  $\mu$ m) using a NANOpureUV Ultrapure water system Model D7331 (Barnstead / Thermolyne, Dubuque, IA). A VWR Scientific (West Chester, PA) Model 8005 pH meter was used for pH measurements and a Beckman (Palo Alto, CA) DU 640 Spectrophotometer was used for measuring UV absorbance and optical density of spore suspensions.

Culture media were sterilized at 121°C for 20 min in a Castle autoclave Model ES30 (Sussman, Long Island City, NY). Manipulations requiring aseptic conditions were performed in a BioKlone laminar flow hood Model BK-2-4 (Microzone Corporation, Nepean, ON). Both semi-solid and shake liquid cultures were grown in the dark at 27°C inside a Sanyo growth cabinet model MLR-350H (Sanyo, Japan). Liquid cultures were shaken at 150 rpm using a Lab-Line bench top orbit shaker Model 3520 (Lab-Line Instruments, Melrose Park, IL). Micro-centrifugations at room temperature were performed using a Beckman Microfuge 18 centrifuge with a maximum centrifugal force of 18000 $\times$ g, while micro-centrifugations at 4°C were performed using an Eppendorf 545R centrifuge (Eppendorf, Germany). Preparative scale centrifugations were done at 4°C on a Sorvall Superspeed RC2-B centrifuge (Kendro, Newton, CT) equipped with a GSA rotor with a maximum centrifugal force of 27485 $\times$ g.

Freeze drying was performed using a Virtis freeze drier Model 10-020 (Virtis Company Inc., Gardiner, NY) connected to an Edwards pump Model E1M8 (Edwards High Vacuum Int., England).

### 3.2. Chemicals

All solvents used for high pressure liquid chromatography (HPLC) were HPLC quality and were used without further purification (EM Science, Gibbstown, NJ). Acetyl coenzymeA (AcCoA), L-3,4-dihydroxyphenylalanine (L-dopa), D-3,4-dihydroxyphenylalanine (D-dopa), L-phenylalanine, N-acetyl-L-phenylalanine, L-tyrosine, N-acetyl-L-tyrosine, 5-hydroxytryptamine (serotonin), 5-methoxytryptamine, N-acetyl-5-methoxytryptamine (melatonin), 3-hydroxytyramine (dopamine), 2-aminofluorene (2-AF) and 4-nitroaniline were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). The bisubstrate inhibitor CoA-S-N-acetyltryptamine was obtained from SRI International (Menlo Park, CA) through the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (NIMH). N-acetyldopamine monohydrate (Regis P48-93-1) was obtained from Dr. Steven L. Coon, National Institutes of Health, Bethesda, MD. All chemicals were used without further purification.

### 3.3. High Pressure Liquid Chromatography (HPLC)

#### 3.3.1. HPLC System

The high pressure liquid chromatography (HPLC) system consisted of an HP 1100 quaternary pump with vacuum degasser, an HP 1100 autosampler, and a thermostatted column compartment. Detection was accomplished with a HP 1100 diode-array detector. The entire HPLC was controlled by a PC running HP ChemStation software.

### 3.3.2. Ion Pairing-Buffer

Ion pairing-buffer was prepared by adding tetrabutylammonium hydrogen sulfate (TBAH, Sigma Chemical Co., 10.0 mM) and  $\text{KH}_2\text{PO}_4$  (BDH, Toronto, ON; 2.0 mM) in 700 mL of HPLC grade water (EM Science). The pH was adjusted to 5.0 using dilute NaOH prior to the addition of 200 mL of methanol (EM Science). The final volume was adjusted to 1L with HPLC grade water and the buffer was vacuum filtered and degassed through a teflon filter membrane (TMF 1.5-10, Aura Industries, Staten Island, NY).

### 3.3.3. HPLC Chromatographic Conditions

N-Acetyl-L-dopa was analysed on a Zorbax-SB-C18 column ( $4.6 \times 100$  mm,  $3.5 \mu\text{m}$ ; Agilent Technologies Canada, Mississauga, ON). The column was eluted with ion-pairing buffer using a 6 minute isocratic method at a flow rate of 1 mL/min and  $30^\circ\text{C}$ . Absorbance was monitored at  $206 \text{ nm} \pm 2$  ( $450 \text{ nm} \pm 40$ ). N-Acetyl-L-dopa had a retention time of 2.6 min under these conditions and could be verified by comparison of its UV spectrum to a library of spectra.

Other N-acetylated substrates were analysed on a Zorbax Eclipse XDB-C18 column ( $4.6 \times 150$  mm  $3.5 \mu\text{m}$ ; Agilent Technologies Canada). This column was equilibrated with either 100% water or 20% acetonitrile in water at  $30^\circ\text{C}$ . Elution was accomplished with a gradient 12% to 100% or 20% to 100% of acetonitrile in water at a flow rate of 1 mL/min over 30 min. Column eluant was monitored at 206 nm and products were identified by comparison of their retention time and UV spectra with that obtained from synthetically prepared samples. Retention times for acetylated amines were as follows: acetylserotonin, 10.0 min; acetyltryptamine, 18.4 min; melatonin, 17.5

min; acetyldopamine, 8.3 min; N-acetyl-4-nitroaniline, 22.9 min and N-acetyl-2-amino-fluorene, 25.4 min.

### 3.4. Development of Optimal Assay Conditions

#### 3.4.1. Synthesis and Purification of N-Acetyl-L-dopa

N-Acetyl-L-dopa was synthesized according to the method described by Smith and White (1995) with minor modifications. L-dopa (105.9 mg) was dissolved in water (10 mL) and portions of acetic anhydride (0.1 mL x 12, BDH, Toronto, ON) were added with continuous stirring over 2 h. The reaction mixture was stirred at room temperature (22°C) for an additional 2 h. The resulting solution was evaporated to dryness under reduced pressure (Rotavapor R110, Büchi Laboratorums-Technik AG, Flawil, Switzerland). The remnants of acetic anhydride and acetic acid were removed by repeated additions of water (3 × 3 mL) followed by removal *in vacuo* as indicated above.

The residue was dissolved in water and passed through a Dowex 50W-X2 (Bio-Rad Laboratories, Richmond, CA) column using water as eluant to remove any remaining acetic acid. An aliquot of the eluate (10 µL) was analysed by HPLC to confirm purity.

#### 3.4.2. N-Acetyl-L-dopa Standard Curve

Solutions of N-acetyl-L-dopa (5 µM-1.2 mM) were prepared in HPLC grade water. Portions of each solution (10 µL) were analysed in quartet by HPLC as described in section 3.3.3.

### 3.4.3. Linearity of the Assay at 30°C and 37°C

Assay reactions were performed in triplicate and consisted of enzyme preparation (150  $\mu\text{L}$ ), AcCoA (75  $\mu\text{L}$ , 4 mM) and L-dopa (75  $\mu\text{L}$ , 8 mM). Substrates were prepared in sterile HPLC grade water. The reactions were started by the addition of the enzyme preparation and incubated at 30°C or at 37°C for up to 60 min. Portions (30  $\mu\text{L}$ ) were removed at time intervals, quenched by the addition of 5  $\mu\text{L}$  of 3% (v/v) trifluoroacetic acid (TFA) and centrifuged (18000 $\times$ g, 5 min, 22°C). An aliquot of the supernatant (10  $\mu\text{L}$ ) was analysed by HPLC. The area under the peak for N-acetyl-L-dopa was integrated and correlated to concentrations of product based on the standard curve.

### 3.4.4. Standard Enzyme Assay Conditions

The standard assay mixture, with a final volume of 30  $\mu\text{L}$ , contained enzyme preparation (15  $\mu\text{L}$ ), AcCoA (7.5  $\mu\text{L}$ , 4 mM) and L-dopa (7.5  $\mu\text{L}$ , 8 mM). The reaction was started by the addition of the enzyme preparation and allowed to proceed for 20 min at 37°C. The activity assay was quenched by addition of 5  $\mu\text{L}$  of 3% (v/v) TFA and centrifuged (18000 $\times$ g, 5 min, 22°C). The supernatant was transferred to a 250  $\mu\text{L}$  HPLC vial insert and a portion (10  $\mu\text{L}$ ) was analysed by HPLC. One enzyme unit was defined as the amount of enzyme producing 1  $\mu\text{mol}$  of N-acetyl-L-dopa per min. Each enzyme assay was performed in duplicate.

### 3.5. Microorganism

*Streptomyces akiyoshiensis* L-138, HON<sup>-</sup> mutant strain was prepared by chemical mutagenesis using N-methyl-N<sup>7</sup>-nitro-N-nitrosoguanidine (NTG). This strain was prepared and provided by L. C. Vining (Le *et al.*, 1996)

### 3.6. Microbiological Media

#### 3.6.1. MYM Agar

MYM agar medium was prepared with malt extract (10.0 g/L, Bacto, Difco Laboratories, Detroit, MI), yeast extract (4.0 g/L, Bacto, Difco Laboratories), maltose (4.0 g/L, Bacto, Difco Laboratories) and agar (20 g/L, Becton Dickinson and Company, Sparks, MD) in water.

#### 3.6.2. MYG

MYG medium was prepared with malt extract (10.0 g/L), yeast extract (4.0 g/L) and dextrose (4.0 g/L, all Bacto, Difco Laboratories) in water. The final pH was adjusted to 6.0 prior to autoclaving.

#### 3.6.3. Casein-Starch

Casein-starch medium was prepared with casein (4.0 g/L, technical grade, Sigma Chemical Co.) and soluble starch (30 g/L, Difco, Becton Dickinson and Company) in basal medium. After autoclaving the media was swirled to prevent the starch from solidifying on cooling.

#### 3.6.4. Basal

Basal medium was prepared with  $\text{MgSO}_4$  (0.20 g),  $\text{KH}_2\text{PO}_4$  (0.81 g),  $\text{K}_2\text{HPO}_4$  (1.75 g), 4.5 mL of  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$  solution (7.2 mM), 1 mL of salt solution ( $\text{NaCl}$ , 171.0 mM;  $\text{CaCl}_2$ , 901 mM), 4.5 mL of stock mineral solution ( $\text{H}_3\text{BO}_3$ , 26  $\mu\text{M}$ ;  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4 \text{H}_2\text{O}$ , 8  $\mu\text{M}$ ;  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ , 40  $\mu\text{M}$ ;  $\text{ZnCl}_2$ , 294  $\mu\text{M}$  and  $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ , 444  $\mu\text{M}$ ) and water to a total volume of 1L. The pH was adjusted to pH 6.5 prior to autoclaving.

### 3.7. Buffers

#### 3.7.1. Tris-Glycerol Buffer (TG)

Tris-glycerol buffer (TG) was prepared with tris (hydroxymethyl)aminomethane (100 mM, OmniPur, EM Science) and glycerol 10% (v/v) in water. The pH was adjusted to 8.0 at room temperature (22°C). Prior to use,  $\beta$ -mercaptoethanol was added to the buffer to give a final concentration of 20 mM.

#### 3.7.2. Tris-Glycerol-EDTA Buffer (TGE)

Tris-glycerol-EDTA buffer (TGE) was prepared as the TG buffer above but with the addition of 2 mM of ethylenediaminetetra acetic acid dipotassium salt (EDTA, BDH Laboratory Supplies, Poole, England). The pH was adjusted to 8.0 at room temperature (22°C). Prior to use,  $\beta$ -mercaptoethanol was added to the buffer to give a final concentration of 20 mM.

### 3.8. Stock Spore Suspension

The protocol used was described by Glazebrook *et al.* (1993), with only slight modifications. MYM agar plates were inoculated by streaking spore suspension (10  $\mu$ L) using a sterile inoculating loop. After seven days of growth at 27°C in the dark, spores were harvested by flooding plates with sterile water (5 mL) and the spores were dislodged with the aid of a sterile inoculating loop. The suspension was filtered through a layer of sterile miracloth (Calbiochem, La Jolla, CA) to remove mycelial fragments and the resulting filtrate was centrifuged (9100 $\times$ g, 15 min, 4°C). The pelleted spores were resuspended in sterile 20% (v/v) glycerol to give a final optical density of 0.16 (measured at 640 nm after a 1:50 dilution) and were stored in small aliquots (1.5 mL) at -20°C.

### 3.9. Seed Cultures

Seed cultures were prepared by inoculating sterile MYG medium (30 mL in 250 mL baffled flasks) with 30  $\mu$ L of spore suspension. Cultures were incubated at 27°C in the dark for 72 h with continuous shaking (150 rpm).

### 3.10. Production Cultures

Production cultures were prepared by inoculating casein-starch medium (300 mL in 2 L Erlenmeyer flasks) with 1% (v/v) of seed culture and incubating at 27°C in the dark for 72 h with continuous shaking (150 rpm).

### 3.11. Harvesting and Storage of Cells

Production cultures were distributed into centrifuge bottles (250 mL capacity) and cells were pelleted by centrifugation (9100×g, 15 min, 4°C). Cells were resuspended in water and centrifuged (9100×g, 15 min, 4°C). Pelleted cells (approximately 50 g wet mass) were transferred onto aluminum foil making a thin layer (3-4 mm thick maximum), wrapped and stored at -80°C.

### 3.12. Cell Free Extract

Cells were thawed and resuspended in cold TGE buffer containing 20 mM of  $\beta$ -mercaptoethanol using a ratio of cell mass to TGE buffer of 1:1.2. All subsequent steps were carried out at 4°C, unless otherwise stated. The cells were homogenized using a Branson Sonifier Model 450 (40 s at 53 watts (50% duty cycle followed by 10 s of cooling, repeated 5 times), Danbury, CT). The homogenate was centrifuged (9100×g, 15 min, 4°C) to pellet cell debris and the resulting cell-free supernatant was partitioned into 15 mL falcon tubes (10 mL per tube), frozen using liquid nitrogen and stored at -80°C. The crude cell-free preparation could be stored for several months without any appreciable loss of activity.

### 3.13. Partial Purification of *S. akiyoshiensis* NAT

The partial purification scheme for *S. akiyoshiensis* NAT was developed after labourious trials using various other techniques. In order to reduce the volume of extract and achieve partial separation bulk methods such as precipitation techniques using

ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), ethanol, acetone and polyethylene glycol (PEG) were evaluated.

Affinity chromatography was investigated using various approaches to achieve a highly selective capture. Affi-gel®102 (Bio-Rad Laboratories) and ArgoGel®-NH<sub>2</sub> (Argonaut Technologies Inc., Foster City, CA) were coupled to L-dopa in order to capitalize on the specificity of *S. akitoshiensis* NAT for this arylalkylamine substrate. Affi-agarose-CoA (Bio-Rad Laboratories) tested the possible affinity of this enzyme for CoA. To explore the affinity of this enzyme for cibacron blue F3GA, various approaches using affi-gel blue (Bio-Rad Laboratories) were tried. Finally, in order to attempt further purification of this NAT an econo-pac DEAE blue cartridge (Bio-Rad Laboratories) was also used.

Ion-exchange chromatography was evaluated aiming for a good recovery and resolution. Cation exchangers econo-pac High S cartridge (methyl sulphonate, Bio-Rad Laboratories), and econo-pac CM (carboxymethyl, Bio-Rad Laboratories) as well as anion exchangers econo-pac high Q cartridge (quaternary ammonium, Bio-Rad Laboratories) and macro-prep DEAE (Bio-Rad Laboratories) were tested. In addition, a weak anion-exchange HPLC column, SynChropack AX300 column (4.6 × 250 mm, 6 µm, Eichrom Technologies, Inc., Darien, IL) was also tried. This column consisted of crosslinked polyethyleneimine silica based support compatible with aqueous and many organic solvents in the pH range of 2-8.

Gel filtration chromatography was also tested using a Bio-Silect SEC 250-5 column (Bio-Rad Laboratories) with a molecular weight fractionation range of 10-300 kDa and a Zorbax Bio Series GF-250 column (9.4 × 250 mm, 4 µm; Agilent

Technologies Canada). Both of these columns were run using HPLC systems and very concentrated protein preparations.

Further purification of *S. akiyoshiensis* NAT was also attempted using hydrophobic interaction chromatography (HIC). An econo-pac methyl HIC cartridge (Bio-Rad Laboratories) was tested for selectivity and binding strength. Adsorption chromatography was also tried using an econo-pac CHT II cartridge (ceramic hydroxyapatite, Bio-Rad Laboratories). This column explored the mixed mode ion-exchanger hydroxyapatite as a possible method in the purification of *S. akiyoshiensis* NAT.

After a careful evaluation of all these techniques a three-step procedure was established for the partial purification of *S. akiyoshiensis* NAT. Only additional methods that allowed some progress in the purification of this protein will be discuss further. Unless otherwise indicated all buffer solutions used in the purification were at pH 8.0 and supplemented with 20 mM  $\beta$ -mercaptoethanol. All operations described below were carried out at 4°C.

#### 3.13.1. Affi-Gel Blue Gel Chromatography

Affi-gel blue (Bio-Rad Laboratories) was used according to the specifications of the manufacturer. This resin was loaded into a column (1.3 × 3.0 cm, 5 mL) and equilibrated with TGE buffer. To take advantage of the affinity of *S. akiyoshiensis* NAT for cibacron blue F3GA the cell free extract was loaded in five portions of 10 mL each. Each portion of cell free extract was equilibrated with the resin batchwise with gently suspension (Rocking Platform shaker, Model 200, VWR) for 20 min. A wash consisting

of 10 mL of TGE buffer with 0.5 M NaCl followed each application to remove protein weakly bound to the affi-gel blue resin. NAT activity was eluted from the resin by suspending the resin in 10 mL of 10% (v/v) glycerol for 10 min, allowed the resin to settle and collected the glycerol eluate. This procedure was repeated 5 times and each portion was assayed for enzyme activity and protein content. Portions containing the majority of activity were pooled and diluted with one-tenth the volume of 1 M Tris buffer, pH 8.0 containing 0.5 M NaCl to obtain a final concentration of 100 mM Tris and 50 mM NaCl in the sample. These combined portions were immediately re-assayed and used in the next step.

### 3.13.2. First Macro-Prep DEAE Column Chromatography

The pooled enzyme preparation from affi-gel blue chromatography was applied to a macro-prep DEAE (Bio-Rad Laboratories) column ( $0.7 \times 1.5$  cm, 0.6 mL) pre-equilibrated with 3 column volumes of TG buffer containing 50 mM NaCl. The column was washed with an additional 3 mL of this buffer and elution was accomplished with TG buffer containing 0.35 M NaCl at a flow rate of 0.4 mL/min. Five fractions were collected and assayed for enzyme activity as well as for protein. The fractions showing the majority of activity were pooled and diluted to 50 mM NaCl final concentration using TG buffer. Initial experiments using dialysis to concentrate this enzyme preparation rendered the enzyme inactive. Therefore the enzyme solution was concentrated to a final volume of 1 mL using Amicon stirred cells Model 8010 (Millipore Corporation, Billerica, MA).

### 3.13.3. Second Macro-Prep DEAE Column Chromatography

The concentrated protein preparation from above was loaded onto a macro-prep DEAE (Bio-Rad Laboratories) column (1 × 42 cm) pre-equilibrated with TG buffer containing 50 mM NaCl. The column was washed by addition of buffer (40 mL) and the bound proteins were eluted with a linear NaCl gradient generated between 0.20 and 0.50 M NaCl solution in TG buffer (25 mL of each). The column was operated at a flow rate of 0.5 mL/min using a peristaltic pump. Fractions of 2 mL were collected and assayed for NAT activity and protein.

### 3.13.4. Other Chromatographic Techniques

#### 3.13.4.1. *Hydrophobic Interaction Chromatography (HIC)*

Ammonium sulfate was added to a pooled enzyme preparation from affi-gel blue chromatography (40 mL) to reach a final concentration of 2 mM. This enzyme preparation was applied to an econo-pac methyl HIC cartridge (5 mL, Bio-Rad) pre-equilibrated with TGE buffer containing 2 mM ammonium sulfate. The column was washed with 10 mL of this buffer and elution was accomplished with a linear  $(\text{NH}_4)_2\text{SO}_4$  gradient generated between 2 and 0 mM  $(\text{NH}_4)_2\text{SO}_4$  in TGE buffer (20 mL of each). The column was operated at a flow rate of 0.4 mL/min. Fractions of 2 mL were collected and assayed for NAT activity and protein.

#### 3.13.4.2. Gel Filtration Column Chromatography

One injection of 100  $\mu\text{L}$  of a 25-fold concentrated enzyme preparation from affi-gel blue chromatography (freeze-dried and resuspended in 50 mM Tris containing

200 mM NaCl, pH 7.75) was applied to a Zorbax Bio Series GF-250 column (9.4 × 250 mm, 4 µm; Agilent Technologies Canada) pre-equilibrated with 50 mM Tris containing 200 mM NaCl, pH 7.75. Elution was accomplished with a flow rate of 0.2 mL/min over 2 min followed by 1 mL/min over 25 min. Fifty fractions of 0.2 mL were collected and assayed for NAT activity and protein.

#### **3.14. Protein Determination**

Protein assays were performed using the Bio-Rad (Hercules, CA) protein microassay based on the Bradford's protein dye-binding method (Bradford, 1976), with gamma globulin as the standard.

#### **3.15. SDS-Polyacrylamide Gel Electrophoresis**

Electrophoresis was performed using the Bio-Rad Mini Protean III apparatus. Gels were 1 mm thick (12% total acrylamide concentration; 2.7% N'N'-bis-methylene-acrylamide concentration). The total monomer concentration of the stacking gel was 4%. Samples were mixed with 0.5 M Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 25% (w/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue and were denatured by heating for 4 min at 98°C prior to electrophoresis.

Molecular masses of proteins were estimated by comparison to standard proteins (Bio-Rad Laboratories) run on the same gel: myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase (97.40 kDa), bovine serum albumin (66.20 kDa), ovalbumin (45.00 kDa), carbonic anhydrase (31.00 kDa), soybean trypsin inhibitor

(21.50 kDa), lysozyme (14.40 kDa), and aprotinin (6.50 kDa). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) or Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories).

### **3.16. Characterization of Partially Purified *S. akiyoshiensis* NAT**

#### **3.16.1. Apparent Optimum pH of *S. akiyoshiensis* NAT**

The apparent optimal pH of *S. akiyoshiensis* NAT was determined using both Tris buffer and bis-Tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane, Sigma Chemical Co.) prepared using final pH values ranging from 6.0 to 10.0 at room temperature (21°C). Initially, the enzyme preparation (30 µL, post affi-gel blue fraction containing only 10% (v/v) glycerol) was mixed with either Tris or bis-Tris buffer solution (60 µL of a 150 mM solution). The resulting enzyme mixtures at different pH values were used to perform enzyme assays. These assays consisted of the enzyme preparation at various pH values (15 µL), L-dopa (7.5 µL, 8 mM) and AcCoA (7.5 µL, 4 mM). Reactions were started by the addition of the enzyme preparation, stopped by the addition of 3% TFA, and were run in duplicate. The exact same approach was followed using tris or bis-tris buffer supplemented with 375 mM NaCl to get a final concentration of 250 mM NaCl in enzyme preparation.

#### **3.16.2. Apparent Optimum Temperature of *S. akiyoshiensis* NAT**

Enzyme assays were performed at different incubation temperatures (20 - 50°C) and pH 8.0. The reactions were run in duplicate and consisted of the enzyme preparation (15 µL), AcCoA (7.5 µL, 4 mM) and L-dopa (7.5 µL, 8 mM). The reactions were started

by the addition of the enzyme preparation and stopped after 20 min by the addition of 3% TFA (5  $\mu$ L).

### 3.16.3. Renaturation of *S. akiyoshiensis* NAT following Heat Denaturation

For the analysis of the temperature stability of *S. akiyoshiensis* NAT, two enzyme preparations (post-affi-gel blue and post-first DEAE fractions) were tested. These were aliquoted into eight microfuge tubes respectively (50  $\mu$ L per tube, eight tubes per enzyme preparation) and incubated at boiling water temperature ( $\sim 98^{\circ}\text{C}$ ) for 5 min. After this treatment, one set of four tubes (for each of these enzyme preparations) was incubated on ice. Standard assays were performed with these enzyme solutions at 5 min, 30 min, 60 min and 120 min of incubation on ice respectively. The other set of four tubes (for each of the enzyme preparations) was centrifuged (18000 $\times$ g, 10 min,  $4^{\circ}\text{C}$ ) to pellet precipitated protein, then incubated on ice. Standard activity assays were performed with these enzyme samples at 5 min, 30 min, 60 min and 120 min of incubation on ice respectively.

### 3.16.4. Effect of Metal Ions on *S. akiyoshiensis* NAT Activity

The effect of metal ions on *S. akiyoshiensis* was examined using two final concentrations of metal ion salts (1 mM and 5 mM). Enzyme reaction mixtures consisted of enzyme preparation (8  $\mu$ L of a post-affi-gel blue fraction containing 100 mM tris buffer, pH 8.0), AcCoA (10  $\mu$ L, 4 mM), L-dopa (10  $\mu$ L, 8 mM) and the metal ion salt solution (12  $\mu$ L of either 16.7 mM or 3.34 mM aqueous solutions). The assay reactions

were initiated with the addition of L-dopa, incubated at 37°C for 20 min and quenched with 3% TFA (5 µL).

#### 3.16.5. Effect of KCl, NaCl, and CaCl<sub>2</sub> on *S. akiyoshiensis* NAT Activity

The effect of different concentrations (0 - 500 mM) of KCl, NaCl, and CaCl<sub>2</sub> on the activity of *S. akiyoshiensis* NAT was evaluated. Enzyme reactions consisted of the enzyme preparation (10 µL), AcCoA (10 µL of a 4 mM solution), the metal ion salt (10 µL of a 4-fold concentrated solution), and L-dopa (10 µL, 8 mM). Assay reactions were initiated by the addition of L-dopa, incubated at 37°C for 20 min and stopped with 3% TFA (5 µL).

#### 3.16.6. Kinetic Studies of *S. akiyoshiensis* NAT

##### 3.16.6.1. Determination of Apparent $K_m$ and $V_{max}$ for L-Dopa

To obtain estimates for the Michaelis constants ( $K_m$  and  $V_{max}$ ) for L-dopa, initial rates of N-acetylation were measured at pH 8.0 and various initial concentrations of this substrate (0.014 - 0.05 mM at 37°C and 0.012 - 0.05 mM at 30°C) while using a final constant concentration of AcCoA (1 mM). The assays were run in triplicate. Each reaction consisted of the enzyme preparation (5 µL), L-dopa (25 µL of a range 0.027 - 0.8 mM or 0.023 - 0.8 mM respectively), and AcCoA (10 µL, 4 mM, fixed concentration). The reactions were started by the addition of the enzyme preparation, incubated at 37°C or 30°C for 20 min, and stopped by addition of 3% (v/v) TFA (5 µL).

### 3.16.6.2. Determination of Apparent $K_m$ and $V_{max}$ for AcCoA

In order to determine kinetic constants for AcCoA, initial rates of N-acetylation were measured at pH 8.0 using a constant final concentration of L-dopa (2 mM) and different concentrations of the cofactor AcCoA (0.02 - 0.2 mM at 37°C and 0.022 - 0.1 mM at 30°C). Activity assays were run in triplicate. Each assay reaction contained the enzyme sample (5  $\mu$ L), L-dopa (15  $\mu$ L, 4 mM, fixed concentration) and AcCoA (10  $\mu$ L of a range of concentration 0.06 - 0.6 mM or 0.07 - 0.3 mM respectively). Reactions were started by the addition of the enzyme preparation, incubated at 37°C or 30°C for 20 min and quenched with 3% TFA (5  $\mu$ L).

### 3.16.7. Substrate Specificity for Partially Purified *S. akiyoshiensis* NAT

#### 3.16.7.1. Synthesis and Purification of N-Acetylated Amines

The protocol for the synthesis and purification of N-acetylated amines, other than L-dopa, was adapted from Vara *et al* (1985) for N-acetylpuromycin with minor modifications. Amines (25 mg) were dissolved in 5% (v/v) methanol (2 mL) and portions of acetic anhydride were added with continuous stirring. The reactions were allowed to proceed and monitored using analytical TLC plates 60 F<sub>254</sub> (EM Science). The resulting solutions were evaporated to dryness under reduced pressure (Rotavapor R110, Büchi) and 35°C. The remnants of acetic anhydride and acetic acid were removed by repeated additions of 5% (v/v) methanol followed by removal *in vacuo* as indicated before.

The resulting residues were purified by preparative TLC on silica gel 60 F<sub>254</sub> (EM Science) support developed with solvent systems consisting of CH<sub>2</sub>Cl<sub>2</sub>–methanol–acetic

acid (95:5:0.5). Major bands were scrapped and eluted with CH<sub>2</sub>Cl<sub>2</sub>-methanol (10:3). N-acetylated amines were identified by <sup>1</sup>H NMR.

#### *3.16.7.2. HPLC Standard Curves for N-acetylated Amines*

Solutions of N-acetylated amines (0.05-0.8 mM) were prepared in HPLC water or 5% (v/v) ethanol. Two aliquots (10 μL each) from each of these solutions were analysed by HPLC as described in the section 3.3.3.

#### *3.16.7.2. Enzyme Assay with other Amine Substrates*

To investigate substrate specificity, enzyme activity assays were performed using a) arylalkylamine substrates that structurally resemble L-dopa, such as: phenylalanine, tyrosine, and D-dopa, b) arylalkylamine substrates characteristic for AANATs, such as: 5-methoxytryptamine, dopamine, tryptamine, and serotonin; and c) arylamine substrates, such as: 4-nitroaniline and 2-aminofluorene (2-AF).

The enzyme reactions were performed in duplicate at pH 8.0 and consisted of the enzyme preparation (50 μL), AcCoA (25 μL, 4 mM) and the amine substrate (25 μL, 4 mM). AcCoA was prepared in sterile HPLC water while the amines were prepared in sterile HPLC water or in 5% (v/v) ethanol (serotonin, tryptamine, 5-methoxytryptamine, 4-nitroaniline and 2-AF). The reactions were started by the addition of the amine substrate, incubated at 37°C for up to 3 h and stopped by the addition of 3% TFA (5 μL). Aliquots (30 μL) of these reactions were taken at 0 min, 20 min and 180 min. HPLC analysis was performed as described in the section 3.3.3 using a gradient from 12% to 100% acetonitrile in water (serotonin, tryptamine, 5-methoxytryptamine, dopamine, 4-

nitroaniline and 2-AF), a gradient from 20% to 100% of acetonitrile in water (tyrosine and phenylalanine) at a flow rate of 1 mL/min over 30 min or ion-pairing buffer using an isocratic method at a flow rate of 1 mL/min over 6 min (D-dopa).

#### 3.16.8. Effect of AANAT Bisubstrate Inhibitor (Coenzyme-A-S-acetyl tryptamine)

The effect of the specific bisubstrate inhibitor for serotonin AANAT was tested as an inhibitor against N-acetyltransferase activity. Four reactions were prepared at pH 8.0 and consisted of the enzyme preparation (20  $\mu$ L) and bisubstrate inhibitor (20  $\mu$ L of a 2 mM solution). Four control reactions were also prepared using enzyme preparation (20  $\mu$ L) and water (20  $\mu$ L). These mixtures were incubated at 37°C for up to 20 min. Each pre-incubated mixture was used to perform standard assays at 5 min-intervals during 2 h.

## 4. RESULTS AND DISCUSSION

N-Acetyltransferases are ubiquitous and versatile enzymes with diverse roles in the living cell. The amide bond formed between a primary amine and reactive ester constitutes part of critical steps in protein synthesis, antibiotic detoxification, chromatin remodelling, melatonin biosynthesis as well as other important biochemical processes. A comprehensive study and characterization of NAT from *S. akyoshiensis* has not been previously described. This investigation focussed on the partial purification and characterization of this *S. akyoshiensis* NAT, which would help to advance the understanding of the structural and functional roles of this enzyme in *Streptomyces*.

In this study *S. akyoshiensis* NAT was partially purified using various chromatographic techniques. Biochemical methods have been used to identify and characterize this enzyme utilizing partially purified enzyme preparations. Additionally, this investigation will help in developing strategies for further purifying this enzyme. The data presented here may ultimately make it possible to elucidate the nature of this enzyme and determine whether it is an ANAT or an AANAT.

### 4.1. Development of Optimal Assay Conditions

#### 4.1.1. N-Acetyl-L-dopa Standard Curve

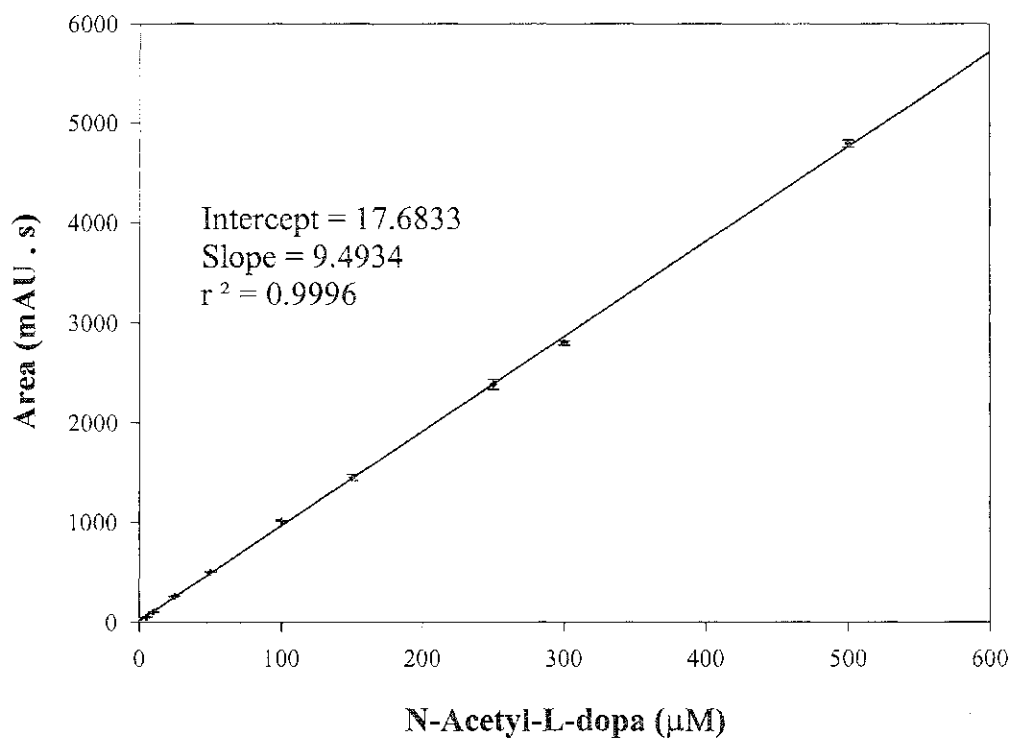
This study utilized an HPLC based assay method to assess N-acetyltransferase activity of cell free extracts and chromatographic fractions. The sensitivity of this method allowed for small assay volumes which reduced the cost of reagents. Since AcCoA is over \$5.00/mg, reducing assay volumes resulted in significant savings. The

chromatographic separation utilized a reverse-phase HPLC column (4.6 × 100 mm, 3.5 μm) to which tetrabutylammonium ion (TBAH) was bound to act as an ion pairing agent. The short run time (6 min) and the utilization of computer controlled automation of injections allowed for the analysis of up to 160 samples per day with exceptional day-to-day reproducibility. In addition, the use of a diode array detector permitted UV spectral verification of product identity. This method avoids the use of radioactive labelled substrates but still maintained high sensitivity and selectivity.

In order to verify the linearity and reproducibility of the chromatographic response, a standard curve was created for N-acetyl-L-dopa as shown in Figure 13. A series of solutions of N-acetyl-L-dopa of varying concentrations were injected and plotted against their areas. Figure 13 shows the linear and reproducible relationship between the concentration of N-acetyl-L-dopa and peak area, which allows for the accurate quantification of enzyme activity. The standard curve created for N-acetyl-L-dopa shows the sensitivity and reproducibility of the HPLC method as a concentration as low as 5 μM of N-acetyl-L-dopa could be detected and quantified accurately in terms of product formed. In addition, the reproducibility of the method is evident by the small error bars among quadruplicates.

#### 4.1.2. Linearity of the Enzyme Assay at 30°C and 37°C

To allow the *S. akiyoshiensis* NAT to be compared to a range of NATs isolated from other organisms, it became necessary to assay the enzyme at both 30°C and 37°C.

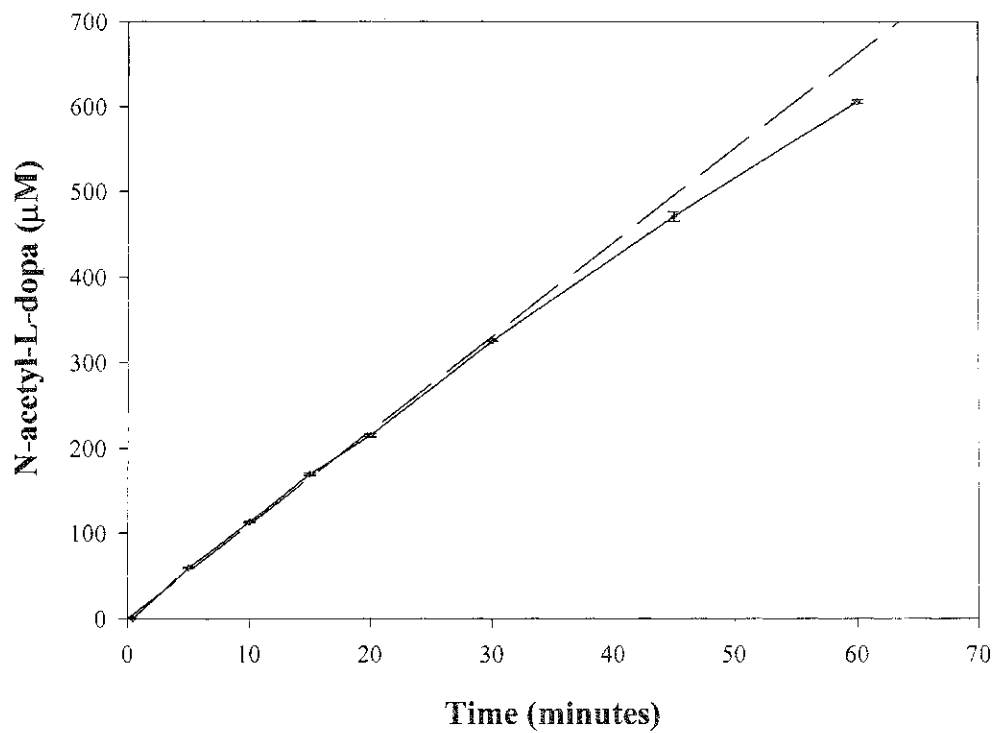


**Figure 13.** HPLC Standard Curve for N-acetyl-L-dopa. Concentrations ranged from 5 µM to 500 µM. Each data point represents the average of four injections to a reverse-phase HPLC column (Zorbax-SB-C18 Column, 4.6 x 100 mm, 3.5 µm, Agilent Technologies Canada). The chromatography was performed according to “Materials and Methods” section 3.4.2., and error bars represent the standard deviation of the mean for each data point.

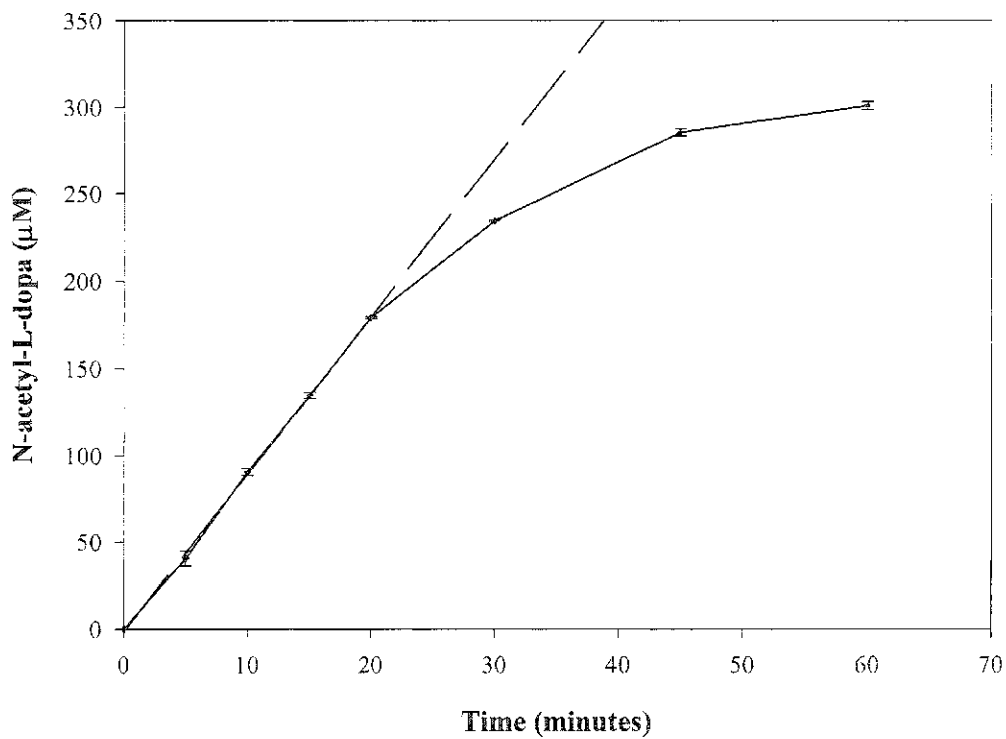
This allowed the comparison against *Streptomyces alboniger* puromycin AANAT (Vara *et al.*, 1985) as well as other NATs, which were characterized at 37°C (Chung, 1998; Ferry *et al.*, 2000; Ganguly *et al.*, 2001; Hsieh *et al.*, 1998; Muimo and Isaac, 1993; Zhan-Poe and Craft, 1999). As can be observed from Figures 14 and 15, the formation of N-acetyl-L-dopa is linear over time up to a concentration of about 180  $\mu$ M and 200  $\mu$ M, which correlate to approximately 20 min at 30 and 37°C, respectively. Although these reaction rates were obtained using different enzyme preparations, product concentrations at or below 180  $\mu$ M at 30°C and 200  $\mu$ M at 37°C can be assumed to be equivalent to initial rates. The non-linearity of the reaction rates above 180  $\mu$ M and 200  $\mu$ M might reflect changes in substrate concentration such as substrate depletion. Another possibility is that the concentration of active enzyme might be changing due the presence of inhibitors or the effect of temperature. This enzyme may become unstable after 20 min at 30 or 37°C.

#### 4.1.3. Standard Activity Assay

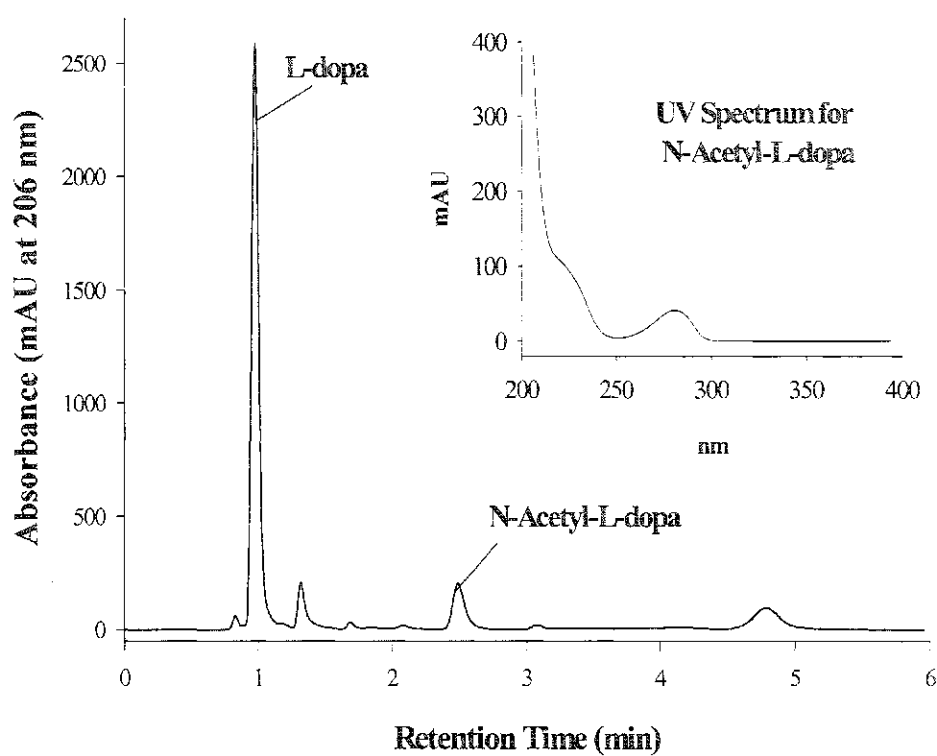
The standard activity assay conditions were established as a 20 min incubation at 37°C. The termination of the enzymatic reaction was initially done using a boiling water bath. However, further experiments indicated that some enzyme preparations conserved some N-acetyltransferase activity after heat treatment. Initially, this was thought to be due to the *S. akiyoshiensis* NAT being a heat stable enzyme. Therefore, the assay was modified to use TFA to terminate reactions. This acid did not interfere with the chromatography as seen in Figure 16 and was efficient in terminating the reaction using



**Figure 14.** Reaction Rate for *S. akioyoshiensis* NAT at 37°C. The enzyme preparation used consisted of a post-first DEAE fraction (total protein 0.19 mg/mL). Reactions were run according to “Materials and Methods” section 3.4.3. Linear regression line (long dash) created for points between 0 and 20 min. Each point represents the mean of triplicate assays. Error bars represent the standard deviation of the mean for each point.



**Figure 15.** Reaction Rate for *S. akiyoshiensis* NAT at 30°C. The enzyme preparation consisted of a post-first DEAE fraction (total protein 0.18 mg/mL). Reactions were run according to “Materials and Methods” section 3.4.3. Linear regression line (long dash) created for points between 0 and 20 min. Each point represents the mean of triplicate assays. Error bars represent the standard deviation of the mean for each point.



**Figure 16.** Chromatogram for a Standard Activity Assay. The chromatographic profile for the analysis of N-acetyltransferase activity was estimated by absorbance at 206 nm (UV spectrum). Partially purified *S. akiyoshiensis* NAT (post-affi-gel blue fraction) was incubated in the presence of AcCoA and L-dopa as described under “Materials and Methods” section 3.4.4. An aliquot of the incubation medium was injected on a reverse-phase HPLC column and resolved using an isocratic method. The concentration of N-acetyl-L-dopa in this chromatogram corresponded to 150  $\mu$ M.

only 5  $\mu\text{L}$  of a 3% (v/v) solution. As can be seen from Figure 16, L-dopa (retention time, 0.99 min) and the product of the reaction, N-acetyl-L-dopa (2.58 min) are well resolved with excellent peak shape. In addition, matching of the UV spectrum with those in the spectral library facilitated the identification of the product of this reaction. This latter factor allowed discrimination of N-acetyl-L-dopa from other compounds which might have a similar retention time.

#### **4.2. Partial Purification of *S. akiyoshiensis* NAT**

Numerous trials were necessary to develop the purification scheme presented in this thesis. Initially, 100 mM MOPS (3-(N-morpholino)propane-sulfonic acid), EM Science) with 0.2 mM PMSF (phenylmethylsulfonyl fluoride) was used as the extraction buffer. However, the N-acetyl-transferase activity recovered was lower than that with TGE buffer with 20 mM  $\beta$ -mercaptoethanol (averages of 0.76  $\mu\text{mol}/\text{min}$  and 1.34  $\mu\text{mol}/\text{min}$ , respectively). In the same manner, the cell free extract obtained with MOPS was not as stable under storage at  $-80^\circ\text{C}$  (about two months), while that obtained with TGE buffer had better stability under storage (about six months). Considering that MOPS is a more expensive buffer than Tris and PMSF is a highly toxic compound that provides uncertain protein protection due to its poor stability and solubility in aqueous solutions, TGE was selected as the extraction buffer.

In order to improve the activity recovered and stability under storage of cell free extracts, the use of protease inhibitors was examined. PMSF and a protease inhibitor cocktail (Sigma-Aldrich Canada) containing 4-(2-aminoethyl)benzenesulfonyl fluoride

(AEBSF), E-64, bestatin, leupeptin, aprotinin, and sodium EDTA were evaluated. Neither of these protease inhibitors provided an increase in activity recovered nor extended stability under storage. On the other hand, TGE with  $\beta$ -mercaptoethanol offered better stability under storage as well as higher recovery of activity. Therefore, the cell free extracts were prepared using TGE buffer supplemented with 20 mM of  $\beta$ -mercaptoethanol.

A great deal of effort went into designing the steps to obtain the most highly purified enzyme preparation. Initially, bulk precipitation techniques using ammonium sulfate, ethanol, acetone and PEG were evaluated. None of these offered a significant fractionation of the cell free extract and all resulted in poor enzyme activity recovery.

Column chromatography utilizing a number of modes of differential binding was also explored. Affinity chromatography which takes advantage of the protein binding selectivity for one or a small number of molecules with high affinity was evaluated. One approach used L-dopa and CoA as ligands. Affi-agarose CoA was purchased from Sigma-Aldrich while affi-gel-L-dopa and argogel-L-dopa were prepared by coupling L-dopa to Affi-gel®102 gel (aminoalkyl agarose, Bio-Rad Laboratories) and to ArgoGel®-NH<sub>2</sub> (Argonaut Technologies Inc.) respectively. Unfortunately, the enzyme activity was not retained on either of these resins. There may be several factors involved in the failure of this techniques. First, as CoA is the product released during this reaction, one should expect that the binding would be considerably weaker than for AcCoA. There has only been a single literature account of this resin being utilized for the purification of an N-acetyltransferase from hen oviduct (Kamitani *et al.*, 1989). The attempt at using L-dopa

affinity chromatography was also a venture as the attachment of the carboxylate to the resin linkers have very possibly sacrificed binding effectiveness. As results will later show, all structural aspects of L-dopa including the presence of the carboxylate, the o-catechol and the stereochemistry are all critical for substrate acetylation. There may be a direct correlation between these structural aspects and substrate access to the binding site.

Of all the fractionation procedures tried during *S. akiyoshiensis* NAT purification, affinity chromatography on affi-gel blue affinity resin (Bio-Rad Laboratories) resulted in the highest recovery of activity. Interestingly, the optimal conditions for elution from this affinity column were quite unconventional. According to the literature a chromatographic buffer containing 5 mM ATP, 0.2 M KCl or 1 M NaCl are among the most adequate eluants of proteins from dye-binding affinity columns (Ngai *et al.*, 2003; Stellwagen, 1990; Worrall, 1996). Remarkably, NaCl in a concentration of 0.5 M was found to be appropriate to elute undesired proteins but did not elute *S. akiyoshiensis* NAT. In an attempt to elute NAT activity a step pH gradient elution was used with no elution of activity observed. Interestingly, an attempt to elute *S. akiyoshiensis* NAT using phosphate buffer pH 5.5 led to the accidental discovery of an appropriate eluant for this protein. This attempt consisted of the application of the cell free extract to the affi-gel blue column, followed by a wash consisting of an aqueous solution of 10% (v/v) glycerol in order to prepare the column for the phosphate buffer pH 5.5. HPLC analysis showed that the majority of the activity (approximately 90%) was found in this wash and no activity was detected in any of the eluant fractions. Therefore, these observations were transformed into a protocol that used NaCl in TGE buffer to remove undesired protein and

10% (v/v) glycerol to elute NAT activity.

In order to find other suitable chromatographic techniques to fractionate the NAT containing preparation further, hydrophobic interaction column chromatography (HIC) was also attempted. Hydrophobic interactions are greatly significant in the stabilization of the three-dimensional structure of proteins (Kennedy, 1990). HIC is based on the ability of a protein to undergo hydrophobic interactions with an insoluble, immobilized hydrophobic group. An econo-pac methyl HIC cartridge (5 mL, Bio-Rad Laboratories) was examined and although the activity recovered through hydrophobic interaction was comparable to those of DEAE chromatography, the presence of ammonium sulfate in the fractionated protein preparations complicated storage and further purification steps. For instance, these samples could not be run directly on a SDS-PAGE gel due to the “smeared” migration in fractions with high concentrations of ammonium sulfate. In addition, fractions eluted from HIC were unstable under storage at -80°C with all activity lost over two weeks of storage. Dialysis of these fractions against TGE overnight at 4°C resulted in significant loss of activity.

The major difficulty during the purification of *S. akiyoshiensis* NAT has been the coelution of proteins. In an attempt to resolve proteins using the size exclusion approach, gel filtration chromatography was examined. A polyacrylamide gel Bio-gel P60 (Bio-Rad Laboratories) and a Bio-Silect SEC 250-5 HPLC column (Bio-Rad Laboratories) were used for these experiments. For both of these columns, there appeared to be no further fractionation of proteins as shown by SDS-PAGE. On SDS-PAGE, each fraction containing activity contained the same protein, but just at different amounts. However, a

Zorbax Bio Series GF-250 gel filtration column (Agilent Technologies Canada) achieved separation, recovered activity and increased the fold purification. The approach tested started with an affi-gel blue fraction (Fraction 4, 13 mL, Table 1) eluted using water with 20 mM  $\beta$ -mercaptoethanol. This initial fractionation step provided a 64.5-fold purification with 27% of initial activity recovered, as seen in Table 1. This affi-gel blue fraction was buffered with tris, lyophilized and resuspended in a small volume (400  $\mu$ L). An injection (100  $\mu$ L) of this concentrated fraction was resolved in a Zorbax Bio Series GF-250 gel filtration column using 50 mM tris with 200 mM NaCl, pH 7.75. As shown in Table 1, an impressive 354-fold purification with 0.6% of initial activity was accomplished with this chromatographic step. However, various limitations with the gel filtration chromatography prevented its routine use in purification. First, the injection volume was limited to a maximum of 100  $\mu$ L/injection by both injector capability and column design. Therefore, scaling up this procedure would not be very practical as it would require multiple injections. The collection of eluant fractions had to be performed manually, which made this approach not very reproducible in addition to labourious. The fact that the HPLC system used was not biocompatible may explain the loss of protein and the instability of active fractions during storage. Finally, protein in eluant fractions was very dilute and could not be visualized on SDS-PAGE, even with the use of a silver stain kit (BioRad Laboratories). The use of NANOSEP microconcentrators (10 K, Pall Gelman Laboratories, ON) was attempted to concentrate these fractions but no correlation of protein bands on SDS-PAGE with activity was possible. NAT activity was lost during this concentration approach possibly due to the binding of the enzyme to the membrane .

**Table 1.** Summary of the Partial Purification of *S. akiyoshiensis* NAT using Gel Filtration. The purification approach consisted in two steps: an affi-gel blue chromatography followed by gel filtration chromatography on a Zorbax Bio Series GF-250 gel filtration HPLC column (Agilent Technologies Canada). Enzyme activity was determined under standard assay conditions according to “Materials and Methods” section 3.4.4.

Purification step	Vol (mL)	Total Act. U (%)	Protein (mg/mL)	Spec. Act. (U/mg protein)	Purification (x-fold)
Cell free extract	110	1.38 (100 %)	18.13	$6.94 \times 10^{-4}$	1.0
Affi-gel blue	13	$3.8 \times 10^{-1}$ (27 %)	$6.5 \times 10^{-1}$	$4.46 \times 10^{-2}$	64
GP-250 F24	0.2	$6.39 \times 10^{-3}$ (0.6 %)	$1.3 \times 10^{-1}$	$2.46 \times 10^{-1}$	354

GP-250 F24 = Zorbax Bio Series GF-250 gel filtration HPLC column fraction 24.

Note: 1993.97 mg of total protein/110 mL of cell free extract.

8.46 mg of total protein/13 mL of affi-gel blue fraction

$2.6 \times 10^{-2}$  mg of total protein/0.2 mL of GP-250 F24

and/or plastic of these microconcentrators

Ion-exchange chromatography was also explored using various approaches. A strongly acidic cation-exchanger, econo-pac High S cartridge (5 mL, Bio-Rad Laboratories) as well as a weakly acidic cation-exchanger, econo-pac CM cartridge (5 mL, Bio-Rad Laboratories) did not retain N-acetyltransferase activity under conditions investigated. On the other hand, a strongly basic anion-exchanger, econo-pac High Q cartridge (5 mL, Bio-Rad Laboratories), retained all the enzyme activity but did not provide a good separation of proteins. A weak anion-exchanger consisting of a SynChropack AX300 HPLC column (Eichrom Technologies Inc.) was tested. However, the activity on eluant fractions was barely detected possibly due to either loss of activity or loss of protein during the chromatography. Finally, the weak anion-exchanger macro-prep DEAE (Bio-Rad Laboratories) was shown to retain all the N-acetyltransferase activity, provided significant separation of proteins and yielded a stable active fraction. Therefore, macro-prep DEAE was chosen as part of this purification scheme.

A summary of the partial purification of *S. akiyoshiensis* NAT is given in Table 2. Chromatography on affi-gel blue constituted the first step of the partial purification scheme and produced a 98-fold purification. The SDS-PAGE of fractions from this first purification step (Figure 17) shows the enrichment of two major bands, one around 31 kDa and a second one at about 14.4 kDa. As seen in Figure 17 and Appendix Table 1, around 95% of undesired protein was removed in the first step of this purification scheme providing a very impressive “capture” step of around 0.4% recovery of the protein and the majority of NAT activity. Affi-gel blue chromatography actually produced an even higher

**Table 2.** Summary of the partial purification of *S. akiyoshiensis* NAT. The purification scheme consisted of three steps as described in “Materials and Methods” section 3.14. The enzyme activity was determined under standard assay conditions (L-dopa as substrate) according to “Materials and Methods” section 3.4.4.

Purification step	Vol (mL)	Total Act. U (%)	Protein (mg/mL)	Spec. Act. (U/mg protein)	Purification (x-fold)
Cell free extract	80	1.67 (100 %)	19.76	$2.11 \times 10^{-3}$	1.0
Affi-gel blue *	40	$6.8 \times 10^{-1}$ (41 %)	$1.7 \times 10^{-1}$	$2.07 \times 10^{-1}$	98
1 <sup>st</sup> DEAE *	3	$3.4 \times 10^{-1}$ (21 %)	$8.7 \times 10^{-1}$	$2.64 \times 10^{-1}$	125
2 <sup>nd</sup> DEAE F11	2	$9.50 \times 10^{-3}$ (0.6 %)	$2.0 \times 10^{-2}$	$1.97 \times 10^{-1}$	93

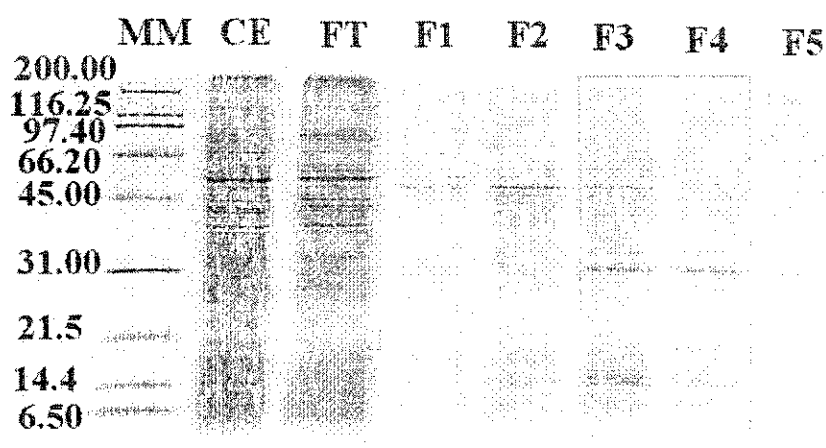
2<sup>nd</sup> DEAE F11 = Fraction 11 from second-DEAE chromatography.

Note: 1581.24 mg of total protein/ 80 mL of cell free extract

6.62 mg of total protein/ 40 mL of affi-gel blue fractions

$5.0 \times 10^{-2}$  mg of total protein/ 2 mL of 2<sup>nd</sup> DEAE F11

\* = two set of columns were run and fractions with the majority of the activity were combined.



**Figure 17.** SDS-PAGE Gel for Affi-Gel Blue Step. SDS-PAGE gel (12% w/v) of enzyme preparations during purification of *S. akiyoshiensis* NAT stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories). All wells contained 20  $\mu$ L of enzyme preparation and 5  $\mu$ L of loading buffer. MM = molecular marker (kDa); CE = cell free extract (1/10 dilution); FT = flow-through and wash (1/10 dilution); F1, 2, 3, 4, and 5 = fraction 1, 2, 3, 4 and 5, respectively. Fractions 3 and 4 presented most of the activity.

purified fraction (10 mL) with 112-fold purification (Appendix Table 1).

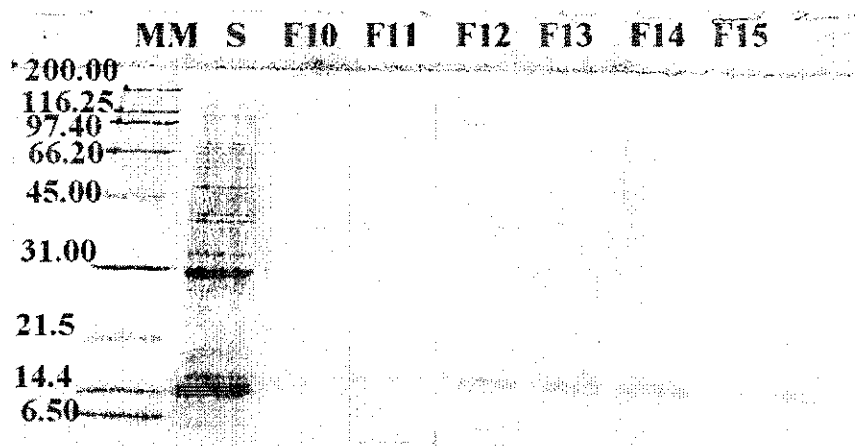
As mentioned previously, the enzyme activity was eluted from the affi-gel blue column using 10% (v/v) glycerol with 20 mM  $\beta$ -mercaptoethanol and therefore, was not strongly buffered. Attempts to store eluant fractions at  $-80^{\circ}\text{C}$  resulted in complete loss of enzyme activity over 24 h. Consequently, fractions containing the majority of enzyme activity (usually fractions 3 and 4) were pooled, supplemented with tris buffer and taken to the next step of the purification immediately.

The second step in the purification of *S. akiyoshiensis* NAT consisted of a chromatography on the ion-exchange resin macro-prep DEAE (Bio-Rad, Laboratories). This weak anion-exchange resin was able to capture all of the N-acetyltransferase activity using a very small bed volume (600  $\mu\text{L}$ ). This complete capture is supported by the absence of detectable NAT activity in the flow-through (Appendix Table 2). Although TG containing 350 mM NaCl eluted *S. akiyoshiensis* NAT, a pH gradient (pH 8.0 to 5.5) elution was undertaken to see if a better performance could be achieved. Unfortunately, the use of pH to elute *S. akiyoshiensis* NAT failed as no activity was recovered in any of the eluate fractions. Therefore, elution of NAT activity from macro-prep DEAE chromatography was established using TG buffer supplemented with NaCl.

This second column chromatography step on macro-prep DEAE accomplished three main objectives. First, it eliminated a pink-violet pigment which presented characteristics of melanin pigments. This pigment was readily soluble in alkali but precipitated under acidic environments, which is consistent with other melanins (Prota, 2000). In addition, the possibility of this pigment being related to melanins is justified

since tyrosinase is very active in *S. akiyoshiensis*, especially in the culture broth, and catalyses the conversion of L-dopa to dopachrome from which melanins are synthesized (Smith, 1995). This pigment precipitated on top of the DEAE support columns slowing the flow thereby increasing the backup pressure and reducing chromatographic resolution. The second objective was to provide stabilization of N-acetyltransferase activity so that the enzyme preparations could be stored. As mentioned previously, fractions from affi-gel blue chromatography were not buffered and could not be stored. Consequently, the first-DEAE chromatographic step, using TG buffer with 350 mM of NaCl to elute NAT activity, also resulted in a buffer exchange that stabilized the enzyme activity. Although only 21% of initial activity was recovered (Table 2) using this first-DEAE column, active fractions stored at -80°C for up to 9 months exhibited no loss of activity even on repeated thawing and freezing. The third objective accomplished with this step was the concentration of NAT activity. Post-affi-gel blue pooled (20 mL) fractions were concentrated to 1.5 mL containing about 50% of the activity (only 21% of initial activity, Table 2) and with 125-fold purification. Several other techniques were explored in order to stabilize and concentrate affi-gel blue fractions. Experiments involving buffer exchange using dialysis and gel filtration proved to be inadequate as most of the activity was lost during these procedures. Attempts to concentrate NAT activity were also investigated using ultrafiltration and lyophilization. However, these techniques resulted in decreased activity (about 30% loss in each treatment) possibly due to non-specific binding of protein to membranes and/or to instability of the enzyme under the experimental conditions.

The final step of the purification of *S. akiyoshiensis* NAT utilized pooled active fractions from the first-DEAE chromatography. As previously mentioned, these fractions contain 350 mM of NaCl. Therefore, in order to continue with another DEAE chromatography step, the concentration of salt would need to be lowered to achieve binding. A concentration as low as 50 mM NaCl was sufficient to retain NAT activity on the column and remove some undesired protein in the flow-through. Consequently, fractions from the first-DEAE were diluted and applied to a second-DEAE column. The main objective of this second larger DEAE column was to increase the fold purification by further fractionating the protein. However, this further purification step drastically lowered the recovery of NAT activity as observed in Table 2. This second-DEAE chromatography consisted of a longer column and elution was accomplished using a linear salt gradient (200 mM - 500 mM NaCl in TG buffer). Activity was eluted at approximately 300 mM NaCl (Fractions 11, 12 and 13). Although the activity recovered in fraction 11 contained only 0.6% of initial activity, SDS-PAGE showed an enrichment of a band at around 14.4 kDa (Figure 18). An explanation for this loss of activity observed might be the loss of a required component for catalytic activity such as a cofactor or stimulator or even dissociation of an enzyme subunit during chromatography, which resulted in the loss of the enzyme activity (Pohl, 1990). Another possibility could be the loss of acetylating enzyme due to denaturation of the enzyme, or the binding of the protein to column and plastic surfaces. Finally, the last possibility is the actual loss of activity due to denaturation of the enzyme, or the binding of the protein to column and plastic surfaces. Considering that the concentration of protein in these preparations was



**Figure 18.** SDS-PAGE Gel for Second-DEAE Step. SDS-PAGE gel (12% w/v) of enzyme preparations during the purification of *S. akiyoshiensis* NAT. Bands were visualized by staining with Coomassie brilliant blue R-250 (Bio-Rad Laboratories). All wells contained 20  $\mu$ L of enzyme preparation and 5  $\mu$ L of loading buffer.

MM= molecular marker (kDa); S = fraction 1 from 2 sets of first DEAE (concentrated to 1 mL); F10, 11, 12, 13, 14, 15 = fraction 10,11, 12, 13, 14 and 15, respectively. F10 represents a 55.8-fold purification. F11 represents the highest purified fraction through this chromatographic step with 93.3-fold purification. F12 and F13 represent 90.8 and 72.7-fold purification, respectively. F14 and 15 represent a 49.3 and 39.8-fold purification, respectively.

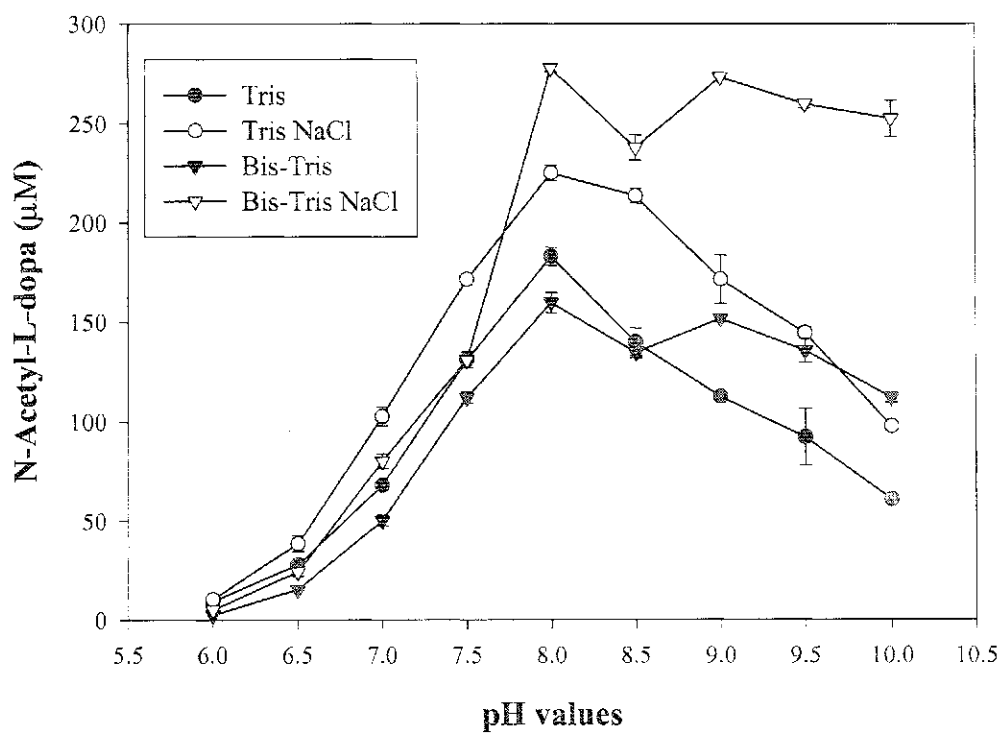
only 0.02 mg/mL, binding to a minor extent could cause serious losses. Attempts to confirm the identity of the 14.4 kDa band were performed using electroelution techniques. Unfortunately, only traces of activity were observed which might indicate that this enzyme was not stable under the conditions used.

### **4.3. Characterization of Partially Purified *S. akiyoshiensis* NAT**

#### **4.3.1. Optimum pH for *S. akiyoshiensis* NAT**

The study of the effect of pH on *S. akiyoshiensis* NAT was performed using a fraction from affi-gel blue chromatography. The maximum acetylation activity occurred at pH 8.0 as seen in Figure 19 (Raw data in Appendix Table 3). The same trend was observed with tris and bis-tris propane buffers with or without NaCl. However, as seen in Figure 19, the addition of NaCl at 250 mM significantly increased activity over most of the pH range. These observations indicate that NaCl plays an important role in stabilizing and/or activating this N-acetyltransferase.

Interestingly, the pH profile for this enzyme in bis-Tris buffer exhibited an apparent double-maximum (Figure 19). The maximum activity was observed at pH 8.0, while a second increase in activity was observed at about pH 9.0. This observation could suggest the presence of two NATs in this *S. akiyoshiensis* preparation. This idea is consistent with a report by Ichihara *et al.* (2001) which presented evidence on the existence of two forms of an arylalkylamine N-acetyltransferase (AANAT) in *Periplacenta americana*. These two types of AANAT generated a double-peaked pH profile when crude extracts from various organs were assayed. Further purified samples



**Figure 19.** Optimum pH for *S. akiyoshiensis* NAT. Activity assays were performed as described in “Materials and Methods” section 3.16.1. Partially purified enzyme (post-affigel blue preparation) was mixed with Tris or bis-Tris buffer with and without the addition of NaCl and at different pH values (6-10). This mixtures were used to perform activity assays at 37°C and 20 min. Enzyme reactions were stopped using 3% TFA. Each point represents the average of duplicate assays. The error bars represent the standard deviation of the mean for each point. Tris = 100 mM Tris buffer, Tris NaCl = 100 mM Tris with 250 mM NaCl, Bis-Tris = 100 mM bis-Tris buffer, Bis-Tris NaCl = 100 mM bis-Tris with 250 mM NaCl.

indicated two enzymes with equivalent molecular mass (28 kDa) but very different pH optima (6 and 10 respectively). *S. akiyoshiensis* NAT, however, has the double maxima much closer and therefore might be the case of a broad pH range profile as supported by the results obtained with Tris buffer.

As can be seen in Table 3, the apparent pH optimum value for *S. akiyoshiensis* NAT of 8.0 agree with those reported for *Saccharomyces cerevisiae* AANAT (scAANAT) and *P. americana* basic AANAT. However, the optimal pH values for *S. akiyoshiensis* NAT differ from data reported for mammalian, pike and trout AANATs. A comparison of the optimal pH value for *S. akiyoshiensis* NAT of 8.0 with those reported in the literature for ANATs isolated from *Enterobius vermicularis*, *Lactobacillus acidophilus*, *Aeromonas hydrophilia* and *Pseudomonas aeruginosa* show similarity.

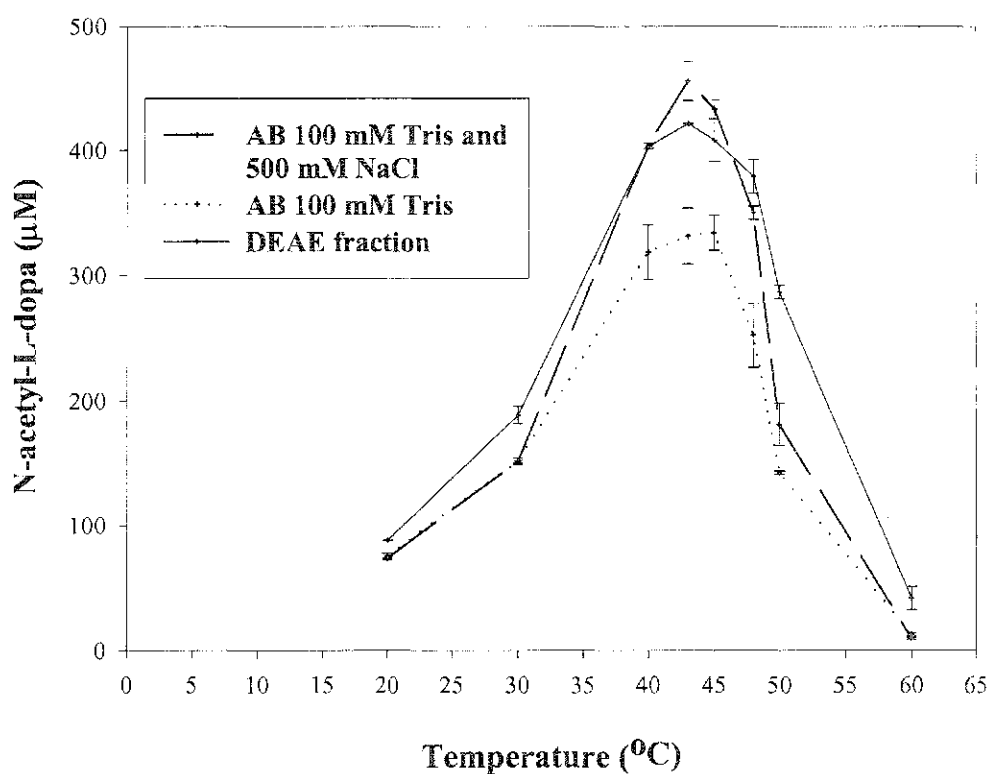
In summary, the apparent optimal pH values found for *S. akiyoshiensis* NAT are within the range of values reported for AANATs and ANATs in the literature. Interestingly, the maximal activities observed with tris and bis-tris indicate that tris is a more suitable buffer for this enzyme. On the contrary, the maximal activities observed for *S. akiyoshiensis* NAT in Tris and bis-Tris containing NaCl show that bis-Tris was a more appropriate buffer for this enzyme. This contradiction suggests that bis-Tris allows a better interaction of NaCl with the enzyme, producing a better stabilization and/or activation and that bis-Tris alone is not activating this enzyme. Therefore, bis-Tris with NaCl constitutes a better buffer for *S. akiyoshiensis* NAT but the higher cost of bis-Tris prevented its routine use. However, although these differences mentioned are statistically significant, the differences are fairly small and may result from other factors.

**Table 3.** Comparison of Optimal pH Found for *S. akiyoshiensis* NAT with Literature Data for AANATs and ANATs.

Source	pH	References
AANATs:		
<i>Saccharomyces cerevisiae</i> AANAT	8.6	Ganguly <i>et al.</i> , 2001
<i>Periplacenta americana</i> AANAT	6.0 & 10.0	Ichihara <i>et al.</i> , 2001
Rat pineal AANAT	6.8	Nambodiri <i>et al.</i> , 1979
Pike and trout AANATs	6.0	Falcon <i>et al.</i> , 2001
ANATs:		
<i>Enterobius vermicularis</i> ANAT	7.5	Chung, 1998
<i>Lactobacillus acidophilus</i> ANAT	7.0	Chen <i>et al.</i> , 1999
<i>Aeromonas hydrophilia</i> ANAT	7.0	Chung <i>et al.</i> , 1998
<i>Pseudomonas aeruginosa</i> ANAT	8.5	Hsieh <i>et al.</i> , 1998
<i>Streptomyces akiyoshiensis</i> NAT	8.0	This study

#### 4.3.2. Optimum Temperature for *S. akiyoshiensis* NAT

The effect of temperature on *S. akiyoshiensis* NAT activity was examined using an affi-gel blue and a first-DEAE fraction. In order to add buffering capacity to the affi-gel blue fraction, Tris was added to give a final concentration of 100 mM and pH 8.0. In addition, to confirm the increase in activity observed in the presence of NaCl during the determination of the optimal pH, the affi-gel blue fraction was evaluated with and without the addition of this salt. The temperature curves (Figure 20) for the enzyme fractions tested are in close agreement and show a maximal activity at 43°C. A very



**Figure 20.** Optimum Temperature for *S. akiyoshiensis* NAT. Partially purified enzyme preparations included: **AB 100 mM Tris 0.5 M NaCl** = Post-affi-gel blue preparation in 100 mM Tris with 0.5 M NaCl, pH 8.0; **AB 100 mM Tris** = Post-affi gel blue preparation in only 100 mM Tris buffer, pH 8.0; and **DEAE fraction** = post-first DEAE fraction containing TG buffer with 0.35 M NaCl and diluted 1:3 using water. Enzyme assays were performed according to “Materials and Methods” section 3.16.2. Temperatures tested ranged between 20 and 60°C. Each point represents the mean of duplicate assays. Error bars represent the standard deviation of the mean for each point.

interesting finding was the fact that the post-affi-gel blue fraction containing 500 mM NaCl exhibited at maximum 37.6 % more activity than the same fraction without this salt. This is concordant with previous observations (optimum pH experiment) and indicates an important role for NaCl in the activity of *S. akiyoshiensis* NAT. There is not significant information on an activating role of NaCl on N-acetyltransferases. Namboodiri *et al.* (1979) have found that NaCl not only protected but activated rat pineal AANAT, but no mechanism to explain this observation was discussed. Conversely, there is extensive information on the role of NaCl in halophilic bacteria. For instance Oren and Mana (2002) have determined the relationship between amino acid composition of bulk protein in *Salinibacter ruber* and salt. *S. ruber* proteins present a high content of acidic amino acids and serine but a low abundance of basic and hydrophobic amino acids. Acidic residues are more highly hydrated than other amino acids and can coordinate hydrated salt ions at the surface of the protein (Dennis and Shimmin, 1997). Moreover, acidic residues can form salt bridges with strategically positioned basic residues. These salt bridges provide structural rigidity and are important in the stabilization of the three-dimensional structure of halophilic proteins (Oren and Mana, 2002). The low content of hydrophobic amino acids is balanced by a high frequency of serine due to its compact size and borderline hydrophobic-hydrophilic character. Consequently, the activating effect of NaCl on *S. akiyoshiensis* NAT may suggest that this protein may present lots of acidic amino acids on its surface and/or this salt might be responsible for the formation of catalytically important salt bridges.

As seen in Table 4, the apparent optimal temperature for *S. akiyoshiensis* NAT is

in good agreement with that reported for *S. cerevisiae* AANAT . However, this value differs from values reported for the mammalian, *Esox lucius* L. pineal organ and retina, and *Ascaridia galli* AANATs. In the same manner, the optimal temperature for *S. akiyoshiensis* NAT differs from those of *Pseudomonas aeruginosa*, *Aeromonas hydrophilia*, *Lactobacillus acidophilus*, and *S. cerevisiae* ANATs.

**Table 4.** Comparison of Apparent Optimal Temperature found for *S. akiyoshiensis* NAT with Literature Data for AANAT and ANATs.

Source	Optimal Temperature (°C)	References
AANATs:		
<i>S. cerevisiae</i> AANAT	42	Ganguly <i>et al.</i> , 2001
rat pineal AANAT	37	Namboodiri <i>et al.</i> , 1979
<i>Ascaridia galli</i> AANAT	55	Muimo and Isaac, 1993
Pineal <i>Esox lucius</i> L. AANAT	20	Falcon <i>et al.</i> , 2001
Retina <i>Esox lucius</i> L. AANAT	37	Falcon <i>et al.</i> , 2001
ANATs:		
<i>Pseudomonas aeruginosa</i> ANAT	37	Hsieh <i>et al.</i> , 1998
<i>Aeromonas hydrophilia</i> ANAT	37	Chen <i>et al.</i> , 1999
<i>S. cerevisiae</i> ANAT	25	Lee <i>et al.</i> , 1998
<i>Lactobacillus acidophilus</i>	37	Chen <i>et al.</i> , 1999
<i>S. akiyoshiensis</i> NAT	43	This study

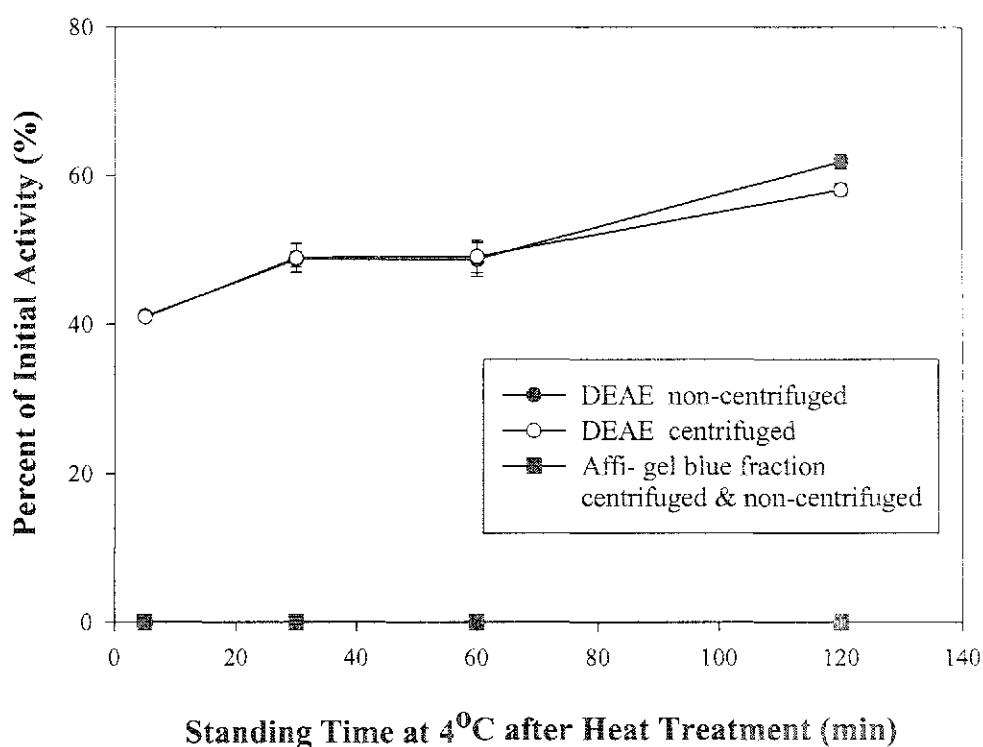
In summary, the apparent optimal temperature for *S. akiyoshiensis* NAT was within the range of values found for AANATs and ANATs. The highest rate for the *S. akiyoshiensis* NAT was observed with tris buffer containing NaCl. This observation reaffirms an important role of NaCl in stabilizing and/or activating this enzyme.

#### 4.3.3. Renaturation of *S. akiyoshiensis* NAT following Heat Denaturation

As previously mentioned, the attempt to terminate the enzyme assay by heating in a boiling water bath led to the suspicion that *S. akiyoshiensis* NAT was heat stable. In order to evaluate this previous observation, an affi-gel blue fraction and a first-DEAE fraction were used in an experiment. In order to stabilize the affi-gel blue fraction, tris buffer pH 8.0 to a final concentration of 100 mM was added. This fraction showed complete denaturation after the pre-incubation period of 5 min at boiling water temperature (~98°C) as seen in Figure 21. Furthermore, denaturation was irreversible over a 120 min period at 4°C.

On the other hand, the first-DEAE fraction containing TG buffer with 350 mM NaCl showed 41% of initial activity remaining after heat treatment (98°C, 5 min). This result was consistent for both centrifuged vs. non-centrifuged tested for this fraction (as described in “Materials and Methods” section 3.16.3.). Unexpectedly, after the 120 min incubation at 4°C both sets of the first-DEAE fraction recovered up to 61% of initial activity.

These results suggest that this enzyme was not heat stable. However, depending on its environment, it recovered some of its activity after standing at 4°C. Therefore,



**Figure 21.** Renaturation of *S. akiyoshiensis* NAT after Heat Treatment. Partially purified enzyme preparations (affi-gel blue and 1st-DEAE fractions) were treated as specified in “Materials and Methods” section 3.16.3. DEAE non-centrifuged = 1st-DEAE fraction heated at 98°C for 5 min, and incubated on ice (4°C) for up to 120 min; DEAE centrifuged = 1st-DEAE fraction heated at 98°C for 5 min, centrifuged at 4°C (18000×g, 10 min) and stored on ice for up to 120 min. Affi-blue fraction = Affi-gel blue fraction treated the same as 1<sup>st</sup>-DEAE fraction. Each point represents the mean of duplicate standard assays. Error bars represent the standard deviation of the mean for each point.

some component(s) in the first-DEAE fraction was protecting enzyme activity against complete denaturation as well as preventing protein precipitation. Evaluating the environment of the first-DEAE fraction, the presence of NaCl and glycerol might have a role in stabilizing this enzyme. As mentioned previously, the role of NaCl in protection against thermodegradation has been reported mainly referred to halophilic proteins. For example, Tehci *et al.* (2002) have confirmed that the surface of *Haloarcula marismortui* malate dehydrogenase is highly acidic with negative charges that bind water and salt ions providing thermostability. Interestingly, the homologous mesophilic enzyme (pig heart malate dehydrogenase) did not exhibit this property. The results presented in this study show that NaCl stabilized *S. akiyoshiensis* NAT and protected it from irreversible heat denaturation.

#### 4.3.4. Effect of Metal Ions on *S. akiyoshiensis* NAT Activity

The possible effects of inhibition and activation of various metal ions were tested on *S. akiyoshiensis* NAT and results are shown in Figure 22 (numerical data in Appendix Table 4). As seen on Figure 22 A, the effect of metal chlorides on *S. akiyoshiensis* NAT activity was varied. N-acetylating activity was inhibited by the following chlorides at 1 mM,  $\text{CdCl}_2 < \text{FeCl}_3 < \text{MnCl}_2 < \text{CoCl}_2 = \text{ZnCl}_2 = \text{HgCl}_2 < \text{FeCl}_2 = \text{NiCl}_2 < \text{CuCl}_2$ . This inhibitory effect was stronger at 5 mM. A relative stabilization of the enzyme activity was observed in the presence of  $\text{CaCl}_2$ ,  $\text{MgCl}_2$  and NaCl at both concentrations

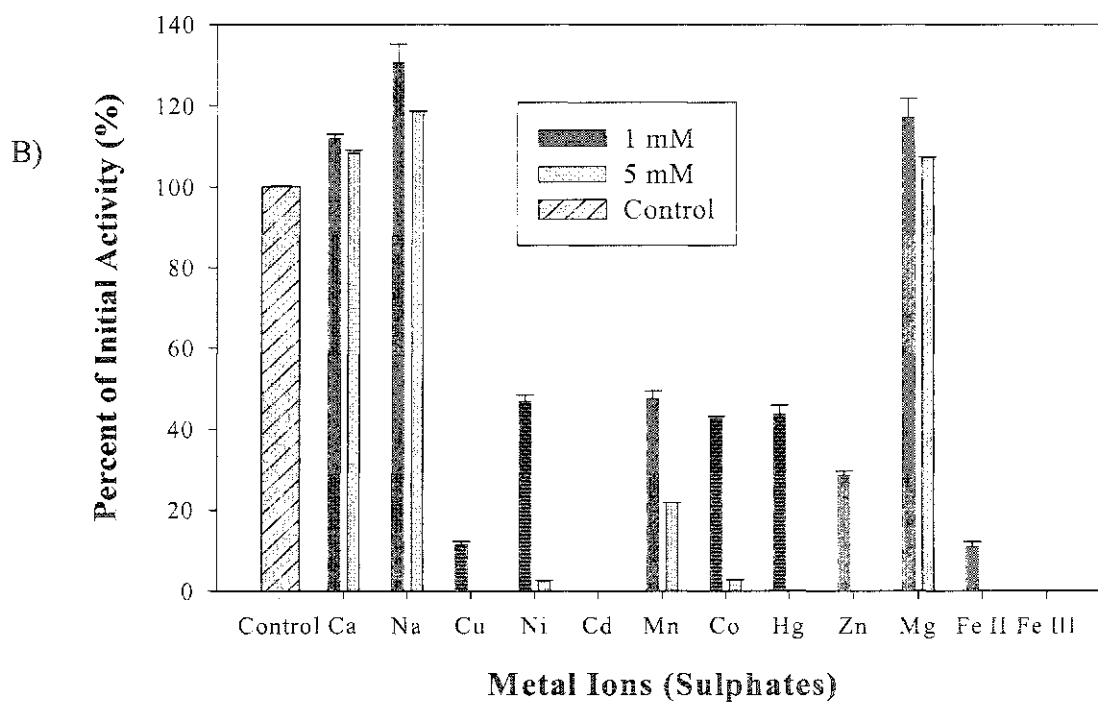
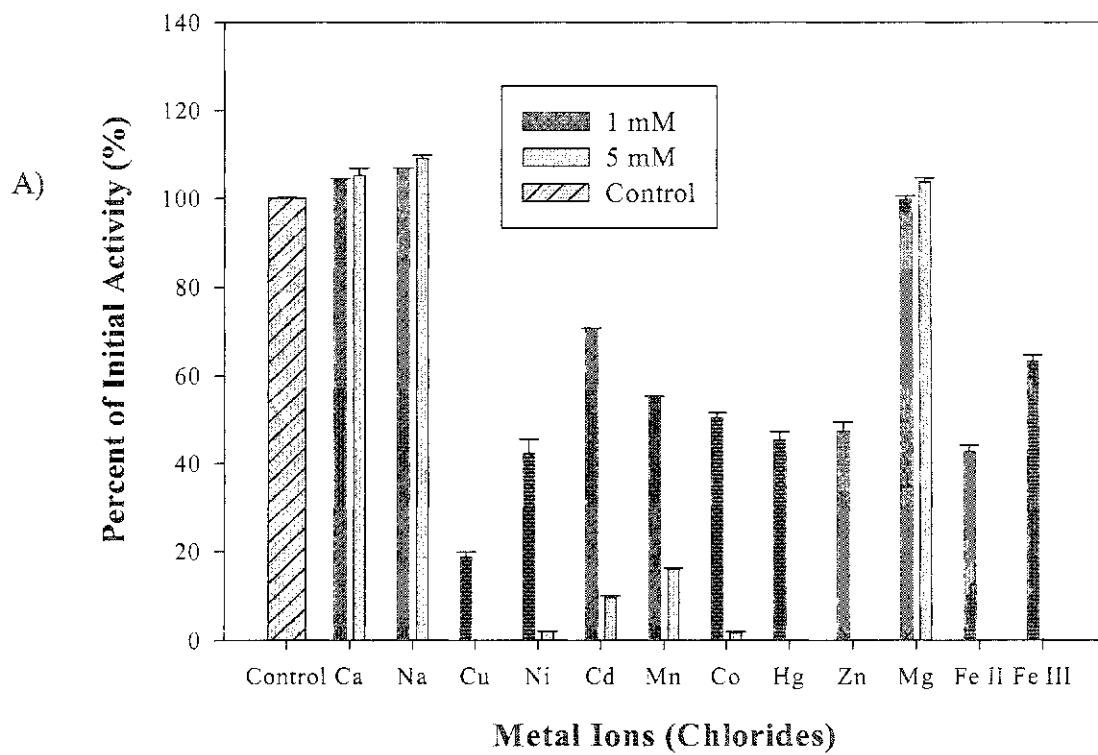
**Figure 22.** Effect of Metal Ions on *S. akiyoshiensis* NAT Activity. Partially purified enzyme preparation (affi-gel blue fraction) was incubated in the presence of various monovalent and divalent cations at 37°C for 20 min in tris buffer pH 8.0. The enzyme activity was determined according to “Materials and Methods” section 3.16.4. Metal ion salts were assayed at 1 mM and 5 mM final concentrations. A) chlorides and B) sulphates. The absence of bars represents no enzyme activity detected. Each bar represents the mean of duplicate assays. Error bars represent the standard deviation of the mean for each point.

Control = no salt added; Ca = Ca<sup>2+</sup> ; Na = Na<sup>+</sup> ; Ca = Ca<sup>2+</sup> ; Ni = Ni<sup>2+</sup> ; Cd = Cd<sup>2+</sup> ;

Mn = Mn<sup>2+</sup> , Co = Co<sup>2+</sup> ; Hg = Hg<sup>2+</sup> ; Zn = Zn<sup>2+</sup> ; Mg = Mg<sup>2+</sup> ; Fe II = Fe<sup>2+</sup> ; Fe III = Fe<sup>3+</sup>.

Addition of salts with Cu, Ni, Cd, Co and Zn oxidized L-dopa during incubation period.

Addition of salts with Hg, Fe II and Fe III readily oxidized L-dopa.



tested. In the case of the metal sulfates (Figure 22 B), an inhibitory effect of NAT activity was noticed in the presence of  $\text{MnSO}_4 < \text{NiSO}_4 < \text{HgSO}_4 < \text{CoSO}_4 < \text{ZnSO}_4 < \text{CuSO}_4 < \text{FeSO}_4 < \text{Fe}_2(\text{SO}_4)_3 < \text{CdSO}_4$ . A very distinct effect was observed in the presence of  $\text{CaSO}_4$ ,  $\text{MgSO}_4$  and  $\text{NaSO}_4$ , which stabilized the activity of *S. akiyoshiensis* NAT at 1 mM and 5 mM. From these results, the inhibition of N-acetylating activity was stronger in presence of the anion sulphate since  $\text{FeSO}_4$ ,  $\text{Fe}_2(\text{SO}_4)_3$ ,  $\text{CuSO}_4$ ,  $\text{CdSO}_4$  and  $\text{ZnSO}_4$  than the respective chlorides.

The relative stabilization of *S. akiyoshiensis* NAT activity in the presence of the cations  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  agrees with results reported by Zhan-Poc and Craft (1999) for rat recombinant AANAT at 1 mM of  $\text{MgCl}_2$  and  $\text{CaCl}_2$ . Similarly, Vara *et al.* (1985) found that  $\text{MgCl}_2$  slightly activated *S. alboniger* puromycin AANAT at 5 mM. Furthermore, Morton (1989) discovered that calcium and magnesium activated rat pineal AANAT at 10 mM. Consequently, the stabilizing effect of the cations  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  on *S. akiyoshiensis* NAT correlates with that observed for other AANATs. On the contrary,  $\text{CaCl}_2$  and  $\text{MgCl}_2$  were shown to be strongly inhibitory on ANATs of *Enterobius vermicularis* (Chung, 1998) and *Lactobacillus acidophilus* (Chen *et al.*, 1999) at 0.5 mM.

The stabilization of *S. akiyoshiensis* NAT activity in the presence of  $\text{NaCl}$  and  $\text{Na}_2\text{SO}_4$  suggests an important role for the monovalent cation  $\text{Na}^+$ . This result is in good agreement with the data reported by Namboodiri *et al.* (1979) on rat pineal AANAT. This team of researchers found that  $\text{NaCl}$ , sodium citrate and sodium phosphate, stabilized AANAT. In addition,  $\text{NaCl}$  was found to not only protect but activate this enzyme. The effects attributed to  $\text{NaCl}$  were considered to be non-specific changes in the

conformation of the enzyme, encouraging the exposure of hydrophilic regions and internalization of hydrophobic areas. Assuming that the hydrophobic region of AANAT is involved in inactivation, its internalization would result in the activation or stabilization of the enzyme. Morton (1989) agree that a likely explanation for the increase of NAT activity observed in AANATs in presence of NaCl indicates a conformational change and/or charge distribution which increases the capacity of AcCoA to bind to the enzyme and improve catalysis. However, the actual mechanism that explains the activating and protective effect of NaCl on AANATs is not clear. The effect of NaCl on *S. akiyoshiensis* NAT correlates with the stabilization observed for *S. alboniger* puromycin AANAT at 5 mM (Vara *et al.*, 1985). In the same manner, the stabilizing effect of Na<sub>2</sub>SO<sub>4</sub> on *S. akiyoshiensis* NAT agrees with data reported by Hsieh *et al.* (1998) for *P. aeruginosa* ANAT at 50 mM. Conversely, the stabilizing effect of Na<sup>+</sup> on *S. akiyoshiensis* activity differs from the inhibitory effect of Na<sub>2</sub>SO<sub>4</sub> on *L. acidophilus* ANAT (Chen *et al.*, 1999) and *E. vermicularis* ANAT (Chung, 1998). An inhibitory effect of NaCl was also observed for *P. aeruginosa* ANAT at 50 mM (Hsieh *et al.*, 1998).

The N-acetylating activity of *S. akiyoshiensis* NAT was inhibited by the following cations Hg<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, and Mn<sup>2+</sup> (Figure 22). Most of these cations exhibited an increased inhibition at higher concentrations. However, these results might be misleading. L-dopa was readily oxidized in the presence of Fe<sup>2+</sup>, Fe<sup>3+</sup> and Hg<sup>2+</sup> resulting in an immediate darkening of the reaction mixture. Other salts (Cd<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup> and Zn<sup>2+</sup>) oxidized L-dopa during the 20 min incubation period. Nevertheless, this observed inhibitory effect agrees with reports for ANATs such as *P. aeruginosa* ANAT

(Hsieh *et al.*, 1998), *E. vermicularis* ANAT (Chung, 1998), and *L. acidophilus* ANAT (Chen *et al.*, 1999) which were inhibited by  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Co}^{2+}$ . In addition the results of this study agree with data obtained for AANATs.  $\text{Cu}^{2+}$  was shown to be strongly inhibitory of rat pinal AANAT at 0.05 mM (Morton, 1987).  $\text{Cu}^{2+}$  and  $\text{Hg}^{2+}$  were shown to be strong inhibitors of *Streptomyces alboniger* puromycin AANAT (Vara *et al.*, 1985), recombinant rat AANAT (Zhan-Poe and Craft, 1999), and *A. galli* AANAT (Muimo and Isaac, 1993). However,  $\text{Mn}^{2+}$  and  $\text{Fe}^{2+}$  were only slightly inhibitory at 1 mM in recombinant rat AANAT (loss of activity was 20% and 36%, respectively).

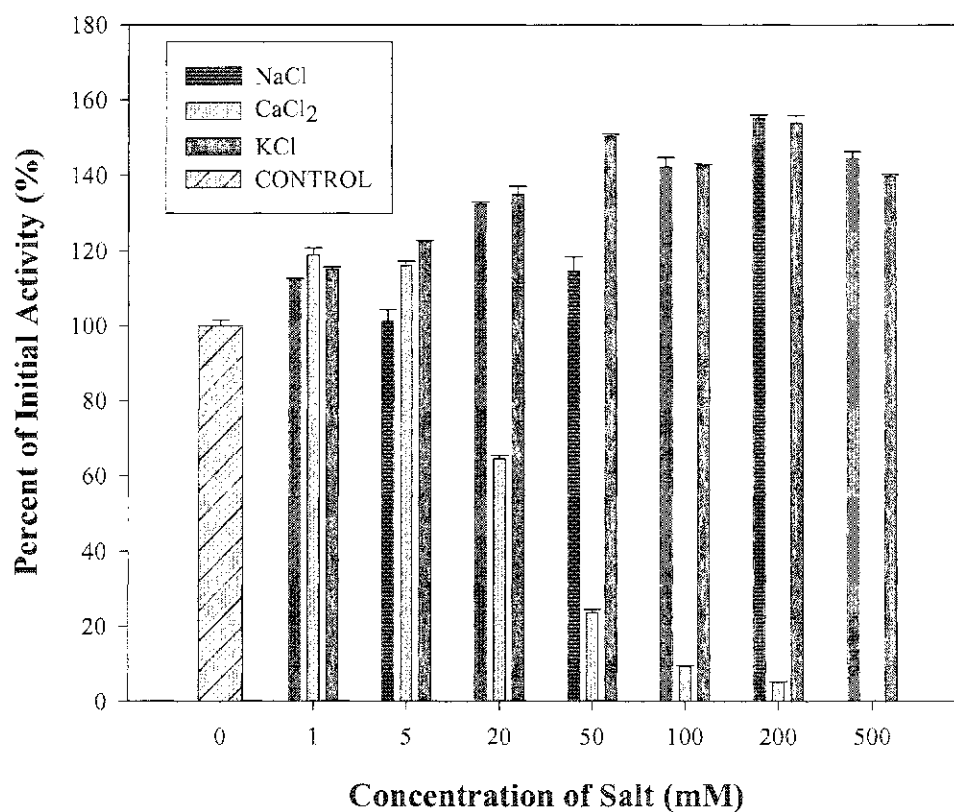
From these results, it suggests that *S. akiyoshiensis* NAT may have cysteine and histidine residues which are important for catalytic activity. Copper salts are known to precipitate proteins with multiple surface histidines, apparently by a mechanism that involves network formation (Van Dam *et al.*, 1989). In addition, Vara *et al.*, (1985) described  $\text{Cu}^{2+}$  and  $\text{Hg}^{2+}$  to strongly interact with sulfhydryl (-SH) groups of cysteine residues in the catalytic site of *S. alboniger* puromycin AANAT and inhibit activity. This is concordant with both ANATs (Upton *et al.*, 2001) and AANATs (Tsuboi *et al.*, 2002). ANATs present a catalytic triad constituted of Cys<sup>69</sup>, His<sup>107</sup> and Asp<sup>122</sup> (*S. typhimurium* NAT). Similarly AANATs present Tyr<sup>168</sup>, His<sup>120</sup>, and His<sup>122</sup> (*Ovis aries* AANAT) as part of the catalytic site. More recently, Tsuboi *et al.* (2002) have shown that two cysteine residues (Cys<sup>61</sup> and Cys<sup>177</sup>) in serotonin AANAT are involved in the regulation of NAT activity. These residues are localized in loops 1 and 3, respectively, and have been shown to confer sensitivity to sulfhydryl reagents in addition to form a disulfide bond that acts as a switch for the catalysis. This finding suggests that AANAT is regulated by the

formation (reduced active form) and cleavage (oxidized inactive form) of a disulfide bond which might allow or prevent AcCoA from gaining access to the catalytic site.

#### 4.3.5. Effect of KCl, NaCl and CaCl<sub>2</sub> on *S. akiyoshiensis* NAT Activity

To provide direct evidence on the role of salt on *S. akiyoshiensis* NAT activity, the effects of a higher range of concentrations of NaCl, KCl and CaCl<sub>2</sub> on this enzyme were compared. The results obtained were dependent on the cations rather than anions, since NaCl and KCl showed moderate activating properties on this NAT (Figure 23 and numerical data in Appendix Table 5), while CaCl<sub>2</sub> showed a concentration-dependent inhibitory activity above 5 mM concentration.

As can be seen from Figure 23, NaCl and KCl (chlorides of monovalent cations) increased enzymatic activity in a concentration-dependent manner up to a maximum of about 50% above the activity observed for the control (no salt added). This result is in agreement with data reported by Morton (1989) on rat AANAT using concentrations of 50 to 100 mM for NaCl and 50 to 200 mM for KCl which showed activation of this enzyme. On the other hand, CaCl<sub>2</sub> (a chloride of a divalent cation) stimulated NAT activity at lower concentrations (1 - 5 mM), while at higher concentrations it became increasingly inhibitory (Figure 23). Similarly, an activating effect of Ca<sup>2+</sup> was reported on rat pineal AANAT between 2.5 and 10 mM (Morton,1987; Morton,1989). All concentrations above 10 mM were inhibitory.



**Figure 23.** Effect of NaCl, KCl and CaCl<sub>2</sub> on *S. akiyoshiensis* NAT Activity.

N-acetyltransferase activity was measured at various concentrations (0 - 500 mM) of these chlorides according to “Materials and Methods” section 3.16.5. Each point represents the mean of duplicate assays. Error bars represent the standard deviation of the mean for each point.

Control = no salt added.

These results suggest that NaCl and KCl might be important for the activity of *S. akiyoshiensis* N-acetyltransferase. Although there is not much literature information on the role of NaCl and KCl on N-acetyltransferases, Namboodiri *et al.* (1979) reported a non-specific protective and activating role of NaCl from 25 to 100 mM. It has been speculated that activation results from a conformational change in the presence of NaCl, which leads to exposure of hydrophilic areas and internalization of inhibitory hydrophobic regions, but the actual mechanism is not yet clear. Fan *et al.* (2000) have found that monovalent cations allow the formation of an essential salt-bridge, which is important for allosteric communication between subunits in tryptophan synthase from *S. typhimurium*.

In any case, the increase of *S. akiyoshiensis* NAT activity with increasing concentration of NaCl and KCl (up to 50% of initial activity) was not as drastic as that observed for rat pineal (Namboodiri *et al.*, 1979) which showed a 4-5-fold increase. Therefore the possibility of these salts acting as activators is low, while their role in stabilizing the *S. akiyoshiensis* NAT activity is more reasonable.

### 3.3.6. Kinetic Studies of *S. akiyoshiensis* NAT

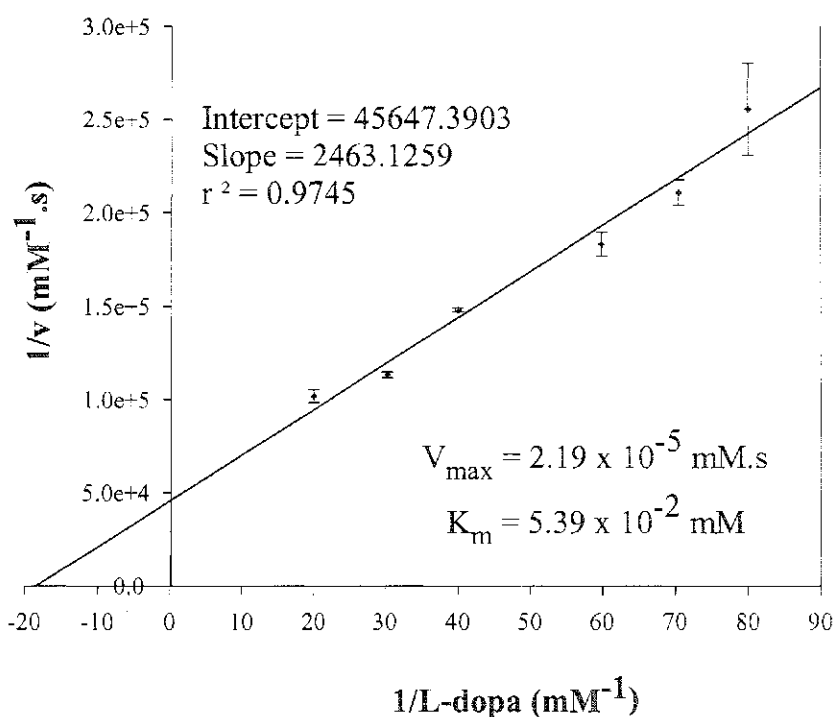
#### 3.3.6.1. Determination of Apparent $K_m$ and $V_{max}$ for L-dopa

The apparent  $K_m$  and  $V_{max}$  values of *S. akiyoshiensis* NAT for L-dopa were determined at pH 8.0 using two temperatures, 30 and 37°C (Figure 24 and 25). These kinetic parameters were obtained by the double-reciprocal plot (Lineweaver-Burk), which is well accepted and has been employed in previous literature (Chung, 1998; Chung *et al.*,

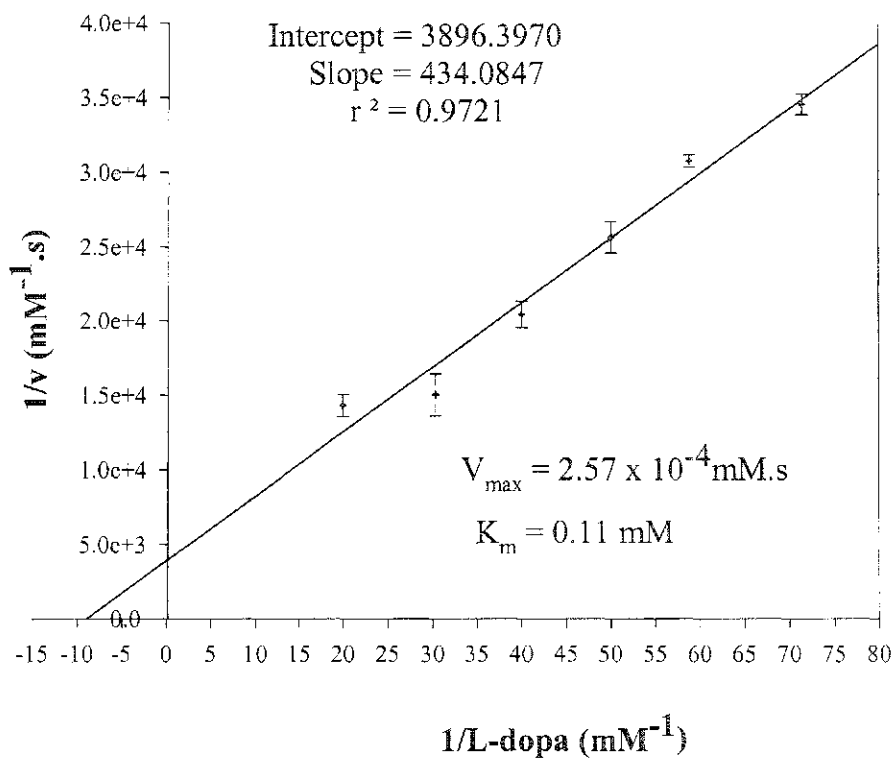
1998; Craft and Zhan-Poe, 2000; De Angelis *et al.*, 1998; Falcon *et al.*, 2001; Ferry *et al.*, 2000 Muimo and Isaac, 1993; Tsou *et al.*, 2001). As seen in Figure 24 the apparent  $K_m$  of this enzyme for L-dopa at 30°C was  $5.39 \times 10^{-2}$  mM and the corresponding  $V_{max}$  value was calculated to be  $2.19 \times 10^{-5}$  mM·s. On the other hand, the  $K_m$  value of this of this enzyme for L-dopa at 37°C was 0.11 mM with a  $V_{max}$  of  $2.57 \times 10^{-4}$  mM·s. As can be seen from these values, the  $K_m$  value at 37°C is two-fold higher than that observed at 30°C. The same trend was observed with the  $V_{max}$  value at 37°C being 10-fold higher than that at 30°C. These observations show that the apparent  $K_m$  increases as a positive function of temperature. The increased binding affinity of this enzyme for substrate at a lower temperature will compensate for a reduction in environmental kinetic energy (Hazel and Prosser, 1974). Hochachka and Somero (1968) found that the  $K_m$  value for lungfish lactate dehydrogenase (M-LDH) was 25-fold higher at 45°C than that calculated at 33°C and they suggested that this showed adaptation of the enzyme to a different environment.

There is no previous report on a N-acetyltransferase activity using L-dopa as an amine substrate. However, a protein that utilises L-dopa as a natural substrate such as rat liver dopa/tyrosine sulfotransferase (Sakakibara *et al.*, 1995) reported an apparent  $K_m$  value of 0.76 mM determined at 37°C and pH 9.25 (Table 5). Since the  $K_m$  value of *S. akiyoshiensis* NAT at the same temperature was much lower, a much higher specificity for this substrate has been observed.

The apparent  $K_m$  value of *S. akiyoshiensis* NAT for L-dopa at 37°C was similar to



**Figure 24.** Lineweaver-Burk Plot for L-dopa as Variable Substrate at 30°C. The N-acetyltransferase activity was determined at different concentrations of L-dopa and constant AcCoA concentration of 1 mM. Assays were performed as described in “Materials and Methods” section 3.16.6.1., at 30°C for 20 min and stopped using 3% TFA. Each data point represents the mean of triplicate assays. Error bars represent the standard deviation of the mean for each point.



**Figure 25.** Lineweaver-Burk Plot for L-dopa as Variable Substrate at 37°C. The N-acetyltransferase activity was determined at different concentrations of L-dopa and constant AcCoA concentration of 1 mM. Assays were performed as described in “Materials and Methods” section 3.16.6.1., at 37°C for 20 min and stopped using 3% TFA. Each data point represents the mean of triplicate assays. Error bars represent the standard deviation of the mean for each point.

the value of human and sheep AANATs for tryptamine of 0.12 mM (Ganguly *et al.*, 2001; De Angelis *et al.*, 1998). Moreover, there is only a 2-fold discrepancy of the  $K_m$  value found for L-dopa of *S. akiyoshiensis* NAT at 37°C and that for 5-methoxytryptamine in human and ovine AANATs ( $6.50 \times 10^{-2}$  and  $6.20 \times 10^{-2}$  mM, respectively, Table 5) (Ganguly *et al.*, 2001). Interestingly, the  $K_m$  value for *S. akiyoshiensis* NAT at 30°C agrees with those of human and ovine AANATs for 5-methoxytryptamine. These findings show that the specificity of *S. akiyoshiensis* NAT for L-dopa was in the same range as AANATs toward their natural substrates.

#### 4.3.6.2. Determination of Apparent $K_m$ and $V_{max}$ for AcCoA

The apparent  $K_m$  and  $V_{max}$  values for AcCoA were determined by analysis of double-reciprocal plots (Lineweaver-Burk) (Figures 27 and 28). These kinetic parameters were investigated at pH 8.0 and again, at 30°C and 37°C. The apparent  $K_m$  at 30°C was determined to be 0.42 mM and at 37°C, 0.58 mM. The corresponding  $V_{max}$  values were calculated to be  $4.34 \times 10^{-4}$  mM·s and  $1.15 \times 10^{-4}$  mM·s, respectively. From these values, the  $K_m$  for AcCoA remains relatively constant at the two temperatures used, while the  $V_{max}$  increased 4-fold. This lack of response observed in the  $K_m$  value might be related to the function of this enzyme and complexity of its catalytic involvement (Hazel and Prosser, 1974).

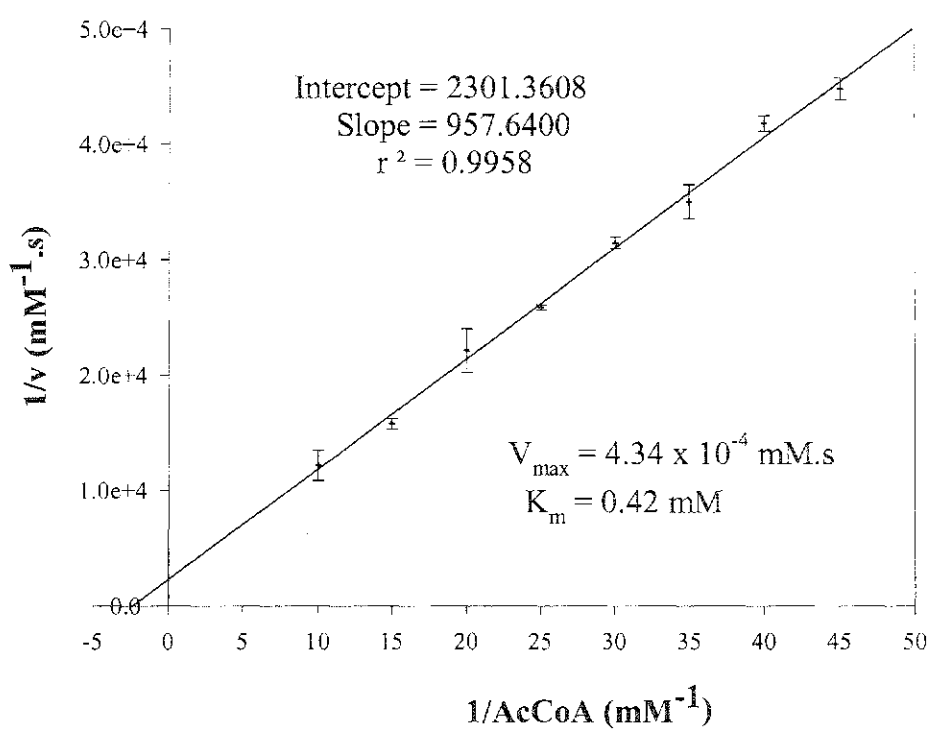
The  $K_m$  value of *S. akiyoshiensis* NAT for AcCoA at 37°C was in good agreement with the values observed for *Essox lucius* pineal organ AANAT at 0.34 mM (Falcon *et al.*, 2001), *S. cerevisiae* AANAT at pH 8.60 with 0.34 mM and pH 6.80 with

**Table 5.** Comparison of  $K_m$  Values for *S. akiyoshiensis* NAT (saNAT), Recombinant *S. cerevisiae* AANAT (scAANAT), Human AANAT (hAANAT), Sheep AANAT (oAANAT), *S. alboniger* Puromycin AANAT (purAANAT), and Rat Liver Dopa/Tyrosine Sulfotransferase (DOPA/Tyr). The  $K_m$  value for L-dopa was determined in 100 mM tris buffer pH 8.0 with AcCoA concentration fixed at 1 mM. The  $K_m$  value for AcCoA was obtained with L-dopa fixed at 2 mM. Assays were incubated at 37°C for 20 min. The  $K_m$  values for the amines and/or AcCoA with recombinant scAANAT, hAANAT, oAANAT and rat liver dopa/tyrosine sulfotransferase were taken from literature (Ganguly *et al.*, 2001; Sakakibara *et al.*, 1995; De Angelis *et al.*, 1998; Vara *et al.*, 1985). Values from this study are the means of triplicates. NA = not available.

Substrate	$K_m$ (mM)			
	sa NAT (pH 8.0)	scAANAT (pH 8.6)	scAANAT (pH 6.8)	hAANAT (pH 6.8)
L-dopa	0.11	NA	NA	NA
tryptamine	NA	4.7	5.6	0.12
serotonin	NA	5.1	6.5	1.3
5-methoxytryptamine	NA	2.7	4.5	0.065
AcCoA	0.58	0.34	0.51	0.11

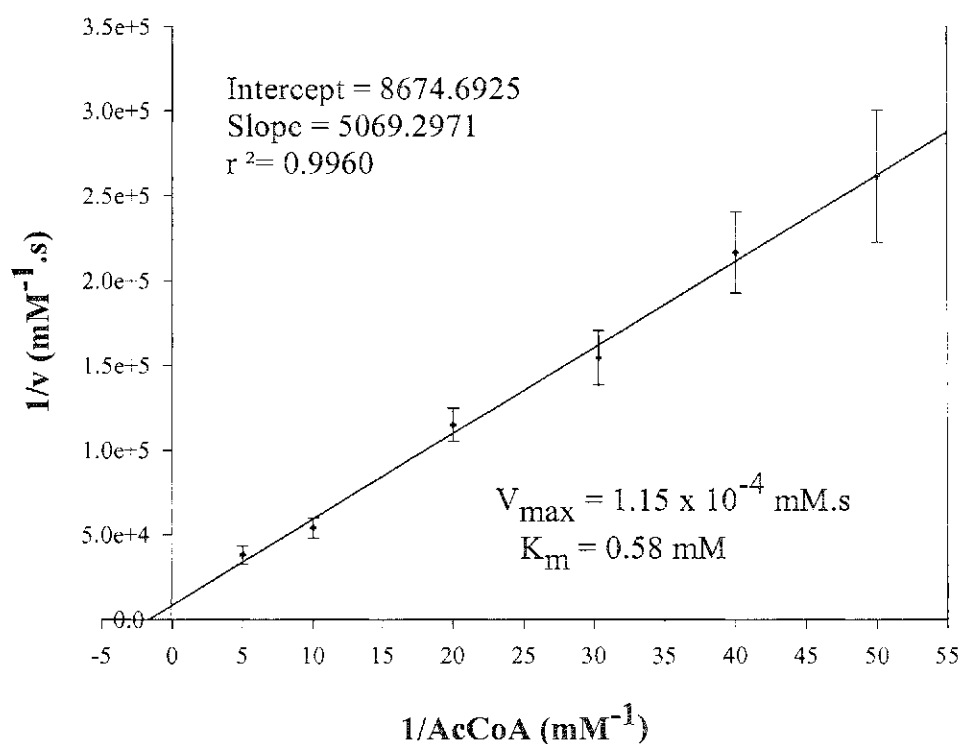
  

Substrate	$K_m$ (mM)		
	oAANAT (pH 6.8)	DOPA/Tyr (pH 9.2)	purAANAT (pH 8.5)
L-dopa	NA	0.76	NA
tryptamine	0.20	NA	NA
serotonin	0.31	NA	NA
5-methoxy tryptamine	0.062	NA	NA
AcCoA	0.13	NA	$2.1 \times 10^{-2}$



**Figure 26.** Lineweaver-Burk Plot for AcCoA as Variable Substrate at 30°C.

N-acetyltransferase activity was determined in the presence of various concentrations of AcCoA at a constant L-dopa concentration of 2 mM. Assays were performed as described in “Materials and Methods” section 3.16.6.2., at 30°C for 20 min and stopped using 3% TFA. Each point represents the average of triplicate assays. Error bars represent the standard deviation of the mean for each point.



**Figure 27.** Lineweaver-Burk Plot for AcCoA as Variable Substrate at 37°C.

N-acetyltransferase activity was determined in the presence of various concentrations of AcCoA at a constant L-dopa concentration of 2 mM. Assays were performed as described in “Materials and Methods” section 3.16.6.2., at 37°C for 20 min and stopped using 3% TFA. Each point represents the average of triplicate assays. Error bars represent the standard deviation of the mean for each point.

0.51 mM (Table 5) (Ganguly *et al.*, 2001), and sheep AANAT with 0.21 mM (Table 5) (De Anlgelis *et al.*, 1998). However, the  $K_m$  value determined for AcCoA of *S. akiyoshiensis* NAT was 5-fold higher than that of human and *Esox lucius* retina AANATs with 0.10 mM and 0.11 mM respectively (Falcon *et al.*, 2001; Ganguly *et al.*, 2001). Interestingly, the  $K_m$  values found for *S. akiyoshiensis* NAT are about 25-fold higher than that of *S. alboniger* puromycin AANAT with 0.021 mM (Table 5) (Vara *et al.*, 1985).

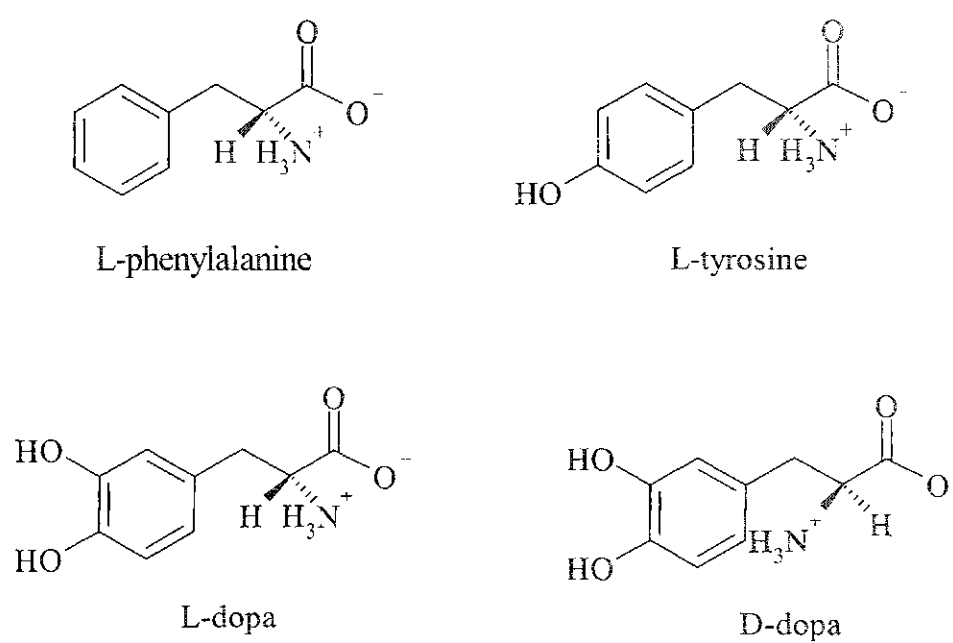
These results strongly suggest an affinity of *S. akiyoshiensis* NAT for AcCoA in the same range as eukaryotic AANATs. This clear similarity should be kept in mind whenever structure-activity relationships are investigated.

#### 4.3.7. Substrate Specificity Studies for Partially Purified *S. akiyoshiensis* NAT

##### 4.3.7.1. Amines Structurally Similar to L-dopa

In order to investigate the substrate specificity of *S. akiyoshiensis* NAT some amines structurally similar to L-dopa were evaluated (Figure 28). The results for the enzyme assays performed using either D-dopa, L-tyrosine and L-phenylalanine at 1 mM concentration in the assay are listed in Table 6. No acetylated product was observed for any of these amines after 3 hr of incubation at 37°C. Interestingly, this enzyme showed a very high specificity for L-dopa as acetylated D-dopa could not be observed. This may suggest that L-dopa is indeed the natural substrate for this enzyme. The stereochemistry of the amine substrate might play an important role in facilitating its fitting into the binding

pocket of the enzyme. In any case, a crystal structure of the protein showing the substrate binding pocket would be necessary to further study these observations.



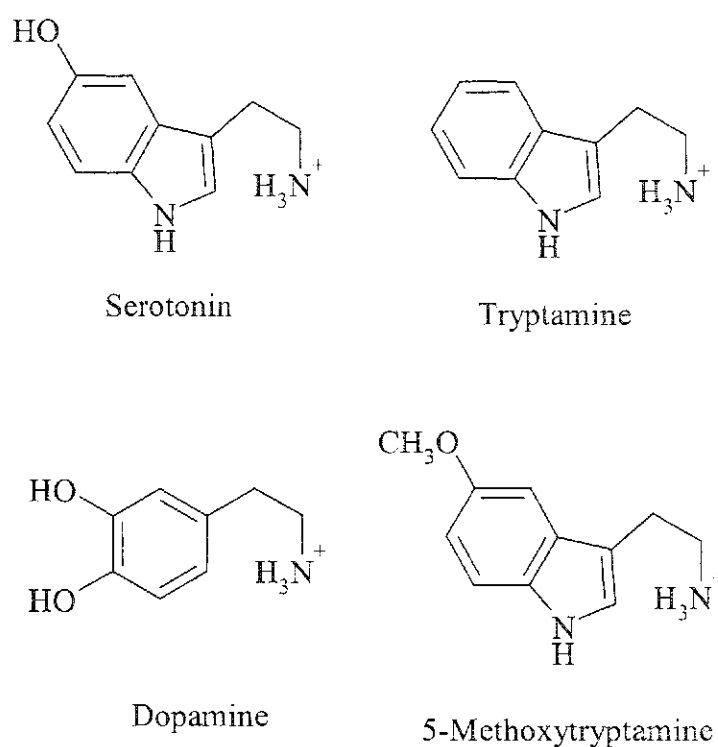
**Figure 28.** Arylalkylamines Structurally Similar to L-Dopa. These amines were used to investigate the substrate specificity of *S. akiyoshiensis* NAT.

**Table 6.** Arylalkylamines Tested as Potential Substrates for *S. akiyoshiensis* NAT. Amines as substrates (1 mM) were incubated in the assay in the presence of the cosubstrate (AcCoA) and enzyme preparation. Assays were analysed by HPLC as described under “Materials and Methods” 3.16.7.2. All the reactions were done in duplicate.

Arylalkylamine tested	N-acetylated arylalkylamine ( $\mu\text{mol}$ product formed/20 min/ mg protein produced within 20 min at 37°C)
L-tyrosine	0.00 (0.0%)
L-phenylalanine	0.00 (0.0%)
D-dopa	0.00 (0.0%)
L-dopa	169.93 (100%)
dopamine	1.81 (1.0%)
serotonin	4.28 (2.5%)
tryptamine	6.90 (4.1%)
5-methoxytryptamine	22.4 (13.2%)

#### 4.3.7.2. Amines Substrates Characteristic of AANATs

The substrate specificity of *S. akiyoshiensis* NAT was explored using natural substrates for serotonin AANAT (Figure 29). As a result of a trial using appropriately dilute enzyme (second-DEAE fraction), very low concentrations of N-acetylated amines were detected on the HPLC chromatograms. Moreover, the actual quantification of these concentrations was impossible due to the extremely small peak which made confirming structures by comparing the UV spectra impossible. For this reason, a very concentrated



**Figure 29.** Structures of Arylalkylamines Related to Serotonin AANAT and used to Explore the Substrate Specificity of *S. akiyoshiensis* NAT.

enzyme preparation was used in all the assays dealing with possible alternative substrates for *S. akiyoshiensis* NAT. The chosen enzyme preparation was a first-DEAE fraction which, ordinarily, had to be diluted 15-fold in order to be inside the linear region using L-dopa as amine substrate. As seen in Table 6 all of the amine substrates of serotonin AANAT tested were acetylated. However, the acetylation reaction proceeded at a very slow rate compared to that observed for L-dopa. Acetylation of dopamine was only 1% the rate of N-acetylated amine as that observed when L-dopa was substrate (20 min at 37°C). Serotonin and tryptamine were acetylated at a similar rate by *S. akiyoshiensis* NAT with

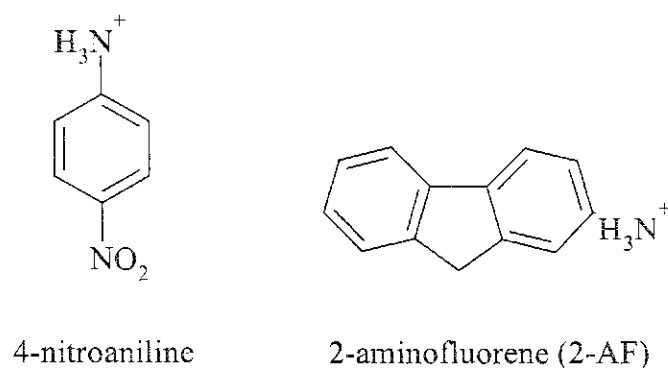
values of about 2.5% and 4.1%, respectively, the rate of L-dopa under the same conditions. The acetylation of 5-methoxytryptamine occurred at the highest rate among the group of arylalkylamines tested. In a similar pattern *Saccharomyces cerevisiae* AANAT preferentially acetylated 5-methoxytryptamine over serotonin, tryptamine and dopamine (Ganguly *et al.*, 2001). After 20 min at 37°C, the concentration of melatonin was 13.2% of that of N-acetyl-L-dopa under the same conditions.

These results show that *S. akiyoshiensis* NAT was able to acetylate arylalkylamines that are considered substrates of AANATs. However, the lower rate of acetylation observed for these amines might indicate that the catalytic domain amino acid composition of *S. akiyoshiensis* NAT differs from that of mammalian AANAT (oAANAT). This is concordant with observations by Ganguly *et al.* (2001) on *S. cerevisiae* AANAT (ScAANAT). The binding pocket for the arylalkylamine substrate is only partly conserved between scAANAT and oAANAT and might determine the substrate specificity of these enzymes. In consequence, the actual structure of the amine substrate binding pocket in *S. akiyoshiensis* NAT might justify the selectivity of this enzyme for L-dopa over other arylalkylamines.

#### 4.3.7.3. Arylamines as Potential Substrates for *S. akiyoshiensis* NAT

In order to determine if *S. akiyoshiensis* N-acetyltransferase could acetylate arylamines, 4-nitroaniline and 2-AF, two of the most common substrates for ANATs, were tested (Figure 30) (Chen *et al.*, 1999; Chung, 1998; Hasmann *et al.*, 1986; Hsieh *et al.*, 1998, Rougraff and Paxton, 1987). As summarized in Table 7, there was no acetylation

observed for 4-nitroaniline as a substrate under the experimental conditions used. The result for 2-AF shows very marginal activity at 37°C for 20 min since only 0.12% of N-acetylated amine was produced compared to L-dopa as substrate. This result is much lower than any of those obtained using AANAT substrates. Although acetylation of arylamines is considered not to be mediated by AANATs, there is evidence that this might occur to a small degree. Zhan-Poe and Craft (1999) have shown that rat recombinant AANAT was able to acetylate aniline and phenetidine but it was a marginal activity (0.85 and 1.74%, respectively) compared to what was observed for tryptamine. In addition, Ganguly *et al.* (2001) have found that scAANAT was able to acetylate aniline but only at a rate of 4% of that observed for 5-methoxytryptamine. In the same study, oAANAT was found to acetylate aniline but only 0.4% the rate observed for 5-methoxytryptamine. Therefore, *S. akioyoshiensis* NAT is following the pattern of acetylation of very specific AANATs and arylamines do not appear to be appropriate substrates for this enzyme.



**Figure 30.** Structures of Possible Arylamine Substrates of *S. akiyoshiensis* NAT.

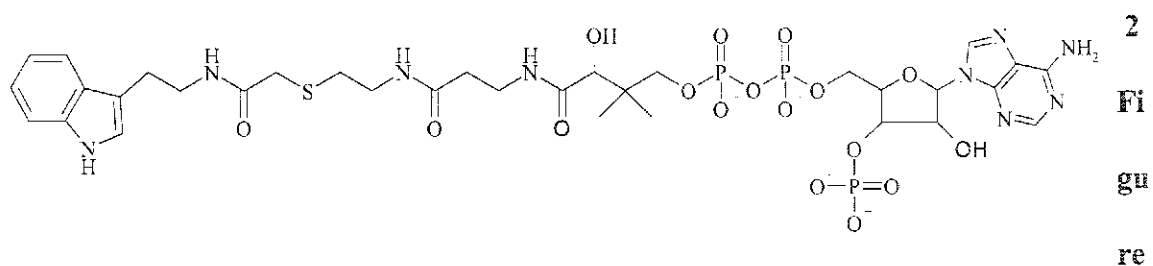
**Table 7.** Arylamines as Potential Substrates of *S. akiyoshiensis* NAT. Compounds as substrates were incubated in the presence of the cosubstrate (AcCoA) and enzyme preparation at 37°C for up to 3 h. Assays were analysed by HPLC as described under “Material and Methods” 3.16.7.2. The data reported represents the average of duplicate assays.

Amine Tested	N-acetylated amine ( $\mu\text{mol}$ product formed/20 min/mg protein) produced within 20 min at 37°C
L-dopa	169.93 (100%)
4-nitroaniline	0.00 (0.0%)
2-aminofluorene (2-AF)	0.20 (0.12%)

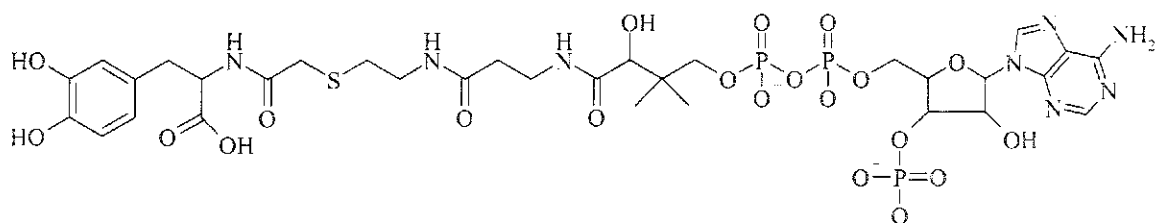
#### 4.3.8. Effect of AANAT Bisubstrate Inhibitor

In an attempt to evaluate if the active site and catalytic mechanism of *S. akiyoshiensis* NAT and vertebrate AANATs are similar, the bisubstrate inhibitor (CoA-S-N-acetyltryptamine) for AANATs was tested. As previously mentioned AANATs follow an ordered “Bi-Bi” ternary complex kinetic mechanism (De Angelis *et al.*, 1998). This mechanism implies that both substrates should bind to the enzyme prior to the release of either product. Therefore, there is no covalent acetyl-enzyme intermediate. Based on this knowledge, a bisubstrate analog, which links tryptamine and CoA via an acetyl bridge (CoA-S-N-acetyltryptamine, Figure 31) was synthesized and is considered a

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31. Bisubstrate inhibitor for AANATs (coenzyme-A-S-N-acetyltryptamine) (1) and

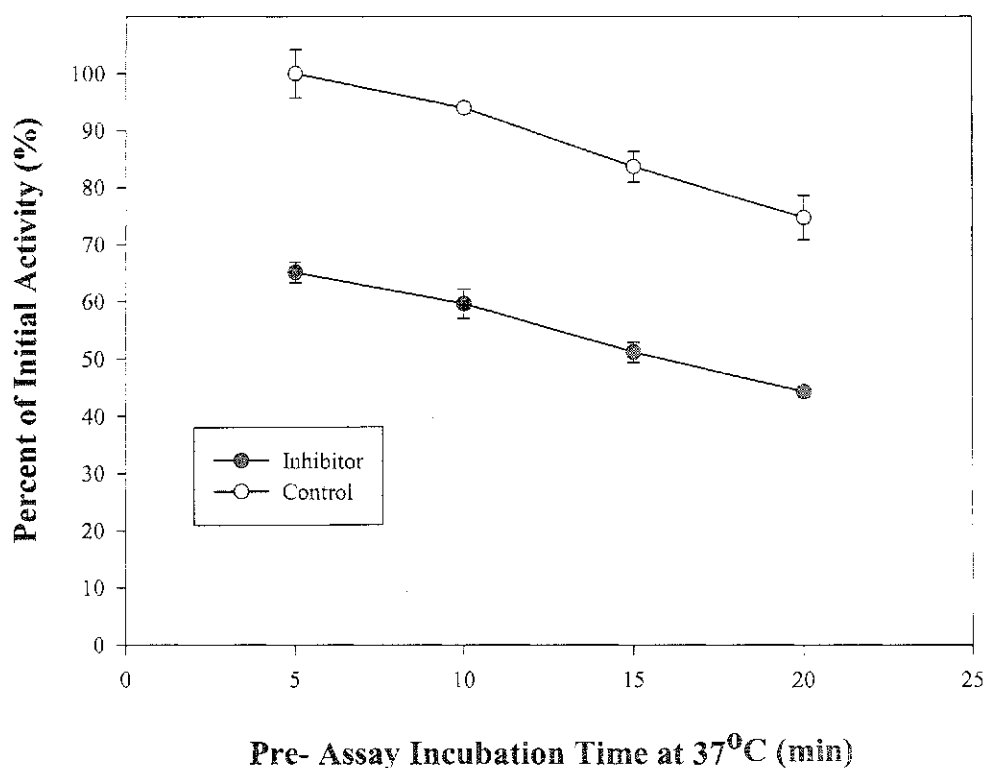


Bisubstrate Inhibitor Proposed for *S. akiyoshiensis* NAT (coenzyme-A-S-N-acetyl-L-dopa)

(2).

potent inhibitor of AANAT activity. On the other hand, ANATs follow a ping-pong mechanism which involves the formation of a covalent acetyl-cysteine intermediate (Dupret and Grant, 1992). Khalil and Cole (1998) showed that ANATs are resistant to the bisubstrate analog inhibitor for AANATs. The  $IC_{50}$  was of about 140  $\mu$ M, while the  $IC_{50}$  of this bisubstrate on AANATs was 150 nM. As seen from Figure 32, *S. akioyoshiensis* NAT was only partially inhibited at 0.50 mM of this bisubstrate inhibitor. A first-DEAE fraction was pre-incubated with the bisubstrate analog at 37°C for 5 min and reported 35% loss of activity, while the control showed no major effect after 5 min of pre-incubation at 37°C. After 10 min of pre-incubation with the bisubstrate analog this fraction showed 34% activity lost compared to the control which lost 6% of initial activity. After 15 min of pre-incubation in presence of the bisubstrate analog this fraction showed 32% activity loss compared to the control which lost 16% of initial activity at 37°C. Finally, the fraction pre-incubated with the inhibitor at 37°C for 20 min reported a 30% activity loss compared to that of the control at 37°C which loss 25% of initial activity. Assuming the  $IC_{50}$  for *S. akioyoshiensis* NAT to be 0.5 mM, this result is about 50-fold higher than that observed for sheep and human AANATs (0.2  $\mu$ M) (Khalil *et al.*, 1999) and *S. cerevisiae* AANAT (10  $\mu$ M) (Ganguly *et al.*, 2001).

Although the inhibitory effect of the bisubstrate analog specific for AANATs was not strong against *S. akioyoshiensis* NAT activity, the low acetylation rate for tryptamine might be responsible for this effect. Bisubstrate analogs are potent inhibitors because they mimic the transition state (Kim and Cole, 2001). Studies on substrate specificity



**Figure 32.** Bisubstrate Inhibitor (coenzyme-A-S-N-acetyltryptamine) Effect on *S. akiyoshiensis* NAT. Partially purified enzyme preparation (post- first DEAE fraction) was incubated with the bisubstrate analog (1 mM final concentration) or water (control). This mixture was used to run activity assays as described in “Materials and Methods” section 3.16.8. Each data point is the mean of duplicate assays. Error bars represent the standard deviation of the mean for each point.

of AANAT have revealed that the indole function is particularly important for efficient turnover (Khalil *et al.*, 1998). Most of the bisubstrate analogues designed for AANATs

are based on tryptamine, a preferred substrate. Therefore, in order to test the mechanism of *S. akiyoshiensis* NAT a bisubstrate analog based on L-dopa should be synthesized and evaluated such as the one shown in Figure 31.

## 5. CONCLUSIONS AND FUTURE DIRECTIONS

The aim of the purification procedure presented in this thesis was to isolate an N-acetyltransferase from *S. akiyoshiensis* in sufficient purity and yield to complete further studies. In effect, the highest purity (354-fold) for this partial purification was accomplished with a gel filtration chromatography step using a Zorbax Bio Series GF-250 column. However, limitations of this technique that could not be overcome during this study prevented its routine utilization. Therefore, the second best recovery (125-fold) was achieved with a first-DEAE chromatography. Although the fold purification achieved with this anion-exchange chromatography was the maximum reached within this purification scheme, the second DEAE chromatography (93-fold) showed the enrichment of a band on SDS-PAGE. Both post-DEAE enzyme preparations were stable under storage at -80°C for around 8 months and could be used as a starting point to attempt further purification techniques, followed by blotting and sequencing of this protein.

*S. akiyoshiensis* NAT represents a very specific enzyme with a remarkably high preference for L-dopa. This specificity was observed against other arylalkylamines that are known to function as substrates for eukaryotic AANATs. In addition, the fact that this protein acetylates arylalkylamines but does not acetylate arylamines to any significant extent strongly supports the suggestion that *S. akiyoshiensis* NAT is more closely related to AANATs than to ANATs. These observations are concordant with the kinetic parameters determined for this enzyme, which agree with values reported for AANATs and their natural substrates. It will be important to investigate if this enzyme is capable of acetylating other GNAT superfamily substrates as well, such as histones. Unfortunately,

in order to confirm the extent of similarities with AANATs, the amino acid sequence and ultimately three-dimensional structure are needed.

The high specificity of *S. akiyoshiensis* for L-dopa has also further supported the biosynthetic pathway to N-acetyl-L-dopa suggested by Smith (1995). Since the acetylation of L-tyrosine by *S. akiyoshiensis* NAT was not detected, the most plausible pathway proceeds *via* hydroxylation of L-tyrosine to L-dopa catalysed by tyrosinase, followed by the N-acetylation of L-dopa. This latter step has been observed in this study for partially purified enzyme by the isolation of *S. akiyoshiensis* NAT from cell free extracts.

While enzyme activity increases with the addition of NaCl, it is not clear if this is an activation or strictly a salt related stabilizing effect. This enzyme may contain specific structural elements that enable proper folding at relatively high salt concentrations and further investigation should be aimed at elucidating fully the mechanisms involved in order to determine the physiological significance.

Partially purified *S. akiyoshiensis* NAT showed significant changes in activity in the presence of a variety of divalent cations. The strong inhibitory effect observed in the presence of copper and mercury salts agree with the occurrence of cysteine and histidine residues important in catalysis or in structural conformation of the active enzyme. This finding correlates with the presence of two histidine residues in the catalytic site of AANATs. In addition, two cysteine residues have been recently found to regulate the activity in AANATs. The optimal temperature and pH were found to be 43°C and 8.0, respectively. These values are also similar to those for fungal AANATs. As far as the

molecular weight of this enzyme is concerned, SDS-PAGE showed a strong band at around 14.4 kDa in the most highly purified preparation. However, it should be noted that this preparation is not homogeneous and further investigations are required. Although this size *S. akioyoshiensis* NAT is below the range for AANATs, this could indicate that this protein is an ancestor of the eukaryotic AANATs. In any event, the purification of this protein to homogeneity is necessary to further characterize it and confirm the data reported in the present document.

This thesis has described the partial purification and characterization of *S. akioyoshiensis* NAT. Most of the data gathered from this study strongly suggest that this enzyme is related to AANATs. However, further experimental evidence is required to draw a final conclusion. One important question remains to be answered : How is the acetylation of L-dopa functionally relevant for *S. akioyoshiensis*? Is it intervening in the production of an antibiotic other than HON? Although it has been shown that *S. akioyoshiensis* produces only one antibiotic (Vining, L., personal communication), this analysis used only several complex growth media. As the production of HON is very dependent on the growth media, it could be that *Streptomyces akioyoshiensis* has the genes for the biosynthesis of another as of yet unidentified antibiotic but is dependent on the necessary environmental factors (specific nitrogen source, etc) before those genes are fully expressed.

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## APPENDIX

**Appendix Table 1.** Summary of the First Step in the Purification of *S. akiyoshiensis*

NAT. Cell free extract was applied to an affi-gel blue column as described in “Materials and Methods” section 1.13.1. The enzyme activity was determined under standard assay conditions (L-dopa as substrate). CE = crude extract (cell free extract); FT = flow through; W = wash; F1 = fraction 1; F2 = fraction 2; F3 = fraction 3, F4 = fraction 4 and F5 = fraction 5. One enzyme unit was defined as the amount of enzyme producing 1  $\mu\text{mol}$  of N-acetyl-L-dopa per min.

Volume (mL)	Sample	Total Act. (U)	% recovery	Protein (mg/mL)	% protein	Specific activity (U/mg of protein)	Fold Purification
80	CE	1.67	100	19.76	100	$2.11 \times 10^{-3}$	1.00
80	FT	0.10	6.25	12.93	65.45	$2.01 \times 10^{-4}$	0.10
120	W	0.19	10.89	4.74	35.99	$6.49 \times 10^{-4}$	0.30
20	F1	0.02	1.46	0.11	0.14	$2.85 \times 10^{-2}$	13.50
20	F2	0.04	2.62	0.16	0.20	$2.81 \times 10^{-2}$	13.32
20	F3	0.26	15.76	0.20	0.26	$1.30 \times 10^{-1}$	61.83
20	F4	0.28	16.90	0.12	0.16	$2.36 \times 10^{-1}$	111.72
20	F5	0.06	3.36	0.07	0.09	$9.49 \times 10^{-2}$	37.85

**Appendix Table 2.** Summary of the Second Step in the Purification of *S. akiyoshiensis*

NAT. Fractions post-affi-gel blue presenting most of the activity were combined and applied to a DEAE column as described under “Materials and Methods” section 2.13.2.

The enzyme activity was determined according to standard conditions. Data are the average of duplicate assays. F3-4 = fractions 3 and 4 from affi-gel blue column

chromatography; FT = flow through; F1= fraction 1, F2 = fraction 2; F3 = fraction 3,

F4 = fraction 4 and F5 = fraction 5.

Volume (mL)	Sample	Total Act. (U)	% recovery	Protein (mg/mL)	% protein	Specific activity (U/mg of protein)	Fold Purification
40	F3-4	0.68	100	0.16	100	$2.07 \times 10^{-1}$	97.80
46	FT	0	0	0.08	50.34	0	0
3	F1	0.34	50.11	0.87	39.50	$2.64 \times 10^{-1}$	125.02
2	F2	0.02	2.25	0.19	0.20	$8.08 \times 10^{-2}$	38.3
2	F3	$9.80 \times 10^{-2}$	1.43	0.15	0.16	$6.37 \times 10^{-2}$	30.19
2	F4	$8.50 \times 10^{-3}$	1.24	0.14	0.12	$6.94 \times 10^{-2}$	32.90
2	F5	$5.80 \times 10^{-3}$	0.84	0.08	0.09	$7.19 \times 10^{-2}$	34.08

**Appendix Table 3.** Effect of Different pH Values on the Activity of *S. akiyoshiensis*

NAT. Enzyme preparations were mixed with tris or bis-tris buffers as indicated in “Materials and Methods” section 2.16.1. Enzyme activity was assayed under standard conditions except for the final pH of the reaction. A value of 100% of enzyme activity was used for the highest value obtained.

pH value	Enzyme Activity (%)			
	Tris (37°C)	Tris-NaCl (37°C)	Bis- Tris NaCl (37°C)	Bis-Tris –NaCl (37°C)
6	5.12	4.59	1.45	1.8
6.5	15.17	17.1	9.64	8.74
7	37.21	45.62	31.5	28.86
7.5	71.6	76.11	70.21	47.12
8	100	100	100	100
8.5	76.44	94.8	89.39	85.6
9	61.49	76.11	95.01	98.39
9.5	50.38	64.13	84.97	93.51
10	33.2	43.35	70.16	90.84

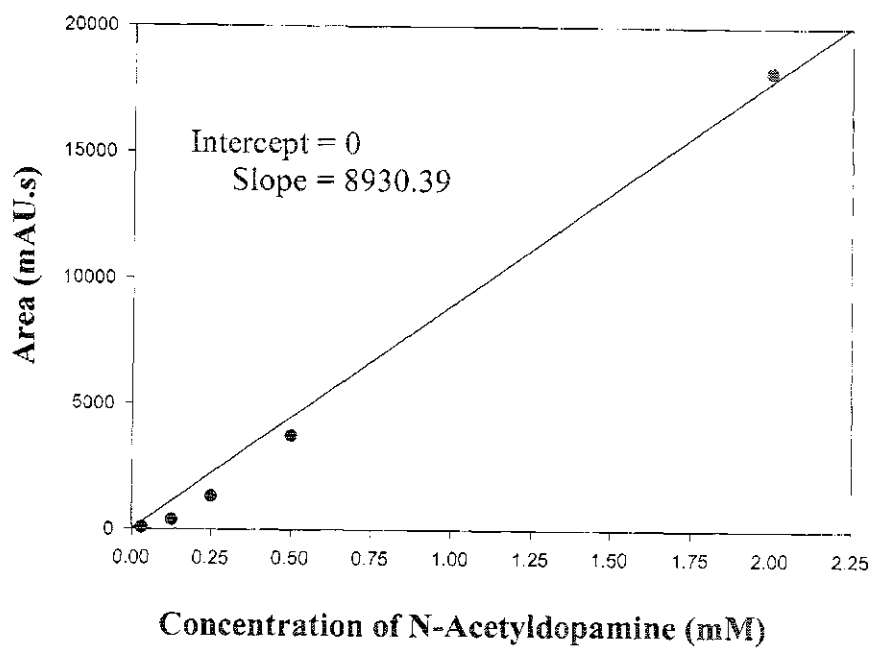
**Appendix Table 4.** Effect of Metal Ions on Enzyme Activity of *S. akiyoshiensis* NAT.

Post-affi-blue enzyme preparation was incubated in the presence of metal ions as described in “Materials and Methods” section 2.16.4. 100% = no salt added.

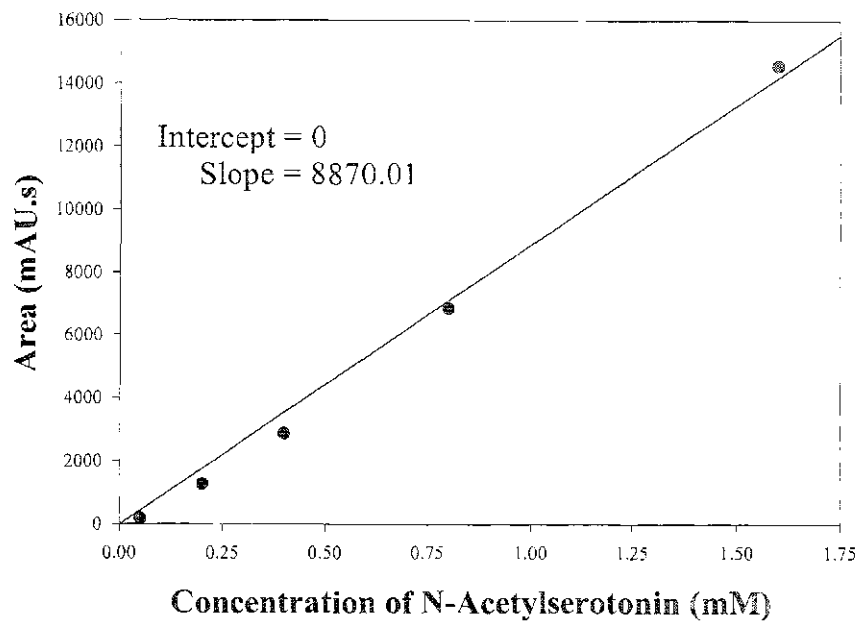
Metal Salt Added	Enzyme activity (%)	
	1 mM	5mM
None (Control)	100	100
FeCl <sub>3</sub> · 6 H <sub>2</sub> O	63.2	0
FeCl <sub>2</sub> · 4 H <sub>2</sub> O	42.67	0
HgCl <sub>2</sub>	45.33	0
CaCl <sub>2</sub>	104.32	105.18
MnCl <sub>2</sub> · 4 H <sub>2</sub> O	55.36	16.04
CdCl <sub>2</sub> · 2½ H <sub>2</sub> O	70.47	9.57
NiCl <sub>2</sub> · 6 H <sub>2</sub> O	42.27	1.87
CoCl <sub>2</sub> · 6 H <sub>2</sub> O	50.28	1.76
CuCl <sub>2</sub> · 2 H <sub>2</sub> O	18.72	0
MgCl <sub>2</sub> · 6 H <sub>2</sub> O	99.72	103.78
NaCl	106.54	109.13
ZnCl <sub>2</sub>	28.56	0
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> · 7 H <sub>2</sub> O	0	0
Fe(SO <sub>4</sub> ) · 7 H <sub>2</sub> O	10.81	0
HgSO <sub>4</sub>	43.62	0
CoSO <sub>4</sub>	42.46	2.62
MnSO <sub>4</sub>	47.4	21.79
CdSO <sub>4</sub>	0	0
NiSO <sub>4</sub>	46.8	2.42
CaSO <sub>4</sub>	111.94	108.33
CuSO <sub>4</sub> · 5 H <sub>2</sub> O	11.39	0
CoSO <sub>4</sub>	42.46	2.62
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	117.04	107.24
NaSO <sub>4</sub>	130.57	118.58
ZnSO <sub>4</sub>	0	0

**Appendix Table 5.** Effect of different concentrations of KCl, NaCl and CaCl<sub>2</sub> on *S. akiyoshiensis* NAT. The enzyme preparation (post-affi-gel blue) was incubated in the presence of various concentrations of KCl, NaCl and CaCl<sub>2</sub> (0 - 500 mmol/L) as described in “Materials and Methods” section 2.16.5. A value of 100% for enzyme activity was used for the control (no addition of salt).

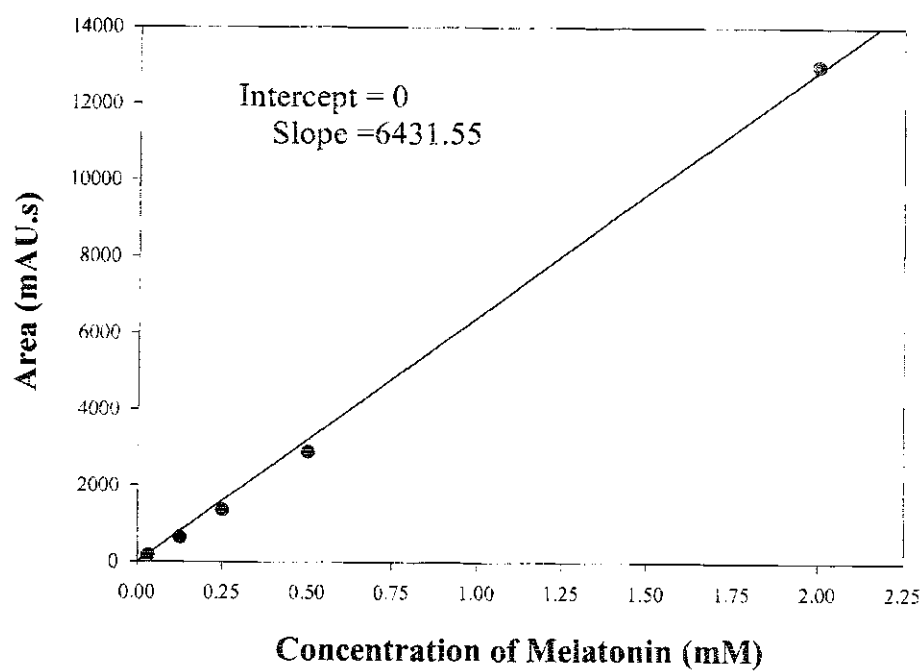
Concentration (mmol/L)	Enzyme Activity (%)		
	KCl	NaCl	CaCl <sub>2</sub>
0	100	100	100
1	115.06	112.17	118.9
5	122.48	101.22	116.04
20	134.95	132.41	64.51
50	150.26	114.54	23.59
100	142.72	142.18	9.36
200	153.79	155.05	5.09
500	139.61	144.38	0



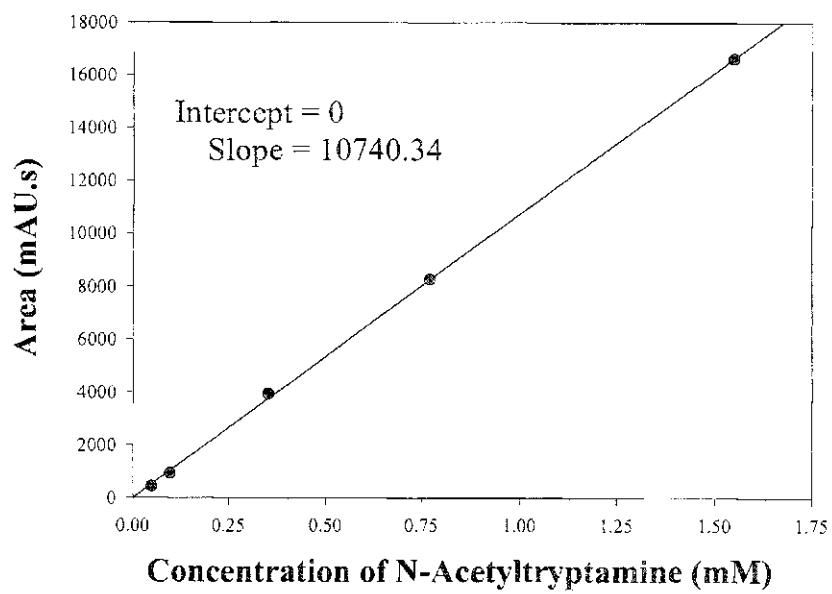
**Appendix Figure 1.** Standard Curve for N-acetyldopamine. The curve was forced through zero in order to analyse low concentrations.



**Appendix Figure 2.** Standard Curve for N-acetylserotonin. The curve was forced through zero to be able to analyse low concentrations.



**Appendix Figure 3.** Standard Curve for Melatonin. The curve was forced through zero in order to analyse low concentrations.



**Appendix Figure 4.** Standard curve for N-acetyltryptamine. The curve was forced through zero in order to analyse low concentrations.