COMPARATIVE CHARACTERIZATION AND MUTATIONAL ANALYSIS OF TYPE III PANTOTHENATE KINASES

by

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Thesis

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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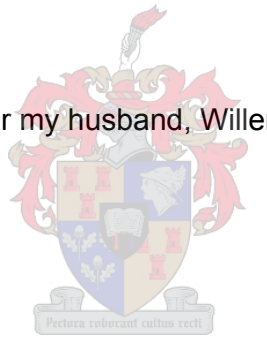
Signature Date

Summary:

This thesis reports the cloning, overexpression and characterization of the *coaX* gene product from *Bacillus subtilis* and its homologue from *Helicobacter pylori.* It demonstrates that these proteins have pantothenate kinase activity. Compared to the two pantothenate kinase analogues classified to date, these two enzymes exhibit distinctly different characteristics, suggesting that they are the first characterized examples of a third pantothenate kinase analogue. In addition, mutational studies are presented that probe the importance of conserved aspartate residues within the active sites of these newly characterized analogues. The results show that these residues are important for the activity of the protein.

Opsomming:

Die klonering, uitdrukking en karakterisering van die *coaX* geenproduk van *Bacillus subtillis* en sy homoloog van *Helicobacter pylori* word in hierdie werkstuk gerapporteer. Daar word getoon dat beide hierdie twee proteïene pantoteensuurkinase aktiwiteit besit. In teenstelling met die twee pantoteensuurkinase analoë wat tot dusver bestudeer is, toon hierdie twee ensieme duidelik onderskeidende karaktereienskappe. Hierdie verskeinsel ondersteun die veronderstelling dat hierdie ensieme die eerste gekarakteriseerdie voorbeelde van ’n derde pantoteensuurkinase analoog is. Mutasie studies bevestig verder die belang van gekonserveerde aspartaat residue binne-in die aktiewe sentrum van die nuut gekarakteriseerde analoog en dui aan dat hierdie residue van spesiale belang is vir die aktiwiteit van die proteïen.



For my husband, Willem

Acknowledgements

- Doctor Erick Strauss, my supervisor, for his constant guidance and assistance throughout the duration of my Masters course. I would also like to thank Erick for his friendship, which has come to mean a great deal to me over the last couple of years.

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- I would like to thank my husband, Willem, whose support throughout this endeavour has been nothing short of remarkable.

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Abbreviations

A	Absorbance
ACP	Acyl carrier protein
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulphate
Asp	Aspartate
ATP	Adenosine triphosphate
Bs	*Bacillus subtilis*
BSA	Bovine serum albumin
CoA	Coenzyme A
CoaA	Pantothenate Kinase
CTP	Cytidine triphosphate
D	Aspartate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Glutamate
Ec	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
FAS	Fatty acid synthase
Glc	Glucose
Glu	Glutamate
GTP	Guanidine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane sulphonic acid
Hp	*Helicobacter pylori*
Hsp	Heat shock protein
k_{cat}	Turnover number
kDa	Kilodalton
K_M	Michaelis constant
LB	Luria Bertani
MIC	Minimal inhibitory concentration
N	Asparagine

NADH	Nicotinamide adenine dinucleotide (reduced)
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PanCOOH	Pantothenate
PanK	Pantothenate kinase
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
Sa	*Staphylococcus aureus*
SDS	Sodium dodecyl sulphate
spp.	Species (plural)
TCA	Tri-carboxylic acid cycle
TEMED	*N*,*N*,*N*',*N*',-tetramethyl-ethylene diamine
Thr	Threonine
TRIS	Tris(hydroxymethyl)aminomethane
TRIS-HCl	Tris(hydroxymethyl)aminomethane-HCl
UTP	Uridine triphosphate
V_{max}	Maximal velocity

Chapter 1

A General Introduction

Pantothenate is a member of the B group of vitamins and is an essential component of the acyl group carriers coenzyme A (CoA or CoA-SH) and acyl carrier protein (ACP). These two acyl group carriers are present in all organisms and take part in more than 100 reactions in metabolism (*1, 2*). This study presents an overview of the biosynthesis of coenzyme A. In particular it concentrates on pantothenate kinase, the enzyme catalysing the first reaction in the five-step biosynthesis of CoA.

Chapter 2 concentrates on coenzyme A, giving an in depth review of its structure and function in metabolism. It also looks at the biosynthesis of this cofactor in both prokaryotes and eukaryotes and the regulation of this pathway.

Chapter 3 takes a closer look at the first reaction in the biosynthetic pathway to CoA, namely that catalyzed by pantothenate kinase (PanK). This chapter gives a detailed account of the currently available literature on this enzyme. The analogues of PanK from various organisms are compared and the apparent absence of this enzyme function in certain pathogenic bacteria is noted.

Following on from the information presented in chapter 3, chapter 4 reports our findings of a possible third analogue of pantothenate kinase. We cloned and classified this analogue from *B. subtilis* and *H. pylori*. Protein activity was tested by functional complementation and an independent kinetic assay for pantothenate kinase activity. We make the suggestion that this is a third analogue of pantothenate kinase present in a subset of mainly pathogenic bacteria.

The protein classified in chapter 4 was assigned to a protein fold family based on information gleaned by protein structure prediction servers. Alignments with proteins with the same folds whose structures have already been solved identify certain aspartate residues as conserved. These conserved aspartate residues are suspected of playing a role in substrate and inhibitor binding. This hypothesis is tested by mutagenesis studies in chapter 5.

Finally, chapter 6 summarizes the main findings of this study and highlights some interesting possibilities for future research with respect to this newly characterized pantothenate kinase enzyme.

Chapter 2

Coenzyme A, Central to Metabolism

2.1. The Importance of Coenzyme A:

Coenzyme A is a ubiquitous and essential cofactor in all living organisms. It functions as an acyl group carrier and acyl activating group in a number of central metabolic transformations, including the tricarboxylic acid cycle and fatty acid metabolism. Along with its thioesters, CoA is in demand as a substrate for approximately 9% of all enzyme activities, where it participates in a variety of acyl transfer reactions (*2*). It has been estimated that CoA is involved in over 100 different reactions in intermediary metabolism (*1, 2*). CoA is the source of 4'-phosphopantetheine, which is the prosthetic group of carrier proteins of fatty acid, polyketide and nonribosomal peptide synthases in mammals, bacteria and plants (*2, 3*). CoA and its esters also function as regulators in several important reactions in intermediary metabolism such as pyruvate dehydrogenase and phosphoenolpyruvate carboxylase (*4*).

As a result of CoA's ubiquitous nature and its role as a cofactor in metabolism its levels must be stringently regulated. In addition to this, the CoA biosynthetic pathway is an energetically expensive pathway (the production of one molecule of CoA uses three ATP equivalents), so it makes sense that the pathway itself be regulated as not to waste cellular energy.

The biosynthetic pathway from pantothenate to CoA is essential in both prokaryotes and eukaryotes. CoA is produced through a series of five enzymatic reactions from pantothenate or vitamin B_5. All the genes coding for the enzymes that catalyze the reactions in the biosynthetic pathway are known. This chapter is a detailed account of coenzyme A's structure, function and biosynthesis. It also highlights this cofactors' suitability as a drug target.

2.2. Structure of CoA

The German-born biochemist Fritz Lipmann discovered Coenzyme A in 1945 (*5*). He was the first to show that a coenzyme was required to facilitate biological acetylation reactions (the A in Coenzyme A stands for *acetylation).* In 1953, Lipmann was awarded the Nobel Prize in physiology and medicine for his pioneering work in elucidating the role of this very important coenzyme. CoA's structure was first reported in 1953 (*6*). Although CoA is a structurally complex molecule, it is functionally simple (Figure 2.1).

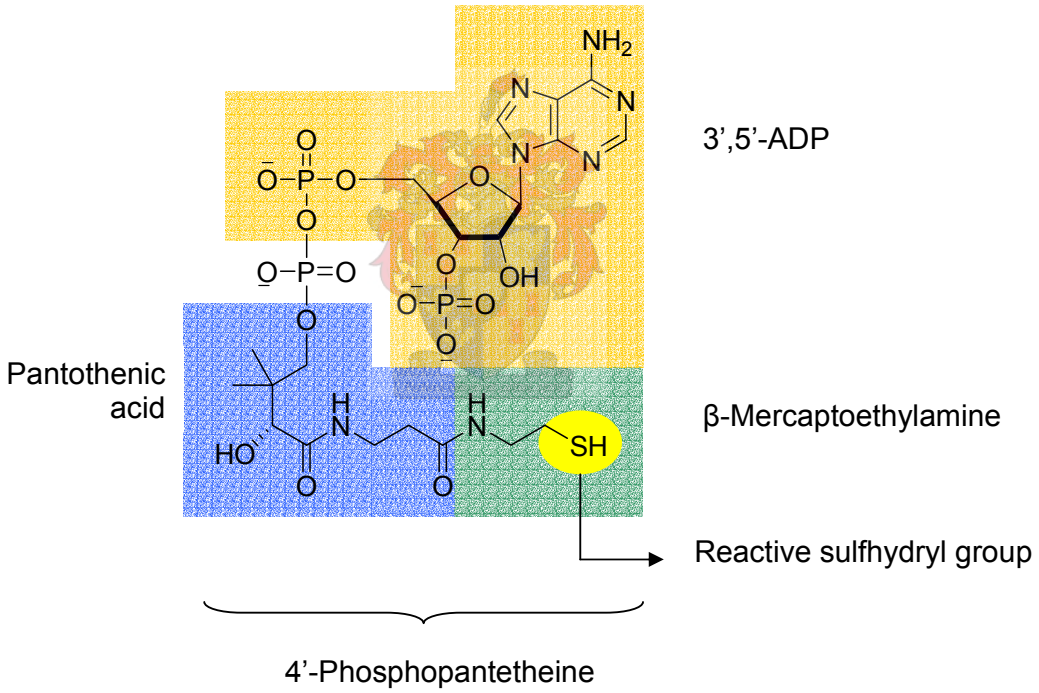


Figure 2.1. The structure of coenzyme A. The coloured areas represent the different functional groups comprising the structure of CoA. The area coloured in orange is 3',5'-adenosine diphosphate (ADP); the area coloured in blue represents pantothenic acid; the green area represents β-mercaptoethylamine and the area in yellow highlights the reactive sulfhydryl group that forms thioester linkages with acyl groups.

CoA is made up of 3',5'-adenosine diphosphate joined to 4'-phosphopantetheine in a phosphoric anhydride linkage. The phosphopantetheine part of CoA consists of β-mercaptoethylamine and pantothenic acid, a member of the vitamin B family (vitamin B_5). The adenine moiety of CoA serves as the recognition site for enzyme to bind CoA. This increases the affinity and specificity of CoA when it binds to the enzyme in question (*5, 7*). The sulfhydryl group of the β-mercaptoethylamine moiety is the key functional group of the molecule as noted in the following section (*8*).

2.3. Function of Coenzyme A

CoA has two main functions:

- It activates acyl groups for transfer by nucleophilic acyl substitution
- It activates the α-hydrogen of the acyl group for abstraction as a proton.

These two functions are illustrated in Figure 2.2.

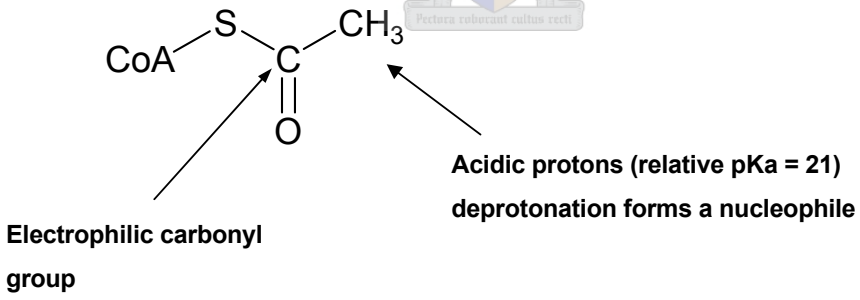


Figure 2.2. The two general modes of reactivity of Acetyl-CoA. The thioester carbonyl can act as an electrophile toward attack by a nucleophile cosubstrate. The thioester α-carbon upon deprotonation can react as a nucleophile (*5*).

It is pertinent to consider why acyl groups are carried in the form of thioesters rather than oxygen esters. The most important consideration is the difference in resonance stabilization between these two functional groups.

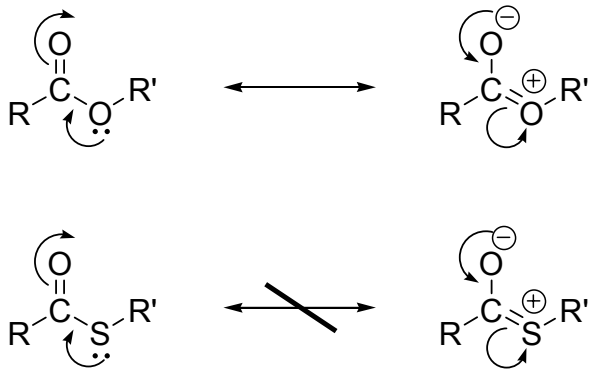


Figure 2.3. Resonance forms of the type that are important in the stabilization of esters do not contribute to the resonance stabilization of thiol esters.

Sulphur is a third-row element with a limited ability to donate a pair of 3*p* electrons into the carbonyl π system. With an electronegativity that is much less than oxygen, however, its destabilizing effect on the carbonyl group is slight, and thioesters lie in the middle of the group of carboxylic acid derivatives in respect to reactivity (*9*). This translates to the fact that resonance forms of the type that are important in the stabilization of esters do not contribute to the resonance stabilization of thioesters (Figure 2.3).

Carbanion formation at the α-carbon atom of the thioesters is more favourable than for oxygen esters. Both of the carbanions are resonance stabilized in essentially the same way (Figure 2.4) (*8*). However, because of the poor π-overlap between sulphur and the carbonyl group (as mentioned above) in the case of the thiol ester, its carbonyl group has more double bond character than that of the oxygen ester. Consequently, the resonance stabilization of the type shown here will be more favourable in stabilizing the α-carbanion of the thioester (*8*).

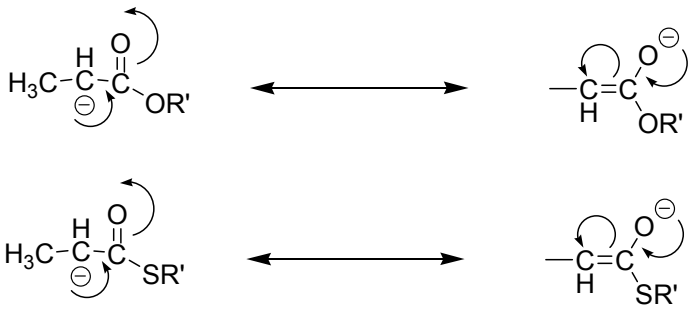


Figure 2.4. The carbanions of thiol esters and oxygen esters are resonance stabilized in essentially the same way, although carbanion formation at the alpha carbon of the thiol ester is more favourable.

Acetyl-CoA is the most common CoA thioester. Several enzymes are responsible for the formation of acetyl-CoA including acetyl-CoA synthetase, phosphotransacetylase, ATP citrate lyase and thiolase (*5*). The enzymes that utilize acetyl-CoA can be divided into two main classes. These are the Claisen enzymes, which catalyse reactions involving deprotonation of the α-carbon, and the acetyltransferases, which catalyse the nucleophilic acyl substitution reactions at the carbonyl carbon.

2.3.1. The Claisen Enzymes

The Claisen enzymes utilize acetyl-CoA as a nucleophilic substrate via deprotonation of the methyl group. The electrophile is the carbonyl group of an aldehyde, ketone, or thioester or the carboxy group of carboxybiotin. Claisen enzymes are involved in a variety of biological pathways and the reactions catalysed by Claisen enzymes could occur through either a stepwise or concerted pathway (*5*).

2.3.2. Acyltransferases

Acyltransferases, as their name suggests, catalyse the transfer of the acyl group from a CoA thioester to a nucleophile acceptor, most commonly an alcohol or amine, or in the case of thioesterases, water. Acyltransferases that specifically use acetyl-CoA are called acetyltransferases, which is the second major class of acetyl-CoA utilizing enzymes (*5*).

The biological significance of acetyltransferases is broad. Bacterial acetylation of antibiotics renders the drugs inactive, thus conferring antibiotic resistance to many bacteria. Acetylation also plays a key role in the transmission of nerve impulses (e.g. acetylcholine is a major neurotransmitter). Acetylation of histones catalysed by histone-*N*-acetyltransferase is a vital control element in gene transcription, and several additional DNA-binding proteins may be acetylated as part of the transcriptional process (*5*).

2.3.3. Other CoA Ester-utilizing Enzymes

Enzymatic reactions of longer chain CoA esters may involve functionality beyond the α-carbon. Some examples of this are provided by the fatty acid β-oxidation cycle (Figure 2.5). The reaction of acyl-CoA dehydrogenase results in oxidation of the α,β-unsaturated CoA ester. Addition of water across the double bond catalysed by enoyl-CoA hydratase forms the β-hydroxy ester. Fatty acid biosynthesis by fatty acid synthase is the approximate reversal of this β-oxidation cycle except that it is catalysed by a multi-enzyme complex. Here the acetyl groups are not carried by acetyl-CoA but by acyl carrier protein (ACP) (*5, 10*). The portion of ACP to which the acyl derivatives are bound as thiol esters is the 4'-phosphopantetheine moiety of CoA, thus acyl carrier protein (ACP) may be considered as chemically equivalent to CoA (*8*).

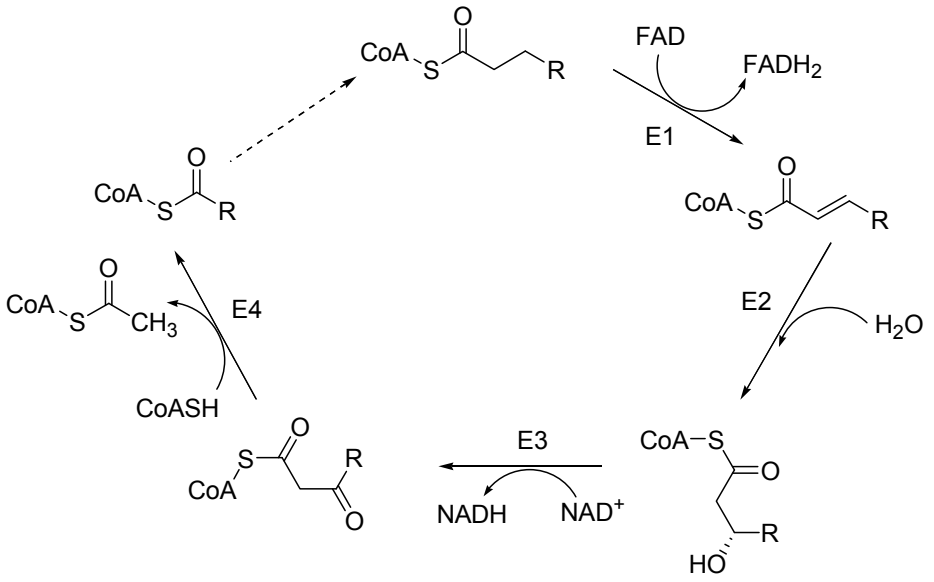
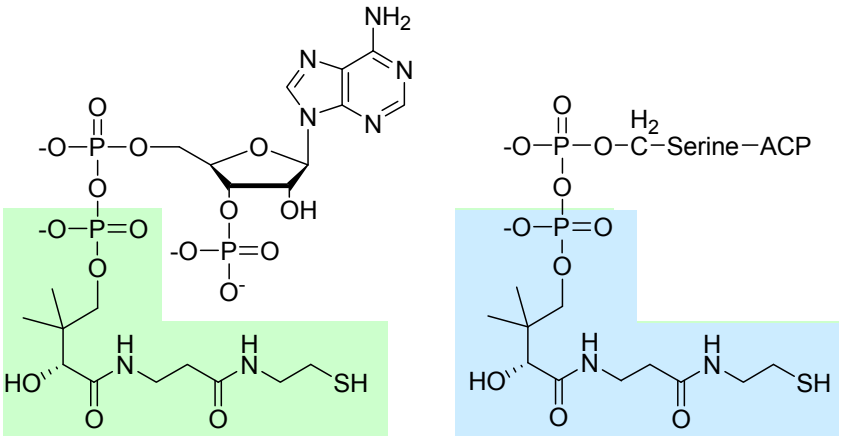


Figure 2.5. The fatty acid β-oxidation cycle. E1 = acyl-CoA dehydrogenase, E2 = crotonase, E3 = β-hydroxyacyl-CoA dehydrogenase, E4 = thiolase *(5)*.

Coenzyme A serves as a precursor to ACP via the transfer of its 4'-phosphopantetheine moiety to apo-ACP (product of the *acpP* gene (*11*)) by the enzyme holo-ACP synthase (product of the *acpS* gene (*12*)). In other words, holo-acyl carrier protein is generated when apo-ACP is phosphopantotheinylated by the displacement of the adenine monophosphate moiety of coenzyme A by an active site serine in a reaction catalyzed by ACP synthase (*1, 2, 5*).

ACP is a larger version of CoA, also using the phosphopantetheine group as a functional group for essentially the same purpose (Figure 2.6). Intermediates in fatty acid synthesis are linked covalently to the sulfhydryl groups of ACP. Fatty acid chains are constructed by the addition of two-carbon units derived from acetyl-CoA. The acetate units are activated by the formation of malonyl-CoA (at the expense of ATP). The addition of two-carbon units to the growing chain is driven by decarboxylation of malonyl-CoA.



Coenzyme A ACP

= phosphopantetheine group of CoA

= phosphopantetheine prosthetic group of ACP

Figure 2.6. Fatty acids are conjugated to both coenzyme A and acyl carrier protein through the sulfhydryl of the phosphopantetheine prosthetic group (*7*).

The building blocks of fatty acid synthesis, namely acetyl and malonyl groups, are not transferred directly from coenzyme A to the growing fatty acid chain. Rather they are first passed on to ACP and form acyl carrier protein conjugates. ACP serves as the “transporter” of fatty acid biosynthesis intermediates. The elongation reactions are repeated until the growing chain reaches 16 carbons in length (palmitic acid). Other enzymes then add double bonds and additional carbon units to the chain. In summary, ACP is used in fatty acid biosynthesis whereas CoA is used in β-oxidation of fatty acids, however, the formation of ACP is reliant the dephosphopantotheinoylation of coenzyme A (*7*).

2.4. Biosynthesis of CoA

The biosynthesis of coenzyme A can be divided into two parts in bacteria. First, pantothenate is synthesized, where after the universal biosynthesis of coenzyme A from pantothenate occurs. The second part of the pathway is present in most organisms, even those that are not capable of de novo pantothenate synthesis. Unless stated to the contrary, the biosynthesis discussed below refers to *E. coli.*

2.4.1. Pantothenate in Nutrition

Pantothenate is one of the B complex of vitamins (vitamin B_5) (*13*). During the 1930's a number of research programs were concentrating on growth factors for microrganisms and chick antidermatitis factor. These studies resulted in the isolation, synthesis and characterization of the vitamin pantothenate. Thereafter, pantothenate was found to play a fundamental role in all organisms (*14*).

Animals and some microbes lack the capacity to synthesize pantothenate and are totally dependent on the uptake of pantothenate in their diets. However, most bacteria, (*2, 13, 15*) plants and fungi are capable of synthesizing pantothenate (*1*). As a result of pantothenate being found virtually everywhere in biology it was designated pantothenate, which is derived from the Greek "*pantothen"* meaning "from everywhere" (*1, 14*). It has been reported that *E. coli*, for example, produce and secrete 15 times more pantothenate than is required for intracellular CoA biosynthesis (*16*). This highlights the abundance of pantothenate available to organisms that harbour microorganisms. It has been demonstrated that ruminants are capable of surviving without pantothenate supplementation from the pantothenate supplied to them by intestinal microorganisms (*17*). As a result of pantothenate's ubiquitous nature a clinical deficiency of vitamin B_5 has not been reported in humans (*1, 14*).

2.4.2. The Pantothenate Biosynthetic Pathway in E. coli

2.4.2.1. A General Overview

Bacteria synthesize pantothenate (Figure 2.6 and 2.7) from aspartate, α-ketoisovalerate and ATP. The biosynthesis begins with the decarboxylation of aspartate to give β-alanine. Pantoic acid is formed by the hydroxymethylation of α-ketoisovalerate followed by reduction of ketopantoate. Pantoic acid and β-alanine are then condensed to generate pantothenic acid. This section of the pathway occurs only in microbes and plants.

Bacteria divert amino acids and intermediates from central metabolism to produce pantothenate. Pantothenate is used in the biosynthesis of coenzyme A and the formation of ACP, the two predominant acyl group carriers in cells. Most of what is known of the biosynthesis of pantothenate and coenzyme A has been learnt through research involving *E. coli* and *Salmonella typhimurium* (*13*).

2.4.2.2. Biosynthesis in Detail

α-Ketopantoate Biosynthesis

The first step in the biosynthesis of D-pantoic acid is the transfer of a hydroxymethyl group from $N^{5,10}$-methylenetetrahydrofolate to α-ketoisovalerate by α-ketopantoate hydroxymethyltransferase. α-Ketopantoate hydroxymethyltransferase is the product of the *panB* gene (*18*). The conversion of α-ketoisovalerate to pantoate proceeds stereospecifically, with inversion of the configuration at the C-3 carbon of α-ketoisovalerate (*19*). It has been experimentally shown that the only pathway to pantothenate is from α-ketoisovalerate (*20*).

Pantoate Biosynthesis

D-Pantoate is synthesized from α-ketopantoate by α-ketopantoate reductase, which is the product of the *panE* gene *(13)*. It has been determined that acetohydroxy acid isomeroreductase (the *ilvC* gene product) is also capable of catalyzing the reduction of α-ketopantoate. This means that mutants that do not contain the *panE* gene can still survive provided that the *ilvC* gene is expressed abundantly,

however, when this gene is not expressed at significant levels, the mutants do require pantoate for growth (*21*).

β-Alanine Biosynthesis

On the basis of a study of intact cells it was suggested that aspartate was the precursor of β-alanine (*22, 23*). Two independent scientific groups, Williamson and Brown (*24*) and Cronan (*25*) characterized an L-aspartate-1-decarboxlase enzyme used in *E. coli* to convert aspartate to carbon dioxide and β-alanine. The gene responsible for the expression of this enzyme is *panD.* It has been shown that *panD* mutants require supplementation with β-alanine in order to grow (*25, 26*).

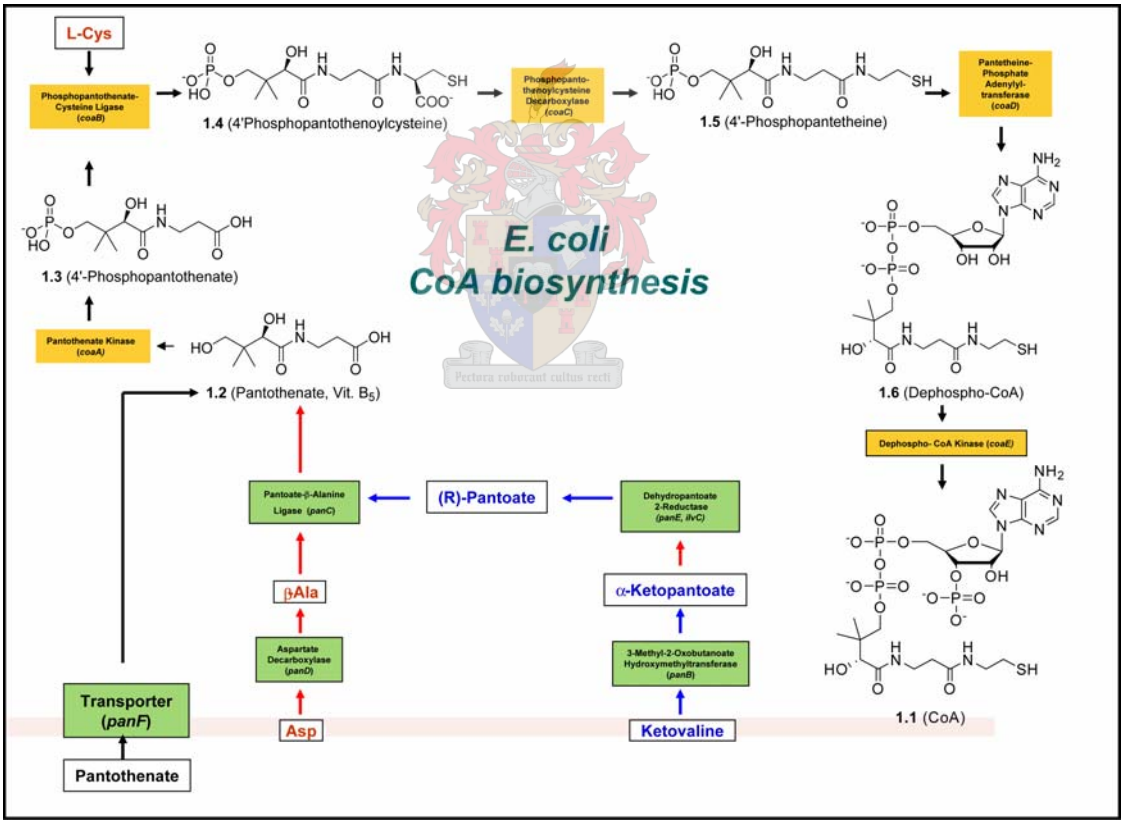


Figure 2.6. A schematic illustration of the complete biosynthetic pathway of CoA in *E. coli*. .

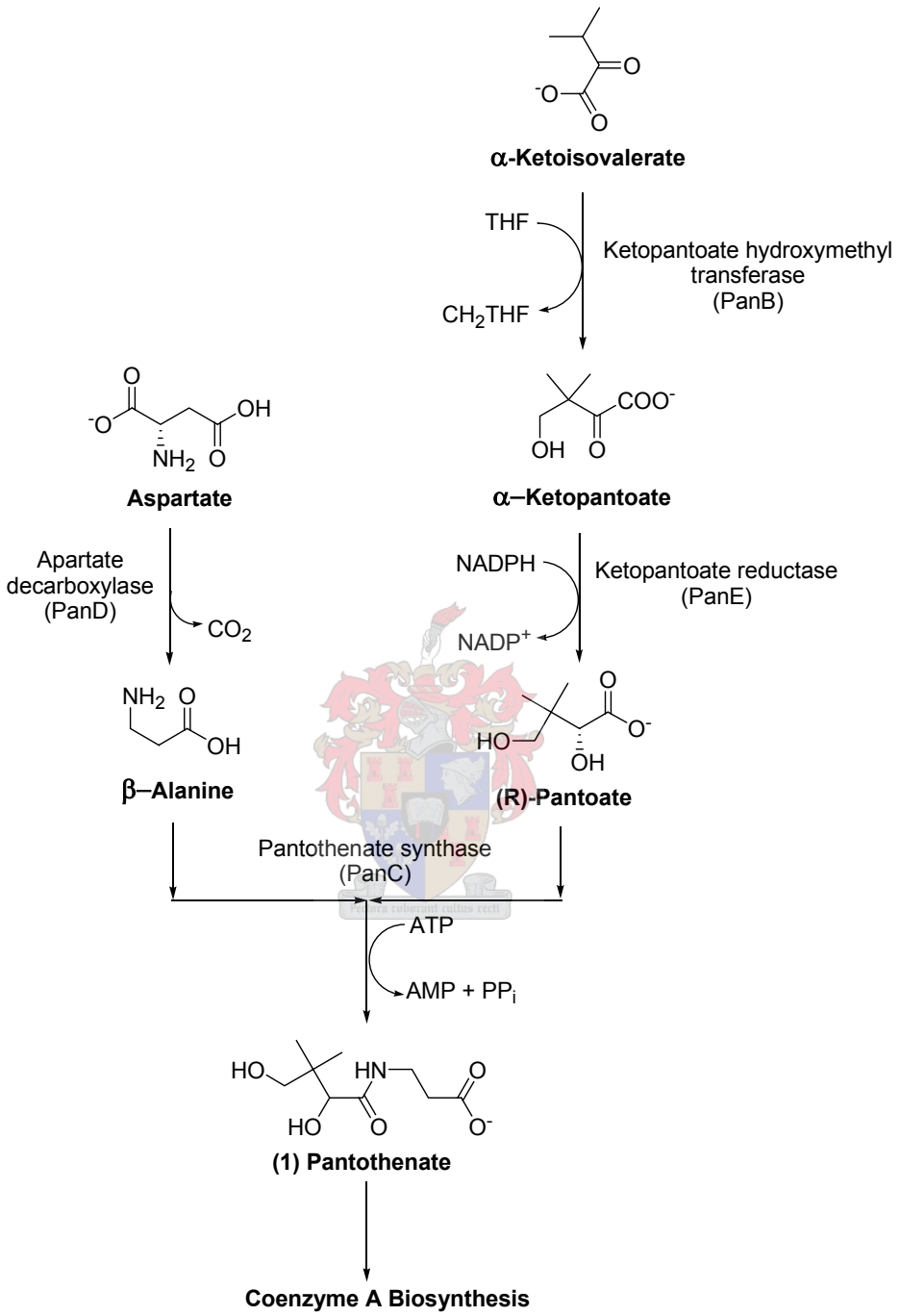


Figure 2.7. The pantothenate biosynthetic pathway in *E. coli*

Pantothenate Biosynthesis

Pantothenate synthetase is the enzyme responsible for the condensation of pantoate with β-alanine. Pantothenate synthetase exists as a homotetramer (*27-29*). It has been established that pantothenate synthase is a product of the *panC* gene (*30, 31*). Since the activity of pantothenate synthase is not tightly regulated, *E. coli* secretes most of the synthesised pantothenate into the medium in vivo (*32-34*). The significance of this overproduction of pantothenate points to a role of intestinal flora in providing this vitamin to the mammalian host (*32*).

2.4.3. Pantothenate and Transport

Plants and microbes synthesise pantothenate de novo as discussed above (*35*). Bacteria are capable of moving pantothenate across membranes bidirectionally. The best-characterized transport system exists in *E. coli*. This is the high-affinity pantothenate permease, which catalyzes the concentrative uptake of pantothenate by a sodium ion cotransport mechanism (*36, 37*). Pantothenate permease is a member of inner membrane permeases that catalyze active cation-dependent symport. A similar transport system is present in *S. typhimurium* (*38*). Pantothenate permease is also called the PanF protein and is encoded by the *panF* gene (*36*).

PanF is predicted to contain 12 transmembrane hydrophobic domains connected to each other by short hydrophilic chains. This is a topological motif that is characteristic of other cation-dependent permeases of the major facilitator superfamily of proteins (*16, 39*). This transport system is extremely specific for pantothenate, with a K_t of 0.4 μM and a maximum velocity of 1.6 pmol/min/10^8 cells (*36, 37*). Overexpression of the PanF protein in *E. coli* produced a 10-fold increase in the amount of pantothenate incorporated into the cell as well as an increase in the steady state intracellular concentration of pantothenate (*16*). However, the levels of coenzyme A biosynthesis remain unaffected. This clearly indicates that CoA biosynthesis is not regulated by the amount of pantothenate available (*16*).

In bacteria not capable of synthesizing pantothenate the pantothenate permease transport system is indispensable (*16*). Pantothenate permease is only responsible

for transporting pantothenate into the cells while another, still uncharacterized, transport system is responsible for expulsion of pantothenate from the cells. This efflux system may play a role in the kinetic control of pantothenate phosphorylation by ensuring that the intracellular concentration of pantothenate remains low (*13, 40*).

2.4.4. Coenzyme A Biosynthesis From Pantothenate.

Unless stated to the contrary, the following section describes the biosynthetic pathway in *E. coli* (Figure 2.8).

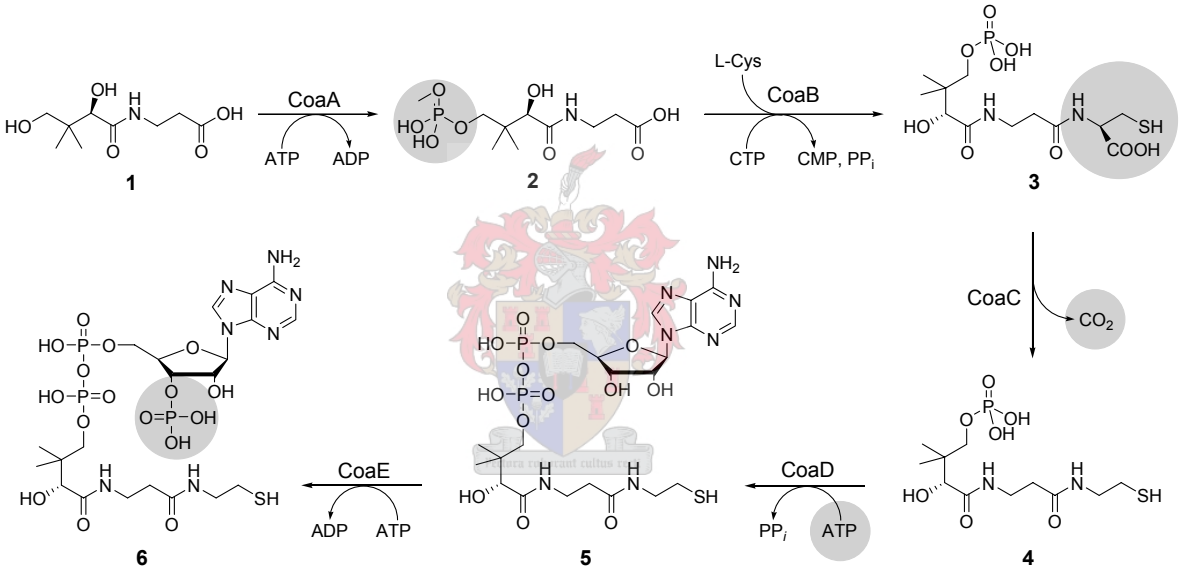


Figure 2.8. Biosynthesis of Coenzyme from pantothenate (1). CoaA, Pantothenate kinase (PanK); CoaB, Phosphopantothenoylcysteine synthetase; CoaC, Phosphopantothenoylcysteine decarboxylase; CoaD, Phosphopantetheine adenylyltransferase; CoaE, Dephospho-Coenzyme A kinase. Numbers indicate the following: **1** pantothenate, **2** 4'-phosphopantothenate, **3** 4'-phosphopantothenoylcysteine, **4** 4'-phosphopantetheine, **5** dephospho-Coenzyme A, **6** Coenzyme A.

2.4.4.1. Phosphorylation of Pantothenate

Pantothenate kinase, also known as PanK or CoaA, catalyses the ATP-dependent phosphorylation of pantothenate to form phosphopantothenate. This is the first committed step in the biosynthesis of coenzyme A, since none of the phosphorylated intermediates formed in the subsequent reactions can enter the cell. Pantothenate kinase is encoded by the *coaA* gene.

Because of CoA's metabolic centrality, the enzyme(s) that regulates its production is of paramount importance. This study concentrates on the pantothenate kinase enzyme, the most probable candidate to fill this role. In particular, we are interested in the apparent absence of a pantothenate kinase analogue in some organisms. Chapter three focuses on PanK and discusses the enzyme in greater detail.

2.4.4.2. Formation of 4'-Phosphopantetheine

Phosphorylation of pantothenate **(1,** numbers correspond to structures in Figure 2.8 above) to form 4'-phosphopantothenate **(2)** is followed by condensation of 4'-phosphopantothenate with cysteine, catalysed by an enzyme known as 4'-phosphopantothenoylcysteine synthetase, (PPCS/CoaB, *coaB* gene product) producing 4'-phosphopantothenoylcysteine **(3)**. Thereafter, decarboxylation of 4'-phosphopantothenoylcysteine by 4'-phosphopantothenoylcysteine decarboxylase (PPCDC/CoaC, *coaC* gene product) yields 4'-phosphopantetheine **(4)** (*2*).

The activities of these two proteins are fused in most prokaryotes such as *E. coli.* The *coaBC* gene (originally *dfp* (*41, 42*)) encodes a flavin mononucleotide (FMN)-containing bifunctional enzyme responsible for both the 4'-phosphopantothenoyl-cysteine synthetase and the 4'-phosphopantothenoylcysteine decarboxylase activities (*4, 43*).

The eukaryotic counterparts of these two enzymes are monofunctional and show very little sequence similarity to the bacterial proteins (*44-46*). Neither 4'-phosphopantethenolycysteine nor 4'-phosphopantothenate is detected in vivo (*32*) owing to the rapid conversion of both to 4'-phosphopantetheine.

2.4.4.3. Conversion of 4'-phosphopantetheine to Coenzyme A

There are two enzymatic steps involved in the conversion of 4'-phosphopantetheine to CoA (*13*). Adenylation of 4'-phosphopantetheine by 4-phosphopantetheine adenyltransferase (CoaD, expressed by the *coaD* gene) and ATP yields dephospho-coenzyme A **(5)**. During this step the enzyme transfers the AMP moiety from ATP to 4'-phosphopantetheine with the release of PP*i* (*13*). Phosphorylation of the 3'-hydroxyl of dephospho-coenzyme A by dephospho-coenzyme A kinase (CoaE, product of the *coaE* gene) completes the biosynthesis of coenzyme A **(6)**.

In mammals these two enzymes are copurified and exist as a bifunctional protein called CoA synthase.

Metabolite labelling experiments[1] have detected intracellular and extracellular 4'-phosphopantetheine. This suggests that 4'-phosphopantotheine adenyltransferase is a secondary control point in the biosynthesis of CoA (*32, 47*). Experiments indicate that extracellular 4'-phosphopantetheine is derived from the degradation of ACP, and reutilization of this intermediate (before it is excreted) is regulated at the adenylyltransferase step (*48*). Excretion of 4'-phosphopantetheine is an irreversible way to reduce the intracellular CoA and ACP content as *E. coli* is incapable of assimilating CoA from exogenous 4'-phosphopantetheine (*48*).

[1] The concept of metabolite labelling refers to stable isotope labelling of an intracellular chemical precursor or metabolite and allows the direct detection of downstream metabolites of that precursor, arising from novel enzymatic activity of interest, using metabolite profiling liquid chromatography-mass spectrometry (LC-MS). This approach allows the discrimination between labelled downstream metabolites produced from novel enzymatic activity from the unlabeled forms of the metabolite arising from native enzyme activity.

2.5. Regulation of Coenzyme A Levels.

Coenzyme A and its thioester derivatives are important cofactors participating in over 100 different reactions in intermediary metabolism of microorganisms. Moreover, they regulate several key metabolic reactions (*49*). This being the case, their production must be tightly controlled to prevent metabolic activity running awry. CoA levels can be regulated in one of five ways:

- The compartmentalization of CoA
- Feedback regulation by pantothenate kinase
- Secondary regulation by 4'-phosphopantetheine adenyltransferase
- Regulation of CoA levels by gene expression
- Regulation by degradation

The next section describes the above means of regulation in little more detail.

2.5.1. Compartmentalization of CoA

Sequestered pools of CoA exist in eukaryotic cells. These are essential for activating carboxylic acid metabolites. Intracellular CoA levels are limited by the membranes surrounding the mitochondria and peroxisomes. In mammals, mitochondrial CoA is used as a cofactor in the Krebs cycle and fatty acid β-oxidation, thus the concentration of free CoA and its thioesters regulate the rates of these processes (*50*). CoA also donates the 4'-phosphopantetheine prosthetic group to activate mitochondrial ACP that is involved in mitochondrial fatty acid synthesis (*51-55*). Peroxisomes are involved in the β-oxidation of very long-chain fatty acids and therefore have high concentrations of CoA (*56, 57*).

2.5.2. Feedback Regulation by Pantothenate Kinase

Pantothenate is not a rate limiting intermediate because bacteria produce 15 times more pantothenate than is required for CoA biosynthesis. In addition to this,

overexpression of pantothenate permease (responsible for transport of pantothenate into the cell) does not increase the production of coenzyme A in the cell (*16*). Pantothenate kinase is the key regulatory point in the control of CoA levels in the cell. It is subject to feedback inhibition by CoA itself and to a lesser extent by CoA thioesters (*58, 59*). This is evidenced by the work done by Song and Jackowski who discovered that a 76-fold overexpression of pantothenate kinase only resulted in a 2.7 fold increase in the cellular concentration of CoA (*60*). This feedback inhibition of PanK by the different CoA molecular species controls the overall CoA availability in response to the cell's metabolic status. In *E. coli*, the CoA pool consists mainly of acetyl-CoA, followed by nonesterified CoA, succinyl-CoA and malonyl-CoA. The total amount of CoA and the variety distributed through the cell is dependent on the carbon source in which the *E. coli* bacteria are cultured (*61*).

The crystal structure of *E. coli* PanK in complex with either ATP or CoA has been determined (*62*). Based on this structural data, Rock *et al.* (*63*) set about designing three site-directed mutants of PanK that were predicted to be resistant to feedback inhibition by CoA based on decreased binding efficiencies of this inhibitor. These mutants CoaA(R106A), CoaA(H177Q), and CoaA(F247V) were shown to retain significant activity and be refractory to inhibition by CoA. CoaA[R106A] retained 50% activity while the other two mutants were less active. The presence of Arg 106 is postulated to be an important and specific requirement for CoA binding since it forms a salt bridge with the phosphate attached to the 3'-hydroxyl of the CoA ribose. The authors state that this residue does not have a role in catalysis. The authors show that the mutants that are refractory to feedback inhibition accumulate intracellular phosphorylated pantothenate-derived metabolites, thus translating into a higher CoA content (*63*). This data confirms that the feedback inhibition is operating *in vivo* to limit the amount of CoA being produced.

The feedback inhibition of the PanK enzyme by CoA is competitive with ATP binding at the active site (*58, 61, 62*). Thus CoA biosynthetic activity can be modulated depending on the energy state of the cell. This means that a reduction in the ATP level would allow for more binding of the feedback inhibitor and a

reduction in the rate of CoA biosynthesis (*1*). In this way, the levels of CoA are collectively controlled by the amount of the predominant CoA species and the ATP levels in the cell (*61*).

The eukaryotic PanK enzymes are also feedback regulated by CoA and CoA thioesters. Acetyl-CoA and palmitoyl-CoA selectively and strongly inhibit the *Aspergillus nidulans* PanK in a competitive way with ATP (*64*). Human PanK2 protein is very sensitive to long-chain acyl-CoA, acetyl-CoA and malonyl-CoA, whereas nonesterified CoA is a much less effective inhibitor (*65*).

Pantothenate kinase is the subject of chapter 3, where the different analogues of PanK are discussed and compared in detail.

2.5.3. Secondary Regulation by CoaD

Experiments using metabolite labelling have detected intracellular and extracellular 4'-phosphopantetheine, suggesting that 4'-phosphopantotheine adenyltransferase (CoaD) is a secondary control point in the biosynthesis of CoA (*32, 47, 66*). While the primary means of regulation is through PanK, regulation by CoaD becomes more important when the regulation at the PanK site is disrupted (*63*) or when the PanK protein is overexpressed (*60*). In both instances the amount of intracellular and extracellular 4'-phosphopantetheine increases and this reflects restriction of the flux through the coenzyme A biosynthetic pathway by CoaD. 4'-phosphopantetheine cannot be transported back into the cells (*66*). It is predicted that the CoaD protein is feedback regulated by free CoA, much like PanK (*32, 47, 63, 67*). This hypothesis is based on the time- and concentration-dependent correlation between accumulation of intracellular CoA and the exit of 4'-phosphopantetheine from the cells (*32, 47*). This is supported by biochemical studies of the CoaD protein. When CoaD is purified from *E. coli*, CoA remains bound to the CoaD protein in a ratio of 1 mole per 2 moles of protein (*68*). In addition to this, the crystal structure of CoaD bound to CoA indicates that the CoA binds to the 4'-phosphopantetheine site (*67*). *S. aureus* does not show the same inhibition by CoA of the homologous CoaD protein (*1, 69*). In mammalian cells the pool of 4'-phosphopantetheine is almost as high as that of pantothenate and when

PanK1 is overexpressed, the 4'-phosphopantetheine pool increases almost 3-fold (*1*).

2.5.4. Regulation of CoA Levels by Gene Expression

CoA biosynthesis is regulated enzymatically by negative feedback of PanK. However, the upper threshold of the intracellular CoA concentration is set by the levels of *coaA* gene expression in *E. coli* and most bacteria. The *coaA* promoter has poor sequence homology with the consensus *E. coli* promoter sequences. In addition to this, the *coaA* coding region contains a relatively large percentage of low usage codons (*60*). This means that in relation to the average *E. coli* protein, PanK protein levels are low. When the *coaA* is amplified in a multi-copy plasmid in *E. coli* a 76-fold higher enzyme activity and a 3-fold increase in the steady state levels of CoA is the result (*60*). However, feedback inhibition of the PanK enzyme prevents the concentration of CoA rising unchecked. No evidence has been found for the regulation of the *coaA* gene on a transcriptional level and it seems that bacteria control the CoA levels sufficiently on a biochemical level (*1*).

In contrast, mammalian cells and tissues modulate PanK expression to modify CoA levels in long-term response to diet and disease. The mechanism by which this occurs is, however, still unknown (*1*).

2.5.5. Regulation by Degradation

CoA levels can also be regulated by degradation of the coenzyme. CoA can be dephosphorylated to dephospho-CoA or hydrolyzed by cleavage of the phosphodiester bond yielding 4'-phosphopantetheine. As an alternative to direct CoA degradation, the 4'-phosphopantetheine moiety of CoA can be transferred to carrier proteins like the acyl carrier protein (ACP) of bacteria or the fatty acid synthase (FAS) in eukaryotes. This 4'-phosphopantetheine is the prosthetic group that activates these proteins and allows them to form thioester linkages with carboxylic acids (*1*).

2.6. Coenzyme A as a Drug Target

Research involving CoA has always been popular due to its biochemical centrality; however, a discovery in 1999 heightened this interest. The cloning of the first eukaryotic pantothenate kinase from fungus revealed a sequence completely different to that of *E. coli* PanK (*64*). However, this protein from *Aspergillus nidulans* is homologous to several proteins encoded by mammalian genes (*70-73*). This discovery (that the bacterial PanK was different to mammalian PanK) led to the prediction that the pantothenate kinase step was a prime target for the identification and design of novel antibacterial drugs.

The development of new antibiotics designed as inhibitors of CoA utilizing enzymes (such as CoA antivitamins or antimetabolites) would be very beneficial. In this context, antivitamins and antimetabolites are analogues of pantothenate that will be incorporated into the CoA biosynthetic pathway producing CoA analogues that are inactive. Even though many CoA analogues have been designed as CoA utilizing inhibitors *in vitro* they have not been useful as antibiotics because bacteria are unable to transport CoA across the cell membrane.

The significance of extensive research focussing on CoA cannot be overstated. This subject is extended in the following chapter highlighting the relevance of pantothenate kinase's role in the regulation of CoA.

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Chapter 3

Pantothenate Kinase, an Overview

3.1. Introduction

From chapter 1, it is clear that coenzyme A plays a central role in metabolism. As a result of this, enzymes involved in this cofactors' biosynthesis are also important as research candidates. We are interested in one enzyme in particular, pantothenate kinase (ATP:D-pantothenate 4'-phosphotransferase). Pantothenate kinase catalyses the first committed step in the Coenzyme A biosynthetic pathway. It catalyzes the transfer of the terminal phosphate from ATP to pantothenate, forming phosphopantothenate.

This chapter focuses on coenzyme A biosynthesis and concentrates on pantothenate kinase in particular, giving an overview of the different analogues[2] of this enzyme and how they differ from one another in terms of inhibition, sequence and structure.

Currently two analogues of the enzyme have been characterized: the first (Type I) is found predominantly in prokaryotic organisms and is exemplified by the *Escherichia coli* PanK enzyme (*2*). The second (Type II) occurs mainly in eukaryotic systems, of which the murine enzyme (MmPanK1β) has been the best characterized (*3*).

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[2] In this study we refer to *analogues* as genes or proteins that display the same activity but lack sufficient similarity to imply common origin. The implication is that analogous proteins followed evolutionary pathways from different origins to converge upon the same activity. Thus, analogous genes or proteins are considered a product of convergent evolution (*1*). In contrast, a protein *isoform* is a version of the same protein with some small differences, usually a splice variant or the product of some posttranslational modification, and normally refers to the same protein occurring in different locations in the cell Due to these differences, we shall refer to the different PanK proteins in this study as analogues.

Recent studies have indicated that this classification is not unambiguous, as the *Staphylococcus aureus* PanK has a primary structure that is more closely related to Type II pantothenate kinases than to the Type I PanK's. In addition to this, it is not regulated by feedback inhibition by CoA or its thioesters (*4*).

The Type I and II analogues show very little sequence homology and are predicted to be structurally distinct. Despite this, they share a common regulation mechanism based on feedback inhibition by CoA and its thioesters, although the degree of inhibition is system- and inhibitor-dependent (*3, 5-10*). This feedback mechanism is primarily responsible for controlling the intracellular CoA concentration (*5, 7, 10*).

Because of the lack of sequence homology between the two analogues, it has been predicted that they adopt different three-dimensional structures. This chapter serves as an overview of the currently available information pertaining to the pantothenate kinase enzymes classified thus far and intimates the likelihood of two additional, unclassified analogues of this enzyme.

3.2. Type I Pantothenate Kinase

The pantothenate kinase enzyme was first identified in *S. typhimurium* (*11*) and *E. coli* (*12*) and thereafter in numerous other bacteria by comparative genomics (*13, 14*). The PanK protein from *E. coli* (*13*) has been expressed and purified. This prokaryotic pantothenate kinase enzyme is what we refer to as the Type I pantothenate kinase. In this study, the pantothenate kinase enzyme from *Escherichia coli* will represent the Type I PanK and when Type I is mentioned, the *E. coli* analogue is implied.

3.2.1. E. coli Pantothenate Kinase, the Prototypical Bacterial PanK

The PanK from *E. coli* is structurally distinct from its eukaryotic counterparts (*8, 13, 14*). The *E. coli* enzyme has been extensively studied and characterized, in fact, it is considered the prototypical bacterial PanK. *E. coli* is capable of de novo pantothenate biosynthesis, but can also actively transport exogenous pantothenate into the cell through a sodium-dependent permease (PanF) (*15-17*). Metabolic

labelling experiments have established that it is the utilization of pantothenate rather than the supply of pantothenate that controls the rate of CoA biosynthesis (*18*). Temperature sensitive *E. coli* PanK mutants have a growth phenotype that causes a temperature-dependent inactivation of pantothenate kinase activity at temperatures higher than 42˚C (*12*). The pantothenate kinase gene of *E. coli* (*coaA*) was cloned by functional complementation of the temperature sensitive mutant and (*12*) and was found to be identical to a previously sequenced allele called *rts* (*19, 20*). The *E. coli coaA* transcript has two translation initiation sites and the PanK protein was first identified as a mixture of two peptides. The shorter protein lacks the first eight N-terminal amino acid residues (*20*).

There is a single nucleotide-binding site on each monomer. The binding of ATP to the homodimer is highly cooperative and mediates sequential ordered catalysis with ATP as the leading substrate (*9*). CoA and its thioesters inhibit bacterial PanK activity by binding competitively to the ATP binding site (*9, 10*). Nonesterified CoA is the most potent inhibitor of bacterial PanK in vitro and in vivo, whereas acetyl-CoA is about 20% as effective as CoA (*10*). It is postulated that the additional acyl chain in acetyl-CoA (steric hindrance) is the reason that it is a less potent inhibitor than CoA, which fits snugly into the pantothenate binding pocket (*10*). The crystal structure of PanK in complex with ATP or CoA has been determined (Figure 3.1) (*6*). These structures revealed that ATP and CoA bind to the enzyme in different ways. Despite this their phosphate binding sites overlap at Lys 101. This explains the competition between the CoA regulator and the ATP substrate (*21*). This negative feedback mechanism of inhibition is the primary manner in which bacteria control the cellular level of CoA.

A survey conducted by Cheek *et al* (*22*) has characterized all known kinases into specific groups and families based on their three dimensional structure. Based on the crystal structure of the Type I PanK the authors place this analogue into the Rossmann-like fold group (group 2). Within this group they are classified into the P-loop kinase family. The P-loop family constitutes the largest family in the Rossmann-like fold group (*22*).

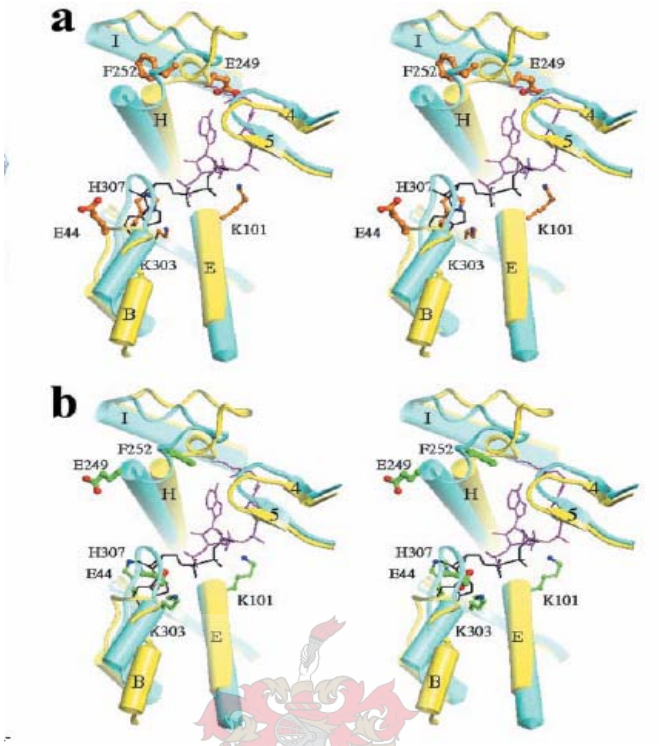

Figure 3.1. Close-up stereoview of the overlapping site of two ligands, AMPPNP and CoA. The binding pockets of AMPPNP and CoA are shown in *yellow* and *cyan*, respectively. CoA (*magenta*) and AMPPNP (*black*) are shown in *lines*. ***a***, residues of the AMPPNP-bound enzyme are shown in *brown*, and residues of the CoA-bound enzyme are not shown for clarity. Note that the carboxyl group of Glu249 of the AMPPNP-bound enzyme coincides with the pantetheine moiety of CoA. ***b***, residues of the CoA-bound enzyme are shown in *green*, and residues of the AMPPNP-bound enzyme are not shown for clarity. Note that the carboxyl group of Glu44 of the CoA-bound enzyme coincides with the adenine base of AMPPNP (*6*). Figure reproduced with permission from Yun *et al.* (2000). *J. Biol. Chem.* ***275,*** 28093-28099.

Although the *E. coli* PanK is considered the model bacterial pantothenate kinase, this analogue is not universally expressed in bacteria (*23*). For example, the eubacteria *Pseudomonas aeruginosa* and *Helicobacter pylori* do not have recognizable pantothenate kinases in their genomes, although all other components of the biosynthetic pathway are present.

3.3. Type II Pantothenate Kinase

Mammalian pantothenate kinase belongs to the group of Type II pantothenate kinases. Of the mammalian pantothenate kinases, perhaps the best characterized is the murine pantothenate kinase. The murine pantothenate kinase (*PanK1)* gene consists of seven introns and eight extrons and is located on chromosome 19. Two biochemically distinct PanK1 isoforms, namely, PanK1α and PanK1β, are encoded by this *PanK1* gene. The two isoforms differ with regards to their regulatory properties. The PanK1α isoform is inhibited by free and long-chain acyl-CoA and more distinctly inhibited by acetyl-CoA and malanoyl-CoA in comparison to the PanK1β isoform. This infers distinct regulatory properties by the α and β isoforms, in fact, the PanK1β isoform is only weakly inhibited by acetyl-CoA and stimulated by CoA. The isoforms are differentially expressed in mouse tissue as a result of the metabolic status of the tissue (*3, 7)*. Cells modulate PanK expression to modify tissue CoA levels in response to diet and disease. The mechanism in which they do this is largely unknown. However, it is clear that the metabolic state of the animal (*24-28*), pathological condition such as diabetes (*29, 30*), and hypolipidemic drugs (*27, 31, 32*) all significantly effect the cellular levels of coenzyme A.

The *Aspergillus nidulans* PanK, also characterized as a Type II PanK, is sensitive to inhibition by acetyl-CoA but not effected, positively or negatively, by CoA. This indicates that the properties of the fungal enzyme are intermediate between the mPanK1α and mPanK1β. It is clear that the characteristics of the eukaryotic (Type II) pantothenate kinases are in sharp contrast to the prokaryotic (Type I) PanK in terms of inhibition, with Type I PanKs being inhibited predominantly by CoA and to a lesser extent acetyl-CoA, and the Type II PanK's being inhibited more potently by acetyl-CoA (*9*).

Unlike the Type I pantothenate kinases, which belong to the P-loop family of kinases, the Type II PanK's are predicted to belong to the ribonuclease H-like kinase group. The ribonuclease H-like group contains the ASKHA (acetate and sugar kinase/hsc70/actin) superfamily (*33*).

3.3.1. Pantothenate from Staphylococcus aureus, an Atypical Type II PanK

Even though *S. aureus* is a bacterium, its PanK primary sequence is distinctly different from that of *E. coli*. In fact, it is more closely related to mammalian pantothenate kinases than to those of its fellow bacteria (*4*). *S. aureus'* PanK primary sequence (*8, 14, 34*) shares 18% identity with the murine PanK isoform 1β (MmPanK1β) and only 13 % identity with *E. coli* PanK.

The native molecular mass of *S aureus* PanK is 59 kDa, which is consistent with the existence of a homodimer (*4*). All organisms characterized to date share a common mechanism to regulate CoA biosynthesis, feedback inhibition by CoA and/or its thioesters. The key difference between *S. aureus* PanK and other pantothenate kinases is that it is refractory to feedback inhibition by CoA and its thioesters (*3, 7, 8, 10*). *S. aureus* produces CoA in proportion to the input of β-alanine with no indication of regulation at PanK or in downstream reactions. This observation may be understood in the light of the physiology of *S. aureus.* Prokaryotes and many eukaryotes use glutathione as the major low-molecular weight thiol, which, together with an NADPH-dependent glutathione reductase, constitutes the primary thiol/disulphide redox system (*35, 36*). This system is essential for maintaining the intracellular reducing environment and protects the organism from oxidative assaults by functioning in the detoxification of peroxides, epoxides and other products of reactive oxygen (*4*). *S. aureus* does not contain glutathione (*37*). In *S. aureus*, CoA is the primary intracellular thiol and together with a unique CoA disulphide reductase functions as the reducing system (*38, 39*). Because of this the concentration of CoA in *S. aureus* can reach millimolar levels (*38, 39*), and the lack of CoA feedback regulation in this organism means that the upper limit of CoA is set by the availability of the starting materials of CoA biosynthesis, namely pantothenate and cysteine. As a result CoA levels are limited by the supply of pantothenate produced by the biosynthetic pathway encoded for by the *panBCDE* genes (*40*).

3.4. Type III Pantothenate Kinases

As discussed, at least two analogues of PanK exist in nature. These analogues show very little sequence homology and are predicted to be structurally distinct. However, sequence analysis of bacterial genomes, using these two analogues as templates, fails to locate a homologue of either analogue in some species. Since PanK activity is required for the biosynthesis of CoA in all living systems such a gene must exist in these species. The group of species lacking analogues of the currently identified pantothenate kinases can be separated into two subgroups: those belonging to the family of archeabacteria, and those that include well-known pathogenic eubacteria such as *Bordetella* spp., *Helicobacter pylori* and *Pseudomonas* spp. Since these organisms possess sequences that are homologous to all the other CoA biosynthetic genes, it is highly likely that at least one other analogue of PanK exists that represents this activity in these bacteria. This analogue would have little or no sequence homology to the well-characterised analogues (*14*).

A recent patent application has claimed to have identified a gene sequence which, when cloned in *trans*, can suppress the effects of an *E. coli* PanK temperature sensitive mutant (*41*). This sequence was identified from the genome of *Bacillus subtilis.* This organism does already possess a gene homologous to the Type I pantothenate kinase. In support of the suggested duplication of PanK activity, interruption of the putative *B. subtilis coaA* gene gave a normal-growing phenotype. However, experiments aimed at the additional deletion of the identified gene sequence failed. This indicates that the simultaneous deletion of both genes is lethal to *B. subtilis*. This strongly supports that the identified gene, dubbed *coaX*, encodes a protein that exhibits pantothenate kinase activity. Homology searches based on the *coaX* gene sequence identified orthologues in several bacterial genomes, including those eubacteria that do not possess another PanK analogue. Included among these are various pathogenic bacteria like *Bordetella pertussis* (the causative agent for whooping cough) and the category A biodefence pathogen, *Francisella tularensis.* Some organisms, such as *B. subtilis*, contain a gene sequence homologous to both *coaA* (Prokaryotic, Type I) and *coaX.* This indicates

that the putative duplication of PanK activity is not unique (*42*). The results of these searches are summarized in Table 3.1.

Interestingly, the *coaX* homologue in *B. pertussis* has been studied before and was found to be an essential gene in this organism (*43, 44*). However, these studies concluded that the gene product was involved in pertussis toxin production via interaction with the two-component transcriptional regulator BvgAS.

Bordetella pertussis is a coccobacillus that causes the upper respiratory tract disease pertussis (whooping cough) in humans (*44*). *B. pertussis* produces multiple attachment factors and toxins that together produce the symptoms of the disease. The attachment factor and toxin production are innitiated by the products of the *bvgAS* locus, namely BvgA and BvgS. BvgS is a transmembrane sensor protein that in response to the absence of environmental stimuli ($MgSO_4$, nicotinic acid, or growth at low temperatures) activates BvgA. BvgA is a cytoplasmic protein which, when activated by BvgS, binds upstream to virulence genes and positively influences their transcription. BvgS activates BvgA by phosphorylation. Two of these virulence genes are termed *ptx* and *cya*, and they require an accessory factor in order for BvgA to activate their transcription. This accessory protein was cloned and expressed and termed Bvg accessory protein (Baf) (*44*).

Baf does not have any significant sequence homology to any of the known bacterial transcriptional regulators. What is unusual is that it shows homology (28% identity and 49% similarity) to CoaX from *B. subtilis*. The results presented by DeShazer *et al.* suggest that a Baf mutation may be lethal (*43, 44*). Thus, Baf may regulate the expression of some essential *B. pertussis* genes, or it may be essential itself.

Bordetella falls into the group of eubacteria that do not have a gene sequence homologous to either the known PanK analogues. The Baf protein, however, is homologous to CoaX, the protein with putative PanK activity. Although the transactivation experiments that lead to its annotation are completely unrelated to CoA biosynthesis, the same study did aim to create a Baf deletion mutant in *Bordetella,* but failed (*43*). This indicates that Baf is essential in *B. pertussis*,

supporting its suggested role in CoA biosynthesis rather than implicating its involvement in a non-vital cellular process.

Table 3.1. **Sequence homology analysis of bacterial genomes to confirm the presence of putative CoA biosynthetic enzymes**

			"UNIVERSAL PATHWAY, 5 steps					
	Genome	taxon	2.7.1.33 CoaA (PanK)	6.3.2.5 CoaB	4.1.1.36 CoaC	2.7.7.3 CoaD	2.7.1.24 CoaE	CoaX-like PanK
1	Methanobacterium thermoautotrophicum	A		+	+	+		
2	Methanococcus jannaschii	A		+	+	+		
3	Methanopyrus kandleri AV19	A		+	+	+		
4	Thermoplasma acidophilum	A		+	+	+		
5	Agrobacterium tumefaciens C58	B	+	+	+	+	+	
6	Bacillus anthracis A2012	B	+	+	+	+	+	+
7	Bacillus cereus ATCC 14579	B	+	+	+	+	+	+
8	Bacillus subtilis 168	B	+	+	+	+	+	+
9	Bacillus thuringiensis israelensis ATCC-35646	B	+	+	+	+	+	+
10	Corynebacterium glutamicum ATCC-13032	B	+	+	+	+	+	
11	Enterococcus faecalis	B	+	+	+	+	+	
12	Escherichia coli K12 MG1655	B	+	+	+	+	+	
13	Haemophilus influenzae KW20	B	+	+	+	+	+	
14	Lactococcus lactis str. IL 1403	B	+	+	+	+	+	
15	Leuconostoc mesenteroides ATCC-8293	B	+	+	+	+	+	
16	Mycobacterium tuberculosis H37Rv	B	+	+	+	+	+	+
17	Rhodobacter capsulatus	B	+	+	+	+	+	+
18	Salmonella typhimurium LT2	B	+	+	+	+	+	
19	Staphylococcus aureus ATCC-29213	B	+	+	+	+	+	
20	Staphylococcus epidermidis ATCC 12228	B	+	+	+	+	+	
21	Streptococcus pneumoniae 23F	B	+		+	+	+	
22	Streptomyces coelicolor A3(2)	B	+		+	+	+	+
23	Vibrio cholerae El Tor N16961	B	+	+	+	+	+	
24	Yersinia enterocolitica 8081	B	+	+	+	+	+	
25	Yersinia pestis CO92	B	+	+	+	+	+	
26	Acinetobacter calcoaceticus ADP1	B		+	+	+	+	+
27	Azotobacter vinelandii AvOP	B		+	+	+	+	
28	Bacillus stearothermophilus 10	B		+	+	+	+	+
29	Bordetella pertussis	B		+	+	+	+	+
30	Borrelia burgdorferi B31	B		+	+	+	+	
31	Burkholderia cepacia J2315	B		+	+	+	+	
32	Campylobacter jejuni NCTC-11168	B		+	+	+	+	+
33	Caulobacter crescentus CB15	B		+	+	+	+	+
34	Clostridium botulinum ATCC-3502	B		+	+	+	+	+
35	Cytophaga hutchinsonii ATCC-33406	B		+	+	+	+	
36	Deinococcus radiodurans R1	B		+	+	+	+	+
37	Haemophilus influenzae 86028NP	B		+	+	+	+	
38	Helicobacter pylori 26695	B		+	+	+	+	+
39	Neisseria gonorrhoeae FA 1090	B		+	+	+	+	+
40	Neisseria meningitidis ser. A strain Z2491	B		+	+	+	+	+
41	Porphyromonas gingivalis	B		+	+	+	+	+
42	Pseudomonas aeruginosa PAO1	B		+	+	+	+	+
43	Pseudomonas fluorescens Pf0-1	B		+	+	+		+
44	Pseudomonas putida KT2440	B		+	+	+	+	
45	Ralstonia eutropha CH34 (JGI)	B		+	+	+	+	+
46	Synechocystis sp. PCC6803	B		+	+	+	+	
47	Thermotoga maritima MSB8	B		+	+	+	+	+
48	Xanthomonas campestris ATCC-33913	B		+	+	+	+	

Note: Taxon refers to archaea (A) or eubacteria (B)

3.5. Type IV PanK in Archaeabacteria

Ulrich Genschel has recently conducted a study (*13*) where he attempted to systematically elucidate the evolutionary relationships of the CoA biosynthetic genes among bacteria, archaea, and eukaryotes. He discovered that based on the phylogenetic distribution and the evolutionary histories of the CoA biosynthetic genes, two phases can be distinguished in the evolution of the pathway: CoA biosynthesis from phosphopantothenate was complete in the universal ancestor, while pathways for the synthesis of phosphopantothenate arose only after the separation of bacteria and archaea (*13*). Genschel found that the conserved genes encoding for PanK (Type I and Type II) are generally lacking in archaea. He investigated the possibility that the archaeal genomes may contain a more distantly related homologue to these enzymes by using iterative Psi-Blast searches against the archaeal subset of the NCBI peptide sequence database.

The presence of conserved CoA biosynthetic genes indicates that several non-methanogenic archaea produce pantoate in the same way as bacteria and that all archaea convert 4'-phosphopantothenate into CoA by using CoaB, CoaC, CoaD and CoaE activities. Thus, at least one subset of archaeal species should be able to convert pantoate into phosphopantothenate. However, no candidate genes were found by homology searches to PanC or PanK that could perform these enzyme functions (*13*). This simplest explanation for this finding is that unrelated genes encode for PanC and PanK activities in archaea. This possibility was explored by inferring functional links to archaeal genes for PanB, PanE, and CoaB/CoaC using the functional proximity method (refer Genschel 2004 (*47)*). The results gained by these experiments indicate that COG1701 (uncharacterized protein that is conserved in archaea) and COG1289 (a predicted archaeal kinase) are very likely to represent archaeal forms of PanC and PanK, respectively. The author states that this confirms that the set of enzymes for the synthesis of phosphopantothenate in methanogens is unrelated to the corresponding bacterial enzymes. *Thermoplasma* lack bacterial or archaeal enzymes for the synthesis of pantothenate and may be dependent on exogenous pantothenate. All the archaea considered in Genschel's study contained bifunctional CoaB/CoaC, CoaD, and

CoaE enzymes for the conversion of phosphopantothenate to CoA. This means that in several non-methanogenic archaea, the topology of the synthesis of CoA from α-ketoisovalerate is conserved with that in bacteria and eukaryotes.

To summarize, from comparative genomic analysis of the genes coding for PanK it is clear that no homologous gene coding for this enzyme has been discovered in the archaeabacteria (*13*). The archaeabacteria have all the other genes that code for the remaining four steps in the universal biosynthesis of CoA (*13, 23*). The PanK analogue in archaeabacteria is thus distinct from the Type I and II PanK analogues classified thus far. In addition, it is distinct from the Type III PanK present in the predominantly pathogenic bacteria (Table 3.1). This points to the existence of yet another analogue of pantothenate kinase. This, together with the fact that CoA is essential indicates that there is a Type IV analogue in the archaeabacteria that has not yet been identified and characterized (*13*).

3.6. Pantothenate Antimetabolites

In the context of the heading above, an antimetabolite is an alternative substrate for PanK that is phosphorylated and incorporated into the CoA biosynthetic pathway resulting in an analogue of coenzyme A. The resulting CoA analogue may then act as an inhibitor of CoA and acetyl-CoA utilizing enzymes (*45*).

The pantothenamides are such antimetabolites and are competitive with respect to pantothenate. *N*-Pentylpantothenamide (*N*5-Pan) and *N*-heptylpantothenamide (N7-Pan) are effective inhibitors of bacterial cell growth (*21*). The PanK-ADP-pantothenate ternary complex structure in *E. coli* can explain how the bulky pantothenamide antimetabolites (Figure 3.2) are capable of being phosphorylated by pantothenate kinase.

Their inhibitory action is thought to be due to pantothenate kinase converting them to their phosphorylated intermediates that are then incorporated into the CoA biosynthetic pathway. The end result is the biosynthesis of CoA analogues that are inactive. *N*5-Pan is metabolised through the CoA biosynthetic pathway to the cofactor analog ethyldethia-CoA (*45*).

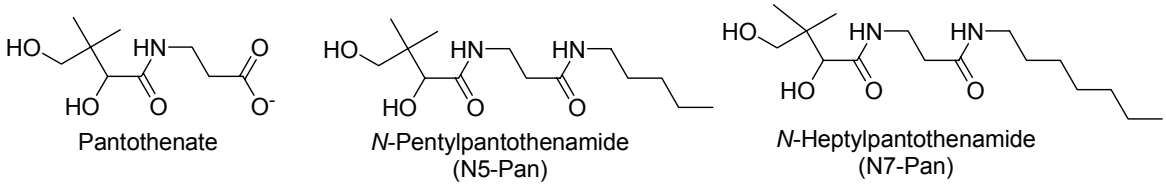

Figure 3.2. Structure of pantothenate compared to the pantothenamide antimetabolites. Pantothenate is the substrate for the reaction catalyzed by PanK, however, PanK phosphorylates both *N*5-Pan and *N*7-Pan with similar efficiency.

The incorporation of these analogs into ACP, where they block fatty acid synthesis by the accumulation of inactive ACP, has been demonstrated. This is the main cause for growth inhibition of *E. coli* when these pantothenamides are used as substrates (*46*). This, coupled with the fact that the affinity of PanK for these antimetabolites is comparable to its affinity for pantothenate, is unusual because enzymes that utilise small molecules are usually highly selective for their substrates (*21*). The flexibility of the pantothenate kinase binding site may account for its ability to phosphorylate the pantothenamides (*21*).

Based on the interaction of ligands with the pantothenate-binding pocket in *E. coli*, the pantothenate-binding site is also part of the binding site for CoA (Figure 3.3). Despite the structural differences between these two molecules the binding of CoA induces a similar conformational change in the enzyme (*21*). The pantothenate moiety of bound CoA has the same location and orientation as the bound pantothenate substrate. The β-mercaptoethylamine moiety of CoA extends into the hydrophobic dome over the pantothenate-binding pocket. Therefore, pantothenamide binding is predicted to induce a similar conformational change in the pantothenate-binding site. A modelling solution of *N*5-Pan with the PanK-ADP-pantothenate structure (with the pantothenate removed) supports the prediction that the hydroxyl of the pantothenamide is correctly orientated for the phosphorylation by binding in the same conformation as the pantothenate substrate. The hydrophobic tail of the antimetabolite interacts with the hydrophobic domain, which binds the β-mercaptoethylamine moiety of CoA (*6*). This is an exciting development as the model predicts that the design of future pantothenamide derivatives with hydrophobic groups that interact more strongly with the aromatic

residues of the hydrophobic roof over the pantothenate binding pocket will increase the affinity and specificity of the inhibitors/substrates (*6*).

Like in *E. coli*, *N*5-Pan and *N*7-Pan are also effective antimicrobial agents against *S. aureus* but have a different mechanism of action (*4*). The differences in the regulation of CoA in *S. aureus* and *E. coli* account for the difference in responses of these organisms to pantothenamides in the presence of pantothenate. It has been shown (*4*) that the simultaneous addition of *N*5-Pan or *N*7-Pan and 50 μM pantothenate to the growth medium resulted in an upward shift of the minimal inhibitory concentrations (MICs) (8-fold for *N*5-Pan and 64-fold for *N*7-Pan). This is an indication that the pantothenamides compete with pantothenate as a substrate in *S. aureus.* When the same experiment was conducted in *E. coli* the addition of pantothenate only resulted in a 2-fold shift in the MIC of *N*5-Pan against *E. coli* (*46*). This suggests that the lack of feedback inhibition in *S. aureus* allows the accumulation of larger amounts of CoA and thus allows the organism to overcome the inhibitory effect of the pantothenamides more effectively (*4*).

There are multiple targets for the pantothenamides in *S. aureus* and there are several important differences between this Gram-positive bacterium compared with the model *E. coli* system (*46*). *N*7-Pan (MIC = 0.16 μM) is considerably more potent that *N*5-Pan (MIC = 25 μM) in *S. aureus (4)*. However, the efficacy of the two compounds is reversed in *E. coli* because of the export of *N*7-Pan from the cell via a TolC-dependent pump (*46*). This means that the differences in the cell wall and the outer membrane define the selectivity of pantothenamides in Gram-positive and Gram-negative bacteria (*4*). In both systems the pantothenamides are phosphorylated by PanK and incorporated into CoA and ACP analogs (*4*). The accumulation of *N*7/*N*5-ACP results in inactivation of ACP and the inhibition of fatty acid synthesis. Fatty acid synthesis is the most critical pathway blocked in both cases as shown by the ability of exogenous fatty acids to ameliorate the toxic effects of the pantothenamides (*46*). However, Zhang *et al.* suggest the possibility of other targets of pantothenamides in *S. aureus* (*4*). These researchers made another new discovery in that *N*7-Pan is also incorporated into *Dcp* (*4*). *Dcp* is required for the biosynthesis of D-alanyl-lipoteichoic acid, a macroamphiphile

component of the Gram-positive cell wall-membrane complex (*47*). Deactivation of *Dcp* would block D-alanine incorporation into the cell wall and this is critical for the function of the cell wall in pathogenic bacteria (*36, 47, 48*). The inhibition of D-alanine in *S. aureus* increases the sensitivity of this pathogen to charged antimicrobial peptides such as defensins as well as vancomycin (*49, 50*).

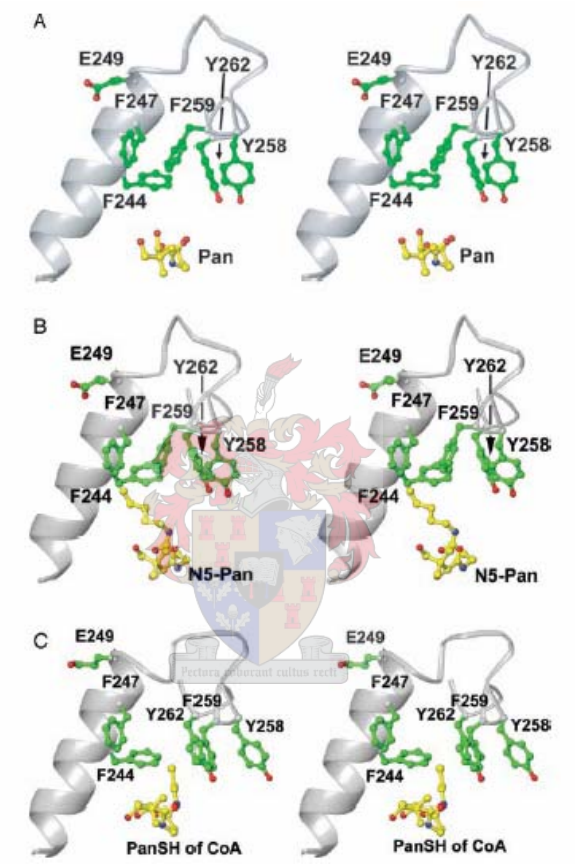

Figure 3.3. Interaction of ligands with the pantothenate-binding pocket. *A*, bound pantothenate (*yellow*) does not interact with the aromatic pocket formed by the side chains of residues Phe-244, Phe-247, Tyr-258, Phe-259, and Tyr-262 (all *green*) at the "top" of the pantothenate binding groove. *B*, N5-Pan (*yellow*) is shown docked into the pantothenate binding groove, and its binding is predicted to cause minimal perturbation of the aromatic pocket relative to pantothenate binding. *C*, bound CoA (*yellow*) interacts with several aromatic residues that rearrange to accommodate the relatively large bulk of the CoA molecule. For clarity, only the pantetheine moiety (*PanSH*) of CoA is shown (*21*). Reproduced with permission from Ivey *et al*. (2004) *J. Biol. Chem.,* ***279*****,** 35622-35629.

Virga *et al.* (*51*), have recently conducted a study where they have synthesized a number of new antimetabolites based on the structures of the alkylpantothenamides and screened them as inhibitors against the pantothenate kinase enzymes of *E. coli, A. nidulans, S. aureus* and the murine analogue *MmPanK1*α. In this paper the authors report that the pantothenamide analogues act as inhibitors of the pantothenate kinase reaction (*51*). It is our belief that instead of inhibitors, they act as alternative substrates and that is why there is a decrease in the observed amount of phosphopantothenate formed. We believe that this is also why the authors observe inhibition under assay conditions but not in live cells.

3.7. Significance of the Discovery of a Third PanK Analogue

The discovery of a third analogue of pantothenate kinase would be a significant contribution to our understanding of how identical enzyme activities arise through convergent evolution. Additionally, since genomic analysis studies suggest that the CoaX homologues account for the only PanK activity found in the pathogenic bacteria listed in Table 3.1, the immediate implications for the development of specific and directed antibacterial agents are evident.

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Chapter 4

Characterization of the First Type III Pantothenate Kinase*

4.1. Introduction

In spite of our current knowledge of CoA biosynthesis in general and PanK enzymes in particular the identification of this activity remains elusive in a subset of pathogenic bacteria, including *Helicobacter pylori*. This fact was highlighted in two recent studies, both of which used a comparative genomics approach to reconstruct the universal CoA biosynthetic pathway in representative organisms of all kingdoms (*1*, *2*). Among these, *H. pylori* and *Pseudomonas aeruginosa* are examples of bacteria in which no putative PanK similar to either known analogue could be found, even though the four remaining CoA biosynthetic enzymes were clearly represented. Since PanK is an essential activity in these organisms, this suggested that at least one additional, uncharacterized analogue of PanK exists.

A recent patent application has claimed to have identified a gene sequence which, when cloned in *trans*, can suppress the effects of an *E. coli* temperature-sensitive mutant defective in PanK activity (*3*). Interestingly, this gene (*yacB*) was identified from the *Bacillus subtilis* genome; although similarity-based searches predict that its *yqjS* gene encodes the expected model prokaryotic Type I PanK.

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In support of the suggested duplication of PanK activity interruption of the *B. subtilis yqjS* gene gave a normal growing phenotype. However, a *B. subtilis* double deletion mutant lacking both the *yqjS* and *yacB* genes was not viable. These results indicate that simultaneous deletion of both genes is lethal to *B. subtilis* and that the *yacB* gene encodes for a protein with pantothenate kinase activity (*3*).

Similarity-based genome searches using the *yacB* gene sequence identify homologues in a number of bacteria (Table 4.1). A subset of these is similar to *B. subtilis* in that they also have gene sequences encoding for Type I PanKs. The rest consists of mainly pathogenic bacteria in which the *yacB* homologue accounts for the only recognizable PanK-encoding gene sequence. These include *H. pylori* and *P. aeruginosa* in which the PanK-encoding gene was missing (*2*) (see section 3.4), as well as others like *Bordetella pertussis* (the causative agent of whooping cough) and the category A biodefence pathogen, *Francisella tularensis*. Interestingly, the *yacB* homologue in *B. pertussis* has been studied before, and was found to be an essential gene in this organism (*4, 5*). However, these studies concluded that the gene product was involved in pertussis toxin production via interaction with a two component transcriptional regulator, BvgAS. The gene was subsequently named *baf*, for *B*vg *a*ccessory *f*actor (Section 3.3). Most *yacB* homologues are currently annotated as Bvg accessory factors, or as putative transcriptional regulators.

In this chapter we report the cloning, overexpression and characterization of the *yacB* gene product from *B. subtilis* and its homologue from *H. pylori*, and demonstrate that these proteins have pantothenate kinase activity. We chose to study *H pylori* because it is a medically relevant organism causing peptic ulcer disease and inflammation in the stomach known as gastritis. *H. pylori* causes more than 90% of duodenal ulcers and up to 80% of gastric ulcers. Before 1982, when this bacterium was discovered, spicy food, stress and lifestyle were considered the major causes of ulcers. After the discovery of this bacterium treatment measures were far more beneficial as patients were

treated with antibiotics instead of medication to relieve ulcer related symptoms. Shockingly, approximately two-thirds of the world's population is infected with *H. pylori* (*6*). This statistic highlights *H. pylori's* importance as an organism for further research.

Table 4.1

Selection of organisms predicted to have a Type III pantothenate kinase [a]

In addition to a Type I PanK	As the only PanK activity
Bacillus halodurans	*Aquifex aeolicus*
Bacillus subtilis	*Bordetella pertussisi* Tohama
Mycobacterium avium	*Borrelia burgdorferi*
Mycobacterium bovis	*Campylobacter jejuni*
Mycobacterium leprae	*Clostridium botulinum*
Mycobacterium microti	*Deinococcus radiodurans*
Mycobacterium tuberculosis	*Francisella tularensis* (Livermore)
Listeria monocytogenes	*Helicobacter pylori*
Rhodobacter capsulatus	*Neisseria meningitidis*
Rhodobacter sphaeroides	*Porphyromonas gingivalis*
Streptomyces coelicolor	*Pseudomonas aeruginosa*
	Synechocystis sp. PCC 6803
	Thermotoga maritima
	Treponema pallidum
	Xylella fastidiosa

[a]Predictions were made using the SEED tool (http://TheSEED.uchicago.edu/FIG/index.cgi)

Our results show that in comparison to the Type I and Type II PanKs these CoaX[3] enzymes exhibit distinctly different characteristics, suggesting that they are the first characterized examples of a third (Type III) PanK analogue. These findings have important implications for our continued effort in identifying new drug targets in pathogenic bacteria, as well as for our understanding of the convergent evolution[4] of key metabolic enzymes.

[3] The authors of the patent referred to above (*7*) called the product of the *yacB* gene CoaX. We shall also use this nomenclature to refer to Type III PanK enzymes.

[4] Convergent evolution is the process whereby a similar character evolves independently in two species. It is also a synonym for *analogy*; that is, an instance of a convergently evolved character, or a similar character in two species that was not present in their common ancestor (*8*).

4.2. Results and Discussion

4.2.1. Cloning, Purification and Expression of Type III PanKs

The putative CoaX proteins from *B. subtilis* (*Bs*CoaX) and *H. pylori* (*Hp*CoaX) were prepared by independently cloning the respective genes into overexpression vectors coding for N-terminal His_6-tagged fusion proteins. In addition to the CoaX genes, *E. coli* PanK and the *S. aureus* PanK were also cloned according to published methods (*9, 10*). The vectors were transformed into *E. coli* BL21 Star(DE3) and the proteins overexpressed and purified using immobilized metal affinity chromatography. The purity of the proteins was verified SDS-PAGE analysis (Figure 4.1).

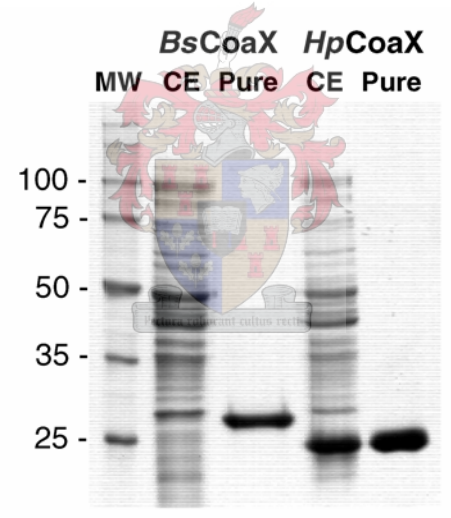


FIGURE 4.1. SDS-PAGE analysis of the overexpression and purification of *Bs*CoaX and *Hp*CoaX. The *B. subtilis yacB* gene and the HP0862 gene from *H. pylori* were cloned and overexpressed in *E. coli*. The resulting proteins, named *Bs*CoaX and *Hp*CoaX respectively, were purified by immobilized metal ion chromatography. *Lane 1*: Molecular weight markers (kDa). *Lane 2*: *Bs*CoaX cell-free extract. *Lane 3*: Purified *Bs*CoaX fraction. *Lane 4*: *Hp*CoaX cell-free extract. *Lane 5*: Purified *Hp*CoaX fraction.

4.2.2. Complementation

The *Hp*- and *Bs*CoaX genes were subcloned from pET28a-*Hp*CoaX and pET28a-*Bs*CoaX into pENTR4NT producing the plasmids pENTR4NT-*Hp*CoaX and pENTR4NT-*Bs*CoaX respectively. By means Gateway technology (Invitrogen) these two genes were subsequently cloned into pBAD-DEST49. While pET28a is our preferred expression vector, we decided that for complementation the pBAD-DEST49™ Gateway™ vector would be more suitable due to a number of special features. Chief among these is the use of the *ara*BAD (P_{BAD}) promoter that provides tight, dose-dependent regulation of heterologous gene expression based on the concentration of L-arabinose in the media. In addition, the tight regulation of P_{BAD} by araC is useful for the expression of potentially toxic or essential genes.

Once the gene of choice is cloned into this vector it becomes an expression vector designed for the regulated expression of N-terminal HP-thioredoxin fusion proteins in *E. coli.* The resulting plasmids, pBAD-Exp-*Hp*CoaX and pBAD-Exp-*Bs*CoaX were transformed into competent *E. coli* DV62 (*Ec*DV62) cells. In addition, pBAD-Exp-pGUS (negative control) was also transformed into *Ec*DV62. The *E. coli* strain DV62 is temperature sensitive with respect to pantothenate kinase function. This means that at temperatures of 42˚C and above the mutant is unable to biosynthesize CoA and cannot survive, while it grows normally at 30˚C. Therefore, unless an active PanK-encoding gene is cloned in *trans* in this cell strain it will not survive when grown at 42˚C.

We performed the complementation experiment by streaking the transformants onto two identical plates, and incubating them separately at 30˚C and 42˚C. At 30˚C all transformants survived, while at 42˚C only those containing the *coaX* gene survived (Figure 4.2). This observation supports the hypothesis that the c*oaX* gene encodes a protein with pantothenate kinase activity.

The plate-based complementation experiment highlights one of the drawbacks of doing complementation studies on solid media. It is apparent that the cell

density in the separate thirds of the plate is not equal (Figure 4.2). Although this is more apparent on the 42˚C plate it is also evident on the plate at 30˚C. We drew the conclusion that this may be due to an unequal number of cells being transferred to the plate when it is initially streaked. This could give a skewed and inconclusive result. However, it is also possible that *Hp*CoaX expresses at higher levels or is more active than *Bs*CoaX and for this reason is better able to complement the temperature sensitive PanK mutant.

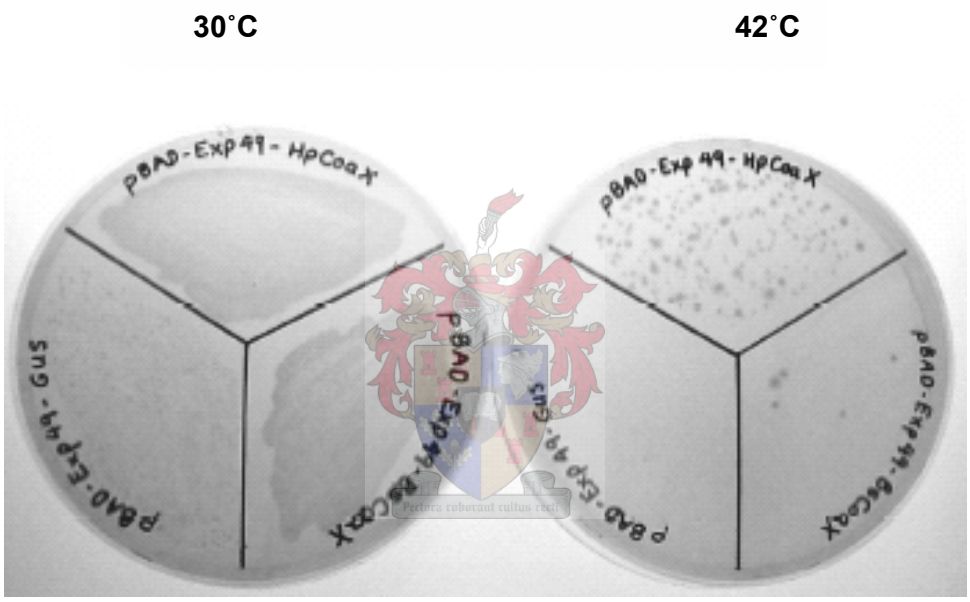


Figure 4.2. Complementation studies. *Bs*CoaX (bottom right), *Hp*CoaX (top center) and a negative control (pGUS, bottom left) were transformed into an *E. coli* strain that is temperature sensitive in terms of pantothenate kinase activity. At 30˚C the temperature sensitive PanK could still function and all transformants were able to grow. At 42˚C only the transformants containing the *coaX* gene survived. This means that the *coaX* gene is capable of complementing a temperature sensitive PanK mutant and therefore has PanK activity.

We decided that it was more reliable to conduct complementation in liquid media and to measure the increase in optical density of the growth media at 600 nm. The results indicate that both *Hp*CoaX and *Bs*CoaX complement for pantothenate kinase activity. As expected of the negative control, pGUS does not complement the temperature sensitive mutant and the cells do not grow at 42˚C (Figure 4.3). The results indicate that *Hp*CoaX complements the temperature sensitive mutant far sooner than *Bs*CoaX. This supports the hypothesis that the *Hp*CoaX protein is either more soluble or expresses at higher levels than the *Bs*CoaX protein, and indicates that the difference in cell density observed in the plate-based complementation study was a real event, and not due to a streaking inconsistency.

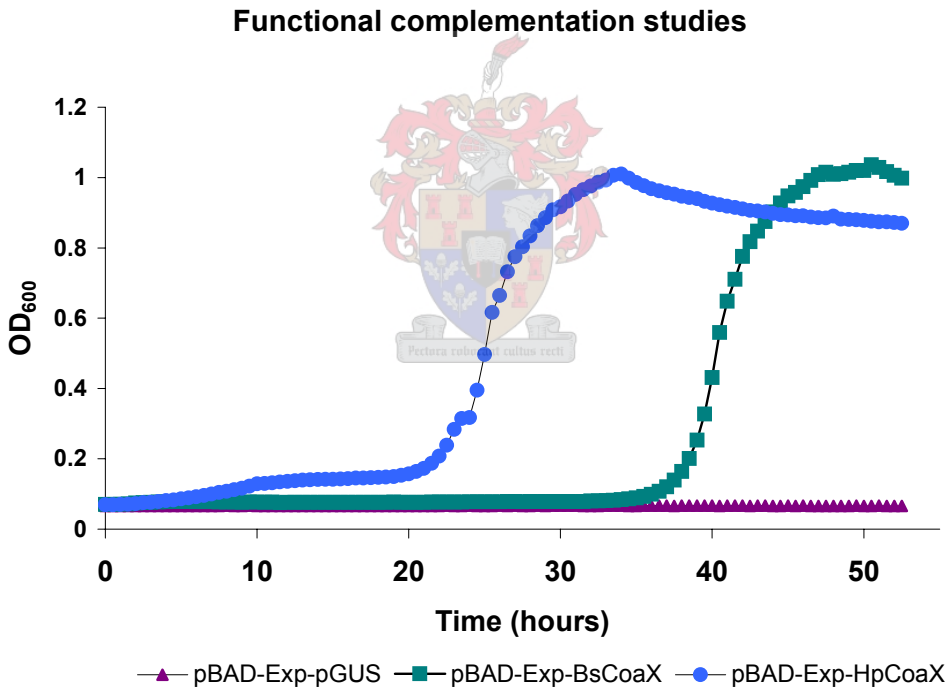


Figure 4.3. The *coaX* genes from *B. subtilis* and *H. pylori* are able to complement the pantothenate kinase activity of a temperature sensitive mutant. Expression vectors containing *Bs*CoaX, *Hp*CoaX and pGUS (negative control) were transformed into the *E.coli* DV62 temperature sensitive mutant cell line. These vectors were cultured in minimal at 42°C and the increase in absorbance was measured over time.

4.2.3. Kinetic Characterization of Type III PanKs

The purified proteins were assayed for their ability to catalyze the ATP-dependent phosphorylation of pantothenate using a two enzyme-coupled assay that links the production of ADP to the oxidation of NADH. This allows the enzyme activity to be measured continuously by monitoring the change in A_{340}. The assay was performed by varying the pantothenate concentration at a number of set ATP concentrations, and determining the initial rates of the reaction for each protein (Figure 4.4). The results clearly show that both CoaX proteins have PanK activity.

The initial rate data were used to determine the kinetic parameters of the enzymes by performing global non-linear fits using the most general form of the equation describing a bireactant mechanism (*11*). The results are summarized in Table 4.2 which also shows the kinetic parameters of the model prokaryotic Type I PanK, *E. coli* (*Ec*CoaA) (*12*), the eukaryotic PanK from *Aspergillus nidulans* (*An*PanK) (*13*) and the recently characterized atypical Type II PanK from *S. aureus* (*Sa*CoaA).

Table 4.2. Kinetic parameters of the three characterized pantothenate kinase isoforms

Analogue	Enzyme	k_{cat} (s^{-1})	Substrate	K_M (μM)	kcat/K_M ($mM^{-1}.s^{-1}$)
Type I (Prokaryotic)	*Ec*CoaA[a]	0.30 ± 0.13	Pantothenate	36 ± 4	8.38 ± 3.69
			ATP	136 ± 15	2.22 ± 0.98
Type II (Eukaryotic)	*An*PanK[b]	1.95	Pantothenate	60	32.6
			ATP	145	13.5
	*Sa*CoaA[c]	1.73	Pantothenate	23	75.2
		1.56	ATP	34	45.9
Type III (CoaX-like)	*Bs*CoaX	2.12 ± 0.17	Pantothenate	168 ± 27	12.58 ± 2.27
			ATP	3050 ± 520	0.70 ± 0.13
	*Hp*CoaX	2.09 ± 0.26	Pantothenate	101 ± 26	20.6 ± 5.9
			ATP	9590 ± 2140	0.21 ± 0.05

[a] *E. coli* CoaA; apparent kinetic parameters taken from ref. (*12*)
[b] *A. nidulans* PanK; apparent kinetic parameters taken from ref. (*13*)
[c] *S. aureus* CoaA; apparent kinetic parameters taken from ref. (*14*)

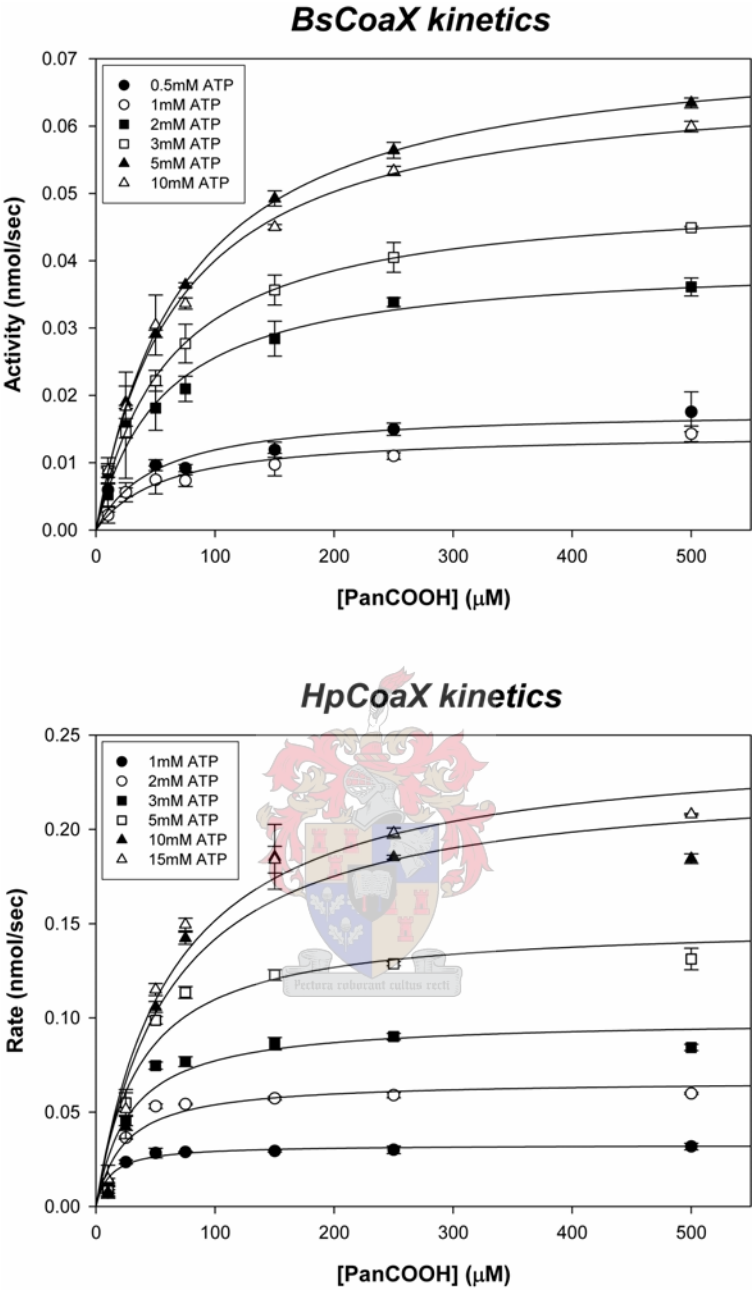


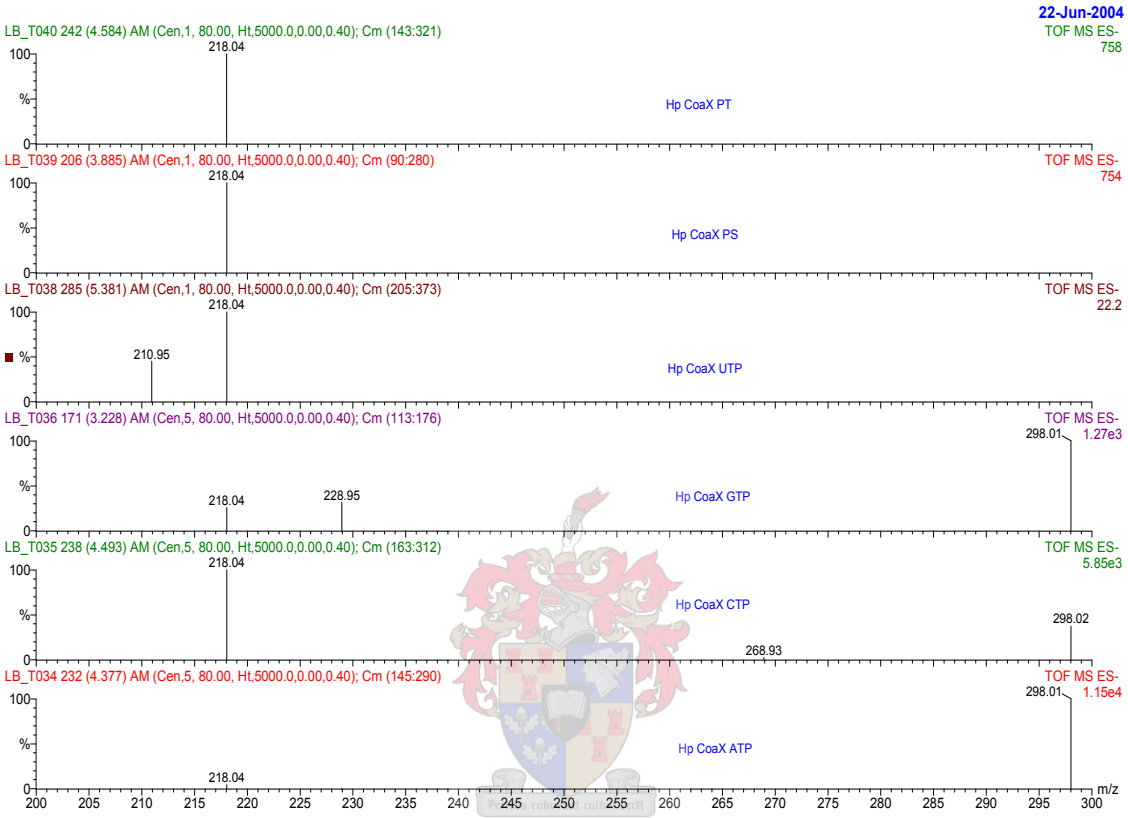
Figure 4.4. Initial rate plots of *Bs*CoaX (top) and *Hp*CoaX (bottom). Iinitial rates of *Bs*CoaX against pantothenate concentration at an ATP concentration of 0.5 mM (●), 1.0 mM (○), 2.0 mM (■), 3.0 mM (□),5.0 mM (▲) and 10.0 mM (△) respectively. Initial rates of *Hp*CoaX against pantothenate concentration at an ATP concentration of 1.0 mM (●), 2.0 mM (○), 3.0 mM (■), 5.0 mM (□), 10.0 mM (▲) and 15.0 mM (△) respectively. Symbols show the average of three replicates, with error bars indicating the standard deviation. 4.5 μg of PanK enzyme was used per reaction. Curves show fits of the individual data sets to the Michaelis-Menten equation.

When one examines the numerical kinetic data from the various PanK analogues, the K_M values for pantothenate are all within the same range. The CoaX enzymes K_M values are slightly higher but not significantly so. However, the same cannot be said for the ATP, where the K_M-values for the CoaX proteins are in the millimolar range. The affinity of *Hp*CoaX for ATP is especially low with a K_M value of nearly 10 mM. The K_M of *Hp*CoaX differs from that of the well-characterized *E. coli* PanK analogue (*Ec*CoaA) by a factor of over 70! Such large K_M values can be rationalized in the case of the *Bs*CoaX enzyme that duplicates an activity already embodied by the more typical Type I PanK. However, since *Hp*CoaX depends exclusively on this PanK for its CoA biosynthetic needs the question arises as to whether it uses an alternate phosphate donor in the reaction catalyzed by this enzyme.

4.2.4. Testing the Efficacy of Alternate Phosphoryl Donors

The low ATP specificity constant of *Bs*CoaX and *Hp*CoaX suggested that these enzymes might have a preference for other phosphoryl donors in the biosynthesis of 4'-phosphopantothenate. This proposal was tested by incubating the enzymes with pantothenate and a range of activated and non-activated phosphate-containing substrates. These included the three other ribonucleotide 5'-triphosphates (GTP, CTP and UTP), PEP, acetyl- and carbamoylphosphate, and phosphoserine and -threonine. The latter two molecules were tested because of the recent discovery and characterization of a phosphotransferase enzyme, which catalyzes the transfer of a phosphoryl group from phosphoserine to homoserine via an activated phosphoenzyme intermediate (*15*). Other examples of similar reactions have also been described (*16*). The reaction mixture with PEP was subsequently analyzed by the two enzyme-coupled assay, while the rest of the reaction mixtures were analyzed by ESI-MS for the presence of 4'-phosphopantothenate. The results were compared to mixtures containing ATP as well as to control reactions with *E. coli* PanK. Only CTP and GTP could substitute for ATP in the case of *Hp*CoaX, albeit to a lesser extent (Figure 4.5). No other phosphoryl donor was active in the reactions catalyzed by *Bs*CoaX. This strongly points to ATP

as the cognate phosphoryl donor in the PanK reaction catalyzed by CoaX enzymes. Results for *B. subtilis* and *E. coli* are not shown.



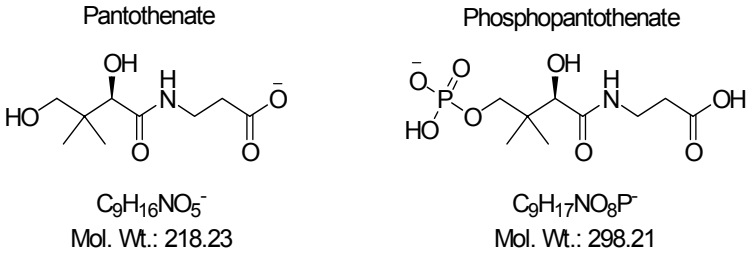


Figure 4.5. ESI-MS result for *Hp*CoaX using phosphothreonine (line 1), phosphoserine (line 2), UTP (line 3), GTP (line 4), CTP (line 5) and ATP (line 6) as phosphate donors for the pantothenate kinase reaction. Only GTP and CTP can substitute for ATP, albeit to a lesser extent (note that the intensity of the base peak for these two nucleotides is an order of magnitude lower than that of ATP). Also shown are the structures and molecular weight analysis for pantothenate and phosphopantothenate.

4.2.5. Inhibition of Type III PanKs by CoA and Acetyl-CoA

With the exception of the recently characterized *S. aureus* PanK enzyme (*14*), all other known PanKs are inhibited by CoA or its thioesters. The extent of inhibition is system dependent: prokaryotic Type I PanKs exhibit greater inhibition by free CoA than its thioesters, while the activity of eukaryotic Type II PanKs is affected more by the CoA thioesters acetyl- and malonyl-CoA. In both cases the inhibition serves to regulate the intracellular CoA concentration by affecting the flux through the pathway. To determine whether a similar effect can be observed with the CoaX enzymes reactions containing increasing amounts of CoA or acetyl-CoA were assayed for PanK activity (Figure 4.6). The experiment was also performed with *E. coli* PanK, which has a well-described inhibition profile (*17*), for comparison. Our results show that while *E. coli* PanK demonstrates inhibition by CoA and to a lesser extent by acetyl-CoA as expected, neither of the CoaX enzymes is affected. This result is surprising, since the inhibition of PanK by CoA and its thioesters was until recently considered to constitute a common mechanism for the regulation of intracellular CoA levels in all organisms. The fact that the *S. aureus* PanK enzyme is not subject to such regulation has been rationalized in terms of the unique physiology of this organism that depends on CoA and a NADPH-dependent CoA reductase to maintain the intracellular redox balance (*18, 19*) (Discussed in Section 3.3.1).

A possible explanation for the unusual steady state kinetic properties, especially the high K_M value for ATP, of *H. pylori* is that the low specificity for ATP may exert some effect on the rate of CoA biosynthesis in this organism. Futhermore, the CoA biosynthesis may be directy affected by the cellular ATP and pantothenate levels and thus coupled to the energy status of the cell.

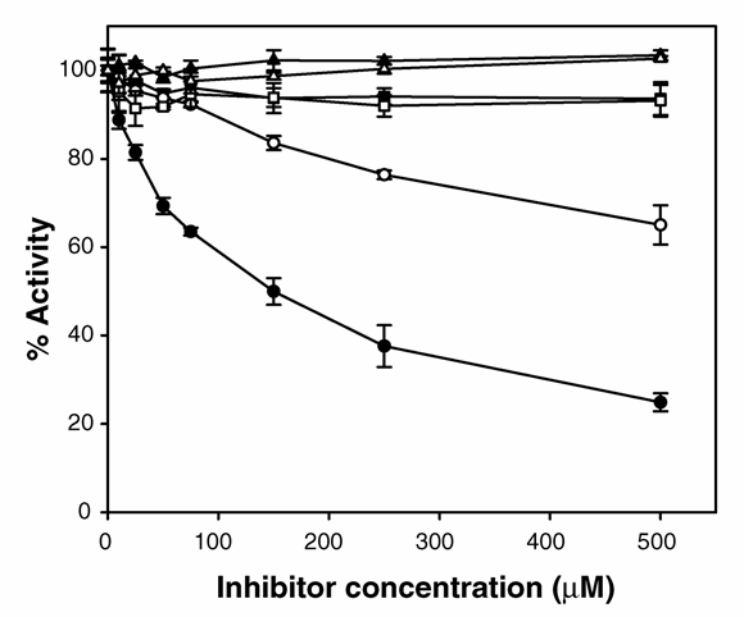


Figure 4.6. Inhibition of *Ec*CoaA, *Hp*CoaX and *Bs*CoaX by CoA and acetyl-CoA. Inhibition of the pantothenate kinase activity of *Ec*CoaA (●,○), *Bs*CoaX (■,□) and *Hp*CoaX (▲,△) in the presence of CoA (*closed symbols*) and acetyl-CoA (*open symbols*). Symbols show the average of three replicates, with error bars indicating the standard deviation. Reaction mixtures contained 500 μM pantothenate, ATP (5.0 mM for *Bs*CoaX, 10.0 mM for *Hp*CoaX and 1.5 mM for *Ec*CoaA), 20 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 0.3 mM NADH, 5 U of lactate dehydrogenase, 2.5 U of pyruvate kinase and 4.5 μg of the PanK enzyme in 100 mM HEPES (pH 7.6). CoA and acetyl-CoA were added at concentrations between 10 and 500 μl and the reaction was initiated by the addition of pantothenate.

4.2.6. Effect of Pantothenamide Antimetabolites on Type III PanKs

Recent studies on the *E. coli* PanK and *S. aureus* PanK enzymes have shown that *N*-alkylpantothenamides like *N*-pentyl and *N*-heptylpantothenamide (Figure 3.2, Section 3.4) act as antimicrobial agents through their action as CoA antimetabolites (*9, 10, 14*). These compounds were found to act as substrates of the CoA biosynthetic enzymes in both *E. coli* and *S. aureus*, where they are converted to inactive CoA analogues which inhibit a number of cellular targets, especially fatty acid biosynthesis (*10, 14, 20*). To test whether this was also the case with CoaX enzymes, *N*-pentylpantothenamide was substituted for pantothenate in reaction mixtures and the reactions assayed by

the two enzyme-coupled system. No activity could be detected using *N*-pentylpantothenamide at concentrations of up to 0.5 mM (data not shown). To test if the compound was an inhibitor of the enzyme, the activity of CoaX enzymes was assayed in the presence of increasing concentrations of *N*-pentylpantothenamide. No effect was observed (data not shown). It has been suggested (*14*) that the two enzyme-coupled system is not as sensitive as the radioactive-based assay employed in other studies as it reportedly failed to detect that *N*-pentylpantothenamide was a substrate for *S. aureus* PanK (*9*). However, in our hands we clearly detect activity when assaying *S. aureus* PanK in the presence of *N*-pentylpantothenamide by this method, suggesting that sensitivity is not a factor.

4.2.7. Gene Cluster Analysis in Support of Functional Characterization

The conservation of gene clusters across genomes can often be applied to infer gene function (*21*). To apply this technique to CoaX enzymes the genes coding for these proteins were aligned across a set of genomes and the respective gene clusters analyzed for the presence of other CoA and pantothenate biosynthetic genes (Figure 4.7). The results show that in *Mycobacterium* spp., *Streptomyces coelicolor, Moorella thermoacetica* and *Francisella tularensis* genes encoding CoaX proteins are found associated with two genes involved in pantothenate biosynthesis (combinations of pantothenate synthetase, aspartate 1-decarboxylase or ketopantoate hydroxymethyltransferase), while in *Bdellovibrio* the cluster contains these three pantothenate biosynthetic genes as well as the putative phosphopantothenoylcysteine synthetase/decarboxylase-encoding gene. Such clustering provides strong genetic support for the direct involvement of CoaX proteins in CoA biosynthesis.

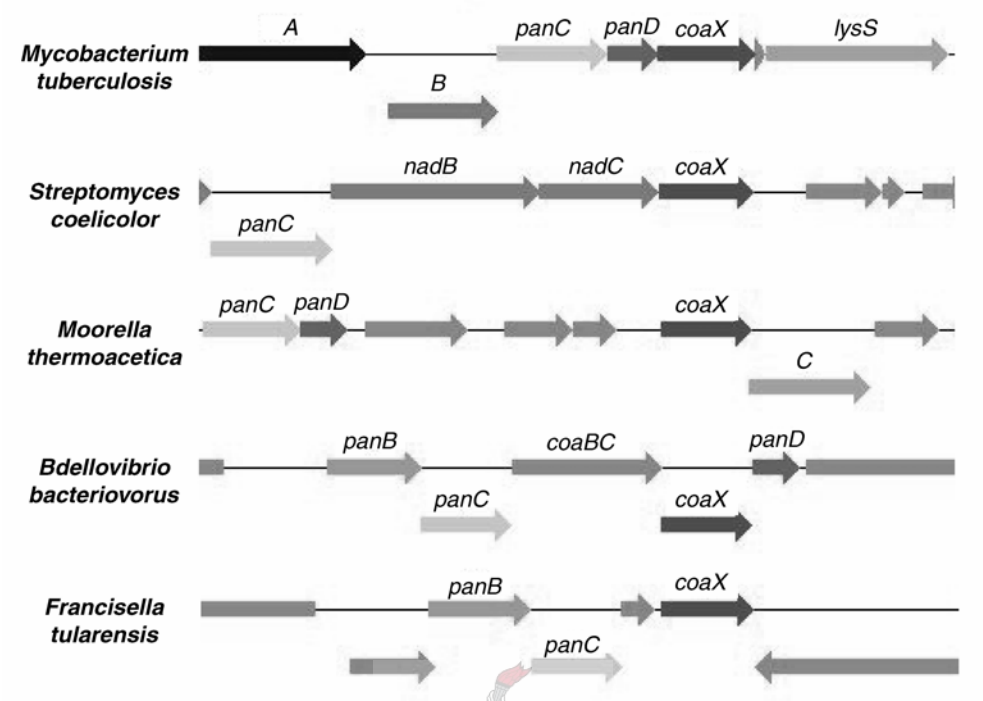


Figure 4.7. Cluster analysis of the predicted *coaX* gene in selected organisms. Selected chromosomal contigs containing a gene predicted to encode a Type III pantothenate kinase (labelled *coaX*) were aligned to visualize gene clustering on the chromosome. Other genes involved in pantothenate and CoA biosynthesis are *panB* (ketopantoate hydroxymethyltransferase), *panC* (pantothenate synthetase), *panD* (aspartate 1-decarboxylase) and *coaBC* (phosphopantothenoylcysteine synthetase/decarboxylase). Other unrelated genes are also labelled using standard nomenclature for sequences with known or predicted functions, or using letters A, B, and C for unknown hypothetical proteins which share high sequence-similarity. The alignment was prepared from data modified from the SEED tool (http://TheSEED.uchicago.edu/FIG/index.cgi).

4.3. Conclusion

In this chapter we have cloned, overexpressed and characterized two homologous proteins from *B. subtilis* and *H. pylori* and demonstrated that they have pantothenate kinase activity. However, these proteins do not share sequence similarity with the other two known PanK analogues. They also exhibit unique kinetic characteristics, showing low specificity constants for ATP and no regulation by CoA and its thioesters. They do not utilize the

alkylpantothenamides as substrates, nor are they inhibited by them. These properties all distinguish them from the other PanK analogues, and strongly suggest that they are the first characterized examples of a third analogue of the PanK enzyme. These Type III PanKs represent the only recognizable pantothenate kinase activity in a variety of organisms, including the pathogens *H. pylori* and *P. aeruginosa*, in which no candidate PanK-encoding genes could be identified to date. It is still unclear what advantage, if any, these enzymes confer upon the mainly pathogenic organisms that harbour them.

4.4. Experimental Procedures

4.4.1. Materials and Methods

All chemicals were purchased from Aldrich, Sigma or Fluka and were of the highest purity. Other materials were from the following suppliers: Pyruvate kinase and lactate dehydrogenase enzymes from Roche; restriction endonucleases, *Pfu* and *Taq* DNA polymerase from Fermentas; Quick ligation kit from New England Biolabs (NEB), Bradford dye reagent and BSA protein standards from Biorad; oligonucleotides from Inqaba Biotechnology (Pretoria, South Africa); and HiTrap columns from Supelco. *B. subtilis* genomic DNA was purified from *B. subtilis* 168, obtained from ATCC. *H. pylori* genomic DNA was a gift from Paul van Helden and Rob Warren (Department of Medical Biochemistry, Stellenbosch University). Automated DNA sequencing was performed by Inqaba Biotechnology (Pretoria, South Africa). Proteins were purified on an ÄKTA Prime instrument (Amersham Biosciences). Large-scale centrifugation was done on a Heraeus Multifuge® 3S/3S-R. Centrifugation on a smaller scale was conducted on a Heraeus Biofuge pico centrifuge. Enzyme assays were performed on a Multiskan Spectrum multiplate spectrophotometer (ThermoLabsystems), using an extinction coefficient of 6220 $M^{-1}.cm^{-1}$ for NADH. ESI-MS analyses were performed at the Central Analytical Facility at Stellenbosch University on a Waters Micromass Q-TOF Ultima API mass spectrometer. The *Sa*CoaA expression plasmid was a gift from Cynthia Kinsland (Department of Chemistry and

Chemical Biology, Cornell University). The *Sa*CoaA protein was purified according to published methods, using BL21 (DE3) (Novagen) as expression strain (*9*). *Ec*CoaA was prepared by published methods (*10*). All curve-fitting analyses were performed using SigmaPlot 9.0 (Systat software). *E. coli* DV62 was obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University)

4.4.2. Construction of Expression Vectors

The *B. subtilis coaX (yacB)* gene was amplified by PCR from *B. subtilis* 168 genomic DNA using *Pfu* DNA polymerase and the following primers: 5'-CAAAAGTGGTGACATATGTTGTTACTGGTTATC-3' (forward primer), introducing an *Nde*I site (underlined) at the start of the gene, and 5'-CCATATCAGTCGTTCTCGAGGCATAAGCCCGAAC-3' (reverse primer), introducing an *Xho*I site (underlined) at the end of the gene. The *H. pylori* HP0682 gene was amplified by PCR from *H. pylori* genomic DNA using *Taq* DNA polymerase and the following primers: 5'-ATAAGAAGTAGGCATATGCCAGCTAGGC -3' (forward primer), introducing an *Nde*I site (underlined) at the start of the gene, and 5'-ATGCCCAAAAAACTCGAGTTGTGCATC-3' (reverse primer), introducing an XhoI site (underlined) at the end of the gene. The resulting PCR products were digested with NdeI and XhoI and ligated to NdeI/XhoI-digested pET28a expression vector (Novagen) using the NEB Quick Ligation Kit. The sequences of the resulting plasmids, named pET28a-*Bs*CoaX and pET-28a-*Hp*CoaX respectively, were verified by automated DNA sequencing. The expression plasmids allow for the production of the proteins as *N*-terminal His_6-tagged fusion proteins. The detailed method for the cloning of these plasmids is given below in 4.4.2.1.

4.4.2.1. Standard Cloning Procedure

Design of Primers

When cloning from genomic DNA, in most cases (unless stated otherwise) we introduced an NdeI (CAT-ATG) site in the forward primer and an XhoI (CTC-GAG) site in the reverse primer. The NdeI site is inserted in the forward primer so that the ATG is the starting Met of the protein. If a mutation is introduced then it is kept 12 or more bases from the ‘3 end of the primer. All primers are checked for self-annealing. If possible, the ‘3- end of the primer ends in G or C. The ‘3 end of the primer is not terminated in more than 2 of the same base pairs in a row.

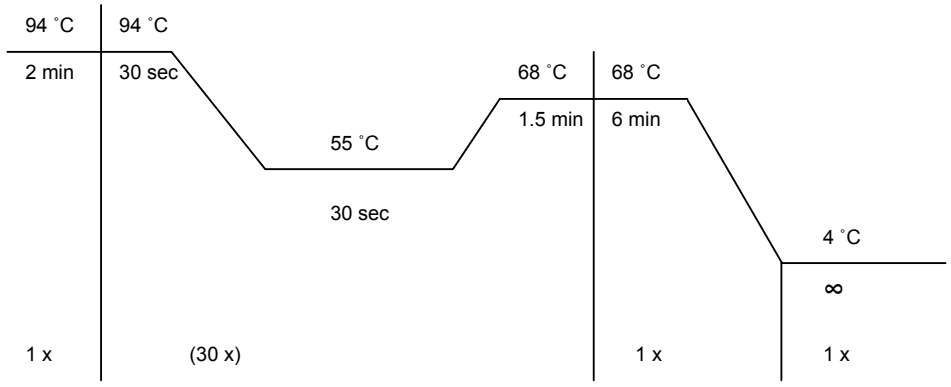
PCR Reaction

The PCR reaction is set up as follows. If the reaction yields no products at the suppliers specified magnesium concentration, then it is repeated with differing $MgSO_4$ concentrations. If this is the case then the amount of water is decreased so that the total volume remains 25 µl. The PCR reactions were performed in a 250 µl reaction vessel as follows:

2.5 µl	10 x amplification buffer
1 µl	dNTP mix (10 mM)
1 µl	$MgSO_4$ (25 mM)
1 µl	Forward Primer (25 µM)
1 µl	Reverse Primer (25 µM)
X µl	Genomic DNA (2 ng)
0.5 µl	Pfu DNA polymerase (X U/ µl)
18 – X µl	Sterile water
25 µl	TOTAL

The PCR products were run on a MyCycler™ Thermal Cycler (BioRad) using the protocol illustrated below:

PCR Program:



DNA Electrophoresis

Unless stated otherwise, 10 x loading buffer (0.25 % bromophenol blue, 49.5 mM TRIS pH 7.6 and 60 % glycerol) was added to samples loaded on agarose gels. All gels were 1 % agarose made up with 1 x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and run at 75 V (PowerPac from Biorad). The samples were run against 1 µg 2-log ladder (New England BioLabs Inc.) The gels were stained in 50 ml of TAE buffer supplemented with 5 µl SYBR® Gold (Molecular probes, Invitrogen detection technologies). DNA visualization took place with the help of a Dark Reader Transilluminator (Molecular probes Inc.) The products of the PCR were excised from the gel and purified with the Perfectprep® Gel Cleanup Kit (Eppendorf).

Digestions:

The purified PCR product and the pET28a expression plasmid (Novagen) were digested with NdeI (New England Biolabs) and XhoI (Inqaba Biotechnology). The digestion mixture contained the following:

2 µl	XhoI 10 x reaction buffer (contains BSA to final concentration of 0.1 mg/ml)
14 µl	Purified PCR product/plasmid (~ 250 ng)
1 µl	sterile water
1 µl	NdeI (20U/µl)
2 µl	XhoI (10U/µl)
20 µl	TOTAL

The digests were incubated at 37˚C for 2 hours.

After incubation, the NdeI/XhoI cut plasmid and PCR products underwent electrophoresis and were excised from the gel. The gel bands were purified with the Perfectprep® Gel Cleanup Kit (Eppendorf).

In order to determine the concentration of the digested plasmid and PCR DNA, 5 µl of each were run on a gel. They were run against the 2-log ladder containing bands of known concentration. By comparing the intensity of the sample DNA with that of the ladder it is possible to determine the concentration of the sample DNA in ng/µl. This value was converted to fmoles/µl for the purpose of the ligation reaction.

Ligation of plasmid and PCR DNA

The concentration of the digested plasmid is not always equal to that of the digested PCR product. We used a 1:1 concentration ratio of plasmid to insert DNA in our ligation reaction. Ligations were performed using the Quick Ligation™ Kit (New England BioLabs). This kit ensures that the entire ligation protocol is completed in 5 minutes and is highly reliable. Except for halving the reaction volume, the protocol was carried out according to the manufacturer's specifications.

Transformation

The products of the ligation reaction were transformed into Mach1 competent cells (Invitrogen).

Making Competent Cells:

From frozen stock the cell line of ones choice is streaked on Luria Bertani (LB) agar (biolab – Merck) plates. The plates are incubated at 37 ˚C overnight. A single colony is selected from the plate and used to inoculate 5 ml of (LB) broth (biolab – Merck). This culture is grown overnight at 37 ˚C in a shaking incubator (200 rpm) and in turn used to inoculate 500 ml of LB broth in a 2L flask (at a ratio of 1:1000, i.e. 5 ml in 500 ml of media). This is grown at 37 ˚C in a shaking incubator (200 rpm) until the OD_{600} = 0.5 – 0.6 nm (~ 2 hours). The cells are collected by centrifugation at 4500 x g for 30 minutes at 4 ˚C. The supernatant is discarded and the cells resuspended in 100 ml of ice cold

100 mM $MgCl_2$. Thereafter they are incubated on ice for 30 minutes. The cells are centrifuged at 4500 x g at 4 ˚C for 10 minutes, the supernatant discarded, and re-suspended in 10 ml of 100 mM $CaCl_2$-15 % glycerol (ice cold). The competent cells are then aliquoted (170 µl/tube) into the pre-chilled tubes and stored at –80 ˚C.

Transformation Procedure

The frozen competent cells are thawed on ice for 5 to 10 minutes. 80 µl of the competent cells are added to the ligation reaction. The reaction is incubated on ice for 30 minutes and then heat shocked at 42 ˚C for 45 seconds. This is followed by a cooling step on ice for 5 minutes. Thereafter, 900 µl of LB is added to the transformation mixture and it is incubated in a shaking incubator (200 rpm) for 1 hour at 37 ˚C. The cell pellet is collected by centrifugation at 8000 rpm for 4 minutes. Using a pipette 850 µl of the supernatant is removed (being careful not to disturb the cell pellet) and the cells are then re-suspended in the remaining media. This is plated onto an LB agar plate containing the relevant antibiotic. Unless stated otherwise the concentrations of antibiotics used are as follows:

Kanamycin:	30 mg/L
Ampicillin:	100 mg/L
Tetracycline:	12.5 mg/L

The cells are spread evenly on the plate by means of sterile glass beads. The plate is Incubated at 37 ˚C overnight.

Screening of Clones

Two screens are conducted to check for the insertion of the gene of interest into the plasmid. Firstly, a screening gel is run. From the results of the screening gel, colonies are chosen and plasmids prepared from these colonies. The second method follows the first and involves the digestion of these plasmids with the original restriction enzymes to check for an insert of the correct size.

Screening Gel

Firstly, 25 µl of lysis solution 1 (30 mM Tris pH 8.0, 5 mM Na_2EDTA, 50 mM NaCl, 20 % sucrose, 0.05 mg/ml lysozyme and 0.05 mg/ml RNAse) is added to each 1.5 ml microcentrifuge tube. Using a sterile toothpick, a single colony is scraped from the LB plate and re-suspended in solution 1. Thereafter the toothpick is streaked on a new LB plate that is marked with a numbered grid so that each screen can be identified at a later stage. This plate is called the reporter plate. The process is repeated with each colony. The tubes are vortexed briefly followed by addition of 10 µl of solution 2 (1 x TAE, 2 % SDS, 5 % sucrose and 2 mg/ml bromophenol blue). The microcentrifuge tubes are vortexed to evenly mix their contents. Thereafter, 20 µl from each tube is loaded on a 1 % agarose gel. Loading must be done very carefully as the solution becomes viscous and is difficult to load. Undigested pET28a is loaded as a standard. The gel is run at 75 V.

Positive screens are those that are bigger than the plasmid standard loaded as a marker. A representative of the positive screens (recorded on the reporter plate) is used to inoculate 5 ml of media (containing the relevant antibiotic) and grown in a shaking incubator (200 rpm) at 37 ˚C overnight.

Digestion Screen

The above overnight culture is used to purify a plasmid. This is done with the help of the FastPlasmid™ Mini kit (Eppendorf). The purified plasmid is digested with the original restriction enzymes used to cut it before ligation (in most cases NdeI and XhoI). The digestion is conducted in the same was as above. The digestion products are run on a 1 % agarose gel against the 2-log ladder. Positive clones are those that produce inserts the size of the gene of interest.

Sequencing

The positive clone is transformed into Mach1 and plated onto a LB plate containing the relevant antibiotic. The plate is sent to Inqaba Biotechnology (Pretoria) who confirm the clone by automated DNA sequencing.

On confirmation the clone is stocked at –80 ˚C (900 µl of overnight culture, 100 µl 80 % glycerol). In addition, the plasmid is transformed into *E. coli* BL21 (Star)DE3 for expression.

4.4.3. Complementation Studies

4.4.3.1. Cloning of the Expression vectors

The pENTR4 vector is commercially available from Invitrogen. This vector is used as an Entry clone for LR reactions in the Gateway™ cloning system. The "L" and "R" of the LR reaction are derived from the *att*L and *att*R sites of the plasmids involved in the reaction.

Gateway™ Cloning Technology is a universal system for the cloning and subcloning of DNA sequences. This facilitates gene function analysis and protein expression. The LR reaction is used to create an Expression clone. The first step is to get the gene of interest into an Entry clone. Thereafter, the gene is cut on either side within the *attL* sites in the Entry clone and ligated into the corresponding *attR* sites in the Destination vector. This single step reaction creates an Expression clone and is very convenient.

We needed a modified pENTR4 entry clone for our LR reaction. Our genes were cloned into pET28a and flanked by NdeI and XhoI. The commercially available pENTR4 does not contain an NdeI site. We performed a mutagenesis PCR reaction to replace the NcoI site in pENTR4 with NdeI. We used the QuikChange® Site-Directed Mutagenesis Kit from Stratagene. The primers used for the mutagenesis PCR were as follows (NdeI site underlined):

Forward Primer: 5'-AAGCAGGCTCCCATATGGGAACCAATTCAGTCGACTGGATC-3'

Reverse Primer: 5'-GATCCAGTCGACTGAATTGGTTCCCATATGGGAGCCTGCTT-3'

The resulting plasmid was named pENTR4N (N for NdeI). This plasmid was further modified to contain the tetracycline resistant gene. The tetracycline resistant gene was cloned from pBR322 vector (Promega) by PCR reaction.

The forward primer introduced a PstI site (underlined), while the reverse primer introduced a PfIm site (underlined):

Forward primer: 5'-GGCCCTTTCGTCTGCAGGAATTCTCATGTTTG-'3

Reverse primer: 5'-GATTGGCTCCAAAGGTTGGAGTGGTGAATCCG-'3

The resulting PCR product was digested with PstI and PfIm and ligated to PstI/PfIm-digested pENTR4N Entry clone using the standard cloning procedure described in 4.4.2.1. The resulting plasmid was designated pENTR4NT ("N" for NdeI and "T" for tetracycline resistant gene)

Thereafter, the *coaX* genes from *H. pylori* and *B. subtilis* were excised using NdeI and XhoI from pET28a-*Hp*CoaX and pET28a-*Bs*CoaX respectively and subcloned into NdeI/XhoI-digested pENTR4NT. The resulting plasmids were designated pENTR4NT-*Hp*CoaX and pENTR4NT-*Bs*CoaX.

All that remained was to perform the LR reaction to get the *coaX* genes into the Expression clone, pBAD-DEST49. For each (*Hp*CoaX and *Bs*CoaX) the Entry clone (pENTR4NT-*Hp*CoaX and pENTR4NT-*Bs*CoaX respectively) was combined with pBAD-DEST49 as specified by the manufacturer and the LR reaction conducted. Two Expression clones were created and termed pBAD-Exp49-*Hp*CoaX and pBAD-Exp49-*Hp*CoaX. These expression clones were used in the complementation studies. In addition to these two clones, a third was made to serve as a negative control in the complementation studies. An Entry clone named pENTR-gus™ comes with the LR reaction kit and this was used in an LR reaction with pBAD-DEST49 in the same way as the other two LR reactions. All the Expression clones are ampicillin resistant.

4.4.3.2. Complementation

The pBAD-Exp49-*pGUS*, pBAD-Exp49-*Hp*CoaX and pBAD-Exp49-*Bs*CoaX vectors were transformed into chemically competent *E. coli* DV62 cells (*metB1, PanD2, CoaA15, zij*::Tn*10*) (*17*). This strain of *E. coli* is temperature sensitive in terms of pantothenate kinase activity and will only grow at

temperatures above 42˚C if a gene coding for PanK activity is cloned in *trans* in these cells.

The transformations were plated onto LB ampicillin agar plates and grown overnight at 37˚C. From the plates obtained, overnight cultures were made from single colonies in 5ml LB ampicillin broth and these overnight cultures were used to stock the plasmids at -80˚C.

Complementation on Minimal Media Plates:
Minimal media (E salts (1%), 0, 5% glycerol, 1 mg/ml methionine, 0.1 mg/ml thiamine) plates supplemented with β-alanine (10μM), 0.2% L-arabinose and 100 mg/L ampicillin were divided into three. Two plates were each streaked with pBAD-Exp-p*GUS*, pBAD-Exp-*Hp*CoaX and pBAD-Exp-*Bs*CoaX in *Ec*DV62 from the -80˚C freezer stock. One plate was incubated at 30˚C and the other at 42˚C for 48 hours. The pGUS vector was used as negative control.

Complementation in Liquid Media
Corning 6-well, flat-bottomed cell culture plates (Sigma) were used. To each well 4 ml of sterile minimal media (E salts (1%), 0, 5% glycerol, 1 mg/ml methionine, 0.1 mg/ml thiamine) supplemented with 10μM β-alanine, 0.2% L-arabinose and 100 mg/L ampicillin was added. The minimal media was inoculated with 10 μl of an overnight culture of the respective expression clones (the experiments were done in duplicate). The absorbance readings were read by a Varioskan™ spectrophotometer (ThermoLabsystems) at 600 nm every 30 minutes for 52 hours.

4.4.4. Expression and Purification of Recombinant Proteins

pET28a-*Bs*CoaX and pET28a-*Hp*CoaX were transformed into *E. coli* BL21 Star(DE3) (Invitrogen). LB media (500 ml) was supplemented with 30μg/ml kanamycin sulfate and inoculated with overnight cultures of either BL21 Star(DE3)/pET28a-*Bs*CoaX or BL21 Star(DE3)/pET28a-*Hp*CoaX. Cultures were grown at 37°C to an A_{600} of ~0.6, and subsequently induced by the

addition of IPTG (a final concentration 800 μM for *Bs*CoaX and 100 μM for *Hp*CoaX). After growing overnight at 37°C, the cells were harvested, suspended in sonication buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9), using 10 ml/g cell paste), disrupted by sonication, and centrifuged at 15 000 x g for 30 min to clarify the cell-free extract. The extract was applied to a 1 ml HiTrap chelating column and prepared as described by the suppliers. Weakly bound proteins were removed by washing with sonication buffer, followed by sonication buffer containing 60 mM imidazole. *Bs*CoaX was eluted from the column by using strip buffer (100 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 7.9). *Hp*CoaX was eluted by using sonication buffer containing 500 mM imidazole. The purified protein solutions were exchanged to gel filtration buffer (5 mM $MgCl_2$, 25 mM Tris pH 8.0 and 5% glycerol) using HiTrap desalting columns pre-equilibrated in the same buffer. Aliquots of the purified proteins were stored at -80°C. The concentration of the proteins was determined by the Bradford method according to the instructions of the supplier.

SDS-polyacrylamide gels determined the purity of the proteins. The gels consist of a running gel and a stacking gel:

12% Running gel: 40 % (v/v) 30 % acryl-bisacrylamide mix (Sigma), 0.75 M Tris pH 8.8, 0.1 % SDS (v/v), 0.1% APS (v/v), 0.04 % TEMED (v/v).

Stacking gel: 17% (v/v) 30 % acryl-bisacrylamide mix (Sigma), 0.063M Tris (pH 8.8), 1 % SDS (v/v), 1 % APS (v/v), 0.33 % TEMED (v/v).

A vertical slab gel was cast consisting of a stacking gel and a running gel. This was done on a Hoefer Mighty Small II SE 250 assembly (AEC Amersham). All the samples were treated with 2 X loading buffer (0.125 M Tris-HCl pH 6.8, 4 % SDS, 30 % glycerol, 1.5 % β-mercaptoethanol and 0.02 % bromophenol blue). Thereafter, they were incubated in a heating block at 95 ˚C for 5 minutes. The molecular weight marker (Broad Range Protein Molecular Weight Marker from Promega) was not heat shocked and loaded as

instructed by the supplier. 5 µg of sample protein (we loaded 0.5 µl when loading crude extract) was applied to each well. Electrophoresis was carried out in 1 x SDS-PAGE running buffer (10 x buffer consists of 1 % SDS, 0.25 M Tris and 1.92 M glycine) at 20 mA until the front of the samples reached the bottom of the gel. The gel was stained for half an hour with coomassie blue R-250 staining solution (2.5 % Coomassie brilliant blue R-250, 45 % methanol and 10 % acetic acid). De-staining was carried out in two changes of de-staining solution (45 % methanol, 10 % acetic acid).

4.4.5. Determination of Kinetic Parameters

Pantothenate kinase activity was measured in a continuous fashion by coupling the production of ADP to the reactions catalyzed by pyruvate kinase and lactate dehydrogenase, and monitoring the decrease in NADH concentration spectrophotometrically at A_{340} (Figure 4.8).

All reactions were performed at 25°C. Kinetic parameters were determined by global non-linear fitting of the initial rate data at varying ATP and pantothenate concentrations to the general equation for a steady-state bireactant model (*11*):

$$\frac{V}{[E]} = \frac{k_{cat} \cdot [A] \cdot [B]}{(K_{iA} \cdot K_B + K_A \cdot [B] + K_B \cdot [A] + [A] \cdot [B])}$$

where A is ATP, B is pantothenate, K_A and K_B are the Michaelis constants for ATP and pantothenate respectively and K_{iA} is the enzyme inhibition constant for the reverse reaction.

Each 300 µl reaction mixture contained 100 mM HEPES (pH 7.6), 20 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 0.3 mM NADH, 5 U of lactate dehydrogenase, 2.5 U of pyruvate kinase and 4.5 µg of pantothenate kinase. ATP concentrations were varied between 0.5 and 15 mM and pantothenate concentrations between 5 and 500 µM. Reactions were initiated by the addition of pantothenate.

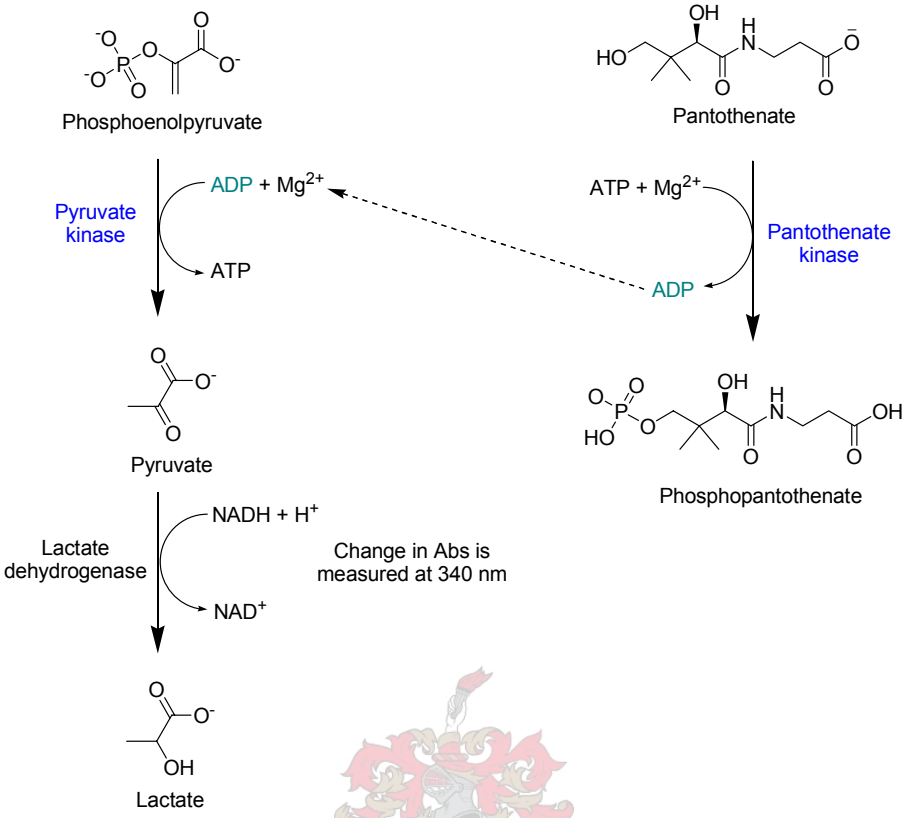


Figure 4.8. Schematic representation of the reactions involved in the pantothenate kinase assay. The ATP-dependent phosphorylation of pantothenate to phosphopantothenate produces ADP and uses Mg^{2+}. The ADP produced is fed into the reaction catalysed by pyruvate kinase, which dephosphorylates PEP producing pyruvate. Finally pyruvate is reduced to lactate in a reaction that oxidises NADH. This oxidation of NADH can be monitored at A_{340} and this is how the activity of pantothenate kinase is quantified.

4.4.6. Inhibition Studies

Reaction mixtures for inhibition studies were identical to those described above except that the pantothenate (500 μM) and ATP (5.0 mM for *Bs*CoaX, 10.0 mM for *Hp*CoaX and 1.5 mM for *E. coli* PanK) concentrations were kept constant. Inhibitors (CoA, acetyl-CoA or *N*5-pantothenamide) were added at concentrations between 10 and 500 μM and the reaction was initiated by the addition of pantothenate.

*N*5-pantothenamide was also tested as a substrate for *Bs*CoaX, *Hp*CoaX and *S. aureus* PanK. These reactions were performed as described above but pantothenate was substituted with *N*5-pantothenamide and ATP concentrations remained constant at 10.0 mM for *Hp*CoaX and *Bs*CoaX and 1.5 mM for *S. aureus* PanK.

4.4.7. Testing of Alternate Phosphoryl Donors

To determine whether other phosphate-containing compounds could substitute for ATP in the pantothenate kinase reaction catalyzed by *Bs*CoaX and *Hp*CoaX, reaction mixtures containing 50 mM Tris pH 7.6, 20 mM KCl, 10 mM $MgCl_2$, 5 µg of enzyme, phosphate donor (1.5 mM of either ATP, UTP, CTP, GTP, phosphoserine or phosphothreonine or 10 mM of either acetylphosphate or carbamoylphosphate) and 500 µM pantothenate were incubated at 37ºC for 2 hours. The reactions were placed at 95ºC for 5 min to denature the protein, followed by centrifugation at 13,000 rpm for 5 min. The supernatant was applied to a column of Dowex 50WX8-100 resin and rinsed with deionized water. The solvent was subsequently evaporated from the combined eluate in a speed vac concentrator. The dried samples were resuspended in 100 µl of 50% aqueous acetonitrile and analyzed by ESI-MS by direct infusion into the instrument at a rate of 20 µl/min. Phosphoenolpyruvate was tested as a phosphate donor by the two enzyme coupled assay described in 4.4.5. The exception was that ATP was replaced with 1.5 mM PEP, pyruvate kinase was left out and only one pantothenate concentration was used, namely 500 µM.

4.5. References

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Chapter 5

Mutation Studies of the Type III Pantothenate Kinase from *H. pylori*

5.1. Introduction

This chapter reviews the literature available to date on the classification of the Type I pantothenate kinase into the Rossmann-like fold group of kinases. In addition, we present evidence for the placement of the Type II and III PanKs into the ribonuclease H-like family of kinases. By placing these PanKs into this fold group certain conclusions can be drawn with regard to their active sites when compared to other members of this fold group with solved structures. In particular, amino acid residues within the active site are identified as conserved in members of this fold group. This chapter investigates the consequences of mutational studies on these conserved amino acid residues.

5.2. Structural Analysis of Kinases

Kinases are a group of enzymes that catalyze the phosphoryl transfer reaction from a phosphate donor (usually ATP) to a receptor substrate (*1*). In order to investigate the relationship between structural fold and functional specifications in all known kinases Cheek *et al*. (*1*) carried out a comprehensive analysis of all the available kinase structures and sequences. These were then classified into structure/sequence families with evolutionary implications. In addition to this they predicted the structures of some kinases with unknown structures. In so doing, possible biochemical roles are suggested by the function of these proteins' homologues (*1*).

In order to create this kinase classification the authors constructed a list of all the Pfam[5] profiles and COGs that describe catalytic kinase domains (*2, 3*). After extensive analysis to remove redundant entries a list containing 57 kinase profiles was created (44 from Pfam and 12 from COG). After all the kinases sequences were assigned to Pfam/COG profiles or to novel groupings, PSI-BLAST[6] (*4*) was used to detect possible evolutionary links between the profiles. Sequences from different Pfam/COG profiles with statistically significant similarities were identified and assembled into families. In this way, homology is inferred to all sequences in the same family. The authors found that in most cases finding these links was trivial (a trivial link is one that is established by three iterations of PSI-BLAST (*4*) with *E*-value cut-off 0.001). For orphan Pfam/COG profiles or sequence groupings, multiple alignments were constructed in order to reveal conserved active-site motifs. Secondary structure predictions with Jpred[7] (*5*) and manual inspection of PSI-BLAST (*4*) search results were performed. In some cases, orphan groupings could be placed into existing families while in others they had to be assigned to novel kinase families. Only kinases that use ATP as a phosphate donor were used as subjects in this study (*1*).

A total of 30 kinase families were classified. Of these kinase families, 19 contained at least one member with solved structure. These 19 families could be assembled into seven fold groups on the basis of similarities of structural fold. Families in the same fold group share structurally similar nucleotide-binding domains that are of the same architecture and topography for at least

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[5] Pfam is a web-based database containing a large collection of protein multiple sequence alignments and profile hidden Markov models for finding domains in new sequences. COG is a database of Clusters of Orthologous Groups of proteins. The function of this database is to attempt to phylogenetically classify the proteins encoded by complete genomes.

[6] BLAST is an algorithm that is used to search protein and DNA databases for sequence similarities. PSI in PSI-BLAST stands for Position-specific iterated BLAST and runs at the same speed per iteration as gapped BLAST, but in many cases is more sensitive to weak but biologically relevant sequence similarities.

[7] Jpred is a server for the prediction of a consensus secondary sequence structure from an amino acid sequence.

Table 5.1. Classification of kinase activity by fold group (*6*).

Group	Examples of Kinase activities
Group 1**: Protein S/T-Y kinase/ atypical protein kinase/ lipid kinase**	**Choline Kinase**
Group 2**: Rossmann-like**	**Pantothenate kinase Type I (P-loop kinase family)**
Group 3**: Ferredoxin-like fold kinases**	**Creatine kinase**
Group 4**: Ribonuclease H-like**	**Pantothenate kinase Type II Pantothenate kinase Type III Hexokinase**
Group 5**: TIM β/α-barrel kinases**	**Pyruvate Kinase**
Group 6**: GHMP kinase**	**Galactokinase**
Group 7**: AIR synthetase like**	**Thiamine-phosphate kinase**
Group 8**: Riboflavin kinase**	**Riboflavin kinase**
Group 9**: Dihydroxyacetone kinase**	**Glycerone kinase**
Group 10**: Putative glycerate kinase**	**Glycerate kinase**
Group 11**: Polyphosphate kinase**	**Polyphosphate kinase**
Group 12**: Integral membrane kinases**	**Dolichol kinase**

the core of the domain. The remaining ten groups are composed of the 11 families that contain no members with solved structures (*1*). A revision was made to this study in 2005 (*6*) where the number of kinase families was reduced to 25, wherein 22 families for which the three-dimensional structures are known fall into 10 groups. In addition, two novel kinase structural folds were highlighted bringing the total number of fold groups to 12 (Summarized in Table 5.1) (*6*).

To summarize, comparative analysis of the protein structural folds allows for the inference of biochemical and biological functional properties among kinases. Structure analysis methods are able to detect evolutionary relationships that sequence similarity searches miss because protein structure conservation persists after sequence similarity disappears. One must still consider however, that similarity of fold alone does not necessarily indicate a common ancestor (*1*).

5.3. Fold predictions for Type I, II and III Pantothenate Kinases

5.3.1. Type I PanK

The crystal structure of the Type I pantothenate kinase identifies it as a member of the P-loop kinase family within the Rossmann-like fold group (Group 2) of kinases. The P-loop family constitutes the largest family in the Rossmann-like fold group. This family contains one three-layered (α/β/α) domain. In the majority of the P-loop kinases, the central parallel β-sheet is five-stranded with strand order 23145. Nucleotide binding in this family is distinguished by the presence of the conserved Walker A (GXXXXGKT/S) and Walker B (ZZZZD, where Z is any hydrophobic residue) motifs (*7*). The Walker A motif forms a phosphate binding loop (P-loop) and is found in a variety of different proteins that bind nucleotides (*8*). In this family of kinases, the P-loop is located at the end of the first β-strand and includes the first half turn of the following α-helix. The conserved lysine residue of the Walker A motif binds to and orientates oxygen atoms of the β- and γ-phosphate groups of ATP. The essential magnesium cation is coordinated directly by the hydroxyl group of the conserved threonine/serine of the Walker A motif and indirectly by the conserved aspartate residue of the Walker B motif. The Walker A and B motifs are common in proteins that bind nucleotides such as pantothenate kinase (*9*).

5.3.2. Type II PanK

The crystal structure of the eukaryotic pantothenate kinase is unknown. Due to the lack of sequence identity between the prokaryotic (Type I PanK) and eukaryotic (Type II PanK) analogues of pantothenate kinase (*10*) in conjunction with dissimilar predicted secondary structure patterns, the Type II PanK is predicted to adopt a different fold pattern to its Type I counterpart.

Cheek *et al.* (*6*) report that standard sequence similarity search methods failed to obtain any reasonable structural assignment; however, several fold recognition servers strongly suggest that the Type II PanK adopts a ribonuclease H-like fold.

The ribonuclease H-like group contains the ASKHA (acetate and sugar kinase/hsc70/actin) superfamily (*11*). These structures are characterized by the duplicate domains of the ribonuclease H-like fold. The ribonuclease H-like fold is composed of three layers (α/β/α). The five-stranded mixed β-sheet has strand order 32145, with strand 2 antiparallel to the rest of the sheet. The topology of the core of this fold is βββαβαβα. Nucleotide binding and divalent metal coordination are achieved by interactions of the ATP with several motifs conserved within the ASKHA superfamily (*11*). These conserved motifs include the ADENOSINE motif that interacts with ribosyl and the α-phosphoryl group of ATP, the PHOSPHATE 1 motif that interacts with the magnesium ion through coordinated water molecules, and the PHOSPHATE 2 motif that interacts with the β- and γ-phosphoryl groups of ATP. The mechanism of kinases in this group is presumed to be acid-base catalysis.

Thus, based on the presence of these conserved motifs in addition to the similarity of secondary structure patterns (Figure 5.1) within the Type II PanKs, they have been classified as a member of the ribonuclease H-like family (*6*).

5.3.3. Type III PanK

In the same way that the Type II panK enzymes were assigned to the ribonuclease H-like family (*1*), so too were the Type III PanKs. This was based on the lack of sequence identity between the Type I and Type III PanKs as well as their dissimilar predicted secondary structure. All the characteristics of the ribonuclease H-like fold family can be obtained from 5.3.2 above.

Figure 5.1. Type II pantothenate kinase is a ribonuclease H-like kinase. Multiple sequence alignment for representative sequences of the pantothenate kinase family and two related ribonuclease H-like families with known structure (2-hydroxyglutaryl-CoA dehydratase component A (PDB 1hux (*12*)) and hexokinase I (PDB 1dgk (*9*)) is shown. Residue conservation is denoted with the following scheme: uncharged, highlighted in yellow; charged/polar, highlighted in grey; small, red; identical, bold. Dashed boxes indicate the PHOSPHATE 1, PHOSPHATE 2 and ADENOSINE motifs. Abbreviations of species names are as follows: Af *Acidaminoccus fermentans,* At *Arabidopsis thaliana,* Bc *Bacillus cereus,* Ca *Clostridium acetobutylicum,* Ce *Caenorhabditis elegans,* Dm *Drosphila melanogaster,* En *Emericella nidulans,* Hs *Homo sapiens,* Mm *Mus musculus,* Mt *Methanothermobacter thermautotrophicus,* Pa *Pichia angusta,* Ta *Thauera aromatica,* and Tt *Thermoanaerobacter tengcongensis.* Locations of predicted (gi|4191500) and observed (pdb 1hux_A, PDB 1dgk_N) secondary structure elements (E, β-strand; H, α-helix) are marked above the sequences in green and blue font respectively (*6*). Reproduced with permission from Cheek *et al.* (2005) *BMC Structural Biology* ***5***, 6.

Our collaborators[8] have constructed a multiple sequence alignment for representatives of the CoaX proteins (Type III), Type II pantothenate kinase families as well as members of the ribonuclease H-like fold family with known structures. The conservation of the motifs of the ribonuclease H-like families between the CoaX and Type II proteins is noted in Figure 5.2. This multiple sequence alignment helps to identify a number of conserved amino acids within the conserved motifs. Specifically, these alignments identify key aspartate residues that are putatively involved in the binding of Mg^{2+} and the stabilization of the phosphate group of ATP during the course of the pantothenate kinase reaction, and are based on the known interactions of other ribonuclease H-like proteins. Based on the numbering of the amino acids of *Hp*CoaX (line 1 of I in Figure 5.2) it is possible to identify aspartate residues number 17 (in PHOSPHATE 1), 87 and 102 (in PHOSPHATE 2) in particular as conserved. The following section details a representative of the ribonuclease H-like family, hexokinase I. The purpose of this is to highlight the importance of certain amino acid residues in the active site of this protein. Based on the importance of these residues in hexokinase I, comparisons will be made with the corresponding amino acids within the *Hp*CoaX protein. Through mutational studies, we hope to elucidate the importance of these corresponding amino acids in the active site of a representative of the Type III pantothenate kinases, namely *Hp*CoaX. This will allow us to ascertain the behaviour and interaction between enzyme and substrate. The information gained from these studies will provide insight into the function of the Type III pantothenate kinases.

To summarize, even in the absence of solved structures of either the Type II or CoaX-like (Type III) pantothenate kinase proteins it is possible to identify key residues through sequence alignments with proteins of known structure. These are based on the predicted structural homologies identified in

[8] This sequence alignment was compiled by Prof Nick Grishin and Dr Krzysztof Ginalski of the Department of Biochemistry, University of Texas Southwestern Medical Centre, USA. Together with Prof Hong Zhang, a structural biologist from the same department, they have been our key collaborators on the structural aspects of this project.

preliminary studies using fold prediction servers (Figure 5.2). In this chapter, extensive mutagenesis studies of the aspartate residues that are conserved and predicted to be involved in catalysis will be carried out.

5.3.4. Hexokinase I – An Example of a Ribonuclease H-like kinase

Hexokinase I is the pacemaker (*9*) of glycolysis in brain tissue. It is composed of two structurally similar halves, connected by an α-helix. Hexokinase I catalyzes the phosphorylation of glucose by ATP to produce glucose 6-phosphate (G6P). In brain tissue and red blood cells hexokinase I regulates glucose metabolism. When the active site of hexokinase I (*H. sapiens,* PDB code 1dgk) is in the open conformation, productive association with the adenine nucleotide takes place (*9*) (Figure 5.3).

A loop of amino acids corresponding to residues 532-539 is involved in the opening of the active site. A model has been constructed to explain the binding that takes place within the active site (Figure 5.4). The model for the Mg^{2+}-ATP/glucose (Glc) complex of hexokinase I shares features of metal-ATP complexes of related enzymes (glycerol kinase, actin and hsp70). Thr863 of the ADENOSINE motif (*11*) interacts with the ribosyl and α-phosphoryl groups of ATP, Asp532 (PHOSPHATE I motif, corresponds to Asp17 in *Hp*CoaX) interacts with the Mg^{2+} through coordinated water molecules, and loop 679-681 (PHOSPHATE II motif, this region corresponds to the region housing Asp102 in *Hp*CoaX) has conserved interactions with the β and γ-phosphoryl groups of ATP.

Asp657 (corresponds to Asp87 in *Hp*CoaX) hydrogen bonds with the 6-hydroxyl group of glucose as a proton acceptor, and is a putative catalytic base. The mutation of Asp657 to alanine reduces the k_{cat} by at least 100 fold (*13*). Clearly, these amino acid residues (mentioned above) within the active site of this protein are important to the activity of the enzyme. By comparing the active site of hexokinase, an example of a ribonuclease H-like kinase with a solved structure, with that of the *Hp*CoaX active site we were able to identify

Figure 5.2. Multiple sequence alignment for representative sequences of the CoaX and eukaryotic (Type II) pantothenate kinase families, Group I and II, respectively, mapped on the representatives of the actin/hsp70/sugar kinase superfamily with known structures (Group III). Sequences are labelled according to gi number or PDB code and species name. The first and last residue numbers are indicated before and after each sequence with the lengths of insertions specified in parentheses and the total sequence lengths of raptor proteins following in square brackets. Residue conservation is denoted with the following scheme: uncharged, highlighted in yellow; charged/polar, highlighted in grey; small, red; identical, bold. The PHOSPHATE 1, PHOSPHATE 2, and ADENOSINE motifs are indicated at the bottom of the alignment. Locations of predicted (gi15611833 and gi|4191500) and observed (PDB|1hux_A) secondary structure elements (E, β-strand; H, α-helix) are marked above the sequences in green and blue capital font, respectively. Abbreviations of species names are as follows: Hp *Helicobacter pylori*, Ps *Pseudomonas syringae*, Dv *Desulfovibrio vulgaris*, Ch *Cytophaga hutchinsonii,* Bc *Bacillus cereus*, En *Emericella nidulans*, Mm *Mus musculus*, Ce *Caenorhabditis elegans*, Hs *Homo sapiens*, Sa *Staphylococcus aureus*, Af *Acidaminococcus fermentans*, Tm *Thermotoga Maritima,* Ec *Escherichia coli*

Asp residues within the *Hp*CoaX active site that we suspected of being involved in catalysis. Mutation of these aspartate residues would enable us to ascertain their relevance in the active site of *HpCoaX* and infer this importance to other as yet unclassified homologues of the *Hp*CoaX protein.

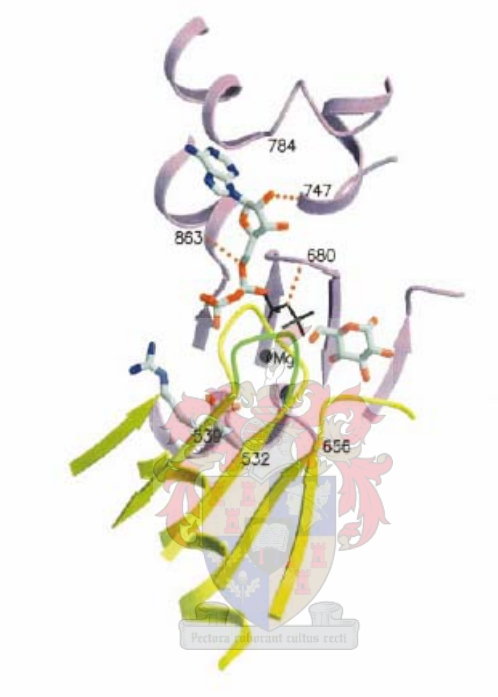


Figure 5.3. The ADP/Glc complex of hexokinase. The large domain of Hexokinase is light purple and the small domain yellow. Dotted red lines indicate donor-acceptor interactions. The β-phosphoryl group of ADP is not productively bound in the ADP/Glc monomer complex due to the steric influence of loop 532-539. In the conformation observed for the open C-half (green), however, loop 532-539 allows productive binding of the β and γ-phosphoryl groups of ATP (black lines) with Mg^{2+} (black sphere) (*9*). (Figure reproduced with permission from Elsevier, *J. Mol. Biol.*, ***296***, Aleshin *et al.,* Crystal Structures of Mutant Monomeric Hexokinase I Reveal Multiple ADP Binding Sites and Conformational Changes Relevant to Allosteric Regulation, 1001-1015, (2000)).

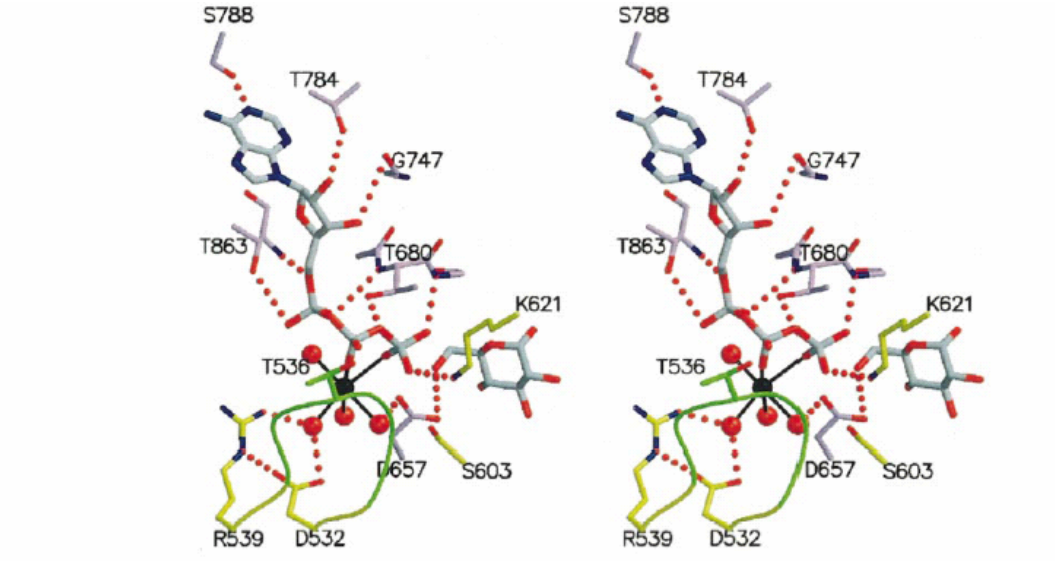


Figure 5.4. Stereoview of the Mg^{2+}-ATP/Glc complex of the active site of hexokinase I. The illustration is based on a model derived from the ADP/Glc monomer complex of Hexokinase I. Structural elements in purple and yellow are from the large and small domains, respectively, of the C-terminal half of Hexokinase I. Elements in green represent loop 532-539 in its open conformation. Dotted red lines designate donor-acceptor interactions. Mg^{2+} and its coordinate bonds are black, with four red spheres representing coordinated water molecules. The 6-hydroxyl group of Glc is in line with respect to the γ-phosphoryl group of ATP, with Asp657 acting as a catalytic base (*9*). (Figure reproduced with permission from Elsevier from Aleshin *et al.* (2000) Crystal Structures of Mutant Monomeric Hexokinase I Reveal Multiple ADP Binding Sites and Conformational Changes Relevant to Allosteric Regulation, *J. Mol. Biol.*, ***296***, 1001-1015).

5.4. Results and Discussion

5.4.1. Critical Residues Involved in Enzyme Activity

Although their properties are very different, the Type II and III PanK's are predicted to belong to the same fold group, namely the ribonuclease H-like fold group. Sequence alignments were performed between representatives of the CoaX Type III pantothenate kinases, their Type II counterparts and proteins belonging to the same fold superfamily with known structures. The resulting sequence alignment (Figure 5.2) identifies a number of aspartate residues that are highly conserved in many of sequences compared.

Aspartate residues corresponding to *H. pylori* (Line 1 of group I in Figure 5.2) amino acid residues number 17, 87 and 102 are highly conserved and possibly involved in catalysis.

5.4.2. Mutational Analysis

As stated above, three aspartate residues were identified for mutation studies. These aspartate residues were mutated to both asparagine and glutamate (Figure 5.5).

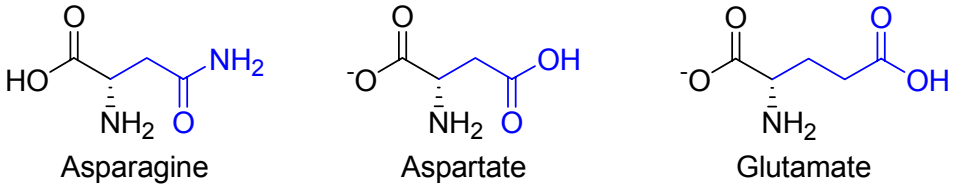


Figure 5.5. A comparison of the structures of asparagine, aspartate and glutamate. The side chains of the amino acids are coloured in blue (*14*).

Mutation from aspartate to asparagine is experimentally meaningful as these two amino acids are identical except that in asparagine, an amide group replaces the γ-carboxyl group of aspartate and results in the side chain being polar but uncharged. In contrast, aspartate is negatively charged at physiological pH. This change clearly has repercussions with regard to the chemical property of the side chain and therefore could influence the activity of the protein if the selected amino acids are involved in catalysis. If the net negative charge was the pre-requisite for enzyme action then making this change should seriously hamper enzyme activity. Negatively charged amino acids play several roles in proteins. For example, proteins that bind metal ions (like PanK) often possess metal binding sites containing one or more aspartate or glutamate residues. Thus, by making this change we are probing for the importance of the ionic nature of the aspartate residue in question.

Mutation from aspartate to glutamate retains the acidic nature of the amino acid side chain but increases the tether length by one methylene group (Figure 5.6). Glutamate, like aspartate, is also negatively charged at

physiological pH. Thus by making this change we are probing for the importance of the negative charge at exactly the right position within the active site. The binding pockets of most proteins are highly specific and uniquely designed to accommodate and bind their substrates. Any interference in the spatial arrangement of the substrates in the active site could hamper the activity of the enzyme in question.

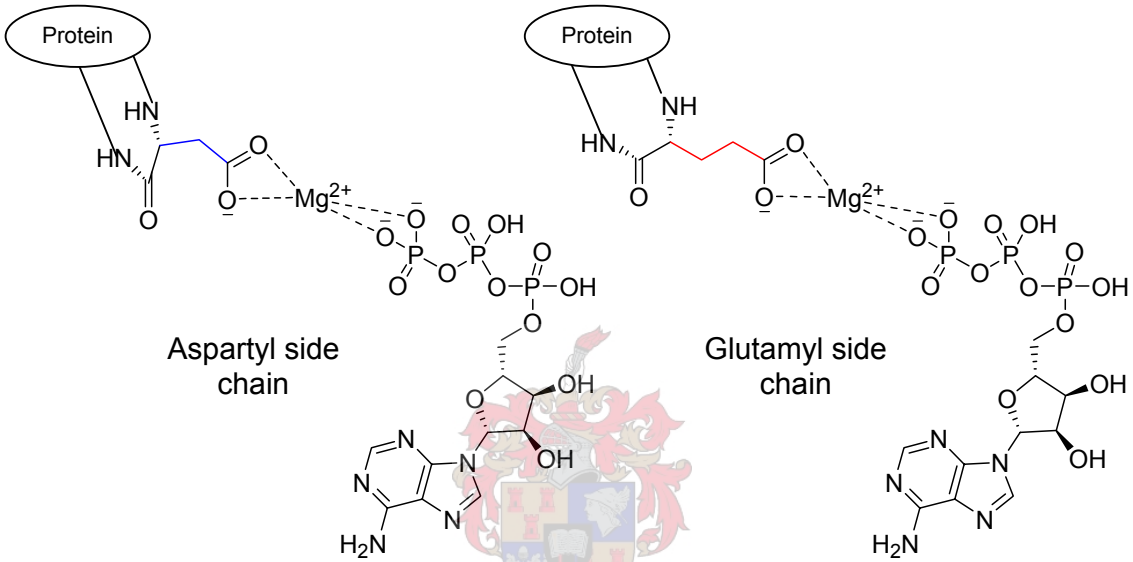


Figure 5.6. A rudimentary diagram of the interaction between an aspartate side chain (left) and glutamate side chain (right) and the γ-phosphoryl of ATP, facilitated by a magnesium ion. Because of the additional methylene group in glutamate (highlighted in red), the distance between the amino acid and the ATP molecule is shorter, while in the case of aspartate (highlighted in blue) the distance is longer. This could cause a small measure of steric hindrance in the active site of the enzyme and explain why, in the case of pantothenate kinase, aspartate is the acidic amino acid of choice.

Thus, the increased tether length of the side chain of glutamate could have spatial implications in the active site of the protein. Consider Figure 5.6 above, where one can see that replacing an aspartate residue with a glutamate residue increases the space required in the binding pocket of the enzyme. While this is a miniscule difference, the architecture of enzymology is so acutely specific that perhaps the addition of just a single methylene group in the side chain required to bind the magnesium ion could hamper enzyme activity.

5.4.3. Purification of Mutant Proteins

Mutagenesis PCR was conducted on the original overexpression vector (pET28a-*Hp*CoaX) containing the *H. pylori coaX* gene insert. The mutagenesis PCR reactions changed the residues Asp17, Asp87 and Asp102 to either asparagine or glutamate.

The success of the mutagenesis reactions was confirmed by automated DNA sequencing. In the case of the mutation of residues 17 and 87 from aspartate to glutamate we experienced difficulties with the above procedure. As the asparagine mutants had already been cloned and verified by automated sequencing at this point, we used these plasmids as templates for the mutational PCR reaction. This proved successful for both of the outstanding mutants.

The mutant plasmids were transformed into *E. coli* BL21 Star(DE3) and the proteins overexpressed and purified using immobilized metal affinity chromatography. The purity of the mutant proteins in relation to native *Hp*CoaX is depicted in Figure 5.7. For one protein, namely *Hp*CoaX(D17E), expression conditions could not be created where the protein expressed successfully. The presence of the mutated gene was verified through sequencing, but under no conditions was there protein present in the crude extract. We investigated the possibility that the replacement of the aspartate with a glutamate residue resulted in the introduction of a protease site. However, this was not the case in our *E. coli* expression system. Other possibilities for the lack of expression include instability of the protein, the formation of inclusion bodies after expression or degradation of the protein after expression. These factors have to be investigated further as we would like to be able to include this protein in our mutant analysis. Whatever the cause of the lack of expression may be, this protein was not purified and thus was not part of our study as initially planned. The purity of the remaining mutants as compared to native protein is illustrated in the SDS-PAGE gel depicted by Figure 5.7.

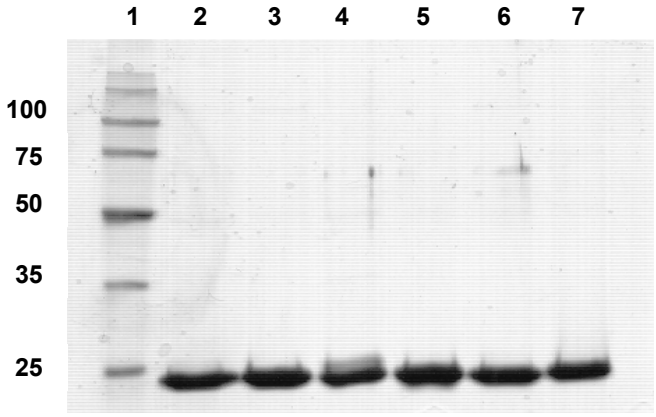


Figure 5.7. SDS-PAGE analysis of the purity of the *Hp*CoaX mutants as compared to native protein. *Lane 1*: Molecular weight markers (kDa). *Lane* 2: native *Hp*CoaX. *Lane* 3: *Hp*CoaX(D17N). *Lane* 4: *Hp*CoaX(D87N). *Lane* 5: *Hp*CoaX(D87E). *Lane* 6: *Hp*CoaX(D102N) and *Lane* 7: *Hp*CoaX(D102E).

5.4.4. Activity of CoaX Mutants

The purified proteins were assayed for their ability to catalyze the ATP-dependent phosphorylation of pantothenate using the same two enzyme-coupled assay that links the production of ADP to the oxidation of NADH. This allows the enzyme activity to be measured continuously by monitoring the change in A_{340}. We chose a final ATP concentration of 15 mM and the reactions were initiated by the addition of 500 µM pantothenate.

The results indicate that when compared to native protein activity, mutation of the aspartate residue (to either asparagine or glutamate) at any of the three sites seriously hampers the activity of the protein (Table 5.2 and Figure 5.8).

One would expect to see a marked decrease in activity when an aspartate is mutated to an asparagine if the negative charge on the γ-carbonyl of aspartate is required for metal binding and catalysis, while a decrease in activity in the case of the glutamate mutants would indicate the importance of the location of the negative charge in the active site.

Table 5.2. Rate and percentage remaining activity of mutants compared to native *Hp*CoaX.

Hp variant	Activity (nmoles.sec^{-1}.µg^{-1})	% Relative remaining activity
***Hp*CoaX**	0.035	100
***Hp*CoaX(D17N)**	0.000	0
***Hp*CoaX(D87N)**	0.011	32
***Hp*CoaX(D87E)**	0.012	35
***Hp*CoaX(D102N)**	0.010	28
***Hp*CoaX(D102E)**	0.015	43

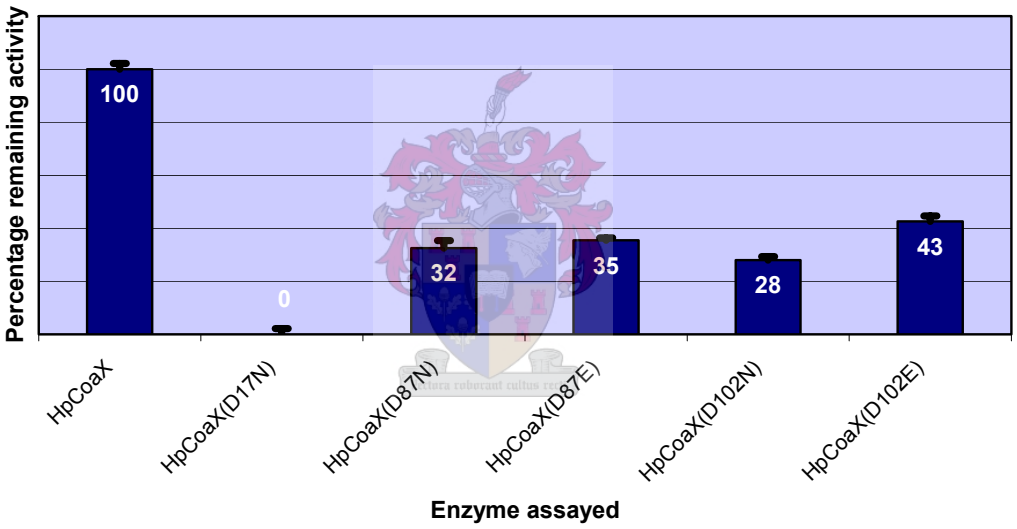


Figure 5.8. The percentage remaining activity of mutant CoaX proteins compared to that of the native CoaX protein. None of the mutated proteins maintains even 50 % of the activity of the native protein. In fact, mutation at aspartate residue 17 completely abolishes enzyme activity.

We found that the proteins where the aspartate residues were mutated to asparagine show a greater decrease in activity when compared to the same residues mutated to glutamate (32 % for *Hp*CoaX(D87N) as compared to 35 % for *Hp*CoaX(D87E) and 27 % for *Hp*CoaX(D102N) as compared to 42 % for *Hp*CoaX(D102E). However, mutations to glutamate were still far less active

than the native protein. This supports the suggestion that the absolute position of these aspartate residues at the chosen sites also plays a role in the optimum function of the *Hp*CoaX enzyme. The differences in remaining activity between asparagine and glutamate mutations at any one site were not at all significant suggesting that changing the charge or the position of the negative charge of the amino acid side chain was sufficient to decrease activity of the *Hp*CoaX protein by more than 50 %.

In particular, mutation of the aspartate residue at position 17 to asparagine completely abolishes protein activity. This aspartate residue is clearly essential to enzyme function. This aspartate residue is predicted, like Asp 532 in hexokinase I, to interact with the Mg^{2+} through coordinated water molecules. Magnesium is a vital metal cofactor for the activity of pantothenate kinase and it comes as no surprise that blocking its interaction with the active site abolishes enzyme activity.

According to Figure 5.2 the amino acid at the position corresponding to *H. pylori* Asp87 is glutamate in all but one of the kinases with known structure. In the case of hexokinase I (subheading 5.4) mutation of this glutamate residue reduced the k_{cat} of the enzyme 100 fold (*9*). This residue in hexokinase I is a suspected catalytic base. It is possible that the corresponding aspartate in *Hp*CoaX (Asp 87) has a similar function. Whatever the case may be mutation of this residue to either glutamate or asparagine decreases the activity of the enzyme dramatically. This could be due to the lack of charge of the asparagine mutant or the incorrect placement of the charge of the glutamate mutant.

The smallest difference in remaining activity is present in the mutations at aspartate position 87, with *Hp*CoaX(D87N) having a remaining activity of 32 % as a compared to 35 % remaining activity for *Hp*CoaX(D87E). In hexokinase I, the corresponding amino acid (Asp657) is involved with binding of glucose, the enzymes' main substrate (*13*). However, it is not the only amino acid residue that is involved in this function. It is possible that the same is true for

Asp87 in *Hp*CoaX and mutating this residue alone is not enough to abolish this function of the enzyme.

Aspartate residue 102 is putatively involved in phosphate binding (Figure 5.2). Hexokinase I does not have an aspartate residue at this corresponding site, instead it has an isoleucine (non-polar/hydrophobic) (*6, 14*). The loop of residues from 679-681 containing this isoleucine make up part of the PHOSPHATE II motif. This motif is responsible for the conserved interactions between the β and γ-phosphoryl groups of ATP. All the other kinases in the group with known structure have an aspartate at this position. As a result of the implied structural homology, we can conclude that Asp102 of *Hp*CoaX is also involved with phosphate binding. Asparagine cannot perform this function and that may be why its activity is lower than that of the same glutamate mutant. It is possible that steric hindrance is the reason that the glutamate residue is not as successful in this role as the native aspartate. Because of the presence of another aspartate residue involved in phosphate binding (Asp17 in PHOSPHATE I), mutation of just the single residue at Asp102 (PHOSPHATE II) may not be enough to abolish activity. It appears that the level of importance of phosphoryl binding is not equal between the two residue positions. Mutation of the aspartate at residue 17 completely abolishes activity, while mutation of the aspartate residue at position 102 simply lowers activity. This points to a hierarchy, where Asp17 is more important than Asp102 in phosphoryl binding.

Taken together, the results indicate that all three of the residues chosen for mutation are involved in the functionality of *Hp*CoaX. This is evidenced by the fact that a mutation at any one of these residues causes a decrease of activity of more than 50 %.

5.5. Conclusion

By comparison to proteins within the ribonulcease H-like family we were able to identify the presence of conserved aspartate residues within the active site of *H. pylori* PanK. Aspartate residues numbers 17, 87 and 102 in *H. pylori* were mutated to both asparagine and glutamate. We expected residual activity to remain in the glutamate mutants as both aspartate and glutamate are acidic amino acids and have a very similar structure. However, even in the case of the two glutamate mutants (D87E and D102E), activity was significantly reduced. This is explained by the fact that glutamate has an additional methylene group and does not comply with the spatial constraints of the active site and thus cannot bind substrate as efficiently as aspartate can. The total eradication of activity caused by mutation of Asp residue 17 implies the absolute necessity of an aspartate residue at this position in the active site of *Hp*CoaX.

As predicted, mutation to asparagine at the identified aspartate sites decreased the activity of the enzyme further. In fact, mutation of aspartate residue number 17 to asparagine completely abolishes enzyme activity. The effect of mutating Asp102 to asparagine also had a more pronounced effect than the same mutation to glutamate. This aspartate residue is predicted to be involved in interactions with the β and γ-phosphoryl groups of ATP. Mutation of this residue to asparagine decreases the activity of the protein. This means that even though the binding of the phosphates of ATP to the enzyme is impaired it is not abolished and the phosphorylation of pantothenate can still take place, albeit more weakly.

It is unclear what the exact function of aspartate residue number 87 is. It is possible that, like its counterpart in Hexokinase I, it is involved in the binding of its main substrate, namely pantothenate. It clearly does have some relevant function as mutation of this residue seriously hampers the function of the protein. This mutant showed the highest of levels of remaining activity in both the asparagine and glutamate mutants. This is surprising as the majority

of residues corresponding to this one in group II and III (Figure 5.2) are naturally glutamate residues. All the corresponding amino acids in the CoaX like group (group I Figure 5.2) are aspartates. It is possible that this is a key difference in the structure of the pathogenic bacteria as compared with the other, mainly eukaryotic pantothenate kinases, belonging to the ribonuclease H-like group of proteins.

These mutation studies indicate that the placement of the Type III PanKs into the ribonuclease H-like fold family is justified. This is supported by the fact that the mutation of conserved amino acids identified by sequence homology impairs the activity of the resulting proteins.

5.6. Methods and Materials

5.6.1. Materials

All chemicals were purchased from Aldrich, Sigma or Fluka and were of the highest purity. Other materials were from the following suppliers: Pyruvate kinase and lactate dehydrogenase enzymes from Roche; restriction endonucleases and *Pfu* DNA polymerase from Fermentas; Bradford dye reagent from Biorad; oligonucleotides from Inqaba Biotechnology (Pretoria, South Africa); and HiTrap columns from Supelco. Automated DNA sequencing was performed by Inqaba Biotechnology (Pretoria, South Africa). Proteins were purified on an ÄKTA Prime instrument (Amersham Biosciences). Enzyme assays were performed on a Multiskan Spectrum multiplate spectrophotometer (ThermoLabsystems), using an extinction coefficient of 6220 $M^{-1}.cm^{-1}$ for NADH.

5.6.2. Mutant Construction

The *coaX* gene in plasmid pET28a encoding the wild type *H. pylori* CoaX protein was mutated by PCR to introduce the mutations at aspartate positions 17, 87 and 102 in the protein sequence. The pET28a-*Hp*CoaX plasmid cloned in section 4.4.2 was used as template for the PCR reactions except for

the PCR reactions creating the mutants D17E and D87E, where the plasmids pET28a-*Hp*CoaX(D17N) and pET28a-*Hp*CoaX(D87N) respectively, were used as templates. The primers for the above PCR reactions were as follows:

D17N:
Forward Primer: 5'-TTTGAAAAACCTGGTTTTATGCAATATAGGTAATACGCGCATCC-'3
Reverse Primer: 5'-GGATGCGCGTATTACCTATATTGCATAAAACCAGGTTTTTCAAA-'3
D17E:
Forward Primer: 5'-AACCTGGTTTTATGCGAGATAGGTAATACGCGCATC-'3
Reverse Primer: 5'-TGCGCGTATTACCTATCTCGCATAAAACCAGGTT-'3
D87N:
Forward Primer: 5'-GGGCTTGGGATAAACCGGCAAATGGCGTG-'3
Reverse Primer: 5'-GCCATTTGCCGGTTTATCCCAAGCCCTAC-'3
D87E:
Forward Primer: 5'-TTGGGATAGAGCGGCAAATGGCGTG-'3
Reverse Primer: 5'-GCCATTTGCCGCTCTATCCCAAGCC-'3
D102N:
Forward Primer: 5'-GTGGTGGTGAATTCGGGGAGCGCGA-'3
Reverse Primer: 5'-CGCTCCCCGAATTCACCACCACGCC-'3
D102E:
Forward Primer: 5'-GTGGTGGAGGCGGGGAGCGCG-'3
Reverse Primer: 5'-GCTCCCCGCCTCCACCACCAC-'3

The resulting expression vectors were named pET28a-*Hp*CoaX(D17N), pET28a-*Hp*CoaX(D17E), pET28a-*Hp*CoaX(D87N), pET28a-*Hp*CoaX(D87E), pET28a-*Hp*CoaX(D102N) and pET28a-*Hp*CoaX(D102E).

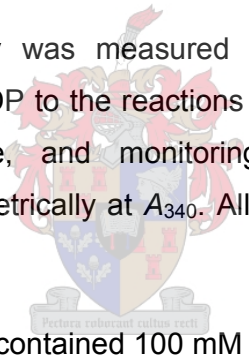
5.6.3. Protein Purification

The pET28a-*Hp*CoaX mutants were transformed into *E. coli* BL21 Star(DE3) (Invitrogen) and overnight cultures (500 µl) of these transformants were used to inoculate LB media (500 ml) supplemented with 30µg/ml kanamycin sulfate. Cultures were grown at 37°C to an A_{600} of ~0.6 and subsequently induced by the addition of 100 µM IPTG. After growing overnight at 37°C and shaking at 200 rpm, the cells were harvested by centrifugation, suspended in sonication buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9), using 10 ml/g cell paste), disrupted by sonication, and centrifuged at 15 000 x g for 30

min to clarify the cell-free extract. The extract was applied to a 1 ml HiTrap chelating column prepared as described by the suppliers. Weakly bound proteins were removed by washing with sonication buffer, followed by sonication buffer containing 60 mM imidazole. Protein was eluted by using sonication buffer containing 500 mM imidazole. The purified protein solutions were exchanged to gel filtration buffer (5 mM $MgCl_2$, 25 mM Tris pH 8.0 and 5% glycerol) using HiTrap desalting columns pre-equilibrated in the same buffer. The protein concentration was determined by the Bradford method using bovine serum albumin as standard. Aliquots of the purified proteins were stored at -80°C. SDS-PAGE gel analysis was conduced as described section 4.4.4.

5.6.4. Pantothenate Kinase Assays

Pantothenate kinase activity was measured in a continuous fashion by coupling the production of ADP to the reactions catalyzed by pyruvate kinase and lactate dehydrogenase, and monitoring the decrease in NADH concentration spectrophotometrically at A_{340}. All reactions were performed at 25°C.



Each 300 μl reaction mixture contained 100 mM HEPES (pH 7.6), 20 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 0.3 mM NADH, 5 U of lactate dehydrogenase, 2.5 U of pyruvate kinase 15 mM ATP and 4.5 μg of mutant pantothenate kinase. Reactions were initiated by the addition of 500 μM pantothenate.

5.7. References

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Chapter 6

Concluding Remarks and Future Research Possibilities

6.1. Summary of Findings

Pantothenate kinase (PanK) catalyzes the first step in the biosynthesis of the essential and ubiquitous cofactor coenzyme A (CoA) in all organisms. Two well-characterized analogues of the enzyme are known: Type I, a prokaryotic PanK, exemplified by the enzyme from *E. coli*, and Type II, a eukaryotic analogue that has primarily been characterized from mammalian sources. Curiously, the genomes of certain pathogenic bacteria, including *H. pylori* and *P. aeruginosa*, do not contain a pantothenate kinase similar to either analogue, although these organisms possess all the other biosynthetic machinery required for CoA production. In this study we cloned, overexpressed and characterized an enzyme from *B. subtilis* as well as its homologue from *H. pylori* and show that they catalyze the ATP-dependent phosphorylation of pantothenate. However, the enzymes do not share sequence homology with any known PanK, and neither CoA nor acetyl-CoA inhibits their activity. This stands in contrast with the bacterial and eukaryotic PanK analogues which both share a common mechanism of feedback regulation by CoA and its thioesters. The *B. subtilis* and *H. pylori* enzymes also do not accept the pantothenic acid antimetabolite *N*-pentylpantothenamide as a substrate, nor are inhibited by it, which also distinguishes them from the atypical *S. aureus* enzyme that has been characterized recently. Taken together, these results point to the identification of a third distinct analogue of PanK that accounts for the only known pantothenate kinase activity in pathogens such as *H. pylori* and *P. aeruginosa*.

The Type III pantothenate kinase has unusually high K_M values for ATP. Other possible phosphate donors were tested and although some measure of activity was detected with CTP and GTP none of the donors tested could substitute for ATP.

Aspartate residues involved in catalysis were identified and mutational studies indicate that these residues are critical for the complete functionality of the protein. Mutation to glutamate allows residual activity to remain whereas the same mutation to asparagine causes the activity of the protein to decrease further. Increasing the substrate concentration does not affect the activity of the mutant proteins.

6.2. Future Research Possibilities

6.2.1. High K_M of Type III PanKs

The CoaX enzymes exhibit a very low specificity for ATP. This is due to their surprisingly high K_M values, which is nearly 10 mM in the case of *Hp*CoaX. Such large K_M values can be rationalized in the case of the *Bs*CoaX enzyme that duplicates an activity already embodied by the more typical *B. subtilis* Type I PanK. However, since *H. pylori* depends exclusively on *Hp*CoaX for its CoA biosynthetic needs, the question arises whether the enzyme's low specificity constant for ATP affects the flux through the pathway. Further studies are currently underway to address this question (*1*).

6.2.2. Finding Inhibitors for Type III PanK

The Type III pantothenate kinase is refractory to inhibition by CoA or its thioesters (*1*). This is one of the main differences between it and the other pantothenate kinase analogues classified to date. The only other PanK that shows similar lack of inhibition is that of the recently classified *S. aureus* (*2*).

There is currently a paper in press (*3*) that concentrates on the synthesis of inhibitors of the pantothenate kinase enzyme. The authors synthesized three

series of novel pantothenamide-type analogues based on the known *S. aureus* PanK inhibitors, *N*-pentyl-, and *N*-heptylpantothenamide. The first series of analogues were designed as molecular probes of the PanK binding site. The reason for this was to elucidate the important structure-activity relationships (SAR) within the binding site. The second set of analogues was designed using structural information obtained from the *E. coli* PanK structure by targeting the pantothenate binding site and the adjacent phenylalanine-lined lipophilic binding pocket. The construction of the third series of analogues was driven by the antimicrobial effect of *N*-pentylpantothenamide (*N*5-Pan). The inhibitory effect of this inhibitor is primarily through its conversion to the anti-metabolite ethyldethia-CoA and its further incorporation into an inactive from of the acyl carrier protein. Each of the inhibitors designed was tested on a number of pantothenate kinase analogues including *E. coli* PanK, *S. aureus* PanK, *Aspergillus nidulans* PanK and the murine analogue *Mm*PanK1α (*3*).

Series one demonstrated only modest inhibitory activity, but revealed some relevant structure-activity relationship findings, including stereospecific binding. Series two demonstrated a much higher degree of inhibition for all the PanK analogues tested and the authors reported significant inhibition for analogues containing alkyl substituents. Series three exhibited the most preferential inhibition profile with the highest degree of inhibition in *S. aureus* PanK and *Mm*PanK1α. The results indicate that the *Mm*PanK1α is highly promiscuous in terms of its inhibitors whereas *E. coli* PanK shows far greater selectivity (*3*). This means that selectivity will be a challenge when discovering potent inhibitors directed solely at the bacterial enzyme (*3*).

These researchers measured the efficiency of inhibitors based on the decrease in product (phosphopantothenate) formation. However, if the inhibitors could in fact be phosphorylated by pantothenate kinase then the formation of product would be reduced without the enzyme being inhibited. This highlights the difference between the inhibition of product formation (i.e. competitive substrates) and enzyme inhibition (*3*).

The assay that the authors used to test for inhibition of pantothenate kinase activity was a radioactive assay that used radiolabelled pantothenate and tested for the presence of radiolabelled phosphopantothenate (*3*). This assay does not take into account the possibility that PanK may phosphorylate the inhibitors themselves as alternate substrates. This is indeed the case with *N*5-Pan, which is an alternate substrate for the PanK reaction and which is eventually transformed by the CoA biosynthetic enzymes to the CoA analogue ethyldethia-CoA. This is the template antimicrobial agent that the authors based their inhibitor design on. Nevertheless, the research is valuable insofar as the synthesis of new inhibitors and we would like to conduct a similar study taking into account the possibility of the inhibitors being competitive substrates for the PanK enzymes. As of yet, no inhibitors have been found for the Type III pantothenate kinases. As many of members belonging to this group are pathogenic bacteria and have no alternative pantothenate kinase enzyme in their genomes, finding an inhibitor that specifically targets this group and not other analogues of pantothenate kinase would be invaluable to the formulation of new drugs against pathogenic bacteria. The increasing bacterial resistance to commonly used antibiotics is of growing concern to the healthcare industry. The development and design of new inhibitors would go a long way in developing a means to overcome this dilemma.

6.2.3. Alternate Metal Ions Used by Type III PanK

Our assay for measuring the activity of the Type III PanK relies on its ability to catalyze the ATP-dependent phosphorylation of pantothenate using a two enzyme-coupled assay (lactate dehydrogenase and pyruvate kinase) that links the production of ADP to the oxidation of NADH. This allows the enzyme activity to be measured continuously by monitoring the change in A_{340}. The norm is to use magnesium ions in the buffer when monitoring this reaction. Mg^{2+} is an important metal cofactor in the phosphorylation reaction whereby pantothenate is phosphorylated to phosphopantothenate by pantothenate kinase and ATP. Due to the very high K_M values for the CoaX enzymes we postulated that this unique enzyme might use a metal ion other than

magnesium. We would like to explore this possibility. However, we cannot use our current assay system as we would be using different metal ions and pyruvate kinase uses Mg^{2+} exclusively We will have to adopt an alternative assay in order to follow the reactions with other metal ions.

Jackowski (*4*), who is very well known for her work on the prokaryotic and eukaryotic PanKs, has always used a radioactive assay for monitoring the pantothenate kinase reaction. In this method, pantothenate is radiolabelled and the quantity of radioactive phosphopantothenate (product) formed in the reaction is measured. This method of assaying the activity of the enzyme is highly accurate and reliable. Reactions testing the efficiency of alternative metal ions in *A. nidulans* have been conducted by Calder *et al.* (*5*) using this assay. Here, magnesium was replaced with calcium, cobalt, manganese or zinc. The authors measured the activity of the enzymes in the presence of these metal ions as a function of the activity of the reaction with magnesium. Every alternative metal ion resulted in a decrease in the activity as compared to the activity obtained with magnesium. Zinc and calcium in particular rendered the enzyme almost inactive with percentages of remaining activity less than 3 %. Manganese and cobalt were able to maintain a certain amount of activity, however, neither enabled the enzyme to function above 50 % of its normal activity with the magnesium cation (*5*).

The results indicate that magnesium is the cation of choice for *A. nidulans. A. nidulans* PanK belongs to the Type II pantothenate kinases. Type II and III PanKs are expected to have a similar fold structure and thus functional domains, one of which is involved with magnesium and phosphoryl binding specifically. This being the case, it seems unlikely that CoaX (Type III) will use an alternative cation. However, it is worth exploring and we will conduct experiments to test whether magnesium is the cation of choice for the CoaX proteins.

6.2.4. Crystal structure of the Type III PanK

Complete characterization of the novel CoaX-like pantothenate kinase would rely on the elucidation of the crystal structure of the protein. The crystal structure of CoaX with substrates and inhibitors bound would create an even clearer picture. Our collaborators are currently undertaking to solve the crystal structure of one of the CoaX proteins.

Initial studies aimed at determining the structure of the *Hp*CoaX protein failed due to the production of low diffraction quality crystals. As a result of this we revisited our sequence alignments for the CoaX-like proteins (see Table I in Chapter 3). We decided to clone the c*oaX* genes from three additional putative Type III PanKs, namely, *Thermus thermophilus, Treponema pallidum* and *P. aeruginosa.* Having cloned and determined the expression conditions for these proteins successfully, the plasmids were sent to our collaborators who are currently busy with the structural analysis.

One of the aims of this collaboration is to compare the CoaX protein to its Type I counterparts in regards to structure and mechanism. We would like to elucidate the mechanistic implications of the diverse PanK structures in regards to phosphate transfer and the binding of inhibitors of the enzyme. Our collaboration will not only expand our understanding of the role of small metabolite kinases in central metabolism, but it will also provide for the characterization of an analogue of the PanK enzyme that is structurally and mechanistically distinct from the known PanK analogues.

6.2.5. Cloning and Characterization of Additional Type III PanKs.

As mentioned in the section above we have cloned additional PanKs with predicted Type III structure. Cloning and characterization of these CoaX-like proteins will further broaden our understanding of this new analogue of pantothenate kinase. We can compare the results obtained from *H. pylori* with these new members of the Type III PanK family. Will these PanKs also be refractory to product inhibition; will they also show a high K_M for ATP? This

could lead to a better understanding of the high K_M value recorded for *Hp*CoaX. The more test subjects we have, the more likely it will be that we can draw reliable conclusions about the characteristics of the Type III PanK. The additional three organisms are all interesting in their own right:

Thermus thermophilus

Thermus thermophilus is an extremely thermophilic, halotolerant bacterium that was originally isolated from a natural thermal environment in Japan. The organism has considerable biotechnological potential; many thermostable proteins isolated from members of the genus *Thermus* are indispensable in research and in industrial applications. The reason for this is the genus' high maximum growth temperature of 85 °C. They show high transformation competence and are therefore amenable to genetic manipulation. One particular point of interest is that because of its thermostability, it can produce proteins that will make ideal candidates for use as biocatalysts. This would be particularly interesting if one were to create a system whereby CoA could be produced on an industrial scale. As a predicted member of the Type III PanKs this enzyme is not inhibited by CoA. This is an additional advantage since this would provide an organism that can potentially withstand high temperatures and be refractory to feedback regulation by CoA. This enzyme could be a candidate (along with CoaBCDE) for the "one pot" enzymatic biosynthesis of CoA.

Treponema pallidum

Treponema pallidum is the causative agent of the sexually transmitted disease syphilis. It is a Gram-negative spirochete that is an obligate human parasite. The organism is microaerophilic and requires low concentrations of oxygen. Although the organism initially causes an ulcer at the site of infection, over time it can move throughout the body and cause wide spread damage to the vital organs. Usually the disease is treated with penicillin or tetracycline (*6*). The dose of antibiotic administered depends on the stage of syphilis. There is no vaccine against this bacterium.

Because of this organisms' importance as a human pathogen, it would make a very interesting test species. We would not have to physically culture the bacteria and all expression work would be conducted in *E. coli.* For this reason, we can gain a great deal of insight into an organism that threatens thousands of people, while maintaining a high level of security. Specifically, if this organism has a CoaX-like PanK, it would be very interesting for the testing of novel inhibitors. A recent publication has identified the penicillin binding protein (named Tp47) in *T. pallidum.* In this study the authors concluding remarks indicate their fear that a mutation of this protein would lead to the resistance of the bacterium to the administration of penicillin (*6*). This means that like so many diseases, our current means of treatment would no longer be effective. This lends additional impetus to the design of novel inhibitors for the CoaX enzymes (as discussed in 6.3) above.

Of special interest is the fact that this organism does not have the *coaBC* gene. *CoaBC* encodes a flavin mononucleotide (FMN)-containing bifunctional enzyme responsible for both the 4'-phosphopantotheinoylcysteine synthetase and the 4'-phosphopantotheinoylcysteine decarboxylase activities (*7, 8*) in coenzyme A biosynthesis. It is possible that this enzyme uses pantetheine as a starting substrate for CoA biosynthesis instead of pantothenate and uses the enzyme CoaD to process this further to CoA. We would like to explore this possibility experimentally.

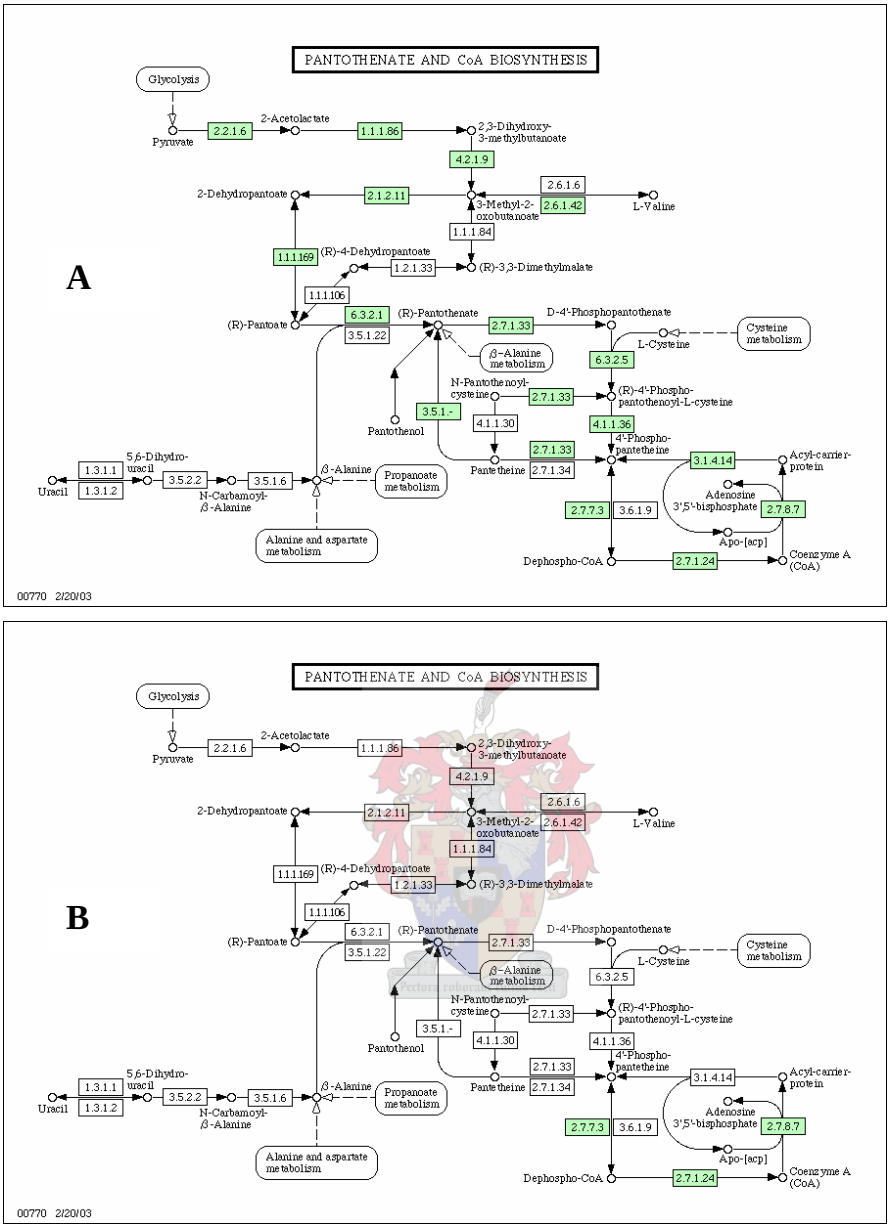

Figure 6.1. A comparison of the CoA biosynthetic pathway between *Escherichia coli* (A) and *Treponema pallidum* (B). Note that *E. coli* is capable of pantothenate biosynthesis, whereas *T. pallidum* is not. In addition to this, *T. pallidum* does not have the gene encoding for the protein CoaBC (genes absent from the organism are unshaded and those present are shaded in green). This means that this organism cannot catalyze the reaction converting 4'-phosphopantothenate to 4'-phosphopantetheine. It does, however, have the gene responsible for encoding the CoaD protein that converts 4'-phosphopantetheine into dephospho-CoA. It is plausible then that *T. pallidum* is capable of CoA biosynthesis with pantetheine as starting product. Reproduced from **http://www.genome.jp/kegg/pathway.html**

Pseudomonas aeruginosa

P. aeruginosa is a Gram-negative bacterium that is noted for its environmental versatility, its ability to cause disease in susceptible individuals, and its resistance to antibiotics. The most serious condition caused by this bacterium is complications related to cystic fibrosis, a respiratory tract infection. Cancer and burn patients also commonly suffer serious infections caused by this organism, as do other individuals with immune system deficiencies. This organism's remarkable ability to survive and adapt in many ecological niches, from water, to soil, plant and animal tissue, as well as its ability to survive on a range of organic compounds as food sources makes it a formidable cause of disease.

P. aeruginosa is widely studied in the academic as well as pharmaceutical fraternity because of its uncanny ability to survive in diverse circumstances and its resistance to antibiotics. The insights gained will be used to develop new antibacterial drugs to treat the infections caused by this bacterium.

Until recently there was no gene homologous to either the Type I or Type II PanK in *P. aeruginosa.* The only gene accounting for pantothenate kinase activity in this organism is the newly characterized CoaX-like PanK (Type III). As this organism poses such a threat in the health care industry it would make an extremely interesting subject for research.

Our long-term goal will be to test inhibitors against all the organisms discussed thus far in the hopes of designing novel antibacterial agents that are highly specific and effective in targeting the Type III pantothenate kinase without harming the Type II PanK that is present in mammalian systems.

6.2.6. Additional mutation studies

After we have received the crystal structure of one of the Type III PanKs we will be able to identify additional amino acids in the active site of the enzyme responsible for activity. From this information we will construct further mutants and test the consequence of the chosen mutations on the activity of

the protein. This will further contribute to the complete characterization of this new pantothenate kinase.

6.2.7. Drug Design

As mentioned in 6.2.2 and 6.2.5 above, microbes are becoming increasingly resistant to antibiotics. This is of great concern to the healthcare industry. As bacteria evolve new means of defying the effect of antibiotics so we too have to develop novel inhibitors. This current research into pantothenate kinase would go a long way to achieving this.

CoA and its thioesters are inhibitors of the pantothenate kinase enzyme in all known analogues except the newly characterized *S. aureus* PanK and the CoaX-type pantothenate kinases. Analogues of these CoA and CoA thioesters have been made and found to inhibit many of the Type I and II PanKs. These results, however, are purely academic, as these CoA analogues cannot be transported over the cell membrane. For this reason, the design of CoA antimetabolites must be based on pantothenic acid, the most advanced cell-permeable biosynthetic intermediate, or its precursors (*9*).

We would like to conduct further inhibition studies with the CoaX-type PanK. Our aim would be to determine novel inhibitors based on the structure of pantothenate. We would like to synthesize many of the analogues used in the studies conducted by Virga *et al.* (*3*) and use these substances as a stating block for our inhibitory studies. The ideal inhibitor must be able to cross the cell membrane and be inhibitory to the pathogenic CoaX proteins only. This would be the ideal candidate for further development as a species and enzyme specific drug. This would truly be a breakthrough in so far as the design of novel inhibitors for pathogenic bacteria.

6.2.8. The Role of CoaX Proteins

The role of CoaX in organisms that possess another PanK analogue is enigmatic. Does it serve as an additional, unregulated form of the enzyme, which is only active under certain conditions? Or is it a latent enzyme activity, which has only been adapted to PanK activity at a later evolutionary stage? Does the CoaX form of the PanK possess unique characteristics that set it apart from the other two analogues of PanK and in so doing; make it ideally suited for adoption by mainly pathogenic organisms for the synthesis of coenzyme A? We hope that further research into this protein will be able to answer some of these burning questions.

6.3. References

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