Biocatalytic production of Coenzyme A Analogues using Cellulose-based Enzyme Reactors

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 study focuses on the development of an immobilized enzyme-based reactor for the chemo-enzymatic preparation of coenzyme A (CoA) analogues. The concept is based on published research proving that CoA biosynthetic enzymes can be used to catalyze the transformation of chemically-synthesized pantetheine analogues to their related CoA analogues. The three CoA biosynthetic enzymes that are used to catalyze the transformation reaction are pantothenate kinase (CoaA), phosphopantetheine adenylyltransferase (CoaD) and dephospho-coenzyme A kinase (CoaE).

In this study we discuss the construction of fusion proteins of these three biosynthetic enzymes from two different bacterial sources (*Escherichia coli* and *Staphylococcus aureus*) with a cellulose binding domain (CBD) in order to achieve their immobilization on cellulose. Out of the eight biosynthetic enzyme combinations that can possibly be made up by these six CBD-fusion proteins we identified two combinations of three proteins that were fully characterized for their ability to transform the natural substrate and a substrate analogue into CoA and a CoA analogue respectively. One of these combinations is subsequently used in the construction of batch- and column-based reactors that are successfully employed in the preparation of two CoA analogues. This demonstrates that multi-enzyme reactors based on CBD-fusion proteins embody a viable strategy for the production of CoA and its analogues.

Opsomming

Hierdie studie fokus op die ontwikkeling van 'n geïmmobiliseerde ensiem-gebaseerde reaktor wat gebruik kan word om analoë van koënsiem A (KoA) chemo-ensimaties te berei. Die konsep is gebaseer op bestaande kennis wat KoA biosintetiese ensieme gebruik om die transformasie van chemies-gesintetiseerde panteteïen-analoë na hul verwante KoA-analoë te kataliseer. Die drie ensieme wat nodig is om die transformasie te kataliseer is pantotenaatkinase (CoaA), fosfopanteteïen-adenielieltransferase (CoaD) en defosfo-koënsiem A-kinase (CoaE).

In hierdie studie bespreek ons die versmelting van hierdie drie KoA biosintetiese ensieme afkomstig van twee verskillende bakteriële bronne (*Escherichia coli* en *Staphylococcus aureus*) met 'n sellulose-bindingsdomein (SBD) om sodoende die ensieme se immobilisering op sellulose te bewerkstellig. Vanuit die agt biosintetiese ensiemkombinasies wat moontlik saamgestel kan word uit die ses SBD-fusieproteïene, identifiseer ons twee kombinasies van drie ensieme wat ten volle gekarakteriseer is vir hulle vermoë om die natuurlike substraat en 'n substraatanaloog om te skakel na KoA en 'n KoA analoog onderskeidelik. Een van hierdie kombinasies is vervolgens gebruik om lot- en kolomreaktors saam te stel wat suksesvol aangewend is om twee KoA analoë te berei. Hierdie bevinding demonstreer dat multiënsiemreaktore wat op SBD-fusieproteïene gebaseer is 'n lewensvatbare strategie is om KoA en sy analoë mee te produseer.

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SOLI DEO GLORIA

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List of Abbreviations

ACPs	Acyl carrier proteins
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
B. subtilis	*Bacillus subtilis*
CBDs	Cellulose binding domains
Cip	Cellulose-integrating protein
CoA	Coenzyme A
CoaA	Pantothenate kinase
CoaB	Phosphopantothenoylcysteine synthetase (*P*PC-S)
CoaC	Phosphopantothenoylcysteine decarboxylase (*P*PC-DC)
CoaD	Phosphopantetheine adenylyltransferase (also *P*PAT)
CoaE	Dephospho-coenzyme A kinase (also *D*PCK)
C. thermocellum	*Clostridium thermocellum*
CTP	Cytidine 5'-triphosphate
DHAP	Dihydroxyacetone phosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli* (also *Ec*)
EDTA	Ethylenediamine tetra-acetic acid
ESI-MS	Electronspray Ionization Mass Spectroscopy
GAP	D-glyceraldehyde-3-phosphate
GDH	Glycerophosphate dehydrogenase
HAT	Histone acetyl transferase
HEPES	*N*-2-Hydroxyethylpiperazine-*N*'-2-ethane sulphonic acid
His	Histidine
HPLC	High Performance Liquid Chromatography
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl-thiogalactoside
Kan	Kanamycin
k_{cat}	Turnover number
KCl	Potassium chloride
kDa	Kilodalton
K_M	Michaelis constant

LC-MS	Liquid Chromatography Mass Spectroscopy
LB	Luria Bertani
LR	Lawesson's reagent
MeCN	Acetonitrile
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide (reduced)
NH_4OAc	Ammonium acetate
$NiSO_4$	Nickel sulphate
OD	Optical density
PanK	Pantothenate kinase
PEP	Phosphoenolpyruvate
PP_i	Pyrophosphate
PPTase	Phosphopantetheine transferase
PT-linker	Proline-threonine linker
S. aureus	*Staphylococcus aureus* (also *Sa*)
S. cerevisiae	*Sacharomyces cerevisiae*
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPE	Solid phase extraction
TPI	Triosephosphate isomerase
TRIS-HCl	Tris(hydroxymethyl)aminomethane-HCl
V_{max}	Maximum velocity

Chapter 1

Coenzyme A: its Biosynthesis and Utility

1.1 Introduction

The research presented in this thesis was aimed at the development of a new method for the preparation of analogues of Coenzyme A (CoA), a ubiquitous biological cofactor. As an introduction, we will first provide background information on CoA, focusing on its biosynthesis and the variety of applications of its currently available analogues.

CoA is present in all living organisms, where it acts as an essential cofactor to many enzyme-catalyzed conversions. Based on a survey of the BRENDA database it is involved in approximately 9% of all enzymatic reactions (*1*). The molecule was discovered in 1945 by Lipmann *et. al.* and about ten years later its structure was determined by Baddiley (Figure 1.1) (*2, 3*). The pantetheine moiety of CoA is derived from L-cysteine and pantothenic acid, which is also known as vitamin B_5. Pantetheine is the final precursor of the cofactor that can be taken up by cells (*4*).

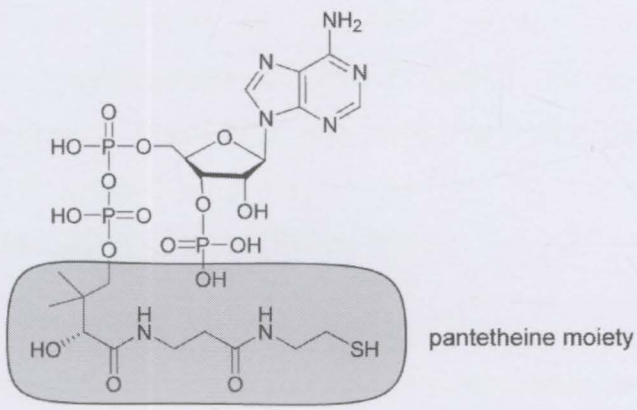


***Figure 1.1*: Structure of Coenzyme A (CoA).** Pantetheine is the final precursor of CoA that can be taken up by cells.

In recent years a great diversity of CoA analogues has been developed as valuable research tools, as they were being used as inhibitors, molecular probes and protein labels. Such applications of CoA analogues have increased the scope and utility of these molecules, and therefore have caused renewed interest in the technologies whereby CoA and its analogues are prepared.

1.2 The CoA biosynthetic pathway

CoA has been known for more than fifty years but the enzymes that are responsible for its biosynthesis have only recently been discovered and characterized (*4-6*). The biosynthesis of CoA is dependent on five enzymes. The enzymes and their substrates are presented in Figure 1.2.

In the first step of the CoA biosynthetic pathway pantothenate kinase (PanK; CoaA) phosphorylates the vitamin precursor pantothenic acid **1.2** to form 4'-phosphopantothenic acid **1.3** (*7*). ATP is the phosphate donor in this step. In the second step **1.3** is activated via a coupling reaction to L-cysteine with the consumption of either CTP or ATP. The enzyme involved in this reaction is phosphopantothenoylcysteine synthase (PPC-S; CoaB). The product of the second step in the pathway is 4'-phosphopantothenoylcysteine **1.4**. The third step in the pathway is a decarboxylation reaction. The enzyme assisting the reaction is 4'-phosphopantothenoylcysteine decarboxylase (PPC-DC; CoaC) and the product of the reaction is 4'-phosphopantetheine **1.5**. In the penultimate step dephospho-CoA, **1.6** is formed by the transfer of an AMP moiety from ATP to **1.5** by phosphopantetheine adenylyltransferase (PPAT; CoaD). Inorganic pyrophosphate is a byproduct of this reaction. In the final step phosphorylation of the 3'-hydroxy group of dephospho-CoA **1.6** yields Coenzyme A **1.1** (*8, 9*). This step utilizes ATP and is catalyzed by dephospho-CoA kinase (DPCK; CoaE).

All of the enzymes constituting this pathway has been characterized and expressed in human and bacterial systems. The enzymes are currently used for the chemo-enzymatic synthesis of CoA analogues.

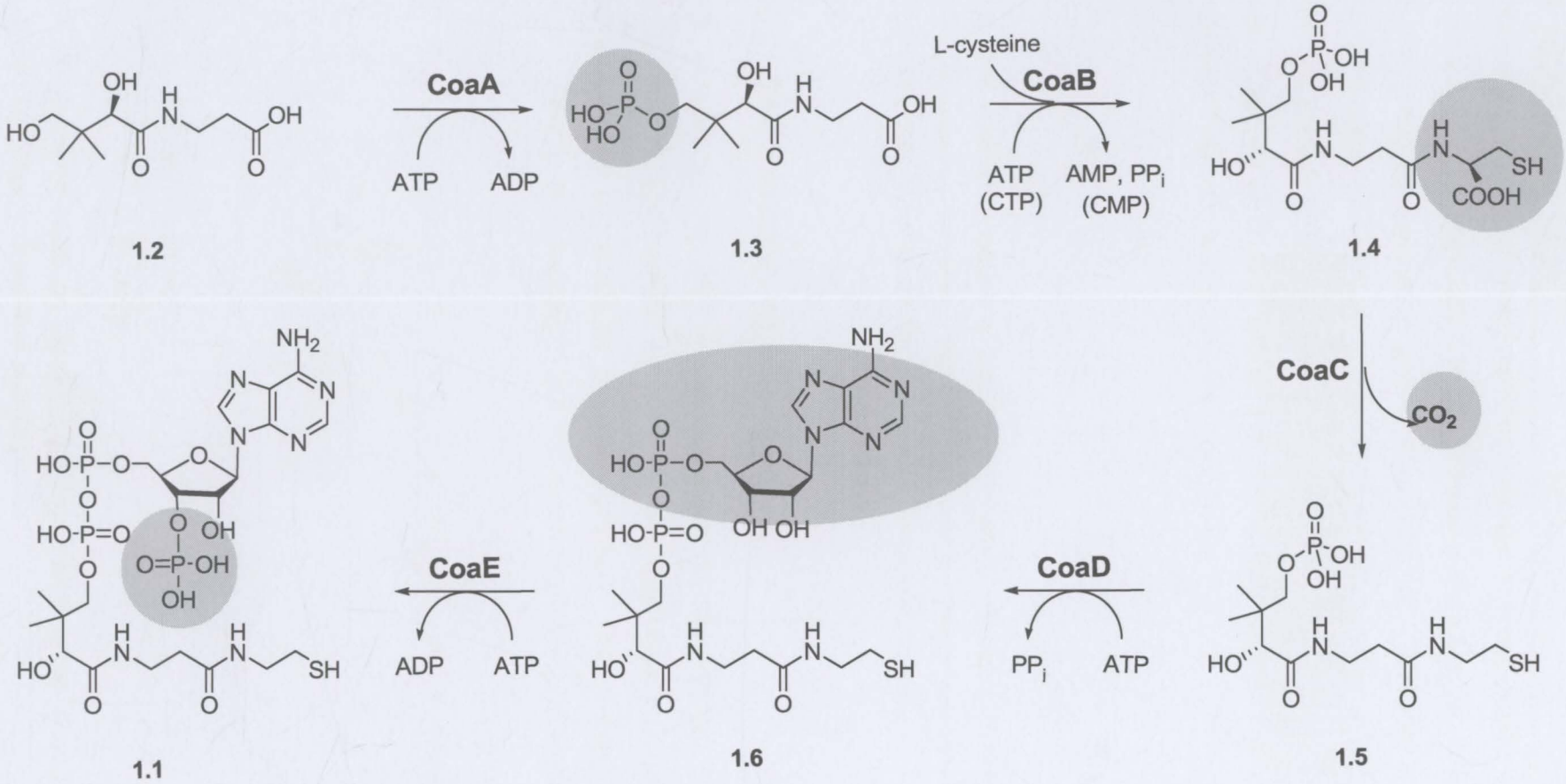


***Figure 1.2*: The universal biosynthetic pathway of Coenzyme A (CoA)**

1.3 The function of CoA

CoA acts mainly as an acyl group carrier in various enzyme-catalyzed reactions (*10*). Despite its complex appearance it is functionally a very simple molecule. The thiol, or sulfhydryl group, is the active part of the molecule. It reacts with the activated carboxylate groups of various carboxylic acid or aldehyde substrates to form thioesters, as shown in Figure 1.3. Acetyl-CoA is the most common of the CoA thioesters. Transfer of an acyl group from a thioester can proceed by either one of the two general methods which are described below (*9*).

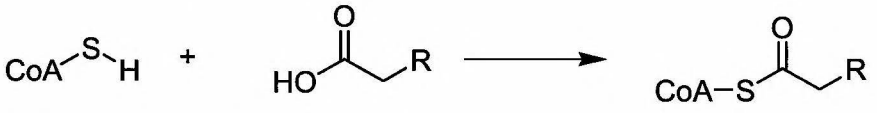
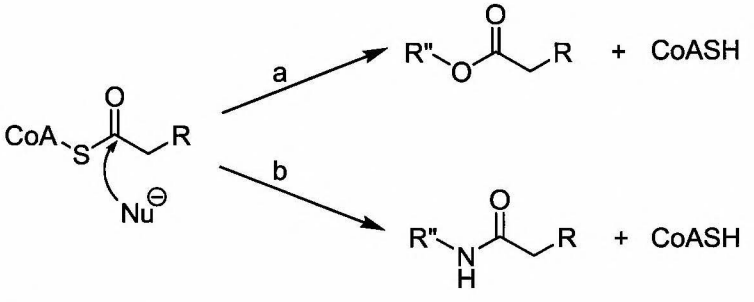


***Figure 1.3*: The general formation of acyl-CoA thioesters.** A thioesterification reaction between CoA and a carboxylic acid derivative. The carboxylic acid is normally activated before the coupling reaction can take place. R represents the main chain of the carboxylic acid.

1.3.1 Acyl-CoA thioesters as electrophiles

Acyl-CoA thioesters can act as electrophiles in the attack of a non-carbon nucleophile on the electrophilic carbonyl of the acyl group (Figure 1.4). These reactions are mostly catalyzed by acyltransferases. The nucleophiles are usually alcohols and amines thus leading to the formation of esters and amides.



a: $Nu^{\ominus}$ as an alcohol

b: $Nu^{\ominus}$ as an amine

***Figure 1.4*: Nucleophilic attack on the electrophilic carbonyl group of an acyl-CoA thioester.** The nucleophiles are either alcohols (a) or amines (b).

1.3.2 Acyl-CoA thioesters as nucleophiles

Acyl-CoA thioesters can also react as nucleophiles in reaction with carbonyl-based electrophiles, leading to the formation of new carbon-carbon bonds (Figure 1.5). The nucleophilic thioester attacks the electrophilic carbonyl groups of ketones, aldehydes, and other thioesters. The Claisen enzymes that catalyze these reactions are named as such due to the Claisen condensation reaction mechanism through which the activation takes place.

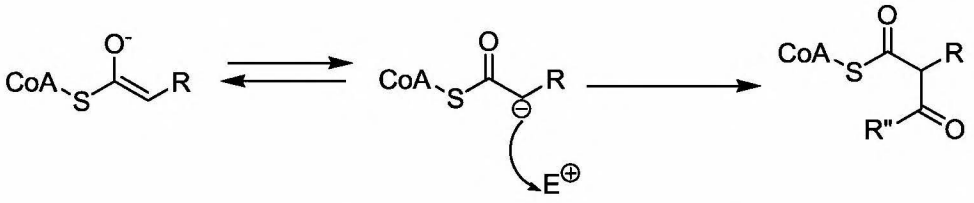


***Figure 1.5*: Nucleophilic attack by the deprotonated α-carbon of an acyl-CoA thioester.** These reactions are catalyzed by Claisen enzymes. The electrophile usually is a carbonyl group, aldehyde, ketone or another thioester.

1.4 Application of CoA analogues

A wide variety of CoA analogues have been developed as valuable mechanistic probes, enzyme inhibitors and protein labels. An overview of some of these analogues and a short discussion of their applications follows below.

1.4.1 Reactivity probes and inhibitors

Table 1.1 presents a variety of CoA analogues that have been developed as reactivity probes and inhibitors of biological reactions. We will shortly discuss the specific applications of each of these.

Both the epoxide-substituted CoA analogues and the unsaturated CoA thioesters are known inhibitors of acyl-CoA dehydrogenases (*11*). The citrate synthase inhibitors are mimics of acetyl-CoA, the natural substrate of this enzyme. They imitate the formation of the possible enol or enolate reaction intermediate that is formed in the presence of oxaloacetate (*12*). *E. coli* fatty acid biosynthesis is inhibited by the formation of an inactive acyl carrier protein (ACP) complex (*10*). Transfer of the

modified phosphopantetheine moiety of CoA to the ACP leads to the formation of an inactive covalently bonded enzyme-substrate complex.

The histone acetyltransferase (HAT) domain of the p300 transcriptional co-activator catalyze the transfer of an acetyl group between acetyl-CoA and the ε–amino lysine atom in histones and other proteins (*13*). The analogue shown in Table 1.1 known as Lys-CoA has the ability to block this activity, but the negatively charged phosphate groups on the molecule prevent it from crossing the cell wall. To increase cell-permeability a cell permeable tag was linked to the analogue by formation of a disulfide bond (*14*). Once the molecule has entered the cell the bond is broken and the inhibitor is trapped inside.

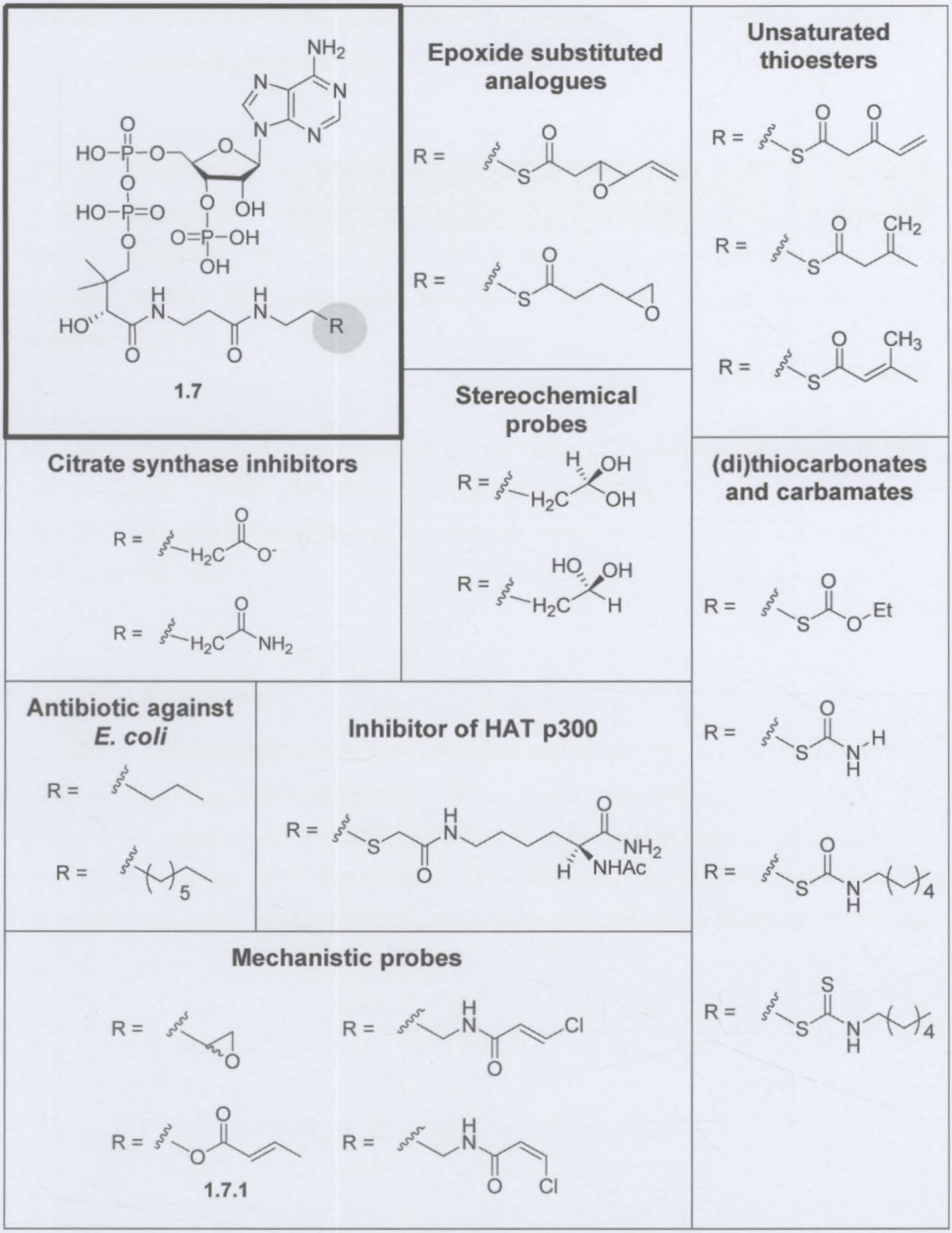


***Table 1.1*: Examples of CoA analogues that have been developed as enzyme inhibitors and mechanistic probes**

The two secondary alcohol stereochemical probes are reactivity probes used to determine the structure of the tetrahedral intermediate in the reaction of acetyl-CoA dependent acetyltransferases (*15*).

Crotonyl-oxy-CoA **1.7.1** is used as a mechanistic probe of the reaction catalyzed by enoyl-CoA hydratase. This reaction concerns the *syn*-hydration of α,β–unsaturated fatty acyl-CoAs (*16*). The other three mechanistic probes are used for investigation of carrier protein (CP) mediated biosynthetic reactions by the inactivation of ketosynthases (*17*).

The thiocarbonates and carbamates are examples of heteroatom-substituted acyl-CoA analogues. They were used to determine the structural requirements of the substrate that prevents oxidation of the reduced flavin of the medium-chain acyl-CoA dehydrogenase (*18*).

1.4.2 Reporter labels

Acyl carrier proteins (ACPs) play and essential role in fatty acid, polyketide and non-ribosome based peptide biosynthesis (*17*). These proteins are activated by the transfer of the phosphopantetheine moiety of CoA to a conserved serine residue on the ACP itself (Figure 1.6). The activated form of the ACP is referred to as *holo*-ACP. The transfer reaction is catalyzed by a phosphopantetheinyl transferase (PPTase) enzyme.

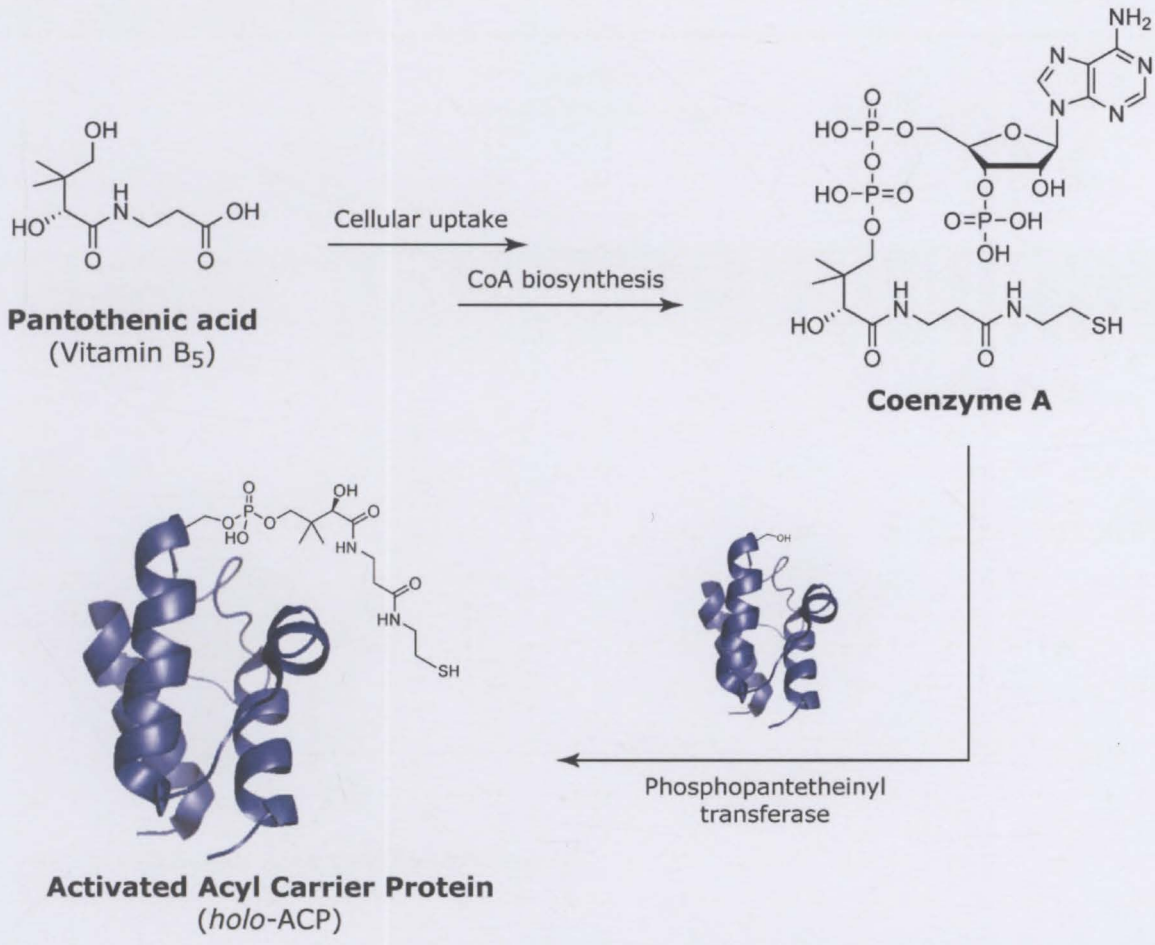


***Figure 1.6*: Activation of acyl carrier proteins.** The 4'-phosphopantetheine moiety from CoA is transferred to a serine residue on ACP. The transfer is mediated by phosphopantetheinyl transferase (PPTase) enzymes.

Research by La Clair *et. al.* has shown that the PPTase from *B. subtilis*, known as Sfp, can catalyze the transfer of phosphopantothenamide moieties from various CoA analogues to ACPs, thus allowing the labelling of these proteins with fluorescent and affinity tags (Figure 1.7) (*19*). These labeled ACPs are named *crypto*-ACPs. *In vivo* and *in vitro* studies conducted in this regard have led to the development of a wide range of new CoA analogues of which some are presented in tables 1.2 and 1.3. The methodologies used in these studies are discussed below.

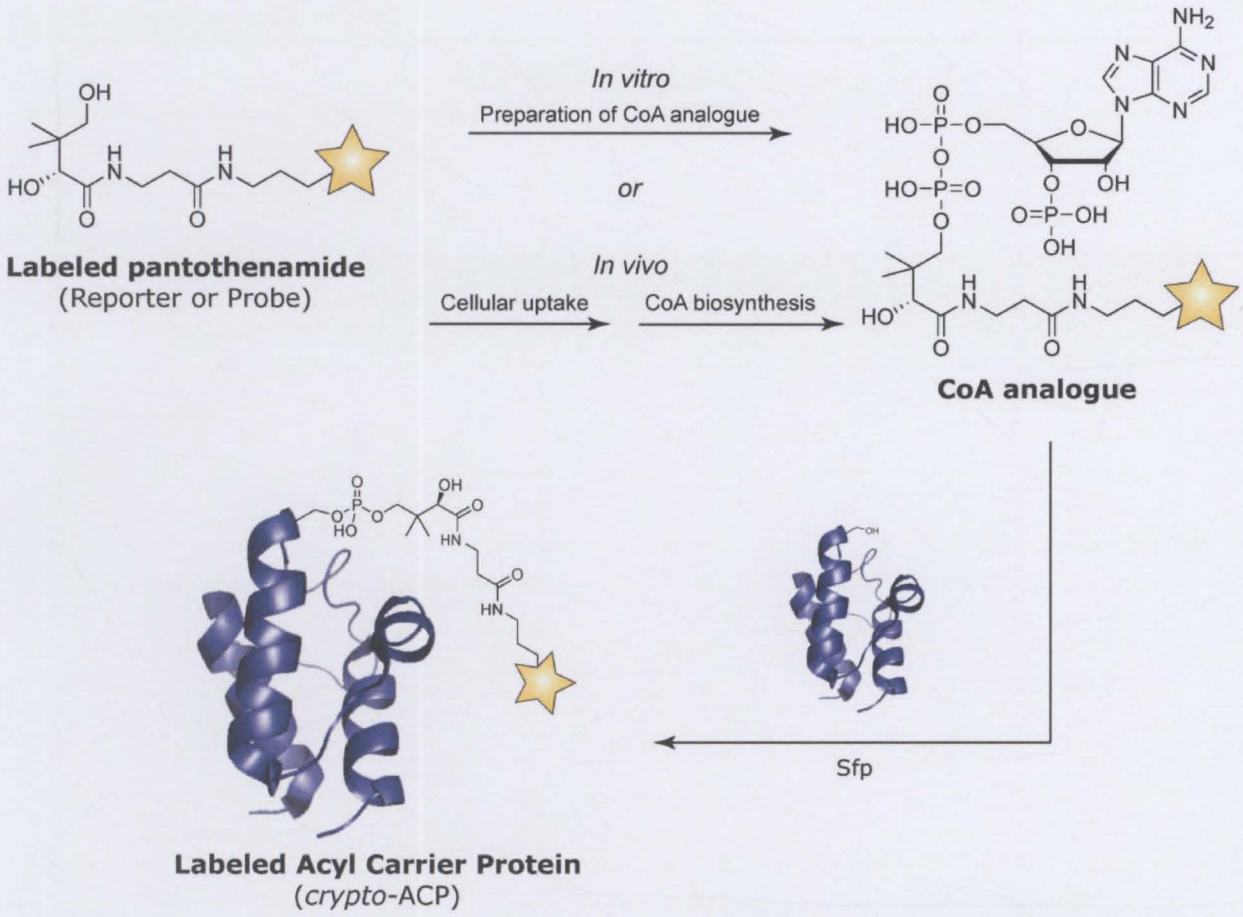


***Figure 1.7*: *In vitro* and *in vivo* labelling of ACP with phosphopantothenamide moieties from CoA analogues carrying fluorescent or affinity labels.** The labeled ACP is in this case referred to as a *crypto*-ACP. Sfp is the promiscuous PPTase that catalyze the transfer of the phosphopantothenamide moiety from CoA to ACP.

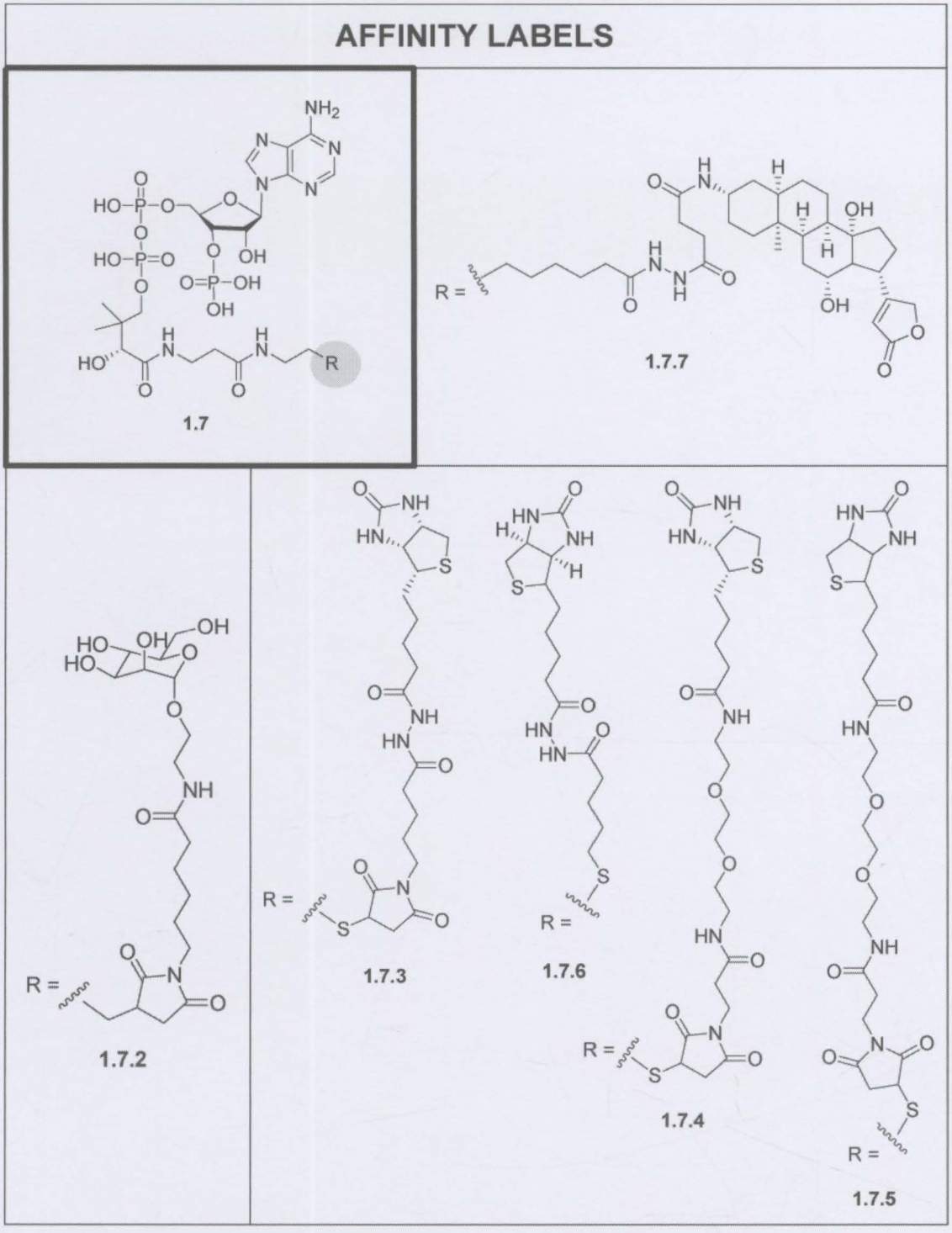


***Table 1.2*: Affinity labels used for the purification of specifically targeted proteins**

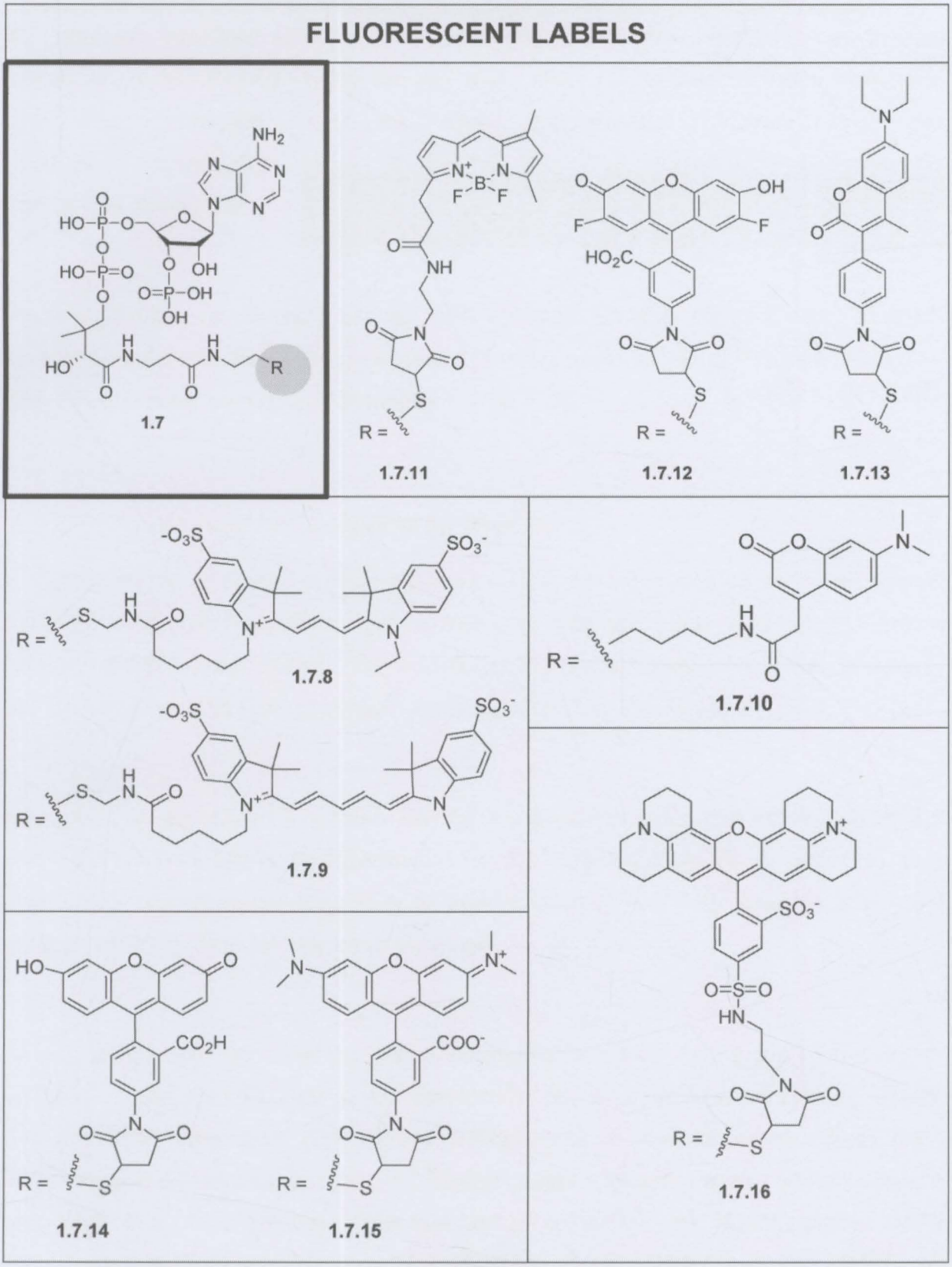


***Table 1.3*: CoA analogues with fluorescent tags used for labelling of targeted ACPs**

1.4.2.1 In vivo protein labelling

To achieve labelling of ACPs inside living cells one needs to synthesize pantothenamides that can cross the cell wall. Once inside the cell these molecules are then accepted into the CoA biosynthetic pathway and the phosphopantothenamide moiety of the resulting CoA analogue is subsequently transferred to an ACP.

Recent studies by Burkart *et. al.* has focused on the development of such phosphopantothenamides and successful *in vivo* protein labelling has been achieved with the coumarin based CoA analogue **1.7.10.** (*20*).

1.4.2.2 In vitro and cell surface protein labelling

For the purpose of *in vitro* protein labelling a CoA analogue can be prepared by any synthetic or chemo-enzymatic method, and only afterwards added to cell free media containing ACP and PPTase. The transfer of the phosphopantothenamide moiety of the CoA analogue to ACP is subsequently catalyzed by the PPTase.

Burkart *et. al.* developed a range of affinity and fluorescent reporter labels **1.7.2-1.7.5** and **1.7.11-1.7.16** based on maleimides for the labelling of ACPs *in vitro* (*19*). He specifically chose maleimides due to their particular reactivity towards sulfhydryl groups, which is ideal for reactions with CoA.

Affinity labels can be used to purify labeled proteins by recognition of receptor proteins. It can also be used to visualize carrier proteins via Western blotting. Biotin and mannose have been used for the development of CoA containing affinity tags. These tags are then transferred to ACPs and receptor proteins are used to recognize and purify them from solution. The receptor proteins for biotin labels used in **1.7.3-1.7.6** are avidin/streptavidin and for the mannose label used in **1.7.2** it is concanavalin (*19*).

Research involving *in vitro* protein labelling is currently at a more advanced stage than *in vivo* labelling. In a study by Yin *et. al.* published in 2004 they have reported the fusion of a protein of interest and an ACP, thus allowing the labelling of these proteins by phosphopantetheine moieties from CoA analogues through their transfer to the ACP (Figure 1.8) (*21*).

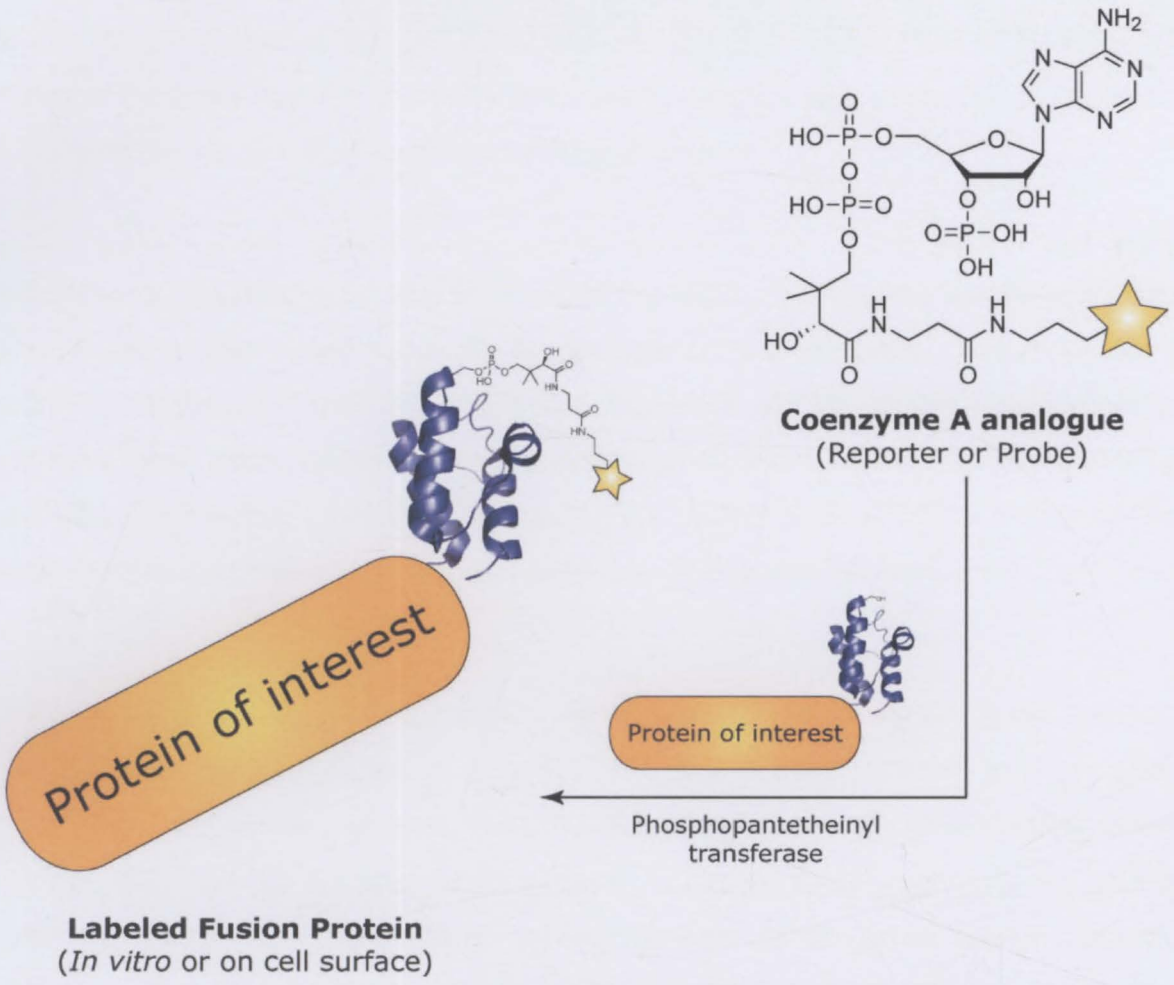


Figure 1.8: *In vitro* labelling of targeted proteins through the post-translational modification of ACP-fusion proteins. The phosphopantothenamide moiety of a CoA analogue is transferred to an ACP that is fused with the protein of interest. Transfer is mediated by the promiscuous PPTase.

This technology has now not only been applied to *in vitro* studies, but also to the labelling of proteins on cell surfaces (*22*). The ACP/PPTase pair from *E. coli* was used in a study to achieve the specific labelling of proteins on the cell surface. The ACP was expressed as a C-terminally attached protein to the α-agglutinin receptor, the protein of interest. These studies were conducted in *S. cerevisiae*. The affinity and fluorescent labeled CoA analogues **1.7.6-1.7.9** were used in this study. Incubation of the modified yeast cells with 6 x His-tagged PPTase and the respective

CoA analogues resulted in a clear fluorescent signal surrounding each of the cells surfaces.

1.5 Outline of this study

Considering the great number of existing CoA analogues and the wide scope of their applications the development of a method that allows the efficient and inexpensive synthesis of such analogues have become critical. In chapter 2 we will review the diversity of methods that are currently available to prepare all known CoA analogues, and indicate the advantages and disadvantages of each.

Our laboratory has been intensely involved in this area, focusing on the development of chemo-enzymatic based methods to prepare CoA analogues. We have been specifically interested in methodologies that make use of CoA biosynthetic enzymes which have been immobilized on a solid support, with the specific aim of developing enzyme-based reactors while also increasing the usable lifetime of the proteins. No published reports currently exists that makes use of such technology.

This thesis will describe our most recent achievements in this regard. It will build on previous studies that attempted to fuse the CoA biosynthetic proteins to a so-called CBD, or cellulose binding domain, with the aim of subsequently immobilizing these fusions on cellulose as a cheap solid support. Chapter three describes our efforts towards the construction, expression and purification of six novel fusion proteins, followed by a full characterization report of these proteins in chapter four. In the final experimental chapter we present and prove the concept of implementing these proteins in a newly developed strategy that utilizes a bioreactor for the continuous production of CoA analogues. Chapter six is a summary of all our achievements and contains a short discussion of our future research plans.

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Chapter 2

A Review of the Available Methods for the Preparation of Coenzyme A Analogues

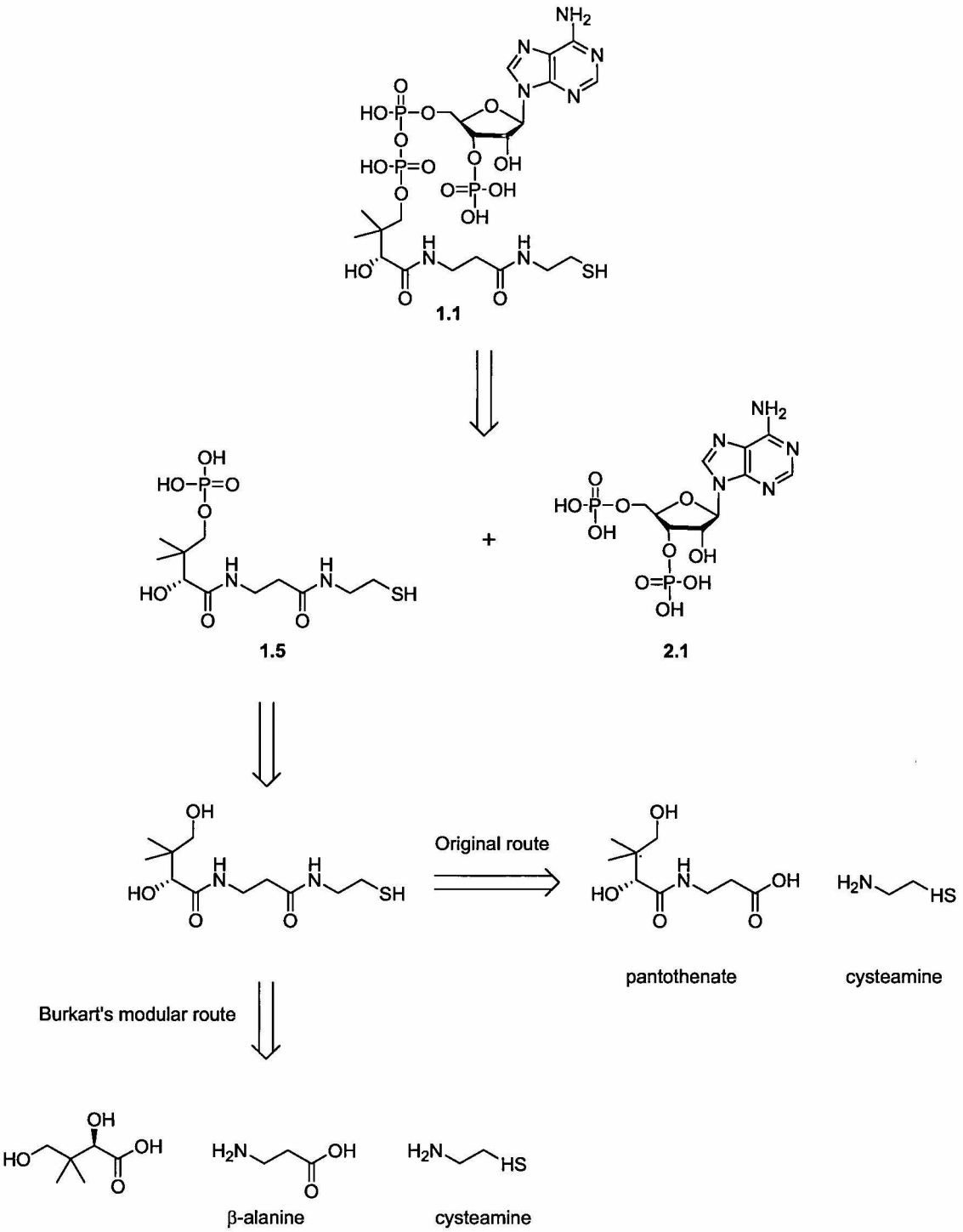
2.1 Introduction

There is a great diversity of methods currently available for the preparation of CoA and its analogues. For the purpose of this review we have divided these methods into two categories, namely synthetic methods and chemo-enzymatic methods. Chemo-enzymatic is a term used to describe methods that are not based on pure chemical synthesis but also include enzymatic transformations. This review will specifically focus on the drawbacks and advantages of the various methods that are currently in use.

2.2 Chemical synthesis of CoA Analogues

2.2.1 Retrosynthesis of CoA

The synthesis of CoA can easily be rationalized in terms of a retrosynthesis of the molecule, as shown in Scheme 2.1. The two parts that form the basic structure of the molecule is the adenosine and 4'-phosphopantetheine moieties (*1*). Synthesis of the adenosine moiety is very challenging and is therefore avoided by using an adenosine diphosphate molecule **2.1** (*2*). On the other hand 4'-phosphopantetheine **1.5** is relatively easily synthesized from pantothenate and cysteamine (*3*). A more recent route, proposed by Burkart *et. al.*, also allows synthesis starting with pantolactone, β-alanine and cysteamine (*4*).



Scheme 2.1*: *Retrosynthesis of Coenzyme A

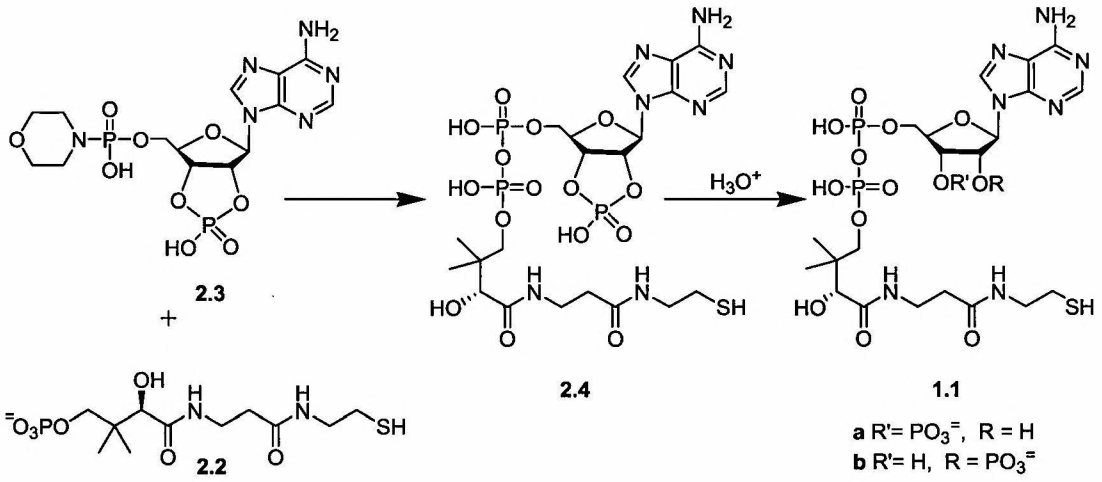
2.2.2 Starting from pantetheine derivatives

The two retrosynthetic routes allow the introduction of modifications in the 4'-phosphopantetheine moiety of CoA. Pantetheine derivatives can be prepared by the reaction of an activated pantothenic acid derivative with a cysteamine analogue, or the reaction of pantolactone and a β–alanylcysteamine analogue (*1*). From this the phosphopantetheine analogue can be prepared and then the same strategy is followed as with the synthesis of natural CoA to prepare the desired analogue.

2.2.3 Total chemical synthesis of CoA

CoA was first synthesized via the method reported in 1959 by Moffatt and Khorana (*3*). Scheme 2.2 summarizes the three main steps of their approach. The reaction is based on a coupling between phosphopantetheine **2.2** and an activated adenosine diphosphate **2.3**.

There are three drawbacks to this strategy. The synthesis of the adenosine moiety of CoA is very difficult and is therefore avoided by starting with 2'(3'),5'-cyclic ADP. Forming the pyrophosphate bridge is also problematic. 2'(3'),5'-Cyclic ADP needs to be activated before it reacts with **2.2**. The activation is achieved via a coupling reaction to morpholine to form **2.3**. The final step of this synthesis is the phosphorylation of the adenosine group. Unfortunately, hydrolysis of **2.4** causes formation of CoA **1.1a** and *iso*-CoA **1.1b** in roughly equal amounts. *Iso*-CoA is the 2'-phosphate isomer of the natural molecule. Separation of these two molecules is difficult and tedious, and this final step is thus one of the major drawbacks of the pure synthetic preparation of CoA.



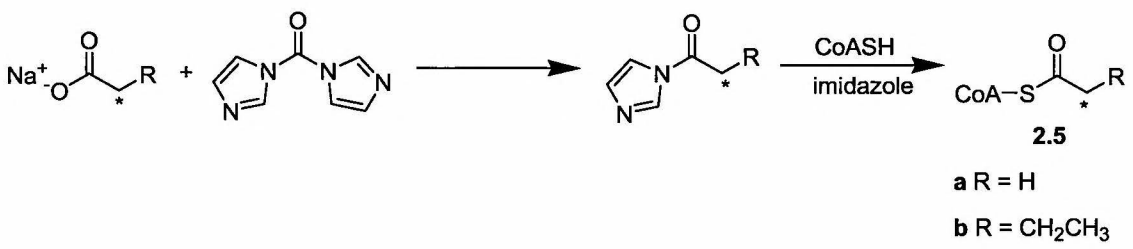
***Scheme 2.2*: The total chemical synthesis of CoA as published by Moffatt et. al. in 1959**

2.2.4 Modifying and derivatizing natural CoA

2.2.4.1 Derivatization of the thiol group

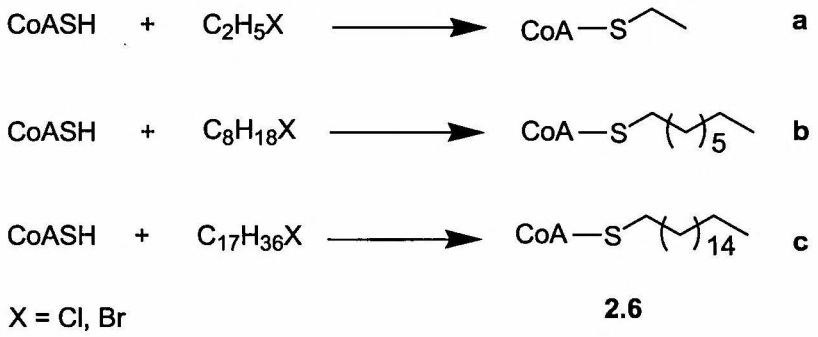
Analogues of CoA can be synthesized from the natural molecule by derivatization of the reactive terminal thiol group of native CoA. Various thioesters have been synthesized by reactions between CoA and acylating reagents. Some of these acylating reagents include activated acid derivatives, symmetric and mixed anhydrides and acyl imidazoles (*1*).

An example of an acylation reaction by an acyl imidazole is the synthesis of radio-labeled acetyl-CoA **2.5a** and propionyl-CoA **2.5b** (*5*). The sodium salts of acetate and propionate were reacted with carbonyldiimidazole before the addition of reduced CoA (Scheme 2.3).



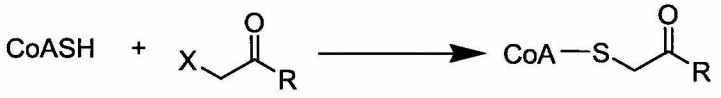
***Scheme 2.3*: Synthesis of radio-labeled acetyl-CoA 2.5a and propionyl-CoA 2.5b.**

Thioether analogues have also been synthesized. This was achieved by alkylation of the reactive thiol group of natural CoA. Examples of CoA thioethers include *S*-ethyl-CoA **2.6a**, *S*-octyl-CoA **2.6b** and *S*-heptadecyl-CoA **2.6c** (*6, 7*). These are all prepared by reaction of CoA with alkyl halides (Scheme 2.4).



***Scheme 2.4*: Synthesis of thioether CoA analogues by alkylation.**

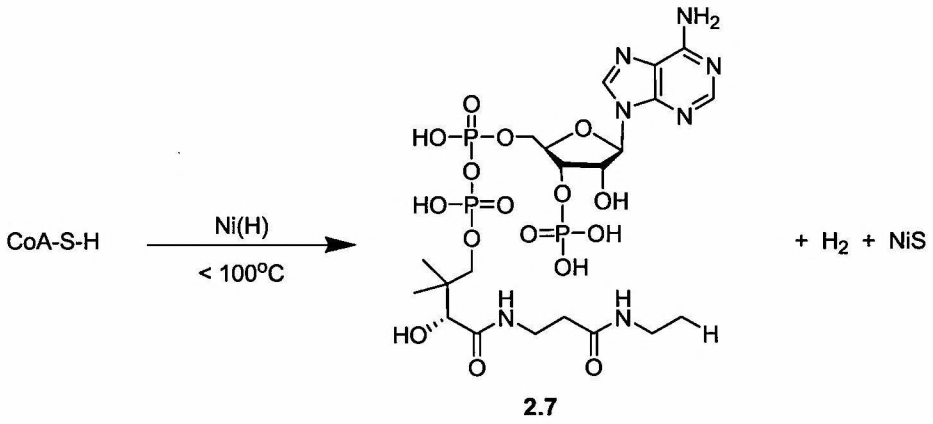
α–Haloketones have been used to synthesize CoA thioethers thus producing CoA derivatives with a methylene group inserted between the carbonyl group and the sulfur atom (Scheme 2.5) (*8, 9*).



***Scheme 2.5*: Synthesis of CoA thioethers with a methylene group inserted between the S-atom and the carbonyl group.**

2.2.4.2 Desulfo-CoA

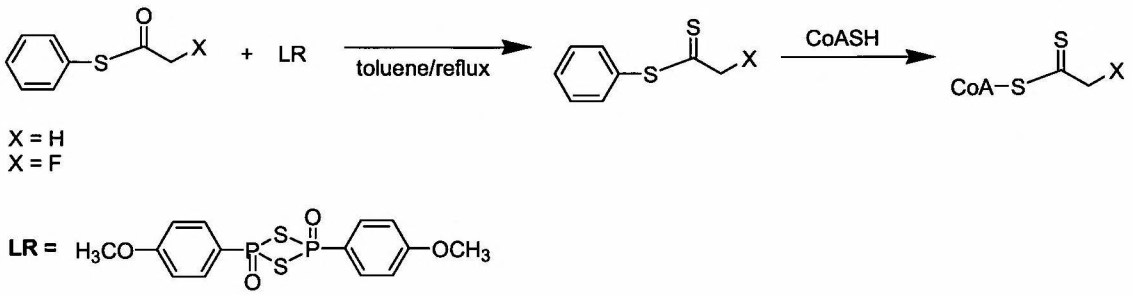
Some synthetic analogues are prepared by directly modifying the thiol group of CoA. Desulfo-coenzyme A **2.7** is a CoA analogue that is prepared by reductively removing the terminal thiol group of natural CoA. A Raney nickel catalyst is used as reducing agent (Scheme 2.6).



***Scheme 2.6*: A general reaction scheme for desulfurization by a Raney nickel catalyst**

2.2.4.3 Dithioesters of CoA

Another alternative synthesis is a reaction in which the oxygen in the thioester carbonyl is replaced by a sulfur atom (*10*). Lawesson's reagent (LR) was used to convert a phenyl thioester to a phenyl dithioester by simply refluxing it in the presence of toluene. LR is a very effective thionating agent of carbonyl compounds (*11*). A transesterification reaction was conducted to yield the corresponding CoA analogue (Scheme 2.7).



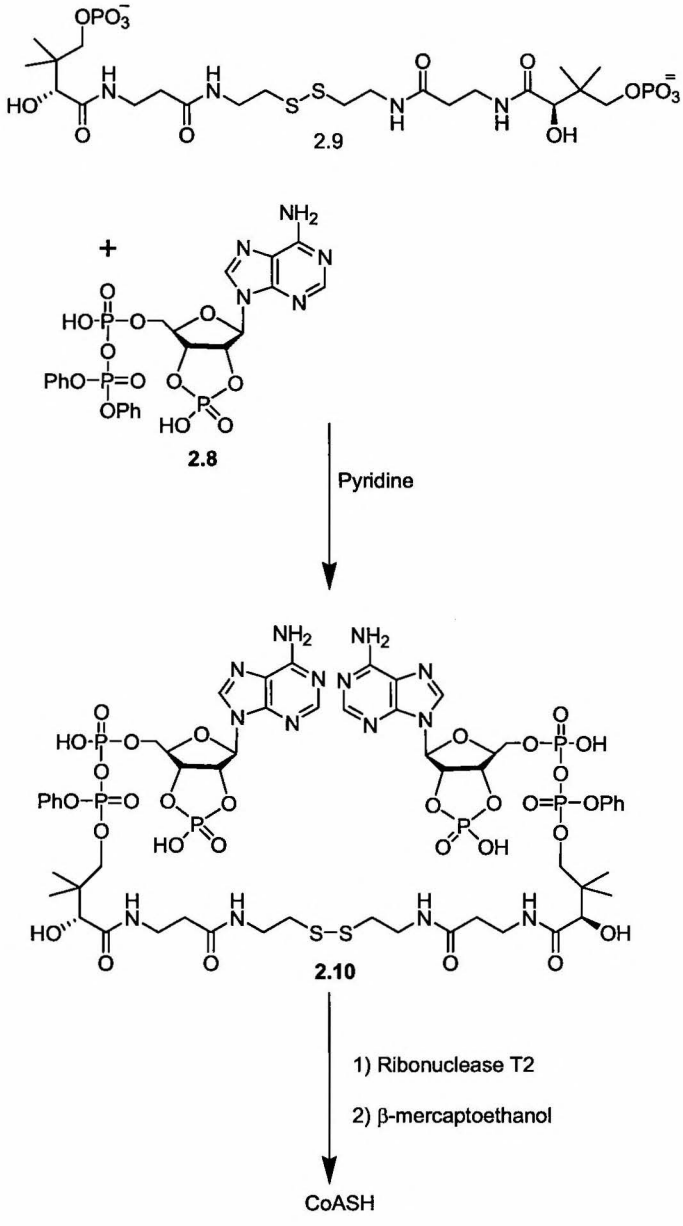
***Scheme: 2.7*: Thionation of a carbonyl compound.** Conversion of a thioester to a dithioester and the subsequent transesterification yields a CoA dithioester analogue.

The major drawback of these methods involving the derivatization and modification of CoA itself in order to prepare its analogues is that they are all restricted to the preparation of one type of analogue. There is no general method available by which CoA can be modified or derivatized to prepare different kinds of analogues.

2.3 Enzymatic synthesis of CoA analogues

2.3.1 Introducing Ribonuclease T2

The first introduction of enzymes into the synthesis of CoA was in 1964 when Michelson presented a modification of the original method published by Moffatt and Khorana (Scheme 2.8) (2).



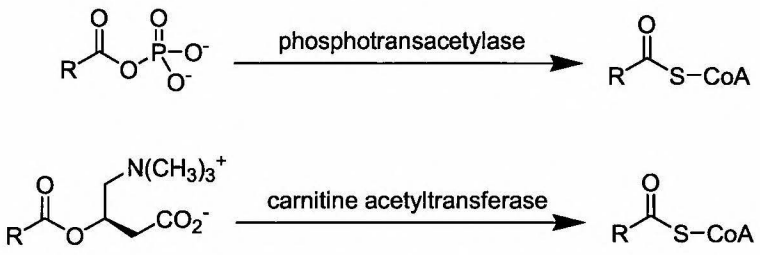
***Scheme 2.8*: The first chemo-enzymatic synthesis of CoA.**

The first modification he introduced was synthesizing P^1-adenosine (2',3'-cyclic phosphate)-5'-P^2-diphenylpyrophosphate **2.8** as a starting material. Using **2.8** instead of **2.1** as starting material simplified the synthesis by eliminating the activation step that was necessary for formation of the pyrophosphate bridge in CoA.

2.8 was reacted with pantetheine 4',4'-bisphosphate **2.9** to form a disulfide **2.10** of the final intermediate from the original synthesis. In the final step Michelson introduced ribonuclease T2 to catalyze the selective hydrolysis of the 2',3'-cyclic phosphate, thus preventing the formation of isomers as in the original method. CoA disulfide was reduced with β–mercaptoethanol to obtain CoASH (reduced CoA). Although this modified method was an improvement on the first synthesis proposed by Moffat and Khorana it could still not fully address the problems with the difficult synthesis of the adenosine moiety and the formation of the pyrophosphate bridge.

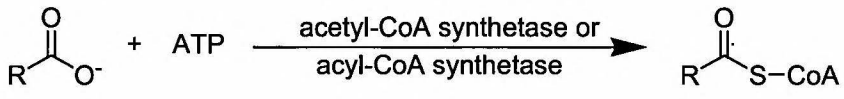
2.3.2 Derivatization of CoA

There are four enzymes that have been used for the derivatization of the CoA molecule to yield CoA ester analogues. Phosphotransacetylase assists the acylation of CoA by various acyl phosphates (Scheme 2.9). Carnitine acetyltransferase assists acylation of CoA with carnitines acting as acyl donors (*12*).



***Scheme 2.9*: Enzymatic acylation of CoA using acyl donors as substrates.**

CoA thioesters have also been formed by a direct coupling reaction between CoA and a carboxylic acid in a simple aqueous medium (*1*). These reactions were catalyzed by ATP utilizing enzymes, acetyl-CoA synthetase and acyl-CoA synthetase (Scheme 2.10). In both cases the ATP is hydrolyzed to AMP and pyrophosphate. Acyl-CoA synthetase may have more general usage since it accepts long chain fatty acids as substrates, whereas the versatility of acetyl-CoA synthetase is limited by its substrate acceptance (*13-15*).



***Scheme 2.10*: A general scheme for the enzyme-assisted acylation of CoA via a direct coupling reaction to a carboxylic acid.**

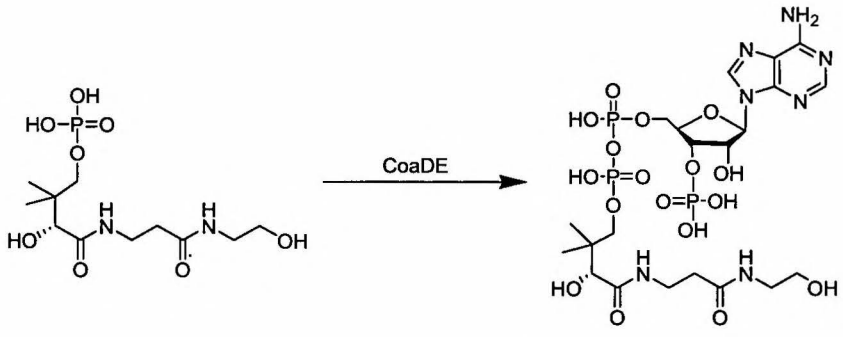
2.3.3 Introducing CoA biosynthetic enzymes

The drawbacks of the chemical synthetic strategy used for the synthesis of CoA forced researchers to investigate alternative methods of producing the molecule and its analogues.

Analogues of CoA can be synthesized using isolated enzymes from the CoA biochemical pathway. The addition of the adenosine moiety and formation of the pyrophosphate bridge which was the main drawbacks of the original synthetic strategy could now be achieved in one enzymatic step. Further, no racemic mixture of the product is formed since specific hydrolysis yielding only the CoA isomer could be achieved.

2.3.3.1 Introducing phosphopantetheine adenylyltransferase (CoaD) and dephospho-CoA kinase (CoaE)

In 1966 Stewart and Ball presented a method by which D-Oxypantetheine 4'-phosphate was chemically synthesized and then enzymatically transformed to Oxy-CoA (Scheme 2.11) (*16*). They isolated a bifunctional enzyme complex from beef liver containing phosphopantetheine adenylyltransferase (CoaD) and dephospho-CoA kinase (CoaE) capable of this transformation. This method has been expanded to the production of various CoA analogues including desulfo-CoA **2.7** which was previously produced by the Raney nickel reduction of CoA (*17*).

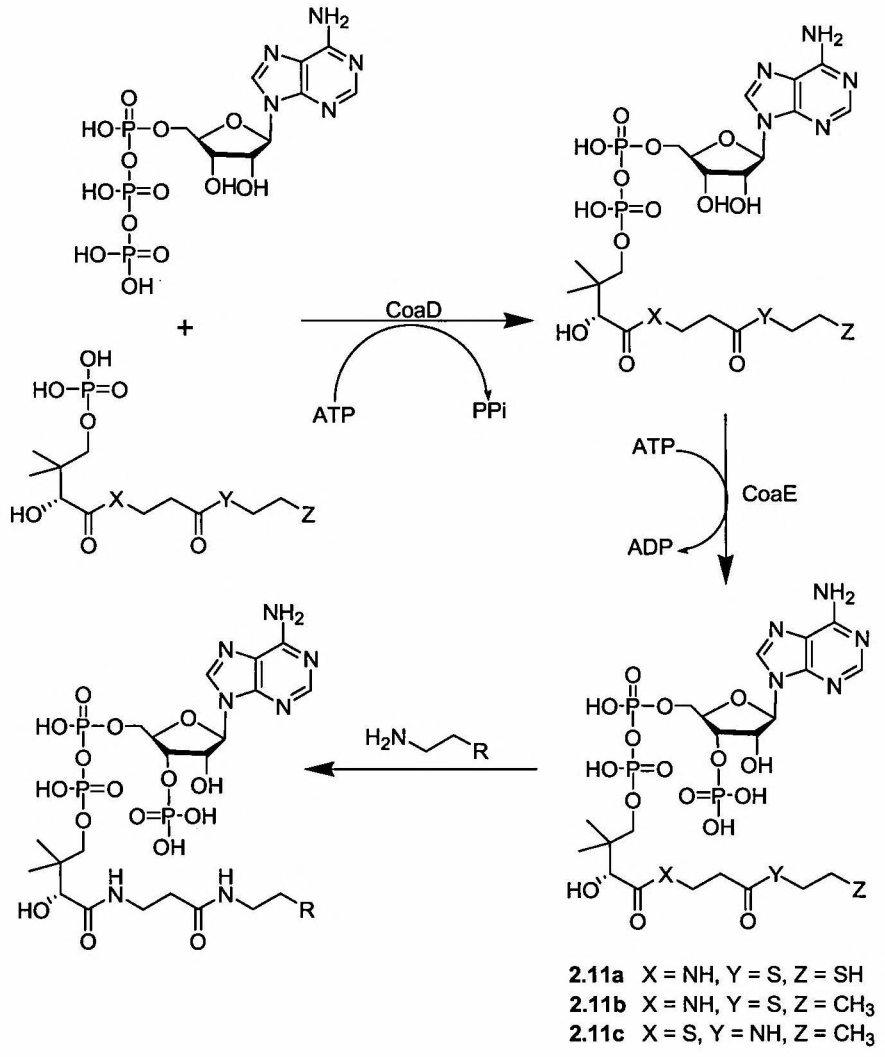


***Scheme 2.11*: Biosynthesis of Oxy-CoA.**

In 1992 Martin and Drueckhammer presented a method based on the research of Stewart and Ball for the chemo-enzymatic synthesis of a CoA analogue synthon **2.11a** (*18*).

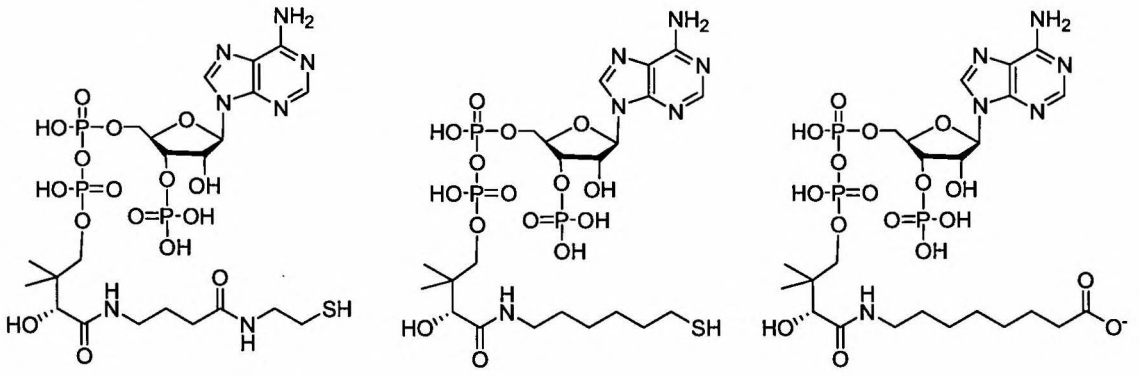
A synthon is a template molecule that can very easily be modified to produce a number of related molecules. They used a phosphopantetheine analogue in which the amide bond furthest from the phosphate group was replaced by a thioester group. This was enzymatically transformed to the related CoA thioester analogue (Scheme 2.12). *Brevibacterium ammoniagenes* was the enzyme source for these reactions.

By aminolysis of **2.11a** with various primary amines a variety of CoA and CoA ester analogues could be produced, but the very reactive terminal thiol group of the synthon caused problems. It formed unwanted disulfide bonds and caused interfering thiolysis reactions. To overcome this problem the thiol group was replaced by a methyl group forming a propyl thioester synthon **2.11b** (*19*).



***Scheme 2.12*: Synthesis of the three CoA analogue synthons 2.11a, 2.11b and 2.11c.**

A second generation CoA analogue synthon has later been developed in which the amide bond more distant from the terminal thiol group was replaced by a thioester **2.11c** (*20*). A racemic phosphopantetheine analogue was synthesized. Thereafter enzymatic conversion to the corresponding CoA analogue was achieved using the same enzyme system as before. CoaD selectively yielded the natural *S*-isomer. Three new analogues were synthesized by this method, for the first time introducing modifications in the β-alanylcysteamine moiety (Scheme 2.13).



***Scheme 2.13*: CoA analogues containing modifications in the β–alanylcysteamine moiety.** The three analogues synthesized from the second generation CoA analogue synthon 2.11c.

2.3.3.2 Introducing pantothenate kinase (CoaA)

2.3.3.2.1 Characteristics of bacterial pantothenate kinase (CoaA)

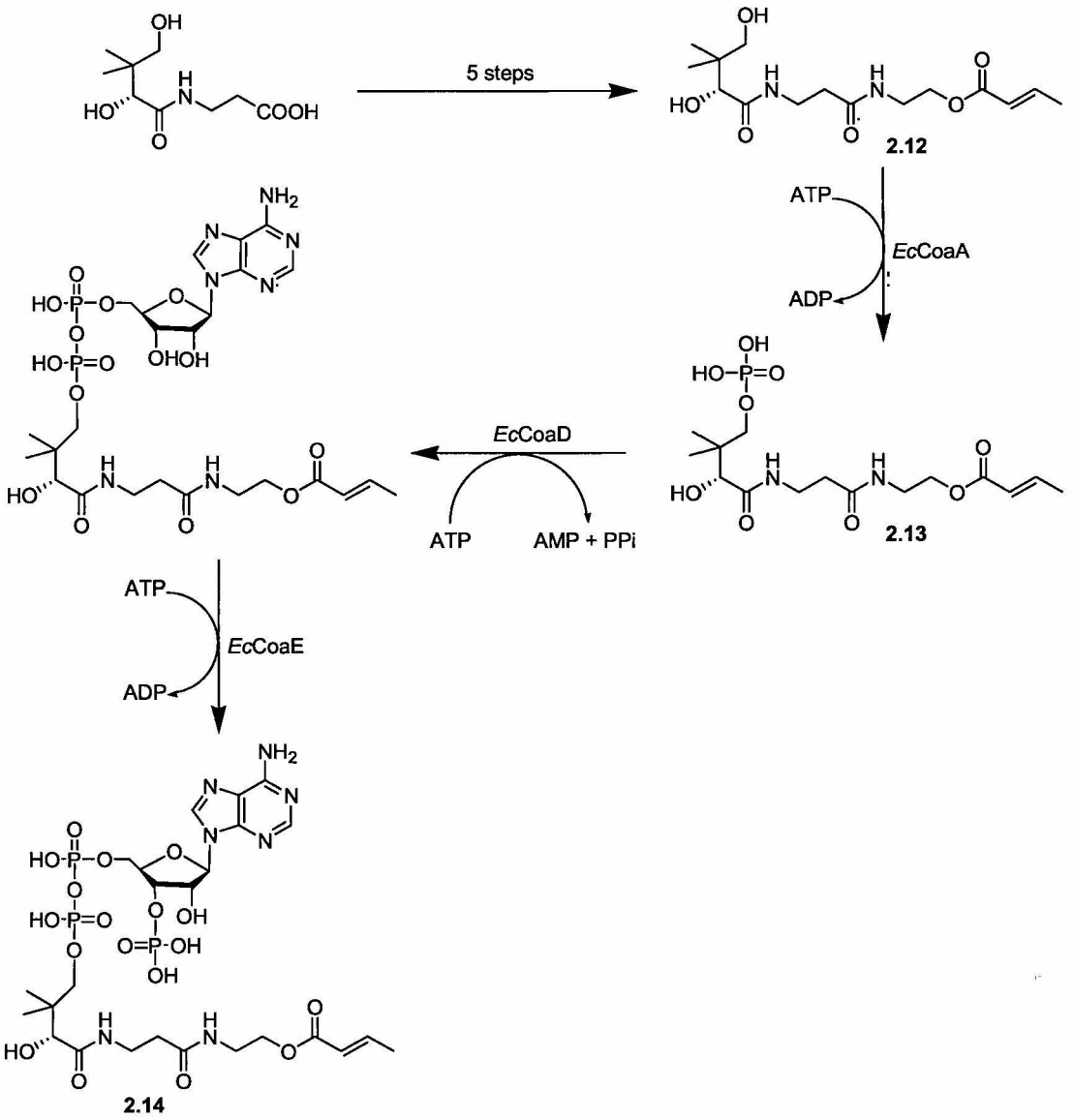
Pantothenate kinase (CoaA), acts as the gate-keeper in the biosynthetic pathway of CoA. It catalyzes the ATP-dependent phosphorylation of pantothenate which is the rate-controlling step of this pathway (*21*). *Ec*CoaA, pantothenate kinase isolated from *E. coli*, was the first pantothenate kinase enzyme to be extensively characterized and is considered the prototype of bacterial CoaA (*22*).

*Ec*CoaA lacks substrate specificity which makes it ideal for accepting various pantetheine derivatives. However, it is subject to feedback inhibition by CoA and its thioester analogues. This is due to competitive binding between CoA and ATP to the ATP binding-site on the protein (*23*). The disadvantage of feedback inhibition is that it prevents the use of *in vivo* and one pot *in vitro* methods for the overproduction of CoA or CoA thioesters.

2.3.3.2.2 Pantothenate kinase (CoaA) in a step-wise synthetic strategy

In more recent years the chemo-enzymatic synthesis of CoA analogues has developed to include CoaA as the first enzyme in the transformation process. By this method pantetheine derivatives can be chemically synthesized and then phosphorylated by CoaA to produce the corresponding phosphopantetheine

derivatives. This can further be transformed by CoaD and CoaE to yield the CoA analogue with a modified pantetheine moiety.



***Scheme 2.14*: Chemo-enzymatic synthesis of Crotonyl-Oxy-CoA 2.14 starting from a chemically synthesized pantetheine derivative 2.12.**

One of the first analogues to be synthesized by this method was Crotonyl-Oxy-CoA **2.14** (Scheme 2.14) (*24*). The pantetheine derivative **2.12** was synthesized from D-pantothenic acid in 5 consecutive steps. Three separate enzymatic steps followed to obtain the final product. *E. coli* enzymes were used in this reaction. Crotonyl-oxypantetheine **2.12** was found to be an excellent substrate for *Ec*CoaA. Pyruvate kinase and phosphoenol pyruvate was added to the separate steps for the recycling of ATP (*25*). Pyrophosphatase was added to the reaction catalyzed by *Ec*CoaD to

hydrolyze the inorganic pyrophosphate (PP_i) formed from the coupling reaction. The overall yield for the enzymatic part of the synthesis with purification after each step was 62%.

2.3.3.2.3 A one-pot synthesis of CoA analogues

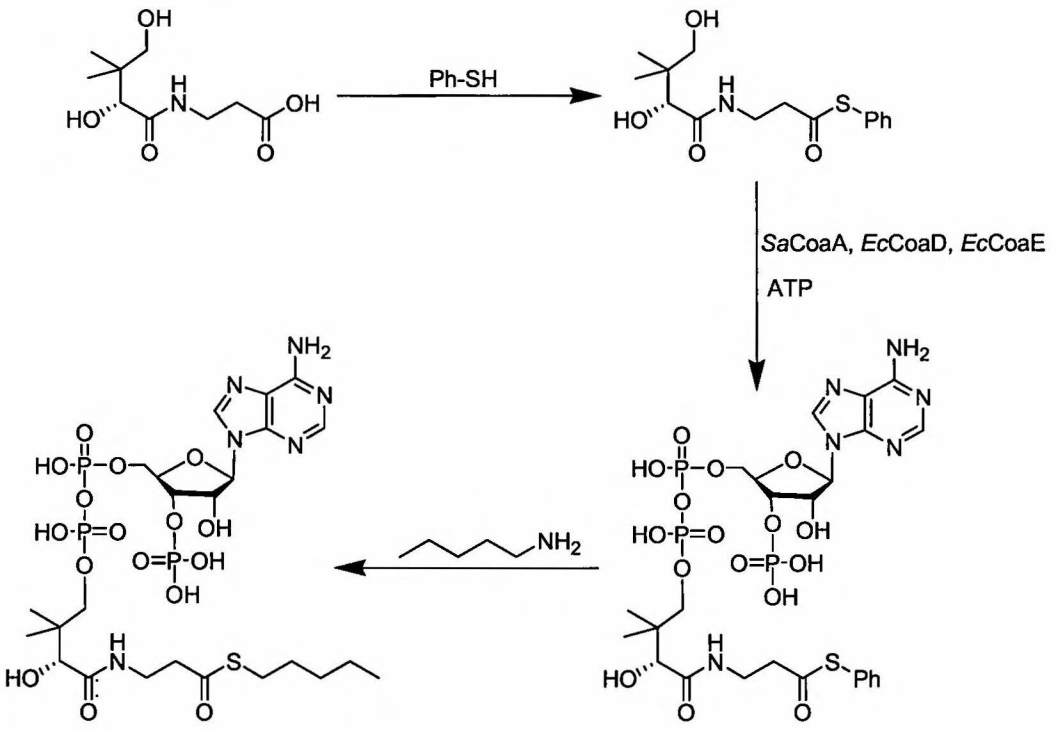
In 2004 Nazi *et. al.* reported another improved strategy in which he proposed a one-pot chemo-enzymatic synthesis, thus eliminating the purification step after each transformation (*26*). Enzymes from *E. coli* were purified before usage to eliminate the interference of possible side reactions. Each transformation reaction was followed by HPLC and the next enzyme added only after complete conversion by the previous one was achieved.

Nazi also showed that complete conversion to the CoA analogue could be achieved by simultaneous addition of all three enzymes and the required amount of ATP to the pantetheine derivative, followed by a 90 min incubation period at 37°C.

2.3.3.2.4 SaCoaA in a one-pot synthesis of CoA analogues based on a new CoA synthon

Research from van Wyk and Strauss published in 2006 showed a new improved CoA analogue synthon from **2.11b** synthesized by Martin *et. al.* (*27*). They synthesized phenyl thiopantothenate via a one step transesterification reaction with unprotected pantothenic acid. *Sa*CoaA, pantothenate kinase from *S. aureus*, together with *Ec*CoaDE was used in a one pot reaction to catalyze the total conversion of the phenyl thiopantothenate to the CoA analogue synthon (Scheme 2.15).

*Sa*CoaA is the only known CoaA protein that is not feedback regulated by either CoA or CoA thioesters (*28*). It also shows a lack of substrate specificity (*27*). Thus by using *Sa*CoaA this method allows one pot preparation of CoA analogues without the poor yield due to inhibition by the product.



***Scheme 2.15*: Chemo-enzymatic synthesis of ethyldethia-CoA via a phenyl-CoA thioester synthon.**

2.4 Conclusion

This review of literature methods whereby CoA is currently prepared showed that these methods can be divided in two basic categories. The first group consists of methods which are purely based on chemical synthetic methods and transformations, while the second also uses enzymatic transformations to prepare the CoA analogues. We identified the major drawbacks of the synthetic strategy and showed how the chemo-enzymatic based strategies have developed to address these drawbacks.

The current best-practice chemo-enzymatic method is based on a one-pot reaction using *Sa*CoaA, *Ec*CoaD and *Ec*CoaE to transform pantetheine analogues to their related CoA analogues. However, this methodology still has a major drawback in that it is solution-based, causing large amounts of protein to be destroyed during work-up of the product. This makes the method very expensive and thus not suitable for large scale synthesis. This drawback may be addressed by immobilization of the enzymes on a solid support, and subsequent construction of a multi-enzyme reactor

to prepare the CoA analogue. Not only would this allow the enzymes to be reused, but may also significantly improve the separation and purification of the product.

In the next chapter we will introduce the concept of protein immobilization and discuss the construction, expression and purification of six novel fusion proteins aimed at achieving this goal.

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Chapter 3

Expression and Purification of CoA Biosynthetic Enzymes as Novel CBD-fusion Proteins

3.1 Introduction

In a previous study from our laboratory we have looked at the construction of fusion proteins to achieve the immobilization of CoA biosynthetic enzymes on a solid support. Cellulose was identified as an ideal supporting material for enzyme immobilization since it is inexpensive, biodegradable and approved for use in most pharmaceutical products (*1, 2*). Cellulose binding domains (CBDs) were identified as a means whereby immobilization on cellulose could be obtained.

CBDs are carbohydrate binding modules found in cellulases. The primary function of these domains is to bind the cellulases to cellulose. There are more than 150 known CBD sequences, which have been divided into 13 families based on sequence similarity (*3*).

In the previously mentioned study several attempts were made at the construction of fusion proteins of different CBD domains with the CoA biosynthetic enzymes from *E. coli*. One of the major struggles that we encountered with this work was that the CBD-fusions were often only insolubly expressed, a result which was in agreement with the observation that CBDs tend to form inclusion bodies during expression. Finally a CBD was identified that does not promote inclusion body formation and whose fusions are often found to be soluble (*4*). This CBD, CBD_{cipA} is a cellulose binding domain from *Clostridium thermocellum* and forms part of the third family of CBDs.

A vector encoding for the expression of CBD_{CipA} with a proline-threonine enriched linker (PT-linker) and an *N*-terminal 6 x Histidine tag was subsequently constructed, and used as base vector for the cloning of CoA biosynthetic enzymes as His-CBD-fusion proteins. The first protein that was solubly expressed in this manner was the fusion construct CBD_{cipA}-*Ec*CoaD.

In this chapter we describe how we built on this result by determining expression conditions for all three the CBD fusion proteins of the *E. coli* and *S. aureus* CoA biosynthetic enzymes that are currently employed in the preparation of CoA analogues. We also express the native counterparts of these proteins in order to compare their characteristics to those of the fusion proteins.

3.2 Results and Discussion

3.2.1 Construction of expression vectors

Soluble expression of CBD*Ec*CoaD has been reported in a previous study from our lab (*5*). We used this expression vector as a template for the construction of expression vectors for CBD*Ec*CoaAE and CBD*Sa*CoaADE. As shown in Figure 3.1 this vector encodes for an N-terminal histidine tag on the CBD domain. This allows purification of the proteins using nickel based IMAC (*6*).

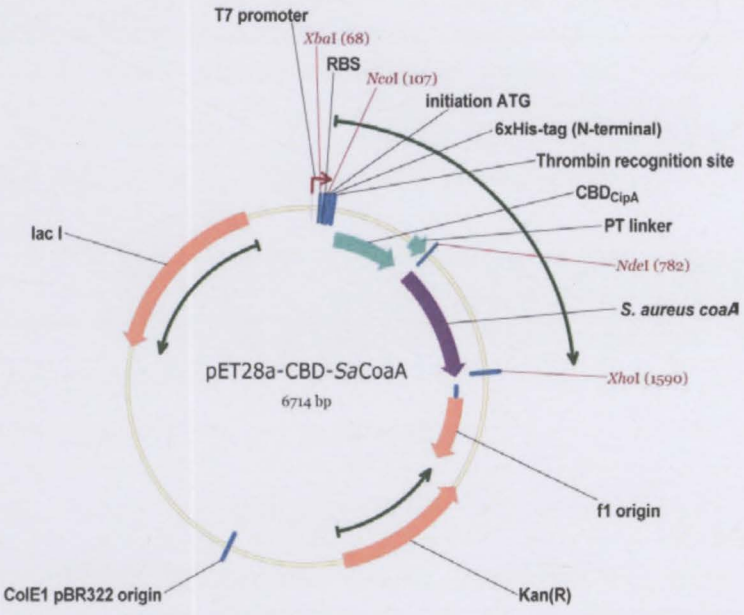

***Figure 3.1*: Newly constructed expression vector of pET28a-CBD$_{CipA}$-*Sa*CoaA**

We also constructed expression vectors for the expression of native *Sa*CoaDE with an N-terminal histidine tag, so that the same purification method could be used. The expression of the native *Ec*CoaADE and *Sa*CoaA proteins has been reported previously (*7, 8*).

3.2.2 Expression trials

Expression trials were conducted on all the newly constructed expression vectors. Various parameters were investigated to determine the optimal conditions for achieving soluble protein:

- IPTG induction concentrations
- Growth time after induction
- Cell strains
- Expression media

3.2.2.1 *First attempt at expressing CBD-fusion proteins*

We first conducted small scale expression trials with CBD_{CipA}*Sa*CoaA and CBD_{CipA}*Ec*CoaD fusion proteins. The plasmids were transformed into *E. coli* BL21-star(DE3) cells and induced with different IPTG concentrations.

Expression trails were run at a constant temperature of 37°C and induced with IPTG concentrations varying between 50 µM and 1 mM. The growth time after induction was alternatively 3 hours or 18 hours (overnight).

Soluble expression of both fusion proteins was achieved. The SDS-PAGE gel below shows the results of the various trials that were conducted with CBD_{CipA}*Sa*CoaA (53 kDa, Figure 3.2). The best expression for CBD_{CipA}*Sa*CoaA was obtained with induction with 200 µM IPTG. For CBD_{CipA}*Ec*CoaD 250 µM IPTG gave the best results. Both these results are for expression trials that were conducted overnight.

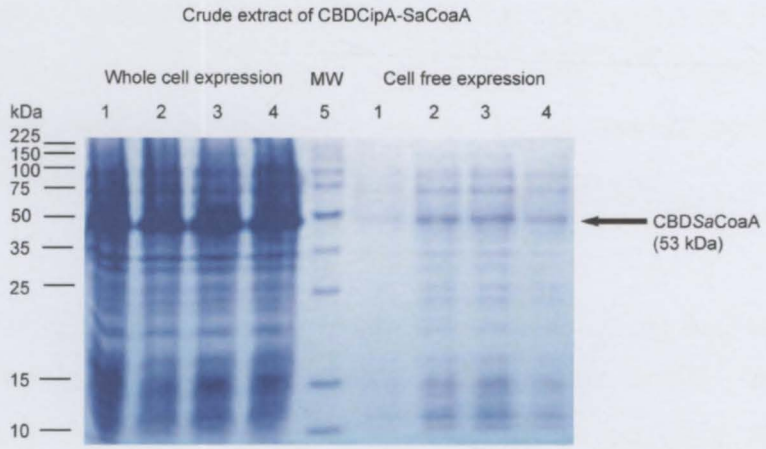

Figure 3.2. **Expression trials for pET-28a-CBD_{CipA}-*Sa*CoaA with different IPTG concentrations at 37°C, overnight**. *Lane 1*: 750 µM IPTG. *Lane 2*: 500 µM IPTG. *Lane 3*: 200 µM IPTG. *Lane 4*: 100 µM IPTG. *Lane 5*: Molecular weight markers. Whole cell expression represents the expression of insoluble protein. Cell free expression represents the expression of soluble protein.

3.2.2.2 *Expressing native S. aureus proteins*

Various strategies were investigated to achieve the expression of the native *Sa*CoaD and *Sa*CoaE. Expression conditions for *Sa*CoaA were already available (*8*).

In our first attempt we expressed the *S. aureus* proteins in *E. coli* BL21-star(DE3) cells using IPTG induction concentrations ranging between 50 µM and 1 mM. Expression was conducted at 37°C with growth times after induction being chosen as either three hours or 18 hours (overnight). The optimal expression conditions for *Sa*CoaD and *Sa*CoaE was determined as 50 µM IPTG and 100 µM IPTG grown overnight respectively. However, the poor yields compared to that achieved for the expression of the *E. coli* proteins urged us to investigate other methods as well (Table 3.1).

We decided to use Tuner competent cells instead of *E. coli* BL21-star(DE3) to see if any improvement could be obtained.. Tuner cells are more sensitive to variation in IPTG concentrations than BL21-star(DE3). This is because these cells are dependent on passive diffusion of the IPTG into the cells whereas BL21-star(DE3) cells are dependent on an active transport system to carry IPTG across the cell wall. Transforming the plasmids into the Tuner cells proved somewhat problematic and no improvement in the expression of soluble protein was achieved.

Previous reports have promoted auto-induction media as being simpler to work with and giving better expression yields than IPTG induction media (*9*). As stated by the name this media is auto-inducing, thus no IPTG is added after expression has started. It is also less sensitive to variation in growth time.

We decided to attempt the use of ZYM-5052 a routine complex auto-inducing media. It is prepared in phosphate buffer, is versatile over a range op conditions and provides high density cultures. The *Sa*CoaDE plasmids were therefore once again transformed into *E. coli* BL21-star(DE3) and incubated overnight in the auto-inducing media. This produced much better expression levels (Table 3.1).

3.2.2.3 *Second attempt at expressing CBD-fusion proteins*

Since expression with auto-induction media proved so successful we decided to express all the fusion proteins in auto-induction media and compare the results to that achieved by IPTG induction.

A summary of the results obtained from the two expression methods are presented in Table 3.1. From this it was very clear that auto-induction is the more efficient method of expression. In all instances better yields were obtained than when IPTG inducing media was used.

Proteins	IPTG induction mg/L (IPTG concentration)	Auto-Induction media mg/L
	3 hours at 37°C	
Ec CoaA	59.4 (800 µM)	-
Ec CoaD	51.8 (1 mM)	-
Ec CoaE	58.0 (1 mM)	-
Sa CoaA	19.4 (500 µM)	-
	overnight at 37°C	
Sa CoaA	-	20.4
Sa CoaD	2.9 (50 µM)	12.3
Sa CoaE	3.2 (100 µM)	14.8
CBD*Sa* CoaA	2.4 (200 µM)	4.1
CBD*Sa* CoaD	-	8.8
CBD*Sa* CoaE	-	17.6
CBD*Ec* CoaA	-	45.4
CBD*Ec* CoaD	1.8 (250 µM)	9.2
CBD*Ec* CoaE	-	28.6

***Table 3.1*: Summary of the expression conditions and over-expression yields for all *Ec*CoaADE, *Sa*CoaADE, CBD*Ec*CoaADE and CBD*Sa*CoaADE proteins.**

3.2.3 Purification of native and newly constructed fusion proteins

After successful expression of the six fusion proteins and their native counterparts we had to develop a purification strategy. As was mentioned previously all the proteins were expressed with an N-terminal 6 x His-tag, allowing their purification using nickel-based IMAC. In our first attempt at purification, using this protocol, we used a Tris-HCl (pH 7.6) buffer system which caused precipitation of the proteins. To resolve this matter we tested different buffer systems at various pH values and concentrations.

Tris-HCl (10 mM, pH 7.6 and pH 8.0), HEPES (20 mM, pH 8.0), phosphate buffer (10 mM, pH 8.0 and pH 7.4) and phosphate buffer (20 mM, pH 7.2) were tested. We left the enzyme in the buffer solution at 4°C overnight. Most of the enzyme precipitated out of the Tris-HCl and HEPES buffer solutions, while the enzymes in the phosphate buffers (10 mM) stayed perfectly soluble. For our second attempt at protein purification we used the 10 mM phosphate buffer (pH 7.4). This time no precipitation occurred and pure protein solutions were obtained for the native *E. coli* and *S. aureus* proteins (Figure 3.3).

In spite of this success there was one consistent peptide band visible in all the purified CBD-fusion protein extracts. To improve the purification of our target proteins we tried to elute the protein using an imidazole gradient and various imidazole concentrations, but still failed to remove the unknown peptide band from any of the solutions. We therefore decided to first determine whether the purified protein – containing the single major contaminant – showed any activity before attempting to optimize the purification protocol.

For the activity assays we estimated the purity of the fusion protein stocks by analysis of SDS-PAGE gels used to separate the target protein from the contaminant. We used a computer program, UN-SCAN-IT *gel 6.1*, to determine the densities of each band, from which the overall purity could be determined. The percentage purities of the *E. coli* CBD-fusion proteins were found to be: CoaA 89.8%, CoaD 68.8%, and CoaE 91.7%. For the *S. aureus* CBD-fusion proteins the

percentage purities were determined as CoaA 57.8%, CoaD 78.4% and CoaE 73.9% (Figure 3.3).

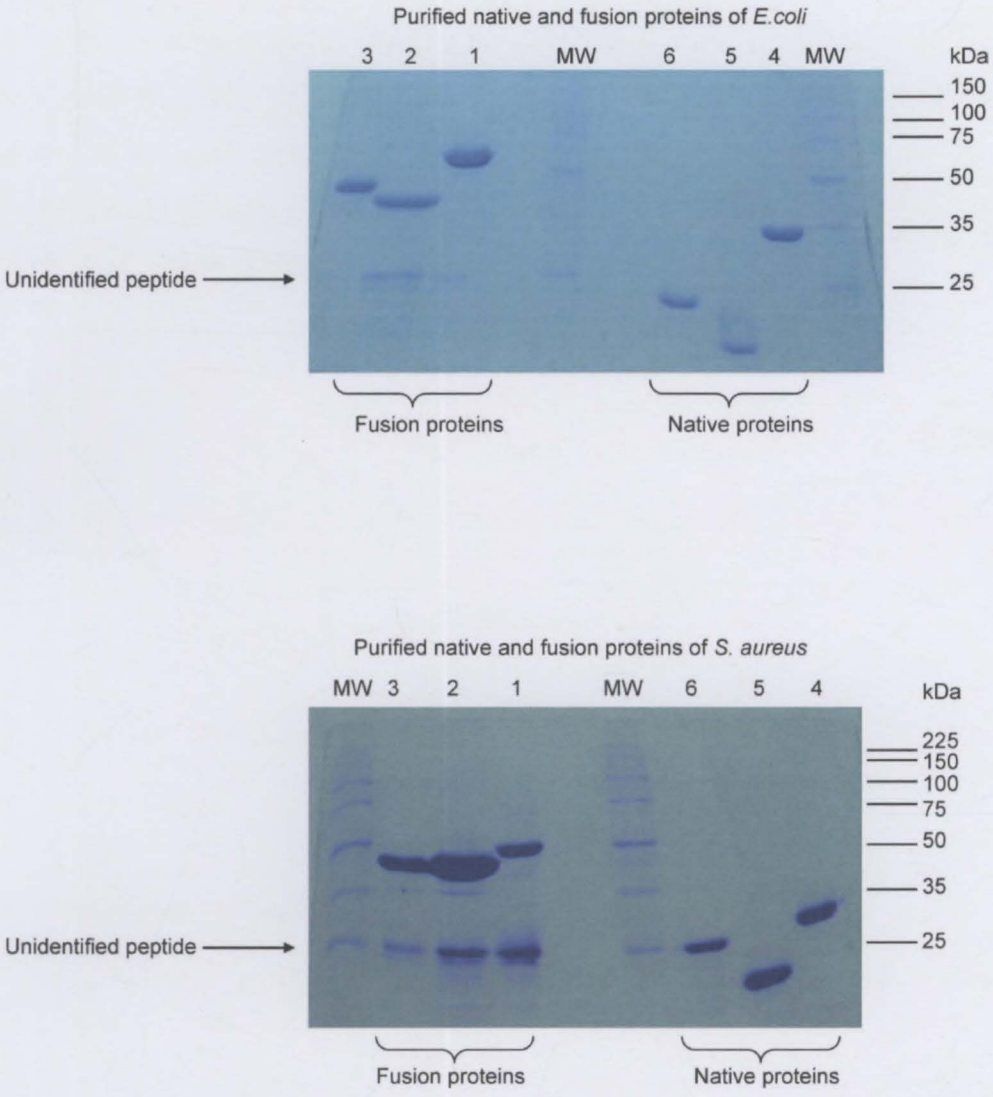

***Figure 3.3:* *E. coli* native and fusion proteins after purification (top):** *Lane 1*: CBD*Ec*CoaA (61 kDa, 90 % pure). *Lane 2*: CBD*Ec*CoaD (42 kDa, 69 % pure). *Lane 3*: CBD*Ec*CoaE (47 kDa, 92 % pure). *Lane 4*: *Ec*CoaA (39 kDa). *Lane 5*: *Ec*CoaD (20 kDa). *Lane 6*: *Ec*CoaE (25 kDa). ***S. aureus* native and fusion proteins after purification (bottom):** *Lane 1*: CBD*Sa*CoaA (53 kDa, 58 % pure). *Lane 2*: CBD*Sa*CoaD (43 kDa, 78 % pure). *Lane 3*: CBD*Sa*CoaE (48 kDa, 74 % pure). *Lane 4*: *Sa*CoaA (32 kDa). *Lane 5*: *Sa*CoaD (21 kDa). *Lane 6*: *Sa*CoaE (25 kDa).

The unknown peptide band must either be an N-terminal fragment of the fusion proteins, or another His-tag containing protein that is co-expressed with the fusion constructs. From the SDS-PAGE gels in figure 3.3 we can see that the unknown peptide band is about 25 kDa in size. This strongly suggests that cleavage occurs somewhere between the PT-linker and the protein itself, since the size of the cellulose binding domain plus the 6 x His-tag and the PT-linker is estimated to be 27.23 kDa.

3.2.4 Confirming the retained activity of the modified proteins

After the successful expression and purification of the CBD-fusion constructs and the native *S. aureus* proteins we had to determine if the modified enzymes had retained their activity. We chose *N*-pentylpantothenamide as the substrate to verify the activity of the various enzymes. This is a substrate known to be active with the native *E. coli* enzymes and since it does not have a thiol group the interference of disulfide forming side-reactions was eliminated.

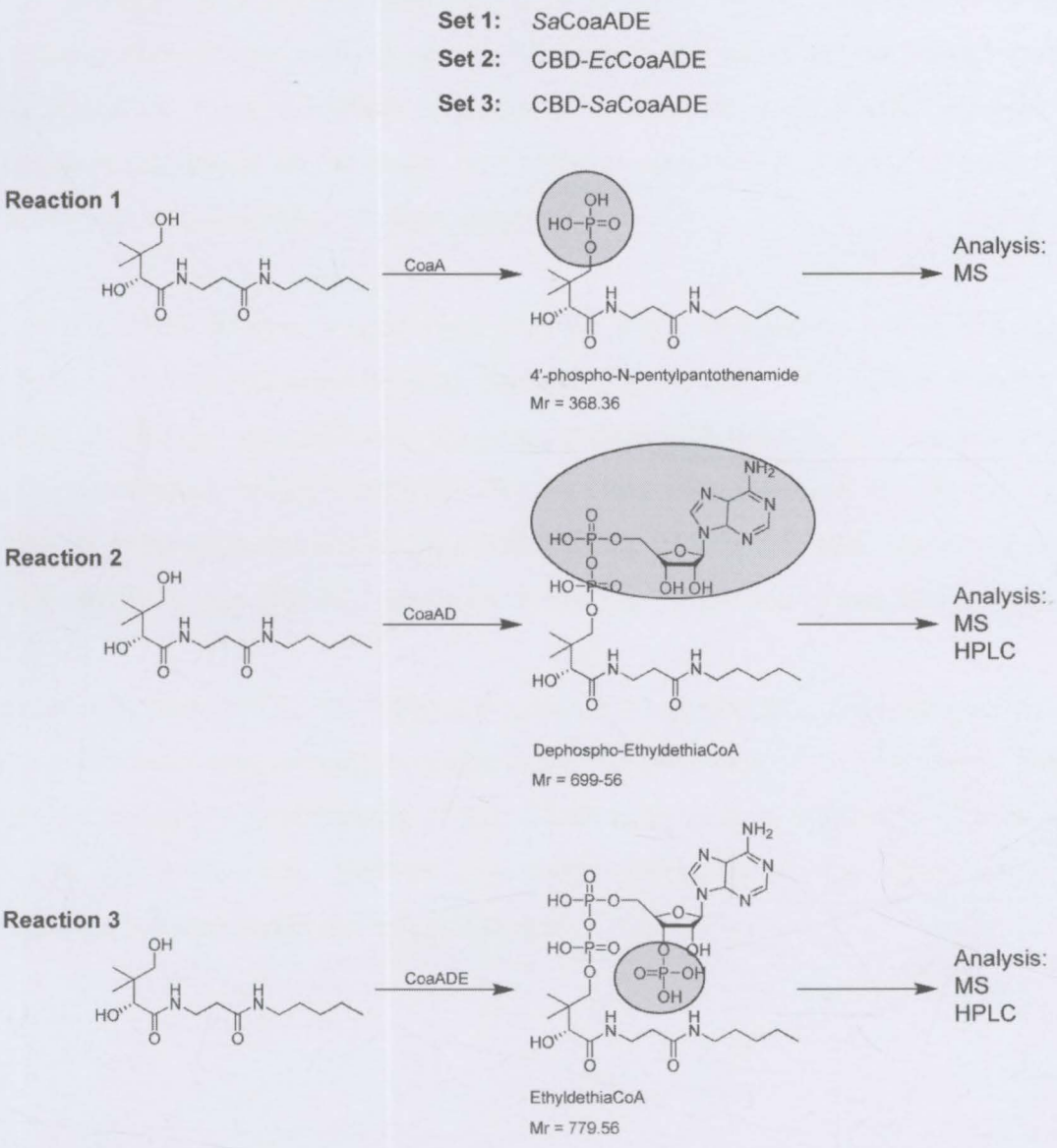

Figure 3.4: Confirming the retaining of activity by the modified CoA biosynthetic enzymes. Three sets of reactions were conducted, each consisting of the three presented reactions. *N*-pentylpantothenamide was the chosen substrate for all the reactions.

To determine the activities of all the newly purified proteins three sets of reactions were conducted (Figure 3.4). Each reaction contained 0.5 mM *N*-pentylpantothenamide and 15 µg of the required enzyme(s) in a 150 µl reaction. The reactions were incubated for 20 minutes at 37°C and then analyzed by HPLC and LC-MS. The first set of reactions was used to determine the activity of all three the native *S. aureus* enzymes. The second set was used to determine the activity of all three the *E. coli* CBD-fusion proteins and the third set was used to determine the activity of all three the *S. aureus* CBD-fusion proteins. Each set consisted of three reactions. The first reaction in every set contained only the CoaA enzyme, thus formation of 4'-phospho-*N*-pentylpantothenamide was assayed. The second reaction contained both the CoaAD enzymes, thus formation of dephospho-ethyldethia-CoA was assayed. The third reaction contained all three the biosynthetic enzymes and thus formation of the CoA analogue, ethyldethia-CoA, was assayed.

HPLC and LC-MS analyses confirmed that all the enzymes do retain activity after being fused with the cellulose binding domain (Figure 3.5). The HPLC spectra were measured at 220 nm and 254 nm, although only the 254 nm spectra are shown. 4'-Phosphopantetheine is not visible at 254 nm and absorbs very weakly at 220 nm therefore its spectra is not shown, but the transformation was still confirmed by ESI-MS. The results of the ESI-MS analyses in the ES- mode are presented below.

This assay has proved that we have succeeded in constructing biosynthetic enzymes fused to a CBD domain without compromising the activities of the enzymes. We also proved that substrate promiscuity of the CoaA enzymes is retained. These results prove that we should be able to use these enzymes in the construction of a bioreactor for the synthesis of CoA analogues.

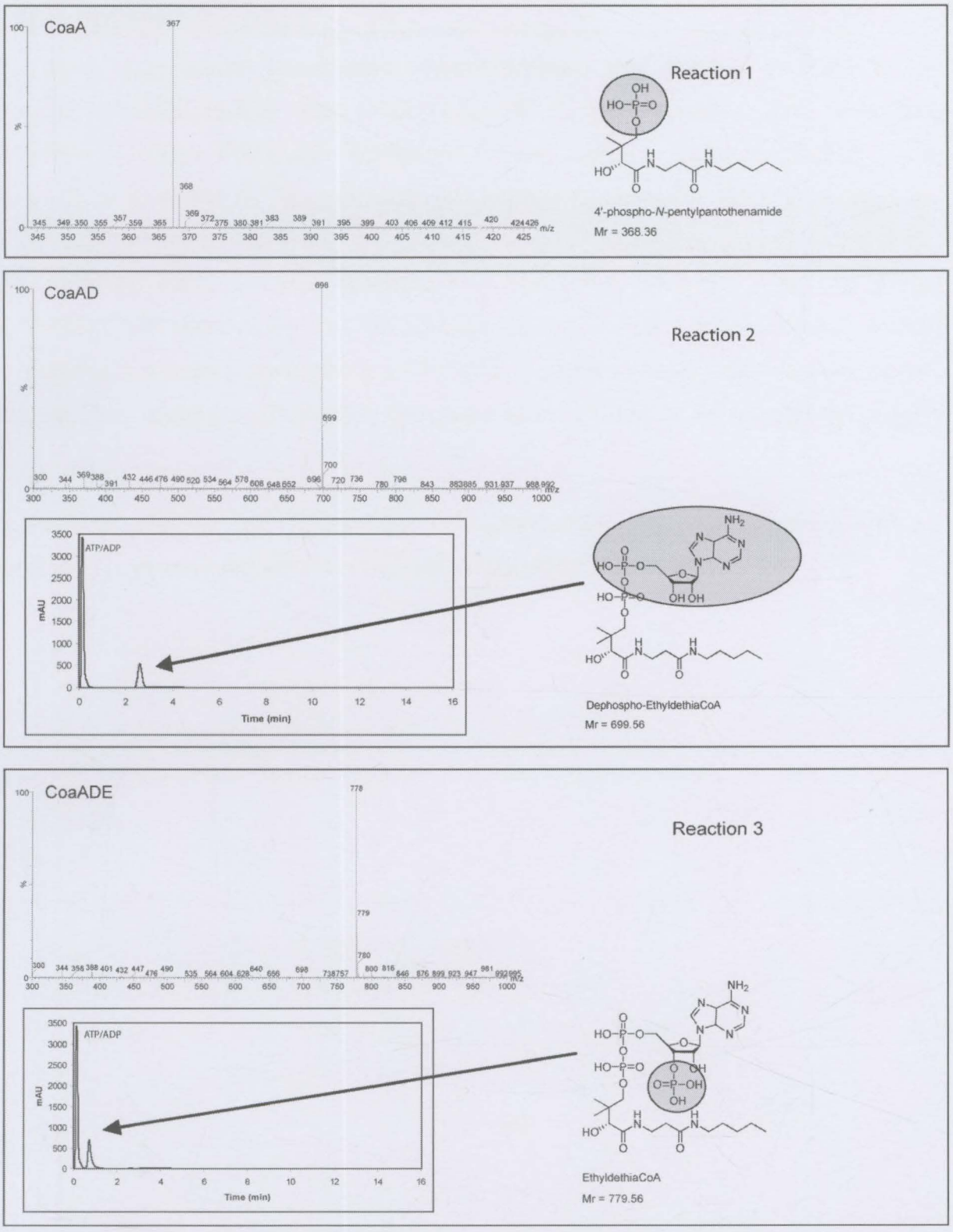

***Figure 3.5*: LC-MS and HPLC analyses of the three biosynthetic reactions confirming formation of both intermediates and the final CoA analogue.** Reaction 1: Assay containing only CoaA. Reaction 2: Assay containing CoaAD. Reaction 3: Assay containing CoaADE.

3.3 Conclusion

We have successfully constructed, over-expressed and purified six new fusion proteins, CBD*Sa*CoaADE and CBD*Ec*CoaADE in good yields. We have also determined optimal expression conditions for *Sa*CoaADE. Total purification of the native *S. aureus* and *E. coli* enzymes was achieved, while the CBD-fusion proteins were only partially purified as a single major contaminant, thought to be the unfused CBD module, could not be separated from the target protein. After this partial purification we determined the percentage purity of the fusion proteins. Most importantly we have shown that the CBD-fusion proteins have retained their activity and that they could thus be used in the preparation of CoA and its analogues.

In the next chapter we discuss the characterization of these enzymes and we determine how immobilization on cellulose may affect their activity.

3.4 Experimental procedures

3.4.1 Materials and Methods

All the expression vectors and competent cell strains were purchased from Novagen. The primers were synthesized by Inqaba Biotechnology (Pretoria, South Africa) and sequencing of the plasmids was done by automated DNA sequencing also at Inqaba Biotechnology (Pretoria, South Africa).

All the purification columns were purchased from Amersham Biosciences. The HiTrap™ Chelating Columns are from Amersham Biosciences. Protein purification was conducted on an ÄKTA*prime* purification system also from Amersham Biosciences. The software used for scanning the protein gels, UN-SCAN-IT *gel* 6.1, was purchased from Silk Scientific. The Quick Start Bradford Protein Assay Kit was purchased from Bio-rad. It contained the Bradford reagent and the Bovine Serum Albumin standard set.

All the chemicals used were purchased from Aldrich or Sigma and is of the highest quality.

A high performance liquid chromatography (HPLC) instrument (Agilent 1100 series) was used for the analysis of the biosynthetic reactions. Analyses were done on a Gemini 5u C18 column (30×2.00 mm) purchased from Phenomenex. A Heraeus Multifuge® 3S/3S-R was used for all the large scale centrifugation. The smaller scale centrifugation was conducted on a Heraeus Biofuge pico centrifuge. All absorptions were measured using a Varioscan multiplate spectrophotometer from Thermo Labsystems.

3.4.2 Expression trials

All the plasmids were cloned and expression vectors constructed by Mrs. L. A. Brand prior to the investigation.

3.4.2.1 IPTG induction

The plasmids pET28a-CBD_{cipA}*Ec*CoaD and pET28a-CBD_{CipA}*Sa*CoaA were transformed into BL21star(DE3). Starting cultures (5 ml) of each were prepared in LB broth supplemented with kanamycin sulphate (30 mg/l) and grown overnight (18 hours). Liquid media cultures, 15 ml LB broth supplemented with kanamycin sulphate (30 mg/l), were induced with 100 µl of the starting cultures and grown at 37°C till an OD_{600} ~0.6 was reached. The liquid media cultures were induced with a range of IPTG concentrations and grown for varying times at 37°C to determine the optimum expression conditions. The cells were harvested at 4500 x g for 20 minutes and whole cell and cell free expression were determined.

The procedure was repeated with the plasmids pET28a-*Sa*CoaD and pET28a-*Sa*CoaE transformed into BL21star(DE3) and Tuner cells.

3.4.2.2 Whole cell analysis

The harvested cells were re-suspended in 2 ml Tris-HCl (10 mM, pH 8) and 200 µl of the cell suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged at 13 000 rpm for 2 minutes. The supernatant was discarded and the pellet re-suspended in 50 µl Tris-HCl (10 mM, pH 8). An equal amount of SDS-PAGE loading buffer was added to the tube and the suspension was boiled for 5 minutes at 95°C. The suspension was spun down before being loaded onto a 12% SDS-PAGE gel that was stained with Coomassie Brilliant Blue R-250.

3.4.2.3 Cell free analysis

The harvested cells were re-suspended in 2 ml Tris-HCl (10 mM, pH 8) and 200 µl of the cell suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged at 13 000 rpm for 2 minutes. The supernatant was discarded and the pellet re-suspended in 50 µl Tris-HCl (10 mM, pH 8). Half a volume of glass beads was added and the suspension was vortexed for 10 minutes to disrupt the cells. SDS-PAGE loading buffer (50 µl) was added to the tube and the suspension was boiled for 5 minutes at 95°C. Afterwards it was spun down before being loaded onto a 12% SDS-PAGE gel that was stained with Coomassie Brilliant Blue R-250.

3.4.3 Overexpression of *E. coli* biosynthetic enzymes

Overexpression of *Ec*CoaADE was conducted at 37°C in 500 ml LB broth supplemented with 30 mg/l of kanamycin sulphate. The cells were grown up to OD_{600} ~0.6, before being induced with different concentrations of IPTG and then left to grow overnight at the same temperature. *Ec*CoaA were induced with 800 µM IPTG, and *Ec*CoaDE with 1000 µM IPTG.

3.4.4 Overexpression of native *S. aureus* and CBD-fusion proteins

3.4.4.1 IPTG induction

*Sa*CoaDE, CBD*Ec*CoaD and CBD*Sa*CoaA were overexpressed according to the optimal conditions determined in the expression trials. All the overexpresssions were conducted at 37°C in 500 ml LB broth supplemented with 30 mg/l kanamycin sulphate. The cells were grown up to OD_{600} ~0.6, before being induced with IPTG and then left to grow overnight at the same temperature. *Sa*CoaD was in induced with 50 µM, *Sa*CoaE with 100 µM, CBD*Ec*CoaD with 200 µM and CBD*Sa*CoaA with 250 µM.

3.4.4.2 Auto-induction media (ZYM-5052)

A complex auto-induction media (ZYM-5052) was used for the overexpression of *Sa*CoaADE, CBD*Ec*CoaADE and CBD*Sa*CoaADE. All the plasmids were transformed into *E. coli* BL21-star(DE3) and overexpressed at 37°C, overnight. The cells were harvested at 4500 × g for 30 minutes and stored at -80°C till used again.

3.4.5 Protein purification

The harvested cells were re-suspended in sonication buffer (20 mM phosphate buffer, 500 mM NaCl, pH 7.4) and disrupted by sonication in 6 × 60 second cycles. It was centrifuged at 15 000 × g for 30 minutes to pellet the cell debris. The collected crude extract was purified on a 1 ml HisTrap™ chelating column pre-loaded with $NiSO_4$ (100 mM) using an ÄKTA*prime* purification system. Weakly bound proteins were removed by washing with sonication buffer and sonication buffer containing 75

mM imidazole, successively. The protein of interest was eluted by increasing the imidazole concentration to 500 mM. Elution of the protein was monitored at A_{280}. After purification the protein was loaded onto a HiTrap™ desalting column and washed with gel filtration buffer (25 mM phosphate buffer, 5 mM $MgCl_2$, pH 7.4) before adding 5% glycerol and storing it at -80°C.

Purity of the native enzymes was confirmed by running a 12% SDS–PAGE gel. SDS-PAGE gels of the fusion proteins were electronically analyzed using gel digitizing software. The gels were scanned in on the computer and the software was used to determine the ratio between the known and the consistent unidentified protein band.

3.4.6 Determining enzyme concentrations

Enzyme concentrations were determined using the Quick Start Bradford protein assay. In this assay Coomassie Brilliant Blue R-250 dye binds to the enzymes to convert it to a stable unprotonated blue form, which can be detected at 595 nm.

Bovine Serum Albumin was used as the standard. It has a linear range of 125-1000 $\mu g.ml^{-1}$. 5 µl of each concentration and of different dilutions of the protein was transferred to a mictrotitre plate. Bradford reagent (250 µl) was added to each well. Distilled water was used as a blank. The plate was incubated at room temperature for 5 minutes after addition of the Bradford reagent. All the measurements were done in triplicate. The Beer-Lambert law was used to convert the measurements to concentrations.

3.4.7 Activity of native *S. aureus* and CBD-fusion proteins

3.4.7.1 Biosynthesis of ethyldethia-CoA

Each reaction mixture contained 50 mM Tris-HCl buffer (pH 7.6), 5 mM ATP, 5 mM $MgCl_2$, 2 mM DTT and 15 µg of the required enzyme in a final reaction volume of 150 µl. The reactions were initiated by addition of 0.5 mM of *N*-pentylpantothenamide and incubated for 20 minutes at 37°C. Three reactions were conducted to confirm

the formation of the intermediates in the pathway. The first reaction contained only CoaA, the second contained both CoaA and CoaD and the third contained CoaADE. The reactions were quenched by transferring the tubes to 95°C for 5 minutes which caused precipitation of the proteins. The precipitated protein was collected by centrifugation for 5 minutes at 13 000 rpm before the supernatant (15 µl) could be injected onto the HPLC column for analysis.

3.4.7.2 Conditions for HPLC analysis

The analysis method took 15 minutes with a flow rate of 1 ml/min. The method started with 95% of a 100 mM NH_4OAc buffer solution (pH 6.6) and 5% acetonitrile. The acetonitrile concentration increased as the method developed: 5-40% MeCN, 1-3 minutes; isocratic, 3-8 minutes; 40-80% MeCN, 8-10 minutes; isocratic, 10-14 minutes; 80-5%, 14-15 minutes; 5% for 5 minutes before the next injection. Absorption was monitored at 254 nm. The measured retention times were: ATP, 0.1 min, 3'-dephospho-ethyldethia-CoA, 2.6 min and ethyldethia-CoA, 0.7 min. LC-MS of the reactions were also conducted which allowed the detection of 4'-phospho-*N*-pentylpantothenamide.

3.4.7.3 Conditions for LC-MS analysis

LC-MS analysis was performed on a Waters 2690 Separations Module with a Waters 996 Photodiode Array Detector, followed by MS analysis on a Waters Quattro Micromass mass spectrometer.

For LC separation the column was equilibrated with 95% of a 100 mM NH_4OAc buffer solution (pH 6.6) and 5% acetonitrile over the first 3 minutes of the analysis. The acetonitrile concentration increased as the method developed: 5-40% MeCN, 3-6 minutes; isocratic, 6-10 minutes; 40-60% MeCN, 10-11 minutes; isocratic, 11-15 minutes; 60-80%, 15-16 minutes; isocratic, 16-18 minutes; 5% up to 25 minutes. A flow rate of 1 ml/min was maintained. The products were detected at A_{220} or A_{254} depending on the absorbance properties of the compounds. Peak identification was done by analysis of the average mass spectrum (negative mode) of each peak.

3.5 References

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Chapter 4

Characterization of the Newly Constructed CoA Biosynthetic Fusion Proteins

4.1 Introduction

We are aiming to build a reactor for the biosynthesis of CoA analogues. In order to achieve this we need to know certain characteristics of the fusion proteins that will form the core of this reactor. In this chapter we focus on the determination of these characteristics.

In chapter three we reported the construction of six novel fusion proteins originating from two sources, namely *E. coli* and *S. aureus*. Thus we have duplicates of each of the biosynthetic enzyme that are needed to produce CoA analogues. This gives us eight possible enzyme combinations that might produce CoA analogues from pantothenamide substrates.

The first objective of the work discussed in this chapter was to identify the best combinations from these eight possibilities for use in the construction of a reactor. Subsequently kinetic parameters had to be determined for the fusion proteins making up these combinations to facilitate the future design and construction of bioreactors. Finally we determined the influence of modification and immobilization of the activity of the fusion proteins by comparison to the activity of the native enzymes.

4.2 Results and Discussion

4.2.1 Comparing the activity of the different fusion protein combinations

In order to compare the activities of all the possible fusion proteins combinations we reconstituted the CoA biosynthetic pathway using a CoaA, a CoaD and a CoaE enzyme from either *E. coli* or *S. aureus* in all possible combinations. The activities of these combinations were determined by analysis of the reaction mixtures by HPLC. An internal standard (guanosine) was co-injected with all these analyses to allow us to compare the yields of the various reactions. Each 150 µl reaction contained 15 µg of each of three fusion proteins and 0.5 mM substrate. The reactions were incubated for 60 minutes at 37°C before analysis was done.

Two substrates were chosen for these reactions namely, pantetheine and *N*-pentylpantothenamide. Pantetheine, although not the natural substrate of the CoA biosynthetic pathway, is an advanced intermediate which can be used by the CoaADE enzymes in a salvage manner. *N*-pentylpantothenamide was used in the previous chapter (Figure 4.1).

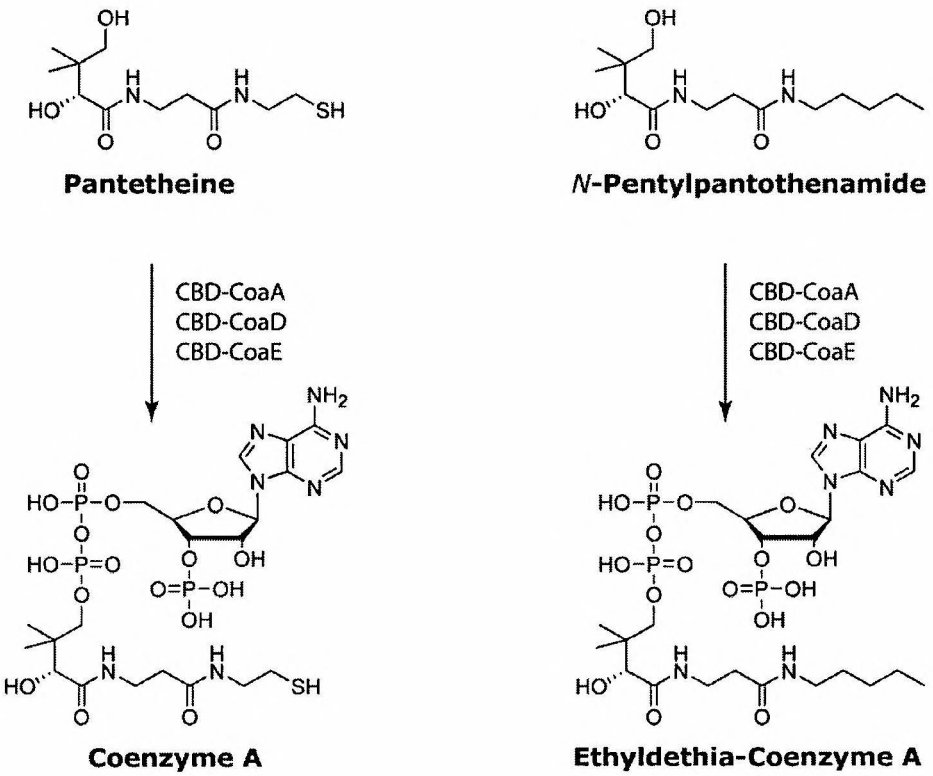


***Figure 4.1*: Reactions used to compare the activity of the different fusion protein combinations.** Each reaction contained a CoaA, CoaD and a CoaE enzyme from either *E. coli* or *S. aureus*.

4.2.1.1 *Comparing E. coli to S. aureus*

The first set of reactions was constructed to compare the activities of the enzymes from the two sources, namely *E. coli* and *S. aureus*. Thus four reactions were conducted, each containing only *E. coli* or *S. aureus* enzymes with either one of the mentioned substrates. All four of these reactions were conducted in triplicate.

In Figure 4.2 we present the average yields of the four reactions as ratios with the internal standard. **C1** represents the *E. coli* proteins and **C2** represents the *S. aureus* proteins. From these results we can conclude that both substrates are formed in higher yields by the *E. coli* enzymes.

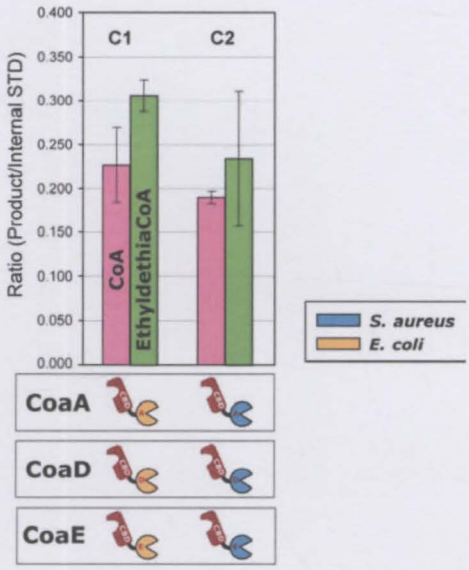


***Figure 4.2*: Comparing the activities of the CBD-fusion proteins from *E. coli* (C1) and *S. aureus* (C2).** CoA was biosynthesized from pantetheine (pink). Ethyldethia-CoA was biosynthesized from *N*-pentylpantothenamide (green). Each of the values represents the average yield of three identical reactions as a ratio to the internal standard, guanosine. The error bars indicate the standard deviations of the average values.

Our results also show that for **C1** ethyldethia-CoA, the product formed from *N*-pentylpantothenamide, is produced in higher yields than CoA from pantetheine. This is in agreement with previous studies of the native enzymes and serves as confirmation of the validity of our assay (*1*).

4.2.1.2 *Comparing combinations containing SaCoaA*

Since *Ec*CoaA is feedback inhibited, while *Sa*CoaA is not, we wanted to see if there is an enzyme combination containing *Sa*CoaA that would show better activity than the combination using only the *E. coli* enzymes. There are three possible enzyme combinations containing CBD*Sa*CoaA (excluding **C2**, which was tested in the previous experiment):

- CBD*Sa*CoaAD and CBD*Ec*CoaE (**C3**)
- CBD*Sa*CoaA and CBD*Ec*CoaDE (**C4**)
- CBD*Sa*CoaA, CBD*Ec*CoaD and CBD*Sa*CoaE (**C5**)

We compared the activities of these combinations using the same two substrates as before. The results are presented in Figure 4.3.

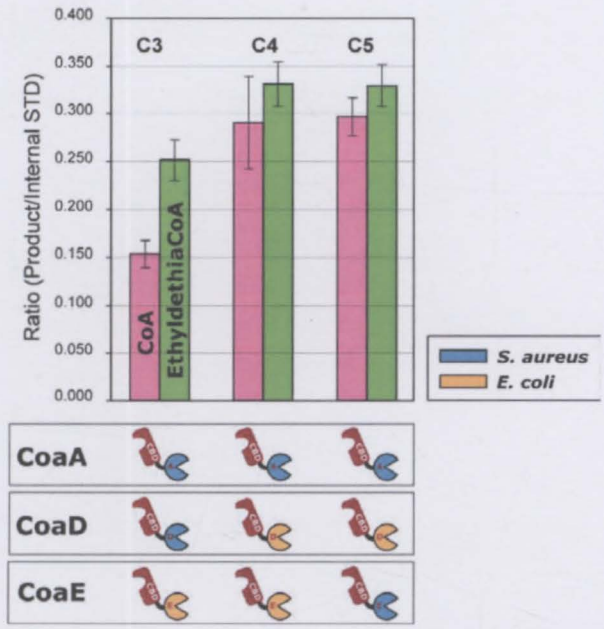


***Figure 4.3*: Comparing the activities of the three CBD-fusion protein combinations that include CBD*Sa*CoaA.** Three enzyme combinations were assayed: CBD*Sa*CoaAD and CBD*Ec*CoaE (**C3**), CBD*Sa*CoaA and CBD*Ec*CoaDE (**C4**), CBD*Sa*CoaA, CBD*Ec*CoaD and CBD*Sa*CoaE (**C5**). CoA was biosynthesized from pantetheine (pink). Ethyldethia-CoA was biosynthesized from *N*-pentylpantothenamide (green). Each of the values represents the average yield of three identical reactions as a ratio with the internal standard, guanosine. The error bars indicate the standard deviations of the average values.

From these results we can conclude that **C3** is the least preferred of the three combinations. This implies that CBD*Sa*CoaA and CBD*Ec*CoaD is a better combination than CBD*Sa*CoaAD. There is very little difference between the activities of **C4** and **C5** indicating that there is not much difference in the activity of the two CoaE enzymes.

4.2.1.3 Comparing combinations containing EcCoaA

Next we compared the activities of the possible fusion protein combinations containing CBD*Ec*CoaA, excluding **C1** which has already been discussed:

- CBD*Ec*CoaAD and CBD*Sa*CoaE (**C6**)
- CBD*Ec*CoaA and CBD*Sa*CoaDE (**C7**)
- CBD*Ec*CoaA, CBD*Sa*CoaD and CBD*Ec*CoaE (**C8**)

Using the same substrates as before the following results were obtained (Figure 4.4):

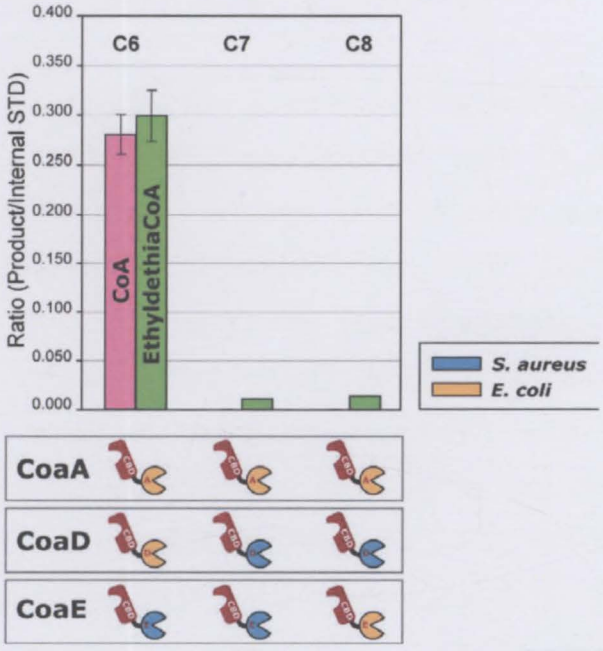


***Figure 4.4*: Comparing the activity of the three CBD-fusion protein combinations that include CBD*Ec*CoaA.** Three enzyme combinations were assayed: CBD*Ec*CoaAD and CBD*Sa*CoaE (**C6**), CBD*Ec*CoaA and CBD*Sa*CoaDE (**C7**), CBD*Ec*CoaA, CBD*Sa*CoaD and CBD*Ec*CoaE (**C8**). CoA was biosynthesized from pantetheine (pink). Ethyldethia-CoA was biosynthesized from *N*-pentylpantothenamide (green). Each of the values represents the average yield of three identical reactions as a ratio with the internal standard, guanosine. The error bars indicate the standard deviations of the average values.

C6 was the only combination that showed significant activity. **C7** and **C8** containing CBD*Ec*CoaA and CBD*Sa*CoaD showed no activity with pantetheine and negligible activity with *N*-pentylpantothenamide.

4.2.1.4 Identifying the preferred enzyme combinations

Using our results from the activity assays we could now identify the two preferred combinations, one each for the biosynthesis of either CoA or ethyldethia-CoA. These two combinations could then be further characterized for use in the construction of a reactor. Since combinations **C7** and **C8** showed no significant activity with either of the substrates we had six combinations to consider.

In considering the biosynthesis of CoA from pantetheine we identified three possible combinations - **C4**, **C5** and **C6** - based on their reaction yields. To choose between these we considered the expression yields and % purity of the various proteins.

In **C6** we used CBD*Ec*CoaA while the *S. aureus* enzyme was used in both **C4** and **C5**. CBD*Ec*CoaA expresses with an average yield of 45.4 mg/l whereas CBD*Sa*CoaA expresses with an average yield of only 4.1 mg/l (Table 3.1). Furthermore, CBD*Ec*CoaA can be obtained in 90% purity, but CBD*Sa*CoaA is only 58% pure (Figure 3.3). This suggests that combination **C6**, made up of CBD*Ec*CoaA, CBD*Ec*CoaD and CBD*Sa*CoaE is the combination of choice for the preparation of CoA. However, a potential problem with this choice is that CBD*Ec*CoaA is used in this combination, an enzyme that experiences feedback inhibition. Nonetheless, we decided on using **C6** in our further studies since the problem of feedback inhibition could still be addressed in the manner in which the bioreactor would be constructed.

In considering the biosynthesis of ethyldethia-CoA we identified four possible combinations - **C1**, **C4**, **C5** and **C6** - based on their reaction yields. Again we considered the expression yields and % percentage purity of the respective proteins to make a selection from among these combinations.

Using the same argument that takes the expression yields of the proteins in question into account we decided to eliminate **C4** and **C5**, which involves using CBD*Sa*CoaA which expresses at low yields and with major impurities. This leaves **C1** and **C6**, which only differs in the source of the CoaE enzyme. Comparing the expression yields and % purity of CBD*Sa*CoaE and CBD*Ec*CoaE we find that CBD*Ec*CoaE can be obtained in 92% purity with an expression yield of 28.6 mg/l. On the other hand CBD*Sa*CoaE can only be obtained in 74% purity with an expression yield of 17.6 mg/l. We thus decided that **C1** will be used for the biosynthesis of ethyldethia-CoA in our further studies.

To conclude we have identified the two preferred enzyme combinations for the biosynthesis of CoA and ethyldethia-CoA to be used in our further studies as **C6** and **C1** respectively.

4.2.2 Determining kinetic parameters of the selected proteins

Next we wanted to determine the kinetic parameters of the four fusion proteins and their native counterparts as are represented by **C6** and **C1**, which would require the availability of pure fusion protein stocks. Up to date we have only managed to determine the percentage purity of the fusion proteins with the putative N-terminal fragment as contaminant (Figure 3.3). Thus we first had to optimize our purification strategy.

4.2.2.1 Further purification of fusion protein constructs

Anion exchange columns have previously been implemented for the purification of CBD-fusion proteins (*2-4*). In these protocols the crude extract is loaded onto a MONO Q or DEAE column that is pre-equilibrated with phosphate buffer, followed by elution of the protein with a salt gradient. In order to optimize our purification strategy we set out to use an anion exchange column, 16/10 DEAE FF column, and succeeded in successfully separating the unknown peptide from the partially purified CBD*Ec*CoaDE and CBD*Sa*CoaE enzyme solution (Figure 4.5).

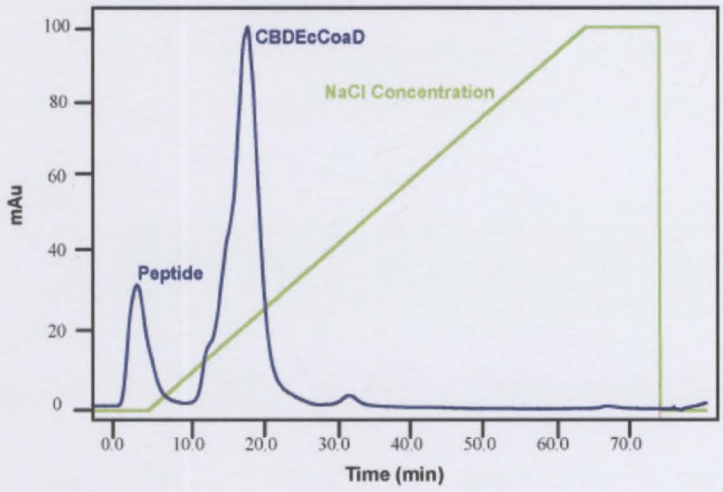


***Figure 4.5*: Enzyme purification with a DEAE anion exchange column showing separation of the unknown peptide band and CBD*Ec*CoaD.** The blue line represents the proteins absorption and the green line represents the NaCl gradient.

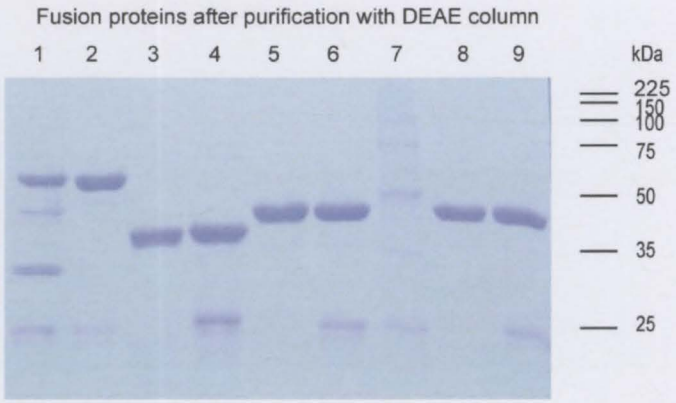
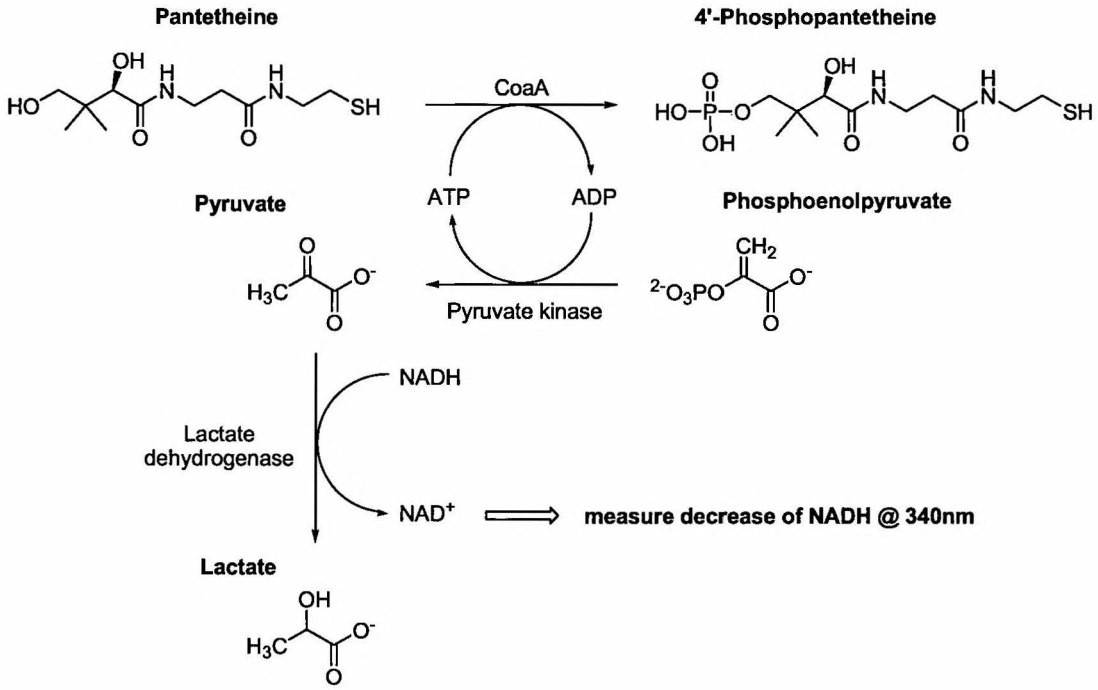


***Figure 4.6*: SDS-PAGE gel of the fusion proteins after purification on an anion exchange column**. *Lane 1*: CBD*Ec*CoaA (after purification). *Lane 2*: CBD*Ec*CoaA (before purification). *Lane 3*: CBD*Ec*CoaD (after purification). *Lane 4*: CBD*Ec*CoaD (before purification). *Lane 5*: CBD*Ec*CoaE (after purification). *Lane 6*: CBD*Ec*CoaE (before purification). *Lane 7*: Molecular weight markers. *Lane 8*: CBD*Sa*CoaE (after purification). *Lane 9*: CBD*Sa*CoaE (before purification).

Unfortunately the same strategy failed to purify CBD*Ec*CoaA (Figure 4.6). Instead we found that the solution was less pure after this procedure, indicating that degradation possibly occurs during purification. We therefore decided to rather use the 90% pure stock that was obtained after the first purification for the kinetic studies.

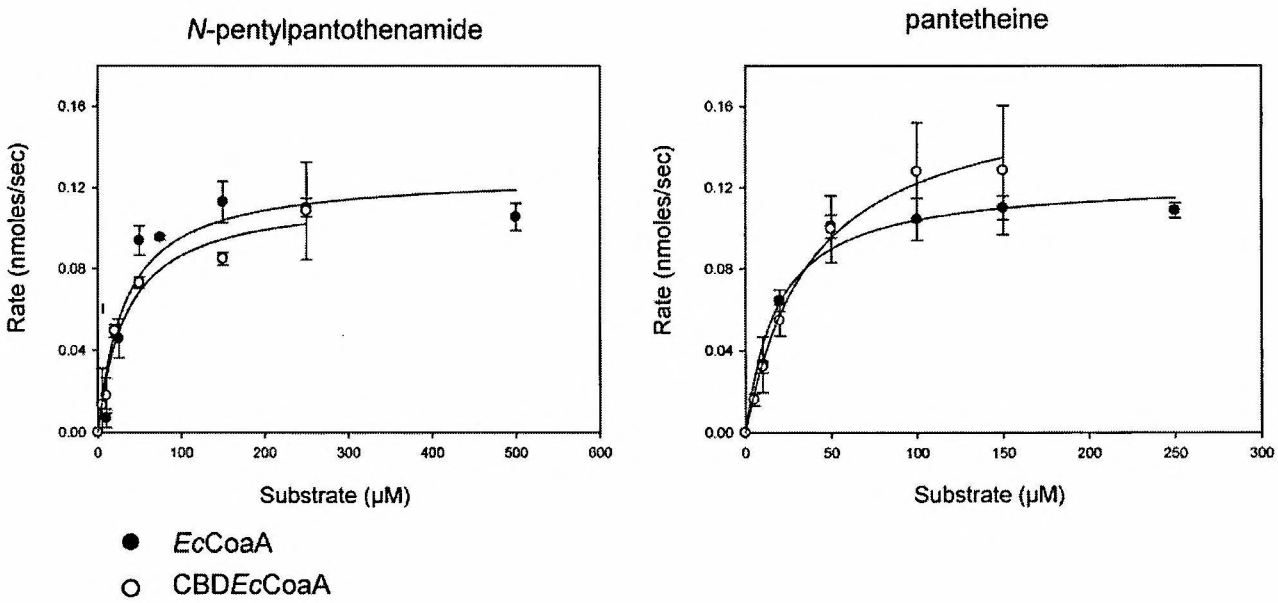
4.2.2.2 *Kinetic parameters of the CoaA enzymes*

The assay that we used measures the decrease in NADH concentration in a coupled reaction (Scheme 4.1) and is a well-established assay of kinase enzymes.



***Scheme 4.1*: The assay used to determine the kinetic parameters of *Ec*CoaA, CBD*Ec*CoaA, *Ec*CoaE, CBD*Ec*CoaE, *Sa*CoaE and CBD*Sa*CoaE.**

Using this assay, kinetic parameters of *Ec*CoaA and CBD*Ec*CoaA were determined for pantetheine and *N*-pentylpantothenamide by fitting the data to the Michaelis-Menten equation (Figure 4.7). These kinetic results show that the addition of the CBD fusion does not negatively impact on the activity of the CBD*Ec*CoaA enzymes; in fact, they have higher specificity constants for both substrates compared to the native enzymes.

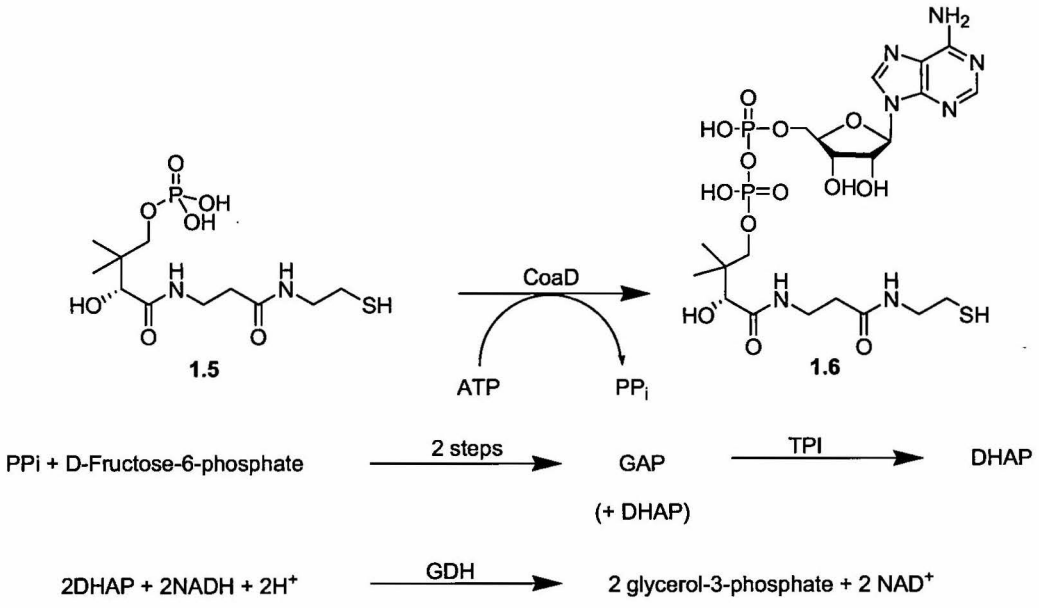


***N*-pentylpanothenamide**	V_{max} (nmoles.sec^{-1})	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M (sec^{-1}.mM^{-1})
EcCoaA	0.127	33.020	1.627	49.269
CBDEcCoaA	0.116	34.588	2.352	67.988
pantetheine				
EcCoaA	0.124	18.969	1.588	83.734
CBDEcCoaA	0.170	39.492	3.446	87.267

Figure 4.7: **Activity of *Ec*CoaA and CBD*Ec*CoaA with pantetheine and *N*-pentyl-pantothenamide.** Initial rate plots of *Ec*CoaA (●) and CBD*Ec*CoaA (O) against increasing substrate concentrations. Substrates used: *N*-pentylpantothenamide (left) and pantetheine (right). Curves show fits of the individual data sets to the Michaelis-Menten equation.

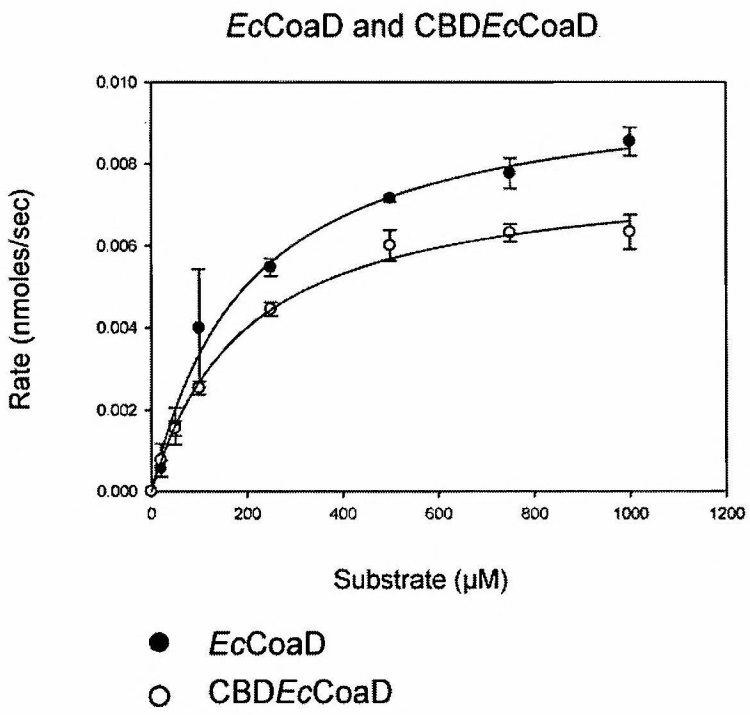
4.2.2.3 Kinetic parameters of the CoaD enzymes

The activity of the CoaD enzymes was assayed using an assay which couples the production of pyrophosphate (which is released during transfer of the adenylyl group to phosphopantetheine) to the consumption of NADH, as shown in Scheme 4.2. The pyrophosphate is used in a three step enzymatic synthesis to yield dihydroxyacetone phosphate (DHAP). Two moles of DHAP consumes two moles of NADH to yield one mole of 2-glycerol-3-phosphate. This implies that for every mole of **1.5** that is consumed two moles of NADH are also consumed. The decrease in the NADH concentration was measured at 340nm.



***Scheme 4.2*: Determining the kinetic parameters of *Ec*CoaD and CBD*Ec*CoaD.** Pyrophosphate (PP_i) is transformed to D-glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). GAP is transformed to DHAP. 2 moles DHAP utilize 2 moles NADH to form 2 moles glycerol-3-phosphate. The decrease in NADH concentration is measured at 340 nm. The two indicated enzymes are triosephosphate isomerase (TPI) and glycerophosphate dehydrogenase (GDH).

The kinetic parameters of *Ec*CoaD and CBD*Ec*CoaD for the natural substrate 4'-phosphopantetheine **1.5** were determined using this assay by fitting the data to the Michaelis-Menten equation (Figure 4.8). Again, the data show that the introduction of the CBD has a negligible effect on the activity of the enzyme.

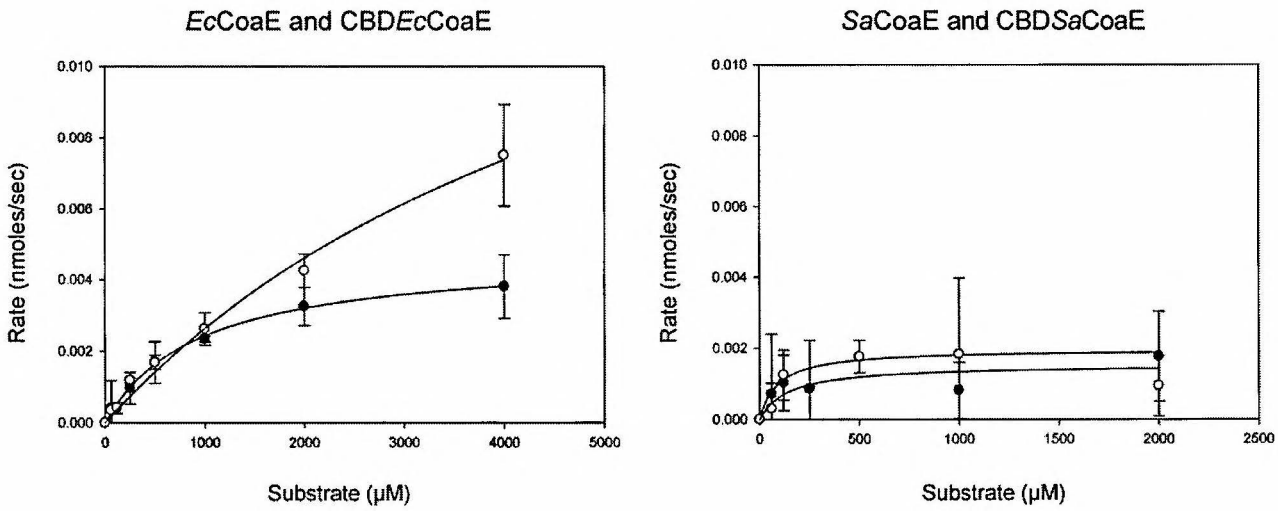


4'-phosphopantetheine	V_{max} (nmoles.sec^{-1})	K_M (µM)	k_{cat} (sec^{-1})	k_{cat}/K_M (sec^{-1}.mM^{-1})
EcCoaD	0.010	198.996	0.119	0.598
CBDEcCoaD	0.008	194.011	0.111	0.573

***Figure 4.8*: Activity of *Ec*CoaD and CBD*Ec*CoaD with 4'-phosphopantetheine.** Initial rate plots of *Ec*CoaD (●) and CBD*Ec*CoaD (○) against increasing substrate concentrations.

4.2.2.4 Kinetic parameters of the CoaE enzymes

For characterization of CoaE the same kinase assay as the one used to determine the kinetic parameters of *Ec*CoaA was employed. CBD*Ec*CoaE, *Sa*CoaE and CBD*Sa*CoaE were subsequently kinetically characterized using dephospho-CoA **1.6** as substrate (Figure 4.9). The CBD fusions proteins again demonstrate slightly improved specificity constants.



dephospho-CoA	V_{max} (nmoles.sec^{-1})	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M (sec^{-1}.mM^{-1})
EcCoaE	0.005	934.906	0.078	0.083
CBDEcCoaE	0.018	5990.958	0.576	0.096
SaCoaE	0.002	90.943	0.034	0.378
CBDSaCoaE	0.002	102.009	0.058	0.564

***Figure 4.9*: Activity of *Ec*CoaE, CBD*Ec*CoaE, *Sa*CoaE and CBD*Sa*CoaE with dephospho-CoA.** Initial rate plots of *E. coli* CoaE (left) and *S. aureus* CoaE (right) against increasing substrate concentrations. Curves show fits of the individual CoaE (●) and CBDCoaE (O) data sets to the Michaelis-Menten equation.

4.2.3 Comparing the rate of biosynthesis between the native and the fusion proteins

Before constructing the reactor we wanted to determine the effect of modification and of immobilization on the activity of the identified CoA biosynthetic proteins. We thus needed to compare the activity of the two chosen combinations of biosynthetic enzymes in their different states as the native proteins and as CBD-fusion proteins – the latter both in solution and immobilized on cellulose.

We used the same HPLC assay that was developed for the comparison of the activity of the different enzyme combinations, except that the reaction's progress was monitored at smaller time intervals.

4.2.3.1 *Native proteins vs CBD-fusion proteins in solution*

In the first assay we wanted to determine the effect of the CBD on the overall biosynthetic activity of the proteins. We thus compared the activity of the native CoaADE proteins to that of the CBD-fusion proteins over a set time period using either pantetheine or *N*-pentylpantothenamide as substrate (Figure 4.10). Enzyme combination **C1** was used for the biosynthesis of ethyldethia-CoA from *N*-pentylpantothenamide and enzyme combination **C6** was used for the biosynthesis of CoA from pantetheine.

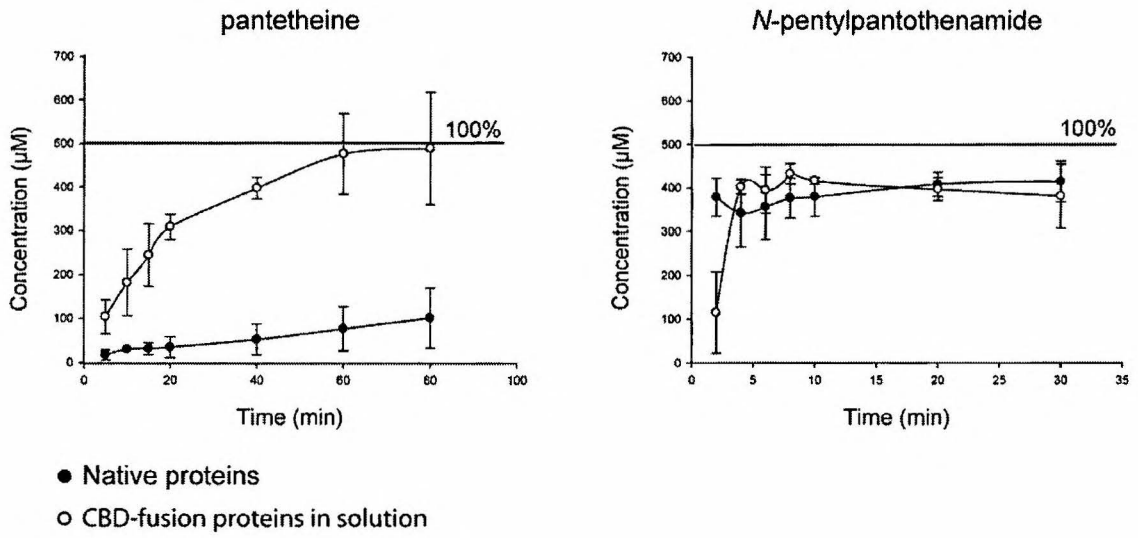


***Figure 4.10*: Rate of biosynthesis for native and solution based CBD-fusion protein combinations.** Enzyme combination **C6** was used for the transformation of pantetheine to CoA (left). Enzyme combination **C1** was used for the transformation of *N*-pentylpantothenamide to ethyldetia-CoA (right). The data sets of the native (●) and the solution based CBD-fusion proteins (O) for the corresponding substrates were plot on the same graphs. Each data point represents an average of three values with the standard deviations indicated by the error bars. A 100% conversion equals 500 µM.

The results show that the rate of biosynthesis of CoA from pantetheine using the **C6** combination of enzymes is drastically increased by the CBD-fusion proteins compared to the native enzymes. While the native enzymes could only achieve a 20% yield after 80 minutes the yield was increased to 100% after 60 minutes by the introduction of the CBD-domain.

For the biosynthesis of ethyldethia-CoA from *N*-pentylpantothenamide using the **C1** combination of enzymes very little difference between the rates of the native and the CBD-fusion proteins could be observed. After 20 min 80% conversion was achieved by both enzymes.

4.2.3.2 CBD-fusion proteins in solution vs CBD-fusion proteins immobilized on cellulose

In the second assay we wanted to determine the effect of immobilization on the activity of the proteins. However to be able to do this we first needed to immobilize the proteins on cellulose. Since we did not want to pointlessly waste protein during the immobilization procedure we set out to determine the binding curves of the various CBD-fusion proteins to cellulose.

4.2.3.2.1 Immobilizing the fusion proteins

To determine the binding capacity of the fusion-proteins to cellulose we added different masses of protein to a constant cellulose mass and determined the percentage immobilization in each case. A known amount of protein (100 µg, 150 µg, 200 µg, 300 µg or 500 µg) was added to 10 mg of cellulose. These mixtures were subsequently incubated at room temperature for one hour to allow immobilization to take place. At the end of the hour the cellulose-bound protein was separated from the protein in solution by centrifugation. The amount of free protein present in the supernatant was determined by conducting a Bradford assay. This value was subtracted from the starting amount to determine the amount of protein that was successfully immobilized on the cellulose. Each of the immobilization reactions were conducted in triplicate.

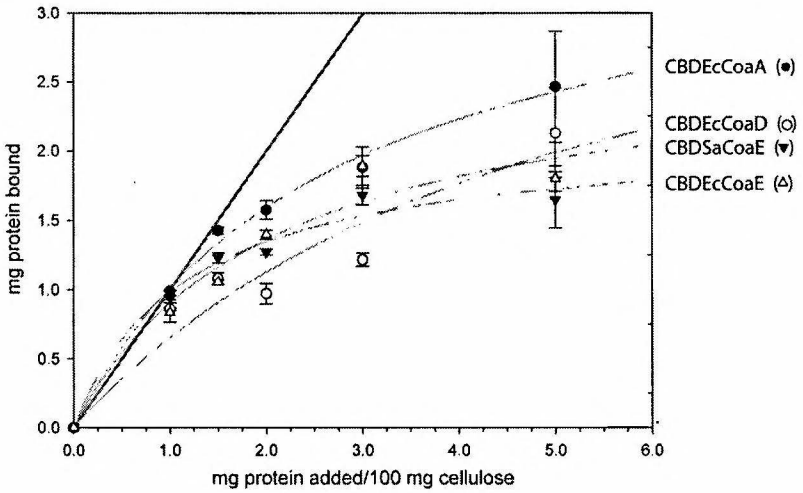


***Figure 4.11*: Determining the % immobilization of the fusion proteins on cellulose.** Different amounts of CBD*Ec*CoaA (●), CBD*Ec*CoaD (O), CBD*Ec*CoaE (△) and CBD*Sa*CoaE (▼) was immobilized onto a constant cellulose mass. Each data point represents the average of three values with the error bars representing the standard deviations of these values.

This data is presented as Figure 4.11. In this graph we have drawn a line through the point that represents a protein to cellulose ratio of 1:1 i.e. when all the fusion protein added to the cellulose remained bound. The results show that this was only the case when protein was added in a ratio of 1 mg of protein to 100 mg of cellulose. We therefore decided to use this ratio in our further studies.

4.2.3.2.2 Determining the effect of immobilization

To compare the effect of immobilization on the overall rate of biosynthesis of the fusion proteins we used the same time-dependent HPLC assay that was developed to determine the effect of the CBD domain on the rate of biosynthesis of these proteins. The enzymes were immobilized on cellulose using the 1 mg protein to 100 mg cellulose ratio determined above. Each of the enzymes was immobilized in a separate reaction before combining for the conduction of the biosynthesis reactions. The results from these biosynthesis reactions were compared to that of the CBD-fusion proteins in solution previously shown in Figure 4.10 (Figure 4.12).

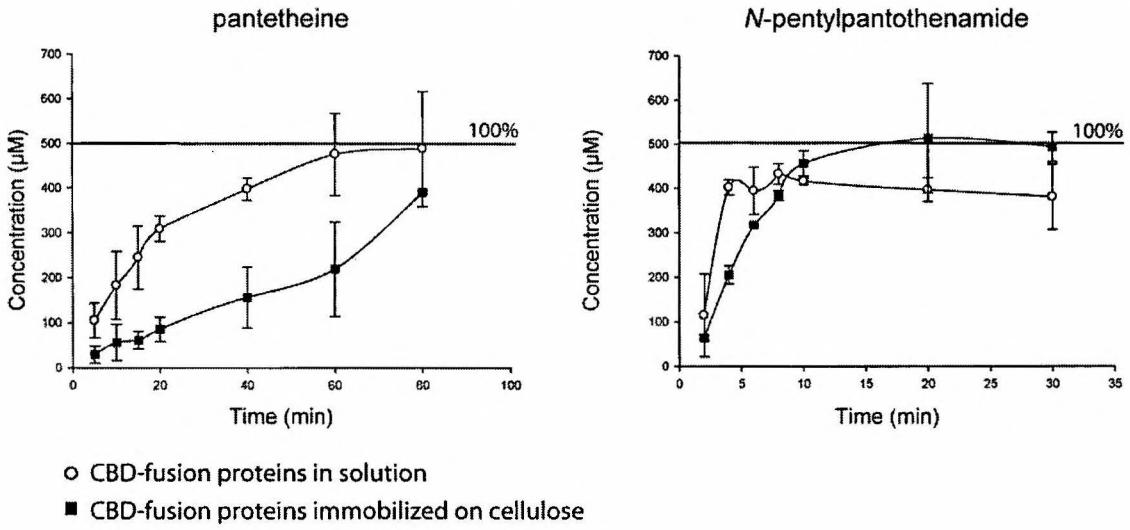


***Figure 4.12*: Rate of biosynthesis for solution based CBD-fusion protein combinations and immobilized CBD-fusion protein combinations.** Enzyme combination **C6** was used for the biosynthesis of CoA from pantetheine (left). Enzyme combination **C1** was used for the biosynthesis of ethyldethia-CoA from *N*-pentylpantothenamide (right). The data sets of the solution based CBD-fusion proteins (O) and the immobilized CBD-fusion proteins (■) for the corresponding substrates were plot on the same graphs. A 100% conversion equals 500 µM.

The result for the biosynthesis of CoA from pantetheine using enzyme combination **C6** shows that by immobilizing the CBD-fusion proteins onto cellulose only an 80% yield of CoA is achieved after 80 min where with the solution based fusion proteins we previously achieved a 100% yield. Still it is 60% more than the yield of the native enzymes after the same period of time (Figure 4.10).

For the biosynthesis of ethyldethia-CoA from *N*-pentylpantothenamide using enzyme combination **C1** the results show an increase in the rate of the overall biosynthesis. While previously an 80% yield was achieved after 30 min this has now increased to 100% by the introduction of the immobilized CBD-fusion proteins.

For both the studied substrates we observed an increase in the overall reaction rates due to the modification and immobilization of the CoA biosynthetic fusion proteins on cellulose. This shows great promise for the implementation of these proteins in an immobilized reactor system.

4.3 Conclusion

Eight possible CBD-fusion protein combinations were identified for the production of CoA and its analogues. Out of these we have chosen the two best possible combinations for the production of CoA and ethyldethia-CoA respectively.

We characterized the four proteins that constitute these combinations by determining their kinetic parameters for their natural substrates and their binding capacities to cellulose. Furthermore, we determined the effect of modification and immobilization on the activities of the two combinations through comparative studies.

From the comparative studies and the kinetic assays we could with great satisfaction deduce that no activity is lost through the introduction of the CBD domain or through the immobilization of the fusion proteins. Instead an increase in the overall reaction rates of the transformation reactions were observed when using the immobilized fusion proteins instead of the solution based native proteins.

In the next chapter we will now use these positive results to guide us in the construction of a bioreactor for the production of various CoA analogues.

4.4 Experimental conditions

4.4.1 Materials and Methods

The HiPrep™ 16/10 DEAE FF anion exchange column was purchased from Amersham Biosciences. Protein purification was conducted on an ÄKTA*prime* purification system.

A high performance liquid chromatography (HPLC) instrument (Agilent 1100 series) was used for the analysis of the biosynthetic reactions. All the analyses were done with a Supelcosil™ LC-18-T 5 µm column (25 cm x 4.6 mm) with a Supelguard™ LC-18-T guard column that was purchased from Sigma. A Hereaeus Multifuge® 3S/3S-R was used for all the large scale centrifugation. The smaller scale centrifugation was conducted on a Heraeus Biofuge pico centrifuge. All absorption measurements were conducted on a Varioscan multiplate spectrophotometer from Thermo Labsystems.

All the chemicals used were purchased from Aldrich or Sigma and is of the highest quality. The fusion proteins were immobilized on Avicel PH-101 from Fluka.

4.4.2 Comparative biosynthesis of CoA and ethyldethia CoA

4.4.2.1 Reaction conditions

Each reaction mixture contained 50 mM Tris-HCl buffer (pH 7.6), 5 mM ATP, 5 mM $MgCl_2$, 2mM DTT and 15 µg of each required fusion enzyme in a final reaction volume of 150 µl. Reactions were initiated by addition of 0.5 mM of either *N*-pentylpantothenamide or pantetheine and incubated for 60 minutes at 37°C. The reactions were quenched by transferring the tubes to 95°C for 5 minutes which caused precipitation of the proteins. The precipitated protein was collected by centrifugation for 5 minutes at 13 000 rpm before the supernatant could be injected onto the HPLC column for analysis.

4.4.2.2 Conditions for HPLC analysis

Analyses were done with 25 µl of a sample containing 60 µl of the reaction mixture and 12 µl of a 6 mM guanosine solution (0.5 M HCl) as an internal standard. The analysis method took 20 minutes starting with 95% of a 100 mM phosphate buffer solution (pH 6.5) and 5% methanol. The methanol concentration increased from 5% to 30% between 3 and 10 minutes and then ran isocratic up to 20 minutes. There was a 5 minute post run to re-equilibrate the column before the next injection took place. Absorption was monitored at 254 nm. The retention times were: ATP, 3.6 min; ADP, 3.8 min; AMP, 4.8 min; internal standard, 8.6 min; Coenzyme A, 9.3 min; Ethyldethia-CoA, 14.4 min.

4.4.3 Purification of the fusion proteins

Purification of the fusion proteins was conducted with a 16/10 DEAE FF column. The column was equilibrated with 50 ml of binding buffer (20 mM phosphate buffer, pH 7.4) before loading the protein. Then it was washed with another 20 ml of binding buffer and the protein was eluted with a gradient that ran over 200 ml, starting at a 100% binding buffer and going up to 50% elution buffer (20 mM phosphate buffer, 1 M NaCl, pH 7.4). Elution was monitored at A_{280}. After purification the protein was loaded onto a HiTrap™ desalting column and washed with gel filtration buffer (25 mM phosphate buffer, 5 mM $MgCl_2$, pH 7.4) before adding 5% glycerol and storing it at -80°C. The purity of the fusion proteins was confirmed with an SDS–PAGE gel (12%).

4.4.4 Kinetic assays

4.4.4.1 *Ec*CoaA and CBD*Ec*CoaA

Each assay contained 50 mM Tris-HCl buffer (pH 7.6), 20 mM KCl, 10 mM $MgCl_2$, 1.5 mM ATP, 1 mM DTT, 0.3 mM NADH, 0.5 mM PEP, 3 units pyruvate kinase, 3 units lactic dehydrogenase, 1.5 µg of the enzyme and 30 µl of the substrate in a final volume of 300 µl. The substrate concentrations varied between 0 µM and 250 µM for pantetheine and between 0 µM and 500 µM for *N*-pentylpantothenamide. The decrease in NADH concentration was measured at 340 nm over a period of 5 minutes. All measurements were taken in triplicate.

4.4.4.2 *Ec*CoaD and CBD*Ec*CoaD

Each assay contained 60 µl pyrophosphate reagent (Sigma, P7275), 50 mM Tris-HCl buffer (pH 7.6), 20 mM KCl, 10 mM $MgCl_2$, 1.5 mM ATP, 1.5 mM DTT, 1.5 µg of the enzyme and 15 µl of substrate in a final volume of 150 µl. The 4'-phosphopantetheine concentration varied between 0 µM and 1 mM. The decrease in NADH concentration was measured at 340 nm over a period of five minutes. Triplicate measurements were taken for each substrate concentration.

4.4.4.3 *Ec*CoaE and CBD*Ec*CoaE

The same assay was used as with CoaA, but with 3'-dephospho-CoA as substrate varying in concentration between 0 µM and 4 mM. All measurements were taken in triplicate.

4.4.4.4 *Sa*CoaE and CBD*Sa*CoaE

The same assay was used as with CoaA, but with 3'-dephospho-CoA as substrate varying in concentration between 0 µM and 2 mM. All measurements were taken in triplicate.

4.4.5 Determining the binding capacity of the cellulose

Cellulose (100 mg) was weighed of in 2 ml Eppendorf tubes. The cellulose was washed twice with phosphate buffer (20 mM, pH 7.4) containing NaCl (0.5 M), by inverting the tubes. The buffer was removed before adding the protein. The tubes were incubated at room temperature for one hour to allow immobilization of the protein and then spun down to collect the loaded cellulose.

The concentrations of the enzymes in the supernatants were determined with a Quick Start Bradford protein assay (Bio-Rad). This was then taken as the concentration of the free protein and was subtracted from the initial protein concentration to determine the amount of immobilized protein per mass cellulose.

4.4.6 Comparing reactivity of native and fusion proteins over time

Each assay was conducted with the native enzyme combination, the fusion proteins in solution and the fusion proteins immobilized on cellulose.

4.4.6.1 Native proteins and CBD proteins in solution

4.4.6.1.1 CoA biosynthesis

Each assay contained 150 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 5 mM ATP, 2 mM DTT, 25 µg of each enzyme (*Ec*CoaA, *Ec*CoaD and *Sa*CoaE) and 0.5 mM pantetheine in a final volume of 500 µl. Aliquots (80 µl) were taken at specific time intervals. The protein was precipitated at 95°C for five minutes, and then the aliquot was centrifuged for 5 minutes at 8 000 rpm. The assay was repeated with the fusion proteins.

4.4.6.1.2 Ethyldethia-CoA biosynthesis

Each assay contained 150 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 5 mM ATP, 25 µg of each *E. coli* enzyme (*Ec*CoaADE) and 0.5 mM *N*-pentylpantothenamide in a final volume of 500 µl. Aliquots (80 µl) were taken at specific time intervals. The protein was precipitated at 95°C for five minutes, and then the aliquot was centrifuged for 5 minutes at 8 000 rpm. The assay was repeated using the fusion proteins instead of the native enzymes.

4.4.6.2 Immobilized fusion proteins

Cellulose (2 mg) was weighed of in 2 ml Eppendorf tubes and washed twice with binding buffer (20 mM phosphate buffer, 0.5 mM NaCl, pH 7.4). The fusion protein (30 µg) was added to the cellulose and the mixture was incubated at room temperature for 1hr. It was washed again with binding buffer to remove all the un-immobilized enzymes before making the volume up to 100 µl. The 3 x 100 µl containing the three required fusion proteins for a specific reaction were combined and the rest of the reaction mixture was prepared in 200 µl to make up the final

reaction volume of 500 µl. The rest of the reaction mixture was identical to that used for the native enzymes. The same assay conditions were used as before.

4.4.6.3 Conditions for HPLC analysis

Analyses were done with 25 µl of a sample containing 60µl of the reaction mixture and 12 µl of a 6 mM guanosine solution (0.5 M HCl) as an internal standard. The analysis method took 20 minutes starting with 95% of a 100 mM Phosphate buffer solution (pH 6.5) and 5% Methanol. The methanol concentration increased from 5% to 30% between 3 and 10 minutes and then it ran isocratic up to 20 minutes. There was a 5 minute post run to re-equilibrate the column before the next injection. Absorption was monitored at 254 nm. The retention times were: ATP, 3.6 min; ADP, 3.8 min; AMP, 4.8 min; internal standard, 8.6 min; Coenzyme A, 9.3 min; Ethyldethia-CoA, 14.4 min.

4.5 References

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(2) Kruus, K., Lua, A. C., Demain, A. L., and Wu, J. H. D. (1995) The anchorage function of CipA (CelL), a scaffolding protein of the *Clostridium thermocellum* cellulosome. *Biochemistry 92*, 9254-9258.

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Chapter 5

Construction of a Continuous Bioreactor for the Chemo-enzymatic Synthesis of CoA analogues

5.1 Introduction

The aim of this project was to employ immobilized CoA biosynthetic enzymes in the construction of a bioreactor for the enzyme assisted transformation of pantetheine analogues to their related CoA analogues. In the previous two chapters we have discussed the preparation and characterization of these required enzymes. We have therefore gathered all the necessary tools and information required to attempt the construction of such a reactor.

In this final experimental chapter we address the construction of a batch and a column reactor. The batch reactor was developed specifically to test the stability of the immobilized fusion proteins while the column reactor was used to test the productivity of the reaction. Finally, we also attempted the purification of the column product. These results allowed us to identify various potential problem areas which may be addressed in the optimization of this system.

5.2 Results and Discussion

5.2.1 Preparing CoA analogues using a batch reactor: Stability tests

The first system that we developed was a batch reactor. The purpose of this reactor was to test the stability of the immobilized fusion proteins over the course of a few days. We chose two substrates for the testing of the batch reactor; *N*-pentylpantothenamide, which has been used in the previous studies, as well as a fluorescent pantothenamide analogue **5.1** (*1*). Compound **5.1** was synthesized according to the method described by Clarke *et. al.* (*2*). We specifically chose **5.1**

since its related CoA analogue **5.2** has previously been used for the successful *in vivo* protein labelling of an acyl carrier protein (Chapter 2). Thus it is a perfect example of the kind of analogue which we are aiming to produce by the development of this new method (Figure 5.1).

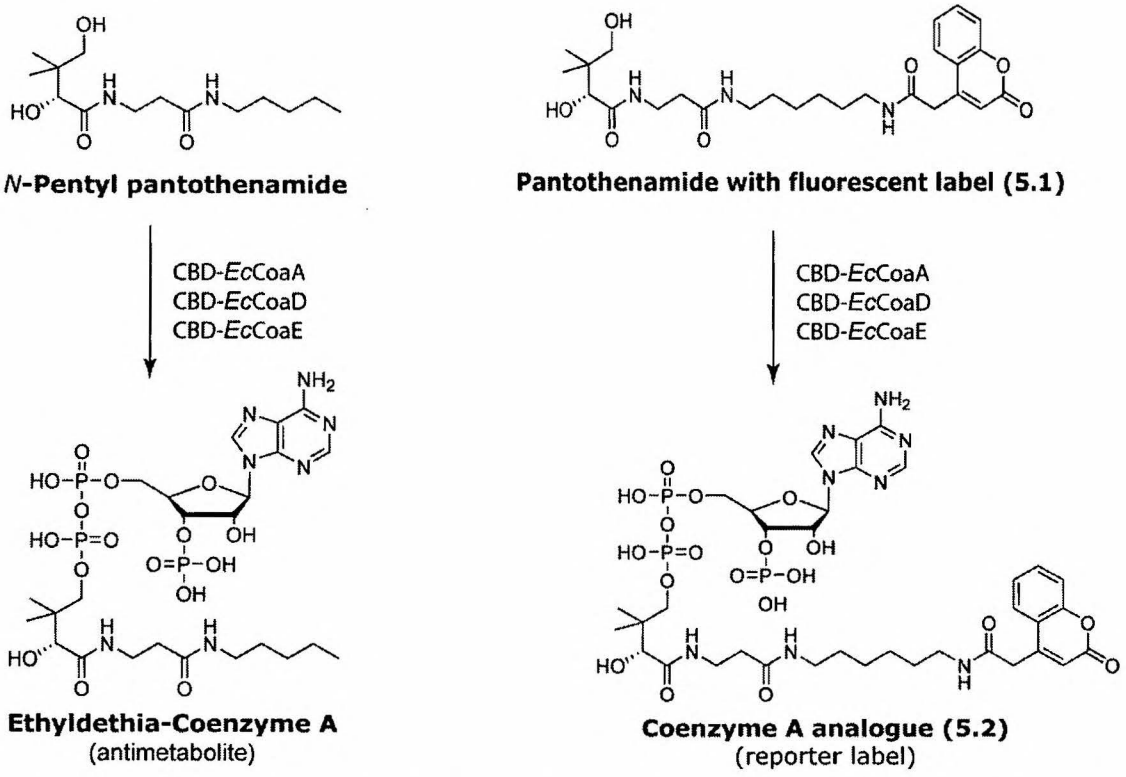

***Figure 5.1*: The two biosynthetic reactions that were used to test operation of the batch reactor.** *N*-pentylpantothenamide is transformed to ethyldethia-CoA. The coumarin based pantothenamide (**5.1**) is transformed to its corresponding fluorescent CoA analogue (**5.2**).

We conducted 1500 µl batch reactions containing 250 µg of each of the three *E. coli* CBD-fusion proteins pre-immobilized on cellulose. The loaded cellulose was kept in suspension for the duration of the reaction to ensure that all the substrate came into contact with the necessary enzymes. The reactions were initiated by the addition of 5 mM of the respective substrates and incubated for 90 min at 37°C. After analyzing the supernatant for product formation by HPLC analysis the cellulose was thoroughly washed and stored overnight at 4°C. An internal standard (guanosine) was co-injected with all the HPLC analyses to allow for the comparison of the yields of the various reactions (Figure 5.2). The reaction was repeated over a period of six days using the same cellulose system. All HPLC analyses were done in triplicate.

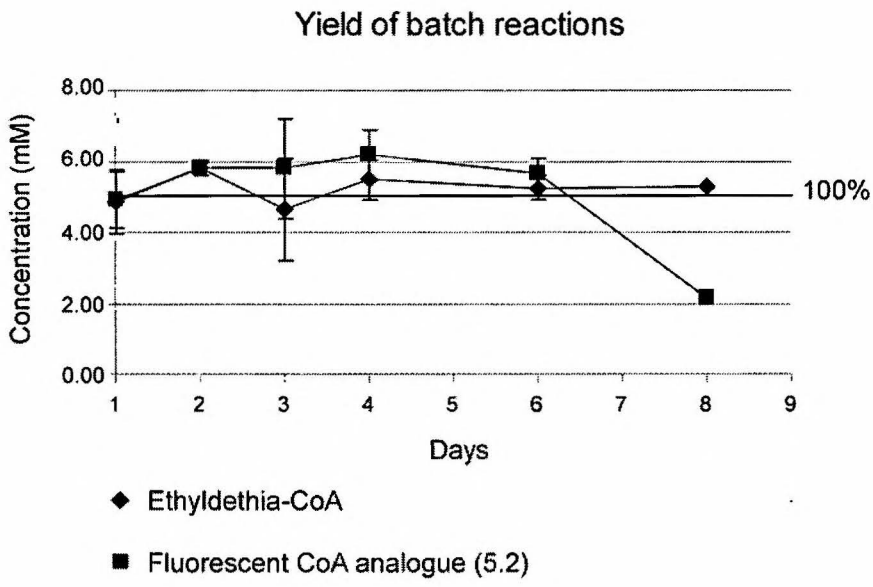

***Figure 5.2*: Evaluating the stability of immobilized CBD-fusion proteins.** The yields of two batch reactions are presented over a period of eight days. Each data point represent the average of three reaction yields determined for the biosynthesis of ethyldethia-CoA (◆) and **5.2** (■) after stating with 5 mM of the required substrates.

The results show that this method was not ideal for stability testing, as the yields of the reactions, as determined by the HPLC analyses, always seemed to be in excess of 100%. This may be due to the fact that it was very difficult to remove all of the product from the cellulose by washing, which would leave a small amount of product behind that would falsely inflate the amount of product determined the next day. Another problem with this method was when taking aliquots from the reactions for analysis we always collected some of the cellulose as well. The amount of protein in the reactions therefore did not remain constant for the duration of the test.

In spite of these problems we can conclude from this study that the immobilized enzymes did not lose much activity for a period of at least six days.

5.2.2 Preparing CoA analogues using a column reactor: Productivity tests

To determine the productivity of a system using immobilized biosynthetic enzymes for the production of CoA analogues we decided to develop a column reactor. However,

there are a great number of variables to consider in such a system. We therefore had to determine what our operating parameters were going to be before starting the experiment. The parameters we considered included:

- Temperature
- pH
- Column dimensions
- Flow rate
- Amount of each enzyme
- Packing of the enzymes
- Substrate concentration

The conditions we decided to use for our first attempt at constructing the column reactor was based on the conditions we used in the conduction of the assays discussed in the previous two chapters. We decided to use 0.5 mM of the fluorescent pantothenamide **5.1** as substrate in a 50 ml reaction. A pH of 7.4 and a constant temperature of 37°C were chosen. We decided to used equal amounts (330 µg) of each of the three *E. coli* CBD-fusion proteins, thus we had a total mass of 990 µg of loaded cellulose that we needed to pack into the column. Protein-free cellulose was added to this mixture to obtain a final column volume of ~5 ml. This was packed into a column with dimensions 10 mm x 50 mm. To obtain a reaction time of about 100 min the reaction mixture was pumped through the column at 0.5 ml/min, giving a residence time of about 10 min (Figure 5.3).

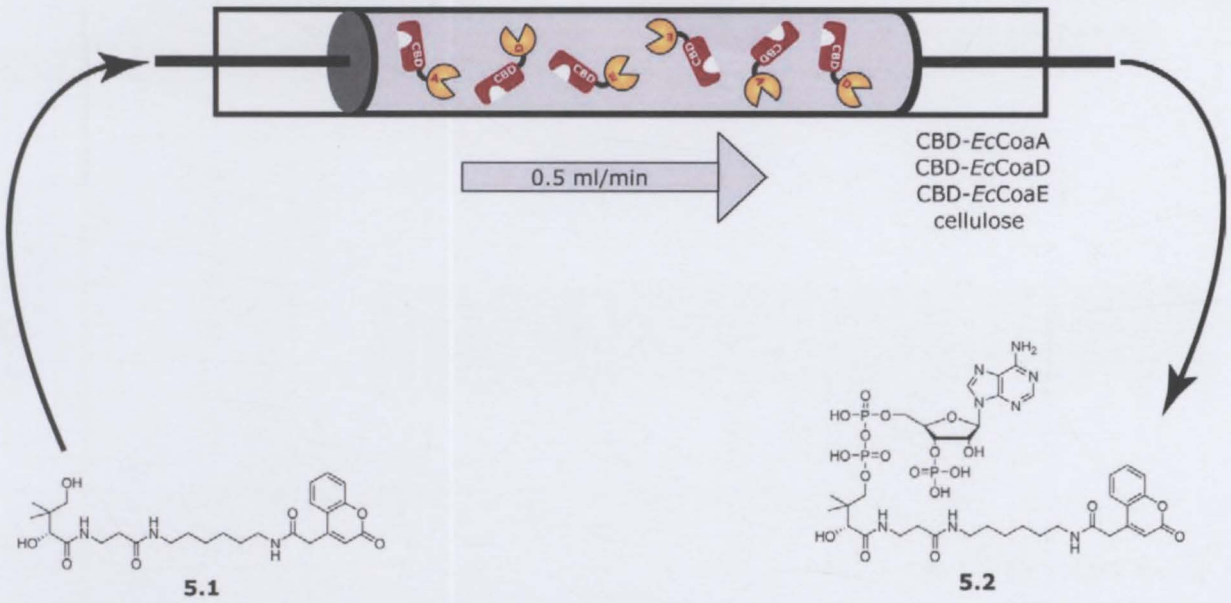

***Figure 5.3*: Schematic representation of the column reactor.** A reaction mixture containing 0.5 mM **5.2**, 1.65 mM ATP 10 mM Mg^{2+} and 50 mM Tris (pH 7.6) is pumped through the cellulose column at 0.5 ml/min. The cellulose was loaded with equal amounts of CBD*Ec*CoaA, CBD*Ec*CoaD and CBD*Ec*CoaE. Product **5.2** was collected and analyzed on HPLC.

After conducting the reaction a fraction of the product mixture was analyzed by HPLC to determine the percentage transformation that had occurred. This was compared to an HPLC of the starting reaction mixture (Figure 5.4, top panel). Two new peaks were visible in the product chromatogram. The one was identified as the expected product **5.2,** while the other was identified as the dephospho-CoA analogue of the product. The peak corresponding to the substrate had disappeared and there was no peak corresponding to the 4'-phosphopantetheine analogue, thus all the substrate was transformed to either **5.2** or the dephospho-CoA analogue. By co-injecting an internal standard (guanosine) with each sample we could determine the ratio between the product and the dephospho-CoA analogue as 9:1 (Figure 5.4, bottom panel).

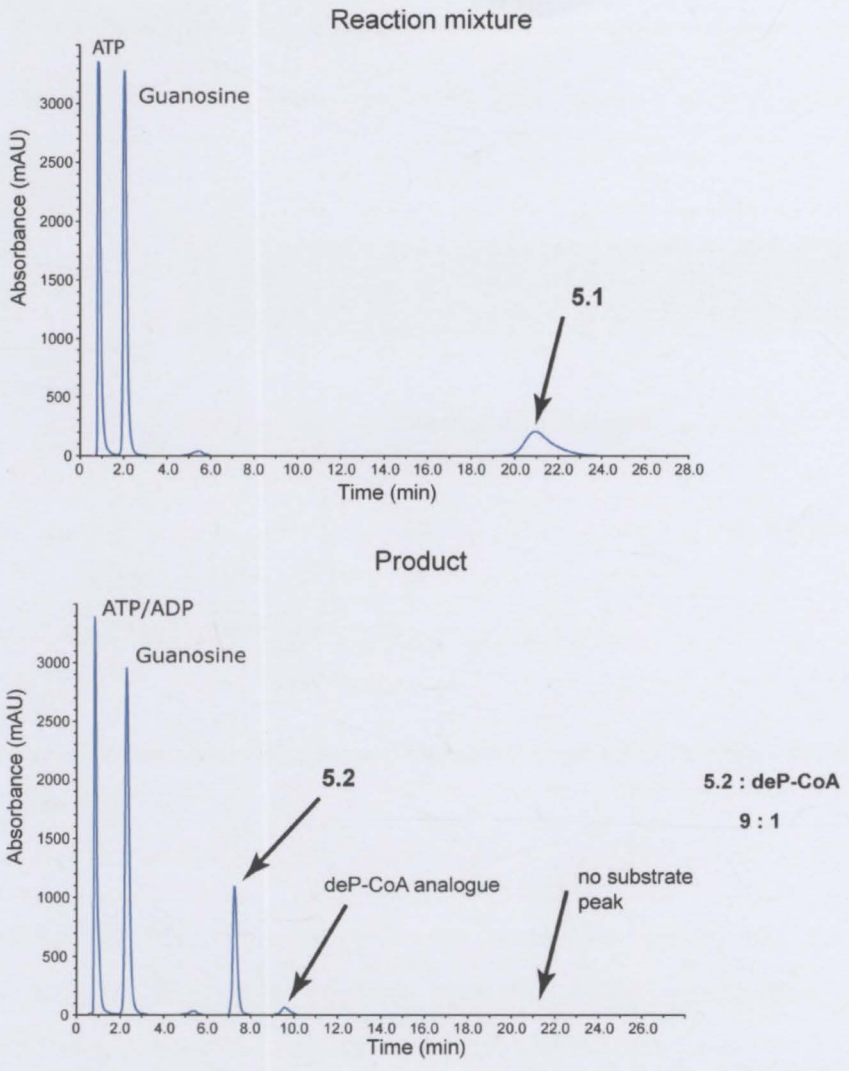

***Figure 5.4*: HPLC analyses of the column reactor.** Analysis of the reaction mixture (Top). Analysis of the product mixture (Bottom). Retention times: substrate (**5.1**), 20.8 min; product (**5.2**) 7.3 min; dephospho-CoA analogue 9.6 min.

To determine the reproducibility of the system we repeated the reaction one week later using the same column. Again the substrate peak was absent when the product was analyzed. We did however observe a decrease of ca. 7% in the ratio between the dephospho-CoA product and **5.2** (Figure 5.5).

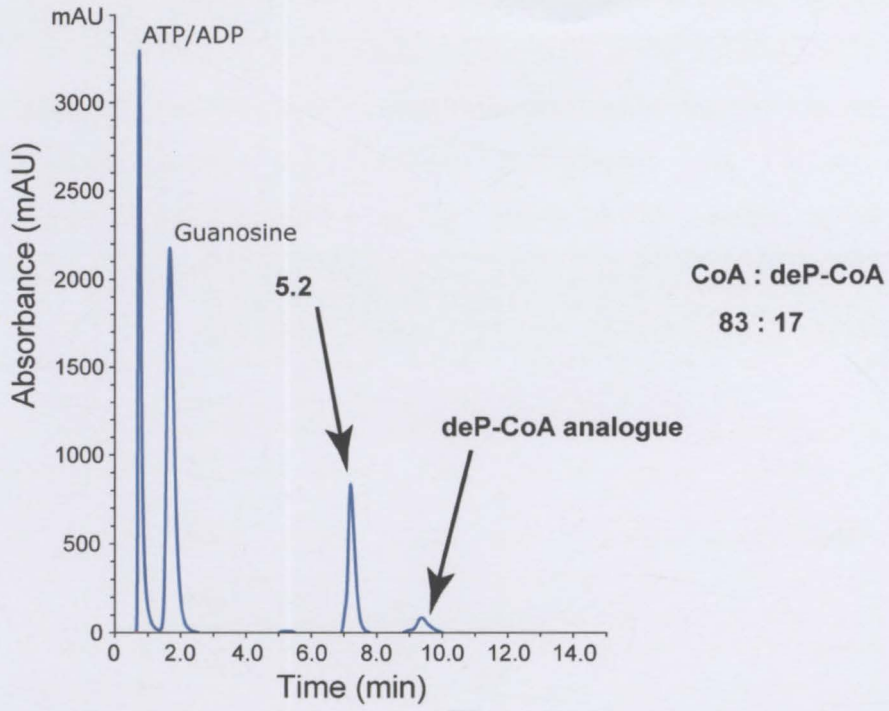

***Figure 5.5*: Product of the second column reaction.** The ratio between the **5.2** and the de-phospho-CoA analogue is 83:17.

The results obtained from the biosynthesis reactions conducted on the column reactor have proved that this system can be used continuously, with good reproducibility for the preparation of various CoA analogues.

5.2.3 Purification of the column product

In the currently available solution-based methods for the preparation of CoA analogues large amounts of cellulose are destroyed during the work-up of the product. Our column-based method now presents a way to overcome this major drawback since the proteins stay immobilized on the column while the product is collected as the column elutes. Thus all that needed to be done to obtain the pure product from the column reaction was to separate the remaining ATP from the product mixture.

We attempted this separation by using a pre-packed C18 SPE cartridge. The column was first activated with methanol and then equilibrated with an NH_4OAc buffer (10 mM, pH 6) solution. After loading the product mixture onto the cartridge the majority

of the ATP was removed with a 3% acetonitrile buffer solution (Figure 5.6). The product was removed with a 40% acetonitrile buffer solution and the ratio between **5.2** and the dephospho-CoA peaks remained unchanged by the procedure. We do however see the appearance of a shoulder in the product peak. We expect this to be iso-CoA analogue of **5.2** formed by isomerization of the product as we have seen similar results in previous reactions. However, this still needs to be confirmed analytically.

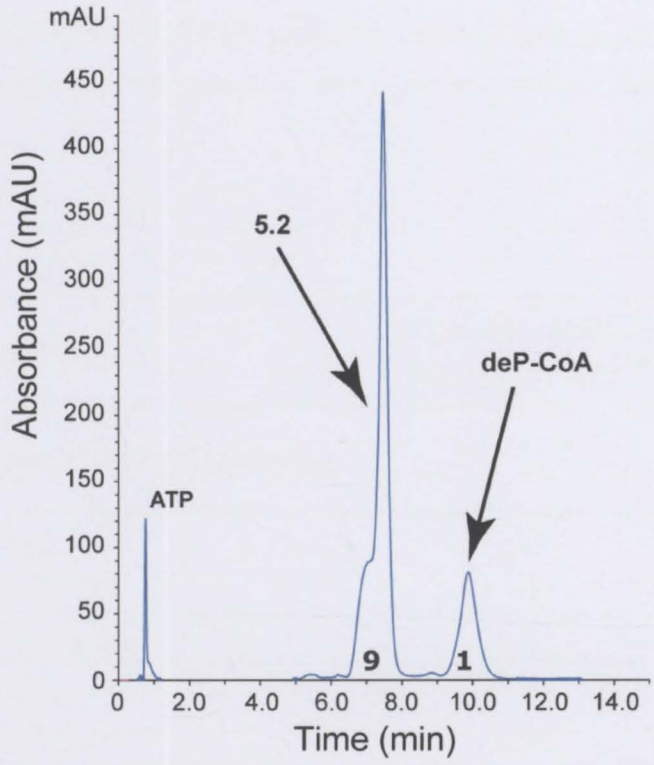

***Figure 5.6*: Purification of the column product.** About 96% of the ATP has been removed. The ratio between **5.2** and dephospho-CoA has stayed unchanged by the purification procedure.

5.2.4 Construction of an optimized column reactor

The results presented above indicate that immobilized enzymes can be successfully employed in a bioreactor for the synthesis of CoA analogues. However, it is clear that the construction of such a bioreactor will have to be optimized, and studies are currently ongoing to determine the optimal operating parameters for the system.

One of the key pieces of information required to prepare an optimized column is the ratio of the three proteins. As to date we have used equal amounts of the three CoA

biosynthetic enzymes to prepare the CoA analogues. We do however need to consider the specific activities of these enzymes to determine the optimal ratio between these proteins that is needed to achieve a 100% conversion of the pantetheine derivatives.

We therefore determined the specific activities of *E. coli* fusion proteins *Ec*CoaA, *Ec*CoaD and *Ec*CoaE (Table 5.1). This data show that if one wanted to use equal amounts of activity (not weight), these proteins would have to be used in a ratio of 1:11:5. This information will be used in the next attempt at the construction of a column reactor.

Substrate	Specific Activity ($\mu g.\mu mol^{-1}.min^{-1}$)
***N*-pentylpanothenamide**	
EcCoaA	395
CBD-EcCoaA	430
pantetheine	
EcCoaA	404
CBD-EcCoaA	294
4'-phosphopantetheine	
EcCoaD	2500
CBD-EcCoaD	3165
dephospho-CoA	
EcCoaE	5319
CBD-EcCoaE	1359
SaCoaE	12500
CBD-SaCoaE	13889

***Table 5.1*: Specific activities of the enzymes that were identified for the production of CoA and its analogues.**

5.3 Conclusion

We have managed to construct two enzyme-based bioreactors for the preparation of CoA analogues. By experimenting with these reactors we have shown that stability is achieved by immobilizing the biosynthetic enzymes on cellulose and that good productivity and reproducibility can be attained. Research is still ongoing to optimize the operating conditions of the column reactor, but we have already succeeded in the object of this thesis by proving that this method can be used for the more efficient synthesis of CoA analogues.

5.4 Experimental Procedures

5.4.1 Materials and Methods

A high performance liquid chromatography (HPLC) instrument (Agilent 1100 series) was used for the analysis of the biosynthetic reactions. All analyses were done using a Supelcosil™ LC-18-T 5 µm column (25 cm x 4.6 mm) with a Supelguard™ LC-18-T guard column purchased from Supelco. Purification of the column product was conducted on a 10 g pre-packed C18 SPE cartridge purchased from Strata. Centrifugation was conducted on a Heraeus Biofuge pico centrifuge.

All the chemicals that we used were purchased from Aldrich or Sigma and are of the highest quality. The fusion proteins were immobilized on Avicel PH-101 purchased from Fluka.

5.4.2 Batch reactor

5.4.2.1 Immobilization of the fusion proteins

500 mg of an over-expression pellet was dissolved in 10 ml phosphate buffer (25 mM) containing EDTA (1 mM, pH 7.4). The cells were disrupted by sonication and centrifuged at 15 000 x g for 20 minutes. The supernatant was added to cellulose in 2 ml Eppendorf tubes in a ratio of 10 µg of enzyme to 10 mg of cellulose.

The tubes were incubated at room temperature for 1 hour after which it was centrifuged at 2 500 rpm for 5 minutes to collect the cellulose. The supernatant was removed and the loaded cellulose was washed twice with the same phosphate buffer. The washed cellulose was re-suspended in 500 mM Tris-HCl buffer (pH 7.6) containing 10 mM $MgCl_2$.

5.4.2.2 Reaction mixture

Each 1500 µl batch reaction mixture contained 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 16.5 mM ATP and 250 µg of each immobilized enzyme and 5 mM substrate.

The two substrates that were used were *N*-pentylpantothenamide and the coumarin pantetheine derivative (**5.1**). The reactions were incubated for 90 minutes at 37°C, while shaking, and then centrifuged at 5 000 rpm for 1 minute before 30 µl aliquots were collected. The aliquots were boiled at 95°C for 5 minutes to insure that the reactions were quenched. The aliquots were centrifuged again, to collect any precipitated protein.

5.4.2.3 Conditions for HPLC analysis

The injection volume for each analysis was 25 µl. Each sample contained 60 µl of a ten time's dilution of the reaction mixture and 12 µl of a 6 mM guanosine solution (0.5 M HCl). Two different methods were used for the analysis of the two substrates.

5.4.2.3.1 Biosynthesis of ethyldethia-CoA

The method used for the analysis of the biosynthesis of ethyldethia-CoA took 20 minutes starting with 95% phosphate buffer solution (100 mM, pH 6.5) and 5% methanol. The methanol concentration increased from 5% to 30% between 3 and 10 minutes and then it ran isocratic up to 20 minutes. The retention times were: ATP 3.4 min; ADP 3.7 min; AMP 4.7 min; guanosine 8.6 min; ethyldethia-CoA 14.4 min.

5.4.2.3.2 Biosynthesis of fluorescent CoA analogue 5.2

The method used for analysis of the coumarin CoA analogue took 25 minutes starting with 90% phosphate buffer solution (100 mM, pH 6.5) and 10% methanol. The methanol concentration increased from 10% to 35% between 3 and 5 minutes and then it ran isocratic up to 25 minutes. The retention times were: ATP 3.1 min; ADP 3.2 min; AMP 3.6 min; guanosine 6.2 min; coumarin CoA analogue 24 min.

5.4.3 Column reactor

5.4.3.1 Preparing the column

CBD*Ec*CoaA, CBD*Ec*CoaD and CBD*Ec*CoaE were immobilized on cellulose in separate tubes using the same procedure as described for the batch reactions. 330

mg of each enzymes was immobilized on 330 mg of cellulose, thus the total mass of loaded cellulose used to pack the column was 990 mg. The three batches of loaded cellulose were combined and clean cellulose was added to obtain a volume of ~5 ml. This mixture was suspended in Tris-HCl buffer (50 mM, pH 7.6) containing $MgCl_2$ (10 mM) and loaded into a glass column. The final dimensions of the packed column were 10 mm x 50 mm. The column was connected to a pump and equilibrated with 50 ml Tris-HCl buffer (50 mM, pH 7.6) containing $MgCl_2$ (10 mM), at a flow speed of 0.5 ml/min.

5.4.3.2 Reaction mixture

A 50 ml reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1.65 mM ATP and 0.5 mM substrate **5.1** was pumped through the equilibrated column at a flow speed of 0.5 ml/min while keeping the temperature constant at 37°C.

5.4.3.3 Conditions for HPLC analysis

The sample prepared for analysis contained 60 µl of the product mixture and 12 µl of the internal standard. A sample containing the reaction mixture was prepared in the same way. Both samples were analyzed with the same method used for analyses of the batch reaction products. The retention times were: ATP/ADP 0.8 min; guanosine, 2.2 min; coumarin CoA analogue (**5.2**) 7.3 min; dephospho-CoA analogue, 9.6 min; coumarin pantetheine analogue 20.8 min.

5.4.3.4 Purification of the column product

A pre-packed C18 SPE cartridge was used to separate the CoA analogue **5.2** from the remaining ATP. The column was activated with MeOH and washed with 10mM NH_4OAc (pH 6) buffer solution before being equilibrated with a 3% MeCN buffer solution. The product was loaded onto the column and the ATP was removed with a 3% MeCN buffer solution. The product was removed with a 40% MeCN buffer solution. The purified product was analyzed by the same HPLC method used to analyze the crude product.

5.5 References

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Chapter 6

Conclusion

6.1 Overview of achievements

CoA analogues are valuable research tools due to the wide variety of biological processes in which natural CoA are involved. Therefore, various methods have been developed to prepare these analogues. This study was aimed at developing a new chemo-enzymatic method utilizing immobilized CoA biosynthetic enzymes to construct a reactor that will allow more efficient preparation of the currently available analogues.

In pursuing this aim we successfully constructed six novel CBD-fusion proteins. CoaA, CoaD and CoaE from *E. coli* and *S. aureus* were each fused with a CBD domain to allow their immobilization on cellulose. We confirmed the activity of all six the fusion proteins and then put together all eight possible enzyme combinations and tested them for the preparation of CoA and ethyldethia-CoA, an *E. coli* antimetabolite. By comparing the activities of the combinations we identified two combinations that would be used for the construction of a bioreactor.

There were four enzymes represented by these combinations. We successfully characterized and immobilized them on cellulose and proved that immobilization caused an increase in the reaction rate of the combinations of biosynthetic enzymes.

The three *E. coli* enzymes, representing one of the identified combinations, were used to construct a batch and a column reactor. Results from the operation of the batch reactor showed that the immobilized enzymes possess stability that allows repeated use of the system. We also proved that yields of up to 90% could be achieved by the operation of the column reactor.

6.2 Future work

6.2.1 Optimizing enzyme usage

At the end of chapter 5 we have commented on current efforts to optimize the operation of the column reactor concerning the specific activities of the CBD-fusion proteins. These studies are still ongoing. We are also aiming to experiment with different ways of packing the immobilized enzymes in the column. To date we have only used mixtures of the enzymes when the packing of the column. We also want to construct a reactor with the enzyme packed in consecutive layers to see if we can decrease the formation of dephospho-CoA and thus optimize the yield of the reaction.

6.2.2 ATP regeneration

ATP is an essential cofactor in the biosynthesis of CoA. It is also very expensive. If we could immobilize a recycling system for this cofactor on the column reactor it would however not only decrease the production cost of the analogues, but also simplify purification of the product. ATP can be regenerated from ADP by any of the four methods presented in Figure 6.1 (*1*).

The efficiency of these systems and the feasibility of their immobilization also using the CBD technology are two concepts that needs to be investigated achieve full efficiency of our newly developed chemo-enzymatic column-based bioreactor.

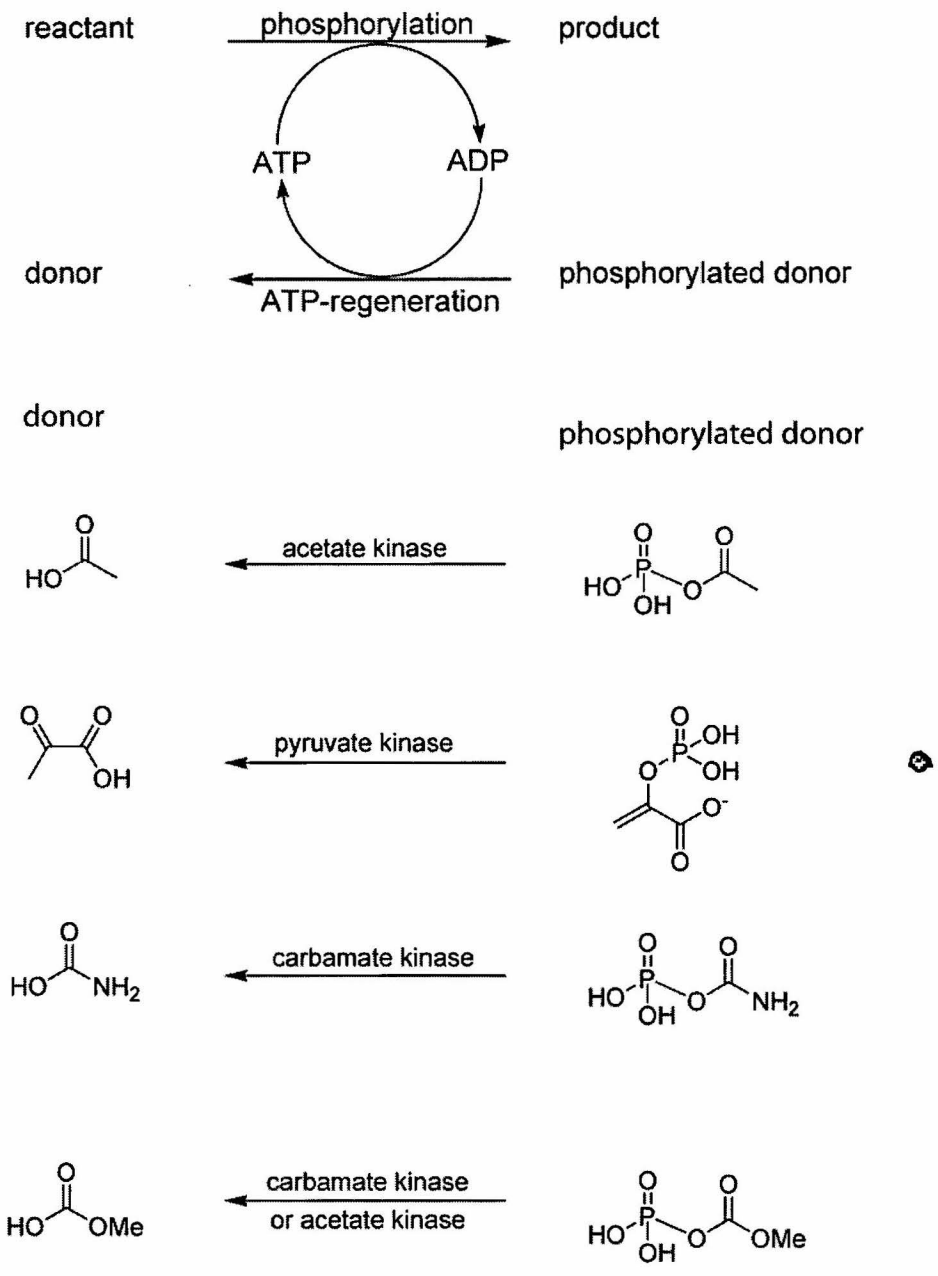

***Figure 6.1*: ATP regeneration.** A schematic representation of an ATP recycling system (Top panel). The systems currently used for ATP regeneration (Bottom panel).

6.3 Conclusion

This thesis presents the successful development of a new chemo-enzymatic method for the preparation of CoA and its analogues. Although much research is still in progress to optimize the operation of the newly constructed column reactor we are positive that this method already is a significant improvement compared to the currently available solution-based methods.

We have introduced a re-usable system by immobilizing the CoA biosynthetic enzymes on cellulose. We also simplified product purification compared to the available solution-based methods, by keeping the enzymes separated from the product through their immobilization. Construction of the column reactor further allows continuous production of CoA analogues and should eventually allow large scale versions of these reactions.

6.4 References

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