Rerouting 'coenzyme A' biosynthesis
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CHAPTER 1

Introduction and aim of the thesis
INTRODUCTION AND AIM OF THE THESIS

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INTRODUCTION

Coenzyme A: an essential metabolic cofactor

Coenzyme A (CoA), an essential metabolic cofactor, was discovered in 1945 by Fritz Lipmann\textsuperscript{1,2}. He created the term “Coenzyme A” to indicate its involvement as an “activator of acetate” and in 1953 Fritz Lipmann was awarded the Nobel Prize for his discovery\textsuperscript{3-4}. Later researchers explored the pivotal role of CoA not only as an acetyl donor, but also its ability to carry other acyl groups as thioesters, together this regulates various biochemical reactions\textsuperscript{5,6}. The biochemistry of CoA biosynthesis and its acyl-derivatives are essential steps in all living organisms ranging from prokaryotes to higher eukaryotes. CoA and acyl-CoA derivatives are involved in diverse functions covering 4% of all metabolic pathways, such as the Krebs cycle, fatty acid regulation, amino acid metabolism and biosynthesis of ketone bodies and sterols\textsuperscript{7,8}.

Among the different CoA derivatives, acetyl-CoA plays an important role both in cellular metabolism and in post-translational protein acetylation\textsuperscript{9}. The de novo biosynthesis and turnover rate of CoA also regulate protein 4’-phosphopantetheinylation. The 4’-phosphopantetheine conjugation to various proteins and enzymes is an essential post-translational modification required for vital cellular processes like fatty acid synthesis, folate metabolism, polyketide synthesis, and lysine metabolism\textsuperscript{10-12}. In-addition to previously mentioned roles, acetyl-CoA is also necessary for the synthesis of acetylcholine\textsuperscript{13,14}. Acetylcholine is an important neurotransmitter, which regulates neuronal excitability in both the sympathetic and parasympathetic system and is required for proper functioning of neuromuscular junctions\textsuperscript{15}. Recently, research groups discovered that CoA is also of influence for various other novel cellular functions such as in autophagy, epigenetics, and signaling\textsuperscript{16-18}. The newly identified role of CoA in cellular physiology also evidently shows that CoA is not just a metabolic cofactor involved in biochemical pathways. Henceforth, it is important to know the molecule CoA in various aspects such as its structure, chemistry, biosynthesis, regulation and function.

In this chapter, the focus is to cover the biochemical and biological interface of CoA, especially in higher eukaryotes and mammals. Firstly, we outline the areas concerning: canonical de novo CoA biosynthesis and discuss how cells and organelles regulate CoA levels. Here, we also highlight the diverse role of CoA in healthy cellular physiology and diseases. Furthermore, we explore the use of model organisms to understand the functions of CoA, especially when there is an impairment in the de novo CoA biosynthesis pathway.

Canonical de novo Coenzyme A biosynthesis

Structurally CoA is comprised of pantothenate, cysteine and an adenosine phosphate group (Figure 1)\textsuperscript{19}. The proper supply of all the components mentioned above are essential for the production of the ubiquitous and indispensable metabolite CoA in the cells. CoA has a bulky structure and contains high electronegative phosphoryl groups, indicating CoA is unlikely
diffuse passively into cells. The biosynthesis of CoA is mainly dependent and derived from the dietary precursor pantothenate (Vitamin B5) in all known organisms. Nevertheless, bacteria, yeast, and plants can synthesize pantothenate from β-alanine by themselves. The occurrence of the conserved and characterized *de novo* CoA biosynthesis pathway among diverse species indicates the inevitable role of CoA\(^7,8\). In this section, the focus is constrained to understand the CoA biosynthesis machinery in higher eukaryotes, especially in mammals. Pantothenic acid is present abundantly in various food sources, and mammals rely on it for the biosynthesis of CoA\(^20\). In addition, the gastroenteric gut harbors diverse microflora, which also helps in the supply of pantothenic acid\(^21\). The surplus amount of pantothenate obtained both from the diet, and intestinal microbes are most likely the reason that pantothenate deficiency is a rare occasion in higher eukaryotes and mammals.

The first step, to initiate the CoA biosynthesis, is the transport of pantothenate into the cells. This process occurs through a sodium-dependent multivitamin transporter (SMVT; the product of the SCL5A6 gene), which belongs to a sodium:solute symporter (SSF) (TC 2.A.21) family. Nevertheless, this transporter also has a substrate affinity for biotin and lipoic acid\(^22,23\). The SMVT is a transmembrane protein with both amino acid and carboxyl-terminal domains oriented towards the cytoplasm. The SMVT has a ubiquitous expression in diverse tissues, including intestine, placenta, liver, brain, kidney and heart. The active transport of pantothenate through SMVT is driven by a sodium electrochemical gradient and intracellularly pantothenate is further processed into CoA by specific enzymes\(^24,25\).

The intracellular CoA biosynthesis from pantothenate is dependent on five enzymatic steps, utilizing pantothenate kinase (PANK; EC 2.7.1.33), 4′-phosphopantothenoylcysteine
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PPCS; EC 6.3.2.5), (R)-4'-phospho-N-pantothenoylcysteine decarboxylase (PPCDC; EC 4.1.1.36), 4'-phosphopantetheine adenyltransferase (PPAT; EC 2.7.7.3) and dephospho-CoA kinase (DPCK; EC 2.7.1.24) (Figure 2)8,26. The first and the foremost rate limiting step in CoA biosynthesis is the phosphorylation of pantothenate by PANK. There are three types of PANK (type I, II and III). Bacteria primarily have PANK type I and III, while higher eukaryotes and mammals have type II PANK8,27,28. In mammals, there are four PANK isoforms (PANK1-4) encoded by four distinct genes, and they occur as homodimers8,29-31. The mammalian putative pantothenate kinase gene was first discovered in mouse32,33, followed by identification and characterization of human PANK isoforms34,35. Among those isoforms in humans, PANK2 is localized in the mitochondrial intermembrane space while others are in the cytosol36,37. In addition, only PANK1-3 isoforms but not PANK4, contributes to overall pantothenate kinase activity38.

The human genes encoding the downstream enzymatic steps following PANK were initially identified using comparative genomics and were further verified8,31. PPCS, a homodimer, catalyzes the second step in CoA biosynthesis utilizing ATP for substrate activation and couples cysteine to 4'-phosphopantothenate leading to 4'-phosphopantothenoylcysteine and the release of AMP. PPCDC, a homotrimeric enzyme, decarboxylates 4'-phosphopantothenoylcysteine into 4'-phosphopantetheine. Moreover, both PPCS and PPCDC are predicted to be cytoplasmic39,40. In eukaryotes and mammals, the last two steps (i.e., both adenyltransferase and kinase activity), are processed by a single bifunctional enzyme PPAT-DPCK (also called CoA synthase, COASY)41. COASY synthesizes CoA from 4'-phosphopantetheine. COASY, a monomeric protein localized in the mitochondrial matrix, is considered to be the second rate limiting enzyme in the CoA biosynthesis pathway42,43. Altogether these five steps comprise the canonical de novo CoA biosynthesis machinery in higher eukaryotes.

**Regulation of Coenzyme A levels in cells and their organelles**

The regulation of CoA levels inside cells and their organelles is a complex mechanism and not completely understood. The reason is that this process is influenced by; 1. The CoA biosynthesis process, 2. The degradation pathway of CoA and 3. The utilization of free CoA into various other CoA adjuncts, including acyl-CoAs (Figure 2). Briefly, the cellular and the tissue distribution of CoA levels depends on the localization and the expression of the enzymes in different tissues. Most of the CoA biosynthesis enzymes are cytoplasmic, but PANK2 and COASY are mitochondrial36,42,43. Nevertheless, COASY completes the last step of the CoA biosynthesis process, which might explain why CoA levels are much higher in mitochondria (2-5mM) as compared to the cytoplasm (0.02-0.14mM)44,45. It is in-line with the fact that mitochondria are central organelles in energy homeostasis, the Kreb’s cycle, and fatty acid β-oxidation utilizing CoA46. Moreover, not all CoA biosynthesis pathway enzymes show a
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Because these enzymes have a high affinity to CoA species and localize in peroxisomes and mitochondria which contain high concentrations of CoA. Nevertheless, it remains unclear how plasma membrane-bound ecto-enzymes such as nucleotide pyrophosphatases and pantetheinases are involved in whole body CoA metabolism. Although, the complete elucidation of the CoA degradation pathway is lacking, it is certainly crucial in the maintenance of intracellular CoA levels.

Figure 2: Overview of CoA de novo biosynthesis, degradation and utilization homeostasis.

Canonical de novo biosynthesis pathway representing the substrate pantothenate which is phosphorylated to 4’-phosphopantothenate by pantothenate kinase (PANK). Subsequently, cysteine is added by 4’-phosphopantothenoylcysteine synthetase (PPCS) to form 4’-phosphopantothenoylcysteine, which is then decarboxylated by (R)-4’-phospho-N-pantothenoylcysteine decarboxylase (PPCDC) producing 4’-phosphopantetheine. Finally, the bifunctional 4’-phosphopantetheine adenylyltransferase/dephospho-CoA kinase (COASY) attaches the adenylyl group and phosphorylates 4’-phosphopantetheine, yielding the product CoA. In a reverse hypothetical scheme, CoA is processed by either single or combined actions of acid phosphatases (ACP), nudix hydrolases (NUDT), and nucleotide pyrophosphatases (ENPP) to form pantetheine, which is further degraded to pantothenate by pantetheinases or vanins (VNN). Thirdly, the utilization of CoA to form acyl-CoA, protein bound or mixed thiol disulfides contributes in CoA levels and its homeostasis inside the cells.

The third prominent system in regulating CoA levels relies on the synthesis of acyl-CoA and other CoA derivatives. The Acyl-CoA synthetase (ACS) class of enzymes utilizes free CoA to form various acyl-CoA species. A similar expression pattern in various tissues and organs. Among functional PANK isoforms, PANK1 is highly expressed in heart, kidney and liver. PANK2 has a notably higher expression in liver and neuronal tissue, whereas PANK3 expresses prevalently in liver tissue. PANK isoforms also significantly differ in their substrate affinity for pantothenate with a Km value.
ranging from 5.7 to 27uM, indicating the complexity in this first important step\textsuperscript{39}. PPCS is found in all tissues, but with the highest expression in kidney and liver. PPCDC has a much higher expression in kidney, temporal lobe and testis compared to other tissues. COASY expression is ubiquitous in all tissues with the lowest expression in peripheral blood leukocytes and highest in kidney and liver. One of its alternative splice isoform: COASY-β is expressed mainly in the brain\textsuperscript{50,51}. The activity of PANK tightly regulates the CoA biosynthesis pathway, and CoA production is also speculated to be dependent on COASY\textsuperscript{52-54}. CoA and acyl-CoAs are known to inhibit the PANK activity, and thereby regulate the \textit{de novo} CoA biosynthesis\textsuperscript{8,33,55}. Although COASY is considered also to be feedback inhibited by CoA, experimental validation is still lacking. In addition to the CoA feedback regulation, changes in the nutritional and metabolic condition, like fasting, alters tissue CoA levels via transcriptional regulation of \textit{Pank1} gene expression by PPARα\textsuperscript{8}.

The second important mechanism which regulates CoA levels is the CoA degradation pathway (Figure 2). The CoA degradation system maintains the turn-over of CoA and keeps the cycle of CoA biosynthesis on-going\textsuperscript{8,56}. However, this pathway has not been extensively studied and remains speculative. The depicted CoA degradation pathway is the reverse of the CoA biosynthesis pathway with the involvement of distinct classes of enzymes, namely lysosomal acid phosphatases\textsuperscript{57}, nudix hydrolases\textsuperscript{58,59}, nucleotide pyrophosphatases\textsuperscript{60,61}, and pantetheinases\textsuperscript{62,63}. CoA, dephospho-CoA, and various other acyl-CoAs degrade into 4’-phosphopantetheine by the enzymatic actions of nudix hydrolases (NUDT), lysosomal acid phosphatases (ACP), and nucleotide pyrophosphatases (ENPP) and are assumed to form pantetheine subsequently\textsuperscript{8,64,65}. Pantetheinases (or Vanin) process pantetheine further into pantothenic acid and cysteamine\textsuperscript{56,62,63}. The cellular localization of these enzymes infers that different CoA pools in each organelle are selectively degraded by particular enzymes, such as acid phosphatases in lysosomes and different nudix hydrolases in cytosolic, mitochondrial, peroxisomal and other compartments. However, NUDT7/NUDT8/NUDT19 gained more attention because these enzymes have a high affinity to CoA species and localize in peroxisomes and mitochondria which contain high concentrations of CoA\textsuperscript{66,67}. Nevertheless, it remains unclear how plasma membrane-bound ecto-enzymes such as nucleotide pyrophosphatases and pantetheinases are involved in whole body CoA metabolism. Although, the complete elucidation of the CoA degradation pathway is lacking, it is certainly crucial in the maintenance of intracellular CoA levels.

The third prominent system in regulating CoA levels relies on the synthesis of acyl-CoA and other CoA derivatives. The Acyl-CoA synthetase (ACS) class of enzymes utilizes free CoA to form various acyl-CoAs, which includes short chain, medium chain and long/very long chain acyl-CoA derivatives\textsuperscript{68}. Acyl-CoAs are essential in major pathways such as the Krebs cycle and the activation of fatty acids for β-oxidation\textsuperscript{69-71}. In mammals, there is a total of 26 putative ACS enzymes that act on different classes of carboxylic acids\textsuperscript{71}. Long-chain
acyl-CoA synthetases (ACSLs) are more actively involved in the activation and channeling of fatty acids. This process is also necessary to regulate toxic xenobiotic carboxylic acids like 2,4,5-trichlorophenoxyacetate (an herbicidal component) from cells. ACS has been shown to be present in various cellular compartments like mitochondria, peroxisomes, endoplasmic reticulum and cytoplasm. Both mitochondria and peroxisomes are crucial organelles in β-oxidation of fatty acids, which also explains why many of the enzymes involved in CoA biosynthesis and its regulation, are found predominant in these cellular compartment. The cellular acyl-CoA pool is also manipulated by the acyl-carnitine system which shuttles various acyl groups between cellular compartments.

In addition to the CoA pools mentioned above and their regulation, recent research shows that CoA can regulate cellular redox homeostasis by forming thiol-mixed disulfides and thereby levels of CoA can be influenced. Like glutathione, CoA is essential for maintaining the proper intracellular redox potential necessary for many metabolic processes. Free CoA has a particular half-cell potential (-234mV) at pH 7 which is comparable to glutathione. The thiol-disulfide forming process can occur either with two molecules of free CoA or one molecule of CoA together with one other thiol-containing molecule. Although, CoA has a pKa value of 9.83 which indicates that it mostly remains in a unreactive thiol form at physiological pH, the role of CoA in oxidative stress remains unclear. Another form of a mixed disulfide exchange process occurs between CoA and various proteins. In dormant spores of bacteria, approximately 45% of cellular CoA is reported to be linked to proteins as mixed disulfides. However, in higher eukaryotes and mammals, the proportion of mixed sulfide CoA has not been explicitly studied. Recent research in eukaryotes shows that CoA binds to specific proteins like calcium/calmodulin-dependent protein kinase II (CaMKII) without making a mixed disulfide exchange, and this regulates cellular survival. Altogether it becomes increasingly apparent that the regulation and utilization of CoA levels is a complex process and requires detailed investigation. In addition, the levels of CoA and the various CoA pools might change and fluctuate according to the metabolic need and physiology of the cellular environment in different tissues. Moreover, this also underlines the requirement for reliable, robust and simple analytical methods to evaluate CoA levels in both a qualitative and a quantitative manner. Research has led to the development of highly sensitive methods using liquid chromatography coupled with mass spectrometry (LC-MS), to measure CoA levels. The reported LC-MS analytical procedures demand laborious sample preparation, and henceforth, a simple and sensitive method, to measure changes in total CoA levels, is a pre-requisite.

**CoA in health and diseases**

Altogether the information stated above, shows that CoA is a central metabolite in energy homeostasis with a broad function in many metabolic pathways. Among the classical
metabolic pathways, CoA and its acyl-derivatives are involved in over 100 cellular metabolic reactions including the Krebs cycle. In particular, acyl-CoA thioesters are crucial in both α- and β-oxidation of fatty acids occurring in peroxisomes and mitochondria. In the next section, we will highlight the important role of CoA in non-metabolic pathways and emphasize the link of CoA to various health aspects and diseases (as summarized in Figure 3).

CoA pools as a regulator of non-metabolic processes
CoA has gained more interest as several research studies revealed a role for CoA in regulating many important non-metabolic processes. These include posttranslational protein modifications, signaling, autophagy, phospholipid and membrane biogenesis, and drug activation. Here, in this section, some of these processes are described in more detail. Post-translational protein modification (PTMs) is an important asset to maintain and manipulate the functions of proteins according to the physiological cellular need. Among those, protein lysine acetylation is an important PTM explored in many research studies. Recently it also became apparent that other acylations like succinylation, malonylation, crotonylation, myristoylation and palmitoylation play a regulating role in biological processes as well. Protein lysine acetylation has now emerged as a key PTM, and it alters chromatin structure by modifying histones and influences transcription. Moreover, research studies in human cells/tissues indicate that there are more than 1000 proteins undergoing one or more lysine acetylation. Interestingly, metabolic enzymes also maintain their function through post-translational acetylation. In humans, almost all enzymes involved in glycolysis, Krebs cycle, gluconeogenesis, urea cycle, fatty acid metabolism and glycogen metabolism are regulated by their acetylation status. Moreover, acetylation does not always lead to activation of protein, but also may lead to inhibition or destabilization of the protein. Two classes of enzymes, namely acetyltransferases (HATs) and deacetylases (HDACs and sirtuins) regulate the protein lysine acetylation. Several core proteins including histones also undergo lysine formylation. Although, the function of this PTM is not clear, studies show that DNA damaging agents increase histone formylation in cells, indicating that histone formylation could have a role in oxidative stress and DNA damage. Lysine residues of histones may also become propionylated, crotonylated or butyrylated. It is not clear whether HATs and HDACs regulate this process as well or whether there are specific additional enzymes involved. Enzyme-independent but pH and acyl-CoA concentration dependent acetylation and succinylation of mitochondrial and non-mitochondrial proteins also occur. More studies have still yet to be done to understand the functions of these PTMs. Many of the metabolic enzymes maintain their function through succinylation and malonylation, including glutamate dehydrogenase. In addition, protein palmitoylation, like in amyloid precursor protein, is recently gaining more interest in health and diseases. These recent findings raised many challenges and interesting questions regarding such PTMs. Nevertheless, it becomes apparent that cells maintain a balanced cross-talk between protein PTMs and metabolism largely influenced by CoA pools.
In addition to protein PTMs, new evidence shows that CoA regulates the activation of drug molecules\textsuperscript{110,111}. Non-steroidal anti-inflammatory drugs like ibuprofen and its related 2-arylpropionic acid derivatives are potentially active in their S-enantiomer configuration, but not in the R-enantiomer configuration. CoA is utilized in-vivo for chiral inversion, a rapid metabolic uni-directional conversion from the R- to the S- state. Long chain acyl-CoA synthases, $\alpha$-methylacyl CoA racemase, and acyl-CoA thioesterases are important in regulating this process\textsuperscript{112}. In cellular physiology, CoA is also involved in the metabolism of phytanic acid, pristanic acid, and bile acid precursor via fatty acid oxidation\textsuperscript{69,113}. There are more biologically relevant enantiomers present inside and outside the cells. Whether CoA is playing a regulatory function in the activation or removal of toxic enantiomers still needs to be elucidated. Moreover many organic acid drugs like valproic, salicylic, phenylbutyric and benzoic acids are metabolized as CoA esters\textsuperscript{70}.

Recently, the direct role of CoA has been revealed which influences other important cellular functions like signaling and apoptosis\textsuperscript{16}. CoA binds directly to CaMKII and regulates its kinase activity. In Xenopus leavis oocytes, CoA activated CaMKII promotes oocyte survival via phosphorylation and inactivation of caspase-2\textsuperscript{17}. Such a diversity of CoA functions clearly indicates that it is not just a metabolite for controlling metabolism, and impaired CoA biosynthesis or imbalance in CoA pools will lead to a complex pathophysiology.

Figure 3: Diverse functions of CoA and its relevance under physiological and pathophysiological conditions.
Diseases related to CoA biosynthesis and regulation

Metabolic diseases

Impaired CoA metabolism has a link to many human pathophysiologies, especially diseases related to metabolism. Most prevalently, CoA and other acyl-CoA levels are considered to play a significant role in inborn errors of mitochondrial and peroxisomal dysfunctional diseases related to fatty acid oxidation. In addition, diseases like diabetes and impaired insulin resistance conditions, are known to be highly associated with a disruption or imbalance of CoA levels. Impaired acyl-CoA metabolism in both mitochondrial and non-mitochondrial systems accompany hereditary and acquired fatty acid related diseases. Some of the peroxisomal β-oxidation inborn errors like Zellweger-like syndrome and X-linked adrenoleukodystrophy are due to a impaired peroxisomal enzymes/proteins which influence acyl-CoA metabolism, leading to altered acyl-CoA levels. In addition, defects like inability to degrade excess of dietary 3-methyl branched chain fatty acid, phytanic acid lead to Refsum disease (an adult-onset peripheral neuropathy) and peroxisomal disorders. Such above mentioned disease conditions caused by deficiencies of acyl-CoA metabolizing enzymes leads to altered acyl-CoA pools and a CoA sequestration, toxicity, and redistribution (CASTOR) condition. However, whether or not the clinical symptoms of these diseases are the direct consequence of altered acyl-CoA levels still needs to be investigated. In eukaryotic model systems, it has also been recently shown that defects in acyl-CoA degradation lead to hyperammonia and a hyperglycemia. Moreover, cardiac hypertrophy is demonstrated to be dependent on the homeostasis of acyl-CoA pools due to defects in cardiac acyl-CoA synthetase and mitochondrial long chain acyl-CoA metabolism. Although many of the above-mentioned metabolic defects relate to altered functions of acyl-CoA synthetases or acyl-CoA regulating enzymes, the most likely cause, and consequence is the imbalance of CoA/acyl-CoA levels and their dependent processes.

Neurodegenerative diseases

The alteration or imbalance in CoA and various acyl-CoA levels due to genetic mutations in the CoA regulating enzymes will affect all tissues, in particular, brain which is one among the highest energy consuming organ in the human body. In addition, the role of acetyl-CoA in the synthesis of acetylcholine and diverse acetylated proteins have also been studied in relation to various neurodegenerative diseases. Altered acetylcholine levels lead to neuronal defects in neurodegenerative diseases like Parkinson’s disease, Alzheimer’s disease, Dementia and Myasthenia Gravis. Because in the brain, a proper balance of various neurotransmitters including acetylcholine, dopamine and serotonin is crucial for many neuronal activities, defects in CoA or acetyl-CoA biosynthesis will lead to disrupted neuronal functions. In many neurodegenerative diseases, altered lipid metabolism and mitochondrial dysfunction has also been shown to have a role in disease progression and pathogenesis. Primarily, alteration in protein acetylation status in brain tissues leads to complex transcriptional
and metabolic dysfunction, as in Huntington’s disease, because manipulation of histone deacetylases is proven to be beneficial in suppressing Huntington’s disease pathology in many model organisms\textsuperscript{27,28}. In all the above-mentioned neuronal diseases, abnormalities in CoA levels and its metabolism can be influential in the pathophysiology and disease progression (Figure 3).

The CoA \textit{de novo} biosynthesis pathway itself has gained renewed attention very recently in the field of neuroscience. Mutations in two of the genes, encoding enzymes involved in \textit{de novo} CoA biosynthesis, lead to a particular rare but severe form of neurodegeneration. Mutations in PANK2 cause pantothenate kinase associated neurodegeneration (PKAN)\textsuperscript{35}, whereas mutations in COASY lead to CoA synthase associated neurodegeneration (CoPAN)\textsuperscript{43}. Interestingly PANK regulates the foremost rate-limiting steps of \textit{de novo} CoA biosynthesis, indicating a direct link between CoA biosynthesis and neuronal function. Both PKAN and CoPAN are rare autosomal recessive disorders that belong to the group of disorders classified as neurodegeneration with brain iron accumulation (NBIA)\textsuperscript{129,130}. PKAN accounts for approximately 50\% of the total reported NBIA cases, and clinical manifestations of PKAN and CoPAN includes dystonia, cognitive decline, spasticity, and dysarthria\textsuperscript{130}.

\textbf{Pathological cause of PKAN and CoPAN}

Metabolic impairment in \textit{de novo} CoA biosynthesis leading to low CoA/acyl-CoA levels is speculated to be the most likely cause for both PKAN and CoPAN diseases. Although, in one of the CoPAN patient’s fibroblasts decreased acetyl-CoA levels were demonstrated, levels of total or free CoA levels were not affected\textsuperscript{43}. More detailed investigations are needed to confirm a direct link between neurodegeneration and abnormal CoA levels in PKAN and CoPAN. In-addition, not all the identified PANK2 mutations in PKAN patients lead to inactivation of pantothenate kinase\textsuperscript{131,132}. Therefore, the detailed pathogenesis in both PKAN and CoPAN remains unclear. In all the NBIA disorders, profound iron accumulation is observed in specific brain areas like the globus pallidus\textsuperscript{130}. The speculation for iron accumulation in PKAN patients was due to the cysteine overload. Because cysteine is normally incorporated to 4’-phosphopantothenate by PPCS, which is downstream of PANK in the \textit{de novo} CoA biosynthesis. Therefore, impairment in PANK activity might accumulate cysteine leading to abnormal iron chelation in cells\textsuperscript{130}. However, this hypothesis is debatable because patients with CoPAN and other forms of NBIA also show iron accumulation. Both PANK and COASY localize in mitochondria, and, therefore, the mitochondrial dysfunction could be one of the major disease contributors in PKAN and CoPAN patients. In many of the other neurodegenerative diseases, mitochondrial abnormalities and impaired mitochondrial function are known to be associated with oxidative stress and neuronal dysfunction\textsuperscript{125,126,133}. Moreover, in mitochondria, iron is utilized for heme synthesis and is required for the synthesis of iron-sulfur cluster containing proteins like aconitase\textsuperscript{134,135}. Therefore, accumulation of defective mitochondria in PKAN and CoPAN may lead to complex effects. Such a complexity could include impaired \textit{de}
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*de novo* CoA biosynthesis, cellular iron overload, increased oxidative stress and reactive oxygen species (ROS) levels, reduced acyl-CoA levels and defects in β-fatty oxidation and TCA cycle.

Treatment options for both PKAN and CoPAN at present are only aimed at symptomatic relief, and there are no curative therapeutic options identified\(^{136}\). Intramuscular botulinum toxin and baclofen treatments have been shown to have beneficial effects to treat PKAN-associated dystonia\(^{37}\). Recently, deep brain stimulation was proven to be effective in treating primary dystonia in PKAN patients\(^{38}\). Sequestering iron accumulation by chelating agents like deferiprone is currently investigated in a double-blinded randomized placebo-controlled clinical trial (www.tircon.eu), and the benefits are yet not known. Researchers speculate beneficial effects of anti-oxidants and pantothenate\(^{139}\), but detailed experimental and clinical proof of such a benefit still has to come. Overall, the lack of detailed knowledge with regards to the pathophysiology and progression of both these diseases and the absence of promising therapeutic options clearly demands more research. With relevant animal model systems, progress can be made in a successful manner. The use of appropriate disease model systems will not only help to understand the cause and manifestations of the disease but also is required to test potential drugs for therapeutic interventions.

**Model organisms to understand de novo Coenzyme A biosynthesis and regulation**

Simple but relevant model systems are essential to study the in-depth role of CoA and the consequence of impaired *de novo* CoA biosynthesis. Especially in the case of rare diseases such as PKAN and CoPAN, there is a limited availability of patient tissue samples. The use of in-vitro model systems consisting of human cell lines with impaired PANK or COASY might provide information to understand the consequence of impaired CoA biosynthesis. However, the overall physiological relevance in a complete organism cannot be concluded with in-vitro studies. Animal model organisms will aid many benefits both in understanding the molecular mechanisms of pathophysiology and also to test therapeutic interventions.

Focus has initially been made on mice model systems which either were formed by creating a PANK2 knock-out mouse or via vitamin B5 depletion to obtain a PKAN mouse model to understand the disease mechanism. The generated PANK2 knock-out mice showed growth retardation, progressive retinal degeneration and male infertility due to azoospermia. However, it did not show any impairment of CoA levels and failed to exhibit movement abnormalities and brain pathology that are characteristics associated with PKAN\(^{140}\). In contrast to PANK2 knock-out mice, a pantothenate deficient diet did elicit movement disorders along with loss of body weight and azoospermia. However, there is no evidence for iron accumulation in the brain\(^{142}\). Recently it was shown that in wild-type mouse, hopanthenate (PANK inhibitor) treatment induced mitochondrial abnormalities, reduced
hepatic CoA levels and altered acyl-carnitine levels. Interestingly, increased expression of enzymes like thioesterases were also observed indicating a compensatory role to generate and maintain elevated CoA levels\(^3\). More recently PANK1/2 double knock-out mice were also generated to understand a CoA depletion effect in a more robust way\(^2\). These mice showed a drastic reduction in liver and brain CoA levels, decreased fatty acid oxidation, and the mice developed severe hypoglycemia and hyperketonemia. Moreover, decreased expression of Nudix hydrolases revealed a cellular compensatory process for regulating CoA levels. Although, this information will help to understand the importance of CoA biosynthesis, whether it reciprocate the PKAN phenotype or not is still an open question. Moreover, experimental evidence is lacking whether or not phenotypic rescue can be achieved by overexpressing human PANK2 in the PANK1/2 double knockout mice to validate the modified mice as a possible PKAN disease models. In addition, so far there is no CoPAN mouse model available to understand the similarity and differences with PKAN regarding the impaired CoA biosynthesis and disease pathophysiology. Nevertheless, there are additional questions remaining such as what is the role of PPCS and PPCDC in maintaining CoA biosynthesis. Addressing all these questions in various mouse models will be laborious and time-consuming. Such a diverse study is possible with organisms like fruit flies, and this will yield interesting information to picture the complexity of diseases in a simple and reliable way.

**Drosophila as a model organism for impaired de novo CoA biosynthesis**

*Drosophila melanogaster* (fruit fly) is a highly suitable model organism to understand disease mechanisms and to find therapeutic approaches for various metabolic and neurodegenerative diseases\(^1\). The availability of various genetic tools, the possibility to manipulate pathways, the relative short life span all allow robust experimental studies. More than 50% of the members of the *Drosophila* proteome have mammalian analogs and these share similarity in functions and cellular localization\(^1\). Enzymes of the CoA *de novo* pathway are also highly conserved in *Drosophila*. Researchers identified the *Drosophila* mutants of the enzymes involved in the *de novo* CoA biosynthesis unexpectedly in a screen for male sterility (for dPANK/fbl)\(^1\) and in a separate screen to identify DNA damage sensitive mutants (for dPPCS)\(^1\). Soon after, a research focus in understanding these mutants explored the *de novo* CoA biosynthesis pathway enzymes in *Drosophila* along with a characterization of mutant phenotypes. Unlike mice and human, there is only one gene encoding for *Drosophila* pantothenate kinase, dPANK/fbl. However, by alternative splicing four different transcripts are generated, among which the longest variant dPANK/fbl E, is localized to mitochondria similar to human PANK2\(^1\). The dPANK/fbl gene is essential for survival because mutations in this gene result in neuroblast abnormality and pupal lethality\(^1\). Interestingly, dPANK/fbl hypomorphic (reduced dPANK/fbl expression) mutants showed sterility, shorter life span, and neuronal defects, which was rescued by overexpression of human PANK2\(^1\). Moreover, downstream CoA biosynthesis mutants (dPPCS and dPPAT-DPCK or dCOASY) also show comparable phenotypes like reduced lifespan, a high sensitivity to
ROS, an impaired lipid homeostasis, increased levels of DNA damage and hypersensitivity to DNA-damaging agents. It signifies that all these mutants might share a common phenotypic mechanism, most likely due to impaired CoA biosynthesis. Consequently, these Drosophila mutants will be a useful tool not only to study the CoA biosynthesis pathway but also to understand the disease pathophysiology and molecular mechanism involved in PKAN and CoPAN. Moreover, it will also enable us to perform drug screening and genetic interventions which will lead to novel therapeutic approaches for PKAN and CoPAN.

**AIM AND OUTLINE OF THE THESIS**

The overall aim of this research project was to understand the de novo CoA biosynthesis and its related cellular function. To aid our research focus, we also developed a simple and reliable analytical method with a selective profiling advantage for CoA and other related thiol molecules. We designed our primary research criteria, in particular, to investigate the physiological implication of impaired de novo CoA biosynthesis pathway using various in-vitro and in-vivo model systems, especially Drosophila melanogaster. Consequently, we also aimed to identify potential compounds which could become useful as an alternative substrate other than vitamin-B5 for CoA biosynthesis. Moreover, we substantiated our effort to understand and improve the applicability of such compounds for therapeutic advantages, which will be beneficial in diseases related to CoA biosynthesis like PKAN.

**Chapter 2: Pantethine rescues a Drosophila model for pantothenate kinase-associated neurodegeneration**

Mutations in the human pantothenate kinase gene, the primary rate-limiting enzyme in the de novo CoA biosynthesis machinery, lead to a specific form of neurodegeneration called PKAN. However, what the downstream effects of PANK impairment are, is not enumerated in details. In this chapter, we discuss the consequence of impaired PANK enzyme using both in vitro and in vivo Drosophila model systems. We demonstrated that PANK impairment led to a decrease in total CoA levels, mitochondrial dysfunction, and increased protein oxidation. Moreover dpANK/fbl Drosophila mutants showed locomotor dysfunction, a neurodegenerative phenotype and a reduced lifespan hereby mimicking characteristics of PKAN disease. Furthermore, selective small molecule screening lead to a remarkable finding that pantethine supplementation can restore CoA levels and also the majority of the phenotypes mentioned above, induced by PANK impairment in Drosophila PKAN model systems, were rescued. Here, we also provide evidence that pantethine can rescue phenotypes induced in mammalian cells due to hPANK2 downregulation. These results are further discussed to underline the importance of pantethine as a possible PANK-independent source for CoA biosynthesis and suggested that pantetheine could be a lead molecule for developing PKAN treatment options.
Chapter 3: Impaired Coenzyme A metabolism affects histone and tubulin acetylation in *Drosophila* and human cell models of pantothenate kinase associated neurodegeneration

In addition to the known metabolic importance of CoA and its derivatives, acetyl-CoA functions as an acetyl donor and thereby regulates protein acetylation homeostasis. Such an essential post-translational protein modification and its precise role in cellular functions are well studied. However, the influence of a perturbed metabolic pathway, especially impairment of the *de novo* CoA biosynthesis pathway, on protein acetylation status was not investigated. In this chapter, we documented our findings to show that decreased CoA levels due to impaired PANK enzyme lead to decreased acetylation of specific proteins like histones and tubulin. Using various model systems and techniques like RNAi, we show both *in vitro* and *in vivo* that the defect in metabolic CoA biosynthesis impairs protein acetylation. Moreover, we also show supportive proof that some of the characteristic PKAN phenotypes like defective DNA damage responses and impaired locomotor function in different model systems are due to a defective protein acetylation status. Our results also confirm that pantethine or histone deacetylase inhibitors (like TSA) supplementation restores protein acetylation and also improves various phenotypes. In this chapter, we also discuss our results bridging the defects in CoA biosynthesis and protein acetylation in the context of PKAN pathogenesis and future therapeutics.

Chapter 4: Synthesis and characterization of 4-thiobutyl triphenylphosphonium-pantetheine, a pantetheine derivative

In both chapter 2 and 3, we described pantethine (which is a disulfide of pantetheine) as a possible therapeutic drug for PKAN. However, the clinical applicability and its efficacy is limited due to the instability of pantetheine in biological systems. Pantetheinase (or Vanin) is a hydrolyzing enzyme that degrades pantetheine rapidly into vitamin-B5 and cysteamine. In this chapter, we explain our approach to synthesize a masked derivative of pantetheine, namely 4-thiobutyltriphenylphosphonium-pantetheine (TBTP-pantetheine) to improving its stability. We also explain the rationale for selecting this particular masking group to improve the stability and therapeutic efficacy of pantetheine. We provide evidence that such masking did not improve the stability of pantetheine. On the contrary, we show that the masking group improved the lipophilicity of pantetheine. We discuss a possible alternative approach not only aimed to improve the stability of pantetheine but also to enable a targeted delivery system to treat PKAN.

Chapter 5: Extracellular 4′-Phosphopantetheine is a novel substrate for intracellular Coenzyme A synthesis

In Chapter 1, we explained the canonical CoA biosynthesis pathway and also the importance of CoA in metabolic pathways and for other essential cellular functions. Finding alternative
ways to efficiently manipulate CoA levels is crucial to envision CoA-based therapeutic intervention. In Chapter 5, we challenge the exclusivity of the well-known canonical CoA supply route starting with Vitamin-B5. Subsequently, we provide proof that cells and organisms possess a mechanism to influence intracellular CoA levels using exogenous CoA via 4’-phosphopantetheine. Our results show that in flies, and in human and mouse serum, CoA is rapidly hydrolyzed by ecto-nucleotide-pyrophosphatases to 4’-phosphopantetheine, a biologically stable molecule. We also show that 4’-phosphopantetheine is able to translocate through membranes passively and is processed by the bi-functional enzyme CoA synthase back to CoA. Furthermore, using various CoA-deprived model systems in flies, worms and human cells, we show that CoA provided via the food or media rescues cell growth, decreased protein acetylation, abnormal locomotor skills, developmental arrest, sterility and decreased lifespan. We discuss further how our findings can answer long-lasting question in fundamental cell biology regarding efficient alternative CoA sources. We also discuss implications of our findings regarding treatment of CoA related diseases like PKAN.

Chapter 6: Summarizing discussion

Here, we summarize the overview of the presented scientific information and our findings regarding the importance of CoA and its biosynthesis pathway. Historically, CoA is considered to be just a metabolic cofactor. However, the recent discoveries in the CoA research field clearly show that CoA is more than a metabolite. In this chapter, we summarize the update of novel functions of CoA and its importance in diverse cellular processes, especially to protein acetylation homeostasis. Moreover, we discuss here the significance of an elegant model system like Drosophila as a powerful genetic tool to investigate the CoA biosynthesis pathway and its related functions. In addition, we explain our exploration of novel alternative CoA sources to manipulate the intracellular CoA levels especially in relation to diseases like PKAN. In this chapter, we also discuss our perspective vision for future research to shed more detailed understanding of CoA biosynthesis and dependent molecular functions. We also underline the importance of such approach in developing a promising treatment strategy for diseases like PKAN. We conclude the summarizing discussion with the expectation that dedicated research efforts are yet to come to reveal the importance of the multifaceted regulator ‘CoA’, which is more than ‘just’ a metabolic cofactor.
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