Gitte Meriläinen

STRUCTURAL AND ENZYMOLICAL STUDIES OF THE THIOLASE ENZYMES

FACULTY OF SCIENCE, DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF OULU, BIOCENTER OULU, UNIVERSITY OF OULU
GITTE MERILÄINEN

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Abstract

In the cells, the last step of the beta-oxidation cycle, aiming at the degradation of fatty acids, is catalyzed by the enzyme named thiolase. It shortens the acyl chain of the acyl-CoA by two carbons. The reaction is reversible, it can proceed for both directions. Thiolases are divided into two categories, synthetic and degradative ones. These two classes of thiolases differ not only by their biological function, but also by their substrate specificity. Degradative thiolases accept substrates with various lengths but synthetic thiolases only accept short chain-acyl-CoAs as a substrate.

In humans, at least six isozymes of thiolases are found. The mitochondrial biosynthetic thiolase, T2, differs from other thiolases by getting activated by potassium. In addition, it accepts branched acyl-CoA, namely 2-methyl-acetoacetyl-CoA, as a substrate. This molecule is an important reaction intermediate in the degradation of the amino acid isoleucine. Many human patients have been diagnosed to have a mutation in the gene of T2, and they are treated with a special diet.

The results of this theses show that potassium ion rigidifies the groups of the T2 protein involved in the substrate binding. The presence of potassium increases the reaction rate and it also raises the affinity towards some of the substrates.

The enzyme mechanistic studies with bacterial thiolase revealed that the oxyanion hole 1, formed by a water molecule and histidine side chain, is important for the synthetic reaction, not so much for the degradative direction. Binding studies showed that both the terminal sulfur of the substrate and the sulfur of the catalytic cysteine are important for the right positioning of the substrate. The electrostatics of the active site also have a significant role in the catalysis. These studies give a good basis for future studies aiming at drug development against this enzyme in pathogenic species.

Keywords: Acetyl-CoA C-acetyltransferase, acyltransferases, Coenzyme A, kinetics, substrate specificity, X-ray crystallography
To My Family
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AcAc-ACP</td>
<td>acetoacetyl-ACP</td>
</tr>
<tr>
<td>AcAc-CoA</td>
<td>acetoacetyl-CoA</td>
</tr>
<tr>
<td>AcAc-OPP</td>
<td>acetoacetyl-(O)-pantetheine-11-pivalate</td>
</tr>
<tr>
<td>AcAc-SPP</td>
<td>acetoacetyl-(S)-pantetheine-11-pivalate</td>
</tr>
<tr>
<td>Ac-CoA</td>
<td>acetyl-CoA</td>
</tr>
<tr>
<td>Ac-OPP</td>
<td>acetyl-(O)-pantetheine-11-pivalate</td>
</tr>
<tr>
<td>ACD</td>
<td>acetyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>ACP</td>
<td>acyl-carrier-protein</td>
</tr>
<tr>
<td>A. thaliana</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C, Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CHS</td>
<td>chalcone synthase</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CT</td>
<td>cytosolic thiolase</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td><em>Candida tropicalis</em></td>
</tr>
<tr>
<td>DESY</td>
<td>Deutsches Elektronen-Synchrotron</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ESRF</td>
<td>European Synchrotron Radiation Facility</td>
</tr>
<tr>
<td>G, Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>(h)</td>
<td>Planck constant</td>
</tr>
<tr>
<td>H, His</td>
<td>histidine</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxyl-3-methylglutaryl-CoA</td>
</tr>
<tr>
<td>HMGS</td>
<td>3-hydroxy-3-methylglutaryl-CoA synthase</td>
</tr>
<tr>
<td>IPP</td>
<td>isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>B-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>LCHAD</td>
<td>long-chain 3-hydroxyacyl-CoA dehydrogenase</td>
</tr>
<tr>
<td>(k)</td>
<td>Boltzmann constant</td>
</tr>
<tr>
<td>(k')</td>
<td>rate constant</td>
</tr>
<tr>
<td>KAS</td>
<td>(\beta)-ketoacyl-acyl carrier protein synthase</td>
</tr>
<tr>
<td>KCS</td>
<td>3-ketoacyl-CoA synthase</td>
</tr>
<tr>
<td>KS</td>
<td>(\beta)-ketosynthase</td>
</tr>
</tbody>
</table>
MES  2-(N- morpholino)ethanesulfonic acid
MPD  2-methyl-2,4-pentanediol
mRNA messenger-RNA
N  asparagine
NADPH reduced nicotinamide adenine dinucleotide phosphate
OPP  O-pantetheine-11-pivalate
PCR polymerase chain reaction
PEG-5000-MME polyethylene glycol 5000 monomethyl ether
PKS polyketidesynthase
PTS peroxisomal targeting signal
R gas constant
S. aureus  *Staphylococcus aureus*
S. cerevisiae  *Saccharomyces cerevisiae*
SCP-2 sterol carrier protein-2
Ser serine
SPP  S-pantetheine-11-pivalate
T absolute temperature
T1 thiolase 1
T2 thiolase 2
TFE mitochondrial trifunctional protein
VLCFA very long chain fatty acid
Wat water molecule
Z. ramigera  *Zoogloea ramigera*
**List of original articles**

This thesis is based on the original articles, which are referred to in the text by their Roman numerals:


* These authors contributed equally to this work.
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1 Introduction

In this thesis structural enzymological studies of the human mitochondrial thiolase 2 (T2) and the bacterial biosynthetic thiolase are reported. The T2 studies address the importance of the $K^+$-ions for its full activity. The bacterial *Zoogloea ramigera* (*Z. ramigera*) thiolase studies concern a quantitative characterisation of important interactions in its active site. (I)

Both of these thiolases belong to the “CNH” subfamily of the thiolase superfamily. “CNH” refers to three important catalytic residues. In the bacterial thiolase it concerns Cys89, Asn316 (the proximal asparagine) and His348 (the distal histidine). Cys89 is the catalytic cysteine and Asn316 and His348 are also important catalytic residues. Asn316 anchors the catalytic water molecule, Wat82, which plays a key role in the reaction mechanism, as it is part of oxyanion hole 1, which stabilizes the transition state of the Claisen condensation reaction. His348 possibly has a dual role; i) it contributes to the oxyanion hole 1 and ii) it activates the reactive cysteine. In these quantitative structural enzymological studies of the bacterial thiolase important determinants of the CoA thiolase interactions (II) as well as the precise roles of Asn316 and His348 have been studied (III).
2 Review of the literature

2.1 Enzyme catalysis

Enzymes are usually protein molecules appearing naturally in all living organisms, but interestingly some enzymes are RNA molecules. Their functions are very specific, they have enormous catalytic power and they work at ambient temperature and pressure. Many vital biological functions are dependent on enzymes and it is reasonable to say that without enzymes there is no life. Different enzymes are used by organisms and cells for various purposes, for example in cell respiration, storage of energy and also in synthesizing secondary metabolites.

Enzymes can enhance reaction speed up to $10^{23}$-fold (Miller & Wolfenden 2002) compared to the reaction occurring in the same conditions without the catalyst (Kraut et al. 2003). The comparison to other catalysts is also favorable for enzymes, since enzyme catalyzed reactions are usually faster and are usually done at lower temperatures (Price & Stewens 1999). Another asset of enzyme catalysis compared to other catalysts is its very high substrate and catalytic specificity. Many enzymes use and produce molecules that are highly specific for them. With enzymes it is possible to specifically produce only one stereoisomer, for example. Enzymes with low specificity are usually degradative enzymes rather than biosynthetic (Price & Stewens 1999).

Many enzyme catalyzed reactions in the cell are regulated at least at some level. The regulation mechanism of the catalytic activity may rely on small ions or other molecules, or on covalent modification of the enzyme (Price & Stewens 1999), such as phosphorylation. For example thrombin, which is a coagulation factor formed in the blood coagulation cascade and affecting the clot formation, is regulated by a Na$^+$-ion. When Na$^+$ is bound to this enzyme, it adopts a “fast” conformation and its protease activity towards procoagulant substrates increases whereas in a “slow” conformation, without bound Na$^+$, it activates the protein C anticoagulant pathway (Huntington 2008). Another very typical regulation mechanism especially in biosynthetic pathways is feedback inhibition (Price & Stewens 1999). This implies that some of the end products of the pathway slow down the catalytic activity of enzymes working earlier in the sequence of reactions if the concentration of the product gets to the level required for inhibition. For example N-acetylglutamate kinase, the second enzyme acting on a
route to synthesize arginine, is inhibited by arginine, the final product of the pathway (Ramón-Maiques et al. 2006).

2.1.1 Energy profile of the enzyme catalysed reaction

In order for a reaction to proceed from substrate(s) to products, the energy barrier ($\Delta G^\ddagger$) must be overcome. The highest point of the traditional reaction energy profile (Fig. 1) is called the transition state of the reaction. Fig. 1 illustrates two key differences between the non-enzyme catalyzed reaction and the enzyme catalyzed reaction: (1) in the enzyme catalyzed reaction a binding event occurs before chemical conversion. After the chemical conversion the product is released. In very efficient enzymes the substrate binding step or the product release step can be the rate limiting step. (2) The transition state barrier is much lower in the enzyme catalyzed reaction as compared to the non catalyzed reaction. There is a relationship between the rate constant $k'$ and the free energy of activation ($\Delta G^\ddagger$), which can be seen from the equation describing the transition-state theory,

$$k' = \frac{kT}{h} \cdot e^{-\Delta G^\ddagger/RT} = \frac{kT}{h} \cdot e^{-\Delta H^\ddagger/RT} \cdot e^{\Delta S^\ddagger/RT}$$

where $k$ is the Boltzmann constant, $h$ is the Planck constant, $T$ is absolute temperature and $R$ is the gas constant. (Price & Stewens 1999.)
The transition state is an unstable structure occurring when substrates are converted into products. It is estimated that the lifetime of the transition states in chemical reactions are about $10^{-13}$ seconds, the time of the single bond vibration (Schramm 1998). In order for a transition state to form, the conformational changes in the catalytic groups of the enzyme and the substrate are required to adopt a very precise coordination. Stabilizing enzyme -transition state interactions (ES‡) must be more favorable than in the Michaelis complex (ES). Destabilizing interactions present in the ground state can also lower the free energy barrier (Whitty et al. 1995). The relative free energy levels are dependent on the substrate concentration: at a low substrate concentration the free energy barrier becomes independent of the enzyme substrate interactions (Fig. 2). At low substrate concentration (low with respect to $K_M$), the catalytic rate is equal to $k_{cat}/K_M$, as illustrated in Figure 3. Therefore the $k_{cat}/K_M$ value is a direct measure of the free energy transition state barrier of the catalyzed reaction. Whenever $k_{cat}/K_M$ values are available in a range of active site mutants, then comparison of these values gives information on the contribution of the mutated side chains to transition state stabilization, as long as the rate determining step does not change. $k_{cat}/K_M$ –value is an apparent second-order rate constant, which is also referred to as the
“specificity constant”. It describes the reaction rate in relation to the concentration of free enzyme, and is thus a measure of specificity of the free enzyme towards an unbound substrate. In the cell the substrate concentrations are very often lower than $K_M$, therefore the $k_{cat}/K_M$ is of great importance for enzyme function in vivo. (Fersht 1999.)

![Diagram](image)

**Fig. 2. Differences in the free energy profile of an enzyme catalyzed reaction at A) high substrate concentrations and B) at low substrate concentrations.** Figures are redrawn from Fersht 1999. Mutations in the active site will generally affect both the free energy of $ES$ as well as of $ES^\ddagger$. These schematic figures illustrate that only at low substrate concentration (as visualized in B) the effect of the mutation to the transition state, $ES^\ddagger$, can be measured.

To increase the reaction velocity without changing the temperature, the $\Delta G^\ddagger$ must be lowered and several tools have been developed in the active sites of enzymes. One way of doing this is, in reactions with more than one substrate, is to bring the reactants close to each other in the active site. The importance of proximity effect is highlighted in DNA-templated synthesis, where synthetic small molecules, not related to DNA structure, are linked to complementary DNA strands. The formation of double-stranded DNA brings the linked molecules close enough for the reaction to happen between them without any catalyst. (Gartner & Liu 2001.)

In enzymes substrates are positioned by the binding residues of the protein in such a way that the catalytic residues are well placed for catalyzing chemical reactions. However, enzymes are not rigid entities and the unliganded enzymes can exist in several conformations. Substrate binding usually involves structural changes, and some reorganization of the enzyme-substrate complexes also happens during the reaction (Benkovic *et al.* 2008).
Catalysis is frequently involves acidic or basic amino acid side chains of the enzyme. Compared to uncatalyzed reactions in solvent, the activation energy is lowered by stabilizing the charges in the transition state (Fersht 1999). Another typical way of reducing the activation energy is a covalent catalysis, where a covalent intermediate is formed and broken down rapidly. Very often this covalent intermediate is formed by nucleophilic attack on an electrophilic center in the substrate. It is generally accepted that the basis of the enzyme catalysis is a tight binding of the unstable transition state structure (Schramm 1998). The selective electrostatic stabilization of the transition state by a preorganized active site is also proposed to be of key importance (Warshel et al. 2006) and is utilized in the development of catalytic antibodies (Ringe & Petsko 2008). Furthermore, dynamical properties of enzymes are of much importance (Hamnes-Schiffer & Benkovic 2006) and when these are taken into account, enzyme catalysis should be presented with multi-dimensional free-energy diagrams (Benkovic et al. 2008).
2.1.2 Applications of enzyme catalysis

The unique properties of enzymes have raised much interest to use them in applications where traditional catalysis has not succeeded or when the circumstances are not optimal for chemical catalysts to work. Enzymes are already widely used for example by detergent and food industry, but their use in fine chemical production is growing, especially due to protein engineering (Luetz et al. 2008). Native enzymes are rarely suitable for chemical industry as they are, but with good resources in research and development, nearly everything is possible. By using computational design it has been possible to create an enzyme that catalyses the breakdown of a carbon-carbon bond in an unnatural substrate (Jiang et al. 2008a) and enzymes with a Kemp-elimination activity (Röthlisberger et al. 2008). Computational methods were also used to modify the thermostability of a model enzyme, yeast cytosine deaminase (Korkegian et al. 2005). By combining rational design and directed evolution Park et al. (2006) changed the original activity of glyoxalase II into activity of β-lactamase without changing the scaffold of the enzyme. It is often necessary to immobilize enzymes onto a surface of some other material to obtain the optimal power of catalysis (Dwevedi & Kayastha 2009, Vasileva et al. 2009).

Another approach to biocatalysis is to re-engineer the metabolic pathways of the organism, to achieve high yields of the product of interest. For example, the production of butanol to be used as a biofuel by fermentation has raised interest in recent years. By metabolic engineering it has been possible to increase the amount of solvents produced by Clostridium acetobutylicum and to lower the amount of the unwanted side product, acetone. This was achieved by upregulating the expression of the enzymes acting in the pathway towards ethanol and butanol production, whereas the enzyme CoA transferase, acting in the acetone forming reaction pathway, was downregulated (Sillers et al. 2008). A combinatorial approach of genetic engineered enzymes and metabolic engineering has been used in the development of improved biodegradable plastics from polyhydroxyalkanoates (Nomura et al. 2004).

2.2 Fatty acid metabolism

Fatty acids are the major source of energy for heart and skeletal muscles and they form the major component of cell membranes. Most nutritional fatty acids are long-chain fatty acids. The enzymes with the thiolase fold have various roles in
fatty acid metabolism. Thiolase itself is particularly important in fatty acid degradation, whereas the other enzymes of the thiolase superfamily are involved in the fatty acid synthesis pathway. Therefore some aspects of fatty acid metabolism will be discussed in this chapter.

2.2.1 Fatty acid biosynthesis

In bacteria, the fatty acid biosynthesis (Fig. 4) is initiated by the acetylation of the β-ketoacyl-[acyl-carrier protein] (ACP) synthase III (KAS III) by acetyl-coenzyme A (Ac-CoA). The acyl group is then condensed with the second substrate, malonyl-ACP, to form β-ketoacyl-ACP. This product is next reduced by reduced nicotinamide adenine dinucleotide phosphate (NADPH), catalyzed by β-ketoacyl-ACP reductase, after which dehydration takes place, followed by the second reduction step. This second reduction step is catalyzed by enoyl ACP reductase and it results in saturated fatty acid elongated by two carbon atoms and attached to the ACP. This cycle of reactions continues until the desired chain length of the molecule is reached. In these subsequent elongation steps the KAS I and KAS II are important. In most bacteria and in all plants the fatty acid synthesis is catalyzed by discrete enzymes (FAS II), whereas in mammalian and fungal cells the same reactions are performed by multienzyme complexes (FAS I) (Brindley et al. 1969, Heath & Rock 2002). In addition, virtually all eukaryotes possess a FAS II system in mitochondria for the synthesis of lipoic acid (White et al. 2005). This FAS II system accounts for a small fraction of the total synthesis of the fatty acids.
2.2.2 Fatty acid oxidation

Stored fats and fatty acids are degraded by a β-oxidation pathway. This is a way to utilize the metabolic energy from the body. β-oxidation is the reversal of the synthesis, but is catalyzed by different enzymes. Fatty acids are activated for degradation by conjugating them with CoA, rather than with ACP as in fatty acid synthesis. This reaction is performed by ATP-dependent enzymes, fatty acid:CoA ligases or acyl-CoA synthetases (Kunau et al. 1995).

In mammals, β-oxidation takes place in mitochondria and peroxisomes. Mitochondrial β-oxidation is directly coupled to the respiratory chain and...
oxidative phosphorylation ensuring the production of ATP. In mitochondria, two subclasses of enzymes catalyze the reactions of β-oxidation. The soluble enzymes are located in the mitochondrial matrix, whereas those specialized in degradation of long-very-long-chain fatty acids are bound to the mitochondrial inner membrane (Kim & Battaile 2002). The speed of the mitochondrial fatty acid oxidation is regulated by the plasma levels of free fatty acids. In animal peroxisomes β-oxidation does not go to completion to yield acetyl moieties. Instead, it produces chain-shortened fatty acids from a variety of fatty acids, including very long- and branched-chain fatty acids, which are further degraded by the mitochondria. (Kunau et al. 1995.)

The cycle of β-oxidation is illustrated in Fig. 5. In the first reaction of β-oxidation the acyl-CoA is dehydrogenated into 2-trans-enoyl-CoA by an acyl-CoA dehydrogenase (ACD) in the mitochondria. Four acyl-CoA dehydrogenases in the mitochondria are involved in β-oxidation. They prefer substrates with different but somewhat overlapping chain lengths and are thus named as short-chain, medium-chain, long-chain and very-long-chain ACDs (Kim & Battaile 2002). In peroxisomes the same reaction is catalyzed by acyl-CoA oxidases, which use molecular oxygen as a substrate.

In the second reaction of β-oxidation 2-trans-enoyl-CoA is hydrated to yield L-3-hydroxyacyl-CoA. This reaction is catalyzed by enoyl-CoA hydratase, which accepts substrates with various lengths (C4-C20) (Kim & Battaile 2002). The hydration of very-long-chain fatty acyl-CoAs is performed by mitochondrial trifunctional protein (TFE). In the third reaction L-3-hydroxyacyl-CoA is dehydrogenated by L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA, the substrate of thiolase is formed. Only one soluble L-3-hydroxyacyl-CoA dehydrogenase is found in the mitochondria, having substrate specificity to short-chain fatty acyl-CoAs and the longer fatty acyl-CoAs are substrates for TFE. The final step of each cycle is to remove an acetyl group from 3-ketoacyl-CoA by a 3-ketoacyl-CoA thiolase, as shown in Fig 5. In addition to soluble thiolases, thiolase enzyme represents the third catalytic activity of TFE. This reaction cycle continues until only Ac-CoA is left, which is a substrate for the Krebs cycle.
Fig. 5. β-oxidation cycle. This figure shows the reactions that take place during the degradation of acyl-CoAs. The last product of the cycle, acyl-CoA, is again a substrate for the subsequent steps of the cycle.

2.3 Thiolase superfamily enzymes

The enzymes belonging to the thiolase superfamily catalyze the formation of a carbon-carbon bond via the Claisen condensation reaction. This reaction is of importance in many biological pathways, such as in a fatty acid, steroid and polyketide synthesis. In the Claisen condensation, a carbon-carbon-bond is formed when a negatively charged C2-atom of a thioester enolate intermediate performs a nucleophilic attack on the carbonyl carbon of the second substrate. This enolate intermediate is stabilized by oxyanion hole 1 of thiolase. The attacked carbonyl carbon is converted into a tetrahedral intermediate, with a
negative charge at the carbonyl oxygen. The charge is stabilized by the oxyanion hole 2 of the active site. (Haapalainen et al. 2006.)

All members of the thiolase superfamily share a similar function and despite their low sequence homology have very similar three dimensional structures and all have a catalytic cysteine. Most of them are dimers (Haapalainen et al. 2006); the only exceptions are thiolases, where dimers of dimers are observed (Modis & Wierenga 1999, Kursula et al. 2005). Each monomer consists of N-terminal and C-terminal halves, which share the βαβαβαβ -topology (Mathieu et al. 1994, 1997). The catalytic cysteine is in the loop between β3 and α3, at the N-terminus of Nα3. These two halves form a five-layered αβαβα -core structure (Fig. 6). The catalytic residues are arranged similarly in space, such that it is possible to superimpose the active sites of the members of the superfamily closely (Dawe et al. 2003, Kursula et al. 2005, Haapalainen et al. 2006). Most of the catalytic residues are located in the C-terminal half but the nucleophilic cysteine is in the N-terminal half (Haapalainen et al. 2006).

Fig. 6. Enzymes sharing the thiolase fold. Figure shows monomers from each enzyme and the αβαβα-layers of the subunits. The structures of A) thiolase from Z. ramigera (PDB entry 1DLV), B) KAS I from E. coli (2VB7), C) KAS III from E. coli (1MZS), D) HMGS from S. aureus (1TVZ) and E) the C-terminal PKS III domain of Steely1 protein from Dictyostelium discoideum (2HR4) are presented.

It is proposed that the thiolase superfamily enzymes have a common evolutionary origin. Phylogenetic analyses suggest that thiolases evolved from an ancestral enzyme, which was probably similar to archaeal thiolases. Later elongation and initiation enzymes evolved from the same ancestor. 3-Hydroxyl-3-methyl-glutaryl-CoA synthase (HMGS) developed from the initiation enzymes. (Jiang et al. 2008b)

Thiolases and HMGS catalyze non-decarboxylative Claisen and aldol condensation reactions, respectively. In these reactions acetyl-CoA is used as a
nucleophilic extender in carbon-carbon bond formation. Several members of the thiolase superfamily are involved in fatty acid and polyketide syntheses, using the decarboxylative Claisen condensation reaction, where malonyl thioesters are used as the nucleophilic extenders (Heath & Rock 2002). Decarboxylative condensing enzymes can be divided into the two groups, elongation and initiation enzymes, according to the catalytic residues in the active site (Jiang et al. 2008b).

The enzymes with the thiolase fold are very interesting from the industrial and pharmaceutical point of view. The antibiotic isoniazid, used in the treatment of tuberculosis, is an inhibitor of KAS A in Mycobacterium tuberculosis (Mdluli et al. 1998). PKSes synthesize a large number of complex molecules, for example new antibiotics (Khosla & Zawada 1996, Olsen et al. 2001) and enzymes catalyzing the fatty acid synthesis are of great interest as drug targets (Kremer et al. 2000, Waller et al. 1998, Olsen et al. 2001, Singh et al. 2008, Lee et al. 2009). It is also emphasized that by transforming KAS genes into crop plants, it is possible to affect the composition of oils produced by plants (Dehesh et al. 1998, Olsen et al. 2001, Stoll et al. 2006, Guo et al. 2009).

All members of the thiolase superfamily posses an active site cysteine that participates in the reaction by acting as a nucleophile. This cysteine is covalently acylated during the first part of the reaction. However, the roles of individual catalytic residues in active sites differ from each other, suggesting different reaction mechanisms (Dawe et al. 2003, Jiang et al. 2008b). The active sites are located close to the dimer interface and some residues of the dimer point into the acyl-CoA-binding pocket of the other monomer (Kursula et al. 2005). These residues are not known to participate in the reaction. The members of the superfamily can be divided further by their superimposable catalytic residues.

Three classes of subfamilies are found: CHH, CHN and CNH. C refers to the catalytic cysteine; the two other residues are either a histidine or an asparagine. These three residues are close together in space, shaping the catalytic site, but rather separated in sequence. The cysteine is part of the N-terminal part, whereas the two other residues are of the C-terminal part of the core. These latter two residues are approximately 30 residues apart, for example in the bacterial thiolase it concerns Cys89, Asn316 and His348. The proximal asparagines, Asn316, is proposed to play a role in shaping the oxyanion hole 1, whereas the distal histidine, His348, is proposed to play a dual role, both in the activation of Cys89, as well as in the shaping the oxyanion hole 1.
2.3.1 Condensing enzymes with a CHH catalytic triad in fatty acid synthesis

The elongation enzymes; ß-ketoacyl-acyl carrier protein synthase (KAS) I and KAS II and a ß-ketosynthase (KS) domain of polyketide synthase (PKS) have cysteine (Cys, C) and two histidines (His, H) as catalytic residues (CHH triad). The two former enzymes use acyl thioesters of ACP as a substrate to condensate with further malonyl-ACP. They differ by the substrate specificity in such a way that KAS I elongates C4 into C16 in plants and KAS II elongates it to C18 (Olsen et al. 1999). In bacteria these enzymes also synthesize the unsaturated fatty acids C16:1 and C18:1 to be used as membrane lipids (Olsen et al. 1999, 2001). An additional elongation KAS enzyme, KAS IV is found in the seeds of some plants. It is involved in the synthesis of C14 from C8 (Olsen et al. 2001).

The enzyme mechanism of elongation enzymes involves a transfer of the growing fatty acid chain to an active site cysteine. In the second step of the reaction malonyl-ACP binds and is decarboxylated with a release of carbon dioxide. In the third part of the reaction the Claisen condensation of acetyl-ACP with the acylated enzyme is catalyzed. An oxanion hole formed by main chain NH groups stabilizes the oxogroup of the bound fatty acid thioester (Olsen et al. 2001). The role of a distal histidine as a base activating the catalytic cysteine has been questioned (Olsen et al. 2001, McGuire et al. 2001). Both histidines have been proposed to be important for the stability of the carbanion formed after decarboxylation of the malonyl-ACP (Heath & Rock 2002), but the precise role of the histidines in the catalysis is not yet fully understood. The missing pieces of information are the structure of a Michaelis complex, visualizing the mode of binding of malonyl-ACP, and the structure of an intermediate complex revealing the binding of the activated, decarboxylated acyl-ACP.

PKSs are responsible for the synthesis of polyketides in bacteria, fungi and plants (Hopwood & Sherman 1990). They form a large group of catalysts and are involved in the formation of complex molecules, such as antibiotics and flavonoids (Hopwood & Sherman 1990). Elongating PKS enzymes are also divided into categories PKS I and II. As in the FAS system, PKS I includes multifunctional enzymes and PKS II enzymes are monofunctional (Austin & Noel 2003). PKS I and II enzymes belong to the CHH-class, like the KAS I and KAS II. PKS III enzymes are also monofunctional. They belong to the subfamily with a CHN catalytic triad.
2.3.2 Initiation enzymes of fatty acid synthesis with a CHN catalytic triad

The initiation enzymes, KAS III and 3-ketoacyl-CoA synthase (KCS) have Cys, His, and asparagine (Asn, N), CHN triad as catalytic residues. Chalcone synthase (CHS), which is a type III PKS, also belongs to this class. These enzymes catalyze decarboxylative condensation reactions as do the elongation enzymes. KAS III is the initiator of fatty acid synthesis in plants and in bacteria, condensing acetoacetyl-ACP (AcAc-ACP) from Ac-CoA and malonyl-ACP (Tsay et al. 1992). It functions as a feedback regulator of the fatty acid biosynthesis pathway, because it is inhibited by the long-chain acyl-ACPs (Heath & Rock 1996) and is a potential target for antibacterial drugs (Qiu et al. 1999). KCS catalyses the initiator step in the production of very long chain fatty acids (VLCFA) to produce wax and seed storage lipids in plants, by the condensation of acyl-CoA with malonyl-CoA (Blacklock & Jaworski 2006).

In the first part of the condensation reaction, the active site cysteine is acetylated by Ac-CoA. The cysteine was initially thought to be activated by histidine (Qiu et al. 1999), but later this was found not to be the case. Based on the studies on KAS III, the formation of a thiolate ion is believed to be assisted by an α-helix dipole effect (Hol et al. 1978, Davies et al. 2000), by a hydroxide ion or by a water molecule (Qiu et al. 2001). In a condensation reaction the decarboxylation requires both His and Asn where they are believed to stabilize the charge formed on the thioester carbonyl (Davies et al. 2000). Another oxyanion hole, formed by main chain amides, stabilizes the tetrahedral intermediate (Qiu et al. 1999, Davies et al. 2000).

CHS enzymes belong to the thiolase superfamily, being in the same subgroup as the type III PKS enzymes (Austin & Noel 2003). These enzymes catalyze multiple reactions in the same active site. CHS is responsible for the biosynthesis of plant phenylpropanoids by providing chalcone to be modified by other enzymes, into a broad range of compounds, including flavonoids and pigments (Ferrer et al. 1999). Unlike PKS I and II, CHS utilizes CoA-derivatives instead of ACP. The structure of CHS is also a traditional thiolase fold, but the loop domain defining the catalytic pocket has unique features. Actually chalcone is synthesized in the sequential condensation reaction of p-coumaroyl-CoA and three malonyl-CoA molecules. In the first part of the reaction p-coumaroyl is captured by the enzyme and the condensation reaction starts with decarboxylation of malonyl-CoA. This last step is repeated twice. Finally the product intermediate is cyclized
by a Claisen condensation to produce an aromatic ring system. All of these reactions are catalyzed by the single active site. (Ferrer et al. 1999.)

3-Hydroxyl-3-methylglutaryl-CoA synthase with a CHN motif

Cytolesmic HMGS catalyses the second step of the mevalonate pathway (Fig. 7), an aldol condensation of Ac-CoA with AcAc-CoA to form 3-hydroxyl-3-methylglutaryl-CoA (HMG-CoA). In mammals, the mevalonate pathway is the only pathway to produce isopentenyl pyrophosphate (IPP). The end products of the IPP metabolism are cholesterol, in higher animals, and in bacteria such vital compounds as ubiquinones (electron transport) and undecaprenol (cell wall biosynthesis) (Wilding et al. 2000b). Genes for the mevalonate pathway are essential for the growth of some pathogenic bacteria, such as *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (Wilding et al. 2000a, b, Campobasso et al. 2004) and *Enterococcus faecalis* (Steuysy et al. 2005). The mitochondrial isoform of HMGS is central for ketogenesis (Hegart 1999). The deficiency of this HMGS has been reported to cause a metabolic disease in humans (Thompson et al. 1997, Wolf et al. 2003, Aledo et al. 2006).

Fig. 7. Mevalonate pathway. The first reaction of the pathway is catalyzed by thiolase and the second by HMGS. The end-product of the pathway, IPP, is vital for many important biological processes in the cell.

The HMGS active site resembles thiolases in a way that there are altogether four catalytic residues. In addition to CHN triad, there is also a glutamate (Glu, E), which participates in the reaction by acting as a base in the condensation step of the reaction (Theisen et al. 2004). This Glu is located on the opposite side of the active site, compared to the Cys378 (base) in the thiolase structure (Campobasso et al. 2004, Haapalainen et al. 2006). In the reaction the nucleophilic cysteine of the CHN-triad is acetylated by the acetyl group of Ac-CoA. The acetylation proceeds via a tetrahedral intermediate which is stabilized by residues in the active site. It is speculated that the HMG-CoA is released from the enzyme via
hydrolysis by water molecules visible in the X-ray crystal structure. (Campobasso et al. 2004.)

2.4 Thiolases with a C(N/H)H motif

Thiolases are divided into degradative (EC 2.3.1.9) and biosynthetic thiolases (EC 2.3.1.16) according to their substrate specificity (Masamune et al. 1989). Degradative thiolases accept substrates with acyl chains ranging from 4 to 22 carbons, whereas biosynthetic enzymes are specific for short chain acyl chains. All biosynthetic thiolases are tetramers; degradative thiolases are either dimers or tetramers (Kursula et al. 2002). They are CoA dependent and, depending on their character, work in either the synthetic or degradative pathways, such as in the mevalonate pathway for synthesis of IPP (Fig. 7) or in β-oxidation (Fig. 5) for the breakdown of fatty acids. All thiolases have a high sequence similarity with each other (Mathieu et al. 1997) and they are able to catalyze the reaction in both directions. A degradative reaction is thermodynamically much more favorable (Masamune et al. 1989).

In mammals there are at least six isoenzymes (Antonenkov et al. 2000a) of thiolases as listed in Table 1. They are localized in peroxisomes, mitochondria and in cytosol and have different functions and expression patterns. Three different 3-ketoacyl-CoA thiolases and one acetoacetyl-CoA thiolase (T2) are found in rat liver. The 3-ketoacyl-CoA thiolases are 3-oxoacyl-CoA thiolase B, 3-oxoacyl-CoA thiolase A and sterol carrier protein 2/3-oxoacyl-CoA thiolase (SCP-2/thiolase) (Hijikata et al. 1987, Seedorf et al. 1994, Antonenkov et al. 1997). Thiolase A and thiolase B are almost identical in sequence and are grouped together as the A/B-thiolases. Errors in the human genome causing thiolase deficiency and diseases have been reported (Hartlage et al. 1986, Tyni et al. 1997, Kamijo et al. 1997, Fukao et al. 1998).
Table 1. Mammalian thiolases; localization and function.

<table>
<thead>
<tr>
<th>Thiolase</th>
<th>Localization in the cell</th>
<th>Substrate specificity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial acetoacetyl-CoA thiolase (T2)</td>
<td>mitochondria</td>
<td>AcAc-CoA, 2-methyl-AcAc-CoA</td>
<td>Ketogenesis (in liver), ketolysis (in extrahepatic tissues)</td>
</tr>
<tr>
<td>Mitochondrial 3-ketoacyl-CoA thiolase (T1)</td>
<td>Mitochondrial matrix</td>
<td>Medium chain-acyl-CoAs</td>
<td>β-oxidation, ketogenesis</td>
</tr>
<tr>
<td>TFE</td>
<td>Mitochondrial inner membrane</td>
<td>Medium and long chain- and branched acyl-CoAs</td>
<td>β-oxidation</td>
</tr>
<tr>
<td>Cytosolic thiolase</td>
<td>Cytosol</td>
<td>AcAc-CoA</td>
<td>Cholesterol synthesis</td>
</tr>
<tr>
<td>3-ketoacyl-CoA thiolase (A/B-thiolase)</td>
<td>Peroxisome</td>
<td>Medium and long chain-acyl-CoAs</td>
<td>Peroxisomal β-oxidation</td>
</tr>
<tr>
<td>SCP2/thiolase</td>
<td>Peroxisome</td>
<td>Medium and long chain-acyl-CoA, 2-methyl-3-ketoacyl-CoA, the side chain of cholesterol</td>
<td>Peroxisomal β-oxidation of branched chain fatty acids, bile acid metabolism</td>
</tr>
</tbody>
</table>

Thiolases catalyze the formation of 3-ketoacyl-CoA and CoA from Ac-CoA and acyl-CoA molecules or vice versa. The two Ac-CoA molecules are treated differently; the first one is utilized as an electrophile at C-1 and the other as a C-2-carbanion (Jordan & Gibbs 1983, Masamune et al. 1989). A conserved catalytic CH dyad is formed by the Cys and the distal histidine. Jiang et al. (2008b) reported that in thiolases the proximal residue is always either His or Asn, suggesting a conserved nature of this residue. In all well documented thiolases the active site has also a second cysteine residue, which acts as a base in the second part of the reaction. The water molecule in the active site plays a role in the reaction forming part of an oxyanion hole during the condensation reaction (Kursula et al. 2005) or as a proton replenisher for the cysteine base in the active site (Sundaramoorthy et al. 2006).

The role of the histidine in the CH dyad is to activate the nucleophilic cysteine (Mathieu et al. 1997, Modis & Wierenga 2000), and to stabilize the negative charge created in the thioester oxygen atom during the condensation reaction (Modis & Wierenga 2000). It has been argued that the role of the catalytic histidine could be no more than to stabilize the reaction intermediate (Dawe et al. 2003) and that the formation of the thiolate ion might be at least partly due to the α-helix dipole effect (Hol et al. 1978, Heath & Rock 2002). For thiolase, at least one mutagenesis study has implied that the active site histidine
has an important catalytic role in the thiolytic direction, suggesting that it is important for activation of the catalytic cysteine (Zeng & Li 2004).

### 2.4.1 Bacterial biosynthetic thiolase

A bacterial biosynthetic thiolase functions in the pathway to produce polyesters. For example, in *Zoogloea ramigera* (*Z. ramigera*) the main energy storage molecule is poly-3-D-hydroxybutyrate, whose synthesis is initiated by the biosynthetic thiolase followed by AcAc-CoA reductase and PHB synthase. PHB is a valuable material because of its low toxicity and biodegradability (Masamune et al. 1989). It has been discovered as an interesting substance for the preparation of bioplastics (Madison & Huisman 1999, Kursula et al. 2002).

The biosynthetic thiolase of *Z. ramigera* has a high homology to mammalian cytosolic thiolase, with, for example, 57% sequence identity with human cytosolic thiolase (Kursula et al. 2005). Structures of *Z. ramigera* thiolase and its complexes with various reaction cycle intermediates have been solved (Modis & Wierenga 1999, 2000, Kursula et al. 2002). It is a dimer of two dimers with a tetramerization motif which is a unique feature in thiolases (Modis & Wierenga 1999).

In addition to AcAc-CoA, *Z. ramigera*’s biosynthetic thiolase cleaves C5- and C6-3-oxoacyl-CoA, with slower turnover rates, but does not cleave 2-methyl-AcAc-CoA. Neither does it react with the oxo ester analog of Ac-CoA or a corresponding amide analog. The pKₐ of the methyl hydrogens of Ac-CoA are about 20 and 26 for the thio ester and oxo ester, respectively. This difference is estimated to be too much for the enolate generation in case of the oxo ester. The triphosphoadenoside portion of CoA molecule is not essential for binding to the bacterial thiolase, since it efficiently catalyses a reaction with AcAcS-pantetheine-11-pivalate (AcAc-SPP), whereas AcAcO-pantetheine-11-pivalate (AcAc-OPP) acts as a reversible inhibitor (Fig. 8). (Masamune et al. 1989.)
In the first part of the catalyzed reaction, Cys89 is covalently acetylated by Ac-CoA. The acyl-enzyme is relatively labile; its half-life is about 2 minutes (Thompson et al. 1989). In the second part the classical Claisen condensation takes place and the acetyl group is transferred to the methyl group of another Ac-CoA to form AcAc-CoA. Cys378 acts as a base by deprotonating the second acetyl-CoA molecule in the Claisen condensation, as confirmed by studies with inhibitors and by site-directed mutagenesis (Palmer et al. 1991, Williams et al. 1992). For the synthetic direction, the C-C-bond formation is rate-limiting, and in the thiolytic reaction the deacylation of the enzyme (the transfer of the acetyl group from acetyl-cysteine to CoA) is the rate determining step (Thompson et al. 1989).

Two oxyanion holes are located in the active site. Oxyanion hole 1 stabilizes the thioester of the bound Ac-CoA, and is formed by a water molecule (Wat82)
and His348, whereas oxyanion hole 2 stabilizes the thioester oxygen of the acetylated Cys89, and is formed by NH-groups of the main chain; N(Cys89) and N(Gly380). Wat82 is connected to a chain of water molecules, starting from the active site and reaching to the back of the molecule. The protein structure is rigid during the reaction cycle: all the captured intermediate structures have very similar structures. The only significant difference is that the reactive cysteine (Cys89) side chain rotates towards the catalytic His348 on ligand binding. (Kursula et al. 2002.)

2.4.2 Cytosolic thiolase (CT)

A cytosolic thiolase (CT) catalyzes the first reaction of the mevalonate pathway (Fig. 7), which leads to the biosynthesis of cholesterol (Antonenkov et al. 2000a). In humans it is encoded by chromosome 6q25.3-q26 (Song et al. 1994, Masuno et al. 1996, Kursula et al. 2005) and is expressed at highest levels in the liver (Fukao et al. 1997) where its activity accounts for 70% of the total thiolase activity, as measured from avian liver (Clinkenbeard et al. 1973). A reduced activity of the cytosolic thiolase has been reported on two patients, both suffering from severe neurological disorders (de Groot et al. 1977, Bennett et al. 1984). Cytosolic thiolases are efficient catalysts in both directions of the reaction (Middleton 1974), emphasizing their important role in isoprenoid and steroid synthesis. Specific inhibitors of this enzyme might serve as drugs to prevent high levels of cholesterol (Bloxham 1975, Wu et al. 2007).

The active site of the human CT resembles that one in the bacterial biosynthetic thiolase. They share the same linear water structure and hydrogen bonding network, with the exception that in human CT the chain of waters is longer by two water molecules. The two oxyanion holes seen in the bacterial biosynthetic thiolase are also found in the structure of human CT. (Kursula et al. 2005).

2.4.3 Mitochondrial acetoacetyl-CoA thiolase (T2)

A mitochondrial biosynthetic thiolase has a role in the metabolism of ketone bodies and isoleucine catabolism. In the liver, it participates in ketogenesis, whereas in extrahepatic tissues its role is in ketolysis (Fukao et al. 1998). Apparently its role in ketolysis is more important than in ketogenesis, because T2 deficiency causes accumulation of ketone body derived acids in the blood. T2 is
synthesized with a cleavable mitochondrial targeting signal in the N-terminus of the protein and has the highest expression pattern in the liver (Fukao et al. 1997). Its activity is increased when the blood ketone-body concentrations are raising (Reed et al. 1977). The mitochondrial acetoacetyl-CoA thiolase differs from other thiolases by requiring a potassium ion for full activity (Middleton 1973).

It was reported quite recently that the reaction thought to be catalyzed by a mitochondrial acetyl-CoA hydrolase, that is, a formation of acetate from Ac-CoA, is actually a side reaction catalyzed by the mitochondrial acetoacetyl-CoA thiolase in a rat liver (Yamashita et al. 2006). Very high Ac-CoA hydrolase activities have been found in a hamster and rat brown-adipose tissue mitochondria (Bernson 1976, Alexson & Nedergaard 1988, Yamashita et al. 2006).

Mitochondrial biosynthetic thiolase deficiency can cause a metabolic disorder (Fukao et al. 1992, 2001) which has been reported more than 60 patients (Sakurai et al. 2007). The disease was first described in 1971 (Daum et al. 1971, Fukao et al. 2001). The major symptoms of this disorder is episodial ketoacidosis, which may lead to unconsciousness, and accumulation of 2-methyl-acetoacetate and 2-methyl-3-hydroxybutyric acid. Some patients died during the attack, some are mentally retarded (Fukao et al. 1998) and neurological disorders have been characterized in few patients (Ozand et al. 1994). Quite recently low activity of mitochondrial acetoacetyl-CoA was detected from the mucosal biopsies of patients suffering from ulcerative colitis (Santhanam et al. 2007). In these patients, the low activity of thiolase is proposed to cause a defect in butyrate oxidation by colonocytes.

The mutations found in patients with mitochondrial acetoacetyl-CoA deficiency are diverse (Fukao et al. 1991, 1992, 1998, 2001, 2007, 2008, Zhang et al. 2006, Sakurai et al. 2007). Most patients are treated for ketoacidosis only once, usually in the childhood, for which they respond well (Fukao et al. 2001). Because the disease is caused by impaired catabolism of isoleucine, a low protein diet is recommended after the diagnosis (Fukao et al. 2001).

Peroxisomal acetoacetyl-CoA (T2-like) thiolase

A biosynthetic thiolase is present in peroxisomes or glyoxysomes of fungi and plants (Peretó et al. 2005). A peroxisomal acetoacetyl-CoA thiolase has been characterized from yeast Candida tropicalis (C. tropicalis) (Kurihara et al. 1992). The analysis has revealed two genes coding for two almost identical (98% identity) proteins, thiolase IA and thiolase IB (Kurihara et al. 1992). Both are
expressed at an equal level in the peroxisomes. Since they are located in the same chromosome, it is possible that these genes are allelic or tandemly aligned (Kurihara et al. 1992). Kanayama et al. 1997 reported later that the same gene products also exist in the cytosol. They share homology with other known thiolases: they are 40% with Z. ramigera acetoacetyl-CoA thiolase, 50% with a rat mitochondrial acetoacetyl-CoA thiolase and 37% with a rat peroxisomal 3-oxoacyl-CoA thiolase (Kurihara et al. 1992) and as expected for the biosynthetic thiolase, its role in β-oxidation is minor (Ueda et al. 2003).

2.4.4 Peroxisomal 3-ketoacyl-CoA thiolase (A/B – thiolase)

Peroxisomal 3-ketoacyl-CoA thiolases are involved in the peroxisomal β-oxidation of fatty acids for long and very long straight chain fatty acids (Antonenkov et al. 2000a). The first solved thiolase structure was from Saccharomyces cerevisiae (S. cerevisiae) 3-ketoacyl-CoA thiolase (Mathieu et al. 1994, 1997). It has a 38% sequence identity with the bacterial biosynthetic thiolase of Z. ramigera (Modis & Wierenga 2000). All peroxisomal 3-ketoacyl-CoA thiolases known so far appear as dimers and they are targeted to peroxisomes by cleavable amino-terminal peroxisomal targeting signal 2, PTS2 (Swinkels et al. 1991, Osumi et al. 1991). After cleavage, the signal peptide is a target for proteolysis by insulin-degrading enzyme in peroxisomes (Authier et al. 1995).

In rat (Hijikata et al. 1990, Bodnar & Rachubinski 1990) and mouse (Chevillard et al. 2004) genome, there are two almost identical genes encoding for peroxisomal 3-oxoacyl-CoA thiolase. The previously found thiolase B of rat has a 10 amino acids shorter N-terminal presequence than thiolase A, found later. In the mature form these enzymes differ only by 6 (Hijikata et al. 1990) or 9 (Bodnar & Rachubinski 1990) residues. Thiolase A is expressed continuously in a rat, whereas thiolase B requires peroxisomal proliferator to be expressed (Hijikata et al. 1990, Bodnar & Rachubinski 1990). In mouse tissues, a thiolase A messenger-RNA (mRNA) is mainly expressed in the intestine and liver, whereas thiolase B mRNA is mainly expressed in the liver (Chevillard et al. 2004). The expression of these thiolase isozymes differ from a mouse to rat, since in a normal mouse, thiolase A and B appear in the relation 60:40, respectively. However, a treatment with peroxisome proliferator induces the expression of thiolase B, as seen in a rat (Chevillard et al. 2004). Only one gene for the peroxisomal 3-ketoacyl-CoA thiolase is present in humans (Fukao 2002).
There are no differences in the reactions or substrate specificities between thiolase A and B, but A form is much more stable than B (Antonenkov et al. 1999b). In a normal rat liver, thiolase A is responsible for the cleavage of 3-oxoacyl-CoAs to yield acetyl-CoA and acyl-CoA, shortened by two carbons (Antonenkov et al. 1997). The difference in the expression of thiolase A and B lies in the promoter region. Thiolase A expression is regulated by a transcription factor NFY, and downregulated by cholesterol (Desaint et al. 2004), whereas thiolase B has two response elements, one in the promoter region for nuclear receptor HNF-4 and the other is located in the sequence of intron 3 and is responsible for the PPARα-activation (Hansmannel et al. 2003).

It has been reported that in humans the deficiency of the peroxisomal 3-ketoacyl-CoA thiolase leads to a lethal disease, with symptoms resembling those seen in a Zellweger syndrome (Schram et al. 1987a, b). Later, however, this was shown to be a misdiagnosis (Ferdinandusse et al. 2002). In the plant Arabidopsis thaliana (A. thaliana) the mutation in this enzyme causes a defect in germination. The plant is no longer able to mobilize its lipids during seeding or to grow without external sugar supply. (Germain et al. 2001.) In yeast Yarrowia lipolytica a point mutation Gly382Glu, just two residues away from the active site base Cys379, makes the yeast incompatible to growth on n-decane as a carbon source (Yamagami et al. 2001).

The structure of the A. thaliana peroxisomal 3-ketoacyl-CoA thiolase reveals a disulfide bond between the nucleophilic Cys138 and another cysteine residue, Cys192 (Sundaramoorthy et al. 2006). This pair of cysteines is conserved in 25 other peroxisomal degradative thiolases, implicating a mechanism for the regulation of peroxisomal fatty acid β-oxidation.

### 2.4.5 Peroxisomal SCP-2/thiolase

A peroxisomal SCP-2/3-ketoacyl-CoA thiolase is synthesized as a single polypeptide chain from the same mRNA. The N-terminal part encodes for 3-oxoacyl-CoA thiolase and the C-terminal part codes for sterol carrier protein-2 (SCP2). Part of the enzyme is cleaved in vivo to yield three thiolase isoforms: 3-oxoacyl-CoA thiolase with SCP2 homodimer, 3-oxoacyl-CoA thiolase homodimer and heterodimers of the previously mentioned chains (Antonenkov et al. 1997, 2000b, Atshaves et al. 1999).

SCP2 is known to participate in the cholesterol metabolism and it promotes the exchange of various kinds of lipids across membranes (Crain & Zilversmit
An overexpression of SCP-2/thiolase increases the uptake of cholesterol from a culture medium in the mouse L-cell fibroblasts (Atshaves et al. 1999) and the expression of this gene is regulated by lauric acid and insulin (Lopez et al. 2008). The thiolase part of this enzyme has a relatively low sequence homology (25%) to other acetoacetyl- and 3-oxoacyl-CoA thiolases, but the active site cysteine is localized in the same place (Seedorf et al. 1994).

The SCP-2/thiolase has the strongest thiolytic activity towards 3-oxooctanoyl-CoA, and has a very low activity on AcAc-CoA (Seedorf et al. 1994). It accepts medium and long straight chain 3-ketoacyl-CoAs C6:0-C18:0, as well as C18:1 as substrates (Seedorf et al. 1994). It also catalyses the reaction with 2-methylbranched fattyacyl-CoAs and bile acid intermediates, such as di- and trihydroxycoprostanic acids (Antonenkov et al. 1997) as well as trihydroxycholestanoic acid (Wanders et al. 1998). It has roles in the peroxisomal β-oxidation of fatty acids and in bile acid synthesis (Antonenkov et al. 1997, 2000a) and its expression is the strongest in the liver and adrenals of a rat (Ossendorp et al. 1996).

One patient with a deficiency of this protein, caused by a frameshift in the gene of SCP2/thiolase, has been characterized. In this patient both the full length SCP2/thiolase and the thiolase part were missing, whereas SCP2 was expressed normally from its own promoter. The patient was diagnosed with several neurological symptoms and infertility. His serum cholesterol and phytanic acid levels were only slightly elevated, but the amount of pristanic acid was over ten times higher compared to the control values. After several months on a phytanic acid-restricted diet, his symptoms had stopped progressing. (Ferdinandusse et al. 2006.) The mouse model which also lacked the SCP2 gene moiety, exhibited even more drastic symptoms, including changes in the peroxisome proliferation and an up to 10-fold accumulation of phytanic acid (Seedorf et al. 1998). When these mice are fed with the diet enriched with phytol, they develop a cardiac phenotype and have a high risk for sudden cardiac death (Mönnig et al. 2004).

### 2.4.6 Mitochondrial 3-ketoacyl-CoA thiolase (T1)

A mitochondrial degradative thiolase of a pig heart is the best characterized of degradative thiolases (Gilbert et al. 1981). It is the only tetrameric degradative thiolase and often referred to as thiolase I (T1). It is expressed with a non-cleavable mitochondrial targeting signal (Mathieu et al. 1994). Its optimal activity is between the pH values 6 and 9 and the rate-limiting step in the thiolytic process.
direction is the formation of the acetylated enzyme. For the condensation reaction, the rate is determined by acetyl transfer (Gilbert et al. 1981). The half-life for hydrolysis of the acetylated pig heart enzyme is 44 minutes, about 20 times longer than observed for the bacterial biosynthetic thiolase (Thompson et al. 1989).

It is reported that 3-pentynoyl- and 3-butynoyl-CoA are irreversible inhibitors of this enzyme from a pig heart (Holland et al. 1973) and that 3-octynoyl-CoA irreversibly inhibits the activity of the rat enzyme (Wu et al. 2007). An isomerase reaction of this enzyme has been observed, it involves the substrates 3-alkenoyl-CoAs that are converted to 2-alkenoyl-CoAs by the active site base, Cys382 in a rat (Wu et al. 2007). It is also reported that mitochondrial 3-ketoacyl-CoA can metabolize 3-oxovalproyl-CoA into propionyl-CoA and pentanoyl-CoA, a substrate that was previously thought to be accumulated in mitochondria. (Silva et al. 2002.)

At least one patient with a partial deficiency of the mitochondrial 3-ketoacyl-CoA thiolase has been described (Kamijo et al. 1997). The patient died at the age of 13 days after several days of vomiting, dehydration and liver dysfunction. At the end he developed rhabdomyolysis with myoglobinurea. T1 might also be important in the ketone body synthesis, in particular in the case of T2 deficiency. T1 is widely distributed in different tissues in humans and it is especially expressed in the liver, heart, kidney and adrenal glands (Fukao 2002).

2.4.7 Mitochondrial trifunctional enzyme (TFE)

A mitochondrial trifunctional protein (TFE) bound to the inner membrane of mitochondria (Uchida et al. 1992) is formed altogether from eight subunits comprising: 4 α-subunits consisting of both long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and trans-2,3-long-chain enoyl-CoA hydratase activity, and of 4 β-subunits having 3-ketoacyl-CoA thiolase activity (Kamijo et al. 1994a). The molecular weight of the recombinant rat enzyme implies, however, that the protein complex is α2β2 (Liu et al. 2008). This might be an artifact caused by the expression host, Escherichia coli (E. coli). The enzyme has a preference towards the long-chain acyl-CoA substrates (Uchida et al. 1992) and is localized to the membrane via α-subunit (Weinberger et al. 1995). The thiolase β-subunit of TFE has CHH motif as a catalytic triad (Liu et al. 2008).

A TFE deficiency is an autosomal recessive disorder of fatty acid β-oxidation, quite important in Finland (Tyni & Pihko 1999). Mutations in the gene encoding
the protein cause symptoms from mild to severe and there is a great variability in
the clinical course of the patients. Some mutations lead only to LCHAD
deficiency, but combinatorial deficiencies have also been described (Jackson et al.
the ß-subunit cause its rapid breakdown and consequently, lability of the α-subunit, because the formation of the multienzyme complex is disturbed (Kamijo
The ß-subunit is a target for anti-anginal drug, trimetazidine (Kantor et al. 2000,
Liu et al. 2008). This drug is an irreversible inhibitor, binding to the Cys105 of
the TFE thiolase, and commonly used in the treatment of heart diseases (Liu et al.
2008): it blocks oxygen dependent fatty acid degradation and switches the cell to
an anaerobic metabolism.

The mitochondrial trifunctional protein catalyzes the three key steps of the
mitochondrial β-oxidation of long-chain fatty acids. It has been shown that
activities of the individual enzymes from this complex, increase when the
complex is formed, which is probably due to the conformational changes (Liu et
al. 2008).

A similar multienzyme complex is also found in bacteria, E. coli. It consists
of 2 α-subunits, carrying activities of enoyl-CoA, hydratase (crotonase) and 3-
hydroxyacyl-coenzyme A dehydrogenase, with a molecular weight of 78 kDa, and
of 2 ß-subunits, which display the activity of 3-ketoacyl-coenzyme A thiolase
with a molecular weight of 42 kDa per subunit (Binstock et al. 1977, Pawar &
Schulz 1981). Thus the bacterial counterpart of the multienzyme complex has an
α2ß2-structure (Binstock et al. 1977).

The structure of the bacterial Pseudomonas fragi trifunctional enzyme has
been solved (Ishikawa et al. 2004). The structure provides a good insight into the
mechanism of substrate channeling between the active sites of the enzymes. It
seems that the enoyl-CoA hydratase and 3-hydroxyacyl-coenzyme A
dehydrogenase utilize a substrate-anchored diffusion mechanism, since they share
the same binding site for the adenine part of the CoA. The active site of the
thiolase is further away and it has its own binding site for CoA-moiety, implying
that different mechanisms are utilized for a substrate transfer, probably requiring
changes in the quaternary structure. In fact, two different crystal structures reveal
a movement between the subunits of the enzyme complex that might be the basis
for the substrate channeling. (Ishikawa et al. 2004.) The complex formation
seems to be a requirement for the thiolase activity to appear (Spratt et al. 1984).
3 Outlines of the present study

The thiolase enzymes are important for the lipid metabolism in the cell. In humans there exist at least six different isozymes of thiolase proteins. These differ in their localization, function and size. The most active thiolase characterized so far is from the bacterium \textit{Z. ramigera}. This bacterial enzyme has some characteristic properties, like a water trail, which is also found in the human cytosolic thiolase, based on the structures in a database to date.

This study for doctoral thesis was aimed at:

1. Characterization of the structure and kinetic properties of human mitochondrial acetoacetyl-CoA thiolase (T2) (I)
2. Detailed analysis of the factors influencing the binding mode of CoA to the wild type \textit{Z. ramigera} thiolase (II) and to
3. Evaluate the role of active site waters and the catalytic residues Asn316 and His348 in catalysis by \textit{Z. ramigera} thiolase (III)
4 Materials and methods

The materials and methods used in this study are described shortly in the following sections. More detailed information is provided in the original articles (I-III).

4.1 Molecular biology

4.1.1 Preparation of plasmid pET-3d::T2

The shuttle vector pCAGGS (Niwa et al. 1991) containing cDNA coding for T2 (Fukao et al. 1990) was used as a template for polymerase chain reaction (PCR). The T2 coding DNA was amplified by the PCR method using following forward and reverse primers (Sigma): 5'-cacttcc atg GcA TCA AAA CCC ACT TTG AAG GGA G-3' and 5'-catctggatcc tca CTA CAG CTT CTG AAT TAG CAT GG-3', respectively. Underlined sequences encode for NcoI-site in forward and BamHI-site in reverse primer. The underscored sequences present mismatches with T2 sequence and the codons are typed with space in between.

PCR products were isolated from agarose gel and subcloned into pMOSBlue vector (GE Healthcare). The sequence of the insert was confirmed by sequencing. The insert of 1209 bp of length was released by restriction digestion with NcoI and BamHI and subcloned into similarly digested pET-3d expression vector (Novagen) to yield a plasmid pET-3d::T2. The more comprehensive protocol is given in the original article (I).

4.1.2 Site-directed mutagenesis of bacterial thiolase

The plasmid encoding for wild type Z. ramigera thiolase (Modis & Wierenga 1999) was used as a template in the reaction for site-directed mutagenesis. The mutagenesis reaction was performed by using the QuickChange site-directed mutagenesis kit (Stratagene) and the presence of the desired mutation was confirmed by sequencing. (III). All the created mutations are listed in the Table I.
Table 2. List of all the mutated variants created for this work.

<table>
<thead>
<tr>
<th>Created mutation and its location in the structure</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active site</strong></td>
<td></td>
</tr>
<tr>
<td>H348A</td>
<td>Low activity</td>
</tr>
<tr>
<td>H348N</td>
<td>Low activity especially to the synthetic direction</td>
</tr>
<tr>
<td>N316A</td>
<td>Low activity especially to the synthetic direction</td>
</tr>
<tr>
<td>N316D</td>
<td>Inactive</td>
</tr>
<tr>
<td>N316H</td>
<td>Low activity to the synthetic direction, high activity to the thiolytic direction</td>
</tr>
<tr>
<td>N316H-H348N</td>
<td>Rescues some of the activity compared to the single mutated variants</td>
</tr>
<tr>
<td>N316L</td>
<td>Problems in purification</td>
</tr>
<tr>
<td><strong>Water channel</strong></td>
<td></td>
</tr>
<tr>
<td>A315S</td>
<td>Active. Purified and crystallized.</td>
</tr>
<tr>
<td>A315V</td>
<td>Problems in purification</td>
</tr>
<tr>
<td>A315I</td>
<td>Problems in purification</td>
</tr>
<tr>
<td>K185A</td>
<td>Active in thiolytic direction</td>
</tr>
<tr>
<td>E317Q</td>
<td>Increased stability</td>
</tr>
<tr>
<td><strong>Interdimer space</strong></td>
<td></td>
</tr>
<tr>
<td>K133A</td>
<td></td>
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<tr>
<td>K208A</td>
<td></td>
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<tr>
<td>R207A-K208A</td>
<td></td>
</tr>
</tbody>
</table>

4.2 Protein expression and purification

4.2.1 Production and purification of recombinant human T2

The expression vector pET3d::T2 was transformed into *E. coli* BL21(DE3)pLysS-strain and cultured in 50 ml of Luria Broth (LB) media supplemented with antibiotics at 37 °C overnight. 10 ml of this culture was used to inoculate 1 liter of M9ZB media. The expression of T2 was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Induction took place at 37 °C for 3 hours, after which the cells were harvested washed and stored at -70 °C.

The protein was purified by cation exchange chromatography (CM Sepharose, Amersham Biosciences) hydrophobic interaction chromatography (Resource ISO, Amersham Biosciences) and gel filtration chromatography (Superdex 200 HR 10/30, Amersham Pharmacia Biotech). The protein
concentration was measured by Bio-Rad protein assay with bovine serum albumin as a standard. Aliquots of 50 µl of the enzyme were frozen in liquid nitrogen and stored at -70 °C for future experiments. More detailed expression and purification protocols are available in the original article (I).

4.2.2 Production and purification of wild type and variants of Z. ramigera biosynthetic thiolase (II, III)

Wild type and the C89A variant of thiolase were expressed and purified as previously described (Modis & Wierenga 1999, Kursula et al. 2002). The variants H348A, H348N, N316A, N316D, N316H and N316H-H348N were treated similarly, except that the induction was performed at 20 °C for overnight. The yields of the mutated variants were much lower than for the wild type protein typically 10–50%. Enzymes were stored at -20 °C with 50% glycerol 1 mM EDTA and 0.1% β-mercaptoethanol (v/v).

4.2.3 Production and purification of human heart recombinant short-chain-L-3-hydroxyacyl-CoA dehydrogenase (SCHAD)

The plasmid pET-28a::SCHAD (Barycki et al. 2000) was introduced into E. coli BL21(DE3)pLysS -strain. This plasmid was a kindly donated by Prof. Banaszak (Minnesota, USA). The cells were induced at 30 °C for 4 hours in the presence of 0.4 mM IPTG. After harvesting and freezing, cells were suspended into lysis buffer containing 50 mM sodium phosphate; pH 8.0 and 50 mM NaCl. Protein was purified as described earlier (Barycki et al. 1999). (I.) In the original article III, the induction was performed as described earlier (Barycki et al. 1999). Otherwise the protocol was same as in the original article I. The protein was stored unconcentrated at 4 °C for several months and its activity was remeasured every time before the using it in the thiolase activity measurements.

4.3 Enzyme activity measurements

4.3.1 Thiolase activity measurements

Thiolase activity measurements for degradation of AcAc-CoA were performed using the Mg²⁺ method described previously (Middleton 1973), with some
modifications. Routine activity measurements with T2 were made in the presence of 40 mM KCl. Exact protocols is given in the original articles (I, III).

Degradation of 2-methylacetoacetyl-CoA by T2 was determined by following the formation of new thioester bond at 232 nm (Antonenkov et al. 1999a) at 25 C. Products, Ac-CoA and propionyl-CoA were identified by mass spectrometry. Detailed information is available in the original article (I).

The synthetic reaction of thiolase, the formation of acetoacetyl-CoA from Ac-CoA, was measured by using the linked assay (Thompson et al. 1989) with the human SCHAD (Barycki et al. 2000) as the linker enzyme by minor modifications (I, III).

For all measurements, two control reactions were performed, one without the enzyme and the other without the substrate. Concentrations of the ligands were measured by Ellman’s test (Riddles et al. 1983) and the protein concentrations were measured at 280 nm by using the NanoDrop spectrophotometer (Thermo Scientific).

4.3.2 Activity measurements of SCHAD

Activity of SCHAD was measured by following the disappearance of acetoacetyl-CoA by using the Mg2+ -method (Middleton 1973). The protocol is described in detail in the original article (I). One unit of SCHAD was determined to be the amount of enzyme that converts 1 µmol of acetoacetyl-CoA into L-3-hydroxybutanoyl-CoA in one minute.

4.4 Chemical synthesis

4.4.1 Synthesis of 2-methylacetoacetyl-CoA

2-methylacetoacetyl-CoA was synthesized from 2-methylacetoacetate to yield 2-methylacetoacetyl chloride. This compound was used to acylate CoA. Synthesized 2-methylacetoacetyl-CoA was purified with Silica RP C18 column (32-62 µm, 60 Å) and fractions were analyzed with thin layer chromatography. Fractions containing the 2-methylacetoacetyl-CoA were pooled and analyzed by MALDI-TOF mass spectrometry (Voyager-DE STR Biospectrometry Workstation, Applied Biosystems). The dried compound was stored at -20 C. More exact protocol is written in the original article (I).
4.4.2 Synthesis of O- and S-pantetheine-11-pivalate (OPP and SPP) and acetyl-OPP (Ac-OPP)

A detailed description of the chemical synthesis of OPP, SPP and Ac-OPP is given in original article (II). All the intermediates and products of the synthesis were checked by thin layer chromatography for their purity. Identity of the compounds was verified by using ¹H-NMR.

4.5 Protein crystallization

4.5.1 Crystallization of T2

Before the crystallization screens were set up, the T2 sample was diluted into concentration of 5 mg/ml by water. Drops were prepared by mixing 1.5 µl of protein solution with 1.5 µl of well solution [100 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.5) and 17–19% (w/v) polyethylene glycol 5000 monomethyl ether (PEG-5000-MME)]. Crystals appeared by using hanging drop vapor diffusion method, after 10 days of incubation at 4 C. Crystals with CoA were obtained in the similar manner, only 2 mM CoA was included in the T2 protein solution. More detailed information from the crystallization experiments is available in the original article (I).

4.5.2 Crystallization of Z. ramigera biosynthetic thiolase and its variants

Wild type (II)

Wild type Z. ramigera biosynthetic thiolase was crystallized at 22 °C using the vapour diffusion method. For wild type the mother liquor contained 1.0 M Li₂SO₄, 0.9 M (NH₄)₂SO₄, 0.1 M sodium citrate; pH 5.0, 1 mM EDTA, 1 mM NaN₃ and 1 mM dithiothreitol (DTT) (II).

C89A (II)

The crystallization of C89A was performed as described for the wild type protein.
**H348A variant (III)**

Crystals of H348A variant were obtained from microseeding experiments. At first, hanging drops were prepared by mixing 2 µl of protein solution (1.5 mg/ml) with 2 µl well solution [0.95 M Li₂SO₄, 1.2 M (NH₄)₂SO₄, 0.1 M sodium citrate; pH 5.5, 1 mM EDTA, 1 mM NaN₃, 1 mM DTT]. Small crystals were obtained after two days equilibration at 22 °C. Obtained crystals were broken and used for microseeding. This time, 4 mM CoA was added into protein solution (1.5 mg/ml) and the well solution consisted from 0.95 M Li₂SO₄, 1.1 M (NH₄)₂SO₄, 0.1 M sodium citrate (pH 5.5), 1 mM EDTA, 1 mM NaN₃ and 1 mM DTT.

**H348N variant (III)**

H348N was cocrystallized with CoA by using the hanging drop vapour diffusion method. Protein solution (1.5 mg/ml) with 4 mM CoA was mixed (2+2 µl) with well solution [0.75 M Li₂SO₄, 1.7 M (NH₄)₂SO₄, 0.1 M sodium citrate; pH 5.5, 1 mM EDTA, 1 mM NaN₃ and 1 mM DTT], and equilibrated for two weeks at 22 °C.

**N316H-H348N variant (III)**

Crystallization of the double mutated variant N316H-H348N was performed at 22 °C by vapour diffusion method in hanging drops. Protein solution (2.5 mg/ml) was mixed (2+2 µl) with well solution containing 167 mM sodium citrate; pH 6.5 and 1.58 M (NH₄)₂SO₄, 1 mM EDTA, 1 mM NaN₃ and 1 mM DTT. No ligands were included in the buffers. First small crystals appeared in few days.

**N316A variant (III)**

N316A variant was cocrystallized at 22 °C with the same method as previously described for other variants, including the 4 mM CoA, with the exception that the protein concentration was 2 mg/ml and the well solution used contained 1.9 M (NH₄)₂SO₄, 0.1 M sodium citrate; pH 6.5, 1 mM EDTA, 1 mM NaN₃ and 1 mM DTT.
**N316D variant (III)**

N316D variant was cocrySTALLized at 22 °C by hanging drop vapor diffusion method in the presence of 4 mM CoA. Drops were set up by mixing 1 µl of protein solution (1 mg/ml) with 1 µl of well solution [1.9 M (NH₄)₂SO₄, 0.1 M MES; pH 5.7, 1 mM EDTA, 1 mM NaN₃ and 1 mM DTT].

**N316H variant (III)**

Crystals of N316H were obtained by mixing 2 µl of protein solution (2 mg/ml) with 2 µl of 1.9 M (NH₄)₂SO₄, 0.1 M MES; pH 5.7, 1 mM EDTA, 1 mM NaN₃ and 1 mM DTT, followed by equilibration in hanging drops at 22 °C. No ligands were included in the experiments yielding the X-ray crystal structures.

4.6 Data collection and processing

4.6.1 T2

Data was collected for altogether 6 protein crystal structures from different soaking and crystallization experiments. These include apoT2 (T2 without any ligand), apoT2+KCl (T2 crystal soaked with 150 mM KCl), T2+CoA (T2 crystal grown with CoA), T2+CoA+KCl (T2 crystal soaked with CoA and 150mM KCl), T2+CoA+KNO₃ (T2 crystal soaked with CoA and 150 mM KNO₃) and an anomalous data set collected from apoT2+KCl crystal with higher wavelength (λ=1.4022 Å). 17.5% glycerol was used as a cryoprotectant. Data was collected at 100 K at various beam-lines at the European Synchrotron Radiation Facility (ESRF, Grenoble, France), MAX-lab (Lund, Sweden) and Deutsches Elektronen-Synchrotron (DESY, Hamburg, Germany).

Images were processed, and the intensities were scaled and merged using the XDS (Kabsch 1993, Kursula 2004). Program MOLREP (Vagin & Teplyakov 1997) was used for molecular replacement calculations for T2+CoA data set, by using human cytosolic thiolase as a model (PDB entry 1WL4). The molecular replacement solution was refined by rigid body refinement, simulated annealing and B-factor refinement by using CNS (Brünger 1998). The model was used in the side-chain docking step of WARPTRACE (Perrakis et al. 1999) using the human T2 sequence. Further refinement and model building were performed by using REFMAC5 (Murshudov et al. 1997) and O (Jones et al. 1991). The other
structures were obtained using the protein part of the T2+CoA structure as a starting model. Waters were added by using ARP/wARP (Perrakis et al. 1999).

An ion binding site near the adenine binding region was recognized from the electron density maps and with the help of $B$-values to be a potassium binding site. The structures without the soak in KCl or in KNO$_3$ had an ordered water molecule in the corresponding position. The other ion binding site found in the dimer interface was recognized to be a chloride ion by using the anomalous Bijvoet difference map and the ionic radius as judgments. More precise description about data collection and processing is available in the original article (I).

### 4.6.2 OPP, SPP and Ac-OPP with bacterial thiolase

OPP and Ac-OPP were soaked into thiolase crystals, both the wild type and C89A variant. These substrate analogs were dissolved (to concentration of 100 mM) into cryosolution containing 12% 2-methyl-2,4-pentanediol (MPD) and 12% glycerol in well solution. The crystals were soaked into this solution for approximately 1 minute. Soak with SPP was performed in a solution containing approximately 20% MPD and 20% SPP in the mother liquor. The length of the soak was 4 days. These soaking experiments are also described in the original article (II).

Data was collected at various beamlines at DESY (Hamburg, Germany), except the complex with Ac-OPP, which was collected on home source. Data processing was performed by using XDS (Kabsch 1993) and XDSi (Kursula 2004) and the refinement with REFMAC5 (Murshudov et al. 1997). Waters were added by using ARP/wARP (Perrakis et al. 1999) and model building was performed by using O (Jones et al. 1991) and COOT (Emsley & Cowtan 2004). More information is available in the original article (II).

### 4.6.3 Structures of the oxidized bacterial thiolase and C89A in complex with CoA

The soaking of the wild type thiolase crystal with 5mM $\beta$-hydroxybutyryl-CoA for 1 minute resulted in a structure of wild type thiolase with oxidized Cys89 and CoA bound in the active site. For structure of C89A with CoA, C89A crystal was soaked with 5 mM CoA. These experiments are described in the original article (II). The data collection, processing and model building were performed as described for OPP, SPP and Ac-OPP complexes in previous chapter.
4.6.4 Structures of the active site variants of bacterial thiolase (III)

The crystal structures of H348A, H348N, N316A, and N316D with CoA were obtained by soaking the crystals in the cryosolution containing 20–25% glycerol and 4 mM CoA in the constituents of mother liquor for 1 minute before freezing. Double mutant N316H-H348N and N316H were frozen in the cryosolution containing 25% glycerol in the mother liquor. N316H crystals grown with CoA were too fragile to be mounted and frozen.

Data for these structures were collected at ESRF (Grenoble, France) at the beamlines ID14-1 and ID14-2. Data was processed by using XDS (Kabsch 1993) and XDSi (Kursula 2004) software package, except for the double mutant N316H-H348N, iMOSFLM (Leslie 1992), and SCALA (Evans 2006) were used. Molecular replacement was made either by using MOLREP (Vagin & Teplyakov 1997) or PHASER (McCoy et al. 2007). Refinement was performed using REFMAC5 (Murshudov et al. 1997) and model building and addition of waters was made with COOT (Emsley & Cowtan 2004). Variants with single point mutation were further refined with PHENIX (Adams et al. 2002).

4.7 Isothermal titration calorimetry (ITC)

ITC experiments, to estimate the binding affinities of CoA to apoenzymes, were performed with VP-ITC Microcalorimeter (MicroCal, Northampton, MA, USA). The samples were prepared or dialyzed against 100 mM Tris-HCl; pH 7.5, 5% glycerol and 1 mM DTT. The protein concentration used was 50 µM, except for variant C89A, 100 µM concentrations were used. All the samples were degassed just before the experiment. Titration with 5 mM CoA solution was performed at 25 °C. The heat of dilution for 5 mM CoA solution was estimated from blank experiments. The injection peaks were integrated and fitted to appropriate binding models by using the ORIGIN software (MicroCal). The stoichiometry was set to be 1 per active site. The titration curve was fitted by the nonlinear least-square method and the thermodynamic parameters were determined. All the experiments were performed at least twice and the binding constants were derived from at least two independent measurements. This information is available in the original articles (II, III).

The affinity of Ac-CoA for the C89A variant was measured in the same way.
4.8 Circular dichroism (CD) spectroscopy

Circular dichroism spectroscopy measurements were performed, by using the Jasco J-715 spectropolarimeter, at two independent pHs. For measurements at pH 4.0 the protein was diluted into 10 mM Na-acetate; pH 4.0 into 0.1 mg/ml concentration, and at higher pH, 10 mM potassium-phosphate buffer, pH 7.4, was used. At both pHs the CD spectra was measured from 260 to 190 nm and the melting temperature was determined by heating the protein 1 °C/min from 20 °C to 90 °C and measuring simultaneously the CD-signal at 222 nm. (III.)
5 Results

My personal contribution for this thesis was the kinetic characterization of T2 and the purification of T2 for kinetic measurements in the original article I. For original article II I have purified the proteins, made the ITC measurements, crystallized and solved some structures, although these structures did not end up to the published article due to the low resolution of the obtained structures. For original article III, all the experiments were made by me, together with Visa Poikela, the student I have supervised.

5.1 Results from kinetic measurements

5.1.1 T2 (I)

The kinetics of T2 were measured for both thiolytic and biosynthetic directions. The thiolytic activity and its dependence on potassium concentration was assayed by varying the concentration of potassium and sodium from 0 to 120 mM in a way that the ionic salt concentration was kept constant (120 mM). The activity of T2 reached its maximum at 40 mM KCl and remained constant at higher concentrations. Therefore, all the subsequent measurements were made at 40 mM KCl or 40 mM NaCl.

Presence of KCl caused increase of about 3 times in $k_{cat}$ compared to the 40 mM NaCl control, for degradation of AcAc-CoA and 2-methyl-AcAc-CoA. Potassium had no effect on the $K_M$ of 2-methyl-AcAc-CoA, but instead it lowered the Km value for AcAc-CoA and CoA. As a result of this $k_{cat}/K_M$ values were approximately six times higher in the presence of potassium. The raise in the $K_M$ value without KCl was also observed when measurements were made into synthetic direction. However, the exact value was not measured since it would have required very high amounts of substrate. The degradation of 2-methyl-AcAc-CoA into propionyl-CoA and Ac-CoA was demonstrated by mass spectrometry for the first time. It is also notable that the complete racemic mixture of 2-methyl-AcAc-CoA was cleaved in the reaction.
5.1.2 Active site variants of bacterial thiolase (III)

Synthetic direction

The kinetics for the synthetic direction was measured only at low concentrations of the substrate to give an estimate of the catalytic efficiency $k_{cat}/K_M$ (Fig. 2 and Fig. 3). The variant N316D was found to be inactive, and the N316H-H348N double mutation was found to have the highest catalytic efficiency. The lowest measured catalytic efficiency was with H348A variant.

Thiolytic direction

All the active site variants N316A, N316D, N316H, N316H-H348N, H348A and H348N were used for kinetic measurements in the thiolytic direction. Evaluated on the basis of $K_M$, the affinities for AcAc-CoA are higher for each of the mutations than for the wild type enzyme. However, the catalytic efficiencies are clearly reduced in most of the variants, with the exception of N316H with a catalytic efficiency about half of the wild type enzyme. The lowest catalytic efficiency is in the variant H348A, whereas variant N316D had no activity at all.

5.2 Structures

5.2.1 T2 (I)

Six crystal structures of T2 were solved. The resolution limit varied from 1.85 to 2.05 Å. In all cases the protein crystallized in the same space group with one tetramer per asymmetric unit. Electron density is continuous for main chain atoms from Pro37 to Leu427 with some small exceptions (I). The subunit structures of the tetramer are essentially the same. The rmsd difference of the main chain ca atoms are 0.4 Å. Ramachandran statistics show that 93% of the residues are in the most favored region and none in the disallowed region.

The overall core of the subunit is a typical thiolase fold with an $\alpha\beta\alpha\beta\alpha$-layered structure. In human T2 the N-terminal domain is formed by residues Pro37-Ser155 together with Asn287-Leu309. The loop domain is formed by residues Met156-Leu286 and the C-terminal domain by residues Ala310-Leu427. The loop domain covers the N- and C-terminal domains and it has two protruding
loops; the tetramerization loop and a cationic loop pointing towards the substrate binding site of the opposing dimer.

**Binding site for potassium**

The binding site for potassium is located near the adenine binding pocket where it is coordinated by six oxygens; main chain oxygens of Ala280, Ala281, Ala283 and Val381 and side chain hydroxyl group of Tyr219 and a water molecule. The OH group of Tyr219 and a water molecule are also in contact with the adenine moiety of CoA. Binding of K+ does not induce conformational changes, but judging by the B-factor plots, it rigidifies its surroundings, including one of the catalytic residues, His385.

**Binding mode of CoA**

In CoA-complex structures the electron density is well defined for the substrate in all of the subunits. Binding of CoA does not induce any conformational changes to the protein structure. The bound CoA has a characteristic bend at the pyrophosphate moiety. The binding site of the adenine ring is aligned by side chains of Tyr219, Arg258, Val259, Asp260, Lys263, Val264, Leu267, Ala280 and Ala281. Hydrogen bonding between the main chain and adenine loop involve the Arg258, Val259 and Asp260. In addition one hydrogen bond is observed with the side chain of Tyr219. One salt bridge between the 3'-phosphate of the ribose part and Lys263 was found.

The pantetheine part of CoA binds into a long (17 Å) and narrow tunnel aligned by the residues 179-194 and 280-285 of the loop domain. The active site is located at the bottom of this tunnel. In addition Ala355, Phe356 and Ile387 have interactions with the pantetheine moiety. An almost complete ring of hydrophobic side chains form the narrowest part of the tunnel. These residues are Ala283, Phe356, Met193, Leu184 and Leu286. The ring is completed by Ser284. Two hydrogen bonds are formed between the pantetheine part and the protein, for side chain and main chain oxygens of Ser284. There are also eight water-mediated hydrogen-bonds between the protein and pantetheine moiety. Several van der Waals interactions supplement the mentioned polar interaction between the pantetheine part and T2.
Binding site for chloride ion

The affinity of chloride ion to T2 is high because it is copurified. Ions are coordinated by three oxygens, from which two are from water molecules and the third is from O(Cys119) from the neighbouring dimer. The N(Cys119) and CA(Pro118) from the neighbouring subunit take also part of the coordination of the chloride ion. Surrounding atoms from the same subunit are ND2(Asn414), CA(Gly418), CE(Lys124). The chloride ion is under hydrogen bonding distance for the two water molecules (3.1 Å), and N(Cys119) (3.4 Å) and ND2(Asn414) (3.5 Å). Rest of the listed interactions are at the range of van der Waals contacts. The closest charged residue (4.2 Å) is positively charged Lys124 from the catalytic Nβ3-Nα3 loop. Because the Asn414 is part of the catalytic Cβ4-Cβ5 loop, and it is also located between the catalytic base Cys413 and the Gly415, components of the oxyanion hole 2, it is clear that the chloride ion has a stabilizing role for the active site loops.

Properties of the active site

Active site’s catalytic residues Cys126, Asn353, His385 and Cys413 protrude out from the four catalytic loops. Oxyanion hole 1 is formed by NE2(His353) and Wat98, whereas the oxyanion hole 2 is formed by N(Cys126) and N(Gly415). Two of the four active site waters of the apo structure, are kept in place when CoA is bound. These are Wat98 and Wat135 and they are weakly hydrogen bonded to each other. Binding of CoA causes only two structural changes in the structure, compared to the apoT2. Cys126 has two conformations in the apoT2 structure and it is not oxidized, whereas in the T2+CoA structure the conformation is fixed and near to the His385 side chain and it is oxidized to a sulfenic acid.

Comparison of the active site of T2 with CT showed some major differences. Firstly, the trail of waters from the active site to the bulk solvent is missing in T2. Instead the waters are replaced by hydrophobic residues; Trp350, Val352, Val360, Ile364 and Val374. Secondly, The Met293 in CT is replaced by Phe325 in T2, and the following residue, glycine in CT, is a proline in T2 and this results in a T2 active site that is a bit wider, than that in CT. All the other thiolase structures have also a Met-Gly in this position, and the change in the T2 structure is probably involved in the recognition of the 2-methyl-group of branched fatty acids, namely 2-methyl-AcAc-CoA.
Modelling of the binding of 2-methyl-AcAc-CoA indicates that the 2-methyl group indeed points to the Phe325 and the two oxygen atoms of the 2-methyl-AcAc moiety are positioned near the oxyanion holes 1 and 2. According to the docking experiment, both $S$ and $R$ isoforms of 2-methyl-AcAc-CoA are in van der Waals contacts with the Phe325.

### 5.2.2 Bacterial thiolase

**CoA binding mode to the wild type Z. ramigera thiolase (II)**

The binding tunnel for the pantetheine part of CoA is shaped by the following residues of the loop domain: Leu148, His156, Met157, Ala234, Phe235, Ala243, Ala246, Ser247, Gly248 and Leu249. In addition Ala318 and Phe319 of the C-terminal domain and Met134 from the neighboring subunit are surrounding the pantetheine moiety of CoA. Only two of these residues are hydrophilic, His156 and Ser247. Ser247 has two conformations; in apostructures it points away from the tunnel whereas in liganded structures it points into the tunnel to interact with the substrate. In addition to protein atoms, there are also water molecules in the pantetheine binding tunnel, both in liganded and unliganded structures. One of these waters is conserved (Wat149). It is located almost at the bottom of the tunnel and it is hydrogen bonded to the O(His348) and O(Gly248). The hydrogen bonds between the CoA and protein include hydrogen bonding with OG(Ser247) and O(Ser247). In addition there exists a water mediated hydrogen bond between the CoA and His156.

The binding surroundings of terminal sulfur atom of CoA is particularly rich with sulfur containing residues, Cys89, Met157, Met288 and Cys378, and also Ala318 and Phe319. The structural water molecule, Wat82, and the catalytic His348 are also within 5 Å distance from the reactive sulfur atom. Water 82 is hydrogen bonded to Asn316 and to Wat49.

The binding mode of CoA is not affected by covalent modification of the catalytic Cys89, for example when acetylated to acetylcysteine or oxidized to sulfenic acid. The location of the sulfur atom of CoA is not changed even when the Ac-CoA is bound to acetylated enzyme.
**Binding mode of SPP (II)**

The structure of *Z. ramigera* thiolase was solved in complex with SPP, a functional analog of CoA. The binding mode of SPP is very similar to the binding mode of CoA, for example the distance between the catalytic Cys89 and the sulfur atom of CoA, differs only by 0.2 Å. This explains the reactivity of the substrate analog with the enzyme. The pivalate moiety points out from the pantetheine binding tunnel lying on the hydrophobic surface formed by Leu249 and Phe18 and Met134 from the neighboring subunit. In the pantetheine binding tunnel, Ser247 has adopted the same conformation as in the apostructure: in other words, it has turned away from the tunnel.

**Binding mode of OPP (II)**

The structures of *Z. ramigera* thiolase were also solved in complex with OPP and Ac-OPP. The structure with OPP was refined to 1.51 Å revealing that the binding mode of OPP was different from SPP and CoA. The terminal hydroxyl group of OPP was 5.2 Å from Cys89 and even more further away from the other sulfur-containing residues in the active site (7.4, 6.2 and 7.6 Å for Met288, Cys378 and Met 157, respectively). Instead, it is now located between the NE2(His348) and O(Ala318) and hydrogen bonded to these atoms. The only hydrogen bond between the protein and OPP, is that with O(Ser247), because also the pantetheine moiety has moved in the tunnel, compared to the SPP and CoA. The t-butyryl group of the OPP is bound to a hydrophobic pocket not observed in other structures. This is formed by a rotation of His156 around the X1 axis and by Met156, Ile144, Leu148, Ala234, Phe 235, Leu249 and from the neighboring subunit Met134. Ile144 and Met134 are in different conformations compared to other thiolase structures. On top of all of this, the whole loop with residues 231-240 moves 1 Å in the presence of OPP and its B-factors indicate a more rigid structure for these residues when OPP is bound.

The structure of the complex with Ac-OPP showed the similar way of binding, to OPP. Due to low occupancy of the ligand, detailed analysis has not been made, but it is clear from the electron density that the binding mode differs from that of Ac-CoA.
**Binding mode of CoA in C89A variant of bacterial thiolase (II)**

The structure of C89A variant of bacterial thiolase was solved in complex with CoA to further investigate the importance of sulfur-sulfur interactions in the active site. Apart from the terminal part of CoA, the binding mode is the same as in the wild type. The terminal sulfur atom has protruded further into the active site by 1.7 Å and is buried between the catalytic residues Cys378 and His348. This displacement of CoA in the C89A variant highlights the importance of the sulfur atom of Cys89 for correct positioning of CoA sulfur atom. The distances for other sulfur atoms near the active site have also changed.

**Structural properties of active site variants N316A, N316D, N316H, N316H-H348N, H348A and H348N (III)**

The comparisons of these active site variant structures were made against the B subunit of 1DLV, for the complex structure with CoA, and against 1DLU, for the unliganded structure. Since the electron density maps are of lower quality in subunits C and D, a feature typical for the crystal packing of this protein, subunits A and B are used for structural analysis.

In the structure of N316A variant in complex with CoA, which was solved to 2.0 Å resolution, the CoA is bound 0.5 Å deeper as in the wild type thiolase and the two active site waters are present at high occupancy. An extra water molecule is located in the space created by the absence of OD(Asn316) and the side chain of His348 has rotated by 50°. It has moved towards the new water, and hydrogen bonded, from ND1 atom, to it. There are no changes in the main chain caused by the mutation. The side chain of Ser353 has changed its conformation and the hydrogen bond between the His348 and Ser353 is lost. The catalytic Cys89 is oxidized to a sulfenic acid and and this oxygen atom is sited in oxyanion hole 2. Wat82 is missing one important stabilizing interaction, which is with ND2(Asn316) in the wild type bacterial thiolase.

In 2.5 Å structure of the N316D in complex with CoA, the hydrogen bonding of ND1(His348) to OG(Ser353) is also lost. Wat49 is still present, but Wat 82 is absent. The main chain and side chain of Asp316 has shifted slightly away from His348. The side chain of His348 has rotated by approximately 90°. The catalytic cysteines Cys89 and Cys378 are not oxidized. One water molecule is bound in the oxyanion hole 2 and the CoA is bound about 1 Å deeper into the active site than
in the wild type enzyme. The OD2(Asp316) is at hydrogen bonding distance for Wat49 of the active site and also for the NE2(His348).

The unliganded structure of N316H variant was solved to 2.3 Å resolution. The active site water 82 is not present in this structure, it is replaced by NE2(His316). The bulky side chain of the histidine has also pushed water 49 further away. One extra water molecule is present in the oxyanion hole 1. This water is hydrogen bonded to NE2(His348), nicely demonstrating the formation of the oxyanion hole by the two histidines in the active site. Another new water is replacing the OD1(Asn316). His348 has moved and rotated slightly. The hydrogen bonding between ND1(His348) and OG(Ser353) is intact.

The structure of double mutated bacterial thiolase, N316H-H348N, was solved to the 2.98 Å resolution. At this resolution the waters are not well defined. Only small changes in the main and side chain structures are seen.

H348A structure was solved to a resolution 1.8 Å. In this variant the binding mode of CoA has changed, it is 1.6 Å deeper in the active site. Cys378 is oxidized to cysteine-S-dioxide and Cys89 to sulfenic acid in the subunit B. There are no changes in the main chain structures of Ala348 and Asn316. The side chain of Asn316 has moved towards Cys378. Both active site waters Wat82 and Wat49 are present, but Wat49 with weak density. There is one extra water molecule in the oxyanion hole 2, and some extra water molecules fill up the space created by the histidine to alanine mutation.

The 1.8 Å structure of H348N has no changes in the main chain structure. The CoA is bound as in 1DLV and the position of Asn316 is unchanged. This Asn316 is hydrogen bonded to the Asn348, which has moved towards Asn316 compared to the His348. Asn348 is hydrogen bonded to Ser353, which has again changed its position. Cys89 is oxidized in both subunits and the oxygen is pointing to the Asn348 and hydrogen bonded to Wat82. Cys378 seems to be partially oxidized. There is one water molecule in the oxyanion hole 2 in both subunits.

5.3 Results from ITC (II, III)

The wild type and active site variants of bacterial thiolase were all titrated with CoA and analyzed by isothermal titration calorimetry. The results from these measurements are listed in the Table 2. Variant C89A has the most striking differences in the thermodynamic parameters compared to the wild type. Other active site mutations did not have such a big difference, except the common
nominators of the H348N and N316A-H348N variants; they both have higher affinity towards the CoA and the asparagines at the position of the catalytic histidine. The inactive variant N316D had somewhat lower affinity towards CoA.

Table 3. Results from isothermal titration calorimetry. Active site variants and the wild type protein were titrated at 25 °C with 5 mM CoA (and Ac-CoA for C89A variant).

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_d$ for CoA (µM)</th>
<th>$K_d$ for Ac-CoA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>81 ± 29</td>
<td></td>
</tr>
<tr>
<td>C89A</td>
<td>307 ± 64</td>
<td>496 ± 33</td>
</tr>
<tr>
<td>H348A</td>
<td>128 ± 9</td>
<td></td>
</tr>
<tr>
<td>H348N</td>
<td>49 ± 3</td>
<td></td>
</tr>
<tr>
<td>N316A</td>
<td>85 ± 5</td>
<td></td>
</tr>
<tr>
<td>N316D</td>
<td>64 ± 4</td>
<td></td>
</tr>
<tr>
<td>N316H</td>
<td>30 ± 2</td>
<td></td>
</tr>
<tr>
<td>N316H-H348N</td>
<td>32 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

5.4 Results from circular dichroism (CD) spectroscopy (III)

The active site variants H348A, H348N, N316A, N316H-H348N and the wild type protein were analysed by CD spectroscopy. The secondary structure was shown to be unchanged and the stability of the variants was found to be similar to wild type at pH 7.4, as suspected by the melting point analysis. The stability of the variants was not as good as the wild type at the acidic pH (pH 4.0), except for the double mutated variant N316H-H348N. The analysis of the melting point of the E317Q variant at pH 4.0 revealed better stability than for wild type protein.
6 Discussion

6.1 T2

Kinetic measurements indicated that T2 reached its full activity when the concentration of potassium was 40 mM and remained constant even when the concentration of potassium was tripled from this. The $k_{cat}$ values measured in this study for degradation of AcAc-CoA and 2-methyl-AcAc-CoA were three times higher with potassium than with sodium ions. In the thiolytic reaction, the $K_M$ values were affected for AcAc-CoA and CoA, implicating higher affinity with potassium, but the same was not observed for 2-methyl-AcAc-CoA. The change in the $K_M$ value was also observed when the reaction was measured in the synthetic direction with and without potassium.

Catalytic efficiencies ($k_{cat}/K_M$) were about six times higher in the presence of potassium than without it, both for degradation of AcAc-CoA and 2-methyl-AcAc-CoA. Based on the $K_M$ values the human T2 has very high affinity towards AcAc-CoA and lower for CoA, as in the rat enzyme (Middleton 1973). This result has been later confirmed by Santhanam et al. 2007, measured from the human mucosal homogenates, supporting our results with recombinant enzyme. The degradation of 2-methyl-AcAc-CoA into acetyl-CoA and propionyl-CoA was confirmed by mass spectroscopy.

The overall structure of human T2 is the typical thiolase fold with an $\alpha\beta\alpha\beta\alpha$-bilayer. The binding of CoA does not cause structural changes. In comparison with the structure of human CT, the most significant difference is found in the loop domain. In T2 the loop section shaping the adenine binding site is two residues shorter, and the substrate is bound more tightly. Other sections of the loop domain have also some differences with these two enzymes. For example, the tetramerization loop is one residue shorter and the cationic loop one residue longer in T2 than in CT. In the cationic loop of T2, the amount of cationic residues is less than in CT, but instead it has more cationic residues near the CoA binding pocket. The cationic loop is thought to be important for efficient capture of the substrate and the arrangement seen in T2 is probably serving for the same purpose.

The potassium ion binding site is characteristically coordinated by six oxygen atoms (Hording 2002), namely O(Ala280), O(Ala281), O(Ala283), O(Val381), a water molecule and the hydroxyl oxygen of Tyr219. The coordinating water and
the OH group Tyr219 both interact directly with the adenine moiety of CoA. Even though the potassium does not cause any conformational changes to the protein structure, it rigidifies the region Thr279-Thr285, involved in the binding of the pantetheine moiety, and the region near Val381, including the catalytic His385, based on the analysis of the B-values plots. This information, taken together with the kinetic data, nicely explains the tighter binding of the AcAc-CoA and CoA to the protein in the presence of potassium. It also correlates with higher turnover numbers by stabilization of the active site geometry. In the mitochondria, the potassium concentration may act as a regulator of the citric acid cycle, since also an enzyme in the glycolytic pathway, producing Ac-CoA, is dependent on potassium.

Two of the active site loops are stabilized by a tightly bound chloride ion. This site is occupied by Met388 in human CT. In T2 the corresponding amino acid is Gly418. This is a unique feature of the T2 thiolases. The chloride ion has the same van der Waals interactions with the main chain atoms, as sulphur of the methionine in CT.

Active sites of CT and T2 are very similar. The main difference is the presence of Phe325 instead of the methionine residue in CT and bacterial thiolase. Replacement of the amino acid in this position creates more space in the active site of T2 thus permitting 2-methyl-AcAc-CoA to bind into the active site. The binding of the methyl group into this cavity was confirmed by the modelling experiment. Binding of this molecule is very tight, and the terminal group of CoA probably controls its affinity to the protein, explaining why potassium did not have the effect on the $K_M$ value of 2-methyl-AcAc-CoA as it was found for Ac-CoA and CoA. It is not possible to determine the chiral specificity based on the docking experiments, although they do indicate that both the S- and R-isofoms could be located into the active site. The mass spectrum revealed that the whole racemic mixture of 2-methyl-AcAc-CoA was degraded, indicating that the enzyme could catalyze the reaction with both S- and R-isofoms. However, at higher pHs the isomers could rapidly interconvert into each other (Middleton & Bartlett 1983).

6.2 Bacterial biosynthetic thiolase

The crystal structure of the bacterial biosynthetic thiolase in complex with SPP showed that the binding mode of SPP is similar to CoA. The exact binding mode of the ligand is critical for the catalysis and indeed the SPP and its thioesters have
been reported to be functional substrates for this thiolase (Davis et al. 1987). The unreactiveness of OPP and its oxoesters has been thought to be due to the higher pKₐ value of methyl hydrogens. The 1.5 Å crystal structure of OPP with bacterial thiolase reveals that the binding mode of OPP is unproductive. The oxygen atom of the OPP is hydrogen bonded to NE2(His348) and O(Ala318) and it is buried between these atoms. Binding of OPP also forces the entire loop of residues 231-240 to move by 1 Å. The B-values of these residues show that the loop is more rigid than in the complex with SPP or CoA. It is fair to say that the different binding mode of OPP causes changes also in the protein itself, which may explain the low reactivity of the enzyme with oxo esters. The same binding mode and the changes in the protein are seen also in the complex with Ac-OPP. It was shown, by solving the structure of bacterial thiolase with oxidized Cys89 in complex with CoA that oxidation, like acetylation, have no effect on the binding mode of CoA. This is actually true in all catalytic steps of the reaction.

The binding mode of CoA was changed, however, when the catalytic Cys89 was mutated into an alanine. Apparently the sulphur atom of the cysteine is critical for the coordination of the sulphur of CoA, since the CoA extended deeper into the active site pocket, by 1.7 Å. The calorimetric analyses of wild type and C89A with CoA showed that not only was the binding mode of CoA changed, but the mutation also affected its affinity for CoA. The change in ΔG between the wild type and C89A protein was small, but the enthalpy term driving the interaction was clearly greater in the wild type reaction. The similarity in ΔG is explained by the entropy term. Positioning of the reactive part of CoA is not as well coordinated as in the wild type enzyme, causing a change in the entropy term, with a significant effect on ΔG.

In the condensation reaction the rate limiting step is the formation of the C-C bond, the Claisen condensation. In wild type protein, the negatively charged enolate oxygen, formed in the proton abstraction step of the condensation reaction, is stabilized by the oxyanion hole 1, formed by Wat82 and His348. The Wat82 is hydrogen bonded to Asn316. To test the importance of the oxyanion hole 1 for the catalytic activity of the bacterial thiolase, the amino acids His348 and Asn316 were mutated. Altogether six different mutants of the protein were created; H348A, H348N, N316A, N316D, N316H and N316H-H348N and tested for their activity and binding properties and their X-ray crystal structures were solved.

Of all the variants created, N316D had the lowest activity; in fact, the activity was not measurable under the reaction conditions tested. All the other variants...
had also very much lowered catalytic efficiencies for the synthetic direction of the reaction. For this direction the $k_{cat}/K_M$ value is most affected for H348A (reduced by 1200 times) and less for the double variant N316H-H348N (reduced by factor of 30). When calculating the energy difference of the reactions compared to the wild type (Fersht 1999), the difference is between 3.4-4.3 kcal/mol for the variants containing only one mutation, and 2.1 kcal/mol for the doubly mutated variant. The energy difference in variants containing only one mutation is comparable to estimated energy for the stabilizing effect of one hydrogen bond in an oxyanion hole by quantum mechanical calculations (Hu et al. 1998) and for the presence of one unpaired charged hydrogen bond donor or acceptor in the active site (Fersht et al. 1985). Both kinetic and calorimetric data indicate that the affinity towards the substrate has not been changed remarkably.

For the thiolytic direction the mostly affected ones, apart from the inactive N316D, are those mutated by the His348. This is understandable, because this histidine is a catalytic residue required for activation of Cys89. In the thiolytic direction N316H still has a comparable activity to the native enzyme. This is interesting since in some thiolases, like in TFE, the Asn in this position is naturally a His residue, and the mutated variant probably reproduces this active site geometry. If this hypothesis is correct, one can propose that the thiolases having a histidine in this position are most likely to be degradative thiolases, as is true for TFE.

X-ray crystal structures of these variants reveal only minimal changes in the active site. The mutations have affected the orientation of the neighbouring residue slightly, except in H348N, where Asn316 is in the same position as in the wild type. The binding mode of CoA is changed the most in the H348A variant. In this variant also the Cys378 is oxidized, a feature not observed before.

Because in the His348 mutated variants the rate limiting step of the thiolytic reaction has most probably changed, the Asn316 variants are considered to be more informative in understanding the mechanism of the catalysis. It is clear that the mutations presented do not affect the binding abilities of the enzyme, and that the step affected the most is the Claisen condensation step for the synthetic direction of the reaction. When comparing the competent active sites for the synthetic and thiolytic reaction and the hydrogen bonding schemes (III) in these steps of the reaction, it is apparent that the mutations created at Asn316 have an effect on the oxyanion hole 1 formed by Wat82 and His348. Even though Wat82 is present in N316A structure, its environment has changed in such a way that the catalysis has become inefficient or even impossible. In N316H the Wat82 is
displaced by NE2(His316), which is a competent solution for the degradative reaction, but not so good for the synthetic direction. In N316A Wat82 lacks its stabilizing interaction with Asn316 and in N316D the negative charge of the carboxyl group makes Wat82, if present, to change its role from hydrogen bond acceptor (from Asn316) to a hydrogen bond donor (to Asp316). This, combined with the negative charge of the Asp in the active site, do not facilitate the reactions with negatively charged intermediates.

Altogether these results indicate that the oxyanion hole 1 is very important for an efficient Claisen condensation, but it is not critical for the thiolytic reaction. This study also emphasizes the importance of the correct electrostatics in the active site for the enzyme catalysis to happen. These results can be helpful for future studies in aimed at creating new enzymatic catalysts.
7 Conclusions

In this study, the structure-function relationship of thiolase enzymes has been studied in detail. The work has been focused on human mitochondrial thiolase, T2, and the bacterial biosynthetic thiolase from Z. ramigera. Both enzymes are tetramers and they share the thiolase fold. They are both classified to be biosynthetic thiolases, even though the thiolytic reaction is much more favourable.

The crystal structure of T2 was solved, and the position of the potassium ion was identified. In addition, two chloride ion binding sites were found near the dimer interface. The catalytic efficiency of T2 is higher in the presence of potassium. This is because some of the groups coordinating the potassium ion are also interacting with the adenine part of the CoA molecule and in the presence of potassium these groups have higher rigidity. This is reflected directly in the $K_M$ values of CoA and AcAc-CoA but not for 2-methyl-AcAc-CoA, in which case the fit of the terminal part of the substrate to the active site is tighter.

In bacterial thiolase the active site and its near surroundings are populated by sulphur atoms, mainly from cysteine and methionine residues, but also from the substrate, CoA, itself. Altogether 5 sulfur atoms are spatially close together. In every step of the reaction cycle, the sulphur of the CoA is at the same place, regardless of the fact if it has an acyl group attached to it. The adenine part of the CoA molecule is not needed for the reaction to proceed, since the CoA analog SPP is a functional substrate for this thiolase. However, OPP, a molecule otherwise the same as SPP, differing only by one atom, is not. In this study it was found out that both the sulphur of the CoA and the sulphur of the reactive cysteine, Cys89, are needed for the competent binding mode for CoA.

The third part of this study focused on studying the importance of the oxyanion hole 1 in the active site of bacterial biosynthetic thiolase. Several point mutations were made to study this issue and these mutated variants were carefully studied. It was found out that this oxyanion hole is important especially to the synthetic Claisen condensation reaction, but not so critical for the thiolytic reaction. It was also shown that the electrostatics of the active site have an enormous role in the efficient enzyme catalysis.
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