Doctoral Thesis

Expanding the chemistry of the nicotinamide adenine dinucleotides

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Expanding the chemistry of the nicotinamide adenine dinucleotides

A thesis submitted to attain the degree of DOCTOR OF SCIENCES of ETH ZURICH

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Äiti, olen kiitollinen että aina olit valmis auttamaan minua kuin tarvitsin apua ja kasvatuksesta. אב, אני מודה על עזרתך והסבלנות שלך לאורך הדרכה ולקודם זו.
Abstract

Enzymes make the chemistry of life possible by catalyzing reactions that would take longer than an organism’s lifetime to occur without them, in just milliseconds. To achieve these remarkable rate enhancements, enzymes arrange into three-dimensional folds that carefully place specific chemical entities in a way that stabilizes the highest energy state of the reaction, the transition state. The chemical spectrum of the twenty-two proteinogenic amino acids is sometimes too limited to catalyze all the reactions necessary for life. A common way to circumvent this limitation is the use of cofactors that can tremendously expand the chemical diversity of enzymes. This thesis concentrates on the reactivity of the most abundant redox cofactors of life, the nicotinamide adenine dinucleotides, abbreviated as NAD(P)H. There are several large enzymes families that use NAD(P)H as stoichiometric cofactors. This thesis focuses on two enzyme superfamilies and their reactivity in respect to NAD(P)H, the short and medium chain dehydrogenase (SDR and MDR) superfamilies. These superfamilies are present in all domains of life and catalyze essential redox chemistry. A detailed understanding of their chemistry is important for the design of new pharmaceuticals, biotechnological applications and the de novo design of oxidoreductases.

Crotonyl-CoA carboxylase/reductase (Ccr) is a recently discovered carboxylase with promising applications in synthetic CO₂ fixation pathways as well as extending the functional diversity of polyketides. To understand the unusual reaction catalyzed by Ccr we compare the reduction and carboxylation reactions of Ccr. We detect an intermediate that is a covalent adduct between the substrate, crotonyl-CoA and the cofactor, NADPH, in Chapter 2 the absence of CO₂. The intermediate can be isolated and serves as a good substrate for Etr1p and Yhdh, two proteins from different MDR subfamilies, indicating it might be much more widespread. This result challenges our current understanding of the chemistry of NAD(P)H dependent enzymes and makes a compelling argument that these metal independent MDRs react through an ene-mechanism and not through a direct hydride transfer.

The isolated intermediate represents a state halfway in between the substrates and the products, i.e. the hydride is already transferred but the proton is not. This property makes it a valuable tool to study the second half reaction, the
addition of an electrophile, like $\text{H}^+$ or $\text{CO}_2$. In Chapter 3 we use the intermediate as a molecular probe to show for the first time that a conserved active site tyrosine is the proton donor of mitochondrial enoyl-thioester reductase (ETR). With this knowledge and a phylogenetic analysis of ETRs from polyketide clusters, we engineered a new ETR variant with inverted stereochemistry, paving the way for controlling the stereochemistry of methyl groups in polyketides. In addition, another adduct is characterized in an active site mutant lacking the catalytic proton donor that has similar UV-vis characteristics as NAD(P)H. This observation serves as a warning to many biochemists in interpreting UV-vis data from mutagenesis studies on this class of enzymes and casts doubt about many past publications.

The results in Chapter 3 raise doubts about past mutagenesis studies on ETRs and possibly other enzymes. New tools are necessary to study active site mutants of ETRs because of the overlapping UV-vis spectra of NAD(P)H and the discovered adducts. Chapter 4 describes a new method to study kinetic isotope effects on protonation steps in ETRs and other enzymes. By comparing the incorporation of hydrogen versus deuterium into a non-exchangeable position in the reaction product, the kinetic isotope effect is calculated. This method circumvents the artefacts caused by the accumulation of UV-vis active intermediates that interfere with classical assays. The measured isotope effects give insight into the contributions of residues that line the active site of Etr1p. Lastly, the catalytic function of a conserved active site threonine, thought just to serve water binding in the apo form and found in all MDRs, is proposed. The results are extended to the SDR superfamily member InhA. InhA is an ETR from the SDR superfamily that is essential in the synthesis of mycolic acids that protect Mycobacterium tuberculosis against the host’s immune system. Therefore antibiotics that inhibit this enzyme are effective treatments against the deadliest infectious disease, tuberculosis. Mutating tyrosine 158, which was thought to be involved in electrophilic catalysis, leads to the accumulation of an analogous adduct as observed in Ccr and Etr1p. This result calls for a re-evaluation of the mechanism of InhA that is proposed based on UV-vis measurements that are faulty.

The last chapter presents a phylogenetic analysis to establish the relationship of Etr1p and Ccr. The different reactivities for of both enzymes are compared. The energetic barriers for both the formation of the intermediate that is possibly central to the reaction of both enzymes and the resolving step with a proton, in the case of Etr1p, and a $\text{CO}_2$, in the case of Ccr are presented. This study gives
a quantitative understanding of the two different reactions catalyzed by these enzymes and gives an evolutionary context to their relationship.

Taken together, this thesis provides a large array of different and complementary experiments that challenge our current views of the chemistry of NAD(P)H dependent enzymes. The presented results affect our understanding of the novel CO\textsubscript{2} fixing enzyme Ccr, the engineering of polyketide-based pharmaceuticals and the understanding of important antibiotic targets. Lastly, the presented results will help with future design efforts of new NAD(P)H dependent enzymes.
Zusammenfassung


Im letzten Kapitel wird der Verwandtschaftsgrad sowie die unterschiedliche Aktivität der beiden Enzyme Ccr und Etr1p anhand von phylogenetischen Analysen sowie der Bestimmung von Aktivierungsentenergien beschrieben. Der Reaktionsmechanismus der beiden Enzyme wird in zwei Schritte unterteilt und die Aktivierungsentenergien werden für a) die Bildung des Zwischenprodukts sowie b) für den Zerfall des Intermediates und der Addition des Elektrophils (H⁺ oder CO₂) bestimmt. Diese Resultate führen zu einem quantitativen Verständnis der beiden Reaktionen (i.e. Reduktion und reduktiver Carboxylierung) und stellt dieses in einen evolutionären Kontext.

Zusammengefasst liefert diese Arbeit mehrere unterschiedliche und komplementäre Experimente welche auf einen fundamental neuen Reaktionsmechanismus für NAD(P)H abhängige Enzyme hinweisen. Diese neuen Einsichten können Auswirkungen auf die Entwicklung neuer Carboxylasen zur Fixierung von CO₂, neuer Polyketid-basierender Medikamente, sowie neuer Inhibitoren für wichtige Antibiotokaziele haben.
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1 Introduction

1.1 Redox reactions: a foundation of life

Redox reactions form one of the foundations of life. Both energy generation as well as many biochemical transformations in cells rely on redox reactions that use redox cofactors. To generate energy, a terminal electron acceptor oxidizes nicotinamide dinucleotide (NADH) to generate a proton motive force that can be used to synthesize adenosine triphosphate (1). In biochemical transformations, redox cofactors expand the chemical repertoire of enzymes. The twenty canonical amino acids are very limited in their redox chemistry: only cysteine can undergo facile redox chemistry by forming disulfides (2, 3). Consequently, most biological redox processes depend on cofactors and occasionally on posttranslational modifications (4) with varying redox potentials to expand the redox chemistry of polypeptides, e.g. glutathione (5), flavins (6), nicotinamide adenine dinucleotides (7), and metal ions (8). As such, understanding how these redox cofactors function is key to understand the enzymes that utilize them and will enable future design efforts of reductases and dehydrogenases.

“What I cannot create, I do not understand” is a phrase attributed to Richard Feynman (9). We are in the exciting era in biochemistry where we begin to create enzymes de novo, demonstrating the progress made in understanding enzymes since J. B. Sumner demonstrated that enzymes were polypeptides in 1926 (10). State of the art designer enzymes can catalyze reactions not found in nature including a Diels-Alder cycloaddition (11), Kemp elimination (12) and multistep retroaldol transformations (13). However, the efficiencies of de novo designed enzymes (0.1-100 M⁻¹ s⁻¹) are low in comparison to natural enzymes (10⁶-10⁸ M⁻¹ s⁻¹) which are sometimes only limited by diffusion (14). The challenges in designing new protein catalysts come from our limited understanding of structure function relationships in proteins and the efficient computational sampling of protein sequence space (15). Modest progress has been made in designing enzymes with bound cofactors (16). Nevertheless, cofactors that are not enzyme bound adds an additional level of complexity to the design process, as they require the alignment of yet another molecule. Therefore, future design efforts depend on understanding Nature’s utilization of coenzyme-dependent catalysis that probably predates life itself.
1.2 The origin of the nicotinamide coenzymes

The origin of life remains elusive, but this has not discouraged many scientists from envisioning possible scenarios under which life on earth arose. In 1929, J. B. S. Haldane published a seminal paper on the origin of life setting forth the pivotal proposal of a prebiotic broth or ‘primordial soup’ (17). This theory, adhered to for many decades by the majority of the scientific community, proposes that life arose in a complex aqueous solution organic and inorganic matter by harvesting energy from a rearrangement of these organic molecules, i.e. by fermentation. This theory is currently losing favor for multiple reasons, with the main argument being that such a primordial soup would already be (almost) at chemical equilibrium and that no or very little energy can be gained from fermentation (18-22). A realistic assumption for the first lifeforms, independent of their origin and biochemistry, is that they were less energy-efficient than their modern relatives and therefore needed a more energy-rich source to sustain themselves. Hypotheses that are more recent therefore propose that life arose near so-called white smokers, hydrothermal vents, with continuously maintained chemical gradients of e.g. small molecules, gasses and pH (18, 21). The first reactions to sustain life in recent hypotheses are energy generation from reduction of CO$_2$ with H$_2$ and storing the released energy in the form of energy rich thioester bonds (23, 24). For this reason, Georg Fuchs suggested that the acetyl-CoA pathway, which utilizes exactly this chemistry, is as ancient as it gets in biochemistry (25). Independent of which sequence of events gave rise to the last universal common ancestor (LUCA) of all known life, all theories have in common that the first metabolic reactions were condensation and redox reactions.

Catalysts like metal ions or small molecules, acting on simple reaction cascades or ‘proto-metabolic networks’ could channel reaction fluxes to particular compounds rather than distributed arrays of compounds. Selection would favor “communities of molecules that collectively were best able to catalyze synthesis of their own constituents” (20). Communities of simple catalysts that were able to increase the concentrations of monomeric building blocks would favor the formation of oligomers e.g. of nucleotides. Such a system might then move towards a pool of RNA catalysts that produced the small molecules and cofactors needed to make the nucleotides necessary for their own replication Figure 1 (26). The addition of nicotinamide (ribonucleotide) to such a pool would
be particularly advantageous for the chemical repertoire of the system because of the absence of redox centers in the four RNA nucleotides (Yarus 2011). From such pools of small RNA catalysts, combinatorial oligomerization and selection for longer and more competent catalysts could give rise to ever more complex RNA catalysts. Once a pool of RNA molecules formed that could catalyze the synthesis of its own monomers and polymerize its constituents it would lie on the evolutionary path that led to the RNA world (27-29). An example of how NAD\(^+\) could expand the chemistry of nucleic acids was provided in a laboratory setting. A simple catalytic RNA molecule, a ribozyme, was selected that could, with addition of free NAD\(^+\), catalyze the oxidation of primary alcohols to their corresponding aldehydes (Tsukiji, Pattnaik et al. 2003).

The likelihood of NAD\(^+\) or a congener having arisen very early on in life is emphasized by the fact that nicotinic acid, a precursor of nicotinamide cofactors (see Figure 2), can be formed under prebiotic conditions (30, 31). Also sugar metabolites like dihydroxy acetone phosphate (DHAP) and aspartic acid can form nicotinic acid, a precursor of nicotinamide, in an abiotic fashion (32). DHAP should have been available if riboorganisms could synthesize their own ribose. Aspartic acid was necessarily present if RNA organisms made their own pyrimidines from orotic acid, as is the case in modern lifeforms (33). Ribozymes can catalyze the formation of pyrophosphate linkages in NAD\(^+\) (Huang, Bugg et al. 2000), adenine can be formed from cyanide and various other small molecules (34-36) and sugars can form from aldehydes (reviewed in (37)). These experiments show that a combination of abiotic reactions and very simple catalysts, thought to predate peptide catalysis, cover most of the reactions needed to synthesize NAD\(^+\) from small molecules. Further evidence that this abiotic pathway predated the modern biosynthetic pathways comes from phylogenetic analysis of the present NADH biosynthesis pathways. The most primitive pathway for the synthesis of quinolic acid is the DHAP/aspartic acid pathway (38) present in all kingdoms of life (32), bacteria, archea and eukarya (39). Taken together, it seems likely that nicotinamide or a close congener was already involved in catalyzing redox reactions before LUCA, and probably already at the onset of life itself.

The ubiquity of the pyridine nucleotides NADH and NADPH as hydride donors and acceptors in all domains of life, reflects their early emergence and their almost certain presence in LUCA (Benner, Ellington et al. 1989). The structure
of pyridine nucleotides has therefore probably stopped evolving while the protein catalysts that utilize them have evolved (White lli 1982). The enzymes utilizing pyridine nucleotides belong to the oldest known proteins (40). Over the course of evolution, these enzymes have evolved into diverse oxidoreductases distributed over diverse superfamilies that make up about one-sixth of all characterized enzyme classes known to date. Two of the largest enzyme families to utilize these coenzymes are the short- and medium-chain dehydrogenase reductase (SDR and MDR) superfamilies (41). These two enzyme superfamilies are present in all three domains of life and therefore probably emerged before LUCA diverged into the bacteria and archaea, emphasizing the importance of the reactions catalyzed by these oxidoreductases.

\[ \text{15.8 percent (930/5858 entries) of the Enzyme commission numbers listed in the Braunschweig Enzyme Database (BRENDA) see also Chapter XX and Rosenthal R.G. et al. 2014 Nat Chem Biol 10(1): 50-55.} \]
Figure 1. Hypothetical events that gave rise to the RNA world from which the last universal common ancestor arose. Simple monomers that are formed by abiotic reactions in e.g. thermal vents (monomer world) assemble to multimers (multimer world) that can catalyze simple chemical reactions. The increase of monomer concentrations by the catalytic properties of multimers favors further oligmerization (micro-RNA world) to give RNA-structures that are ever more complex (mini-RNA world), leading to the first self-replicating RNA (RNA world) (26).
Figure 2. Modern NAD$^+$ biosynthetic pathways. Three different biosynthetic pathways of the precursor quinolinate are known. a) The dihydroxyacetone phosphate/ aspartic acid pathway is the most primitive and present in all the domains of life (32). b) The tryptophan pathway is mostly eukaryotic (42) and depends on monoxygenases that could have only emerged after the great oxygenation event 2.5 billion years ago (43), only after the emergence of LUCA (44). c) The anoxic formyl-aspartate/acetyl CoA pathway is limited to certain Clostridium species (45), in line with its late emergence. Adapted from (32).
1.3 The SDR and MDR enzyme superfamilies

The short- and medium-chain dehydrogenase reductase (SDR and MDR) superfamilies are old enzyme families catalyzing various reduction and oxidation reactions in all domains of life. While both enzyme superfamilies appeared early in evolution, SDRs are thought to have appeared earlier than MDRs because of their more simple architecture and greater abundance and divergence (46). SDR genes are among the most abundant genes found in metagenomic studies e.g. (47) reflecting the diversity and ubiquity of these enzymes. With a third of the sequence entries of SDRs, the MDRs are slightly less well represented in the UniProt database but still among the most abundant oxidoreductases (48).

Several SDR and MDR enzymes are prevalent in humans, with an estimated 230 and 81 members, respectively. Some SDRs are targets of clinically relevant pharmaceuticals. Some SDRs like 5α-reductase acts as "enzymatic switches" that control the availability of hormones in the body (49). 5α-Reductase, is a membrane associated SDR that reduces testosterone into dihydrotestosterone. This hormone is a more potent agonist of the androgen receptor than testosterone and is responsible for transcriptional regulation of male sexual characteristics (50, 51). Finasterid, a 5α-reductase inhibitor, slows down the conversion of testosterone to dihydrotestosterone and thereby lessens secondary male sexual characteristics (52). It reduces hair loss in men and is the treatment for benign prostatic hyperplasia (52).

The widespread occurrence and importance as drug targets of the SDR and MDR superfamilies make them interesting topics for mechanistic studies. The Zn-dependent MDRs, a subclass of the MDR superfamily has received great attention in mechanistic studies. This is due to the early purification of yeast and horse liver alcohol dehydrogenases in 1935 and 1937, respectively (53, 54), establishing ADHs as convenient model systems with easily obtained substrate alcohols. This has led to a great body of knowledge on the mechanism of the metal dependent alcohol dehydrogenases from horse liver and from yeast (Klinman 1981, Leskovac, Trivic et al. 2002, de Smidt, du Preez et al. 2008). These enzymes are thought to have evolved from the metal-independent MDRs by acquiring a zinc ion in their active site that acts as a Lewis acid (Jornvall, Hedlund et al. 2010). Whereas the Zn-dependent MDRs have received a great
deal of attention by mechanistic enzymologists, the Zn-independent MDRs have received much less. For the moment, little experimental evidence supports the assumptions that are made about the mechanisms of metal independent MDR and SDR members. By means of analogy, metal-independent MDRs and SDRs are thought to follow the same mechanism as metal dependent alcohol dehydrogenases.

1.3.1 The MDR superfamily

Most MDRs catalyze reduction and oxidation reactions with a few members lacking known catalytic function. Many, but not all, MDRs that catalyze reductions are zinc independent and NADPH dependent and MDRs catalyzing dehydrogenation reactions tend to be zinc and NAD\(^+\) dependent (55). The nicotinamide cofactors carry out the two-electron reduction/oxidation chemistry and the zinc, if present, acts as a Lewis acid to activate the substrate and possibly indirectly the nicotinamide (56).

The distribution of Zn-dependent and Zn-independent MDRs across bacteria as well as archaea suggests that these two subclasses evolved before the divergence of the domains of life (48). It is however still highly speculative whether the zinc dependent or independent branch of MDRs evolved first. Circumstantial evidence suggest that the zinc dependent MDRs arose from zinc independent MDRs because the acquisition of a zinc ion is reasoned to be a more complex state, i.e. further evolved. On the other hand, MDRs containing two zinc ions are more abundant with 48% of the total amount of known MDRs (in 2013) compared to 40% zinc independent MDRs (48). Zinc dependent MDRs generally also show more variability in the dinucleotide-binding domain indicating a bigger divergence and possible a more distant connection and therefore older origin (48). These arguments speak against the hypothesis that zinc dependent MDRs arose after zinc independent MDRs.

Most zinc dependent MDRs have two zinc ions, of which one is directly involved in catalysis and one serves a structural role. Where as the structural zinc is bound by four cysteine residues, the catalytic zinc is bound by only three ligands with one coordination position available for the substrate. The substrates can be grouped into two classes. One class of substrates has carbon-oxygen bonds (alcohols and ketones/aldehydes) that get reduced or oxidized and a second
group contains activated carbon-carbon double bonds like enoyl thioesters, enones, and quinones.

1.3.1.1 Crotonyl-CoA carboxylase

One of the MDR family members central to this thesis is crotonyl-CoA carboxylase reductase (Ccr), the key enzyme in the ethylmalonyl-CoA pathway (57, 58). This is an enzyme with promising applications like new CO₂ fixation pathways (59) and the production of new precursors for antibiotics (60). The reaction catalyzed by Ccr is unique in nature. The fixation of CO₂ is coupled to the reduction of the carbon carbon double bond of enoyl-thiester. In addition to the striking chemistry of Ccr, it also has a turnover number that is more than a magnitude higher than the most abundant carboxylase: RuBisCO (57, 61). As such, a part of this thesis is dedicated to understanding the chemistry of this exceptional enzyme.

Ccr is part of the ethylmalonyl-CoA (EMC) pathway (Figure 3) that is used by organisms that grow on C1 or C2 compounds, e.g. methanol, ethanol and acetate (57). Growth on C1 or C2 compounds requires a dedicated pathway to assimilate these compounds into central intermediates of e.g. the citric acid (TCA) cycle or gluconeogenesis. For over 50 years the glyoxylate shunt (62) was the only known pathway to assimilate C2-compounds into such central intermediates. Erb et al. discovered the EMC pathway as an alternative pathway for the assimilation of C1 and C2 compounds through acetyl-CoA into the central intermediates malate and succinate. In contrast to the glyoxylate shunt, the EMC pathway is a linear pathway that makes use of a series of unique reactions on the level of C5-compounds (57). The pathway starts by assimilating two acetyl-CoA moieties, reducing, and dehydrating the resulting acetoacetyl-CoA to crotonyl-CoA. As one of the key enzymes of the EMC pathway, Ccr catalyzes the unique reductive carboxylation of crotonyl-CoA (C4-compound) to ethylmalonyl-CoA (C5-compound) with NADPH as cofactor. Ethylmalonyl-CoA is converted to methylsuccinyl-CoA by rearranging the carbon backbone. Methylsuccinyl-CoA is oxidized and hydrated to give β-methylmalyl-CoA which is split to give propinyl-CoA and glyoxylate from where conversion to succinate and malate occurs. About 8% of all sequenced bacteria contain the genes for the EMC pathway compared to just 1% containing the glyoxylate shunt, making it an ecologically important C2-assimilation strategy (63).
Figure 3. The ethylmalonyl-CoA pathway for the assimilation of acetate (57, 64). The reaction catalyzed by Ccr in indicated in blue.

Figure 4. The reactions catalyzed by crotonyl-CoA carboxylase reductase. The carboxylation reaction (red) is the physiologically relevant reaction that takes place in the presence of dissolved CO$_2$ and the reduction reaction is a minor side reaction, observed in the absence of CO$_2$ (58).
Ccr is unique in the sense that it is an oxygen tolerant reductive carboxylase. Reductive carboxylases are enzymes that have a redox reaction coupled to the carboxylation step. The most energetically efficient CO$_2$ fixation pathways, the reductive tricarboxylic acid (rTCA) cycle (65) and the dicarboxylate/hydroxybutyrate (DC/HB) cycle (66), are both based on reductive carboxylases (67). The key enzymes in these cycles, isocitrate dehydrogenase, α-ketoglutarate:ferredoxin oxidoreductase, and pyruvate:ferredoxin oxidoreductase (all three enzymes are needed for the rTCA and only the last enzyme is needed for the DC/HB cycle) are sensitive to oxygen (68, 69). Therefore, although Ccr is not used for autotrophic growth, as far as we know, it is a reductive carboxylase that is insensitive to oxygen, providing a unique enzyme for new synthetic CO$_2$ fixation cycles.

Interestingly, Ccr catalyzes two reactions. Besides the physiological carboxylation reaction, it also catalyzes the reduction of crotonyl-CoA to butyryl-CoA (58) (Figure 4). As Ccr is a member of the MDR superfamily it probably evolved from a classical reductase but never lost its ability to catalyze the reduction of crotonyl-CoA. However, it is remarkable that the reduction, unlike in other reductases, does not occur with tight control of the stereochemistry of the added proton indicating poor catalysis or perhaps another mechanism for the protonation step (58).

1.3.1.2 Enoyl thioester reductase

Enoyl thioester reductases (Etrs) are key enzymes in the biosynthesis of fatty acids and polyketides (70). Etrs catalyze the reduction of enoyl-thioester compounds to their saturated counterparts. The thioester link is between the acyl group and pantetheine, bound either to coenzyme A or acyl-carrier protein (70). These enzymes form an interesting model system for Ccrs because they have a reduced complexity. Without the carboxylation function, one less substrate concentration needs to be controlled. In addition, the concentration of protons is much easier to regulate in vitro than dissolved CO$_2$ levels, which are in equilibrium with the atmosphere and cannot be easily buffered like pH. Despite this reduced complexity, the proton donor in these enzymes is not known.

The Etr that is a key part of this thesis is Etr1p, an enzyme found in mitochondria of Candida tropicalis (71, 72). Eukaryotes have two independent pathways for
the synthesis of fatty acids. The cytosolic pathway belongs to type I fatty acid synthases (FAS) that consist of large multifunctional enzymes (73, 74). The mitochondrial pathway on the other hand consists of independent enzymes that catalyze subsequent reactions in the elongation of fatty acids and thereby resembles closely the prokaryotic FAS type II (75-77). It is as of yet unclear exactly why mitochondria have kept their FAS because most of their lipids are synthesized in the cytosol and imported (77). Yeast cells deficient in mitochondrial FAS lose the ability to respire, indicating that the pathway does have an essential role in mitochondria (71, 78). The current hypothesis is that the conserved mitochondrial pathway for fatty acid biosynthesis supplies the lipoic acid precursor oceanic acid, even though this is a nutritional requirement in mammals (79). In addition, respiratory complexes in mitochondria get acylated with mitochondrial fatty acids, giving another explanation why there is a respiratory phenotype (77).

1.3.2 The SDR superfamily

The SDR superfamily is very diverse in substrate specificity and has besides oxidoreductases also NAD(P)(H) dependent dehydratases and epimerases. With this diverse chemical repertoire, these enzymes are involved in a large variety of biological processes from essential primary metabolism to detoxification pathways. They are one-domain enzymes that are composed for the biggest part of a Rossmann-fold (80), 6-7 central twisted β-sheets flanked by 3-4 α-helices. The Rossmann fold accommodates the dinucleotides and determines the specificity for either NAD(H) or NADP(H). The C-terminal part of the enzyme forms the substrate binding pocket and is highly variable reflecting the diverse substrate specificities of the SDR superfamily. Unlike in the MDR superfamily, metal dependence within the SDR superfamily is rare but there are a few examples that need divalent metal ions for dimerization (81-83).

Most SDRs possess a conserved catalytic tyrosine and a downstream lying residue. In some cases the above-cited residues, alongside others and water molecules, create a complex proton relay network that enhances the acid/base properties of the catalytic tyrosine.
1.3.2.1 2-Enoyl-acyl carrier protein reductase of *Mycobacterium tuberculosis*

The 2-enoyl-acyl carrier protein reductase of *Mycobacterium tuberculosis*, InhA, and related enoyl-ACP reductases from other bacteria, generally called FabI, are probably the most extensively studied SDRs. InhA is involved in the biosynthesis of mycolic acids that protect *M. tuberculosis* against the immune system of its host. Inhibitors of InhA like isoniazid and triclosan hinder the synthesis of this protective mycolic acids layer and are therefore effective antibiotics against *M. tuberculosis* infections. The mechanism of these enzymes is thought to involve a direct hydride transfer from NADH to the enoyl-acyl carrier protein substrate and subsequently a proton transfer from an unknown source (84). An active site tyrosine closest to the Cα that is protonated, Y158, is proposed to be involved in electrophilic catalyst polarizing the enoyl bond by hydrogen bonding to the thioester carbonyl rather than in proton transfer (84). The mechanism of InhA is based on conclusions drawn from studies on NAD(P)H reviewed in chapter 1.4 and a limited number of biochemical studies (84-87). The results obtained in this thesis with the MDR members Etr1p and Ccr gave a reason to reinvestigate the mechanism of this important antibiotic target, to facilitate future efforts for rationally designing new antibiotics against this enzyme and to see to what extent SDRs and MDRs overlap in their mechanisms.

1.4 Hydrogen transfers

NAD(P)(H) is involved in hydride transfers essential for biochemical transformations as well as energy generation. The NADH/NAD⁺ redox couple is more commonly involved in hydride transfers in catabolic pathways and has an electrode potential, E’, in *Escherichia coli* of -280 mV whereas E₀’ is -320 mV. The NADPH/NADP⁺ redox couple, on the other hand is more commonly involved in hydride transfers in anabolic pathways and has an electrode potential, E’, in *E. coli* of -370 mV whereas E₀’ is -320 mV. The discrepancy in E’ comes from the different ratios of reduced to oxidized cofactor present in cells of 1/30 for NADH/NAD⁺ and 50/1 for NADPH/NADP⁺ (88). Thus, whereas both NADH and NADPH are equally strong hydride donors, under physiological conditions NADPH is the stronger reducing agent.
In 1951 Westheimer et al. showed that the hydrogen transferred from ethanol to NAD\(^+\) without exchange with water by yeast alcohol dehydrogenase (89). Two year later the same group showed that the hydride transfers from ethanol to the C-4 of nicotinamide (90). The hydride is transferred to or from the C-4 position of the nicotinamide moiety of NAD(P)(H), see Figure 5. Both hydrogens bound to C-4, H\(_R\) as well as H\(_S\), can be transferred to the substrate. Reductases/dehydrogenases that transfer the Pro\(_R\) hydride, H\(_R\), are called A-side specific and ones that transfer the Pro\(_S\) hydride, H\(_S\), are called B-side specific. The stereochemistry of hydride transfer was thought to have an influence on the activation energy of the hydride transfer (91) but is now considered just a byproduct of evolution (92). It takes many mutations to flip the nicotinamide within the active site of an enzyme, therefore the stereochemistry of hydride transfer is ‘frozen’ within enzyme superfamilies.

Eversince the discovery that the hydride transfers between nicotinamide and the substrate without exchange with the surrounding solvent many studies have looked at the mechanism of hydride transfer. Two of the main questions that have been central in the hydride transfer debate are a) how does the hydride transfer and b) how do enzymes catalyze hydride transfer. Concentrating on the former question, the research community has tried to distinguish mainly between two options; is the hydride transferred as a hydride ion H\(^-\) or do single electron transfers (SET) precede and/or follow a proton transfer in a sequence schematically represented by e\(^-\) \(\rightarrow\) H\(^+\) \(\rightarrow\) e\(^-\)? (Figure 6) A publication on the discrepancy between the kinetic hydrogen isotope-effect and the product isotope distribution in model compounds initiated the discussion (93). A stepwise mechanism of SETs seemed the only plausible explanation. Only a decade after this publication, the origin of the discrepancy was attributed to the formation of side products, correcting the scientific record (94, 95).

Nevertheless, in the meantime a considerable body of research gathered results that seemed to support a SET mechanism in model compounds (96-99). The SET mechanism then lost favor after experiments with radical traps ruled it out in enzymes. If single electron transfers indeed take place, radical intermediates are necessarily involved. Detection of radicals are routinely done by spectroscopic methods like electron paramagnetic resonance spectroscopy, but if short lived, can only be detected by chemical methods. Chemical methods to detect radical intermediates rely on radical traps (100) or radical clocks
molecules that react readily with radicals. In some cases, the substrate can be the radical trap itself; in this case, the substrate has a chemical structure that rearranges readily if a radical intermediate is formed. The fastest radical traps have cyclopropane structures that undergo unimolecular ring opening with a

**Figure 5.** Structure of NADH (R=OH) and NADPH (R=OP\(\text{O}_3\)H\(_2\)) with atom numbering of nicotinamide. The hydride transfers from C-4. Depending on the reductase/dehydrogenase, either HR or HS is transferred.

**Figure 6.** Different possible mechanisms for hydride transfer between NADH and a substrate. \(X = \text{CR}_2, \text{NR} \text{ or O}\).
rate constant on the order of $10^8 \text{s}^{-1}$ at 25 °C. Horse liver alcohol dehydrogenase converts alcohol 1 in Figure 7 to aldehyde 2 in Figure 7 -with a minor solvolysis side product- and in the back reaction, aldehyde 2 in Figure 7 to alcohol 1 in Figure 7 -without side products (101). A radical intermediate would open the cyclopropane ring. Therefore, the electron-proton-electron mechanism and its variations are very unlikely to have a role in horse liver alcohol dehydrogenase and probably enzymatic NADH chemistry in general. Other studies with radical traps confirm this result (102, 103). Additional measurements of kinetic isotope effects and corresponding quantum mechanical calculations deemed the SET mechanism unlikely also in model compounds except for substrates with very strong one-electron oxidizing properties (104). This has led the generally depicted mechanism to be drawn as was first postulated in 1957. This is a push-pull mechanism where the lone pair of the nitrogen ‘pushes’ into the pyridine ring and the hydrogen at the C-4 is transferred through a direct nucleophilic attack onto an electrophilic atom on the substrate (see Figure 8).

Figure 7. Substrates converted into each other by horse liver alcohol dehydrogenase without products indicative of radical chemistry like cyclopropane ring opening (101, 102).

Figure 8. The currently prevailing hydride transfer mechanism for NAD(P)H
An alternative mechanism where the hydride transfers through an Alder-ene, or ene-, mechanism (Alder 1943), proposed by G. Hamilton in 1971 (105), received no attention until recently (106). The biggest difference between this mechanism and the two other proposed mechanisms is that it involves a covalent intermediate of the substrate and NADH. In the direction in which the substrate is reduced and nicotinamide oxidized, the hydrogen from C-4 is transferred to the electrophilic center of the substrate through a covalent intermediate. The covalent bond is formed between the more electron negative atom of the substrate (X in Figure 6) and C-2 or C-6 of NADH. The transition state of this step lies on a continuum from completely concerted to biradical to zwitterionic, the exact nature of which depends on the system and conditions (Figure 9). The covalent adduct is then eliminated from the nicotinamide ring to give the reduced substrate and NAD$^+$ (106). This mechanism is well-established in organic chemistry. Unactivated carbonyl compounds reacting with unactivated alkenes require temperatures higher than 150 °C to react while Lewis acids can catalyze the reaction efficiently and bring down the temperature to 0 °C (107).

Recent evidence, largely provided by the laboratories of Klinman and Kohen, support a model for hydrogen transfers that involves tunneling, the phenomenon by which a particle, like a hydrogen, transfers through barriers as a result of its wave-like nature (108-112). From extensive kinetic isotope effect data they have gained support for the hypothesis that protein motions can bring the hydrogen-donor and acceptor in active sites of enzymes momentarily closer to promote hydrogen tunneling (Figure 10). When a reaction involves tunneling, the temperature dependence of the reaction does not obey semi-classical transition state theory (113). In the semi-classical theories, particles have to cross defined energetic-barriers to

![Figure 9](image)

**Figure 9.** Ene reaction with the different possible transition states. Depending on the reaction and the conditions the transition state changes on a continuum between concerted and zwitterionic.
interconvert between local minima in the energy landscape of a reaction. When tunneling is involved in a reaction, the height of the energetic barrier is not of importance. The reaction speed of tunneling depends on the distance between the donor, the atom to which the hydrogen is attached in the reactant state, and the acceptor, the atom to which the hydrogen is attached in the product state. Tunneling does not change observable intermediates of a reaction in the sense that the local minima in the energetic landscape stay the same. Only the means by which the reaction barrier, the local maximum in the energetic landscape, is crossed changes. Instead of requiring a defined amount of vibrational and translational energy to cross the barrier, the donor and acceptor need to come close enough for the wave function of the hydrogen to be present in both the reactant and product state. Collapse of the wave function in the product state leads to a reaction.

**Figure 10.** Schematic representation of tunneling in enzyme catalyzed reactions. 

**a)** Classical energetic barriers in an enzyme catalyzed reaction with a representation of barrier tunneling in the tunneling ready-conformation (TRC) where tunneling is most probable. 

**b)** Schematic representation of thermally activated tunneling. In the TRC, the hydrogen is delocalized between the substrate and product state (adapted from (114)).
1.5 Aims of this thesis

Understanding the metal independent members of both MDR and SDR families is of great importance because they contain pharmaceutical targets and have promising applications in biotechnology (115). A better understanding of the fundamental catalytic properties of these superfamilies would also facilitate the de novo design of coenzyme dependent oxidoreductases. As of yet, computational models fall short in predicting with precision the catalytic properties of enzymes (14, 116, 117), emphasizing the need for more experimental data to elaborate our current models.

Chapter 2 describes the detection of a covalent intermediate in the catalytic cycle of crotonyl-CoA carboxylase reductase that is consistent with an ene-mechanism. The detected intermediate can be isolated and characterized. The isolated intermediate serves as a good substrate for Etr1p and Yhdh, two proteins from different MDR subfamilies. This result challenges our current understanding of the chemistry of NAD(P)H dependent enzymes and makes a compelling argument that these metal independent MDRs react through an ene-mechanism and not through a direct hydride transfer.

As the isolated intermediate represents a state halfway in between the substrates and the products, it can serve as a molecular probe for the catalytic cycle of enzymes that accept it. In the intermediate, the hydride is already transferred but the proton is not. This property makes it a valuable tool to study the proton donation step in enoyl-thioester reductases (Etrs). In Chapter 3 we use the detected intermediate as a molecular probe to show for the first time that a conserved active site tyrosine is the proton donor of mitochondrial Etrs. With this knowledge and a phylogenetic analysis of Etrs from polyketide clusters, we designed new Etr variants with inverted stereochemistry, paving the way to control the stereochemistry of methyl groups in polyketides. In addition, this chapter presents data that serves as a warning to many biochemists in interpreting the UV-vis data obtained from mutagenesis studies on this class of enzymes.

Chapter 4 describes a new method for the study of kinetic isotope effects on protonation steps. The method is based on comparing the incorporation of protons or deuterons into a non-exchangeable position in the reaction product
from a buffer with a defined deuterium to hydrogen ratio. This method circumvents the artefacts caused by the accumulation of UV-vis active intermediates that interfere with classical assays. The measured isotope effects give insight into the contributions of residues that line the active site of Etr1p. Lastly, the catalytic role of a conserved active site threonine, thought just to serve water binding in the apo form and found in all MDRs, is proposed.

A phylogenetic analysis to establish the relationship of Etr1p and Ccr is presented in Chapter 5 and biochemical evidence is provided for the different reactivities of both enzymes. The energetic barriers for both the formation of the intermediate central to the reaction of both enzymes and the resolving step with a proton, in the case of Etr1p, or a CO$_2$, in the case of Ccr, are presented, giving a quantitative understanding of the different reactions.
1.6 References


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2 Chapter II: NADPH-dependent enzymes that react through a covalent ene-adduct as intermediate

At the core of Zn$^{2+}$-free, NADPH-dependent enzymes: a covalent ene-adduct

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Author contributions: R.G.R. and T.J.E. conceived and designed all experiments, with exception of NMR-experiments that were designed together with M-O.E., and MS-analyses that were designed together with P.K. and J.A.V. NMR-experiments were performed by R.G.R. and M-O.E., MS-experiments were performed by R.G.R. and P.K., enzyme kinetic assays, as well as purification of the intermediate were performed by R.G.R. and D.M.P. R.G.R. and T.J.E. wrote the paper.
2.1 Abstract

The pyridine nucleotides nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are ubiquitous redox coenzymes that are present in all living cells. Although about 16% of all characterized enzymes use pyridine nucleotides as hydride donors or acceptors during catalysis, a detailed understanding of how the hydride is transferred between NAD(P)H and the corresponding substrate is lacking to date for many enzymes. Here we demonstrate the formation of a covalent ene-intermediate between NADPH and its substrate in the catalytic cycle of crotonyl-CoA carboxylase/reductase (Ccr), using nuclear magnetic resonance spectroscopy (NMR) in combination with high resolution mass spectrometry (MS). Our results allowed us to directly access the catalytic cycle of other NADPH-dependent enzymes in an unprecedented way, suggesting that formation of NADPH-ene intermediates is a more general principle in catalysis and also operates in type II fatty acid biosynthesis.

2.2 Introduction

About one sixth of all characterized enzymes use pyridine nucleotides as hydride donors or acceptors during catalysis. After the first demonstration of a direct hydride transfer between NAD(P)H and its substrate through an enzyme by Westheimer more than half a century ago, many NAD(P)H-dependent enzymes and reactions have been investigated extensively to understand the details of NADPH-catalysis (1-3). The postulated mechanism, as formulated by Westheimer in 1957, is a one-step hydride transfer (4) that is generally depicted as a nucleophilic attack of the hydrogen nucleus on an electrophilic center of the substrate, with the C-H bond of the nicotinamide and the electrophilic carbon to which the hydride is transferred being almost collinear in the transition state (5, 6). Other mechanisms have been proposed in the past, such as single electron transfers involving the formation of radical intermediates, or electrocyclic ene reactions that proceed through the formation of covalent intermediates between the nicotinamide ring and the substrate (7). Yet, due to the lack of experimental evidence and the apparent absence of any traceable intermediates in the catalytic cycle of NAD(P)H-dependent enzymes (4, 8),
these alternative mechanisms have lost favor and the concerted one-step hydride transfer has prevailed in the literature.

The superfamily of medium chain dehydrogenases/reductases (MDRs) that comprises more than 11,000 classified individual members in distinct subfamilies of different functions (9) (Figure 1). Some fifty percent of the MDR superfamily are Zn$^{2+}$ dependent enzymes that preferentially catalyze dehydrogenation reactions. Many studies have focused on alcohol dehydrogenase, the prototypic enzyme of this family that reacts via a Zn$^{2+}$ stabilized (alkoxide) transition state (10), which is thought to lower the energy barrier to allow a one-step hydride transfer through hydrogen tunneling (11, 12). However, more than forty percent of the MDRs, mainly reductases, lack Zn$^{2+}$-binding motifs (approximately 4,900 members) and consequently must follow other mechanism(s), as noted before (13-17). In the following, we provide evidence for the existence of a covalent ene-intermediate in enzyme catalysis for the very first time by using a novel model system that allowed us to study and dissect distinct catalytic steps during enzymatic hydride transfers in the MDR family in an unprecedented way.
Figure 1. The superfamily of medium chain dehydrogenases/reductases (MDRs). Phylogenetic tree of 117 selected MDR superfamily members belonging to 13 of the largest subfamilies, as defined by Persson (18). The subfamilies of Ccr (CCR), Etr1p (MCAS), and YhdH (YHDH) that react through a covalent ene intermediate according to this study are highlighted in purple. Other subfamilies of Zn2+-independent enzymes (mainly reductases) are labelled in grey. Black branches show Zn2+-dependent enzyme subfamilies (mainly dehydrogenases) that are thought to react via the “classical” one-step hydride transfer. Tree topographies and evolutionary distance are given by the neighbor-joining method. Numbers at nodes represent the percentage bootstrap values for the clades of this group in 50 replications. Similar trees were obtained by using the minimum evolution and the maximum likelihood method. The sequences alignments to create the trees are available online.
2.3 Results

The recently discovered Ccr, which constitutes a distinct subfamily in the MDR superfamily (Figure 1), is a key enzyme in the central carbon metabolism of a number of bacteria, as well as in the biosynthesis of many polyketide natural products (e.g., the antibiotic monensin and the immunosuppressant FK520) (19-22). The purified enzyme catalyzes two distinct reactions, both using NADPH as reducing coenzyme (Figure 2a and 2e): i) the reduction of crotonyl-CoA to butyryl-CoA and ii) the reductive carboxylation of crotonyl-CoA to (2S)-ethylmalonyl-CoA, from here on referred to as the reduction and the carboxylation reaction, respectively. Ccr has evolved to preferentially catalyze the carboxylation reaction, as reflected by a faster substrate turnover and a higher catalytic efficiency compared to reduction (20) (see also Table 1). It is thought that the active site of Ccr is optimized to accommodate a CO₂ molecule, to effectively promote carboxylation, while at the same time it reduces binding of water as proton donor to minimize the competing reduction reaction without carboxylation (21, 23). This unique active site architecture made Ccr a promising model system to study enzymatic hydride transfers in more detail, as the slowed reduction reaction would provide the unique opportunity to analyze steps in the catalytic cycle that might otherwise be obscured in other enzymes.

The carboxylation and reduction reaction of Ccr are routinely monitored spectrophotometrically by following the consumption of NADPH at a fixed wavelength (20, 21, 24). Whereas the reduction reaction is assayed in the absence of CO₂, the carboxylation reaction is monitored in presence of CO₂ and is approximately six times more efficient (Table 1). However, besides the obvious contrast in catalytic efficiency between both Ccr catalyzed reactions we noted more distinct differences between both reactions when following the reactions at multiple wavelengths. The carboxylation reaction appeared directly coupled to the consumption of NADPH, as demonstrated by the consistent decrease of the NADPH chromophore (λ_max = 340 nm, Figure 2a-d). The reduction reaction, on the contrary, showed an initial transient increase in absorbance around 390 nm besides the consumption of the NADPH chromophore (Figure 2e-h), indicating the presence of a short-lived species during the course of the reaction.
<table>
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<tr>
<th>Enzyme (reaction)</th>
<th>substrate</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$·M$^{-1}$)</th>
</tr>
</thead>
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<tr>
<td>Ccr (carboxylation)</td>
<td>NADPH</td>
<td>24.5±1.9</td>
<td>275±160</td>
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<td>44.5±11.5</td>
<td>167±122</td>
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<tr>
<td>Ccr (reduction)</td>
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<td>39±10</td>
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<tr>
<td>Etr1p</td>
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<tr>
<td>YhdH</td>
<td>NADPH</td>
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<td>4.9±1.2</td>
<td>2.0 · 10$^5$</td>
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<td>2.4±0.7</td>
<td>9.1 · 10$^5$</td>
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Figure 2: Spectrophotometric assay of Ccr activity identifies a transient intermediate during the reduction reaction. (a) Carboxylation reaction catalyzed by Ccr in the presence of CO₂. (b) Time dependent wavelength scan of the carboxylation reaction showing the simple consumption of NADPH (c) Carboxylation reaction at 340 nm, following the consumption of the NADPH chromophore ($\lambda_{max}$ = 340 nm) (d) Carboxylation reaction at 390 nm. Assay-conditions for b-d: 1 mM NADPH, 1.5 mM crotonyl-CoA, 50 mM NaHCO₃, 0.7 µM carbonic anhydrase, 0.2 µM Ccr, 100 mM NaHPO₄ pH 7.9, 30°C. (e) Reduction reaction catalyzed by Ccr in the absence of CO₂. (f) Time dependent wavelength scan of the reduction reaction showing consumption of NADPH and transient accumulation of an intermediate around 390 nm. (g) Reduction reaction at 340 nm, following the consumption of the NADPH chromophore ($\lambda_{max}$ = 340 nm) (h) Reduction reaction at 390 nm, showing the transient accumulation of the intermediate. Assay-conditions for f-h: 1 mM NADPH, 1.5 mM crotonyl-CoA, 0.7 µM carbonic anhydrase, 1.0 µM Ccr, 100 mM NaHPO₄ pH 7.9, 30°C.
2.3.1 Kinetic analysis of the reaction intermediate by NMR.

To acquire more information on the photoactive species causing the transient increase in absorbance, the reduction reaction was assayed by $^1$H-NMR. For that purpose, the kinetics of each component in the reaction mixture was followed by integration of characteristic $^1$H-NMR signals during the course of the reaction (Figure 3a, Figure S1). In the first minutes of the reduction reaction, NADPH and crotonyl-CoA were co-consumed rapidly (substrate consumption half-lifes $t_{\text{½}}^{-s} = 17$ and 18 min, Table S1), in line with the spectrophotometric observation that the NADPH-chromophore is lost quickly in this initial reaction phase (vide supra). Interestingly, however, formation of the reaction products was lagging behind, as the NADP+/butyryl-CoA couple was formed almost four times slower as substrate was consumed (product formation half-lifes $t_{\text{½}}^{+p} = 63$ and 59 min, Table S1). Thus, we hypothesized that both substrates initially react through a common intermediate that would explain the apparent lag in product formation. Indeed, we noticed a set of transient $^1$H-NMR signals that could not be assigned to any of the reaction partners (most prominently two putative vinylic protons at $\delta = 6.28$ and 6.49, Figure S1), confirming the existence of a distinct, short-lived intermediate formed during the catalytic cycle of Ccr. Kinetic analysis demonstrated that formation of this intermediate was directly related to NADPH/crotonyl-CoA consumption (intermediate formation half-life $t_{\text{½}}^{+i} \approx 11$ min$^{-1} \approx t_{\text{½}}^{-s}$, Table S1), whereas disappearance of the intermediate mirrored NADP+/butyryl-CoA formation (intermediate consumption half-life $t_{\text{½}}^{-i} = 57$ min$^{-1} \approx t_{\text{½}}^{+p}$, Table S1).

2.3.2 Characterization of the reaction intermediate as ene adduct.

Because the kinetic data suggested that the intermediate is a direct product of the reaction of NADPH with crotonyl-CoA, we aimed at identifying its exact nature. For that purpose, samples were taken during the initial phase of the enzymatic reaction and immediately subjected to high performance liquid chromatography electron spray ionization mass spectrometric (HPLC-ESI-MS) analysis. A sample withdrawn from the reduction reaction at different time points showed the accumulation and disappearance of an ion with an m/z of 791.123 that matched the expected m/z value and the simulated isotopic pattern of a
The covalent adduct of NADPH and crotonyl-CoA (as [M+2H]^{2+} ion, Figure S2). The same assay with heat inactivated enzyme did not show detectable amounts of the 791.123 ion. To gain structural information about the NADPH-crotonyl-CoA adduct and to locate the exact position of the covalent bond(s) formed, the adduct was further characterized by two-dimensional NMR spectroscopy (Figure S3). NMR analysis suggested that carbon 2 of the nicotinamide ring and carbons α, β and γ of the acid moiety of the thioester form a coherent substructure, and indicated that carbons C4-C6 of the nicotinamide ring form an unusual diene-structure that gives rise to the characteristic vinylic signal observed. These data in combination with the HPLC-ESI-MS results suggested that the intermediate is a covalent C2/Cα-ene-adduct of NADPH and crotonyl-CoA (Figure 3b).

2.3.3 The isolated ene intermediate is catalytically competent for Ccr.

To confirm that the unprecedented ene adduct is a true intermediate in the catalytic cycle of Ccr, we sought to probe its function also for the carboxylation reaction. For this purpose, we established a purification protocol to isolate the labile ene adduct from the enzymatic reaction at preparative scale by high-performance liquid chromatography (HPLC), collecting the intermediate directly in liquid nitrogen with subsequent freeze-drying to keep its decomposition to a minimum (Figure 4a-b). The isolated compound was unstable in solution (t_{1/2}, 21°C = 24 min, Figure S4) and absorbed in the UV spectrum with maxima at 260 and 370 nm (Figure 4c). HPLC-ESI-MS analysis showed a highly abundant ion with an m/z of 791.126 (Figure 4d) with distinct NADP^{+} and butyryl-CoA (sub-)fragmentation patterns. Finally, the proposed ene-structure of the adduct was confirmed by NMR on the pure compound (Figure S5). The isolated ene-adduct served as bona fide substrate for the reduction reaction, as monitored by the absorption change at 370 nm, but more importantly also for the carboxylation reaction when incubated with Ccr in the presence of CO\textsubscript{2} yielding ethylmalonyl-CoA (Figure S6). Note that the catalytic efficiencies of Ccr for the isolated adduct were even better than for the enzyme's individual substrates, NADPH and crotonyl-CoA, demonstrating that the ene-adduct is indeed catalytically competent and not a “simple” dead-end product of the reduction reaction (Table 1).
Figure 3: NMR characterization of the transient intermediate. (a) Kinetic behavior of the reaction substrates (NADPH, blue diamonds; crotonyl-CoA, blue dots), reaction products (NADP⁺, gray diamonds; butyryl-CoA, gray dots), and the intermediate (purple stars) during the reduction reaction, as inferred from characteristic ¹H-NMR signals (see Figure S1 for details) (b) Structures of reaction substrates (blue), reaction products (gray) and the intermediate (ene-adduct, purple) as inferred from reference spectra and correlation NMR-spectroscopy (see Figure S3, S5 and S10 for details). Characteristic substructures of the ene-adduct that localize the covalent bond of the intermediate, as discussed in the text are highlighted in bold and labeled (see Figure S5 for full assignment). R = adenine dinucleotide phosphate. The proposed pericyclic mechanism leading to formation of the ene-adduct is shown as dashed lines, for details refer to Figure S8.

Ene-adducts can be formed through electrocyclic ene reactions, which are pericyclic rearrangements that involve transition states of aromatic character, similar to the one discovered by Alder in 1943 (25). An alternative pathway to for the detected ene adduct is a stepwise mechanism where the enolate is
formed by a direct hydride transfer and subsequently attacks the nicotinamide ring at the C-2 position to form the adduct. A few pericyclic reactions are known in biology that either occur spontaneously (e.g., during vitamin D synthesis (26)), or enzymatically catalyzed (27) (e.g., reactions of chorismate and isochorismate mutase (28, 29). As mentioned above, it was proposed more than fifty years ago that the net hydride transfer from NAD(P)H onto substrates could also proceed through an electrocyclic ene reaction mechanism (7). Although such an ene mechanism had never been experimentally demonstrated for NAD(P)H-dependent enzymes before, it was suggested to operate during chemical model reactions of nicotinamide analogues with α,β-unsaturated compounds that resemble enoyl-esters (30-32). We note that the ene-adduct of Ccr observed in our experiments is consistent with these data as being the result of an ene mechanism operating also during enzymatic catalysis. Our findings are further supported by structural arguments, since the recently reported structure of a Ccr homolog co-crystallized with NADP+ and its 2-enoyl-CoA substrate (PDB 4a0s) shows that both reactants are well aligned for an ene reaction (23). The α-carbon of the enoyl-thioester is 3.8 Å from the C2 of the nicotinamide ring, whereas the β-carbon of crotonyl-CoA is 3.4 Å away from the C4 of the nicotinamide ring (Figure S7). This orientation favors interaction of the π-orbital of the C2-C3 bond of nicotinamide with the π*-orbital of the acceptor (33), which has been considered to be crucial for ene reactions before (30, 33). The chain of evidence is completed by our finding that Ccr accepts the isolated ene-adduct with similar (or even better) efficiency than its individual substrates suggesting that it is indeed a true reaction intermediate.
Figure 4: Purification and characterization of the ene adduct. (a) HPLC analysis of the reduction reaction mixture before intermediate purification (b) Isolated intermediate (ene-adduct). Note that the traces of NADP⁺ and butyryl-CoA are due to rapid decomposition of the ene-adduct during handling and HPLC analysis (for a detailed analysis of the time dependent decomposition, see Figure S4) (c) High resolution mass spectroscopic analysis of the isolated ene-adduct and its isotopic distribution pattern, recorded at 12.5 minutes elution time (d) Absorbance spectrum of the isolated ene-adduct, recorded at 12.5 minutes elution time.
2.3.4 Role of the ene intermediate in other enzymes.

Next we turned our attention to the question whether the ene intermediate might serve a more general role in NAD(P)H-enzyme catalysis, i.e. whether other NAD(P)H-dependent enzymes also react through the proposed electrocyclic ene mechanism. To test this hypothesis, we sought to probe the function of the ene intermediate in other MDR subfamilies. First we focused on enzymes of the MCAS subgroup (Figure 1) that operate in polyketide biosynthesis, as well as in the biosynthetic cycle of fatty acids, a central, conserved process in the metabolism of prokaryotic and eukaryotic organisms. Indeed, the ene adduct – as isolated from the catalytic cycle of Ccr – was converted by the 2-enoyl thioester reductase Etr1p that is involved in mitochondrial synthesis of fatty acids (type II) catalyzing the reduction of unsaturated thioesters (16, 34). A more detailed analysis showed that the ene adduct served as true substrate for Etr1p with comparable kinetics as its individual substrates crotonyl-CoA and NADPH (Table 1). Similar results were obtained with YhdH of *E. coli* that is the archetypal member of the YHDH subfamily (Figure 1) conferring resistance to acrylate (35). Enzymes of the YHDH subfamily have recently been reported to function as acryloyl-CoA reductases in prokaryotic 3-hydroxypropionate and dimethylsulfopropionate (DMSP) metabolism (35, 36). As for Etr1p, *E. coli* YhdH accepted the purified ene intermediate with very similar kinetic parameters to the alternative substrates crotonyl-CoA and NADPH (Table 1). In summary, these results demonstrated that the covalent ene adduct is a shared reaction intermediate in the catalytic cycle of distantly related enzymes in three distinct MDR subfamilies of different physiological functions, suggesting that nature has exploited this reaction mechanism for a variety of enzymes. Consequently, we suggest that nature has functionalized the proposed electrocyclic ene reaction mechanism in many more enzymes.

2.4 Discussion

2.4.1 The ene-adduct chromophore

The isolated ene-adduct of NADPH and crotonyl-CoA has a local absorption maximum at 370 nm whereas NADH and NADPH at 340 nm. The conjugated system that is responsible for the chromophoric properties of NAD(P)H are
shown in (Figure 5). In analogy to this structure we can draw a resonance structure to explain the chromophoric properties on the ene adduct of NADPH and crotonyl-CoA. The red shift of 30 nm in the ene adduct compared to NAD(P)H due to one additional carbon-carbon double bond corresponds exactly with the predicted shift from the Woodward-Fieser rules for UV-vis spectra (37). The red shift in the C-2-ene-adduct can be explained by the additional double bond that is added to the chromophore which raises the HOMO and lowers the LUMO in energy, diminishing the energetic gap.

\[ \lambda_{\text{max}} = 340 \text{ nm} \quad \lambda_{\text{max}} = 370 \text{ nm} \]

Figure 5: Comparison of the chromophores of NAD(P)H (left) and the C-2-ene-adduct (right). The bonds that make up the chromophore are highlighted in blue.

In conclusion, our observations have the following implications: First, they demonstrate for the very first time the existence of a distinct, covalent intermediate during the catalytic cycle of NAD(P)H-dependent enzymes (7). Second, they allow the formulation of an alternative mechanism for enzymatic hydride transfers of NAD(P)H (Figure 3b, Figure S8) that might provide the mechanistic basis for numerous (Zn\(^{2+}\)-independent) enzymes, which so far have been considered to follow the “classical” one-step hydride transfer. Third, our findings provide novel tools to directly access the catalytic cycle of Zn\(^{2+}\)-free, NAD(P)H-dependent enzymes, to study, dissect and understand their molecular mechanisms in better detail. Note that these enzymes include many pharmaceutically relevant targets that cover biologically important processes, such as the biosynthesis of polyketides and fatty acids (type II), the conversion of prostaglandins and leukotrienes, or the reduction of quinones. Consequently, our results will affect the development and design of novel antibiotics and enzyme inhibitors against these targets based on structural analogs of the
observed ene-adduct and/or the proposed transition state (38, 39) (N.B.: covalent NAD(P)H-drug adducts are known to be very potent enzyme inhibitors, the prime examples being isoniazid and finasterid that are used in the treatment of tuberculosis and benign prostate hyperplasia, respectively (40, 41)). Last, but not least, the presented results challenge our current understanding of NAD(P)H by providing new insights into the reactivity and (bio)chemistry of Nature’s most abundant redox cofactor that still holds surprises after 100 years of its first discovery (42).
2.5 Methods

Chemicals. NADPH was obtained from Carl-Roth, butyryl-CoA & NADP⁺ were purchased from Sigma-Aldrich. Crotonyl-CoA was either purchased from Sigma-Aldrich AG or synthesized from crotonic anhydride (Sigma-Aldrich) and coenzyme A (Roche Diagnostics). Carbonic anhydrase (from bovine erythrocytes) was obtained via MP Biomedicals. All other chemicals were purchased from Carl-Roth and Sigma-Aldrich. Salts and solvents were of analytical grade, water was of 18.2 MΩ cm⁻¹ grade.

2.5.1 Spectrophotometric enzyme assays.

Enzyme assays were carried out in a Cary-50 UV/Vis spectrometer (Agilent) at 30 °C using quartz cuvettes (1, 3, or 10 mm diameter; Hellma). A typical enzyme assay to follow the reactions of Ccr, Etr1p, or YhdH contained 1 mM NADPH and 1 mM crotonyl-CoA in 100 mM Na₂HPO₄ (pH 7.9). Assays to monitor the reduction of crotonyl-CoA were started by addition of 0.057 μM Ccr, 0.019 μM Etr1p, or 0.025 μM YhdH. Assays to follow the reductive carboxylation reaction of crotonyl-CoA contained additionally 50 mM NaHCO₃ and 0.23 μM carbonic anhydrase in the reaction mixture, and were started by addition of 0.017 μM Ccr. Reduction and reductive carboxylation of crotonyl-CoA was followed at 340 nm, using an absorption coefficient for NADPH of ε₃₄₀=6.2 cm⁻¹·mM⁻¹ (43), to determine the reaction rate. Formation of the ene-intermediate was monitored by scanning kinetics between 275 and 440 nm. Kinetic parameters of individual enzymes were determined by varying the concentration of NADPH or crotonyl-CoA, while keeping the concentration of all other reactants at saturated concentrations. Assays to follow the reduction or reductive carboxylation of isolated ene-intermediate by Ccr, Etr1p, or YhdH were essentially the same as described above, replacing NADPH and crotonyl-CoA in the reaction mixture by purified ene-intermediate that had been freshly dissolved in H₂O just prior to each assay. Reduction and reductive carboxylation of the ene-intermediate was followed at 370 nm, using an absorption coefficient for the ene-intermediate of ε₃₇₀=6.9 cm⁻¹·mM⁻¹ (see below) to determine reaction rate, as well as starting concentration of the ene-intermediate in the reaction mixture. Kinetic parameters were determined by varying the concentration of ene-intermediate.
2.5.2 Intermediate purification.

A mixture containing 50 mM NADPH, 50 mM crotonyl-CoA and 0.3 μM Ccr in 200 mM Na₂HPO₄ (pH 7.9, 100 μL) was reacted at room temperature until absorption at 395 nm reached a maximum. The mixture was separated on a Waters 2690 HPLC, adapting a previously described elution profile (44) by changing the flow rate to 10 mL·min⁻¹ and using a Gemini® 10 μm NX-C18 110 Å, 100 x 21.2 mm, AXIA™ packed column (Phenomenex). Elution of the intermediate was followed at 370 nm with a Waters 996 photodiode array detector. The intermediate was collected into liquid nitrogen, lyophilized (0.01 mbar, -70 °C) and stored at -80 °C.

2.5.3 Determination of the absorbance coefficient of the ene intermediate.

The absorbance coefficient of the ene intermediate was determined experimentally by measuring the ratio of spectral absorbance at 260 nm and 370 nm (1:0.203) with a Waters 996 photodiode array detector from spectra acquired from the pure compound eluting from preparative HPLC. Assuming that the total absorption of the ene-intermediate at 260 nm represents the sum of its individual components \( \varepsilon_{260} \text{ nm intermediate} = 33.8 \text{ cm}^{-1} \cdot \text{mM}^{-1} = [\varepsilon_{260} \text{ nm NADPH} + \varepsilon_{260} \text{ nm CoA}] = [16.9 \text{ cm}^{-1} \cdot \text{mM}^{-1} + 16.9 \text{ cm}^{-1} \cdot \text{mM}^{-1}] \), according to (43), the absorption coefficient of the ene-intermediate at 370 nm can be calculated as \( \varepsilon_{370} \text{ nm intermediate} = 6.9 \text{ cm}^{-1} \cdot \text{mM}^{-1} \), according to \( \varepsilon_{370} \text{ nm intermediate} = 0.203 [\varepsilon_{260} \text{ nm intermediate}] \).

2.5.4 Mass spectrometric analysis.

For high performance liquid chromatography electro spray ionization mass spectrometric (HPLC-ESI-MS) analysis, the method of Peyraud (44) with online desalting as described by Kuntze(45) was used. To follow the transient intermediate in the reaction mixture, 1 μL samples were withdrawn at different timepoints from a 60 μL reaction mixture containing 1 mM NADPH, 1 mM crotonyl-CoA, and 0.043 μM Ccr in 100 mM NaH₂PO₄ (pH 7.9) at 5 °C. The sample was diluted with 99 μL 100 mM NaH₂PO₄ (pH 7.9) at 0 °C and directly subjected to analysis. For product analysis of reactions of Ccr and Etr1p with
the isolated ene-intermediate as substrate, 1 μL samples were withdrawn after 1 h incubation at 30 °C from 60 μL reaction mixtures containing 1 mM NADPH, 1 mM crotonyl-CoA, in the presence of 0.028 μM Ccr, 0.028 μM boiled Ccr, or 0.030 μM Etr1p. For incubations in the presence of CO₂, the mixture contained additionally 50 mM NaHCO₃ and 0.023 μM carbonic anhydrase. Samples were diluted with 9 μL 100 mM NaH₂PO₄ (pH 7.9) at 0 °C. Then, 2 μL of the dilution were mixed with 98 μL ¹³C-labelled metabolite standard mixture and lyophilized. The residual was resuspended in 20 μL H₂O and subjected to analysis.

2.5.5 Nuclear magnetic resonance spectroscopy

Spectra were acquired on a Bruker AVII and AVIII 600 MHz spectrometer at 4.4 °C and water suppression by excitation sculpting. For kinetic analysis of the transient intermediate, parameters were set on a mixture containing 1 mM NADPH, 1 mM crotonyl-CoA in 100 mM Na₂HPO₄ (pH 7.9, 590 μL, 10 % D₂O). Then, Ccr (15 μg, 10 μL) was added and ¹H-spectra were acquired every 5 minutes. For structural analysis of the transient intermediate, a mixture containing 15 mM NADPH, 15 mM crotonyl-CoA and 0.08 μM Ccr in 100 mM NaH₂PO₄ (pH 7.9, 525 μL) was reacted at 25 °C until absorption at 395 nm reached a maximum. Then, NaOH (1 M, 15 μL) and D₂O (60 μL) was added to stop the reaction, and Correlation Spectroscopy (COSY) and Heteronuclear Single Quantum Coherence (HSQC) spectra were immediately acquired. For structural analysis of the isolated intermediate, 5 mg of purified and lyophilized intermediate was dissolved in ice cold 100 mM Na₂HPO₄ (pH 7.9, 600 μL) and immediately lyophilized again. Just before analysis, the intermediate was dissolved in D₂O (600 μL) to acquire ¹H-, ¹³C-NMR, COSY, HSQC and Heteronuclear Multiple Bond Correlation spectra.

2.5.6 Synthesis of crotonyl-CoA.

Crotonyl-CoA was synthesized according to a well established method (46). In short, 320 mg (0.42 mmol) coenzyme A trilithium salt was dissolved in 5 ml ice-cold 0.1M NaHCO₃. Then, 65 μl (0.44 mmol) crotonic anhydride was added and let to react for 45 minutes. The crude product was purified by preparative HPLC on a Gemini® 10 μm NX-C18 110 Å, 100 x 21.2 mm, AXIA™ Packed (Phenomenex) adapting a previously described elution profile (44) by changing
the flow rate to 10 mL·min⁻¹ and using a Gemini® 10 µm NX-C18 110 Å, 100 x 21.2 mm, AXIA™ packed column (Phenomenex). The purified crotonyl-CoA was lyophilized at 0.01 mbar, resuspended in water, lyophilized a second time and stored at -80 °C until use.

2.5.7 Preparation of Ccr.

The ccr gene from *Methylobacterium extorquens* AM1 (gi: 240136948) was amplified using pGS5 (gift from G. Sturm, Albert-Ludwigs-University of Freiburg, Germany) as template using the forward primer 5'-CGTCATATGGCTGCAAGCGCAGCACC-3' and reverse primer 5'-CTGGCAAGCTTATCGCCCTTGAGCG-3' to introduce NdeI and HindIII restriction sites (underlined), respectively. PCR was performed with Phusion polymerase (Fermentas) in HF-buffer, including an initial denaturation step of 2 min at 98 °C, followed by 35 cycles with denaturation for 10 s at 98 °C, annealing for 30 s at 58 °C, polymerization for 90 s at 72 °C, and a final extension step at 72 °C for 5 min. The PCR product was purified and digested with NdeI and HindIII. The digested PCR product was purified by gel extraction and cloned into the expression vector pET-23b (using *Escherichia coli* DH5 α), to add a C-terminal His-tag to the protein, yielding pTE71. For production of the recombinant protein, *E. coli* Rosetta 2 (DE3) cells were transformed with pTE71 and a single transformant was grown overnight in Luria-Bertani broth containing 100 µg L⁻¹ ampicillin and 34 µg L⁻¹ chloramphenicol at 37 °C. Of this overnight culture, 1 ml was used to inoculate 800 ml of auto-inducing medium (47) containing 100 µg L⁻¹ ampicillin and 34 µg L⁻¹ chloramphenicol. Cells were grown for 24h at 28 °C under constant shaking before harvesting by centrifugation (10 minutes at 10,000 x g and 4 °C). Harvested cells (2-3 g) were suspended in 7 mL of 20 mM Tris(hydroxymethyl)aminomethane (Tris-HCl), pH 7.8 containing 0.1 mg·mL⁻¹ of DNase I (AppliChem). The suspension was sonicated for 3x 45s with cooling on ice for 120s between sonication rounds, and the cell lysate was centrifuged (40,000 xg) at 4 °C for 1 h. The supernatant was applied at a flow rate of 1 mL·min⁻¹ onto a 1-mL Ni-Sepharose Fast Flow Column (HisTrap FF; GE Healthcare) that had been equilibrated with 10 volumes of buffer A containing 20 mM Tris-HCl (pH 7.8) and 200 mM KCl. The column was washed with more than 20 column volumes of buffer A and buffer A containing 75 mM imidazole, before Ccr was eluted with buffer A containing 500 mM imidazole. The enzyme
was desalted and concentrated by ultrafiltration (Amicon ultracel 10K membrane; Millipore). The protein (approximately 2 mg per preparation) was stored at -20 °C in 10 mM Na$_2$HPO$_4$ (pH 7.8) with 50% (v/v) glycerol. For NMR assays, the glycerol was removed by subsequent dilution/concentration steps using ultrafiltration. Protein concentrations were determined spectrophotometrically.

2.5.8 Preparation of Etr1p.

The *etr1* gene from *Candida tropicalis* (48) (gi: 51316051) present in pYES2 was a generous gift of A. Kastaniotis (University of Oulu, Finland), and used as a template for a PCR with the forward primer 5’-GTCGTCATATGATCACCAGGCTGTTCTTT-3’ and the reverse primer 5’-CTCTGGATCCATGCTCGAGTTAGTACGTAATC-3’, introducing NdeI and BamHI restriction sites (underlined) and removing the mitochondrial transport tag. PCR was performed with Phusion polymerase (Fermentas) in HF-buffer, including an initial denaturation step of 30 s at 98 °C, followed by 35 cycles with denaturation for 10 s at 98 °C, annealing for 30 s at 60 °C, polymerization for 55 s at 72 °C, and a final extension step at 72 °C for 5 min. The PCR product was cloned into pET-16b (using *E. coli* DH5α), to add an N-terminal His-tag to the protein, yielding pTE115. In a next step, pTE115 was used as template in a PCR with forward primer 5’-CGCAAGATTCATGGGCCATCATC-3’ and reverse primer 5’-ATGCTCGAGTTAGTACGTAATC-3’, introducing EcoRI and XhoI restriction sites (underlined), respectively. The resulting PCR product was cloned into the integrative *Pichia pastoris* expression vector pPICzA (Invitrogen), yielding pTE250. For production of recombinant protein, pTE250 construct was integrated into the mitochondrial genome of *P. pastoris* NRRLY11430 using the EasySelect™ Pichia Expression Kit (Invitrogen) and essentially following the supplier’s protocol, unless otherwise mentioned. To inoculate expression cultures, 15 ml of an overnight culture of a *P. pastoris* clone in BMGY medium was used. Protein was expressed for 3 days at 28 °C in 300 ml BMMY medium containing 0.5 % (v/v) methanol, which was added approximately every 12 hours to compensate for evaporation, and 0.2 % (v/v) of Antifoam C (Sigma-Aldrich AG). Cells were harvested by centrifugation for 10 minutes at 10,000 x g at 4 °C and resuspended in 15 ml of 20 mM Tris-HCl (pH 7.8). Cells were lysed by french press, centrifuged for 30 minutes at 21,000
x g at 4 °C, and the supernatant was purified by weak anion exchange with DEAE Sephacel™ (GE Healthcare, self-packed: 2.6 cm diameter, 37 ml volume) as stationary phase that had been equilibrated with buffer A (20 mM Tris·HCl, pH 7.8) at a flow rate of 4.4 ml·min⁻¹. After washing with buffer A, elution was conducted by a linear gradient of 80 to 180 mM NaCl in 80 minutes at the same flow rate of 4.4 ml·min⁻¹. The presence of Etr1p in eluted fractions was detected by enzyme activity measurements. Etr1p eluted at approximately 100 mM NaCl. The anion exchange elute was pooled and further purified using three 1 ml HisTrap FF columns in a row (GE Healthcare) and following the same purification protocol as for recombinant Ccr. The eluted protein was desalted over a PD-10 column (GE Healthcare) and stored with 50% (v/v) glycerol at -20 °C. Protein concentrations were determined spectrophotometrically. The resulting protein was confirmed by LC MS/MS at the functional genomics center of the University of Zurich, with a purity of more than 99% according to SDS-PAGE analysis. Mitochondrial alcohol dehydrogenase isozyme III, a zinc dependent enzyme from *P. pastoris*, was observed to co-eluted with Etr1p as the only minor contaminant (<1%, i.e., at less than 100-fold lower concentration). To check activity of this alcohol dehydrogenase with crotonyl-CoA and NADPH, the enzyme was expressed separately using wild type *P. pastoris* with the same conditions as mentioned before, and was purified from cell extract using weak anion exchange and HisTrap FF column, as described above. Mitochondrial alcohol dehydrogenase isozyme III was shown to oxidize only ethanol and exclusively with NADH, as indicated by spectrophotometric analysis. The enzyme neither catalyzed the reduction of crotonyl-CoA to butyryl-CoA nor accelerate the decay of the ene-adduct, clearly demonstrating that this minor contaminant did not interfere with the results presented.

2.5.9 Preparation of YhdH.

A strain for expression of the *yhdH* gene of *E. coli* (*E. coli* JW3222) was obtained from the ASKA clone collection of the National BioResource Project (NBRP)-E.coli at the National Institute of Genetics (NIG; Mishima, Japan). *E. coli* JW3222 was grown in 400 ml Luria-Bertani broth containing 34 µg L⁻¹ chloramphenicol at 37 °C and 150-180 rotations per minute. Protein expression was induced when the optical density at 600 nm wavelength (OD₆₀₀) reached 0.6-0.8 by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final
concentration of 0.5 mM. Cells were harvested after additional growth for 3 hours by centrifugation (10 minutes at 10,000 x g and 4° C). Harvested cells (1-2 g) were suspended in 3-5 mL of 20 mM Tris·HCl, pH 7.8 containing 0.1 mg·mL⁻¹ of DNase I (AppliChem). The suspension was passed twice through a chilled French pressure cell at 137 MPa, and the cell lysate was centrifuged (40,000 xg) at 4 °C for 1 h. The supernatant was applied at a flow rate of 1 mL·min⁻¹ onto a 1-mL Ni-Sepharose Fast Flow Column (HisTrap FF; GE Healthcare) that had been equilibrated with 10 volumes of buffer A containing 20 mM Tris·HCl (pH 7.8) and 200 mM KCl. The column was washed with more than 20 column volumes of buffer A and buffer A containing 75 mM imidazole, before YhdH was eluted with buffer A containing 500 mM imidazole. The enzyme was desalted and concentrated by ultrafiltration (Amicon ultracel 10K membrane; Millipore). The protein was stored at -20 °C in 10 mM Na₂HPO₄ (pH 7.8) with 50% (v/v) glycerol. Protein concentrations were determined spectrophotometrically.
2.6 References


**Figure S1.** $^1$H-NMR kinetic analysis of the transient intermediate in the reaction mixture, recorded at 600 Mhz. Conditions: 1 mM crotonyl-CoA, 1 mM NADPH, 0.67 µM Ccr in 100 mM Na$_2$HPO$_4$ (pH 7.9, 10% D$_2$O) at 4.4 °C. (a) assigned $^1$H-NMR reference spectra of NADPH (purple), NADP$^+$ (blue), crotonyl-CoA (green) and butyryl-CoA (yellow), aligned to the $^1$H-NMR spectrum of the reduction reaction mixture at maximal intermediate concentration (red). Stars indicate peaks of the intermediate that could not be assigned to any compound of the starting material and that were used to follow kinetics of the intermediate by $^1$H-NMR array. (b) $^1$H-NMR array of the reduction reaction. Characteristic $^1$H-signals in the reaction mixture are highlighted as follows: NADPH, blue diamonds; crotonyl-CoA, blue dots; NADP$^+$, gray diamonds; butyryl-CoA, gray dots; intermediate, purple stars. The integrals of the indicated peaks were used to create Figure 2 (main text).
**Figure S2.** HPLC-ESI-MS analysis of the transient intermediate. Conditions: 1 mM crotonyl-CoA, 1 mM NADPH and 3 µM Ccr in 100 mM Na$_2$HPO$_4$ (pH 7.9) at 5 °C. (a) Kinetics of the peak area of the characteristic m/z=791.123 ion at different time points. The first time point (t=0 minutes) was taken before addition of Ccr. (b) Detailed m/z spectrum of t=55 minutes sample.
**Figure S3.** Two-dimensional NMR analysis of the transient intermediate (ene-adduct) in the reaction mixture, recorded at 600 MHz. Conditions: 15 mM NADPH, 15 mM crotonyl-CoA, and 7.5 μg Ccr were mixed in 100 mM NaHPO₄ (pH 7.9, 10% D₂O) were reacted at 25°C to generate the intermediate. Then, the reaction was stopped by raising the pH to 11, and NMR spectra were acquired at 4.4 °C. (a) DQF-COSY spectrum of the intermediate (b) HSQC spectrum of the intermediate in the reaction mixture. For corresponding reference spectra of NADPH, NADP⁺, butyryl-CoA, and crotonyl-CoA, see Figure S10.
Figure S4. (In)-stability of the ene-adduct in solution as followed by HPLC analysis. (a) HPLC analysis of subsequent injections of 0.5 mM ene-adduct dissolved in 100 mM Na₂HPO₄ (pH 7.9) incubated at ambient temperature (21°C) that shows the spontaneous decay of the ene-adduct into NADP⁺ and butyryl-CoA. The times indicated show the absolute time difference between dissolving the isolated, lyophilized ene-adduct in buffer and the time by which the ene-adduct passed the UV/Vis detector. (b) Fraction of the total injection integral at 260 nm for each component in the solution, fitted to a first order decay. The extrapolation to t=0 shows that the intermediate is pure, after lyophilization and before it is dissolved.
Figure S5. NMR analysis of the isolated ene-adduct. Selected correlations of the ene-adduct are highlighted as follows: bold lines, COSY correlations; arrows, $^1$H to $^{13}$C HMBC correlations. For full peak assignment, see Table S2.
Figure S6. HPLC-ESI-MS analysis of reaction products when using purified ene-intermediate as substrate. Products were confirmed by spiking with $^{13}$C-labeled standards. (a-f) Extracted ion chromatogram (EIC) of m/z = 836.162 (butyryl-CoA, [M+H]$^+$) and m/z = 882.154 (ethylmalonyl-CoA, [M+H]$^+$). (g-l) mass spectrum of (a-f) at 20.5 minutes.
**Figure S7.** Active centre of CinF, a Ccr homologue that reductively carboxylates octenoyl-CoA. The structure of CinF co-crystallized with NADP$^+$ and octenoyl-CoA (PDB-code 4a0s) shows that both reaction partners are well aligned for an ene reaction. Distances between the C2 and C4 of NAPD$^+$ to the Cα and Cβ of the 2-enoyl thioester are indicated as measured in structure.

**Figure S8.** Proposed ene-mechanism for the reduction and carboxylation reactions catalyzed by Ccr.
Figure S9. Apparent steady-state kinetic parameters for the reactions of Ccr, Etr1p and YhdH with crotonyl-CoA, NADPH, and isolated ene-adduct. The data are summarized in Table 1 (main text). (a-c) Characterization of the carboxylation reaction of Ccr in the presence of 50 mM NaHCO$_3$ and 0.7 µM carbonic anhydrase. (d-f) Characterization of the reduction reaction of Ccr. (g-i) Characterization of the reduction reaction of Etr1p. (j-l) Characterization of the reduction reaction of YhdH.
Figure S10: Reference NMR spectra, recorded at 600 MHz in 100 mM Na$_2$HPO$_4$ (pH 7.9) at 4.4 °C (a) COSY of butyryl-CoA (b) HSQC of butyryl-CoA (c) COSY of crotonyl-CoA (d) HSQC of crotonyl-CoA (e) HMBC of crotonyl-CoA (f) COSY of NADPH (g) HSQC of NADPH (h) COSY of NADP$^+$ (i) HSQC of NADP$^+$. COSY of butyryl-CoA.
HSQC of butyryl-CoA
COSY of crotonyl-CoA
HSQC of crotonyl-CoA
HMBC of crotonyl-CoA
COSY of NADPH
HSQC of NADPH
COSY of NADP⁺
HSQC of NADP⁺
**TABLES S1-S2**

**Table S1.** Formation and consumption rates of reaction products, substrates and intermediates of the reduction reaction. Rates were calculated from the data in Figure 2 according to first order decay.

<table>
<thead>
<tr>
<th>Rate</th>
<th>compound</th>
<th>( t_{1/2} ) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate consumption ( (t_{1/2}^{-s}) )</td>
<td>NADPH</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>crotonyl-CoA</td>
<td>17</td>
</tr>
<tr>
<td>Product formation ( (t_{1/2}^{+p}) )</td>
<td>NADP⁺</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>butyryl-CoA</td>
<td>63</td>
</tr>
<tr>
<td>Intermediate formation ( (t_{1/2}^{+i}) )</td>
<td>ene-adduct</td>
<td>( \approx 11 )</td>
</tr>
<tr>
<td>Intermediate consumption ( (t_{1/2}^{-i}) )</td>
<td>ene-adduct</td>
<td>57</td>
</tr>
</tbody>
</table>
Table S2. NMR analysis of the isolated ene-adduct. Peak assignment for the isolated ene-adduct recorded at 600 MHz in D$_2$O, 100 mM Na$_2$DPO$_4$ (pH 7.9) at 4.4 °C. The corresponding COSY NMR spectrum and important HMBC and COSY correlations are shown in Figure S5. See Figure S3 for the atom labeling used.

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H-shift</th>
<th>$^{13}$C-shift</th>
<th>HMBC</th>
<th>COSY</th>
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<tr>
<td>α</td>
<td>2.41</td>
<td>61</td>
<td>-</td>
<td>H$_{βa}$, H$_2$</td>
</tr>
<tr>
<td>$β_a$</td>
<td>1.01 (m, 1H)</td>
<td>21$^3_3$</td>
<td>-</td>
<td>H$_γ$, H$<em>α$, H$</em>{βb}$</td>
</tr>
<tr>
<td>$β_b$</td>
<td>1.14 (m, 1H)</td>
<td>21$^3_3$</td>
<td>-</td>
<td>H$_{βa}$</td>
</tr>
<tr>
<td>$γ$</td>
<td>0.29 (m, 3H)</td>
<td>11</td>
<td>C$_α$, C$_β$</td>
<td>H$_{βa}$,</td>
</tr>
<tr>
<td>2</td>
<td>4.38</td>
<td>56</td>
<td>C$_α$, C$_3$, C$_4$, C$_6$, 1'</td>
<td>H$_α$</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>111$^3_3$</td>
<td>-</td>
<td>-</td>
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<tr>
<td>4</td>
<td>6.40 (d, 1H)</td>
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<td>5</td>
<td>4.77 (t, 1H)</td>
<td>100</td>
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<td>6</td>
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<td>1'</td>
<td>4.50 (d, 1H)</td>
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<tr>
<td>2'</td>
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<td>70</td>
<td>1'</td>
<td>H$_1'$</td>
</tr>
<tr>
<td>3'</td>
<td>3.87</td>
<td>70</td>
<td>1'</td>
<td>-$^4$</td>
</tr>
<tr>
<td>4'</td>
<td>3.87</td>
<td>65</td>
<td>-</td>
<td>-$^4$</td>
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<tr>
<td>5$a'$</td>
<td>3.59</td>
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<td>66</td>
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<td>3.17 (dd)</td>
<td>72</td>
<td>-</td>
<td>H$_{1b''}$</td>
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<tr>
<td>1$_b''$</td>
<td>3.47 (dd, 1H)</td>
<td>72</td>
<td>-</td>
<td>H$_{1a''}$</td>
</tr>
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<td>---</td>
</tr>
<tr>
<td>2&quot;</td>
<td>-</td>
<td>38</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3&quot; 5</td>
<td>0.37 (s, 3H)</td>
<td>18</td>
<td>C₁&quot;, C₂&quot;, C₄&quot;, C₅&quot;</td>
<td>-</td>
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<tr>
<td>4&quot; 5</td>
<td>0.51 (s, 3H)</td>
<td>21</td>
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<td>74</td>
<td>C₁&quot;, C₂&quot;, C₃&quot;, C₄&quot;, C₆&quot;</td>
<td>-</td>
</tr>
<tr>
<td>6&quot;</td>
<td>-</td>
<td>175</td>
<td>-</td>
<td>-</td>
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<td>7&quot;</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8&quot;</td>
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<tr>
<td>10&quot;</td>
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<td>174</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11&quot;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12ₐ&quot;</td>
<td>2.81 (m, 1H)</td>
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<td>H₁₃&quot;</td>
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<td>12ₑ&quot;</td>
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<td>C₁₃&quot;, C₁₀&quot;</td>
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<td>13ₐ&quot;</td>
<td>2.50 (m, 1H)</td>
<td>28</td>
<td>C₁₂&quot;</td>
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<tr>
<td>13ₑ&quot;</td>
<td>2.44 (m)</td>
<td>28</td>
<td>C₁₂&quot;</td>
<td>H₁₂&quot;</td>
</tr>
</tbody>
</table>

¹ Diastereotopic protons are indicated with subscript 'a' and 'b', the upfield proton being assigned as proton 'a'. No absolute stereochemistry was measured.

² Where possible multiplicity and integral are given in brackets.

³ Chemical shift assigned from HMBC spectrum.

⁴ COSY signals overlap, therefore no unambiguous assignment was possible.

⁵ No absolute stereochemistry was determined in this study, however the upfield protons were assigned by Dordine et al. as belonging to the pro-S methyl (1).
2.6.1 Supplementary references

Chapter III: The use of ene adducts to study and engineer enoyl-thioester reductases

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This chapter has been published as:


Author contributions: R.G.R., B.V. and T.J.E. conceived and designed all experiments, with the exception of the NMR experiments, which were designed together with M.-O.E., and the MS analyses, which were designed together with P.K. and J.A.V. NMR experiments were performed by R.G.R., B.V. and M.-O.E. MS experiments were performed by B.V., R.G.R. and P.K. B.V. prepared enzyme crystals of Etr1p and mutants, N.Q. and G.C. collected the diffraction data and interpreted the results. Enzyme kinetic assays and stopped-flow measurements, as well as purification of the intermediate, were performed by R.G.R. and B.V. R.G.R., B.V. and T.J.E. wrote the paper.
3.1 Abstract

Enzymes are unmatched in catalytic proficiency and stereoselectivity, which makes them promising catalysts for chemistry, biocatalysis and industrial biotechnology. Here we describe a molecular probing approach that allows uncoupling of single catalytic steps to characterize the molecular basis of stereochemical control in enoyl thioester reductases (Etr), which are essential to fatty acid and polyketide biosynthesis. We could identify the long-sought cryptic proton donor of Etrs and provide an explanation why Etrs appear catalytically active even in the absence of essential residues. Exchanging the original proton donor and screening for new variants by molecular probing allowed us to invert Etr stereochemistry at wild-type like catalytic efficiency. Our results provide novel experimental tools to resolve and manipulate enzyme catalysis at unprecedented detail, call for the re-evaluation of numerous mutagenesis studies on NAD(P)H-dependent enzymes and lay the foundation for obtaining stereochemical control of reduction reactions in biosynthetic engineering and synthetic chemistry.

3.2 Introduction

Synthetic chemistry aims at achieving high regio- and stereoselectivity. Enzymes are Nature's solution to obtain both goals with unmatched catalytic efficiency. The demand for enzymes in green chemistry, biocatalysis and industrial biotechnology is reflected by the great efforts that are currently put into screening for novel enzyme activities and engineering of enzymes by rational or de novo approaches (1, 2). Computational design has proven to be a powerful tool for designing enzyme catalysts de novo (3, 4). Yet, the ab initio design of cofactor-dependent enzyme reactions, which are of great potential in application, is still outside the reach of the current state of the art. Such calculations involve the in silico alignment of multiple entities, including substrates, cofactors and catalytic residues for several subsequent catalytic steps which are not necessarily well understood. To truly functionalize cofactor-dependent enzyme reactions, experimental tools are therefore required that allow resolving catalysis beyond current limitations to open the possibility for novel engineering approaches and to provide the basis for developing novel strategies in computational design.
One of the evolutionarily oldest and largest superfamilies of cofactor-dependent enzymes is the class of medium chain dehydrogenase reductases (MDRs) that contains ketoreductases and enoylreductases (Etrs), which are essential in fatty acid as well as secondary metabolite biosynthesis (5). The ability to engineer the stereochemistry of these reduction reactions harnesses great potential because it enables the tailoring of natural products for pharmacological needs and in synthetic biology (6). The principle reaction catalyzed by Etrs is the NAD(P)H-dependent reduction of \( \alpha,\beta \)-unsaturated-thioesters into their saturated counterparts through addition of a hydride from NAD(P)H to the \( \beta \)-carbon and a proton from an active site acid or water-molecule to the \( \alpha \)-carbon of the enoyl-thioester. Even though this reaction is conserved in all domains of life and appears chemically simple, the biochemistry of Etr catalysis has remained enigmatic so far. Most notably, although several crystal structures of enoyl-thioester reductases have been solved, the nature of the proton donor that determines reaction stereochemistry is still cryptic in these enzymes (7-9). Establishing a structure-function relationship in Etrs is additionally hampered by the fact that no single knock-out of putative catalytic residues renders these enzymes completely inactive (7, 8, 10).

The obstacle to accurately study and manipulate the role of specific active site residues in the large MDR superfamily was recently put in a new perspective by the suggestion that these reductases could follow an ene-mechanism (11), which involves the formation of a transient covalent NADPH-substrate adduct ('C2-ene adduct') for the transfer of the hydride equivalent (Figure 1a; (12)). Besides having mechanistic implications, the observation of these C2-ene adducts opens new options in experimental biochemistry. These adducts represent catalytic intermediates in which the first half of the reduction reaction (i.e., the transfer of a hydride equivalent) has taken place, while protonation has not yet. Here we introduce the use of isolated C2-ene adduct as a molecular probing tool to dissect individual steps of enzyme catalysis by uncoupling enzymatic proton transfers from hydride transfers. Probing the catalytic cycle of Etr1p, a well-studied model from yeast mitochondria with isolated C2-ene adducts, allowed us to resolve the catalytic cycle of Etr1p at unprecedented detail, assigning tyrosine 79 as the proton donor, and controlling the stereochemistry of reduction by rationally inverting the active site chemistry of the enzyme.
3.3 Results

3.3.1 Identification of the cryptic proton donor in Etr1p.

For Etrs, several structures are available, but none as a ternary complex with (7-9). To better assess the role of active site amino acids and to identify the long-sought proton donor, we first solved the structure of yeast Etr1p in complex with crotonyl-CoA and NADP⁺ (protein data base: 4WAS, for data collection and refinement statistics refer to Supplementary Table 1). Inspection of the active site suggested a serine (S70), tyrosine (Y79), and threonine (T324) as possible proton donating residues (Figure 1b). These active site amino acids were individually mutagenized to obtain Etr1p mutants S70V, Y79F, and T324A, which were subsequently tested for enzymatic activity. To directly identify the cryptic proton donor of Etr1p, we used the C2-ene adduct of NADPH and crotonyl-CoA as molecular probe, which was enzymatically prepared in large scale (12). Starting the enzyme reaction with the molecular probe, we expected only mutations that are affected in proton transfer to be severely impaired in

Figure 1. Proposed reaction mechanisms of enoyl-thioester reductases WT and mutant enzymes. a) The classical mechanism for enoyl-thioester reductases (black arrows) assumes a direct hydride transfer from NAD(P)H onto the substrate to create an enolate intermediate that is subsequently protonated to yield the reaction products. The ene-mechanism (blue arrows) proceeds through a distinct C2-ene intermediate that was experimentally verified recently (11, 12). This intermediate is further converted to the consensus enolate intermediate (in brackets) and subsequently protonated. b) Active site of Etr1p WT showing potential proton donors S70, Y79 and T324.
activity (for the natural substrates see Supplementary Table 2). Compared to Etr1p wild type (WT), Etr1p Y79F was affected by more than two orders of magnitude in conversion of the C2-ene adduct, indicating a direct role for this residue in proton donation. In contrast Etr1p S70V and T324A showed only minor losses in catalytic activities (Table 1). This loss in activity corresponds with a secondary role of these active site residues in catalysis (e.g., in transition state stabilization by hydrogen bonding (13, 14)).

Table 1. Kinetic parameters for Etr1p WT and mutants on the C2-ene adduct.

<table>
<thead>
<tr>
<th>Enzyme variant</th>
<th>$k_{cat}^{app}$ (s$^{-1}$)</th>
<th>$K_m^{app}$ (µM)</th>
<th>$k_{cat}^{app}/K_m^{app}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.5 ± 0.5</td>
<td>3 ± 2</td>
<td>1.2±0.8·10^6 (100%)</td>
</tr>
<tr>
<td>S70V</td>
<td>0.73 ± 0.13</td>
<td>4 ± 3</td>
<td>1.8 ± 1.4·10^5 (15.6%)</td>
</tr>
<tr>
<td>T324A</td>
<td>0.35 ± 0.04</td>
<td>3 ± 2</td>
<td>1.2 ± 0.8·10^5 (10.0%)</td>
</tr>
<tr>
<td>Y79F</td>
<td>0.019 ± 0.001</td>
<td>3 ± 1</td>
<td>6.3 ± 2.1·10^3 (0.5%)</td>
</tr>
</tbody>
</table>

3.3.2 A dead-end product is formed by Etr1p Y79F.

In line with previous investigations on the natural substrates(7, 8), but nevertheless surprising, was the observation that Etr1p Y79F still catalyzed the consumption of the molecular probe even though it apparently lacked a key active site residue. To investigate the residual activity of Etr1p Y79F, the reaction with the molecular probe was monitored at multiple wavelengths. In contrast to the WT enzyme that simply consumed the C2-ene adduct as shown by its rapid depletion at $\lambda_{max} = 370$ nm, consumption of the molecular probe by Etr1p Y79F was accompanied with the buildup of a signal at $\lambda_{max} = 328$ nm. This buildup indicated that Etr1p Y79F was not able to protonate the probe as the WT enzyme, but instead formed another UV-vis active product (Figure 2a, b). The UV-vis active product then slowly disappeared, as indicated by the decrease of signal at 328 nm over time. To characterize this novel product, the
compound was purified from the reaction mixture and subjected to ultraviolet-visible spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectrometry, which demonstrated that the species was a covalent C4/Cα-adduct of NADPH and crotonyl-CoA (Figure 3, Supplementary Fig. 1-3). In contrast to the C2-ene adduct that was reported to be a bona fide substrate for Etr1p WT (12), as well as the MDR-family members Ccr, a crotonyl-CoA carboxylase/reductase (15, 16), and YhdH, an acrylyl-CoA reductase (17), the isolated C4/Cα-ene adduct was not converted by these enzymes. Instead, the C4/Cα-adduct acted as a competitive inhibitor for NADPH in Etr1p WT with a Ki.

Figure 2. Scanning kinetic assays of Etr1p WT and mutants using the C2-ene adduct as molecular probe. (a) Etr1p WT showing rapid depletion of the molecular probe. (b) Etr1p Y79F, deficient in proton donation. Formation of a novel photoactive species (C4/Cα-adduct) with an absorption maximum at 328 nm is observed. (c) Etr1p Y79F W322K. Formation of C4/Cα-adduct shows that lysine does not serve as a proton donor. (d) Etr1p Y79F W322E, behaves similar to Etr1p WT suggesting that
of 70 ± 45 nM and as a non-competitive inhibitor with respect to crotonyl-CoA, decaying slowly in solution with a first order constant of $k = 0.02490 ± 0.00001 \text{ min}^{-1}$ at 31 °C (Supplementary Fig. 4). These properties suggested that the C4/C$\alpha$-adduct represents a dead-end metabolite rather than a catalytic intermediate.

### 3.3.3 Resolving catalysis of Etr1p Y79F by NMR.

The detection of the C4/C$\alpha$-adduct in assays started with C2-ene adduct raised the question whether formation of this C4/C$\alpha$-adduct might be also of importance for the complete catalytic cycle of Etr1p Y79F. To that end, we monitored the full reaction of the mutant enzyme by $^1$H-NMR, starting the assay with the original substrates NADPH and crotonyl-CoA. During the initial phase of the reaction, the substrates were consumed quickly and converted into the C2-ene adduct of crotonyl-CoA and NADPH. The C2-ene adduct was then further transformed into the C4/C$\alpha$-adduct, which subsequently decomposed slowly into the final products butyryl-CoA and NADP$^+$. This order of events was independently confirmed by stopped flow measurements (Supplementary Fig. 5), and the reaction kinetics could be modeled according to reactions 1-4 (Figure 3).

$$
crotyl - CoA + NADPH \xrightarrow{Etr1p Y79F} C2-ene adduct \hspace{1cm} (1)
$$

$$
C2-ene adduct \xrightarrow{Etr1p Y79F} C4/C\alpha- adduct \hspace{1cm} (2)
$$

$$
C2-ene adduct \xrightarrow{uncat.} butyryl - CoA + NADPH \hspace{1cm} (3)
$$

$$
C4/C\alpha- adduct \xrightarrow{uncat.} butyryl - CoA + NADPH \hspace{1cm} (4)
$$

While our results reveal a surprising complexity of events in the Etr1p mutant reaction, they also provide the first real explanation for the enigmatic 'residual activity' that has been reported to persist in Etr1p and other NAD(P)H-

---

the glutamate functions as novel proton donor (for all enzymes screened, refer to Figure S3).
dependent reductases in the absence of the putative proton donor (7, 8, 10, 18, 19). First, and in line with an ene-mechanism (12), these enzymes still catalyze the initial transfer of a hydride equivalent, as shown by formation of the C2-ene adduct. However, because the subsequent enzymatic resolution of the C2-ene adduct by protonation is prevented, this leads to the formation of the

![Diagram of the reaction scheme](image)

**Figure 3. Time course of an Etr1p Y79F enzyme assay starting from crotonyl-CoA and NADPH.** The characteristic peaks from 1H-NMR spectra were integrated, to follow the kinetics of the substrates NADPH (dark blue) and crotonyl-CoA (light blue), the labile C2 intermediate species (purple) and the C4/Cα-adduct (dark red) as well as the products butyryl-CoA (red) and NADP⁺ (orange). The dotted lines represent a fit of the data, collected for 500 min, modeled according to reactions (1)-(4) and simulated to 1,500 min. The proposed reaction scheme is shown below with all the compounds in corresponding coloring.
unproductive, dead-end C4/Cα-adduct. Being metastable in solution, the latter compound decays non-enzymatically into the thermodynamically favored products butyryl-CoA and NADP+, which are also the products of the reaction catalyzed by the WT enzyme. To our knowledge, all these fundamental events have gone unnoticed so far. Since activity assays for NAD(P)H-dependent enzymes are routinely carried out at a fixed wavelength, and because the different transient adducts share overlapping spectra with the NAD(P)H chromophore these side reactions have been apparently misinterpreted and simply summarized as 'enzymatic activity' in the past. In the light of these findings, we note that the outcome of many earlier studies on NAD(P)H-dependent enzymes and their mutant variants need to be carefully re-evaluated to truly understand the underlying (enzyme) chemistry.

3.3.4 Screening for a new proton donor with the molecular probe.

A long-standing goal in biocatalysis is the stereochemical control of protonation. This holds especially for MDRs. As mentioned earlier, these enzymes are directly responsible for determining chiral centers in polyketide biosynthesis. Consequently, MDRs have become important targets in synthetic biology and chemistry for the engineering of widely used pharmaceuticals, as put forward recently (10, 20-22). Engineering proton donation in these enzymes, however, requires distinguishing between bona fide protonation events and unproductive formation of dead-end products (vide supra). As demonstrated above, the molecular probing approach represents an exquisite tool to differentiate these different outcomes, allowing for facile engineering of new proton donors. Consequently, we aimed at re-designing the last step of Etr1p catalysis by changing the stereochemistry of protonation from re- to si-face as a proof-of-principle (10, 20). To prevent competition for substrate protonation from the re-face, we started with the Y79F mutant and sought to introduce additional residues that could serve as si-face proton donor. Inspection of Etr1p’s active site suggested position 322 as good candidate for placing the new proton donor. When the corresponding double mutants were screened with the molecular probe, only the Y79F/W322E double mutant was able to directly consume the C2-ene adduct like the WT enzyme, suggesting that this variant contained a competent si-face proton donor (Figure 2c). In contrast, mutants Y79F/W322D, Y79F/W322H, and Y79F/W322K showed only unproductive conversion of the
molecular probe into C4/Cα-adduct, much like the Y79F single mutant (Figure 2d, Supplementary Fig. 6). Without the ability to directly probe proton donation with the C2-ene adduct, these unproductive mutants would have been difficult to distinguish from productive variants (e.g., Etr1p Y79F/W322E). This is because the complex reaction sequence at the active site of Erts cannot easily be reflected by screening for turnover cutoff values. Simply put, screening for the full reaction with standard assays (*i.e.*, conversion of natural substrates) cannot distinguish between slow productive and unproductive mutants due to their 'persistent activity'.

### 3.3.5 The stereochemistry of Etr1p Y79F/W322E is completely inverted.

The successful engineering of a pro-2S-specific enoyl-CoA reductase was confirmed by isotopic labeling, demonstrating that the engineered enzyme incorporated the deuteron with an efficiency of $97 \pm 2\%$ into the 2S-position (compared to $2 \pm 2\%$ for the WT, for Etr Y79F and W322E see Supplementary Table 3). Analysis of a Y79F/W322E crystal structure (protein data base: 4W99, Supplementary Table 1) showed that E322 is held in position by hydrogen bonds to the backbone amide and T324 (Supplementary Fig. 7) and positioned in close proximity to the substrate to either mediate protonation directly or through a water molecule. The relative orientation of E322 to the substrate explains the highly stereo selective transfer of the proton in this variant. The pH optimum of Etr1p Y79F/W322E was lower compared to Etr1p WT, either due to a reduced $pK_a$ of the proton donating *si*-glutamate or a mediating water molecule compared to the *re*-tyrosine of the WT enzyme (Supplementary Fig. 8). When the kinetic parameters of Etr1p Y79F/W322E were assessed at the optimum pH of 5, the *si*-face engineered enzyme showed a catalytic efficiency of $3 \cdot 10^6 \text{ s}^{-1} \text{ M}^{-1}$, which is only a factor four below the *re*-specific WT at its optimum pH (Table 1). Given the fact that our design was solely based on the newly obtained insights into the biochemistry of MDRs and did not involve directed evolution, the high catalytic efficiency achieved is remarkable.

### 3.4 Discussion

The enigmatic residual activity in Etr1p and other reductases has been an open question in biology without a satisfying explanation. Using molecular probing,
we could show that a complex sequence of reactions gives a detailed answer as to why many reductases can still function, even after removal of residues thought to be essential. At the same time, our results suggest that many enzyme studies in the past might have been systematically flawed by the misinterpretation of spectrophotometric assays that are widely used in biology and chemistry. Because of overlapping spectral information of the different transient adducts, the chemical complexity of (mutagenized) enzymes cannot be simply untangled and side reactions cannot be easily distinguished from 'regular' enzymatic activity. We suggest that future mutageneosis studies on NAD(P)H-dependent enzymes should make use of molecular probing, or be at least performed at multiple wavelengths and preferably, in addition, by reaction monitoring with NMR-spectroscopy to minimize the chance of data misinterpretation.

The ability to resolve the biochemistry of Etr1p at unprecedented detail with molecular probing, opened the possibility to rationally engineer catalysis. In a proof of principle, we could completely invert the stereochemical outcome of the reduction reaction with just a minor loss of activity. These results provide the theoretical and practical basis for absolute stereochemical control in reduction reactions and open the door for the (bio)synthetic production and directed manipulation of complex chemical compounds (e.g., by further translating our engineering strategy to enzymes involved in polyketide biosynthesis).

Lastly, our results also have implications for understanding the evolution of novel enzymatic functions. The observation that hydride equivalent transfer and protonation can be separated by transient substrate-NAD(P)H adducts, as shown here for Etr1p by molecular probing, demonstrates an implicit modularity of catalysis that might facilitate the ability of cofactor-dependent enzymes to diverge in both substrate- and reaction-specificity. As soon as novel substrates can be accommodated at the active site of these enzymes that allow for formation of such adducts, 'catalysis' can take place through non-enzymatic decay, even when active site residues for subsequent steps of the catalytic cycle might not be positioned correctly or be completely absent (for the proposed mechanism, see Supplementary Fig. 9). Evolutionary pressure could then select for variants that convert the new substrate with higher efficiency. Notable examples for such scenarios might be the evolution of 2S-
stereoselectivity in methylenoyl-thioester reductase domains of polyketide synthases and FAS I megasynthases from their 2R-specific ancestors, or the emergence of the CO₂-fixation function in the scaffold of ordinary reductases, that are evolutionarily not understood (15, 16).

Taken together, molecular probing, as demonstrated here, provides a promising tool to dissect the catalytic cycle of reductases, and lays the foundation for engineering absolute stereochemical control of reduction reactions.
3.5 Methods

3.5.1 Chemicals.

Butyryl-CoA and NADP⁺ and crotonic anhydride were purchased from Sigma Aldrich AG, coenzyme A from Roche Diagnostics. Crotonyl-CoA and C2-ene adduct were synthesized and purified according to (12). All salts and solvents were of analytical grade.

3.5.2 Cloning and mutagenesis.

The gene encoding for enoyl thioester reductase (etp1p) was codon optimized for expression in Methylobacterium extorquens and synthesized by Eurofins MWG Operon (Ebersberg, Germany), see below for codon optimized sequence. An NdeI restriction site followed by an N-terminal His₁₀-tag was added in front of the start codon and an EcoRI site after the stop codon. The synthesized gene was cloned into the expression vector pT7-7 (23) yielding pTE260. The plasmids carrying desired point mutations were generated using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) with 60 ng of template plasmid and the primers listed in Materials and methods table 1. The resulting mutated plasmids were confirmed by sequencing (Microsynth AG, Balgach, Switzerland). Plasmids carrying the correct mutations were transformed into E.coli BL21(DE3) for protein expression.

DNA sequence of Etr1p WT, His₁₀-tagged and codon optimized for expression in Methylobacterium extorquens:

```
ATGGGACCACCACATCATCACACCACCACCATCACACCACCTCCTCGGGCCACA
TCGAAGGCGGCGCACATGATCAGGGCCAAAGCCGTCTCTCTACACGCAGCA
TGGCGAGCCCAAGGACGTGCTGTTCACGCAGTCCTTCGAGATCGACGAC
GACAACCTGGCGGCCAGGAGGTGATCGTCAAGACCCTGGGCTCGCCG
GTCAACCCGAGCGACATCAACCAGATCCAGGGGTCTATCCGAGCAAGC
CGGCCAAGAACCCCGGTTCCGGCACACCAGCGCCCGGCGCCGCTGC
GGCAACGAGGGCCTCTTCTGAGGATCAAGGTCGGCTCGAACGTGAGC
TCGCTCGAAGCAGCGGACTGGGTGATCCCCTCGACGTAACCTTCGGC
ACCTGGGCGCAGCGCAGCCCTTGggGAACGACGACTTCCATCAAGGCTC
```
Materials and methods table 1. Primers used for quick-change mutagenesis in this study.

<table>
<thead>
<tr>
<th>Primer and mutation introduced</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etr1p S70V forward</td>
<td>CCGGTCAACCCCGGTGACATCAACCACAG</td>
</tr>
<tr>
<td>Etr1p S70V reverse</td>
<td>CTGGTTGATGTCGACCCGGGTTGACCGG</td>
</tr>
<tr>
<td>Etr1p Y79F forward</td>
<td>GATCCAGGGCGTCTTTCCGAGCAAGCCG</td>
</tr>
<tr>
<td>Etr1p Y79F reverse</td>
<td>CGGCTTGTGCTCGAAAGACGCCCCTGGATC</td>
</tr>
<tr>
<td>Etr1p W322K forward</td>
<td>CTCCGGCGGCTTCAAGGTGACCGAGCTG</td>
</tr>
<tr>
<td>Etr1p W322K reverse</td>
<td>CAGCTCGGTCACCTTAAGAGCCCGGAG</td>
</tr>
<tr>
<td>Etr1p W322D forward</td>
<td>CTTCGCGGAGCTGACGTGGCAGCGCTG</td>
</tr>
</tbody>
</table>
3.5.3 Protein production.

A single colony of *E. coli* BL21 (DE3) bearing the correct plasmid (Materials and Methods table 2.) was grown over night in LB medium containing 100 μg/ml ampicillin at 37 °C. Of this overnight culture 1 ml was used to inoculate 1 l of auto-inducing medium (24) containing 100 μg/ml ampicillin. Cells were grown for 24 h at 30 °C under constant shaking before harvesting by centrifugation (10 min at 8000 x g, 4 °C). Harvested cells were resuspended in 15 mL of buffer A (20 mM Tris(hydroxymethyl)aminomethane (Tris-HCl), pH 7.8, 200 mM KCl) containing 0.1 mg/ml of DNase I (*Bovine* pancreas, AppliChem) and 10% glycerol. The solution was sonicated 6 times for 20 s with 40 s cooling on ice in between cycles. The lysate was centrifuged for 1 h at 40,000 x g, 4 °C. The supernatant was applied at a flow rate of 1 ml/min onto an equilibrated HisTrap FF (GE Healthcare, Little Chalfont, UK) and washed with 30 column volumes of buffer A containing 75 mM imidazole. The protein was then eluted with buffer A containing 500 mM imidazole, desalted with a PD-10 desalting column (GE Healthcare, Little Chalfont, UK) and concentrated by ultrafiltration (Amicon® Ultra-4 30 K centrifugal filters, Merk Millipore). For NMR experiments the protein was exchanged into a buffer containing 100 mM Na₂HPO₄ pH 7.9, 200 mM NaCl, stored on ice and used the same day. For crystallization the protein was stored in 20 mM TrisHCl pH 7.9, 100 mM NaCl at 4 °C and for spectrophotometric assays the protein was stored at -20 °C in 50 mM Na₂HPO₄
pH 7.9, 100 mM NaCl with 50% glycerol. Protein concentrations were determined spectrophotometrically \((\varepsilon_{280\ \text{nm}} = 49.5 \ \text{cm}^{-1} \ \text{mM}^{-1})\). Mutations were confirmed additionally by trypsin digestion and subsequent peptide sequencing at the functional genomics center, Zurich.

**Materials and methods table 2.** Primers used for quick-change mutagenesis in this study.

<table>
<thead>
<tr>
<th>Plasmid (and backbone)</th>
<th>Relevant properties/ mutation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT7-7</td>
<td>pT7phi10 promoter, Ampicillin resistance</td>
<td>(23)</td>
</tr>
<tr>
<td>pTE260 (pT7-7)</td>
<td>etr1p codonoptimized for <em>M. extorquens</em>, N-terminal His(_{10}) tag</td>
<td>This work</td>
</tr>
<tr>
<td>pTE261 (pT7-7)</td>
<td>etr1p agc2606gtc</td>
<td>S70V</td>
</tr>
<tr>
<td>pTE263 (pT7-7)</td>
<td>etr1p tat2633ttt</td>
<td>Y79F</td>
</tr>
<tr>
<td>pTE267 (pT7-7)</td>
<td>etr1p acc3368gcc</td>
<td>T324A</td>
</tr>
<tr>
<td>pTE279 (pT7-7)</td>
<td>etr1p tat2633ttt tgg3362gaa</td>
<td>Y79F W322K</td>
</tr>
<tr>
<td>pTE282 (pT7-7)</td>
<td>etr1p tgg3362gaa</td>
<td>W322E</td>
</tr>
<tr>
<td>pTE283 (pT7-7)</td>
<td>etr1p tat2633ttt tgg3362gaa</td>
<td>Y79F W322E</td>
</tr>
<tr>
<td>pTE603 (pT7-7)</td>
<td>etr1p tat2633ttt tgg3362cat</td>
<td>Y79F W322H</td>
</tr>
<tr>
<td>pTE71 (pET23 b)</td>
<td>Ccr C-terminal His(_{6})-tagged (<em>M. extorquens</em>), ampicillin resistance</td>
<td>(12)</td>
</tr>
</tbody>
</table>

### 3.5.4 Spectrophotometric enzyme assays.

Assays were carried out on a Carry-50 UV/Vis spectrometer (Agilent) at 30 °C using quartz cuvettes (1-, 3-, or 10-mm path-length; Hellma). All assays were
carried out in 100 mM Na₂HPO₄, pH 7.9. For determination of the kinetic parameters starting from NADPH and crotonyl-CoA assays contained 200 μM NADPH and were started by adding enzyme to the following end concentrations: 3.3 nM Etr1p WT, 39 nM Etr1p S70V, 11 nM Etr1p T324A or 6.8 μM Etr1p Y79F. Kinetic parameters were determined by varying the crotonyl-CoA concentration and following disappearance of NADPH at 340 nm using an absorption coefficient of $\varepsilon_{340\text{ nm}} = 6.2 \text{ cm}^{-1} \text{ mM}^{-1}$ and for Etr1p Y79F $\Delta \varepsilon_{340\text{ nm}} = 2.5 \text{ cm}^{-1} \text{ mM}^{-1}$ ($\varepsilon_{340\text{ nm}} \text{ NADPH} = 6.2 \text{ cm}^{-1} \text{ mM}^{-1}$, $\Delta \varepsilon_{340\text{ nm}} \text{ C4} = 3.7 \text{ cm}^{-1} \text{ mM}^{-1}$ was used. Assays for determination of kinetic parameters on C2-ene adduct were started by adding varying concentrations of C2-ene adduct and following its disappearance at 370 nm using an extinction coefficient of $\varepsilon_{370\text{ nm}} = 6.9 \text{ cm}^{-1} \text{ mM}^{-1}$ (12).

The screen for protonation of C2-ene adduct was done by monitoring the kinetics between 300 and 500 nm adding 0.033 μM Etr1p WT, 34 μM Etr1p Y79F, 9.3 μM Etr1p Y79F W322K, 3.4μM Etr1p Y79F W322D or 0.62 μM Etr1p Y79F W322E to start the reaction. For these assays the C2-ene adduct was dissolved directly into the assay buffer immediately before the assay was started.

For the inhibition assay C4-adduct determined using $\Delta \varepsilon_{340\text{ nm}} \text{ C4} = 3.7 \text{ cm}^{-1} \text{ mM}^{-1}$ was added to an end concentration of 5 nM to a reaction mixture containing 125 μM crotonyl-CoA and varying concentrations of NADPH or to assays containing 100 μM NADPH and varying the crotonyl-CoA concentration. The reactions were started by adding 3.3 nM Etr1p WT.

3.5.5 Purification of C4/Cα -adduct.

A reaction mixture of 30 mM crotonyl-CoA, 30 mM NADPH and 0.17 mM Etr1p Y79F in 100 mM Na₂HPO₄ (pH 7.9, 100 μl) was reacted at 30 °C for 1.5 h. The mixture was separated on a Waters 2690 HPLC system on a Gemini 10 μm NX-C18 110 Å, 100 x 21.2 mm, AxiA packed column (Phenomenex) using a previously described elusion profile (12). The C4-adduct was collected directly into liquid nitrogen, lyophilized (0.01 mbar, -70 °C) and stored at -80 °C. C4-ene adduct was dissolved in 100 mM Na₂HPO₄ buffer and its absorption spectrum was measured between 200 and 400 nm (Figure S1). The extinction
coefficients at 328 nm and 340 nm were calculated to be \(4.1 \pm 0.2 \text{ cm}^{-1}\text{mM}^{-1}\) and \(3.7 \pm 0.2 \text{ cm}^{-1}\text{mM}^{-1}\) assuming a total absorbance of \(33.8 \text{ cm}^{-1}\text{mM}^{-1}\) at 260 nm \(([\varepsilon_{260} \text{ nm NADPH} + \varepsilon_{260} \text{ nm CoA}] = [16.9 \text{ cm}^{-1} \text{ mM}^{-1} + 16.9 \text{ cm}^{-1} \text{ mM}^{-1}] \text{ (25)}\)).

### 3.5.6 HPLC-ESI-MS analysis.

HPLC-ESI-MS analysis of the C4/Cα-adduct was performed exactly as described earlier for the C2-ene adduct (12).

### 3.5.7 Crystallization of Etr1p WT and Y79F/W322E.

Crystallization was performed similarly to established protocols (26). In short, first crystals were obtained by sitting drop vapor diffusion using 15 mg/mL enzyme in crystallization buffer mixed in a 1:1 ratio with reservoir solution containing 0.1 M N-(2-acetamido)-2-iminodiacetic acid (ADA)/NaOH (pH 6.3), 2 M \((\text{NH}_4)_2\text{SO}_4\), 20 mM crotonyl-CoA and 10 mM NADP+ at 19 °C. Large crystals were obtained by streak seeding 20 h after setting up crystallization plates and they reached their final size after a few weeks. For cryostabilisation a cryosolution containing 0.1 M ADA pH 6.3, 2.4 M \((\text{NH}_4)_2\text{SO}_4\) and 25% glycerol was added and the crystal immediately frozen in liquid nitrogen.

### 3.5.8 Structural data collection and refinement.

The crystals were measured at beamline X06SA at the Swiss light source, SLS. The data processing was carried out using XDS (27) and scaling was done with XSCALE. A set of randomly chosen reflections were set aside for the calculation of the free R factor (Rfree). To obtain initial phases molecular replacement by Phaser (28) was performed using the structure of Etr1p containing NADP+ (1N9G) as a search model, finding two and three monomers in the asymmetric unit for the double mutant and the wild type complex, respectively. The structures were refined with REFMAC5 (29) and Phenix (wt complex) and completed in Coot (30). For the double mutant, clear difference density for the mutated side chains was visible. Water molecules were added and edited manually as well as using the water find tool in Coot with a cutoff of 1 sigma within hydrogen bonding distance to protein residues. A final TLS refinement
step using five TLS groups per monomer as determined by the TLS Motion Determination Server (31) was carried out with REFMAC5 for the double mutant.

3.5.9 Stereospecific incorporation of solvent protons at Cα by Etr1p WT and mutants.

HPLC-ESI-MS analysis was performed as described previously (32). For the isotope labeling experiment the storage buffer of Etr1p WT, Etr1p Y79F, Etr1p W322E and Etr1p Y79F W322E was exchanged by three subsequent ultracentrifugation steps (Amicon® Ultra-4 30 K centrifugal filters, Merck Millipore) diluting the original buffer over 10,000 x with deuterated (98%) 50 mM Na₂DPO₄, pH 7.9. 100 μl assays containing 210 μM NADPH and 200 μM crotonyl-CoA in 100 mM NaD2PO4 buffer (pD 7.9) were started with 0.07 μM Etr1p WT, 0.1 μM Etr1p W322E, 0.5 μM Etr1p Y79F W322E or 5.4 μM Etr1p Y79F. The reactions were followed spectrophotometrically at 340 nm and run at 30 °C until complete consumption of NADPH and decay of formed C4-adduct, in case of Etr1p Y79F, occurred. Butyryl-CoA was purified from the reaction mixture using the above-mentioned HPLC protocol, lyophilized (0.01 mbar, -70 °C) and dissolved in 100 μl 50 mM Na₂HPO₄ (pH 7.9). Label incorporation was checked by HPLC-ESI-MS. The samples were then incubated for 1 h at 37 °C in a reaction mixture containing 2 mM ferrocenium hexafluorophosphate (Sigma Aldrich), 2 μM FAD (Sigma Aldrich) and 2 μM Homo sapiens SCAD (expressed and purified from SGC clone 2VIG, IMAGE:4842286, according to the provided protocols http://www.thesgc.org/). The reaction was quenched by adding 1 μl of 50% formic acid, centrifuged for 3 min at 16,000 x g and the supernatant analyzed by HPLC-ESI-MS.

3.5.10 Nuclear magnetic resonance spectroscopy and curve fitting

NMR analysis was done on a Bruker 600 MHz spectrometer at 4.4 °C. For NMR reaction kinetics of Etr1p Y79F a previously described method was used (12). Water suppression using excitation sculpting was set on a 570 μl mixture containing 50 mM Na₂HPO₄ pH 7.9, 5% D₂O, 10 mM crotonyl-CoA and 5 mM NADPH. 30 μl of a freshly purified 8.16 mg/mL Etr1p Y79F solution was added and ¹H-NMR spectra recorded every 3 min for 500 min. For structural
characterization of isolated C4-adduct, 5 mg of purified lyophilized C4-adduct was dissolved in 600 \( \mu l \) D\(_2\)O to acquire \(^1\)H-NMR, COSY, HSQC, NOE and HMBC-spectra.

Characteristic peaks of all reaction components were integrated and plotted against time. These data points were fitted to equations 1-4 of which equations 1 and 2 were fitted according to enzyme catalyzed Michaelis Menten models and 3 and 4 according to uncatalyzed decay. Fitting was performed in COPASI (33).

3.5.11 Stopped flow spectroscopy.

Measurements were recorded on a thermostated stopped flow unit (SX17MN, Applied Photophysics, Leatherhead, UK) and detected by a 32-channel 0.2 ms photomultiplier array (Hamamatsu Photonics, SZK, Japan). Syringe 1 contained 100 \( \mu M \) NADPH and 100 \( \mu M \) crotonyl-CoA (left out in control experiment) in 100 mM Na\(_2\)HPO\(_4\) buffer pH 7.9 and syringe 2 contained 40 \( \mu M \) of Etr1p Y79F in the same buffer at 5 °C. Data was collected every 0.5 ms for 1 s and the every 5 ms for a total of 15 s covering a range between 300 and 420 nm.
3.6 References


### 3.7 Supplementary Information

**Supplementary Table 1.** Etr1p WT and Y79F W322E data collection and refinement statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Etr1p Y79F W322E apo enzyme</th>
<th>Etr1p/NADP⁺/Crotonyl-CoA Complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein data base accession</td>
<td>4W99</td>
<td>4WAS</td>
</tr>
<tr>
<td>Beamline</td>
<td>SLS X06SA</td>
<td>SLS X06SA</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>C2</td>
</tr>
<tr>
<td>Unit cell dimensions $a$, $b$, $c$ (Å)</td>
<td>66.33, 100.93, 80.54</td>
<td>147.92, 106.14, 94.41</td>
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<tr>
<td>$\alpha,\beta,\gamma$ (°)</td>
<td>90, 102.5, 90</td>
<td>90, 98.41, 90</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.0</td>
<td>1.000</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>39.9-2.0 (2.12-2.0)</td>
<td>50.0-1.7 (1.9-1.7)</td>
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<td>Measured reflections</td>
<td>210226 (30767)</td>
<td>483333 (134383)</td>
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<tr>
<td>Unique reflections</td>
<td>69318 (10914)</td>
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<tr>
<td>$I/\sigma_I$</td>
<td>12.65 (1.72)</td>
<td>10.6 (1.0)</td>
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<tr>
<td>Completeness (%)</td>
<td>98.2 (95.9)</td>
<td>99.0 (98.9)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.03 (2.8)</td>
<td>3.1 (3.0)</td>
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<tr>
<td>$R_{\text{merge}}$ (%)</td>
<td>5.8 (68.8)</td>
<td>4.8 (111.2)</td>
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<tr>
<td>Wilson B factor (Å²)</td>
<td>47.7</td>
<td>40.9</td>
</tr>
<tr>
<td>Monomers in a.s.u.</td>
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<td>3</td>
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<tr>
<td>$R_{\text{work}}$ (%)</td>
<td>17.14</td>
<td>18.9</td>
</tr>
<tr>
<td>$R_{\text{free}}$ (%)</td>
<td>21.41</td>
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<tr>
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<tr>
<td>- Protein</td>
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<td>- Ligand</td>
<td>-</td>
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<tr>
<td>----------------</td>
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<td>-------</td>
</tr>
<tr>
<td>- Solvent</td>
<td>763</td>
<td>517</td>
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R.m.s.d. from ideal geometry

<p>| | | |</p>
<table>
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<tr>
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<tr>
<td>- Bond lengths (Å)</td>
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<td>1.05</td>
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Ramachandran plot (%)

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<tr>
<td>- favoured</td>
<td>97.1</td>
<td>97.2</td>
</tr>
<tr>
<td>- allowed</td>
<td>2.6</td>
<td>2.8</td>
</tr>
<tr>
<td>- outliers</td>
<td>0.3</td>
<td>0</td>
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Supplementary Table 2. Kinetic parameters on crotonyl-CoA and NADPH.
Measured by varying the crotonyl-CoA concentration in presence of 0.2 mM NADPH.

<table>
<thead>
<tr>
<th>enzyme variant</th>
<th>$k_{cat}^{app}$ (s$^{-1}$)</th>
<th>$K_m^{app}$ (µM)</th>
<th>$k_{cat}^{app} / K_m^{app}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etr1p WT</td>
<td>124 ± 9</td>
<td>15 ± 2</td>
<td>8.3 ± 1.3·10$^6$ (100%)</td>
</tr>
<tr>
<td>Etr1p S70V</td>
<td>11 ± 1</td>
<td>48 ± 6</td>
<td>2.3 ± 0.4·10$^5$ (2.8%)</td>
</tr>
<tr>
<td>Etr1p T324A</td>
<td>75 ± 4</td>
<td>81 ± 6</td>
<td>9.3 ± 0.8·10$^5$ (11.2%)</td>
</tr>
<tr>
<td>Etr1p Y79F</td>
<td>0.011 ± 0.002</td>
<td>44 ± 8</td>
<td>1.0·10$^2$ (0.0031%)</td>
</tr>
</tbody>
</table>

Supplementary Table 3. si-face selectivity of different engineered Etr1p variants. The si-face selectivity was measured by treating the 2-[²H]-butyryl-CoA formed by Etr1p and its mutants in [²H]$_2$O with an acyl-dehydrogenase that selectively removes the 2R-proton ((34) the enzyme here was the analogue from *H. sapiens*).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% label retention</th>
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<tbody>
<tr>
<td>Etr1p WT</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>Etr1p W322E</td>
<td>2 ± 3</td>
</tr>
<tr>
<td>Etr1p Y79F</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>Etr1p Y79F W322E</td>
<td>97 ± 2</td>
</tr>
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</table>
Supplementary Figure 1. UV-Vis characterization of the C2-ene adduct, the C4/Cα-adduct and NADPH. Absorption spectra of NADPH, C2-ene adduct and the C4/Cα-adduct. NADPH (dark blue) has a local maximum at 340 nm, C2-ene adduct (purple) at 370 nm and C4/Cα-adduct (red) at 328 nm.
Supplementary Figure 2: High-resolution MS analysis of the purified compound (C4/Cα-adduct) demonstrating its nature as a crotonyl-CoA-NADPH adduct. (a) HPLC-ESI-MS spectra of the purified dead end product showing the [M+2H]^{2+} ion with its isotopic pattern. (b) MS2 spectrum of the parent ion at m/z 791.1244 showing fractioning into butyryl-CoA (838), NADP^+ (744) and NADP^+ without nicotinamide (622, loss of 122).
Supplementary Figure 3: NMR analyses of the purified compound demonstrating its C4/Cα-adduct nature. Conditions: The compound was prepared enzymatically and purified from the reaction mixture by preparative HPLC. After three times lyophilization, 2-3 mg compound were dissolved in 540 µl D$_2$O buffered with 60 µl > 90% deuterated 500 mM NaH$_2$PO$_4$ pD 7.9. NMR spectra were acquired at 4.4 °C. (a) $^1$H-NMR spectrum, (b) DQF-COSY-, (c) HSQC-, (d,e) HMBC- and (f) NOE- spectra of the C4/Cα-adduct. (g) C4/Cα-adduct structure and proton numbering. (h) Assignment table for the C4/Cα-adduct.
<table>
<thead>
<tr>
<th>Position</th>
<th>(^1)H-Shift</th>
<th>(^1^3)C-Shift</th>
<th>HMBC</th>
<th>COSY</th>
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<tbody>
<tr>
<td>(\alpha)</td>
<td>2.35</td>
<td>60.2</td>
<td>6*, 11.0, 20.6, 36.0, (\beta_a, \beta_b, 104.1)</td>
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<tr>
<td>(\beta_a)</td>
<td>0.98</td>
<td>20.6</td>
<td>6*, 11.0, 44*, 46*, (\alpha, \beta_b, \gamma 60.2, 68*)</td>
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<tr>
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* Signals from contaminating Ethanol or decomposition products butyryl-CoA and NADP+
Supplementary Figure 4: Uncatalyzed first order decay of the C4/Cα-adduct. Adduct decay was measured at 328 nm in 100 mM Na₂HPO₄ pH 7.9 at 31 °C. The concentration of the C4/Cα-adduct was calculated using an extinction coefficient of 4.1 ± 0.2 cm⁻¹ mM⁻¹. The resulting decay constant of the C4/Cα-adduct is 0.02490 ± 0.00001 min⁻¹.
Supplementary Figure 5: Stopped flow analysis of the Etr1p reaction. Stopped flow measurements at 382 nm of enzyme assay with Etr1p Y79F (purple) show an initial buildup of C2-ene adduct (note that the C4/Cα-adduct does not show substantial absorption at 382 nm, see also Supplementary Figure 1). The assay contained 20 μM enzyme, 50 μM NADPH and 50 μM crotonyl-CoA in 100 mM Na$_2$HPO$_4$ pH 7.9 buffer at 5 °C. The increase of 12 mAU corresponds to 2.0 μM C2 intermediate, or about 10% of the active sites. A control reaction without crotonyl-CoA is shown in black.
Supplementary Figure 6: Scanning kinetic screening of C2-ene adduct conversion to detect si-face proton donor enzyme variants. All assays were performed in 100 mM Na$_2$HPO$_4$ pH 8 buffer at 30 °C. (A) Reaction with 0.033 μM Etr1p WT showing normal conversion to butyryl-CoA and NADP$^+$ without formation of C4/Cα-adduct. (B) Reaction with 34 μM Etr1p Y79F deficient in proton donation. The formation of C4/Cα-adduct with an absorption maximum at 328 nM is observed. (C) Reaction with 9.3 μM Etr1p Y79F W322K. The formation of C4/Cα-adduct shows that the lysine does not serve as a proton donor. (D) Reaction with 0.62 μM Etr1p Y79F W322E. The glutamate mutation shows similar behavior to Etr1p wt and no formation of the C4/Cα-adduct, suggesting that it serves a productive proton donor from si. (E) Etr1p Y79F W322D, forms mostly C4/Cα-adduct. (F) Etr1p Y79F W322H forms only C4/Cα-adduct.
Supplementary Figure 7: Active site structure the engineered Etr1p Y79F W322E variant that is a si-specific enoyl-CoA reductase. Etr1p Y79F W322E active site showing the mutated WT proton donor Y79 and the catalytic residue E322 with hydrogen bonds to the backbone amide and T324 OH-group (yellow) that position E322 correctly for proton donation.
Supplementary Figure 8: pH dependence of $K_{cat}$ for Etr1p WT (black) and Etr1p Y79F W322E (red). The $k_{cat}$ values of each mutant are fitted with a one proton-donor model (fit shown in respective color) to approximate the pK$_a$ of the donating residue (Y79 for Etr1p WT and E322 for Etr1p Y79F W322E). The fitted values are shown below the graph. All shown data points represent Michaelis Menten fits of a minimum of 18 measurements. Error bars represent the standard deviation of those fits.

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Supplementary Figure 9. Proposed mechanism for formation of C4/Cα-adduct. Steps in bracket take place in the active site that lacks a proton donor (e.g., Etr1p Y79F), protonation of the formed enolate is prevented and instead a Michael addition takes place between the enolate and NADP+, forming the C4/Cα-adduct which can subsequently decay in solution into butyryl-CoA and NADP+. 
4 Chapter IV: Towards understanding mitochondrial enoyl-thioester reductases by novel mass spectrometry based kinetic isotope measurements

Authors: Raoul G. Rosenthal, Patrick Kiefer, Julia A. Vorholt, Tobias J. Erb

Author contributions: All experiments were performed by the RG Rosenthal.
4.1 Introduction

Enoyl reductases (ERs) are key enzymes in fatty acids biosynthesis. These enzymes catalyze the enoyl-thioester reduction step during the iterative biosynthesis process of fatty acids Figure 1. Three types of enoyl-thioester reductases belong to the MDR superfamily, 1) ERs involved in mitochondrial fatty acid biosynthesis (mtFAS II), often called enoyl-thioester reductase (ETR) or mitochondrial enoyl-CoA reductase (MECR) (1); 2) ER domains found in fungal and animal fatty acid synthase multi-enzyme complexes (termed FAS I) (2, 3); 3) ER domains of bacterial and fungal polyketide synthases (PKS) (2). Of these three MDR subfamilies only the mitochondrial ETRs are free standing enzymes: the two other classes are parts of large complexes (2). Mitochondrial ETRs are a part of the mitochondrial fatty acid biosynthesis pathway that closely resembles type II fatty acid biosynthesis found in most bacteria (4). In type II FAS each reaction in the elongation process is catalyzed by a separate enzyme, this modular setup gives the flexibility to synthesize a large variety of fatty acids with different degrees of saturation, hydroxylation and chain length (5). The other two classes of ERs from the MDR superfamily are parts of large enzyme complexes that produce defined products, e.g. palmitic acid in the case of FAS I and defined polyketides in the case of PKSs (2). Because of the stand-alone nature of mitochondrial ETR, we chose to use it as a simplified model system for Ccrs. ETR lacks the complexity of CO₂ binding and has the experimental advantage that the proton concentration, the pH, is much easier to regulate and buffer than the dissolved CO₂ concentration.

The physiological role of mitochondrial ETRs -encoded in the nucleus- is the synthesis of octatonic acid that is a precursor in lipoic acid biosynthesis (4, 6). The biosynthetic pathway of lipoic acid biosynthesis is conserved in all eukaryotes and takes place in the mitochondria. Because mutations in ETRs give phenotypes deficient in respiration, the older name for this enzyme class was mitochondrial respiratory function (MRF) protein (7). Despite numerous studies focusing on the active site of mitochondrial ETRs the contributions of the individual active site residues are not understood (8, 9). The residues close to the catalytic proton donor, Y79 (see Chapter 3) are S70, W322 and T324 (numbered according to Candida tropicalis Etr1p without the mitochondrial signaling tag PDB: 4WAS). Despite the very close proximity to the catalytic
proton donor, S70 and T324 are not well conserved. In the analyzed sequences, alanine can replace S70 and serine or glutamine can replace T324 in some mitochondrial Etrs Figure 2. W322 on the other hand, is strictly conserved. As of yet, the individual contributions of these active site residues to catalysis are unknown.

A closer look at the active site, however, suggests possible contributions for each side chain. Y79 protonates the Cα of the substrate after hydride transfer (see chapter 3). The crystal structure of the ternary complex of Etr1p, crotonyl-CoA and NADP+ shows that two hydrogen bonds hold Y79 in place. One active site water molecule and the indole of W322 hydrogen bond to the phenol of Y79, see Figure 3 (T324 is further away and the angle to form a hydrogen bond to Y79 is worse with about 70°). The crystal structure shows a well-defined water network in the active site that forms hydrogen bonds between active site residues and substrates. For example, S70 makes a hydrogen bond to a water molecule that binds to the thioester carbonyl of crotonyl-CoA (see Figure 3). The substrate, enoyl-CoA, binds across two monomers of the native homodimeric state of the enzyme. The adenine binds to one monomer and the panthenoate to the TYGGM consensus sequence of the other monomer, where

**Figure 1** Fatty acid biosynthesis. During each elongation round one malonyl-ACP enters the cycle, elongating the fatty acid chain with two carbons. The cycle consists four steps: 1) condensation, catalyzed by the keto-synthase (KS), 2) reduction, catalyzed by the keto-reductase (KR), 3) dehydration, catalyzed by a dehydratase (DH) and 4) reduction, catalyzed by the enoyl-thioester reductase (ER or ETR). ACP= acyl carrier protein.
reduction of the enoyl takes place. NADPH binds in the signature Rossman fold, placed on the opposite domain of the active site moiety as in all MDRs. In the active site, one key contact to the nicotinamide C-4 comes from the alcohol group of T175. T175 is located on an alpha helix that connects the catalytic domain and the Rossman fold. Almarsson and Bruice proposed that the amino acid oxygen functionalities which point inwards toward the nicotinamide ring are involved in hydrogen bonds to water molecules in the apo-enzyme to keep the cofactor binding site open (10). Sakowicz et al., however, suggested the analogous threonine in lactate dehydrogenase of *Bacillus stearothermophilus* forces the pyridine ring of NADPH into the right boat conformation for hydride transfer (11). This threonine is conserved in over 85% of MDRs with solved crystal structures in 2012. To put this into perspective, there are only 12 residues in the entire alignment that are conserved at this level (see Supplementary figure 1 for ESPRESSO alignment).

We hypothesized that Etr1p and Ccr would have shared catalytic elements as they both belong to the MDR superfamily and therefore Etr1p could serve as a good model system. In addition, the development of specific inhibitors for NAD(P)H dependent enzymes and the de novo computational design of NAD(P)H-dependent enzymes both depend on an accurate understanding of the individual contributions of active site residues. Therefore, we set to characterize the influence of the above-mentioned active site residues in more detail. To do so we out performed kinetic isotope studies with a newly developed mass spectroscopy based approach to determine the individual contributions of the active site residues to catalysis in Etr1p of *C. tropicalis*.

In addition, we wanted to study whether the discovered adducts observed in Etr1p and Ccr were also relevant for more distantly related ETRs. As such we chose to study the enoyl-ACP reductase from *Mycobacterium tuberculosis* that is the target of many antibiotics. The increasing rates of resistance in *M. tuberculosis* have encouraged the investigations of this enzyme. InhA catalyzes the NADH dependent reduction of enoyl-ACP intermediates in fatty acid biosynthesis (12, 13). The enzyme is especially important in the synthesis of mycolic acids which form an important component of the cell wall of *M. tuberculosis* (12, 13). Targeting this enzyme is therefore an effective way to combat tuberculosis infections.
Figure 2. Multiple sequence alignment of mitochondrial enoyl-CoA reductases. The active site residues closest to the enoyl bond of the substrate S70, Y79, W322 and T324 are indicated with a red arrow. The green arrow marks the consensus sequence that distinguishes the mitochondrial Etrs subfamily within the MDR superfamily. Positions with more than 80% identity have a colored background. The ClustalW algorithm and BioEdit 7.0.9.0 were used to make this figure. Used with permission from Vögeli B. (2014) Understanding and Engineering Enoyl-CoA Reductases (MSc thesis).
Figure 3 Active site waters in Etr1p with crotonyl-CoA and NADP+ bound (PDB 4WAS). All distances between polar neighbors of the selected residues and water within hydrogen bonding distance are shown (<3.5 Å). Red spheres represent waters.
4.1.1 Limitations of the UV-Vis based approach on measuring D$_2$O isotope effects

To investigate the reaction mechanism of Etr1p in more detail we tried to measure the kinetic isotope effect (KIE) on the protonation step by measuring enzyme assays in D$_2$O and following the NADPH consumption in a UV-Vis spectrometer. The results of these measurements, however, were ambiguous because the activity of Etr1p WT was inhibited by the substrate crotonyl-CoA to a different extent in H$_2$O and in D$_2$O. Re-purifying the crotonyl-CoA did not eliminate the observed substrate inhibition effects, see Figure 4.

![Figure 4](image_url)

**Figure 4.** Effects of increasing D$_2$O concentration on substrate inhibition in Etr1p WT. Increasing the D$_2$O content increases the substrate inhibition effect, even after re-purifying crotonyl-CoA; therefore, the classical UV-Vis based approach to measure kinetic isotope effects on protonation in Etr1p cannot be used.
4.1.2 A new mass spectrometry-based kinetic isotope measurement for D₂O effects

In order to measure the KIE on the protonation step, we developed a new mass spectroscopy-based assay that measures the primary isotope effect on the protonation step in a competitive fashion, eliminating artifacts caused by substrate inhibition. The assay measures the incorporation ratios of hydrogen versus deuterium from the solvent. By running the reaction in buffered H₂O as a control and in defined mixtures of H₂O and D₂O the KIE can be calculated. Reductases incorporate a proton or deuteron from the solvent into the product. If not exchanged with the solvent, this can serve as a direct measure of the KIE. The KIE causes the hydrogen to deuterium ratio to be different in the products of the reaction compared to the ratio in the solvent that the reaction took place in. In the case of a normal KIE, deuterium will react slower and therefore the hydrogen to deuterium ratio in the reaction products will be bigger than in the solvent. By measuring the H/D ratio by mass spectroscopy, we can calculate the apparent KIE on the reaction from the following equation:

\[
\frac{\text{product}_H}{\text{product}_D} = \frac{H/D}{KIE} \cdot \frac{f_H}{f_D} = \frac{k_{\text{cat} \ H}}{k_{\text{cat} \ D}} \cdot \frac{K_{m \ H}}{K_{m \ D}} \cdot \frac{f_H}{f_D}
\] (1)

Product\(_H\) and product\(_D\) represent the amounts of product with hydrogen and deuterium, \(k_{\text{cat} \ H}\), \(k_{\text{cat} \ D}\) and \(K_{m \ H}\), \(K_{m \ D}\) are the turnover and Michaelis-Menten constants for the reactions with hydrogen and deuterium respectively and, \(f_H\) and \(f_D\) are the fractions of hydrogen and deuterium in the used buffer. By measuring the H/D ratio by mass spectrometry of assays run in defined H₂O/D₂O mixtures the primary KIE on protonation can be calculated directly. One point is already sufficient to calculate the apparent KIE but measuring several points and confirming linearity is an important control. This greatly facilitates the ease of measurement by omitting buffer exchange for the enzyme and reduces errors by using a competitive method.

There are clear similarities between this method and the competitive methods used to measure heavy atom KIEs. Nevertheless, some important differences are evident. In the case of competitive KIE measurements based on the method of Singleton (14), it is important to stop the reaction at an accurately known
conversion state (e.g. 50 %, 75 %, 95 %) to calculate the KIE. From the stopped reactions with different conversion states, the product or substrate is isolated and their isotopic composition determined. From the different isotopic enrichments/depletions at different conversion states, the KIE is calculated. The presented method does not need to be run to a well-defined conversion factor because the isotopic distribution of the measured substrate (butyryl-CoA) is different from the substrate that undergoes, even if negligibly, isotopic enrichment (H⁺). Therefore the method described here can be run to completion without significant enrichment artefacts because the enrichment of ¹³C in the substrate leads to a negligible deviation, max ~6%, compared to the measured deuterium KIEs which are typically greater than 200% and up to ~700% (when no tunneling is involved). The isotopic enrichment of the solvent is also negligible since the substrate concentration is in the low μM range and the solvent concentration is above 50 M (depending on the exact ratio of H₂O and D₂O). Taken together, this method is independent of the reaction progress. The only requirement is complete separation of substrate and product before measuring the product[H]/product[D] ratio. Without complete separation of substrate and product, the isotopic pattern of the substrate [M+3]+ peak can interfere with the product [M+H]+ peak as the mass shift of the reduction is 2 Da.

Several steps are important when preparing samples. The enzyme needs to equilibrate with the H₂O/D₂O mixture for at least 60 min to equilibrate the backbone and side chain protons with the solvent (protons on the inside of the protein can take much longer to exchange but are not likely to cause a significant solvent isotope effect). The substrate concentration is preferably low, much below the Kₘ to reduce the forward commitment of the reaction (vide infra) to ensure that the measured KIE is as close as possible to the intrinsic KIE. Taken together this method is a straightforward way to measure KIEs on the protonation step in reductases.

Since Etr1p uses the C2-ene adduct as substrate with good kinetics, we assume that it is an on pathway intermediate (as in Chapter 3). With this assumption, the reaction scheme becomes:
In eq. 2, \( k_3 \) and \( k_4 \) represent the hydride transfer (through an ene-reaction) in the forward and backward direction, respectively, and \( k_5 \) represents protonation of the C2-ene-adduct in the active site. The binding of the substrate and release of the product are represented by \( k_1 \) and \( k_2 \), and \( k_6 \) and \( k_7 \), respectively. With this scheme, the kinetic complexity becomes apparent. Kinetic complexity is the masking of the intrinsic isotopic effect, i.e. the KIE on the bond breaking/forming step that is under study, by other kinetic steps such as substrate binding, product release or conformational rearrangements. The steps that precede and follow the chemical step under study contribute to the overall rate of the enzymatic reaction and thereby mask the intrinsic KIE (Cleland, 1975). Even kinetic steps that are not rate limiting can decrease the observed KIE value. These effects are expressed quantitatively by equation 3:

\[
(3)
\]

The nomenclature used here is that recommended by the Standards for the Reporting of Enzymological Data committee and expanded by Francis and Kohen for (15, 16). \( \frac{D V}{K_m} \) represents \( \frac{(H V)/K_m}{(D V)/K_m} \) i.e. the ratio of the catalytic efficiency with hydrogen over deuterium (at the isotopically labeled bond). \( D k \) is the intrinsic isotope effect i.e. the isotope effect on the actual bond breaking/forming step. \( C_f \) represents "the tendency of the enzyme complex poised for catalysis to continue forward as opposed to its tendency to partition back to free enzyme and unbound substrate" (17). The mathematical definition of \( C_f \) is given in eq. 4. \( C_r \) represents the same tendency but in the opposite, backward direction. \( K_{eq} \) is the equilibrium constant. Enoyl-reductions can be considered essentially uni-directional keeping \( C_r \) negligible.
\[
C_f = \frac{k_7}{k_6} \cdot \left(1 + \frac{k_5}{k_4} \left(1 + \frac{k_3}{k_2} [\text{crotonyl} - \text{CoA}] \right) \right)
\]  

(4)

By keeping the substrate concentration much below \(K_m\) the commitment factor, \(C_f\), becomes small because the equilibria before the chemical step will favor dissociation more. With a small commitment factor and negligible reverse commitment to catalysis, the measured KIE estimates the true intrinsic KIE well.

Even though this method only gives KIE values that approach the intrinsic KIE it can provide many useful insights into the rate limiting steps in catalysis. The method presented here can be justified by the fraction of the effort that is needed to measure KIEs compared to Northrop's method (17) that requires a comparison of H/D and H/T isotope effects (nearly impossible when solvent protons are studied due to the properties of \(T_2O\) that can only be handled in diluted form).

### 4.2 Results

To test our strategy we first examined whether the method of data collection has an influence on the outcome of the measured KIEs. We measured the same samples with selected ion monitoring (SIM) as well as with parallel reaction monitoring (PRM) modes. With SIM the mass spectrometer is set to measure only a selected narrow \(m/z\) range of a few \(m/z\) units around the mass of interest, in the case of butyryl-CoA ([M+H]\(^+\) = 838.16 \(m/z\)). PRM mode on the other hand measures the fragmentation products of the parent ion. In this case, the parent ion was butyryl-CoA and the acyl-panthenoate, the fragment ion. By measuring only the isotopically labeled acyl-panthenoate fragment the signal-to-noise ratio should increase. Both methods, SIM and PRM gave similar KIE values for Etr1p Y79F, 5.26 ± 0.16 and 5.48 ± 0.24, respectively (Figure 5). The standard error, however, was much smaller in PRM mode compared to SIM with 0.069 and 0.10. A comparison of the measured values and the fitted curve reflects the same trend. The standard deviation of the error in percent \(((\text{measured} - \text{fitted})/\text{fitted} \cdot 100\%)\) is 4.6 % for PRM and 7.2 % for SIM. Therefore, we measured all KIE samples in PRM mode. Because of the inaccuracy concerned with determining the isotopic distribution of the unlabeled acyl-panthenoate
fragment of 5%, we added 10% ($\approx 2\sigma\%$) to the confidence interval of all the reported values to account for this error.

Figure 5. Comparison between single ion monitoring (SIM) and parallel reaction monitoring (PRM) modes for measuring kinetic isotope effects on the protonation step of Etr1p Y79F in forming butyryl-CoA. The ratio of protonated and deuterated product as measured by either SIM or PRM mass spectrometry plotted against the ratio of H$_2$O/D$_2$O in the buffer.

Although there is a strong substrate inhibition effect in D$_2$O we wanted to have a reference value to calibrate our method with. Therefore, we collected accurate Michaelis-Menten kinetics of Etr1p WT in H$_2$O as well as D$_2$O and modeled the datasets to eq. 5, which is a Michaelis-Menten model that includes substrate inhibition constant, $K_i$:

$$V = \frac{k_{cat} \cdot [S]}{K_m + [S] \left( 1 + \frac{[S]}{K_i} \right)}$$

(eq. 5)
All the variables in this equation are the same as for standard Michaelis-Menten kinetics. V is the initial rate, $k_{cat}$ is the maximal turnover number of the enzyme at saturating conditions, [S] is the substrate concentration, $K_m$ is the Michaelis constant, and $K_i$ is the inhibition constant. With this equation the KIE on protonation, i.e. the $^{\text{H}_2\text{O}}k_{\text{cat}}/^{\text{D}_2\text{O}}k_{\text{cat}}$ at pH=7.0 is $1.6 \pm 0.4$ (95% C.I.) for Etr1p. Due to the difference between the extent of substrate inhibition in $\text{D}_2\text{O}$, $K_i^{\text{H}_2\text{O}} = 380 \pm 350 \mu\text{M}$, versus $\text{H}_2\text{O}$, $K_i^{\text{H}_2\text{O}} = 61 \pm 0.30 \mu\text{M}$, the confidence interval on the KIE is large.

To gain a detailed understanding of the contributions of different residues in the active site of Etr1p we measured the KIEs on the protonation step of Etr1p WT and its mutants (for residues see Figure 6). Mutations that act on residues directly involved in proton transfer from enzyme to substrate should change the measured KIE significantly.

**Table 1.** Observed kinetic isotope effects in Etr1p and mutants as measured by incorporation ratios of H/D from buffers with defined H/D ratios. All values represents averages of two independent values, based on at least four data points each. See methods section for details. Errors given as 95% confidence intervals.

<table>
<thead>
<tr>
<th>Enzyme/reaction</th>
<th>$\text{KIE}_{\text{obs}} \text{ (H/D)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.78 ± 0.08</td>
</tr>
<tr>
<td>S70A</td>
<td>1.11 ± 0.07</td>
</tr>
<tr>
<td>T324A</td>
<td>1.92 ± 0.15</td>
</tr>
<tr>
<td>S70A T324A</td>
<td>1.20 ± 0.08</td>
</tr>
<tr>
<td>Y79F</td>
<td>4.8 ± 0.8</td>
</tr>
<tr>
<td>T175V</td>
<td>4.4 ± 0.8</td>
</tr>
</tbody>
</table>

Etr1p WT has a KIE$_{\text{obs}}$ of 2, indicating a partial rate-limiting protonation step, assuming that the forward commitment, $C_f$, is small due to the low substrate concentration. The KIE$_{\text{obs}}$ of 1.1 in Etr1p S70A indicates either that protonation is not occurring during the transition state in this mutant or, that the forward
commitment to catalysis increased and reduced the KIE\textsubscript{obs} to almost unity. Etr1p T324A does not have a significantly different KIE\textsubscript{obs} from the WT enzyme indicating the absence of a contribution of threonine 324 to the protonation step. In line with the findings in Chapter 3, Etr1p Y79F has a strongly elevated KIE\textsubscript{obs} of 5.5, indicating that proton transfer occurs during the transition state. Alternatively, the commitment to catalysis in the mutant could be strongly reduced compared to the WT but this is unlikely since the low substrate concentration employed should keep the commitment to catalysis low in both cases. Etr1p T175V has the most surprising KIE\textsubscript{obs} of 4.4. Although the mutated residue is more than 6 Å from the C-\(\alpha\) of crotonyl-CoA this variant has one of the largest isotope effects.

**Figure 6** Stereo view of the active site of Etr1p. Three residues that are close to the proton transfer, S70, Y79 and T324 are located above crotonyl-CoA and T175, the residues closest to C4, where the hydride is transferred from, of nicotinamide and highly conserved is shown. Only selected residues are shown and NADP\(^+\) and crotonyl-CoA are truncated for clarity.

In order to put the measured KIEs in perspective we measured the uncatalyzed decay of the C2-ene adduct and the C4-adduct. We purified and isolated both adducts and measured the KIE\textsubscript{obs} under identical conditions as used for the enzyme catalyzed reactions. The KIE\textsubscript{obs} for the C2-ene-adduct and the C4-adduct were 4.5 ± 0.2 and 4.4 ± 0.2, respectively.
For Etr1p WT and S70A we also determined isotope effects on hydride transfer in H$_2$O with a constant crotonyl-CoA concentration. Where the WT has a small normal, isotope effect on $k_{\text{cat}}$ of $2.5 \pm 0.2$, Etr1p S70A has a relatively large isotope effect of $4.5 \pm 0.2$.

As discussed in Chapter 3, of the selected mutants only Y79F has a large effect on the catalytic parameters. The other mutants do contribute to catalysis but have only minimal effects on turnover and catalytic efficiency.

**Table 2.** Kinetic constants of selected mutants on crotonyl-CoA with 125 $\mu$M NADPH at pH 7.0 in 50mM Na$_2$HPO$_4$. n.d. = not determined  

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>180±6</td>
<td>18±2</td>
</tr>
<tr>
<td>S70A</td>
<td>101±10</td>
<td>20±2</td>
</tr>
<tr>
<td>T324A</td>
<td>94±4</td>
<td>107±5</td>
</tr>
<tr>
<td>Y79F</td>
<td>0.011±0.002</td>
<td>44 ± 8</td>
</tr>
<tr>
<td>T175V</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

### 4.2.1 Covalent adduct in the catalytic cycle of InhA Y158F

In analogy to the data obtained from Etr1p we thought that knocking out the proton donor in InhA would lead to buildup of analogous adducts. Although the proton donor in InhA is uncertain, we hypothesized that active site Y158 was the best candidate, given its proximity to the $C\alpha$ of the enoyl-CoA substrate. Indeed, InhA Y158F showed a clear buildup of an absorption signal around 380 nm in the presence of NADH and hexenoyl-CoA. Analogous to the buildup of absorption seen in Ccr WT in the absence of CO$_2$. Injecting this mixture into the mass spectrometer showed a doubly charged species with the expected mass for a covalent adduct between hexenoyl-CoA and NADH (measured: 765.156; calc: 765.148 m/z).
We have developed a new, more accurate method for measuring primary KIEs that is not limited by the shortcomings of absorption-based assays, which in this specific case were especially inaccurate due to substrate inhibition effects. Nevertheless both the mass spectrometry-based method and the UV-Vis assays give the same value within the accuracy of the measurement with KIEs of 1.78 ± 0.06 and 1.6 ± 0.4, respectively. This method greatly facilitates the measurement of primary KIEs on protonation reactions for several reasons: 1) The necessity to exchange enzyme buffers is omitted because the reactions do not need to be carried out at almost 100 % D₂O as in the case of non-competitive methods; 2) Errors are smaller because the reaction with both isotopes occurs under the same conditions including pH, temperature, ionic strength and substrate concentration; 3) Secondary effects like different substrate inhibition constants in H₂O and D₂O do not affect the presented method. 4) The build-up of UV-vis active compounds does not interfere with the measurement, because this mass spectrometry approach directly measures the isotopic distribution of the products. The method can be extended to normal kinetic isotope effect measurements when e.g. NAD(P)H is labeled and used in a large excess so that isotopic enrichment in the substrate can be neglected. The software tool developed specially for quick automated data analysis makes it possible to analyze the primary SIE or KIE of enzymes and their corresponding mutants with medium throughput, i.e. up to twenty mutants per day with most of the work being automated.

With this new method, we determined the primary kinetic isotope effects on the protonation step of Etr1p and selected mutants. The WT enzyme has a small kinetic isotope effect on protonation as well as hydride transfer, 1.8 ± 0.1 and 2.5 ± 0.2, respectively. This indicates either a concerted hydride and proton transfer or two distinct, partially rate limiting, transition states for hydride and proton transfer. To distinguish these two options we can look at the effects of the active site mutations. Removing the OH group of S70 that interacts with the conserved active site water network by mutation to alanine diminishes the KIE on protonation to 1.1 and increases the KIE on hydride transfer to 4.5. To understand this effect we need to look at the polar interactions S70, the OH group of S70 is hydrogen bonded to a water molecule that is hydrogen bonded
to the thioester carbonyl of crotonyl-CoA. This hydrogen-bond network can polarize the enoyl double bond, for either direct hydride transfer or an ene-reaction. In Etr1p S70A this network is likely disrupted, reducing the polarization of the enoyl double bond and increasing the energetic barrier for hydride transfer for both mechanisms. In this mutant enzyme, hydride transfer is rate limiting and protonation is not rate limiting anymore. In terms of commitment factors, this means that once the hydride is transferred in S70A the commitment is very large and no discrimination between hydrogen and deuterium is observed. Therefore, we measure a large $\text{KIE}_{\text{obs}}$ of 4.5 with NADPD and almost no $\text{KIE}_{\text{obs}}$ on the protonation step.

T324A does not have a large enough effect on the KIE, compared to the WT, large enough to draw meaningful mechanistic conclusions. The double mutant S70A T324A does not differ meaningfully from the single S70A mutant either. The largest effect on the protonation step is observed in Y79F with a $\text{KIE} = 4.8 \pm 0.8$. This KIE value is same as decay of the C-2-ene adduct or the C-4-adduct within the error of the measurement the, which are 4.5 and 4.4, respectively. This result confirms previous data, indicating that Etr1p Y79F can form the C2-ene-adduct and convert it into the C-4-adduct but not catalyze subsequent resolution of the C-4-adduct to butyryl-CoA and NADP$^+$. The formation of butyryl-CoA and NADP$^+$ happens in solution from uncatalyzed decay of either the C-2-ene-adduct or C-4-adduct. The large isotope effect on decay of these adducts probably arises from an acid decay mechanism. The protonation of the thioester-carbonyl is rate limiting, followed by a rapid decay and protonation to give butyryl-CoA and NADP$^+$.

The role of T175 in the protonation step is remarkable as it is more than 6 Å away from the C$\alpha$ of crotonyl-CoA and almost 9 Å away from the tyrosine hydroxyl group that serves as the proton donor. We hypothesize that this residue is important in activation of NAD(P)H for hydride transfer, either by direct hydride transfer or by an ene mechanism, in analogy to lactate dehydrogenases from the Rossmann-fold NAD(P)(+)—binding proteins (11). T175 is in close proximity to the C4 of nicotinamide (3.1 Å)$^2$ as well as to the

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$^2$ A note of caution is made here that the reported distances are measured in 4WAS, a structure with NADP$^+$ bound and not NADPH. The structural changes, however, are usually minimal between oxidized and reduced cofactor.
backbone carbonyl of V171 (2.9 Å) and is highly conserved in the MDR family without a clear explanation in the literature. There is support for the presence of a hydrogen bond between the alcohol of T175 and backbone carbonyl of V171. Both the T\textsubscript{175}-O\textgamma--O=C\textsubscript{V171} distance of 2.9 Å, as well as the $\angle$ C\textbeta-O\textgamma--O=C\textsubscript{V171} angle, 95°, are within the empirical boundaries for such hydrogen bonds (18). The second interaction, not mutually exclusive with the first, is one between the alcohol oxygen of T175 and the C4 hydrogen. This is a short distance O⋯H-C bond where the lone pair of oxygen interacts with the slightly positively charged hydrogen, due to carbon’s higher electronegativity compared to hydrogen (19, 20). The highly-conserved nature of T175 indicates an important role for its side chain alcohol and therefore one of the interactions. For the reduction of crotonyl-CoA to occur via direct hydride transfer, it needs to be concerted with proton transfer in order to have a measurable KIE\textsubscript{obs} in T175V. This is possible if an enol intermediate is formed, see Figure 7a. If, however, the hydride transfers through an ene-mechanism, the KIE\textsubscript{obs} in T175V is the result of a concerted cleavage of the C2-C\textalpha bond in the C2-ene-adduct and protonation Figure 7b. In this case, T175 can stabilize the positive charge that forms on the nicotinamide moiety when the C2-ene adduct is resolved into NADP\textsuperscript+ and butyryl-CoA. As the largest positive charge, after N1, is placed on C4 (21), the effect of stabilizing this position gives an important contribution in transition state stabilization of dissociating the C2-ene adduct. If the mechanism is a direct hydride transfer, however, stabilizing a positive charge on the C4 of nicotinamide will also stabilize the transition state. In both mechanisms, Y79 acts as a proton donor that catalyzes the keto-enol tautomerization. The observed KIE in T175V of 4.4 ± 0.8 corresponds well with the KIE on protonation of a carbonyl (22). This supports a mechanism where protonation of the thioester carbonyl is required in the absence of the polarizing effect of T175.

The detection of the analogous intermediate in InhA Y158F is an interesting observation that negates the previously thought role of this intermediate, to act as an electrophilic catalyst and opens new research topics in the development of antibiotics against \textit{M. tuberculosis}. 

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Figure 7 Possible mechanisms of Etr1p that are consistent with the measured isotope effects. a) A classical hydride transfer mechanism, T175 stabilizes the positive charge that forms on the nicotinamide ring. Both hydride transfer and proton transfer are partially rate limiting. Y79 catalyzes the keto-enol tautomerization. In the T175V mutant, the hydride transfer transition state with a partially positive nicotinamide is rate limiting because of the lack of stabilization. b) An ene hydride transfer mechanism leads to the C-2-ene adduct, and hydride transfer is partially rate limiting. The decay of the intermediate is governed by polarization of both ends of the adduct, T175 increases the partial positive charge in the nicotinamide ring and the water molecule bound to S70 increases the partial positive charge on the carbonyl. Decay of the intermediate is coupled to proton transfer to the thioester carbonyl. In T175V the C-2-ene-adduct is less polarized, making the decay more dependent on the proton transfer, increasing the observed KIE for protonation.
In conclusion, the reaction catalyzed by Etr1p is either a direct hydride transfer mechanism where the hydride and proton are added in concerted fashion but not necessarily synchronous fashion or a two-step mechanism with both hydride and proton transfer being partially rate limiting. The formation of an enolate intermediate is ruled out by the fact that T175 is far away from the Cα of crotonyl-CoA but still has a large effect on the protonation step. Therefore, the rate limiting protonation step is probably the protonation of the thioester carbonyl and not the protonation of the Cα by Y79. The role of Y79 is still protonation, not of an enolate but of an enol intermediate, that forms after hydride transfer. Y79 catalyzes here the keto-enol tautomerization of the enol to the keto form in a stereospecific manner to give butyryl-CoA.
4.4 Methods

Protein purification and mutagenesis were performed according to identical methods as described in Chapter 3.5. Crotonyl-CoA was synthesized and purified as described in Chapter 2.5.

4.4.1 KIE Measurements.

Reactions for the measurement of the SIE with Etr1p WT and mutants contained 100 mM Na₂HPO₄, 0.25mM NADH, 1.25 μM crotonyl-CoA, H₂O and D₂O according to the desired percentage of D₂O, between 7 and 80 %. The enzyme was equilibrated with the buffer for at least 60 min before the addition if the last substrate, crotonyl-CoA (to a final volume of 150 μL). Mutant enzymes were added at 5 times higher concentration (125 nM) than the wt (25 nM final). Samples were analyzed on a Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific). Chromatographic separation prior to mass analysis was done with a Kinetex 1.7 μm XB C18 100 Å, 50 x 2.1 mm column (Phenomenex). Reactions were incubated at room temperature and diluted 10 times with CoA-buffer prior to analysis. Reaction products were separated by applying a linear gradient of 25 mM ammonium formate pH=8.1/ methanol (MeOH) at a constant flow of 0.55 mL/min. A flow with 5% MeOH for 1.5 min was diverted to the waste to desalt the analytes. Then a gradient of 5% to 95% MeOH in 6.5 min was applied and kept at this value for 1 min. The column was equilibrated with 100% aqueous phase for 3.1 min before the next injection. The parent ion was 840 ± 3.5 m/z and was fragmented with Ar at 25 eV. The butyryl-panthenoate ion (331.150 m/z) was measured to determine the extent of deuterium incorporation.

4.4.2 Data analysis.

The mass spectrometry software package eMZed 2.0.1(23) was used in combination with the 'kinetic_isotope_effect-0.13-py2-none-any.whl' extension to evaluate the kinetic isotope data.
4.4.3  [2H]-(4R)-NADPH synthesis.

The protocol from (24) was followed exactly to synthesize [2H]-(4R)-NADPH.

4.4.4  Hydride transfer KIE.

Standard UV-Vis assays were performed to measure the KIE on hydride transfer. Briefly, a reaction mixture with 100 mM Na$_2$HPO$_4$ pH =7, 125 µM crotonyl-CoA, 15 nM Etr1p WT or 30 nM mutant enzyme were started by the addition of varying amounts of [2H]-(4R)-NADPH, between 2 and 64 µM. The reaction was monitored in an Agilent Technologies carry 60 UV-Vis spectrometer.
4.5 References


**Supplementary figure 1.** Structure based alignment (ESPRESSO) of all the available MDR structures found with the Dali server in June 2012. Residues conserved in more than 85% of the sequences have a colored background. The conserved active site threonine (T175 in Etr1p) is indicated with a red arrow.
Chapter V: Comparison of the activation energies in the catalytic cycles of Etr1p and Ccr

Authors: Raoul G. Rosenthal, Tobias J. Erb

Author contributions: All experiments were performed by the RG Rosenthal.
5.1 Introduction

The origin of novel functional enzymes is still an open question in molecular evolution. It is unknown how enzymes evolve to catalyze completely new chemistry and whether this process is case specific or general. There are, broadly speaking, two models for the evolution of novel function in enzymes. One model states that new enzymes arise by gene duplication and subsequent divergence of one of the copies with the other one retaining its old function (1). A problem with this model is that after duplication there is no evolutionary pressure on the intermediates to evolve and gain new function. An alternative hypothesis is that new enzymes evolve through bi-functional or promiscuous intermediates (2, 3). An existing enzyme, with only one biological function, deprived of evolutionary pressure to stay or become selective, drifts to a promiscuous catalyst catalyzing different reactions without biological function. If the selection pressure, e.g. by a change of habitat or lateral gene transfer, gives (one of) the side reaction(s) a selective advantage there would be selective pressure favoring a fully bi-functional enzyme that can catalyze both biologically relevant reactions. Gene duplication at this stage allows mutations favoring one reaction to become more proficient at the cost of the other reaction, creating two 'new' specific enzymes.

In this light, it is interesting to ask from what ancestral enzyme the carboxylation reaction of Ccr emerged. By chemical analogy, it is tempting to hypothesize that Ccrs could have evolved from enoyl-thiester reductases that lost their active site proton donor (see Chapter 3) and instead evolved a CO$_2$ binding site. Enoyl-thioester reductases lacking a proton donor can still catalyze the reduction of enoyl-thioesters by formation of the C2-ene adduct of NADPH and substrate and subsequent decay in solution. This mechanism allows the active site of these enzymes to diverge into promiscuous catalysts, including CO$_2$-binding and carboxylating variants. There are examples of enoyl-thioester reductase lacking a catalytic proton donor, e.g. LovC (4), supporting the idea that reductases without a distinct active site acid can act as evolutionary intermediates.

Promiscuous reactions catalyzed by natural enzymes can be completely unrelated to the natural reaction catalyzed by the enzyme, sometimes even varying in the first digit of their enzyme category number (indicating a different
reaction type) (2). Therefore, "least chemical evolution" can be a poor predictor for ancestry and it should not be ruled out that Ccrs evolved from completely different parts of the MDR tree.

To study this hypothesis in more detail we sought to compare two enzymes that belong to the same enzyme superfamily and have a partially overlapping catalytic function. Crotonyl-CoA carboxylase/reductase, Ccr, and enoyl-CoA reductase, Etr1p fulfill these requirements. Both enzymes belong to the MDR superfamily and accept the same substrates, NADPH and crotonyl-CoA, but their reactivity and physiological roles differ. Crotonyl-CoA carboxylase/reductase is part of the ethylmalonyl-CoA pathway and catalyzes the reductive carboxylation of crotonyl-CoA to ethymalonyl-CoA. It still has, however, a rudimentary capacity to reduce crotonyl-CoA to butyryl-CoA. Etr1p, on the other hand, is involved in fatty acid biosynthesis and catalyzes the reduction enoyl-CoAs to their saturated counterparts, among others the conversion of crotonyl-CoA to butyryl-CoA. Both enzymes find their origin, most likely, in a common reductase and not a carboxylase, as all the other members of the MDR superfamily catalyze reduction reactions and not carboxylations. This suggests that somewhere along the evolutionary history of Ccrs the reduction function became less important while the reductive carboxylation function took the upper hand. The fact that reductases have seemingly, fewer constraints on their active site, at least a correctly positioned acidic amino acid or water molecule, compared to carboxylases, which need a CO₂ binding site consisting of several amino acids, speaks in favor of the mentioned evolutionary order. To understand the underlying changes in the active we wanted to compare both enzymes in a quantitative way to understand how the unique evolutionary pressure on each shaped its catalytic properties from the ancestral enzyme that both share. Comparison of the primary sequences of Etr1p from **C. tropicalis** (excluding the 22 amino acid long N-terminal mitochondrial targeting peptide) and Ccr from **M. extorques** have 24% sequence identity and 37% similarity.

Comparison of the active sites of both enzymes reveals several differences. Etr1p has an active site tyrosine strictly conserved among enoyl-thioester reductases, Y79 in the **C. tropicalis** enzyme, which probably acts as an active site acid that catalyzes a keto-enol tautomerization after hydride transfer (see
chapter 4). Y79 is held in place by two hydrogen bonds, one to an active site
water molecule and one to W322.

The active site of crotonyl-CoA carboxylase/reductase is different in its
architecture. There is a CO₂ binding pocket on the opposite side of crotonyl-
CoA relative to NADPH. The binding pocket is proposed to consist of three
amino acids: N79, F156 and E157 in M. extorquens AM1 Ccr (in analogy to (5)).
These three residues are conserved in Ccrs involved in primary as well as
secondary metabolism. There are, however, similarities between the active site
of Ccr and Etr1p. Analogous to T175 of Etr1p (see Chapter 4) Ccr has a
threonine, T195, in van der Waals distance of C-4 of nicotinamide. Also the
binding of crotonyl-CoA is similar. It is bound above the nicotinamide plane,
specifically with the α,β-unsaturated enoyl-moiety of crotonyl-CoA above the C-2,
C-4 vector of nicotinamide (see Figure 1).

![Figure 1](image_url)

Figure 1. Comparison of the active sites of a) Ccr from Streptomyces sp. JS360 with
NADP⁺ and octenoyl-CoA bound (4a0s) and b) Etr1p from C. tropicalis with NADP⁺
and crotonyl-CoA bound. The orientation of the nicotinamide (dark green) and the
enoyl-CoA (pink) is very similar in both enzymes.

Despite the differences in active site architecture, both Ccr WT and Etr1p WT
can use the covalent ene adduct of NADPH and crotonyl-CoA as substrate with
good kinetics. Therefore we assume here that the ene adduct is an on pathway
intermediate and that it can be used to study the catalytic cycles of these
enzymes. This assumption implies that the catalytic cycles of Etr1p and Ccr have at least two distinct steps: 1) transfer of a hydride equivalent from NADPH to crotonyl-CoA through an ene mechanism, forming the C2-ene adduct as intermediate; and 2) Resolving the intermediate into the products, NADP$^+$ and butyryl-CoA or ethylmalony-CoA, by adding a proton or CO$_2$ to the Cα, depending on the conditions and enzyme. Because the isolated C2-ene adduct is a substrate for both Etr1p as well as Ccr it gives a new access point into the catalytic cycle of both enzymes. This halfway point in the catalytic cycle opens the possibility to measure the activation energies for the separate parts of the catalytic cycles of Ccr and Etr1p. The fact that both enzymes, Ccr and Etr1p, share a part of their catalytic cycle, a hydride transfer to the β-carbon but diverge in the second half, raises questions whether the first half of the reaction is catalyzed in the same manner in both enzymes. If not, did both enzymes adapt catalysis of hydride transfer to meet the needs of electrophile addition?

The activation energy of a chemical reaction reflects the amount of kinetic energy that a molecule needs to overcome the energetic barrier that separates the reactants and products. The Arrhenius equation (1) relates speed of a chemical reaction and its temperature dependence based on empirical considerations.

$$k = A \cdot e^{-E_a/RT}$$ (1)

Where $k$ represents the rate constant of the reaction, $A$ is the Arrhenius factor which is an empirical factor and therefore should be interpreted with caution. Since it has the same unit as $k$, s$^{-1}$ for unimolecular reactions and s$^{-1}$.M$^{-1}$ for second order reactions, it is also called the frequency factor, i.e. what fraction of molecular collisions with enough energy lead to a reaction. $E_a$ is the activation energy of the reaction, the minimal energy required to overcome the energetic barrier to react, and $RT$ is the average energy of molecules in the system with $R$ the ideal gas constant and $T$ the absolute temperature.

The Arrhenius equation is derived from empirical observations on simple chemical reactions like gas phase reactions and contains some simplifications (like the assumption that $A$ is temperature independent). The complexity of enzymatic catalysis is difficult to capture in such a simplified model. The theoretically derived transition state theory has a sound theoretical derivation but at the same time has parameters that are very difficult to determine.
experimentally (e.g. the transmission coefficient $\kappa$). Therefore, the absolute values determined in enzyme-catalyzed reactions need to be taken with caution. Nevertheless, the errors inherent to the simplifications made are attenuated when very similar enzymes are compared. Therefore, the $\Delta(\Delta E_a)$ can give meaningful insights into the differences between the enzymes.

Multiple alignments of the MDR superfamily reveals that bacterial Ccrs have a long loop in common with zinc-dependent alcohol dehydrogenases. This loop ranges between 6 and 70 amino acids in length. MDRs that do not have a structural zinc ion have short loops, up to 16 amino acids in length. MDRs that have a structural zinc-binding motif ($\text{CX}_2\text{CX}_2\text{CX}_7\text{C}$) have longer variants of this loop, between 32 and 36 amino acids long (6). There are MDRs that have lost the zinc-binding motif but retained the loop, like many Ccrs. Where some Ccrs (e.g. WP_017495531 and WP_029664753) have retained their zinc-binding motif, most Ccrs have retained a long loop but lack the signature zinc-binding motif. Interestingly, structural data shows that this loop comprises the part of the enzyme that is involved in dimer or tetramer formation. The loop forms the essential contacts for dimerization. In the case of M. extorquenve AM1 Ccr (PDB 4GI2) and tetramerization in the case of Streptomyces sp. JS360 (PDB 4A10). The importance of this loop in oligomerization could be of importance in the selective pressure for retaining the loop in Ccrs.

5.2 Results

In order to understand the evolutionary relationships of Ccr and Etr1p we first set out to make a phylogenetic tree of both enzyme families to infer their evolutionary connection in more detail. For this purpose, we took representative members of several zinc-dependent as well as zinc independent MDR families, and included several Etr1p protein sequences and several Ccrs (see Materials and Methods for details). The construction of an ‘MDR-tree’ is not trivial because of the low sequence homology that the MDRs share < 30 %. Nevertheless, the representative sequences were aligned with the Multiple Alignment tool provided by the Fast Fourier Transform (MAFFT) web server (7), and a maximum-likelyhood tree was calculated.

The phylogenetic analysis shows that all the enzymes that act on carbon-carbon double bonds belong to the same clade, indicating a shared history of these
enzymes. A fraction of the enzyme families that act on carbon-carbon double bonds use enoyl-thiesters (YHDH, MCASTER, PKS ER) as substrates and another fraction on enone substrates (QOR, LTD) Figure 2.

Etr1p belongs to the mitochondrial enoyl-thioester reductases. All the enzymes with a known catalytic function that share the same clade as Etr1p are enoyl-reductases or crotonyl-CoA carboxylases. The closest homologues of Etr1p, part of the mETR clade, are the mycocerosic acid thioester enoyl-reductases that reduce $\alpha$-substituted enoyl thioesters (8).

Ccrs form a clade within MDRs that act on carbon-carbon double bonds. We assume that conservation or close homology of the residues that constitute the CO$_2$ binding-site should be a good predictor of carboxylation activity. In Ccrs with confirmed activity, there are two absolutely conserved motifs which contain the CO$_2$ binding residues. These are an NYN sequence (relatively close to the N-terminus, ~50 amino acids) of which the last N is involved in CO$_2$ binding (9) and an F/YE sequence of which both residues are involved in CO$_2$ binding.

The phylogenetic analysis shows that some of the closest enzymes to bacterial Ccr might also be Ccrs. These sequences come from archaeal MDR members that are found in the Thaumarchaeota, a phylum within the archaea that was first described in 2008. These archaea are mesophilic and found mostly in marine environments but also in mines (10). Comparison of the homologous Ccr sequences from these organisms with their bacterial counterparts show some striking differences. The NYN sequence is less conserved and appears sometimes as NYD, similarly the F/YE dyad is sometimes replaced by F/YQ. The presence of an amide group, when an NYN sequence is present, is strongly correlated with (>0.95) with the presence of a carboxylate in the F/YE sequence and the reverse is true as well. This high correlation between these amino acids important in CO$_2$ binding - and separated by about 80 amino acids- in bacterial homologues suggests that they have the same or a similar role in the Thaumarchaeal enzymes.

This phylogenetic analysis establishes that Etr1p and Ccr are relatively close homologues, and as they share the same substrate, crotonyl-CoA, are well suited for a biochemical comparison of active sites and activities.
Figure 2. Phylogenetic tree of the MDR superfamily. Clades are named according to the nomenclature of reference (11). The phylogenetic tree is roughly split among the MDR members that act on C-O bonds (BurkDH: burkhordia dehydrogenases, PDH: polyol-dehydrogenases, TDH: threonine dehydrogenases, CAD: cinnamyl-alcohol dehydrogenases, TADH: tetrameric alcohol dehydrogenases, ADH: alcohol dehydrogenases) and members that act on C-C bonds (MCASTER: mycocerosic acid thioester enoyl-reductases, mETR: mitochondrial enoyl-thioester reductases, LTD: leukotriene B4 dehydrogenase, YHDH: acrylyl-CoA reductases, PKS ER: polyketide synthase enoyl-reductases, VAT1: vesicle amine transport (has no known reductase function), QOR:quinone oxidoreductases, Ccr:crotonyl-CoA carboxylases, unknown: unknown function). The bootstrap values are reported next to each branch. The sequences were aligned with the MAFFT server and the tree was constructed with a maximum likelihood WAG + G model in MEGA 6.
To get a quantitative understanding of the fundamental differences between Ccr and Etr1p we set to measure the activation energies of these enzymes. With the C-2-ene adduct as a molecular probe to study one half reaction at a time we wanted to compare both half reactions in terms of energetic barriers to understand how Ccr keeps the reduction reaction suppressed. The temperature dependence of both wild type reactions were determined between 5 and 35 °C to calculate the activation energy. When the reaction is started with crotonyl-CoA and NADPH (in case case of Ccr also bicarbonate) both Etr1p and Ccr have the same activation energy for their overall reaction, 33 ± 2 and 33 ± 4 kJ/mol, respectively (figure 3). When the reactions are started with the C-2-ene adduct, however, there is a no significant difference between the activation energies. Etr1p and Ccr have activation energies of converting the C-2-ene-adduct to the products of 20 ± 2 kJ/mol and 25 ± 5 kJ/mol respectively. Both enzymes do have a lower activation energy to convert the C-2-ene adduct to the products, ethylmalonyl-CoA and butyryl-CoA for Ccr and Etr1p respectively. Interestingly, when care is taken to remove CO₂ from all the solutions, the activation energy of Ccr for the conversion of C-2-ene adduct increases to 58 ± 3 kJ/mol. In comparison the uncatalyzed decay of the C-2-ene-adduct under the same conditions, 100 mM Na₂HPO₄ pH 8.0, was 89 ± 2 kJ/mol. Both enzymes give a considerable lowering of the activation energy to resolve the C-2-ene adduct of about 65 kJ/mol.
Figure 3. The energetic landscape of Ccr and Etr1p, both enzymes catalyze similar reactions with similar activation energies. The biggest difference between the enzymes is observed in their cross reactivity, Ccr has a high energetic barrier to form butyryl-CoA and Etr1p does not catalyze the carboxylation of the C-2-ene-adduct.

To measure the activation energy for formation of the C-2-ene adduct, we set out to create a mutant enzyme impaired in the second half reaction. In Ccr of *M. extorquens* AM1, CO\(_2\) is bound between the N77, F156 and E157 residues Ccr (these are the homologues of the side chains that were reported to bind CO\(_2\) in CinF (9). We hypothesized that mutating the CO\(_2\) binding pocket would give an enzyme that could only catalyze the conversion between crotonyl-CoA and NADPH and the C-2-ene adduct and not the second half of the reaction. Mutation of N77 to alanine did indeed increase the build up of intermediate. When starting from the intermediate as substrate and using a high concentration of enzyme (0.5 \(\mu\)M) to make the non-enzymatic decay of the intermediate in solution less significant, an isosbestic point was observed (Figure 3). This result shows that practically all the C-2-ene-adduct is equilibrated with NADPH, and that no forward reaction occurs, or otherwise, there would be no isobestic point. In addition, adding CO\(_2\) gave no change in
activity, and mass spectrometry confirmed the absence of carboxylated product. To confirm the absence of catalysis in the protonation of the C-2-ene-adduct, we measured the stereospecific incorporation of solvent protons at Cα. When the reaction was carried out in deuterated buffer, the ratio of (2S) and (2R)-butyryl-CoA was 45:55 ± 4%. We therefore concluded that Ccr N77A almost exclusively catalyzes the first reaction step, the formation of the C-2-ene-adduct and, to a very small extent, the second protonation or carboxylation step. With the knowledge that Ccr N77A did not catalyze carboxylation of the intermediate, we decided to use this mutant to determine the activation energy needed for the ene reaction. The temperature dependence of the formation of C2-ene adduct from crotonyl-CoA and NADPH by Ccr N77A was measured between 5 and 30 °C. The measured activation energy for this first step in catalysis is 26 ± 3 kJ/mol. The turnover of Ccr N77A is 0.45 ± 0.5 s⁻¹, about ~50 times slower than WT enzyme in the presence of CO₂ at 30 °C. Complementary experiments performed by monitoring the reaction by ¹H-NMR gave comparable results, confirming the validity of the UV-Vis spectral assays.

In Etr1p an analogous mutant that can only catalyze the first half reaction from crotonyl-CoA and NADPH to the C-2-ene-adduct was not possible. When Y79 is knocked out, the enzyme forms another adduct, the C-4-adduct (Chapter 3). Therefore the energetic barrier for the first half reaction could not be measured in Etr1p.

5.3 Discussion

1.1.1 Phylogenetic analysis and putative archaeal Ccrs

The phylogenetic analysis of the MDR superfamily showed that Etr1p and Ccr both cluster together with all the other enzymes that act on activated carbon-carbon double bonds, suggesting this class of MDRs only arose once. The branch of bacterial Ccrs has closely related MDRs that are found in Thaumarchaea. These related archaeal enzymes have the same key residues that bind CO₂ in Ccr and might be reductive carboxylases. Lateral gene transfer from bacteria to archaea is much more likely in the environment. It has even been suggested that major lateral gene transfer events from bacteria to archaea probably led to the emergence of the main archaeal clades (9). In addition, Ccrs are more widely distributed in the bacterial domain of life spread over the
actinobacteria and alpha proteobacteria. Therefore, Ccrs have most likely evolved in actinobacteria or alpha proteobacteria and transferred from these bacterial groups by lateral gene transfer to the Thaumarchaeae. The role of Ccr in Thaumarchaeae is unknown as of yet, but there is a possibility that Ccr is used for autotrophic CO$_2$ fixation. One possible cycle where reductive carboxylation could be found is an alternative to the 3-hydroxypropionate/4-hydroxybutyryl-CoA cycle (Berg I. 2007). A part of the cycle is the conversion of acryloyl-CoA by a NADPH dependent reduction to propionyl-CoA and subsequent carboxylation to methylmalonyl-CoA, by a biotin dependent propionyl-CoA carboxylase. These two steps, double bond reduction and subsequent carboxylation, could be performed in one step by a reductive carboxylase that can use acryloyl-CoA as substrate (Ccr has this side reactivity (12)). Such a substitution would save one ATP for each two CO$_2$ molecules fixed in the cycle, which is a quarter of the ATP in the Thaumarchaeal pathway. The very low detected activities in the measured cell lysates for acryloyl-CoA reductase and propionyl-CoA carboxylase support this hypothesis (13). This would make the Thaumarchaeal CO$_2$ fixation cycle, which is already the most efficient aerobic CO$_2$ fixation pathway, even more efficient.

1.1.2 The energetic landscape of Ccr and Etr1p

The ene adduct gives a tool to dissect the catalytic cycle of crotonyl-CoA carboxylases and enoyl-CoA reductases. For the first time a midpoint in the catalytic cycle of these enzymes becomes available to study the hydride transfer separately from the addition of the electrophile, either a 'H$^+$' or a CO$_2$. We note here that even if the ene adduct would not lie exactly on the reaction coordinate of the 'normal' reactions catalyzed by these enzymes it provides a means to study hydride transfer separately from electrophile addition.

The second half of the catalytic cycle, resolution of the ene adduct into butyryl-CoA or ethymalonyl-CoA, is where both enzymes have experienced different evolutionary pressure. Etr1p evolved to reduce 2-enoyl-CoAs whereas Ccr has evolved to preferentially reductively carboxylate 2-enoyl-CoAs. Differences in the energetic barriers from the ene adduct to butyryl-CoA and NADP$^+$ between the reactions catalyzed by Etr1p WT and Ccr WT and uncatalyzed decay reflect the different origin of both enzymes. The higher activation energy of Ccr WT to resolve the ene adduct into butyryl-CoA and NADP$^+$ compared to Etr1p WT
reflects the rudimentary role of the reduction reaction in Ccr WT. The active site of Ccr WT does not contain a proton-donating amino acid and has a hydrophobic phenyl ring of Y156 ring positioned just adjacent to the Cα-the position of the enolate anion- to avoid protonation by water. This is in contrast to Etr1p WT where the phenolic OH group of Y79 points towards the αC and acts as proton donor. However, the stereospecific incorporation of solvent protons into butyryl-CoA by Ccr WT indicates that the active site cannot exclude water from the active site in an absolute fashion. The hydrogen bonds from N77 and E157, important for CO₂ binding and activation, will bind water in the absence of CO₂.

Accommodation of water in the active site does not, however, explain per se why Ccr WT catalyzes the resolution of the ene-adduct as water is also available during uncatalyzed decay. Catalysis of the decomposition of the ene adduct into product can be explained by a combination of several effects. First, the alignment of the nicotinamide N1 lone pair with the πₚ orbital of the crotonyl-CoA C2 and thioester carbonyl lowers the entropy of activation. In addition, correct positioning of the 2' OH of the nicotinamide sugar, by Ccr, to hydrogen bond to the transitionstate thioenolate, lowers the activation energy for product formation. Possibly, the threonine residue analogous to T175 in Etr1p Chapter 4) also contributes to the polarization and decay of the C-2-ene adduct.

Many parallels can be drawn between the evolution of a carboxylation reaction in the MDR and RuBiSCO superfamilies. In the RuBisCO superfamily, most of the members are canonical enolases that catalyse keto-enol tautomerizations (14). Most MDR superfamily members catalyze reductions of C=O or C=C bonds (or the reverse dehydrogenation reaction). In both enzyme superfamilies, most members form an enolate/enol intermediate/transition state that is subsequently protonated. RuBisCO as well as Ccr have evolved in such a way that protonation of the enolate has become a secondary reaction. Instead, the proton donating catalytic residue has made room for a CO₂ binding site. By placing the CO₂ near the nucleophilic enolate, formation of the C-C bond is facilitated. Although both enzymes are unrelated in the way the enolate transition state is formed as well as in the CO₂ binding pocket the mechanism of C-C bond formation is another example of convergent evolution.
RuBisCO enzymes trade off between catalytic turnover and CO$_2$ over O$_2$ selectivity. A higher turnover number is correlated with a lower selectivity and *vice versa*. The exhaustive data available for RuBisCO’s is lacking for Ccr but it is conceivable that an analogous trade off exists between carboxylation and reduction.
5.4 Methods

Proteins purification and mutagenesis were performed according to identical methods as described in Chapter 3.5. crotonyl-CoA was synthesized and purified like described in Chapter 2.5. The C-2-ene adduct was synthesized as described in Chapter 2.5.

Enzymatic assays and uncatalyzed decay reactions were monitored in a Cary 60 UV-Vis spectrophotometer from Agilent Technologies. Temperature was set by a F12-ED Refrigerated/Heating Circulator from Julabo. All reactions were performed in temperature equilibrated 10 mm Quarz cuvettes. Before each measurement, the machine was blanked due to the different absorptions of the cuvettes.

Activation energies $E_a$ were determined using equation 1. Rate constants were determined at 5, 10, 15, 20, 25, 30 and 35 °C. Lower temperatures are not measurable due to freezing of the solvent whereas higher temperatures caused denaturation of the enzyme.

For the phylogenetic analysis, the MDR sequences from (15) were used. *M. extroquens* AM1 Ccr was used as a seed to BLAST for more Ccr sequences. The top 500 hits were downloaded and reduced with the BLAST Clust server of the University of Tübingen to sequences with 70 % similarities. The reduced set of Ccr and the MDR sequences were combined and aligned with the MAFFT 7 server. The sequence alignment was then used to create a maximum likelihood tree with the WAG + G model in MEGA 6.

The stereochemistry of protonation in Ccr N77A was determined as described in Chapter 3.5.
5.5 References


6 Chapter VI: Conclusions and general discussion

Enzymes are unmatched catalysts that make the chemistry of life possible. To achieve their remarkable chemical proficiencies enzymes rely mainly on a limited set of twenty proteinogenic amino acids. For some reactions, however, this limited set lacks the chemical diversity necessary to sustain life. Therefore, some enzymes need post-translational modifications and/or cofactors to extend their catalytic properties beyond what is possible with proteinogenic amino acids. This thesis focuses on the chemistry of the nicotinamide adenine dinucleotide cofactors NADH and NADPH in several related enzymes. Chapter 2 describes the contribution of NADPH to the catalysis of the recently discovered CO\textsubscript{2}-fixing enzyme, Ccr. In the following chapters the mechanism elucidated is extended to other enzymes, detailing the broader implications of the discovered intermediate.

The work presented in this thesis provides evidence in favor of an alternative chemical mechanism for one of life's most abundant redox-cofactors NAD(P)H/ NAD(P)\textsuperscript{+}. The discovery of a covalent intermediate between the substrate, and C-2 of the cofactor, NADPH in crotonyl-CoA carboxylase/reductase (Ccr), suggests that the hydride could transfer through an ene mechanism (1). This alternative mechanism for hydride transfer by nicotinamide cofactors was proposed by Hamilton in 1971 (2) but was not given much attention by the scientific community. Recently, Libby and Mehl reported evidence for this mechanism in model reactions (3). We detected and characterized similar intermediates for the first time in enzyme-catalyzed reactions. This result challenges the mechanism of previously studied enzymes as well as other NADH- and NADPH-dependent enzymes where similar intermediates can be expected. By chemical analogy with the reaction catalyzed by Ccr, we investigated whether enoyl-thioester reductases (Etrs) also react via the same intermediate. Etrs are involved in fatty acid biosynthesis and add a proton instead of a CO\textsubscript{2} besides a hydride to enoyl-thioester substrates. We investigated two Etrs, one from the MDR superfamily, Etr1p, and one from the SDR superfamily, InhA. Both accumulated analogous intermediates when the
active site proton donor, in both cases a tyrosine, was mutated to the non-acidic amino acid phenylalanine. In addition, Etr1p accepts the intermediate from Ccr as a substrate. As the stereochemistry of the hydride that is transferred from NAD(P)H in SDRs is inverted compared to MDRs the analogous experiment could not be performed with InhA. Taken together these results are consistent with an ene mechanism that would challenge many studies in the field of NAD(P)H chemistry. The current data, however, cannot rule out a classical direct hydride transfer followed by a Michael addition of the formed enolate to the nicotinamide ring at the C-2 position.

Independent of the fundamental question of how the hydride transfers between NAD(P)H and the substrate, these results have broader implications. A common way to study enzyme active sites is to mutate specific residues and measure the effects on substrate binding or catalysis. In Chapter 4 we show that active site mutantions in Etr1p as well as InhA give enzymes that accumulate significant amounts of intermediates that are not converted to the final products in the active sites of these enzymes. Therefore, measuring substrate consumption is not enough in these active site mutants because there is an additional variable, whether or not an intermediate is formed and if it is, how much. Also, the measurements of kinetic isotope effects on the protonation step are skewed. The conventional method for measuring such isotope effects relies on measuring NAD(P)H consumption which is uncoupled from the protonation step by the formation of a covalent intermediate that accumulates. The detection of the intermediates described in this thesis therefore calls for caution in drawing conclusions from biochemical studies on Etrs mutants that only rely on the measurement of substrate consumption at a single wavelength. We recommend that future studies should take care to investigate whether adducts similar to the ones described in this thesis accumulate in the studied mutants and, if so, use NMR spectroscopy to measure activity. To measure kinetic isotope effects on protonation we developed a new facile competitive method based on mass spectrometry (Chapter 4).

As the C-2-ene adduct is a kinetically competent substrate for the Etr1p WT it represents a valuable molecular probe. It represents a state in the reaction where the hydride equivalent is already transferred from NAD(P)H to the enoyl-CoA substrate but not the proton. This property makes the C-2-ene adduct very valuable for studying the protonation step. Numerous studies in the past have
tried to identify the proton donor in the active sites of Etrs, but with little success (4-8). Chapter 3 shows why past studies had little success. Usually, kinetic analyses depend on measuring the initial velocities of an enzyme reaction, to prevent problems like product inhibition. When an active site proton donor is mutated, however, the initial NAD(P)H consumption is due to formation of adducts analogous to the C-2-ene adduct and to a lesser extent the C-4-adduct, and not due to product formation. This gives misleading results when only the NAD(P)H consumption is measured because the observed reaction is not the same as in the WT enzymes. Starting the reaction with the C-2-ene adduct circumvents this issue because the first step of the reaction has already occurred. With this strategy, we used the C-2-ene adduct to engineer an Etr with inverted stereochemistry. Changing the stereochemistry of Etrs brings design of polyketides one step closer. Keatinge-Clay (9) has determined what residues control the stereochemistry in the keto-reductase step in polyketide synthases. Our discoveries in chapter 3 can lead to control of the stereochemistry of the enoyl-reduction step. If our stereochemistry engineering strategy were realized in polyketide synthases this would give full control of the backbone stereocenters. In turn enabling the biosynthesis of altered polyketide variants with different pharmaceutical properties would become possible.

In Chapter 3 we describe another covalent adduct between the Cα of crotonyl-CoA and the C-4 center of NADPH. This adduct forms in Etr1p when the catalytic proton donor, tyrosine 79, is mutated to phenylalanine. Unlike the C-2-ene adduct the C-4-adduct does not serve as substrate for either Ccr WT or Etr1p WT. Moreover, the C-4 adduct is a potent inhibitor of Etr1p WT. There are other reports of adducts with NAD(P)H that serve as potent inhibitors of NAD(P)H dependent enzymes (10-14). Formation of covalent adducts with NAD(P)H is therefore an attractive design principle for new inhibitors. If the reaction of NAD(P)H and a drug can be stopped at the covalent stage, the adduct is likely to be a potent inhibitor, extrapolating from the results of other studies. An attractive approach to stop the reaction at the covalent stage is synthesizing chemical derivatives of the substrate. To trap the reaction with a covalent adduct, the enoyl C=C bond needs to be reactive enough to be reduced, but too inactive to decay if an adduct is formed. In Chapter 5 we show that an Etr from M. tuberculosis InhA forms a covalent adduct when the protonation step is hampered. If a substrate analogue could stop the reaction at this stage this would give a new class of mechanism-based inhibitors for this
important antibiotic target. Possibly this strategy can be extended further to many other NAD(P)H dependent enzymes that are targets of pharmaceuticals (for a list:(15)).

The C-2ene-adduct in Ccr WT in the absence of CO$_2$ also gives some insight into why Ccr does not have oxygenase activity. Nature’s most abundant CO$_2$-fixing enzyme, RubisCO, suffers from a lack of selectivity between CO$_2$ and O$_2$, which have similar electrostatic potential Figure 1. This side reaction with oxygen reduces photosynthetic efficiency (16, 17). The RuBisCO reaction proceeds through several elemental steps yielding an enediolate. This enediolate is the reactive species in both reactions catalyzed by RuBisCO; the carboxylation and oxygenation of 1,5-ribulosebisphosphate. The details of the oxygenase reaction of RubisCO are, however, still unknown (18). Especially how the spin forbidden reaction between ground state triplet oxygen and singlet state ribulose-1,5-bisphosphate or its 2,3-enediolate form occurs, is unclear. The most likely mechanism is a singlet-triplet conversion of the 2,3-enediolate substrate (18). Numerous studies have tried to improve the selectivity of RuBisCO but have failed to give an improved enzyme in a practical sense. An increase of the CO$_2$ versus O$_2$ selectivity comes at the cost of turnover speed (19, 20), which is already low (in the 10$^1$ s$^{-1}$ range) for an enzyme in central carbon metabolism (21). Ccr, on the other hand, is an order of magnitude faster (25 s$^{-1}$ Chapter 2). Despite the resemblance between the enolate intermediate in the Ccr reaction and the enediolate intermediate in the RuBisCO reaction, we did not detect any oxygenase activity for Ccr. This difference could lie in the stability of the triplet state of the enolate of crotonyl-CoA. This makes Ccr an attractive enzyme for the design new CO$_2$ fixation pathways. It is relatively fast, is not sensitive to oxygen, and the side activity of the reduction reaction is small in the presence of CO$_2$. 
The C2-ene adduct only accumulates in the absence of CO₂ in Ccr with a turnover of about one tenth of the physiological carboxylation reaction. This leaves us with two possible conclusions. A) The hydride equivalent transfers through an ene mechanism forming the C-2ene adduct in the active site. The absence of an electrophilic CO₂ molecule in the active site hampers the decay of the adduct, leading to its buildup. B) The mechanism is a classical direct hydride transfer, forming an enolate that in absence of CO₂ adds through a Michael addition to the C-2 of NADP⁺ and forms the C2-ene adduct. If the thermodynamics of CO₂ binding in Ccr are similar to RuBisCO then either explanation can provide insights into the catalytic rational of a covalent ene adduct in Ccr. In RuBisCO there is no Michalis complex of CO₂ and enzyme. Instead when the reactive 2,3-enediolate intermediate forms in the active site, CO₂ diffuses in and carboxylation takes place (23, 24). Attempts to crystallize a Ccr from *Streptomyces* sp. JS360 with CO₂ in the active site failed. Even under

Figure 1. The quadrupole moment of CO₂ (left and O₂ (right) is visualized by showing the molecular surface with mapped electrostatic potential. The color scheme follows commonly accepted conventions: blue, positive; red, negative. The values of the Qzz component of the quadrupole moment are listed with CO₂ having 15 times larger moment than O₂. Reproduced from (22).
a 40 bar CO$_2$ atmosphere there was no detectable CO$_2$ binding (25). This result shows that if there is a binary Michaelis complex of CO$_2$ and Ccr it is very weak. The likelihood that the absence of CO$_2$ binding is a crystal-packing artefact is minimal as the CO$_2$ molecule can be modeled into a clear cavity in the active site (25). Taken together, it seems likely that there is no Michaelis complex of Ccr with CO$_2$ as for RuBisCO. The observed C-2-ene adduct can, independent of the formation mechanism, serve as a high-energy intermediate. The energy released in the hydride transfer is ‘stored’ by forming the C-2-ene adduct until a CO$_2$ molecule enters the active site. In the presence of CO$_2$, the decay of the C-2-ene adduct can be coupled to carboxylation to make the carboxylation thermodynamically favored. This is similar to the carboxylation reaction of RuBisCO where hydrolysis and carboxylation are coupled to make the reaction thermodynamically favored (26).

6.1.1 Ene intermediates in other enzymes

There are several literature reports of detected intermediates in the SDR and MDR superfamilies (27, 28), but the most notable in the context of an ene mechanism is the detection of an intermediate in the reaction of horse liver alcohol dehydrogenase with 3-hydroxy-4-nitrobenzaldehyde (29). When this compound is used as substrate, an intermediate is detected in rapid-scanning stopped-flow experiments. The following reaction scheme is proposed for the overall reaction:

\[
\text{E(NADH)} \rightarrow \text{E(X)} \rightarrow \text{E(NAD$^+$)}
\]

Where E(NADH) and E(NAD$^+$) are the enzyme NADH and NAD$^+$ complexes and E(X) the detected intermediate. The UV-vis spectrum of the intermediate, E(X), deconvoluted from the different absorption spectra of the substrate, product and NADH has an absorption maximum around 370 nm. This absorption spectrum corresponds well with the absorption maximum of the C2-ene adduct of NADPH and crotonyl-CoA. The intermediate is formed in a burst and its formation is mirrored by a burst in the consumption of the substrate aldehyde. The data obtained in this thesis with crotonyl-CoA reductase carboxylase is very similar. The substrate consumption rate mirrors the formation rate of the intermediate.
but not by the formation of the products. The detection of this intermediate can be interpreted as evidence in favor of the formation of an ene-adduct and possibly an ene mechanism in an alcohol dehydrogenase.

**Outlook**

The goal of this thesis was to investigate the mechanism of the recently discovered CO$_2$-fixing enzyme Ccr and to understand the chemistry of different NAD(P)H dependent enzymes. The detection of an unusual C-2-ene adduct in the catalytic cycle of Ccr gives insights into how it catalyzes CO$_2$ fixation. In addition, the detection of this C-2-ene adduct raises questions about the mechanism of the zinc-independent MDRs and SDRs, and possibly also the zinc dependent members of these enzyme superfamilies.

The formation of the C-2-eneadduct does not seem to be governed by very specific catalytic residues. The formation of the adduct seems to be governed by entropic trapping of the substrate in the right orientation. Therefore, this thesis provides a new strategy to design NAD(P)H-dependent enzymes. Binding of the substrate in the correct position compared to the nicotinamide ring can already lead to the formation of a covalent adduct between the substrate and NAD(P)H that can decay in solution to give the products. Such an enzyme could serve as the starting point for directed evolution to place an active site proton donor.

In Chapter 3 we showed that the stereochemistry of protonation in Etr1p can easily be inverted. Extending these results to Etrs found in polyketide synthases would lead to full control of the backbone stereocenters. This can bring designer polyketides with improved biological properties one step closer to reality.

The mechanism of Ccr, which has a relatively high turnover frequency and does not catalyze an oxygenation reaction, can provide interesting insights into improving the most abundant enzyme on earth, RuBisCO. As such, it is interesting to know if the C-2-ene adduct is also formed in the presence of CO$_2$ and if CO$_2$ can catalyze the decay of this catalytically competent adduct. A detailed understanding of these reactions can lead to new small molecule catalysts to fix CO$_2$ as well as improving carboxylating enzymes.
In this thesis the C-2-ene adduct was detected under non-physiological conditions. The intermediate builds up in Ccr in the absence of CO$_2$, in Etr1p and InhA in the absence of the catalytic proton donor and in Yhdh when the substrate is crotonyl-CoA. Therefore, it is interesting to know whether the the C-2-ene-adduct plays a role under native conditions and, if so, what classes of enzymes react with this new mechanism. Measuring the isotope effects on all the carbons in the nicotinamide ring could give conclusive evidence regarding the question whether a covalent adduct is formed at C-2 during catalysis. If the ene mechanism proposed in this thesis is widespread in NAD(P)H chemistry this would revive mechanistic studies on a cofactor that was discovered more than 60 years ago.
6.2 References


7 Curriculum vitae

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Education

Phd in Chemistry and Applied Biosciences, ETH Zürich (CH) and Max Planck Institute for terrestrial microbiology (DE) '12 - '15
-under supervision of Dr. Tobias Erb & Prof. Vorholt
-"Expanding the chemistry of the nicotinamide adenine dinucleotides"

MSc in Biological Chemistry, ETH Zürich (CH) '10 - '12
- Focus on organic chemistry and enzyme catalysis (theoretical and practical) with honors

BSc in Life science & Technology, TU Delft/Leiden University (NL) '07 - '10
- exchange semester at ETH Zürich: gained international experience cum laude
- summer internship in organic chemistry, Leiden University

High school (Athenaeum), JSG Maimonides, Amsterdam (NL) '07

Employment and teaching experience

Visiting scientist computational protein design Weizman Institute for science (IL) '12
- Worked in Dr. Fleishmann's lab on computational protein design.

Teaching assistant in organic chemistry, ETH Zurich (CH) '11

Volunteer at Stichting Rino, Leiden (NL) '08 - '10
- teaching and promoting natural sciences for various audiences
Noteworthy educational experiences and awards

39th International Chemistry Olympiad, Moscow (RU) '07
- Best Dutch team member

Awards
- Travel award ASBMB 15'
- Best paper award '13, Institute of Microbiology, ETH Zürich
- Best poster award '13, Zurich Life science Graduate school
- Huygens Scholarship '10-'12, full scholarship awarded by NUFIC to pursue postgraduate education
- Young talent prize '08, awarded to the best 40 natural science students in the Netherlands by the Royal Dutch academy of science
- Scholarship from The Association of the Dutch Chemical Industry '07

List of publications


Selected oral presentations
