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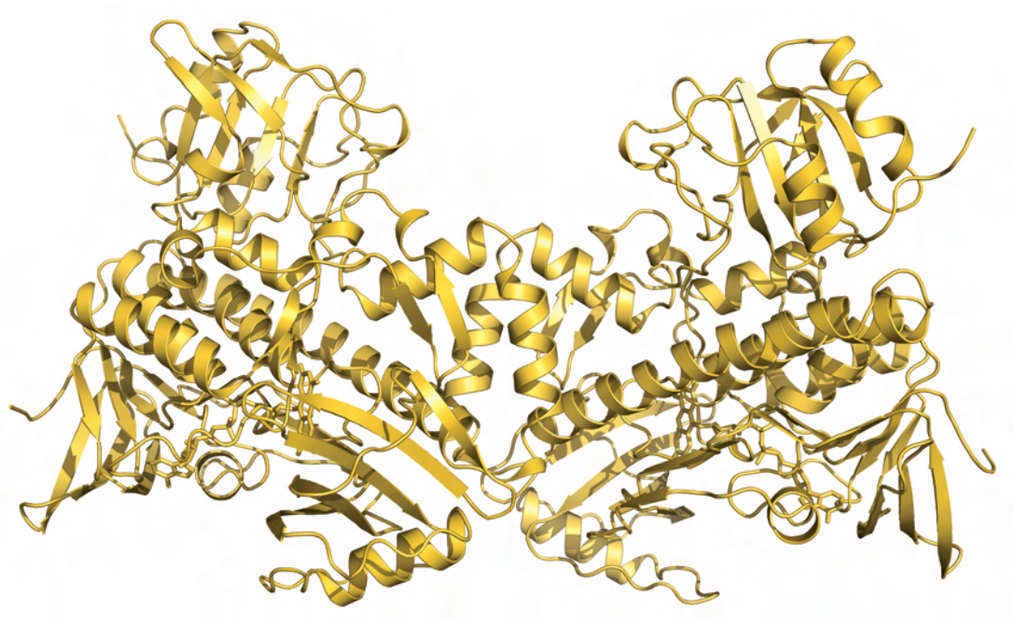

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To my family

En päivääkään vaihtaisi pois…

-Tapio Rautavaara

ABSTRACT

The electron structure of molecular oxygen (O_2) is such that it cannot spontaneously react with organic molecules, a feature that protects organisms from oxidative stress. However, oxygenases can insert O_2 in organic substrates. The enzymes do this after activating O_2 for the reaction, most often with the aid of an organic or inorganic cofactor. A number of different cofactors have been discovered in nature thus far.

In this thesis, five oxygenases with three different catalytic machineries for activation of oxygen have been investigated structurally and biochemically. The enzymes participate in the biosynthesis of aromatic polyketide antibiotics. These natural products possess a range of biological activities, and a number of them have been employed as therapeutic agents as, for instance, antimicrobials and cytostatics in the treatment of cancer. Presently the number of antibiotic-resistant bacteria is increasing alarmingly, and the polyketide-derived cytostatic agents are causing severe side-effects to patients. Thus development of novel polyketide antibiotics with beneficial medicinal properties is highly desirable. Structure-function studies of the biosynthetic enzymes could be of aid in this.

Firstly, three flavin-dependent aromatic hydroxylases, PgaE, CabE and RdmE, were chosen as targets of study. They belong to a family of aromatic hydroxylases, for which *para*-hydroxybenzoate hydroxylase is a well-characterized example. Main features in the catalysis are common to all enzymes of the family and the overall structures are similar. However, there appear to be intriguing differences regarding, for instance, substrate entry and oligomeric state. With RdmE, a ternary complex was obtained with the polyketide substrate, offering insights into substrate recognition.

RdmB exhibits high sequence and structural similarity to SAM-dependent small-molecule methyl transferases, however it hydroxylates its substrate. The structural and biochemical studies revealed the enzyme to indeed utilize SAM as a cofactor in the reaction, but a substrate-carbanion species carries out the activation of O_2. Subtle changes in the orientation of the substrate and the cofactor binding appear to be responsible for the change of activity from a methyl transferase to a hydroxylase. This is a novel function for SAM, and identifies RdmB as a new type of hydroxylase.

SnoaB is a small cofactor-independent oxygenase. Based on structural and biochemical characterization a mechanism proceeding *via* a substrate carbanion intermediate is proposed. This is in accordance with recent results obtained for other cofactor-independent oxygenases.

Despite the very different three-dimensional structures and the different machineries utilized, the five enzymes appear to activate molecular oxygen in a very similar manner: they all form protein- and resonance-stabilized carbanions, which form hydroperoxy-intermediates with O_2. In addition, the study has provided further examples of divergent evolution, a phenomenon which appears to be a common feature in polyketide biosynthesis. Furthermore, some insights into enzymatic recognition of the large hydrophobic polyketide substrates have been obtained.

LIST OF PUBLICATIONS

I. Jansson, A., **Koskiniemi, H.**, Erola, A., Wang, J., Mäntsälä, P., Schneider, G. and Niemi, J. (2005) Aclacinomycin 10-hydroxylase is a novel substrate-assisted hydroxylase requiring S-adenosyl-L-methionine as cofactor. *J. Biol. Chem.* **280**, 3636-3644.

II. **Koskiniemi, H**., Metsä-Ketelä, M., Dobritzsch, D., Kallio, P., Korhonen, H., Mäntsälä, P., Schneider, G. and Niemi, J. (2007) Crystal structures of two aromatic hydroxylases involved in the early tailoring steps of angucycline biosynthesis. *J. Mol. Biol.* **372**, 633-648.

III. Lindqvist, Y., **Koskiniemi, H.**, Jansson, A., Sandalova, T., Schnell, R., Liu, Z., Mäntsälä, P., Niemi, J. and Schneider, G. (2009) Structural basis for substrate recognition and specificity in aklavinone-11-hydroxylase from rhodomycin biosynthesis. *Submitted*

IV. **Koskiniemi, H.***, Grocholski, T.*, Schneider, G. and Niemi, J. (2009) Expression, purification and crystallization of the cofactor-independent monooxygenase SnoaB from the nogalamycin biosynthetic pathway. *Acta Cryst.* **F65**, 256-259.

V. Grocholski, T.*, **Koskiniemi, H.***, Lindqvist, Y., Niemi, J. and Schneider, G. (2009) Crystal structure of SnoaB, a cofactorless monooxygenase from *Streptomyces nogalater*: implications for the reaction mechanism. *Manuscript*

*Equally contributing authors

A RELATED PUBLICATION NOT INCLUDED IN THE THESIS

Jansson, A.*, Koskiniemi, H.*, Mäntsälä, P., Niemi, J. and Schneider, G. (2004)
Crystal structure of a ternary complex of DnrK, a methyltransferase in daunorubicin biosynthesis, with bound products.
J. Biol. Chem. **279**, 41149-41156.

*Equally contributing authors

CONTENTS

LIST OF ABBREVIATIONS

ActVA-Orf6	Monooxygenase from actinorhodin biosynthesis
BH_4	Tetrahydrobiopterin
CabE	Angucycline 11-hydroxylase
CD	Circular dichroism
DbrA	11-deoxy-β-rhodomycin
DmaA	15-demethoxyaclacinomycin
DnrK	Carminomycin 4-O-methyltransferase
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FMO	Flavin containing monooxygenase
MAD	Multiple wavelength anomalous diffraction
MHBH	3-hydroxybenzoate hydroxylase
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced form
PgaE	Angucycline 12-hydroxylase
pHBH	*para*-Hydroxybenzoate hydroxylase
PHHY	Phenol hydroxylase
PhzS	Pyocyanin biosynthetic protein
RdmB	Aclacinomycin 10-hydroxylase
RdmC	Aclacinomycin-15-methylesterase
RdmE	Aclacinomycin 11-hydroxylase
RebC	Hydroxylase from rebeccamycin biosynthesis
SAD	Single wavelength anomalous diffraction
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
SnoaB	12-Monooxygenase from nogalamycin biosynthesis
YgiN	Quinol oxygenase

1 INTRODUCTION

1.1 MOLECULAR OXYGEN

Oxygen is the third most abundant element in the universe and the most abundant in the Earth's crust (Dole 1965; Gilbert, 1972). Molecular oxygen appeared on Earth ca. 2.4 billion years ago, most likely produced as a side product in photosynthesis by blue-green algae (Kerr, 2005). Molecular oxygen in the atmosphere has enabled cellular respiration and the development of multicellular organisms on Earth. Atomic oxygen (O) is extremely reactive and hardly exists in free form, but oxygen is abundant in organic and inorganic molecules and as free gas, as molecular oxygen (dioxygen or O_2). This gas is vital for aerobic life, but toxic in excess and toxic to anaerobic organisms. In the higher atmosphere oxygen exists also as a toxic allotrope, ozone (O_3). Ozone absorbs strongly ultra violet (UV) light, thereby protecting Earth from dangerous UV radiation. The most common isotope of molecular oxygen is the extremely stable $^{16}O_2$, but minute amounts of isotopes $^{17}O_2$ and $^{18}O_2$ do exist as well (Gilbert, 1972).

The story of oxygen is fascinating, and already its discovery was rather dramatic. Molecular oxygen was first scientifically described in the late 18th century independently by two researchers, the Swedish pharmacist Carl Wilhelm Scheele in 1771-1772 and the English Unitarian minister and acknowledged researcher Joseph Priestly in 1774. Scheele was in fact the first to make the discovery and named the novel substance *fire air*. However, as Priestley was the first to publish his results, he has generally been acknowledged for the discovery. Priestley noticed that the novel gas was vital for mice, was needed to keep a candle lit, and was regenerated by plants. The name *oxygen* was coined in 1777 by a Frenchman, Antoine L. Lavoisier. The name originates from Greek oxus (acid) and gennan (generate), as Lavoisier believed oxygen to be present in all acids (Sawyer, 1991; Severinghaus, 2002; Sternbach & Varon, 2005).

Molecular oxygen is produced from water by green plants and cyanobacteria in photosynthesis with energy from the sun. Cellular respiration is essentially the reverse of this: oxygen is reduced to water in the respiratory chain by cytochrome C oxidase, and the released energy is bound in ATP for cellular use (Williams, 1984). Amazingly, cytochrome C oxidase can "harness" as much as 80% of the energy of molecular oxygen, which is more than has been ever achieved in a chemistry laboratory (Sawyer, 1991). Although oxygen is essential to life, it does have its dark side as well: O_2 is toxic to cells at high partial pressures. This is thought to be due to increased generation of free radical oxygen species (ROS). They are formed *via* reductive steps, are very reactive and promote production of further radicals. The radicals formed from oxygen are described in figure 1. These radicals can promote formation of secondary radicals with biomolecules and cause severe damage to cellular membranes, proteins and DNA (Gilbert, 1972; Halliwell & Gutteridge, 1984). To avoid the cellular damage and ultimately cell death, cells have developed protective mechanisms, such as radical scavenging enzymes (superoxide dismutase, peroxidase, catalase) and antioxidants (vitamin E) (Gilbert, 1972; Williams, 1984).

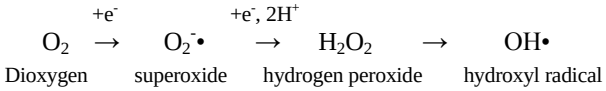


Figure 1. Generation of reactive oxygen species *via* sequential additions of single electrons

Intriguingly, oxygen's great value for aerobic life lies partly in its reactivity and energy, and partly in its inert nature. It reacts with almost any atom given enough time, with noble gases being the only exceptions (Sawyer, 1991). As mentioned above, it provides the thermodynamic basis for cellular respiration: the four-electron reduction of dioxygen to water releases 222 kcal/mol of energy (Sawyer, 1991). This thermodynamic energy is, however, counterbalanced with high kinetic energy of activation, which makes oxygen chemically inert to spontaneous reduction. This is essential for protecting aerobic organisms from harmful generation of oxygen radicals and is brought about by two properties of molecular oxygen (Malmström, 1982; Sawyer, 1991). First, one-electron reduction of dioxygen to superoxide radical has low standard reduction potential. Only few biological systems have a lower reduction potential, capable of reducing it (the subsequent one-electron reductions to water have positive reduction potentials, as well as the overall reduction from dioxygen to water) (Wood, 1988; Sawyer, 1991). Secondly, as will be discussed below, the electron structure of oxygen prevents it from reacting spontaneously with most organic compounds (Malmström, 1982; Sawyer, 1991).

1.1.1 Spin barrier

The exceptional chemical and physical properties of dioxygen are due to its electron structure. It has two unpaired electrons in two antibonding electron orbitals. This arrangement of electrons is unusual: electrons tend to exist in pairs in molecular orbitals with opposite electron spin. Electron spin is a property that describes the rotation of an electron around itself, and it can only have values + ½ or - ½, where the sign +/- denotes the direction of the rotation. The spinning gives electrons magnetic properties, which are usually counterbalanced by the opposite spins of two paired electrons, and the overall spin angular momentum (S) of a molecule is 0. This electron state is called *singlet state*. If a molecule has a single unpaired electron, its total spin angular momentum S is +/- ½ and the spin state is called doublet. The molecule is of radical nature. Molecules in radical state are generally very reactive and short-lived. The total spin angular momentum of molecular oxygen is ½ + ½ = 1 and oxygen's ground state is called *triplet*. As it has two unpaired electrons in molecular orbitals, it has two radical electrons, and oxygen has *diradical* nature. The two unpaired electrons and the total spin of 1 also mean that molecular oxygen has paramagnetic properties, as the spins are not counter-balanced (Malmström, 1982; Fetzner, 2002).

A direct reaction between molecules in singlet and triplet states is forbidden by the physical rule of conservation of angular momentum (Fetzner, 2002, 2007). As most organic molecules are in singlet state, there is a spin barrier for reactions between dioxygen and organic molecules (Malmström, 1982). This is one of the properties of oxygen that protect cells from harmful reactions with it. In addition to the triplet ground state, dioxygen has two singlet forms; with the electrons in opposite spins on either the

same orbital or two separate orbitals. However, the conversion of the ground state to singlet state requires energy, although it can be generated *via* a photochemical reaction. As singlet oxygen can readily react with organic compounds, it is extremely reactive and very short-lived (Gilbert, 1972; Halliwell & Gutteridge, 1984).

1.1.2 Oxygenases and oxygen activation

For years it was believed that oxygen was enzymatically incorporated in organic molecules only from water. However, in the early 1950s, Osamu Hayaishi in Japan was conducting studies on pyrocatechase and Howard Mason in the United States on phenolase. Both researchers showed independently with the oxygen isotope $^{18}O_2$ that the enzymes were utilizing atmospheric oxygen as a substrate (Hayaishi *et al*., 1955; Mason *et al*., 1955). Oxygenases and enzymatic activation of oxygen were thus discovered, and have been subjects of active research ever since.

There are various types of enzymes utilizing oxygen as substrate. Oxidases use oxygen as the final electron acceptor, and water or hydrogen peroxide (H_2O_2) is one of the products (Malmström, 1982). During my doctoral studies I have concentrated on oxygenases, which catalyze insertion of oxygen into organic molecules. There are two main types of oxygenases: dioxygenases add both atoms of molecular oxygen into the organic substrate, whereas monooxygenases add only one of them. The other atom is reduced to water (Malmström, 1982; Waterman, 2005). Bacterial oxygenases are involved in degradation of aromatic compounds and biosynthesis of various organic compounds, including antibiotics. Mammalian oxygenases participate in detoxification of foreign compounds and biosynthesis of, for instance, hormones and neurotransmitters. But how is it possible for enzymes to add molecular oxygen into organic molecules, despite the spin barrier, and what does activation of oxygen mean?

Direct reaction between the triplet oxygen and organic molecules in singlet state is not possible, at least at ambient temperatures. However, as dioxygen is of diradical nature, it can react directly with other molecules with unpaired electrons. It can thus readily react with, for instance, carbon-centered radicals and transition metals (Sawyer, 1991). Furthermore, there are no kinetic barriers for reduction of oxygen by sequential addition of single electrons. The phenomenon of transforming dioxygen into a species that can react with organic molecules is called *activation of oxygen*.

Oxygenases most often use *cofactors* as aid in activation of molecular oxygen. Inorganic cofactors in oxygenases are generally transition-metals and organic cofactors include flavins. The electrons for the reaction are usually obtained from an external electron source. The reduction of the cofactor can be accomplished in the same polypeptide as the oxygenation reaction, but many oxygenases depend on another enzyme, reductase, which derives the electrons from the reducing agent and delivers them to the actual oxygenase. In the following section the various oxygenase types are presented based on their cofactor dependency.

1.1.2.1 Cytochrome P450 monooxygenases

Cytochrome P450 enzymes are among the most common oxygenases in nature. They are abundant in particular in eukaryotic organisms, and for instance in human they are

widely utilized in catabolism of foreign molecules and synthesis of sex hormones. Interestingly, they are less abundant in bacteria. They typically insert a hydroxyl group in their substrates and are able to oxygenate even non-activated carbon atoms. They exist in various isoforms, each specific for certain substrates. P450-enzymes contain a heme cofactor, in which the iron ion is coordinated by an invariant cysteine residue from the enzyme. The electrons for the reaction are typically provided by nicotinamide adenine dinucleotide phosphate (NADPH) *via* a specific flavin-dependent reductase. The cofactor is reduced only after substrate binding (van Berkel *et al*., 2006; Guengerich, 2008; Hamdane *et al*., 2008).

Activation of oxygen in P450 enzymes is thought to occur *via* an intermediate between iron and dioxygen. The reaction is initiated by substrate binding: this increases the redox-potential of the heme-Fe^{+III}, which can subsequently be reduced to heme-Fe^{+II}. The oxygen then binds the heme-iron, and a heme-Fe^{+II}–OO (or heme-Fe^{+III}-OO^{-}) complex is formed. This species is reduced with another electron and protonated to yield a heme-Fe^{+II}–OOH (or heme-Fe^{+III}-OOH^{-}) intermediate. The O-O-bond of this intermediate is broken *via* heterolytic cleavage after second protonation. The products of the cleavage are water and a very reactive cationic radical intermediate, (heme-Fe^{+IV}=O)$\bullet^{+}$. This activated oxygen atom is transferred to the substrate, and the heme is returned to the resting heme-Fe^{+III}-state (Harayama *et al*., 1992; Denisov *et al*., 2005; Groves, 2005; Makris *et al*., 2005; Guengerich, 2008; Hamdane *et al*., 2008).

Cytochrome P450 oxygenases are not the sole proteins possessing a heme as cofactor: for instance, cytochrome c oxidase, peroxidases and hemoglobin possess the same prosthetic group (Williams, 1984; Rydberg *et al*., 2004). It is of particular interest that hemoglobin, the protein that carries oxygen in red blood cells, utilizes heme to bind oxygen: in red blood cells it would be detrimental to risk the escape of activated oxygen molecules (Williams, 1984). The main difference between the hemes in hemoglobin and P450 hydroxylases is the protein ligand of the iron: the cysteine residue in P450 is replaced by histidine in hemoglobin. With a histidine ligand, the redox-potential of the oxygen complex is lower, lowering the activity of the oxygen and reducing the risk of escape of oxygen as hydrogen peroxide or superoxide anion (Rydberg *et al*., 2004).

1.1.2.2 Non-heme metal dependent enzymes

Transition metals may have unpaired electrons in their d-orbitals and can adopt different spin-states, which are the reasons why they can readily react with dioxygen. They also have excited electronic states close to the energy of the ground state, which can facilitate spin-inversion (Jensen & Ryde, 2004; Pau *et al*., 2007). The most commonly utilized metals in oxygenases is non-heme iron, followed by copper (Evans *et al*., 2003) and manganese (Winkler *et al*., 2007). The metal cofactor can be mono- or multinuclear, and it is also common for oxygenases to have an organic cofactor in addition to the metal (Harayama *et al*., 1992). The necessary electrons are often derived from an external reducing agent, or from the substrate, as for example in extradiol chatecol dioxygenases (Mendel *et al*., 2004).

The mechanisms and the reactive intermediates of reactions with oxygen are varied, depending on the metal, the reaction type and the protein environment. For instance, iron can be reduced with two electrons (from Fe^{4+} to Fe^{2+}), and the redox-potential for each pair (Fe^{2+}/Fe^{3+}, Fe^{3+}/Fe^{4+}) is fine-tuned by the protein environment. Iron is a very strong activating agent for oxygen reactions and can generate aggressive oxo-cations. Copper, on the other hand, exists in only two oxidative states in enzymes, Cu^{1+}/Cu^{2+} and is thus capable of transfer of only single electrons. The redox-potential of the Cu^{1+}/Cu^{2+} pair is in a range that is difficult to achieve with the iron cations. Thus it is obvious that the different metals are utilized in different circumstances and different reaction mechanisms are employed (Williams, 1984).

Typically dioxygen binds the metal in the reduced state, after which an electron is transferred from the metal to oxygen within the complex (Malmström, 1982). The second electron is transferred either by the substrate or the metal. Covalent peroxy and hydroperoxy species as well as radical species are common intermediates in metal-catalyzed oxygenations. The reactive oxygen species are often generated by heterolytic or homolytic cleavage of the O-O-bond, as with P450 enzymes described above (Malmström, 1982; Sawyer, 1991; Decker & Solomon, 2005). Dopamine β-monooxygenase provides an example of a binuclear copper enzyme (Evans *et al*., 2003) and the extradiol chatecol dioxygenases are non-heme iron dependent enzymes (Mendel *et al*., 2004). There is also an alternative, less common mechanism for metal-dependent oxygen activation: in some cases the metal in the oxidized state first binds the substrate, and activates it for the reaction with oxygen (Pau *et al*., 2007). Lipoxygenase is an example of such an enzyme: Fe^{+3} together with a catalytic water molecule abstracts a hydrogen from the substrate, thereby generating a radical substrate intermediate. The reaction between the radical substrate species and dioxygen thereafter is spin-allowed (Solomon *et al*., 1997).

1.1.2.3 Flavoenzymes

Flavoenzymes possess an isoalloxazine-ring-containing cofactor, which gives the enzymes bright yellow color (Massey, 2000). Flavoenzymes have been subjects of study for decades and a wealth of information has been obtained, for instance in July 2009, there were 962 protein structures containing an FAD moiety in the PDB and 533 with FMN. Flavoenzymes occur in all life-forms from bacteria to human. Flavin-derived prosthetic groups are generated from riboflavin, which bacteria and plants are capable of synthesizing *de novo*; mammals need to derive riboflavin from diet (De Colibus & Mattevi, 2006). Flavins are utilized extensively as reduction-oxidation (redox) -centers in various energy-demanding tasks, including halogenations of organic substrates, oxidation of aliphatic substrates and hydroxylation of aromatic compounds. Thus flavin-binding proteins are involved in cellular tasks such as energy metabolism, protein folding and apoptosis (Massey, 2000; De Colibus & Mattevi, 2006; Joosten & van Berkel, 2007; Senda *et al*., 2009).

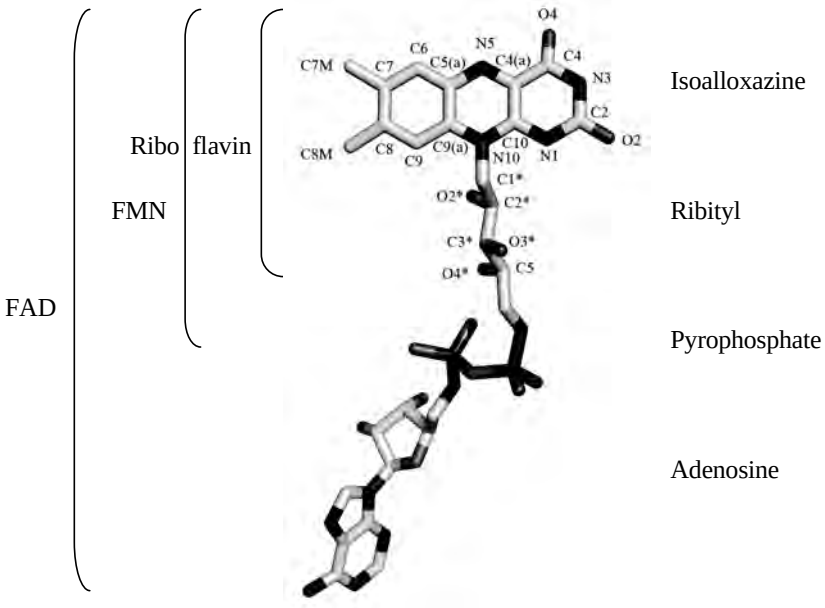


Figure 2. Structure and atom labels of riboflavin and flavin adenine dinucleotide (Adapted from Senda *et al*., 2009)

The two most common flavin derivatives in enzymes are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (figure 2). In riboflavin (or vitamin B_2), there is a ribityl-sugar moiety attached to the isoalloxazine ring. FMN has in addition a phosphate, whereas FAD has a pyrophosphate and an adenosine moiety (Massey, 2000). The isoalloxazine-ring is a fusion of a pyrimidine ring and a dimethylbenzene ring and serves as the redox-center of the cofactor (Fraaije & Mattevi, 2000). The phosphates and the adenosine moieties are mainly used for anchoring the cofactor in the enzyme. Flavins are most often bound noncovalently in proteins, but there are cases where it is bound with one or even two covalent bonds, as for example in aclacinomycin oxidoreductase (AknOx) (Alexeev *et al*., 2007) and vanillyl-alcohol oxidase (VAO) (Mattevi *et al*., 1997). The great chemical potency of flavins is due to their ability to exist in three oxidative states: oxidized, one-electron reduced and two-electron reduced (figure 3) (Massey, 2000). The color of the cofactor depends on the redox-state, which enables detailed mechanistic studies of flavoenzymes with spectroscopic methods (Massey, 1995, 2000; Fraaije & Mattevi, 2000). Furthermore, both the isoalloxazine ring alone and the whole cofactor can adopt different conformations, which in some cases plays an important role in catalysis (Fraaije & Mattevi, 2000; De Colibus & Mattevi, 2006; Senda *et al*., 2009). When discussing flavoenzyme mechanisms, the two sides of the isoalloxazine ring need to be distinguished. They have been thus called *si* and *re*-faces: in the figure 2 the *re*-face is towards the reader (Massey, 1995).

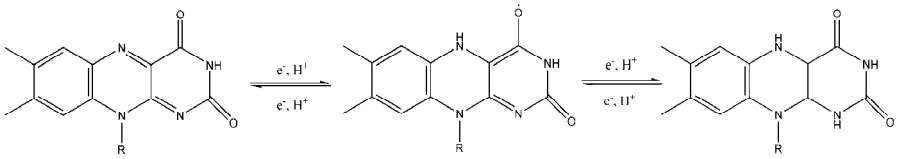


Figure 3. The three oxidative states of flavins

Flavin reacts with molecular oxygen in its fully reduced form in oxygenases. There were originally numerous scientific discussions on how the initial spin-forbidden reaction between flavin and oxygen occurs (Kemal *et al*., 1977). One of the most plausible proposals was the dioxetane-theory suggested in the 1970s by several researchers. According to the proposal, the reduced isoalloxazine ring has a low-occupied triplet-state, which can be populated in contact with the paramagnetic oxygen. First a dioxetane species is formed at carbons C10 and C4a, and this intermediate subsequently rearranges into a hydroperoxide intermediate (Orf & Dolphin, 1974; Massey *et al.* 1988). However, it is presently generally accepted that activation of oxygen is initiated by transfer of one electron from reduced flavin to oxygen, generating *a caged radical pair* of a superoxide anion and a neutral flavin radical. This radical pair collapses with spin-inversion (scheme 1) (Kemal *et al*., 1977; Massey *et al*., 1988; Massey, 1994).

Spin inversion

$$FAD_{red}H^- (\uparrow\downarrow) + O_2 (\uparrow\uparrow) \rightarrow [\ FAD\bullet H \uparrow\uparrow O_2^-\ ] \rightarrow [\ FAD\bullet H \uparrow\downarrow O_2^-\ ] \rightarrow \text{FAD-OO(H)}$$

Caged radical pair — C4(a)(hydro)peroxide

Scheme 1. Spin inversion in the caged radical pair of FAD and molecular oxygen. (The scheme is adapted from Massey *et al*. 1988).

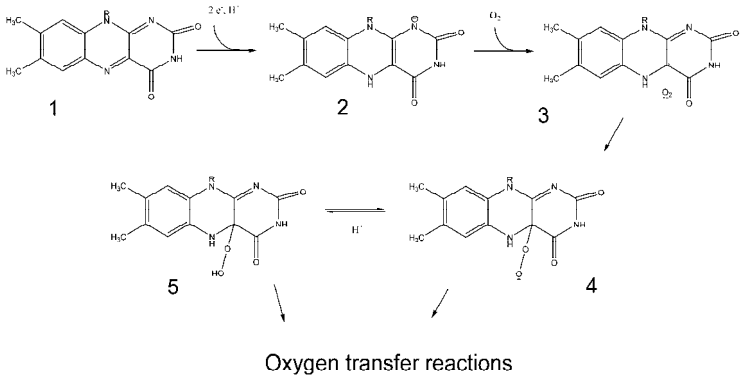


Figure 4. Reactions of flavin with oxygen. 1) Oxidized flavin 2) two-electron reduced flavin
3) Caged radical pair between flavin and superoxide anion. 4) Flavinsuperoxide
5) Flavinhydroperoxide. Both 4 and 5 are used in oxygenation reactions.
(Adapted from Massey, 1994)

A covalent intermediate between the C4(a) atom of the flavin and oxygen is formed, called hydroperoxyflavin or peroxyflavin, depending on its protonation state (figure 4) (Kemal *et al*., 1977; Massey, 1994). This intermediate has been spectroscopically observed in several flavin-dependent monooxygenases (Entsch *et al*., 1976; Beaty & Ballou, 1981; Sheng *et al*., 2001). The intermediate is unstable and prone to dissociate into hydrogen peroxide and oxidized flavin *via* a second electron transfer, but it can be sufficiently stabilized by the environment in the catalytic center of the enzyme. The peroxyflavin attacks the substrate *via* an electrophilic or nucleophilic attack, depending on its protonation state. The terminal oxygen atom is transferred to the substrate and FAD is returned to the oxidized state *via* release of water (Massey *et al*., 1988; Massey, 1994, 2000).

Flavin-dependent monooxygenases catalyze a variety of reactions, including hydroxylations, epoxidation and sulfoxidations. Electrons for reduction of the flavin are in true FAD-dependent monooxygenases provided by an external cofactor, NADPH or NADH, thus they are called *external flavoprotein monooxygenases*. The reduction is stereospecific: usually the interaction with NADPH occurs *via* the *re*-face of the FAD (Ghisla & Massey, 1989). The FAD is reduced either by an NAD(P)H bound to the same polypeptide (*single-component monooxygenases*) or it is reduced by another polypeptide chain, an NAD(P)H-dependent flavin reductase (*two-component monooxygenases*) (Ballou *et al*., 2005).

1.1.2.4 Tetrahydrobiopterin

A small family of amino acid hydroxylases utilizes tetrahydrobiopterin (BH_4) (figure 5) as the cofactor in activation of oxygen. In addition, they possess a single iron atom, or in some cases copper. BH_4 provides the electrons for reaction, thereby being consumed and is therefore categorized as a substrate. The hydroxylation reaction requires both BH_4 and Fe^{+II}. Oxygen is bridged between the two, as a Fe^{+II}-OO-pterin intermediate. The intermediate is protonated and the O-O –bond is cleaved to yield a Fe^{+IV}=O intermediate and 4a-OH-pterin product. Of these, Fe^{+IV}=O is the activated hydroxylating agent. Thus, the two atoms of dioxygen are divided to the substrate to be hydroxylated and to the pterin-cofactor, instead of water (Chow *et al*., 2009).

BH_4-dependent amino acid hydroxylases include phenylalanine hydroxylase, tryptophan hydroxylase and tyrosine hydroxylase. They are involved in biosynthesis of neurotransmitters, and inactivating mutations in these enzymes are involved in a range of neurologic disorders. For instance, phenylketonuria is caused by impairment in the function of phenylalanine hydroxylase, leading to accumulation of phenylalanine and eventually impairments in brain development (Jervis, 1947; Fitzpatrick, 1999; Chow *et al*., 2009). In addition to the amino acid hydroxylases, nitric oxide synthases and a glyceryl ether monooxygenase have been described to be BH_4-dependent (Thönu *et al*., 2000; Watschinger *et al*., 2009).

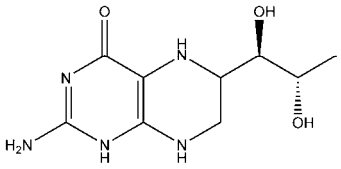


Figure 5. Tetrahydrobiopterin (BH_4)

1.1.2.5 Cofactor-independent oxygenases

Originally it was thought that oxygen could be activated for enzymatic reactions only, if the enzyme possesses one of the coenzymes described above. However, during the last decade an increasing number of mono- and dioxygenases devoid of any coenzyme have been described (Fetzner, 2002, 2007). For instance, DpgC is a dioxygenase that cleaves 3,5-dihydroxyphenylacetate-Co-enzyme A to 3,5-dihydroxyphenylglyoxylate and CoA during vancomycin biosynthesis (Fielding *et al*., 2007; Widboom *et al*., 2007). Another example is provided by the two putative cofactor-free hydroxylases, AclR and SnoaL2, from the biosynthesis of the polyketide antibiotics aclacinomycin and nogalamycin (Beinker *et al*., 2006). The ring-cleaving 2,4-dioxygenases (Hod and Qdo) involved in catabolism of N-heteroaromatic compounds form a third class of cofactor-free oxygenases (Fischer *et al*., 1999; Fischer & Fetzner, 2000). The small monooxygenases participating in antibiotic biosynthesis form another group, a family from which ActVA-Orf6 has been structurally described (Sciara *et al*., 2003). The quinol monooxygenase YgiN from *Escherichia coli* belongs to the same family (Adams & Jia, 2005). In addition to the oxygenases described above, urate oxidase is a more thoroughly studied oxidase capable of activating oxygen without cofactors (Kahn & Tipton, 1998; Colloc'h *et al*., 2008; Gabison *et al*., 2008).

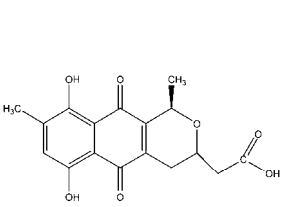
The catalytic mechanisms of cofactor-independent oxygenases are as yet not well understood, but two possible mechanisms for activating dioxygen have been proposed, summarized by Fetzner (2002). According to the first proposal, the enzymes produce substrate and protein radicals which then react with oxygen (Shen & Hutchinson, 1993). Alternatively, the substrate is deprotonated to anionic species, which then activates oxygen *via* electron transfer and formation of a caged radical pair, similarly to flavin-dependent catalysis (Sciara *et al*., 2003; Widboom *et al*. 2007). Based on biochemical and structural studies conducted on these enzymes, Susanne Fetzner proposed in 2007 a substrate carbanion-based mechanism to be the general mechanism for cofactor-independent oxygenases.

1.2 POLYKETIDES

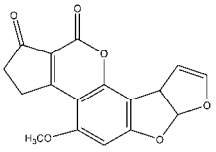
Polyketide compounds are natural products synthesized by certain bacteria, fungi and plants. These chemically very diverse substances are thought to be used naturally as pigments, virulence factors and importantly, antibiotics (Hertweck, 2009). In this context, the term *antibiotics* is used to describe compounds produced by bacteria to compete against other micro-organisms in their growth environment (Waksman, 1947). As polyketides are not vital for the organism in normal life, they are called *secondary metabolites* to differentiate them from primary metabolism necessary for survival.

Thousands of different polyketide compounds have been isolated from soil and marine environments all around the world, and most likely many more remain to be discovered.

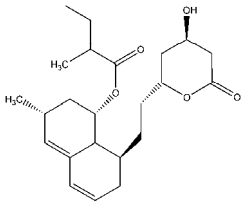
The name *polyketide* originates from the ketone-groups present in alternating carbons in the linear polyketide precursor backbone (Hutchinson *et al.*, 1993). Polyketides are synthesized in the host organism in complicated enzymatic pathways. First a linear poly-β-ketoacyl-chain is synthesized. The linear precursor is assembled into (aromatic) rings in various manners; alternatively, some polyketides retain a linear carbon skeleton (figure 6). The carbon skeleton is subsequently decorated in so-called tailoring steps with various chemical groups.



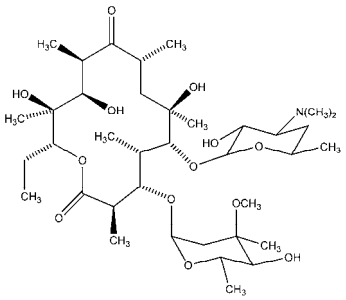
Actinorhodin



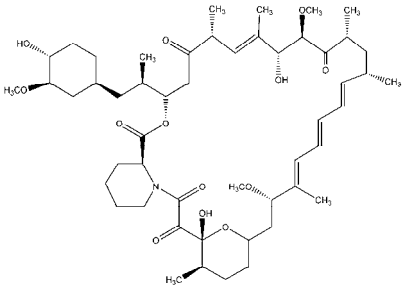
Aflatoxin



Lovastatin



Erythromycin A



Rapamycin

Figure 6. Examples of different polyketide compounds

The great interest in polyketides in academic research as well as in pharmaceutical industry is due to the biologically and medically relevant properties of some of the compounds. These bioactivities have been exploited long before the compounds were actually first identified and studied in a laboratory environment in the late 1800s by James Collie (Bentley & Bennett, 1999; Staunton & Weissman, 2001). For instance, senna and rhubarb which have been traditionally used as laxatives contain anthracene-derived polyketide compounds. The antispasmodics used in treatment of angina in Middle-East contain khellin, another polyketide. Furthermore, the hemlock poison used to execute Socrates contains coniine, a polyketide alkaloid (Bentley & Bennett, 1999). Today polyketides are used, for instance, as antimicrobial agents (tetracycline, erythromycin), cholesterol lowering medicine (lovastatin), immunosuppressant (rapamycin), antifungals (amphotericin) and anticancer agents, such as anthracyclines (doxorubicin, epirubicin) (Bentley & Bennett, 1999; Staunton & Weissman, 2001). In everyday-life, beneficial polyketide compounds are present in, for instance, blueberries

and red wine (Tsai, 2004). Polyketides are divided into groups based on their chemistry. They include macrolides (e.g. erythromycin), polyenes (rapamycin, amphotericin) and polyethers (monensin). Another major class is formed by the aromatic polyketides, such as tetracyclines and anthracyclines (Staunton & Weissman, 2001; Hill, 2006). Some common polyketide polyketide compounds are shown in figure 6.

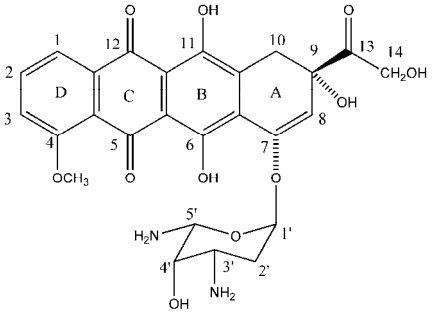
During my doctoral studies I have been interested in the biosynthesis of two types of aromatic polyketides, anthracyclines and angucyclines. They are produced mainly by soil-borne *gram*-positive bacteria of the genus *Actinomycetes*, in particular by members of the subgroup *Streptomycetes* (Rohr & Thiericke, 1992; Hutchinson, 1995: Niemi *et al*., 2002). Both anthracyclines and angucyclines have a characteristic aromatic polycyclic carbon-skeleton (figure 7). The following chapter describes these compounds and their biological properties in more detail.

1.2.1 Anthracyclines and angucyclines

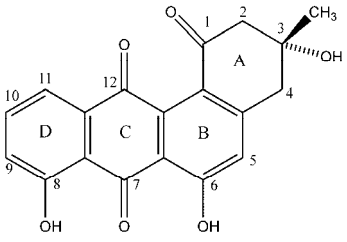
Anthracyclines have a tetracyclic 7,8,9,10-tetrahydrotetracene-5,12-quinone structure, to which one or several aminosugars or amine-free neutral sugars are attached in most of the biologically active compounds. They have an intense color varying from yellow to orange and red, which is due to the polycyclic aromatic chromophore (Hutchinson, 1995; Metsä-Ketelä *et al*., 2008). The first anthracycline compound was discovered in the early 1950s in Germany (Brockmann & Bauer, 1950). Because of its red color, the new compound was given the name *rhodomycin*. Another novel anthracycline compound, daunorubicin, was isolated in 1963 from *S. peucetius* simultaneously in France at Rhône-Poulenc (Dubost *et al*., 1963) and in Italy in the laboratory of Farmitalia (DiMarco *et al*., 1964). In 1969 a closely related substance, doxorubicin (adriamycin), was isolated from a strain of *S. peucetius* exposed to random mutagenesis (Arcamone e*t al*., 1969). The cytotoxic properties of the new compounds were soon discovered (DiMarco *et al*., 1965) and doxorubicin was accepted as a cytotoxic drug in 1974. It has since then been one of the most used therapeutic agents in treatment of cancer worldwide (Cortéz-Funes & Coronado, 2007; Metsä-Ketelä *et al*., 2008). The discovery of daunorubicin and doxorubicin launched efforts to find further anthracycline-based cytotoxic agents, and some 2000 anthracycline compounds are presently known (Niemi *et al*., 2002; Minotti *et al*., 2004). Only a few of them are in clinical use, including daunorubicin, aclacinomycin A and epirubicin. The spectrum of cancer types treated is different for the different anthracyclines, including various leukemias and breast cancer. Taken together, a larger variety of cancer types can be treated with anthracyclines than with any other antitumor agent (Cortéz-Funes & Coronado, 2007). However, their use is limited by drug resistance exhibited by cancer cells, and by serious side-effects, the most serious of which is dose-dependent cardiomyopathy (Minotti *et al*., 2004; Appel *et al*., 2007).

The discovery of the first angucycline compound, tetrangomycin, was published in 1965 (Dann *et al*., 1965), two years after discovery of daunorubicin. Presently relatively few angucyclines are known and include, for instance, jadomycin, urdamycin and landomycin (Rohr & Thiericke, 1992). It has been estimated that many gene clusters encoding for angucyclines are silent, and thus it is possible that many more

compounds of the family will be discovered in the future (Palmu *et al*. 2007). Angucyclines possess a tetracyclic benanthracene carbon skeleton, in which the ring A forms an angle with the three other rings (figure 7): hence the name **angu**cycline (Rohr & Thiericke, 1992). They have been described to possess for instance cytostatic, antiviral and antibacterial properties and they can inhibit platelet aggregation (Rohr & Thiericke, 1992). Despite these promising features, angucyclines have attained significantly less academic and industrial interest than anthracyclines.



Doxorubicin



Rabelomycin

Figure 7. The structure of doxorubicin as an example of an anthracycline compound and that of rabelomycin as an example of an angucycline.

1.2.2 Biosynthesis of aromatic polyketides

Aromatic polyketides are assembled in enzymatic production lines in an iterative manner. First a linear precursor is produced, which is subsequently assembled into an aromatic ring system; a core-compound called aglycone. The aglycone is thereafter modified in tailoring steps by, for instance, methyltransferases, oxygenases and glycosyltransferases. The chemical diversity of polyketides is brought about by varying the starter units, cyclization mode, and tailoring steps (Staunton & Weissman, 2001; Schneider, 2005; Hertweck *et al*., 2007; Das & Khosla, 2009).

The genes encoding for each polyketide are usually clustered together in the *Streptomyces* genome. This facilitates detection of biosynthetic pathways and subsequent studies of the individual gene products (Niemi *et al*., Metsä-Ketelä *et al.*, 2008). During the last two decades, a number of these gene clusters have been cloned, including those of daunomycin (Ye *et al*., 1994) and nogalamycin (Ylihonko *et al*., 1996).

1.2.2.1 Synthesis of the aglycone

The carbon skeleton of aromatic polyketides is synthesized in an enzymatic assembly line in a process reminescent of fatty acid synthesis. Aglycone is produced from simple organic acids, such as malonates, propionates and acetates, which are activated by attachment to coenzyme A (CoA) with a thioester bond. These basic building blocks are condensed into a linear polyketide precursor, poly-β-ketoacyl chain, by the massive type II, iterative polyketide synthetase (PKSII). PKSII is similar to the bacterial type II fatty acid synthetase (FAS): both FASII and PKSII systems consist of individual

polypeptides, encoded by separate genes, and each subunit catalyzes the same reaction multiple times (Hertweck *et al*., 2007; Metsä-Ketelä *et al*., 2008; Das & Khosla, 2009). In addition to the type II PKS, two other PKS systems have been described. Type I PKS is reminiscent of the mammalian fatty acid synthase; all enzyme activities are established within the same polypeptide (Fischbach & Walsh, 2006; Crawford *et al*., 2008; Weissman, 2009). In the iterative type III PKS enzymes all enzymatic reactions are catalyzed within the same active site (Gross *et al*., 2006).

Three enzyme subunits are present in the PKSII of all aromatic polyketides: two ketosynthase subunits (KS_α and KS_β) and an acyl-carrier protein (ACP), which carries the growing polyketide chain. These three proteins thus form the so-called *minimal PKS* (Hertweck *et al*., 2007; Das & Khosla, 2009; Weissman, 2009). The KS_α and KS_β subunits are homologous polypeptides and form a heterodimer (Keatinge-Clay *et al*., 2004). The KS_β subunit lacks catalytic activity, but it is likely to participate in the determination of the polyketide chain length. It is therefore also called the *chain length factor* (CLF) (Hertweck *et al.*, 2007, Das & Khosla, 2009). The PKSII assembles the linear polyketide precursor in multiple rounds of decarboxylative Claisen condensations (figure 8). The nascent polyketide chain is shuffled between the ACP and KS_α subunits and is folded into a cavity between the KS_α and CLF (KS_β). The reaction yields a poly-β-ketoacyl chain, whose folding into an aromatic polycyclic structure is directed and catalyzed by additional PKS enzymes; ketoreductases, cyclases and aromatases (Metsä-Ketelä *et al*., 2008; Hertweck *et al*. 2007; Das & Khosla, 2009).

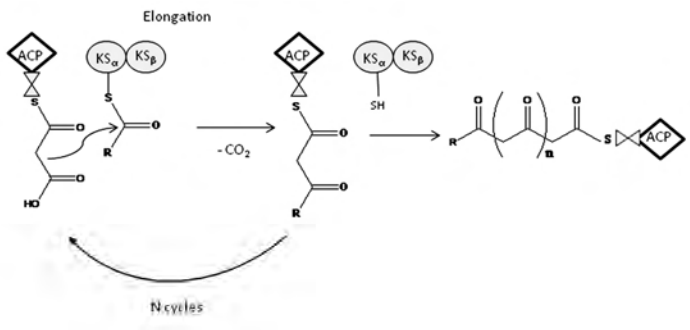


Figure 8. Synthesis of the poly-β-ketoacyl intermediate by minPKSII
(adapted from Staunton & Weissman, 2001; Hertweck *et al*., 2007 and Das & Khosla, 2009)

1.2.2.2 Tailoring steps

After the carbon skeleton has been assembled, it is decorated by various tailoring enzymes. These provide aromatic polyketides with their great chemical variety. The tailoring steps include methylations, various types of oxygenations, reductions and glycosylations. Often the binding of substrates is through extensive hydrophobic interactions rather than specific hydrogen-bonding, and it is believed that the shape of the active site plays a significant role in substrate recognition. Thus it is not surprising that substrate specificity is often rather flexible for tailoring enzymes (Schneider, 2005; Metsä-Ketelä *et al*., 2008).

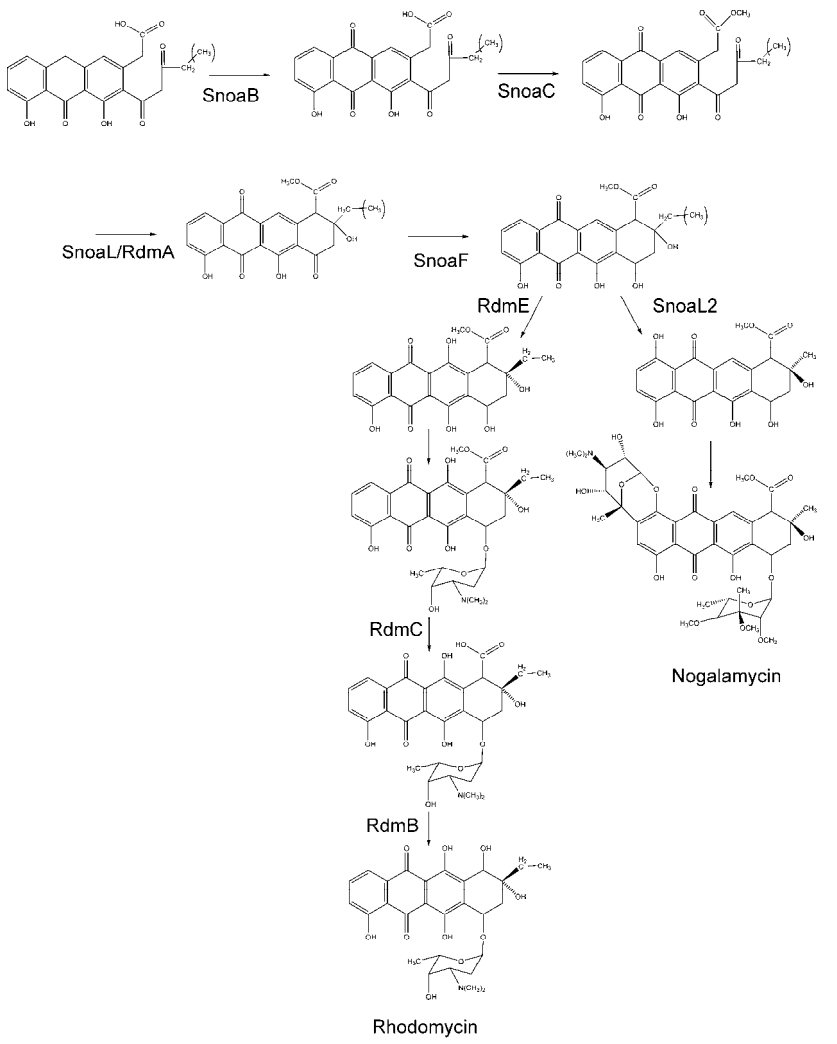


Figure 9. The tailoring steps in the biosynthesis of rhodomycin and nogalamycin (adapted from Niemi *et al*., 2002)

Tailoring steps of the carbon skeleton are catalyzed both prior to and after glycosylation and some steps occur prior to the last cyclization (figure 9). The quinone-forming DnrG and SnoaB are examples of the latter enzymes (Niemi *et al*., 2002). Among the tailoring reactions, oxygenative tailoring is the most common type and includes, for instance, mono- and dioxygenations, epoxidations and Bayer-Villiger reactions (Hertweck *et al*., 2007; Weissman, 2009). Various enzyme-types are utilized in these reactions. Aromatic carbons are often oxygenated by flavoenzymes, for instance, DnrF (Filippini *et al*., 1995) and RdmE (Niemi *et al*., 1999) are examples of FAD-dependent hydroxylases. Non-aromatic carbons are often oxygenated by P450 enzymes, such as DoxA (Lomovskaya *et al*., 1999). Also two-component flavin-dependent oxygenases have been described (Valton *et al*., 2006; Okamoto *et al*., 2009) as well co-enzyme-free

oxygenases (Fetzner, 2002). Recently, two novel oxygenases were discovered from fredericamycin biosynthesis route in *S. griseus* (Chen *et al*., 2009). The cofactor-dependence of these enzymes remains to be elucidated. Thus various types of oxygenases have been harnessed in polyketide biosynthesis, and it is possible that more oxygenase classes will be discovered. Another common tailoring type, methylation, is catalyzed by S-adenosylmethionine (SAM) –dependent methyltransferases, such as DnrK in daunorubicin biosynthesis (Jansson *et al*., 2004; Hertweck *et al*., 2007).

An important step in the biosynthesis of many of the biologically active aromatic polyketides is the incorporation of one or several deoxysugar moieties into the carbon backbone. The sugar moieties are usually reduced hexasacharides, either aminosugars or neutral sugars. The sugars are synthesized separately as nucleotide-activated modules from glucose (Hutchinson, 1995, Niemi *et al*., 2002). They are also decorated in tailoring steps, in a similar manner to the aglycone synthesis. The sugar-tailoring enzymes include aminotransferases, methyltransferases, epimerases and dehydratases. The genes encoding for the sugar biosynthesis are clustered in the *Streptomyces* genome, like the genes of the aglycone biosynthesis. The typical sugars attached to aromatic polyketides include daunosamine (in doxorubicin and daunorubicin) (figure 7) and rhodinose and 2-deoxyfucose (in aclacinomycin). The sugar-moieties are attached to the aglycone by glycosyltransferases, most often *via* O-glycosidic bonds. In the case of anthracyclines, the carbohydrates are usually attached to the position C-7 or sometimes C-10, with nogalamycin being an exception with a sugar attached also to carbons 1 and 2 (figure 9) (Niemi *et al*., 2002). In angucyclines the glycosylation pattern is more varied and also other moieties, such as amino acid molecules occur (Rohr & Thiericke, 1992).

1.2.3 Structural studies on the biosynthetic enzymes

During the present decade, there has been an effort to elucidate the three-dimensional structures of enzymes participating in the biosynthesis of aromatic polyketide antibiotics. The structural studies are important for gaining information on the substrate recognition and enzymatic mechanisms at molecular level (Schneider, 2005). Several subunits of the PKSII have been structurally studied, in particular by Chaitan Khosla and colleagues. The structures of the ACP subunit from several pathways have been determined by nuclear magnetic resonance (NMR) (Crump *et al*., 1997; Li *et al*., 2003, Findlow *et al*., 2003). Furthermore, the complex of the KS_{α} and KS_{β} from the actinorhodin biosynthetic pathway was determined by X-ray crystallography in 2004 by Khosla, Stroud and coworkers (Keatinge-Clay *et al*., 2004), providing insights into the early steps in aromatic polyketide biosynthesis. The structures of three additional PKS subunits have been determined: the PKSII-ketoreductase unit from actinorhodin biosynthesis (Hadfield *et al*., 2004), and the priming β-ketosynthase ZhuH (Pan *et al*., 2002) and priming ketoreductase SCO1815 (Tang *et al*., 2006) from R1128 pathway. In addition, the crystal structures of five cyclases have been determined (Sultana *et al*., 2004; Thompson *et al*., 2004; Kallio *et al*., 2006; Ames *et al*., 2008; Silvennoinen *et al*., 2009).

The first tailoring enzyme participating in the biosynthesis of aromatic polyketides for which the structure was elucidated was the cofactor-independent monooxygenase

ActVA-Orf6 in 2003 (Sciara *et al*., 2003). Since then the crystal structure has been determined for the methyl transferase DnrK (Jansson *et al*., 2004), the methylesterase RdmC (Jansson *et al*., 2003a), and three novel hydroxylases (Jansson *et al*., 2003b; Beinker *et al*., 2006). In addition, the structures of a C-C-bond forming glycosyltransferase UrdGT2 from the biosynthesis route of urdamycin (Mittler *et al*., 2007) and a post-glycosylation sugar-modifying oxidoreductase AknOx (Alexeev *et al*., 2007) have been reported.

1.2.4 Perspectives for novel antibiotics

Development of novel antibiotics is again gaining importance with the alarming number of emerging multidrug resistant (MDR) bacterial strains. Furthermore, there are problems with cancer cells escaping anthracycline-based chemotherapies (Minotti *et al*., 2004). Therefore novel antibiotics and anticancer agents with beneficial toxicity profiles are urgently needed. Natural products, and polyketides in particular, are a great resource for novel therapeutic agents, and several approaches are being employed to discover them. New antibiotics are screened from natural sources (Clardy *et al*., 2006), however, this is a rather expensive approach and nowadays the rate of rediscovery of existing compounds is high (Scherlach & Hertweck, 2009). Traditional methods for generating novel compounds also include random mutagenesis of bacterial strains, a method successfully utilized in generation of doxorubicin (Arcamone *et al.*, 1969). Sequencing of *Streptomyces* genomes has revealed a number of silent biosynthetic clusters. They appear functional, but the end products of these biosynthetic pathways are unknown and difficult to express in laboratory environment. These silent clusters are termed cryptic, and may well yield a number of previously unknown polyketides, once suitable conditions to activate these pathways are discovered (Scherlach & Hertweck, 2009). Another interesting approach is to engineer the biosynthetic pathways to generate novel compounds, an approach termed *combinatorial biosynthesis* or molecular engineering (Weissman & Leadlay, 2005; Baltz, 2006). In combinatorial biosynthesis, genes from different pathways can be mixed, to generate so-called *hybrid polyketides*, an approach facilitated by the relaxed substrate tolerance of the tailoring enzymes. This approach was successfully utilized first by Hopwood and coworkers as early as in 1985 (Hopwood *et al*., 1985). An alternative approach is to modify the biosynthetic enzymes of a specific pathway to either possess novel activities or to accept different substrates, a process that requires detailed information about the reaction mechanisms and three-dimensional structures of the enzymes (Schneider, 2005; Metsä-Ketelä *et al*., 2008). The increasing number of three-dimensional structures of the PKS-subunits as well as tailoring enzymes is likely to facilitate this approach.

2 AIM OF THE THESIS

The aim of the research project was to gain insights into various enzymatic means of activation of oxygen. The enzymes were derived from the biosynthesis of aromatic polyketides, in order to also obtain insights into antibiotic biosynthesis. Three different enzyme-families participating in the biosynthesis of anthracyclines and angucyclines were chosen as study subjects in order to elucidate the structural basis of their mechanism and substrate recognition.

Three enzymes, PgaE, CabE and RdmE, were chosen as examples of FAD-dependent aromatic hydroxylases. FAD-dependent aromatic hydroxylases have been studied for decades, and their general mechanism of action has been characterized in these previous studies. Aside from mechanistic questions the focus of this study was to map the structural basis of recognition of polyketide substrates in this enzyme family.

RdmB is a hydroxylase from the rhodomycin biosynthesis route. Surprisingly, it exhibits high amino acid sequence identity to S-adenosyl-L-methionine (SAM) -dependent methyltransferases. We wanted to ascertain whether the enzyme utilizes SAM or some other cofactor to activate molecular oxygen, and how this is achieved at the molecular level.

SnoaB is an example of a cofactor-independent monooxygenase. It is involved in the biosynthesis of nogalamycin, and it has homologues in biosynthesis routes of many other aromatic polyketides. Cofactor-independent monooxygenases have recently attracted considerable interest and the purpose of this study was to establish the catalytic mechanism of this enzyme and compare it to mechanistic proposals for other cofactor-independent oxygenases.

In addition to gaining insights into mechanisms of oxygen activation, we wished to gather information about tailoring steps in the biosynthesis of various polyketide antibiotics at the molecular level. The long-term aim of the research project is to aid in development of novel antibiotics for treatment of bacterial diseases and cancer.

3 RESULTS AND DISCUSSION

3.1 FLAVOENZYMES - PgaE, CabE and RdmE (papers II and III)

FAD-dependent aromatic hydroxylases are common tailoring enzymes in the biosynthesis of aromatic polyketides. Three such enzymes were chosen for structural and mechanistic investigations: PgaE and CabE participating in angucycline biosynthesis (paper II), and RdmE from anthracycline biosynthesis (paper III).

PgaE and CabE were cloned from *S.* sp. *PGA64* and *S.* sp. *H021*, respectively. They belong to two cryptic gene clusters, producing gaudimycins A and B (Palmu *et al*., 2007). They both catalyze the hydroxylation of carbon 12 of the substrate, UWM6 (paper II, figure 1), which is the first tailoring step in angucycline biosynthesis. The unstable products are directly consumed by the next enzymes of the pathways, PgaM in the case of PgaE and CabM/CabV after CabE (Palmu *et al*., 2007; Kallio *et al*., 2008). PgaE consists of 491 and CabE of 489 amino acids and the amino acid sequence identity between the two enzymes is 70%.

RdmE belongs to the biosynthesis route of rhodomycin in *S. purpurascens*. It catalyzes the hydroxylation of position 11 of aklavinone, a common intermediate in several anthracycline biosynthesis pathways (figure 9; paper III, figure 1). RdmE catalyzes the reaction prior to glycosylation of the aglycone and the natural product is called ε–rhodomycinone (Niemi *et al*., 1999). RdmE consists of 535 amino acids and its amino acid sequence identity to PgaE/CabE is ca. 24%.

3.1.1 Reaction cycle of aromatic hydroxylases

Sequence alignments identified PgaE, CabE and RdmE as members of a family of FAD-dependent aromatic hydroxylases related to *para*-hydroxybenzoate hydroxylase (pHBH). The crystal structures of five members of the family have previously been determined: pHBH from *Pseudomonas fluorescens* (Wierenga *et al*. 1979; Weijer *et al*., 1983) and *P. aeruginosa* (Lah *et al.*, 1994), phenol hydroxylase (PHHY) from *Trichosporon cutaneum* (Enroth *et al*., 1998; Enroth, 2003), 3-hydroxybenzoate hydroxylase (MHBH) from *Comamonas testosteroni* (Hiromoto *et al.* 2006), RebC from *Lechevalieria aerocolonigenes* from rebeccamycin biosynthesis route (Ryan *et al*., 2007) and, most recently pyocyanin biosynthetic protein PhzS from *P. aeruginosa* (Greenhagen *et al*., 2008). Of these, PHHY is the only one from a eukaryotic organism, yeast.

Many oxygenases of the pHBH-family are of special interest as they initiate the metabolism of phenolic compounds and could thus be useful in detoxification of soil. pHBH is fascinating in its capability to regulate a complex hydroxylation reaction with three substrates with little escaping oxygen radicals, all in a single polypeptide. This control and efficiency has encouraged researchers to study the enzyme in great detail, and pHBH has served as a model of the enzyme family for decades. Due to these studies, the elegant reaction mechanism is now known in great detail. The studies have been facilitated by the possibility to follow the various reaction phases

spectroscopically from the changes in the absorption and fluorescence of FAD (Massey, 2000; Entsch *et al*., 2005).

The pHBH-like oxygenases insert a hydroxyl group in aromatic substrates, as the name indicates. The oxygen is derived from molecular oxygen and reducing power is obtained most often from NADPH but in some cases from NADH (Ryan *et al*., 2007; Greenhagen *et al*., 2008). They are single-component oxygenases; NADPH-driven reduction occurs in the same polypeptide. The reaction is divided in two phases, as for all FAD-dependent oxygenases (Massey, 2000): during the *reductive phase*, FAD is reduced, and the subsequent *oxidative phase* entails the steps leading to activation of oxygen and hydroxylation of the substrate. The overall reaction proceeds with an ordered mechanism and is initiated by binding of the substrate. Substrate binding triggers the reduction of FAD – it is only reduced in presence of the substrate to avoid futile consumption of NADPH (Massey, 2000; Entsch *et al*., 2005). There is not sufficient space for NADPH to bind in the active site, where the oxidized isoalloxazine-ring of FAD lies in the so-called "*in-conformation*". Therefore the isoalloxazine-ring swings out from the active site to the so-called "*out-conformation*" to be reduced by hydride transfer from NAD(P)H (figure 12). The movement involves only the isoalloxazine-ring and the ribityl-moieties of the FAD; the pyrophosphate and the adenine remain buried in the enzyme. The out-conformation has been observed in the crystal structures of pHBH (Schreuder *et al*., 1994) and several related enzymes (e.g. Enroth *et al*., 1998; Hiromoto *et al*., 2006). NADPH binds on the surface of the enzyme only transiently and is released directly after reduction (Wang *et al*., 2002), unlike in, for instance, FMOs (Beaty & Ballou, 1981).

The two-electron reduced FAD swings back to the active site and the oxidative phase is initiated. It transfers one electron to O_2, and a superoxide anion –flavin radical caged radical pair is formed. Formation of the caged radical pair requires vicinity of the oxygen to the C4(a)-site of the FAD. The radical pair collapses with spin-inversion and a C4(a)-peroxyflavin intermediate is formed, as described in chapter 1.1.2.3. It is protonated to C4(a)-hydroperoxyflavin, which undergoes electrophilic attack on the substrate. The distal oxygen is transferred to the substrate, and the resulting hydroxyflavin is dehydrated to regenerate the oxidized FAD. The oxygenated substrate is protonated and released from the active site (Massey, 2000; Entsch *et al*., 2005). The electrophilic attack on the substrate is in pHBH enhanced by "activation" of the substrate by deprotonation to yield a more nucleophilic species (Lah *et al*., 1994; Ortiz-Maldonado *et al*., 2004). The deprotonation also triggers reduction of FAD (Palfey *et al*., 1999, Entsch *et al*., 2005). The proton abstraction is accomplished with a hydrogen bonding network from the active site to bulk solvent, consisting of a Tyr-Tyr-His triad (Tyr201, Tyr385, His72) and two water molecules (Lah *et al*., 1994; Entsch *et al*., 2005). However, thus far pHBH is the only member of the enzyme family for which deprotonation and hydrogen bond chain have been observed.

3.1.2 Determination of the structure of PgaE and CabE

Crystals of PgaE belonging to the orthorhombic space-group F222 were obtained with ammonium sulphate as precipitant, whereas CabE crystallized readily with polyethylene glycol (PEG) in space group $P6_522$ (figure 10). From crystals of PgaE,

diffraction data was collected at best to 1.8 Å resolution, and the best crystal of CabE diffracted to 2.7 Å. Both crystals were predicted to contain one molecule per asymmetric unit.

a) b)

Figure 10. a) Crystals of wild-type PgaE b) a crystal of CabE

In order to determine the structure of PgaE, selenomethionine (SeMet) substituted protein was produced. The protein had full incorporation of SeMet, which was confirmed by mass-spectrometry (figure 11). The SeMet-substituted protein crystallized readily in similar conditions to the native protein and a multiple anomalous diffraction (MAD) -dataset was collected at three wavelengths to a resolution of 3.1 Å. Eight of the thirteen selenomethionines were located and used in phasing. After initial refinement, the phases were extended to 2.1 Å and later on to 1.8 Å as a native dataset extending to higher resolution was obtained. The structure of CabE was determined by molecular replacement using the structure of PgaE as the search model.

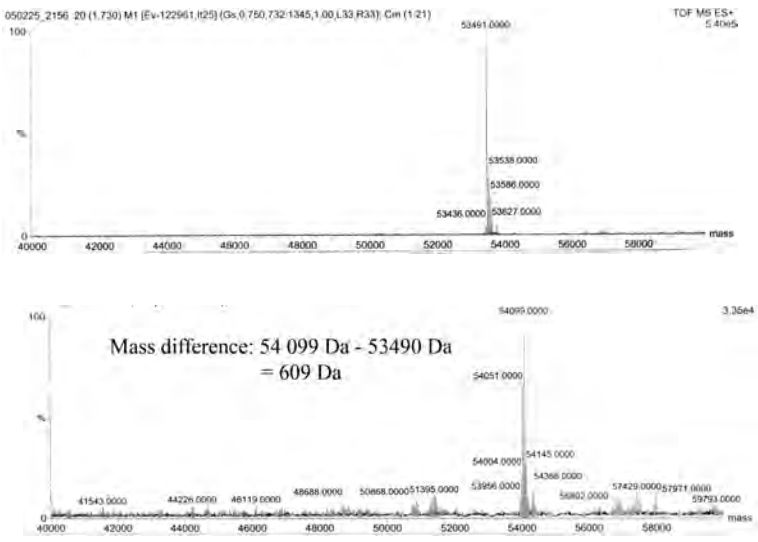


Figure 11. Mass-spectrometric (MS-TOF ES^+) analysis of WT and SeMet-substituted PgaE. The difference in mass between the two protein species, 609 Da, corresponds to incorporation of 13 selenomethionines.

The polypeptide chain could be traced in both enzymes almost completely in the electron density maps. The only exceptions are the polyhistidine tail and a loop

(residues 221-224) in PgaE. In CabE this loop is stabilized by crystal contacts and is visible in the electron density map. In both enzymes, FAD is well defined in the electron density in the in-conformation (figure 12; paper II, figure 6a). No ternary complex with the substrate was obtained despite numerous attempts of soaking and co-crystallization with the natural substrate, UWM6, and substrate analogues, in presence and absence of NADP(H). The substrate was purified from a natural source, and there were difficulties in obtaining it in sufficient purity and yield. Because no ternary complex was obtained, structural and biochemical studies were continued with a homologous enzyme, RdmE.

3.1.3 Determination of the structure of RdmE

RdmE has been studied in our group for many years. However, determination of its structure was initially difficult due to lack of close search models for molecular replacement. SeMet-substituted protein did not yield crystals of good diffraction-quality. Numerous attempts of obtaining heavy-atom derivative were performed without success by Anna Jansson, an earlier PhD student in the group. Phasing was further complicated by suspected merohedral twinning. There was additionally a high peak in the native Patterson map, indicating non-crystallographic translational symmetry. Finally the crystals were suspected to suffer from lattice-translocation defect (Wang *et al*., 2005) and the original diffraction data was corrected by Dr. Tatyana Sandalova according to the protocol described by Wang and co-workers (2005). With the corrected data, determination of the structure was possible with molecular replacement using a search model assembled from the structures of PgaE, CabE and RebC. However, refinement with the corrected data stalled, but successfully continued with uncorrected data when treated as perfectly merohedrally twinned crystal. The final electron density map is well defined for the whole protein and reveals the substrate, aklavinone, bound in the active site. The FAD is in the out-conformation.

3.1.4 Overall structure

The three-dimensional structures of PgaE, CabE and RdmE are very similar, in particular those of PgaE and CabE. The root-mean-square deviation (r.m.s.d.) for the superposition of the main chain atoms of PgaE and CabE is 1.04 Å and 2.6 Å / 2.3 Å between PgaE/CabE and RdmE. All three proteins are composed of three domains (Paper II, figure 3): a large central *FAD-binding domain* containing a Rossmann-type βαβ-fold, a *middle-domain* consisting of an antiparallel β-sheet and a helix, and a thioredoxin-like *C-terminal domain* with two sandwiched β-sheets and two helices. The function of the latter domain is not known. The folding pattern of the central FAD-binding domain and the middle domain are slightly different in the larger RdmE and in PgaE/CabE: in the middle domain of RdmE, there are two additional strands contributing to the β-sheet. Furthermore, RdmE is missing an α-helix which is in PgaE and CabE located in the FAD-binding domain, behind the middle-domain (figure 13). This helix is involved in oligomerization (see below). The active site is located between the FAD-binding domain and the middle-domain; residues from both domains contribute to it. In PgaE the middle domain is less well defined than the other parts of the enzyme, which indicates flexibility and could reflect dynamic movements of this domain during the reaction. It is also plausible that substrate binding is required to properly fix the position of the domain.

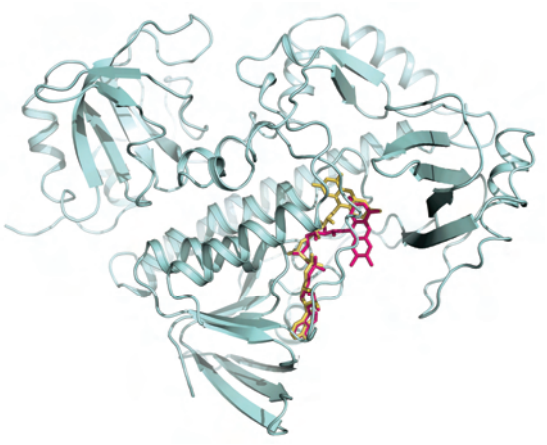


Figure 12. FAD in PgaE, in-conformation in yellow and the modeled out-conformation in pink. Out-conformation was modeled based on the crystal structure of PHHY.

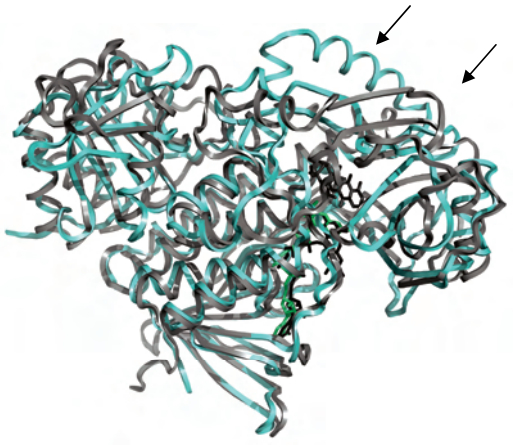
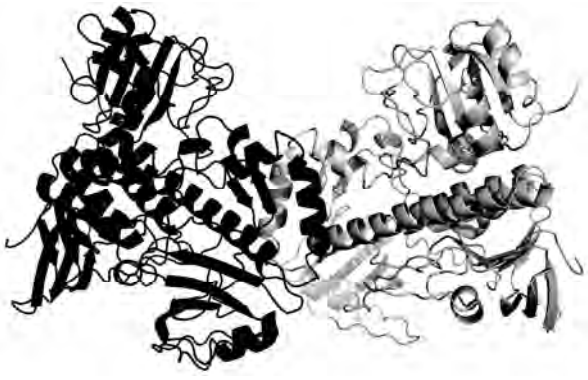


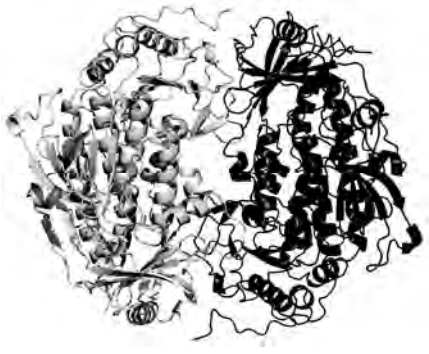
Figure 13. RdmE and PgaE superimposed on each other. RdmE is shown in grey, PgaE in turquose. The most significant differences in secondary structures between the two proteins are indicated with arrows.

3.1.5 Oligomeric state

pHBH, PHHY and MHBH are all dimeric enzymes (Cole *et al*., 2005; Hiromoto *et al*., 2006; Enroth *et al*., 1998). PgaE and CabE are also dimeric both in the crystal structures and in solution. Their mode of oligomerization is different from PHHY and MHBH, which dimerize with a doughnut-like ring *via* the C-terminal domain and the middle-domain (Enroth *et al*., 1998; Hiromoto *et al*., 2006) (figure 14b). Instead, PgaE and CabE form the dimer *via* three helices of the FAD-binding domain (figure 14a). This mode of oligomerization is similar to that of pHBH (Cole *et al*., 2005). RdmE, on the other hand, is monomeric in solution as well as in the crystal (figure 15).



a)



b)

Figure 14. Dimer formation in a) PgaE and b) PHHY

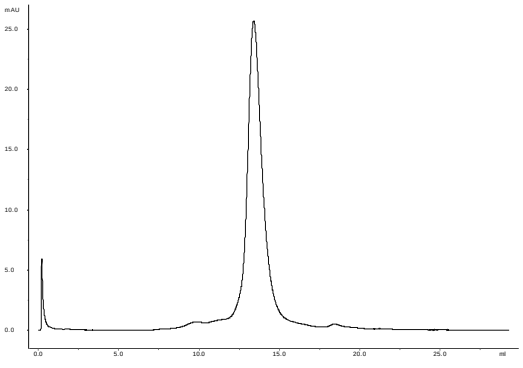


Figure 15. Analytical gel filtration of RdmE. The peak eluted at 13.4 ml, which corresponds to ca. 84 kDa, indicating monomeric quaternary structure for RdmE (expected monomer weight 60 kDa). The molecular weight is calculated with equition (y= -0.199 x+7.59) obtained from calibration.

The two other member of the family that have been structurally characterized, RebC (Ryan *et al.*, 2007) and PhzS (Greenhagen *et al.*, 2008), are also monomeric in their quaternary structure. Thus the family of enzymes has at least three conserved modes of

oligomerization, monomeric and two different modes of dimerization. It remains to be elucidated whether oligomerization has a role in structure stabilization or even catalysis. pHBH has been recently shown to also be active as a monomer, by separating the dimers in reverse micelles and in DMSO-water mixtures (Kudryashova *et al*., 2008). However, fluorescence studies on the FAD in absence of NADPH and substrate indicated that substrate binding in one molecule might induce conformational changes in the other subunit, enhancing adoption of open-conformation and substrate binding (Brender *et al*., 2005).

3.1.6 C-terminal domain

With the exception of pHBH and PhzS, all structurally described members of the enzyme family contain a thioredoxin-like domain in the C-terminus. In MHBH and PHHY this domain is involved in oligomerization (Hiromoto *et al*., 2006; Enroth *et al*., 1998), but not in PgaE, CabE and RdmE. The domain lacks the two conserved catalytic cysteines of thioredoxin (e.g. Chivers & Raines, 1997) and is thus not likely to have any redox-function. With both PgaE and RdmE an attempt was made to test the function of the domain by producing a truncated protein without this domain. No expression of the constructs was observed in cell lysates. It is thus possible that this domain is required for protein folding or stabilization. It is also possible that the truncation exposed a large hydrophobic area which did not allow proper folding.

3.1.7 Substrate entry

Entry of the substrate to the active site is another varying feature between the enzymes of the family. The small substrate of PHHY appears to enter *via* a channel along the *re*-face of the isoalloxazine ring in the out-conformation. In the in-conformation the active site is closed by a large loop (Enroth *et al*., 1998). Also in pHBH the substrate is proposed to enter by the isoalloxazine-ring, although conformational rearrangements are required: a mutant form of pHBH (R220Q) crystallized in a conformation with FAD in an intermediate state between in and out, and the active site is more accessible to solvent. This protein conformation was termed “open” and has been suggested to be the one in which the substrate can enter (Wang *et al*., 2002; Brender *et al*., 2005; Entsch *et al*., 2005).

For the enzymes involved in polyketide biosynthesis there is no equivalent channel adjacent to the isoalloxazine ring sufficiently large for the bulky substrates to enter. PhzS and RebC have a large channel from the opposite site of the active site to bulk solvent (Ryan *et al*., 2007; Greenhagen *et al*., 2008). It appears sufficiently large to allow the entry of the large substrate, but in RebC it is closed by a “melting helix” in the presence of a bound substrate (Ryan *et al*., 2007). In RdmE at an equivalent position there is a channel, which is likely to serve as the substrate entry route (figure 16b). However, there is no equivalent of the “melting helix”. In PgaE and CabE a similar entry route is not possible, as this side of the protein participates in the dimer interface. Instead there is a likely entry route in front of the one described for RdmE and RebC, between the FAD-binding and middle domains (figure 16a). It thus seems that for several enzymes of the family, conformational changes concerning either smaller “lids” or domains are involved in substrate binding. No such features have been observed in PgaE/CabE or RdmE to allow substrate entry, but it is true that none of the

three enzymes was crystallized successfully both in presence and absence of substrate. It is thus possible that the enzymes do undergo domain movements upon substrate binding, in particular PgaE, whose middle domain appears flexible in crystals.

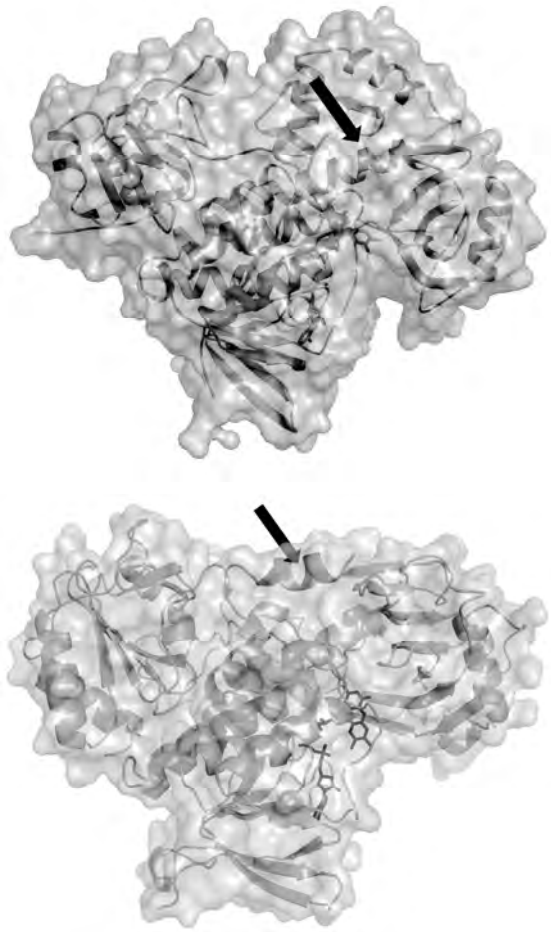


Figure 16. The putative entry route of the substrate indicated with an arrow in a) PgaE and b) RdmE.

3.1.8 FAD-binding

FAD is bound in all enzymes of the family non-covalently, in an extended conformation with similar interactions. The ADP- and pyrophosphates are bound in the FAD-binding domain. There is considerable hydrogen bonding to the protein (paper II, figure 6B; paper III, figure 3) and most of the hydrogen bonding contacts are conserved between the enzymes of the family. Notably, there is a conserved finger print sequence in the N-terminus of the FAD-binding domain participating in FAD-binding: $GxGxxG(x)_{16\text{-}17}D/E$, where x is any amino acid. This sequence motif is known as the dinucleotide binding motif (DBM) and is common among FAD and NADPH-

dependent oxidoreductases (Wierenga *et al*., 1986; Eppink *et al*., 1997; Dym & Eisenberg, 2001). It is also present in all members of the pHBH-type aromatic hydroxylases. Furthermore, in FAD-dependent enzymes reacting with oxygen, there is typically either an amino acid with positive charge, the N-terminus of an α-helix, or a cluster of peptide-amines in the vicinity of the N1 atom. This stabilizes the negative charge on the reduced cofactor (Fraaije & Mattevi, 2000). In case of PgaE/CabE and RdmE, this function is served by the N-terminus of a helix.

FAD has been observed in the out-conformation in the crystal structures of RdmE, MHBH (Hiromoto *et al*., 2006), RebC (Ryan *et al*., 2007), PHHY (Enroth *et al*., 1998), PhzS (Greenhagen *et al*., 2008) and pHBH (Gatti *et al*., 1994; Schreuder *et al*., 1994). In the crystal structure of RdmE, the FAD is in the out-conformation in all three molecules of the asymmetric unit, indicating that this is the preferred conformation in presence of the substrate. The conformation is stabilized by only one hydrogen bond between the atom N3 of the FAD and the carbonyl oxygen of C12 of the substrate (figure 17b). In the complex of pHBH with 2,4-dihydroxybenzoate the FAD is similarly stabilized in out-conformation *via* a hydrogen bond between the substrate and atom N3 of FAD (Schreuder *et al*., 2004), similarly to RdmE. However, in RebC (Ryan *et al*., 2007), MHBH (Hiromoto *et al*., 2006), PhzS (Greenhagen *et al*., 2008) and PHHY (Enroth *et al*., 1998), the out-conformation is stabilized by hydrogen bonding from protein rather than substrate. Notably, in MHBH and PHHY the conformation is stabilized by a hydrogen bond from the atom N3 to a tyrosine residue. In PgaE/CabE there are no protein residues that could directly stabilize the FAD in the modeled out-conformation (paper II, figure 7a & 7b), although it is plausible that conformational changes take place upon substrate binding, enabling hydrogen bonding. It is also possible that like in RdmE, a bound substrate will stabilize the out-conformation. It is attractive to think that the stabilization of the out-conformation is linked to the means of triggering the conformational change and possibly FAD-reduction.

In the out-conformation the *si*-side of the FAD is lined by a conserved arginine. In RdmE, RebC (Ryan *et al*., 2007), PhzS (Greenhagen *et al*., 2008) and MHBH (Hiromoto *et al*., 2006) the *re*-side is lined by an aromatic residue. There would not be enough space for the stacking interaction between the *re*-side of FAD and the pro-*R* side of the nicotinamide moiety of NADPH required for the direct hydride transfer to the N5 of FAD upon reduction (Wang *et al*., 2002). Thus the out-conformation seen in crystal structures cannot be biologically relevant *per se*; additional conformational adjustments concerning the protein and/or FAD are necessary. In PHHY (Enroth *et al*., 1998) and pHBH (Gatti *et al*., 1994; Schreuder *et al*., 1994) the *re*-side is more exposed to solvent, but most likely further conformational changes in the protein are required to allow the stacking interaction with the nicotinamide (Entsch *et al*., 2005).

3.1.9 Active site

The active site of RdmE, PgaE and CabE is large and mainly hydrophobic, and seems even more so in PgaE and CabE. In PHHY, pHBH and MHBH the active site is smaller and more hydrophilic, which is not surprising, as the latter enzymes hydroxylate smaller substrates. In RebC the active site is in the same size-range as with PgaE/CabE and RdmE, and also largely hydrophobic (Ryan *et al*., 2007).

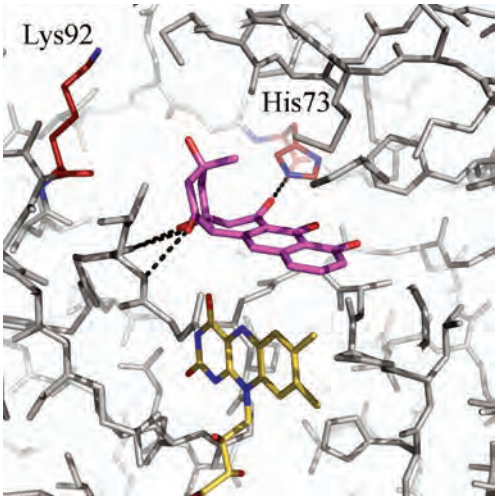
The residues lining the substrate binding pocket are mostly conserved between PgaE and CabE, but not with RdmE. The only significant difference between the active sites of PgaE and CabE is the Tyr89 in CabE: at an equivalent position in PgaE there is the Trp88, which is pointing out from the active site. In PgaE (and CabE), the only two putative catalytic residues identified in the active site are His73 and Lys92 (figure 17a). On the roof of the active site in RdmE, there is Tyr224 which is hydrogen bonded with Arg373, these two residues could have a catalytic function (figure 17b).

3.1.10 Substrate binding

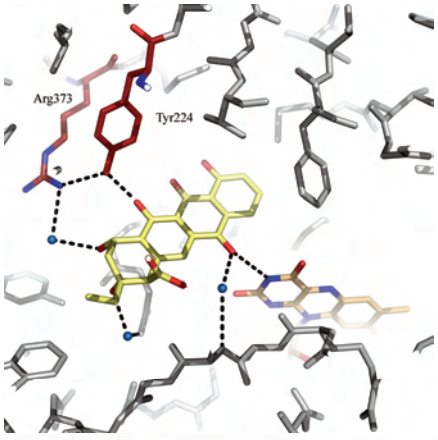
In RdmE the substrate, aklavinone, was well defined in electron density in all three molecules of the asymmetric unit (paper III, figure 4a). Aklavinone is stacked between a structurally conserved polypeptide segment (Pro15-Thr316-Gly317-Gly318) and the side chain of Trp222, and is almost perpendicular to FAD when modeled in its in-position (paper III, figure 7). The locus C-11 is at a distance of about 4.8 Å from the carbon C4(a) of the modeled FAD. Aklavinone is in all three molecules at hydrogen bonding distance to Tyr224 and the N3 of the isoalloxazine ring of FAD in the out-conformation. The ternary complex offers some insights into substrate recognition in RdmE, and possible closely related enzymes. As mentioned before, RdmE cannot hydroxylate glycosylated substrates (Niemi *et al*., 1999). The reason for this is clear in the structure: there is not enough space to allow carbohydrate-moieties to be bound at carbon 7 of the substrate: Arg373, Tyr224, Val95 and Ile81 lining the entry channel to the active site cavity would clash even with a single sugar moiety. RdmE is also not capable of hydroxylating nogalavinone, an anthracycline precursor which differs from aklavinone in the stereochemistry of the carbon 9 (figure 9) (Kallio *et al*., 2006). In the complex of RdmE and aklavinone, the hydroxyl group attached to this carbon is pointing towards a hydrophilic pocket lined by two asparagines and a tyrosine: in addition there are water molecules in this cavity. The ethyl group attached to the same carbon is, however, pointing to a pocket lined by hydrophobic residues; two methionines, a phenylalanine and the hydrophobic part of a tryptophan (figure 17c). Reversal of the stereochemistry would bring the hydroxyl group in a hydrophilic environment and a methyl group in hydrophilic pocket, most likely resulting in repulsions. Thus substrate specificity in RdmE is brought about by the shape and physicochemical properties of the active site in addition to only few specific hydrogen bonding interactions. This is in accordance with tailoring enzymes in general in polyketide biosynthesis (Schneider, 2005).

Figure 17 (following page). a) UWM6 modeled in the active site of PgaE. Lys92 and His73 are indicated in red and potential hydrogen bonds with dashed lines b) Active site of RdmE, with the hydrogen bonds formed by the substrate indicated with dashed lines. The two potential catalytic residues, Tyr224 and Arg373, are shown in red c) a close up in the active site or RdmE, showing the hydrophobic and hydrophilic environments around the C9

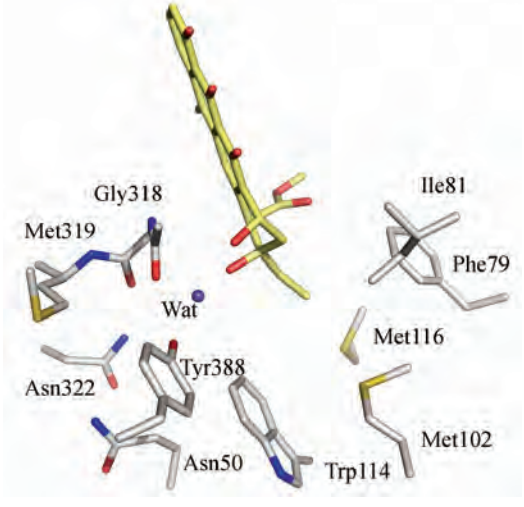
a)


Lys92
His73

b)


Arg373
Tyr224

c)


Gly318
Met319
Wat
Asn322
Tyr388
Asn50
Ile81
Phe79
Met116
Met102
Trp114

As no substrate complex was obtained with PgaE or CabE, substrate binding was originally modeled in PgaE both manually and with the program Autodock3 (Morris *et al*., 1998) (paper II, figures 7c & 7d). Modeling was now repeated from the knowledge of the available ternary complex structure of RdmE. UWM6 was modeled near perpendicular to the isoalloxazine ring, as in RdmE. The angular ring A was modeled to point towards the active site opening; in the opposite orientation, it would protrude into a hydrophobic cavity, which would be difficult with the oxygen atoms attached to carbons 1, 3 and 4a. The distance between the C4(a) locus of FAD in the in-conformation and C-12 of UWM6 in the modeled position is 4.4 Å. The substrate could form hydrogen bonds with main chain atoms of Val44 and with the side chain of His73 through oxygen atoms attached to rings A and B. It is to be mentioned, that modeling is complicated by the possible conformational changes occurring in PgaE upon substrate binding, and by the uncertainty of the conformation the chemically complex substrate adopts in the active site (figure 17a).

3.1.11 Mutational analysis

3.1.11.1 PgaE and CabE

As deprotonation of the substrate has been shown to be an important factor in the catalysis by pHBH, potential catalytic bases were also considered in the active sites of PgaE and CabE. Only two residues, His73 and Lys92, were at an appropriate distance to deprotonate the substrate, even when conformational changes were taken in account. These residues were exchanged into alanine and glutamine, respectively, by site-directed mutagenesis. Both enzymes were expressed in soluble form in *E. coli* and their enzymatic activity was investigated by following the decrease of absorbance of UWM6 at 406 nm. The catalytic activity of the mutant enzymes H73A and K92Q was not decreased as compared with the wild-type enzyme (paper II, table 1). This is surprising, as one would expect the histidine to be involved in binding the substrate *via* hydrogen bonding, and at least a moderate decrease in activity would be expected. However, the apparent absence of a catalytic base is in agreement with the results obtained for PHHY, with which mutational analysis did not reveal any catalytic base (Xu *et al*., 2001).

Problems with purity, solubility and available amounts of the substrate, UWM6, hampered the kinetic characterization: determining reliable Michaelis-Menten -kinetic constants was not possible as the concentration of the substrate was not accurately known. It was also not possible to follow the reaction from the decrease of absorption by NADPH at 340 nm, as the spectrum was overlapping with that of the polyketide product.

3.1.11.2 RdmE

Potential catalytic bases were sought also in the active site of RdmE. There was only one putative catalytic base; Tyr224. This residue forms a hydrogen bond with the substrate (figure 17b) and another one with Arg373, which is in turn in contact with bulk solvent. We wanted to investigate, whether the two residues could form a hydrogen-bonding network, such as the Tyr-Tyr-His-triad described for pHBH. The tyrosine was of special interest as it corresponds to Tyr201 in pHBH, the first residue of

the proton relay network. The tyrosine was thus exchanged into phenylalanine and the arginine into alanine, glutamine and methionine.

The original protein used in crystallization had been purified as native protein in *S. lividans*. For the mutational studies, *rdmE* was cloned *via* ligation-independent cloning (LIC) in a construct encoding for an N-terminal polyhistidine tag. Introduction of the mutations *via* three PCR-reactions and blunt-end ligation is described in paper III. The mutated as well as the wild-type enzymes were all expressed in soluble form in *E. coli* and the color of the proteins was bright yellow. The enzymatic activity of the enzymes was investigated by following the formation of the product, ε-rhodomycinone, spectroscopically at the wavelength 500 nm. No hydroxylation was observed in absence of NADPH. Due to the high concentration of NADPH required for optimal activity and the spectral overlap with the substrate, aklavinone, it was not possible to follow the reaction from the decrease of NADPH. The specific activities of the mutants and the wild-type enzyme are shown in paper III, table 2. The amino acid exchanges of Arg373 had little effect on enzymatic activity, but the effect of the mutation Y224F was dramatic: no formation of the product was observed. Reliable calculation of K_m values was difficult because of the behavior of the product being measured: its absorbance decreased with time. It is possible that this is due to poor solubility or conversion to some other compound.

It is possible that the tyrosine residue is involved in deprotonation of the substrate and thus its activation for electrophilic attack by hydroperoxy-FAD, similarly to pHBH. The high residual activity of the mutants R383A, R383Q and R373M argue against involvement of a proton relay chain to the bulk solvent, unlike in pHBH. It is, however, true that the absence of a specific hydrogen bonding interaction between the substrate and the enzyme is likely to significantly decrease the observed reaction rate. Tyr224 may be necessary to align the substrate in an appropriate orientation for the reaction to proceed and that the observed decrease in the hydroxylation by RdmE is solely due to the impaired binding. Further investigations would be necessary to determine the role of the residue.

In case the effect of the Y224F mutation is indeed due to decreased deprotonation of the substrate, RdmE would be the second member of pHBH-family shown to activate its substrate for hydroxylation by enzymatic deprotonation. pHBH and RdmE would then be rather exceptions in the family with requiring substrate activation. It should be kept in mind, though, that for RebC, MHBH and PhzS probing for putative catalytic bases by mutagenesis has not been reported; PgaE and PHHY have been shown not to require catalytic bases. For pHBH it has been suggested that the deprotonation has evolved to enable substrate discrimination to avoid futile consumption of NADPH and possibly generation of toxic products (Palfey *et al*., 1999; Entsch *et al*., 2005). For RdmE the reason for the possible deprotonation would be unknown.

3.1.12 FAD-reduction

It has been shown that the FAD is reduced in its out-conformation in pHBH (Palfey *et al*., 1997; Palfey *et al*., 1999) and most likely in all enzymes of the family. The reduction is most likely in all enzymes of the family enhanced significantly by substrate

binding; for instance, in pHBH FAD is reduced ca. 10^5 times faster when substrate is bound (Husain & Massey, 1979). The obvious question then is, why does substrate binding enhance the reduction, and how is the conformational change of FAD triggered? With pHBH it has been shown that deprotonation of the substrate enhances the conformational change in FAD and subsequently its reduction (Palfey *et al*., 1999). It has been suggested that the deprotonation of the substrate is sensed by a conserved proline residue, P293, leading to changes in the protein backbone, eventually pushing the flavin out. It is possible that this sensing mechanism is unique to pHBH (Palfey *et al*., 2002). For instance, with PHHY the equivalent residue (P364) has been suggested to have a role in stabilization of the hydroperoxyflavin intermediate (Xu *et al*., 2002). In PgaE mutation of the equivalent Pro282 to serine caused a 2.5-fold increase in catalytic activity (paper II, table 1), and thus the proline does not have a significant role in the hydroxylation or sensing of the substrate binding in PgaE. In PHHY the FAD appears to be in an equilibrium between the out- and in-conformations once the substrate is bound (Enroth *et al*., 1999), whereas in RebC substrate binding attracts the FAD to the in-conformation (Ryan *et al*., 2008). In absence of substrate it is out (Ryan *et al*., 2007), unlike in PgaE and CabE. Also with PhzS the FAD is in the out-conformation in absence of substrate (Greenhagen *et al*., 2008). It is attractive to think that in PgaE/CabE and RdmE binding of the large substrate is enough to trigger the conformational change of FAD to the out-conformation and to thereby facilitate its reduction. This is supported by the fact that the only hydrogen bond stabilizing the out-conformation in RdmE is formed with the substrate. However, further conformational changes concerning the protein must occur to allow reduction by NADPH, as the *re*-side of the isoalloxazine ring is blocked by an aromatic side chain in its out-conformation. Furthermore, the observed out-conformation in absence of substrate in RebC and PhzS shows that mere conformational change of the FAD is not sufficient to promote the reduction; something more complicated happens. The details leading to the induction of FAD-reduction remain to be elucidated, and may be different from one enzyme of the family to another. After being reduced, the FAD has to return to the solvent-secluded active site to prevent futile reactions with the solvent. The $NADP^+$ is released and the positive electrostatic environment of the active site attracts the negatively charged FAD to the in-conformation (Entsch *et al*., 2005).

One remaining open question is the binding mode of NADPH during reduction in pHBH-type of flavoenzymes. NADPH was observed in the crystal structure of the mutant R220Q of pHBH (Wang *et al*., 2002), however, the isoalloxazine ring of FAD and the nicotinamide moiety of NADPH were not in the vicinity of each other, which is required for the hydride transfer. Therefore the crystal structure cannot represent the true reduction complex. It does, however, provide indications of the binding site of NADPH in a groove on the surface of the enzyme, below the middle domain (paper II, figures 7a and 7b).

3.1.13 Comparisons with other FAD-dependent oxidoreductases

There are some unifying mechanistic features in the catalysis of FAD-dependent oxygenases. Firstly, all true FAD-dependent monooxygenases have three substrates, namely the compound to be oxygenated, molecular oxygen and NAD(P)H as electron source (Massey, 1994). They all form with apparent ease an unusually stable covalent

intermediate upon reaction with oxygen, the C(4a)-(hydro)peroxide, which has enabled the spectroscopic detection of these species (Entsch *et al*., 1976; Beaty & Ballou, 1981, Massey *et al*., 1988; Massey, 1994; Sheng *et al*., 2001). Furthermore, the prosthetic group is usually secluded from solvent during the oxidative phase to prevent futile reactions with water and generation of harmful oxygen radicals. Furthermore, the caged radical pair of flavin and superoxide anion undergoes easily a second electron transfer to form hydrogen peroxide and an oxidized flavin. Thus it is necessary to stabilize the unstable caged radical pair and the subsequent peroxyflavin intermediates (Ballou *et al*., 2005). pHBH-like aromatic hydroxylases facilitate this by shuffling the isoalloxazine ring essentially between two active sites for the oxidative and reductive phases, whereas two-component flavin-dependent aromatic oxygenases, such as ActVA-Orf5/ActVB (Valton *et al*., 2006; Okamoto *et al*., 2009), use two active sites in two different proteins to separate the two phases (Ballou *et al*., 2005).

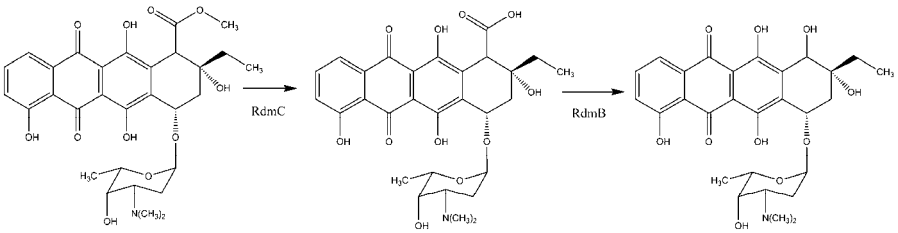
The FAD-dependent monooxygenases have also adopted various means of avoiding futile consumption of NAD(P)H. As described above, PgaE, CabE, RdmE as well as the other pHBH-like aromatic hydroxylases reduce the FAD only after the substrate has bound. For instance, FMOs (flavin containing monooxygenases) reduce FAD to $FADH_2$ in absence of substrate. The reduced flavin can react with oxygen to form a hydroperoxyflavin intermediate, which is then stabilized in the enzyme until substrate binding and subsequent hydroxylation (Beaty & Ballou, 1981). Similarly to FMOs, cyclohexanone monooxygenase (CHMO), a Bayer-Villiger monooxygenase, allows FAD to be reduced and react with oxygen prior to substrate binding. $NADP^+$ participates in stabilization of the peroxyintermediate (Sheng *et al*., 2001). Recently, an ornithine hydroxylase (PvdA) was described to allow FAD to be reduced prior to substrate binding, but substrate binding promotes the subsequent reactions between O_2 and $FADH^-$ (Meneely *et al*., 2009).

FAD can adopt various conformations: for instance in flavodoxin reductase (Ingelman *et al*., 1997) and photolyase (Park *et al*., 1995) it is in a “U”-shaped conformation. Also the isoalloxazine ring undergoes conformational changes; it can adopt a bent conformation in addition to the more common planar one observed in the crystal structures of PgaE, CabE and RdmE. These conformational changes depend on the oxidation state of the cofactor as well as protein environment (Senda *et al*., 2009). However, the shifts between in- and out-conformations observed in the pHBH-family of enzymes have thus far not been reported in other types of flavoenzymes.

FAD-containing oxygenases are structurally and mechanistically varied (van Berkel *et al*., 2006). On the other hand, enzymes with “the pHBH-fold” catalyze a variety of other types of reactions in addition to hydroxylations. These homologous enzymes include, for example, the recently structurally analyzed MtmOIV Bayer-Villiger oxygenase from mithramycin biosynthesis (Beam *et al*., 2009), the mouse axon-guiding enzyme MICAL (Siebold *et al*., 2005) and the 7-tryptophan halogenase PrnA from *Pseudomonas fluorescens* (Dong *et al.*, 2005). Other examples include, for instance, glucose oxidase (Hecht *et al*., 1993) and cholesterol oxidase (Vrielink *et al*., 1991). In fact, the fold is ubiquitous among flavoenzymes, and the pHBH-type aromatic hydroxylases belong to a large family of structurally conserved enzymes with very different catalytic activities (Mattevi, 1998).

3.2 SAM-DEPENDENT HYDROXYLASE - RdmB (PAPER I)

RdmB belongs to the biosynthetic gene cluster of rhodomycine in *S. purpurascens.* It catalyzes a step later than RdmE on the biosynthetic pathway; it is active after glycosylation (figure 9). Amino acid sequence alignments of RdmB suggested that RdmB would be an S-adenosyl-L-methionine (SAM)–dependent methyltransferase: it exhibits 52% amino acid sequence identity to DnrK, an *O*-methyltransferase that catalyzes the methylation of the position C4 of the substrate in the biosynthesis of daunomycin in *S. peucetius* (Madduri *et al.*, 1993). Furthermore, RdmB possesses the fingerprint sequence for binding of the amino acid-part of SAM, E/DXGXGXG (Martin & McMillan, 2002) However, there are no 4-O-methyl groups in rhodomycine or other polyketides produced in *S. purpurascens*, which indicated that the function of RdmB might be other than methyltransfer (Wang *et al*., 2000). Gene-deletion studies and *in vivo* and *in vitro* characterization identified RdmB as a hydroxylase, catalyzing the hydroxylation of the position 10 of the substrate, 15-demethoxyaclacinomycin T (figure 18). It requires SAM and a reducing agent, such as dithiothreitol (DTT) or glutathione (GSH), for the reaction. Interestingly, RdmB is not capable of methylating its substrate (Wang *et al*., 2000). In order to determine how RdmB – an apparent methyltransferase – catalyzes a hydroxylation reaction and activation of oxygen, in absence of any common oxygen-activating groups, we started biochemical and structural studies on this enzyme.



15-methoxy-ε-rhodomycin | 15-demethoxy-ε-rhodomycin | β-rhodomycin

Figure 18. Reaction of RdmB follows that of RdmC, a methylesterase. The product is β-rhodomycin. RdmC/RdmB accepts as substrate also aclacinomycin T, which lacks the hydroxyl group at C-11. The product from the reaction is DbrT. Aclacinomycin A, which was used in tis study, has two additional sugar moieties. The product from this reaction is DbrA.

3.2.1 S-adenosyl-L-methionine

S-adenosyl-L-methionine (SAM or AdoMet) is one of the most common cofactors and substrates in enzymatic reactions in all organisms. It consists of a methionine conjugated with an adenosine moiety (figure 19a). Enzymes utilize SAM extensively in methyltransfer reactions, for instance for methylation of DNA, which is important for regulation of gene expression. SAM-dependent enzymes methylate also hormones and neurotransmitters, to mention few examples. SAM also serves as a cellular source of methylene groups, amino groups, ribosyl groups and aminoalkyl groups (Fontecave *et al*., 2004). In addition, rather recently SAM has been discovered to be extensively utilized in enzymatic radical reactions, including sulphur insertions and anaerobic

oxidations. In these reactions SAM undergoes a cleavage to form a molecule of methionine and a 5'-deoxyadenosyl radical, which initiates further radical reactions. For this the cofactor requires additionally a [4Fe-4S]-cluster as an electron source (Grillo & Colombatto, 2007). SAM can also bind RNA in riboswitches, which are metabolite-sensing stretches of untranslated mRNA. They are involved in regulation of gene expression through transcription and translation mainly in bacteria. SAM-dependent riboswitches are involved in the regulation of sulphur-metabolism (Wang & Breaker, 2008). It is thus obvious that SAM is a real *jack-of-all-trades* in metabolism and most likely all of its functions have not yet been discovered.

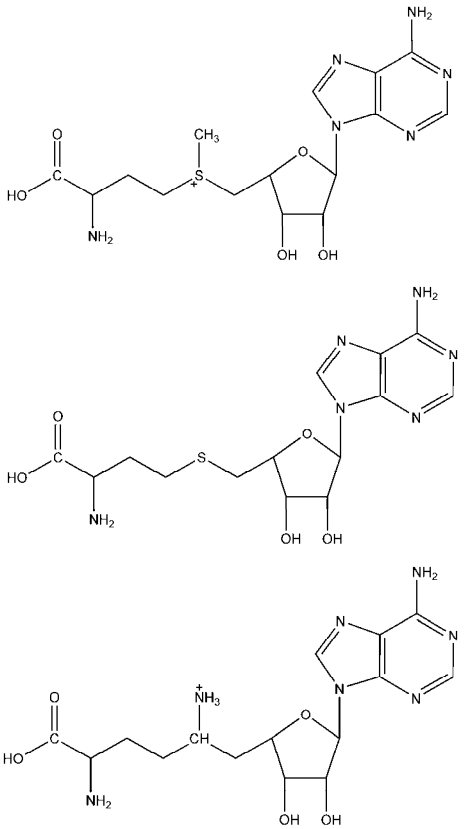


Figure 19. a) SAM b) SAH c) sinefungin

3.2.2 Methyltransferases

Most methyltransferases (MTases) utilize SAM as the methyl donor. In the reaction SAM is converted into S-adenosyl-L-homocysteine, SAH (figure 19b). MTases are poorly conserved at amino acid sequence level, but they typically have a conserved Rossmann-type fold consisting of a central seven-stranded β-sheet flanked by α-helices. SAM and the substrate bind always at equivalent positions in the fold. Interestingly, despite the conservation of the overall protein fold and the binding site of the cofactor, the protein contacts to the cofactor are little conserved: there are only two re-occurring features in the amino acid sequences of SAM-binding MTases. One is the above-

mentioned glycine-rich fingerprint in the N-terminal region of the Rossman-fold type SAM-binding domain, E/DXGXGXG. This region is lining the SAM-binding cavity at the position where the methionine-part is bound. The second conserved feature is an acidic loop, which interacts with the ribityl-moiety of the SAM (Martin & McMillan, 2002). It is to be mentioned that not all SAM-binding proteins possess a Rossmann-fold, for instance, the SAM-dependent radical enzymes possess a TIM (triose phosphate isomerase) barrel domain and lysine methylases have a SET-domain (**S**uppressor of variegation 3-9, **E**nhancer of zest and **T**rithorax) (Kozbial & Mushegian, 2005; Loenen, 2006).

Methyltransfer from SAM is supported by the electrophilic character of the methyl carbon attached to sulphur. Substrates are thus nucleophilic (Fontecave *et al*., 2004) and the reaction typically proceeds *via* S_N2-mechanism. Methyltransferases are divided in *O*-methyltransferases (OMT), which act on hydroxyl and carboxyl moieties of mainly small molecules, *N*-methyltransferases which act on proteins, DNA, phosphoetanolamines and secondary metabolites, and *C*-methyltransferases, which act on, for instance, lipids. Additionally, thiol and halide methyltransferases have been described (Roje, 2006).

3.2.3 Structural characterization of RdmB

Recombinant RdmB was expressed in *E. coli* and the crystal structure has previously been determined (Jansson *et al*., 2003b). The structure of RdmB is very similar to those of small-molecule methyl transferases, OMT's, as is to be expected from the high sequence similarity. RdmB consists of three domains (figure 20). The N-terminal, mainly α-helical domain is involved in forming the dimer interface. The C-terminal α/β Rossmann-fold –like domain and the small helical middle domain bind the SAM cofactor. The SAM-binding fingerprint is located in the Rossmann-type domain. The active site is formed between the C-terminal and middle domains, although residues from all three domains participate in interactions with the substrate. The asymmetric unit of RdmB contains a dimer (figure 20), but in earlier studies Niemi and coworkers suggested from gel filtration studies it to be a tetramer in solution (Wang *et al*., 2000).

The enzyme has little, if any, cofactor bound when purified from bacterial cells, and SAM has to be added for experiments. The structure of the protein has previously been determined as a complex with SAM and SAH (Jansson *et al*., 2003b), and in paper I structures with an inhibitor, sinefungin, and the anthracycline product DbrA are described. SAM, SAH and sinefungin were all bound in a similar manner in the protein, however, the ternary complex with DbrA shows rather large domain rotations compared with the binary complexes (paper I, figure 6). The Rossmann-fold like domain is bent like on a hinge towards the substrate binding site, and in effect, the substrate binding site gets more closed. It thus appears that the enzyme is in an "open" conformation in absence of a bound substrate and closes upon substrate binding.

Figure 20. A dimer of RdmB with the different domains shown in three shades of grey. In the middle with black the N-terminal domain involved in dimer interface. The middle domain is shown in light grey, and the C-terminal SAM-binding domain is shown in darker grey at both extremes of the protein. SAM and the ligand, DbrA, are shown with a stick model.

Amazingly, the triple-glycosylated ligand DbrA is bound with only one hydrogen bond in the active site, between the hydroxyl group of C4 and Asn260. Otherwise the binding is dominated by hydrophobic interactions in a rather narrow active site (Paper I, figure 7). DbrA is the product of RdmB, and the substrate hydroxyl group points to a hydrophobic pocket. The sugar moieties extend from the active site to the bulk solvent along a channel, also without stabilization by hydrogen bonding. Thus it seems that again substrate binding and recognition is governed by hydrophobic interactions and shape of the active site.

3.2.4 Biochemical characterization of RdmB

Compounds that are decarboxylated at position 15 are not substrates for RdmB but both substrates with and without a hydroxyl group at carbon 11 are accepted, which enabled utilization of aclacinomycin as substrate in the study. It also accepts substrates with one or three sugar groups. The enzymatic activity of RdmB was followed by analyzing the reaction products with analytical high pressure liquid chromatography (HPLC). The substrate used for RdmB, 15-demethoxyaclacinomycin (DmaA) is unstable in water. Thus coupled assays with the previous enzyme of the pathway, aclacinomycin-15-methylesterase (RdmC) (figure 18) were used instead. Thus reaction mixtures typically consisted of RdmB, RdmC, aclacinomycin and SAM. In addition, glutathione was added as a reducing agent to the mixture. The reaction mixtures were incubated at 37 ˚C for 1h, isolated and analyzed with HPLC. In some cases, the RdmC reaction was first performed, the product isolated and used subsequently as a substrate for RdmB.

SAM was not significantly consumed during the hydroxylation reaction. To study its role, enzymatic activity was measured also in the presence of SAH and sinefungin, a common inhibitor of SAM-dependent methyltransferases (Pugh *et al*., 1978) (figure 19c). The demethylated SAH inhibits the hydroxylation reaction by RdmB, whereas sinefungin promotes the catalysis. SAM has a positive charge at the sulphur group, and sinefungin has a positive charge at an equivalent position. SAH is not charged, which is the only significant difference between these molecules (figure 19).

The source of oxygen to be incorporated was shown by Anna Jansson and Jarmo Niemi to be molecular oxygen with $^{18}O_2$ labeled oxygen. Remarkably, by removing the reducing agent from the reaction mixture, a hydroperoxide intermediate was captured in mass spectrometric analysis (Paper I, figure 2). Furthermore, the amount of glutathione was shown to be equimolar to the consumed substrate.

3.2.5 Comparisons with DnrK, a true methyltransferase

A DALI search in July 2009 results in several structural homologues, all small molecule *O*-methyltransferases. The closest homologues include carminomycin-4-*O*-methyltransferase (DnrK) (1TW3, Jansson *et al*., 2004), isoflavone-*O*-methyltransferase (1FPX, Zubieta *et al*., 2001) and caffeic acid 3-*O*-methyltransferase (1KYW, Zubieta *et al*., 2002). There are several enzymes homologous to RdmB which are also involved in the biosynthesis of aromatic polyketides, such as TcmN (Summers *et al*., 1992) from tetracenomycin biosynthesis (32% identical), in addition to the above-mentioned DnrK (52% sequence identity). DnrK was characterized biochemically and structurally in a related study (Jansson *et al*., 2004) and the properties of the two enzymes were compared. This was done in order to obtain insights into why RdmB is a hydroxylase, devoid of methyltransferase activity.

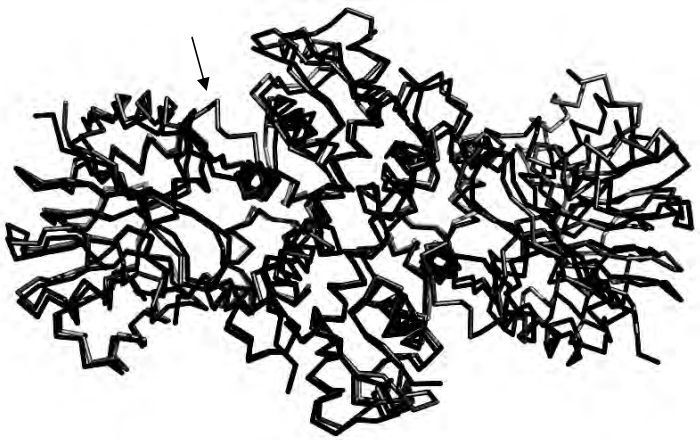


Figure 21. The dimers of RdmB (black) and DnrK (grey) superimposed on each other. The loop that has adopted different orientation in the two enzymes is indicated with an arrow in one subunit.

The overall structures of RdmB and DnrK are very similar, the r.m.s.d. of superposition of main chain atoms is 1.5 Å. The only significant structural difference is the loop formed from residues 292-298 (in DnrK 288-293), which in RdmB is bent closer to the substrate binding site (figure 21; paper I, figure 8). The active sites are also very similar; many residues are conserved between the two enzymes and both are lined mainly with hydrophobic residues. On first sight it seems incredible that the two active sites could be catalyzing two completely different reactions. The only possibly significant amino acid substitution between the two enzymes is Y142 in DnrK, which is replaced by Trp146 in RdmB (paper I, figure 7). However, when these residues were

exchanged, tyrosine to tryptophan in DnrK and *vice versa* in RdmB, neither one of the enzymes lost their catalytic activity, neither did one of them obtained the activity of the other enzyme (paper I, Jansson *et al*., 2004).

However, there is a seemingly subtle additional difference between the active sites of the two enzymes, which can explain the differential catalytic activities. For a methyl transfer reaction to occur *via* S_N2-mechanism, as has been suggested for DnrK (Jansson *et al*. 2004), the methyl group of SAM, the sulphur bound to it, and the substrate oxygen have to be aligned. This is true for DnrK, but in RdmB both the substrate and the cofactor are slightly rotated/ tilted, making the alignment impossible (paper I, figure 9; figure 22). The tilting of the substrate and SAM in RdmB is caused mainly by the change in the orientation of the loop 292-298, which causes changes in overall orientations of secondary structure elements and individual residues in the active site. Partly the changes are also due to changes in the sequence. For instance, DnrK has an arginine residue hydrogen bonded with the adenine moiety of SAM, stabilizing its position. This residue is replaced by an alanine in RdmB. These small changes appear to be sufficient to cause the complete loss in enzymatic activity, or more exactly, the inability of RdmB to function as a methyltransferase. But why is it a hydroxylase?

a)

b)

Figure 22. Alignment of the substrate and cofactor in a) RdmB b) DnrK

3.2.6 Suggested reaction mechanism

RdmB accepts only C15-carboxylated and demethoxylated substrates. At neutral pH, the carboxyl acid group is deprotonated and carries a negative charge. The substrate is decarboxylated in the enzyme, and the active site environment and the resonance from the aromatic ring system will stabilize the resulting negative charge. The positive charge of the cofactor will also aid in stabilization of the anionic species. This proposal is supported by the observation that sinefungin, but not SAH, can facilitate the reaction. The initial decarboxylation occurs easily, as DmaA is readily decarboxylated spontaneously in water solution. Interestingly, decarboxylative hydroxylation has also

been observed with the pyocyanin biosynthetic protein PhzS, which belongs to the above described family of FAD-dependent hydroxylases (Greenhagen *et al*., 2008).

Once in anionic state, the substrate intermediate can transfer an electron to molecular oxygen bound in a hydrophobic pocket near the C10. This results in a caged superoxide anion - anthracycline radical pair, which collapses with spin-inversion to form a peroxide-intermediate which is then protonated to form a hydroperoxide (paper I, figure 10b). Trapping of this intermediate by mass-spectrometry confirms this mechanistic proposal. Dissociation of the intermediate into the substrate and a molecule of water requires an extra electron, which is provided by glutathione. It would also be plausible that SAM is used to initiate a radical reaction chain instead of the mechanism described above. However, sinefungin, an inhibitor unable to undergo a radical cleavage, propagates the hydroxylation reaction of RdmB. Furthermore, RdmB does not possess any 4Fe-4S-cluster, which is conserved in the radical enzymes. The fold of RdmB is also not typical for the SAM-dependent radical enzymes. The active site of DnrK is less hydrophobic around the C10, the site of hydroxylation in RdmB, and does most likely not support binding of hydrophobic molecular oxygen. This may be the reason for DnrK being a methyltransferase devoid of hydroxylase activity.

3.2.7 SAM-binding proteins without methyl transfer activity

RdmB is not the first characterized SAM-binding protein with a methyltransferase Rossman-fold devoid of methyltransfer activity. Spermidine synthase is inactive as a methyltransferase but transfers instead methionine from decarboxylated SAM to putrescine (Korolev *et al*., 2002). Mycolic acid cyclopropane synthases (CmaA1, CmaA2, PcaA, and MmaA2) utilize SAM as methylene- rather than methyl-group donor to unsaturated fatty acids (Huang *et al*., 2002). However, the function of SAM in hydroxylation reaction in RdmB as hydroxylation-facilitating cofactor is novel for SAM.

The fact that RdmB uses its substrate in activation of O_2 is reminescent of cofactor-independent monooxygenases (Fetzner, 2002). However, it is not a pure cofactor-independent oxygenase as it does require SAM for activity. In addition, it requires a thiol-reagent such as DTT or GSH for reducing power. RdmB and DnrK together provide a fascinating example of evolution by supporting very different enzymatic reactions with very similar structures and active sites.

3.3 COFACTOR-INDEPENDENT OXYGENASE – SnoaB (PAPERS IV AND V)

SnoaB is an oxygenase from the biosynthesis route of the anthracycline compound nogalamycin in *S. nogalater* (Ylihonko *et al.*, 1996). It is a small enzyme with only 118 amino acids. It catalyzes the oxygenation of carbon 12 of the substrate, 12-deoxynogalonic acid, yielding a quinone product, nogalonic acid (paper V, figure 1). This catalytic step is important for biological activity of anthracyclines and it is common to many aromatic polyketide biosynthetic pathways: a BLAST search yields hundreds of hits of related enzymes, especially from *Streptomyces* origin, mostly annotated as putative oxygenases. This reflects the biosynthetic importance of the enzyme. Three of the related monooxygenases in antibiotic biosynthesis have previously been characterized: AknX from the biosynthesis route of aclacinomycin in *S. galilaeus* (Chung *et al*., 2002), TcmH from the biosynthesis route of tetracenomycin in *S. glaucescens* (Shen & Hutchinson, 1993) and ActVA-Orf6 from the biosynthesis route of actinorhodin in *S. coelicolor A3(2)* (Kendrew *et al*., 1997; Sciara *et al*., 2003). These three enzymes were shown to incorporate an oxygen atom to the polyketide substrate without utilizing any metal ions or prosthetic groups to activate the oxygen. Only one of these enzymes, namely ActVA-Orf6, had been previously characterized structurally (Sciara *et al*., 2003). However, the amino acid sequence identity between SnoaB and ActVA-Orf6 is rather low, less than 18%.

3.3.1 Cofactorless oxygenases in polyketide biosynthesis

For AknX, TcmH and ActVA-Orf6 and related enzymes, two different mechanisms to activate O_2 have been suggested. Both mechanisms rely on a substrate-intermediate. In 1993, Shen and Hutchinson suggested a mechanism proceeding *via* an organic radical intermediate. In the reaction, a radical intermediate would be formed by abstraction of hydrogen atom from the substrate, which would yield a radical species both in the enzyme and the substrate. This would remove the spin-barrier, and either one of the radicals could readily react with oxygen. The mechanism would then be similar to that of lipoxygenases, where an iron cofactor together with a catalytic water molecule initiates a radical mechanism by abstraction of a hydrogen from the substrate (Solomon *et al*., 1997). In absence of structural data, no potential residues carrying the radical were suggested by Shen and Hutchinson (1993). In general, plausible amino acids for carrying an organic radical species are tryptophans (Morimoto *et al*., 1998; Ruiz-Dueñas *et al*., 2009), tyrosines (Mino *et al*., 2000; Hoganson & Tommos, 2004), cysteines (Andersson *et al*., 2000) and glycines (Eklund & Fontecave, 1999). Usually amino acid radicals are observed in context with transition metals. In addition, histidine-based radicals have been characterized and suggested to be catalytic residues, but to my knowledge, they have not as yet been observed in active enzymes (Fetzner, 2002).

A few years later, Chung and coworkers suggested an alternative reaction pathway proceeding *via* a substrate carbanion in AknX (Chung *et al*., 2002). In this reaction scheme the carbanion substrate donates an electron to molecular oxygen and a caged radical pair is formed, similarly to flavin-dependent monooxygenases. This theory was supported a year later by Sciara and coworkers after determining the crystal structure of ActVA-Orf6 (Sciara *et al.*, 2003). The researchers suggested a catalytic machinery

consisting of a tyrosine and an arginine to be responsible for the initial formation of the substrate carbanion (Sciara *et al.*, 2003). We initiated structural and biochemical characterization of SnoaB in order to determine whether SnoaB and related oxygenases proceed *via* a similar pathway as proposed for ActVA-Orf6.

3.3.2 Crystallization and structure determination of SnoaB

3.3.2.1 Initial crystallization

SnoaB was cloned from *S. nogalater* and produced in soluble form in *E. coli*, initially with an amino-terminal polyhistidine tag. The first crystals of SnoaB were obtained in several PEG-containing conditions in 96-well format screens and were of thin plate-like morphology. The crystals from most crystallization conditions were not reproducible and when analyzed at synchrotron radiation sources, produced smeary diffraction patterns extending only to low resolution. Only one condition, containing PEG monomethyl ether (MME) and KBr, yielded crystals somewhat reliably. Crystal quality and reproducibility were significantly improved by a reagent in a commercial additive screen, pentaerythritol ethoxylate (EO/OH ¾) ($C(CH_2(OCH_2CH_2)_nOH)_4$). Crystals typically appeared after a couple of weeks and continued to grow over a time period of months. Initially the crystals only diffracted to low or medium resolution, at best to ca. 3 Å. They suffered from serious anisotropy, observed by visual inspection and by the anisotropy analysis in the program Truncate (French & Wilson, 1978). However, a dataset extending to 2.4 Å without significant anisotropy was finally obtained by optimization of the initial crystallization conditions and by allowing the crystals to grow for several months. Crystals belonged to a primitive orthorhombic space group with two screw axes.

3.3.2.2 Incorporation of methionines for anomalous phasing (paper IV)

While improving the crystal quality, solutions for obtaining the phases were searched with several methods. Molecular replacement did not work with the initial data sets, most likely because of the anisotropy and the low sequence homology to the closest structural homologue (ca. 18%). Heavy atom derivatization was attempted, but without success. The enzyme contains no natural methionines, except for the initiator one. We decided to introduce two new methionines into the sequence for anomalous phasing with selenomethionine. According to Leahy and co-workers (1994) and Gassner & Matthews (1999), large hydrophobic residues such as leucines, isoleucines and phenylalanines are preferred targets for substitution with methionine to cause as little as possible disturbances in the structure. Ideally, the residues should reside in helices and be buried inside the protein, to reduce their mobility. Based on sequence conservation and secondary structure predictions, Phe29, Phe40 and Leu89 were chosen to be mutated into methionines. They were mutated in pairs to yield in total three double-mutant constructs.

All three constructs were expressed in soluble form and one of them, F40M / L89M, was expressed with selenomethionine-substitution with yield comparable to the native enzyme. The full incorporation of two selenomethionines per molecule was confirmed by mass-spectrometry (figure 23). Using streak-seeding, the SeMet-substituted protein was successfully crystallized in similar conditions as the wild-type protein. The crystals were analyzed at synchrotron source and several SAD (single wavelength anomalous

diffraction) and MAD datasets were collected. Six Se-sites, corresponding to three molecules in the asymmetric unit, were identified with the program ShelxD but the electron density maps were not interpretable. The crystals again suffered from severe anisotropy, which was the likely reason for the poor quality of the maps.

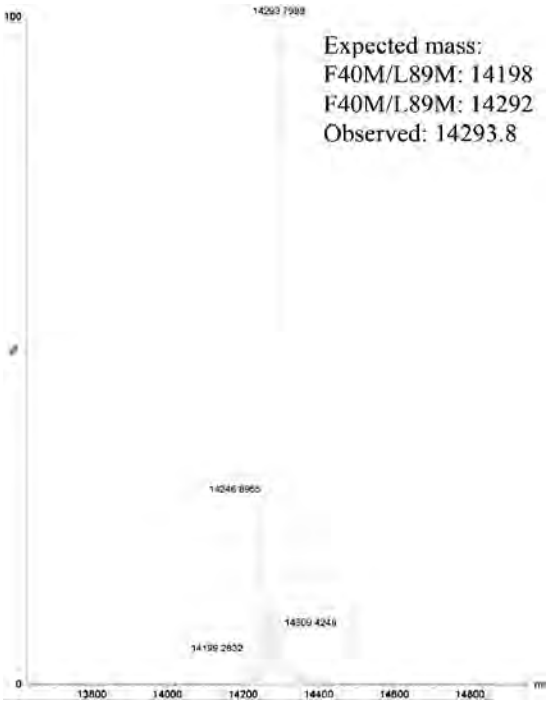


Figure 23. Graph of the mass spectrometric analysis (ES-MS) of the selenomethionine-substituted SnoaB mutant F40M/L89M. The major peak corresponds to protein with two selenomethionines incorporated, the mass is within 2 Da of the expected value. There are also significantly smaller peaks whose mass correspond to single-substituted and non-substituted proteins.

3.3.2.3 Determination of the structure of SnoaB (Paper V)

While crystallizing the selenomethionine substituted protein, a construct with a C-terminal polyhistidine tag (SnoaB-C) was cloned and expressed in *E. coli*. Two crystals were obtained in crystallization screens in a condition containing ammonium sulphate as the precipitant. The crystals could never be successfully reproduced, but one of them diffracted to 1.9 Å at a synchrotron radiation source. The crystal belonged to the space group $P4_12_12$ with two molecules per asymmetric unit. Despite the low homology, the structure could be determined by molecular replacement using the structure of ActVA-Orf6 as model. The structure in the orthorhombic space group was subsequently determined by molecular replacement using the structure of the tetragonal space group as search model.

At a later date, various soaking experiments were made with the native crystals with N-terminal histidine tag. With crystals older than one year, soaking was continued at longest for 10 d. Unfortunately, no ligand complex was obtained, but one of the crystals diffracted to a resolution of 1.7 Å. The improvement in diffraction quality and resolution was most likely partly due to the large size of the crystals after growing for a long time, and partly due to dehydration effect, as the crystals were soaked in 20% (v/v) ligand saturated in methanol and 10% (v/v) ethylene glycol.

3.3.3 Structure of SnoaB (paper V)

3.3.3.1 Overall structure

SnoaB forms a tight dimer both in crystals and in solution, as shown by cross-linking experiments (paper IV). The small enzyme consists of a ferredoxin-like α + β-fold with an antiparallel β-sheet flanked on one side by three short α-helices. The active site cavity is formed between these secondary structure elements and the dimer is formed *via* the β-sheets with a two-fold symmetry axis (figure 24). One of the five strands in the β- sheet comes *via* strand-exchange from the other monomer. The polyhistidine-tails and ca. 10 amino acids from both termini are not visible in the electron density maps in both space groups

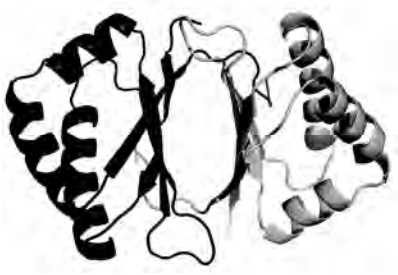


Figure 24. Dimer of SnoaB

Despite co-crystallization and soaking attempts with various compounds, no substrate complex could be obtained for SnoaB, although the crystals turned into the color of the ligand upon soaking. However, the structure of the tetragonal crystals revealed an extended electron density in the active site of one of the two subunits, most likely attributable to a bacterial fatty acid bound in the enzyme during expression (figure 25). Most likely this fatty acid was present only in one batch of the protein, making reproduction of the crystals difficult. In the active site of the other molecule there is uninterpretable electron density for a large planar molecule, at low occupancy, which could be reminiscent of danthrone, a product analogue that was present in the crystallization solution.

3.3.3.2 Modeling of substrate binding

As the attempts to obtain a substrate complex with SnoaB were not successful, substrate binding was modeled manually. The experimentally obtained substrate complex structures of ActVA-Orf6 as well as the electron density of the putative fatty acid bound in the tetragonal structure were used as aid in the modeling. The active site pocket is narrow, which restricts the possible binding modes of the large planar substrate. In the modeled complex, the only hydrogen bond is formed with Trp67. The

natural substrate may form an additional hydrogen bond with the His97 located at the mouth of the active site with the side chains of the ring B (figure 26).

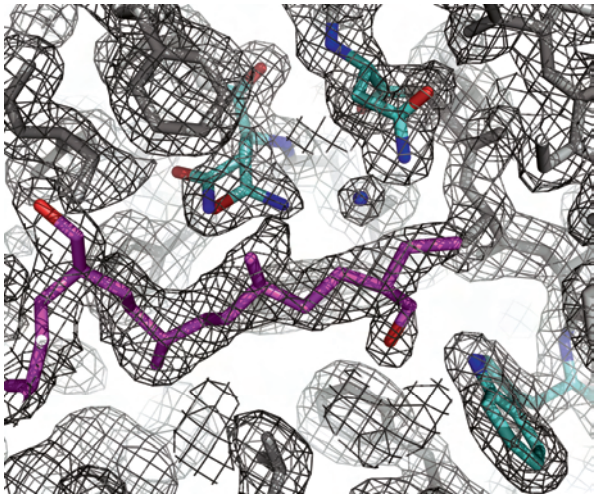


Figure 25. Composite omit map (σ-level 1.0) of SnoaB-C showing the putative fatty acid bound in the active site. The putative catalytic water is bound between the two asparagines.

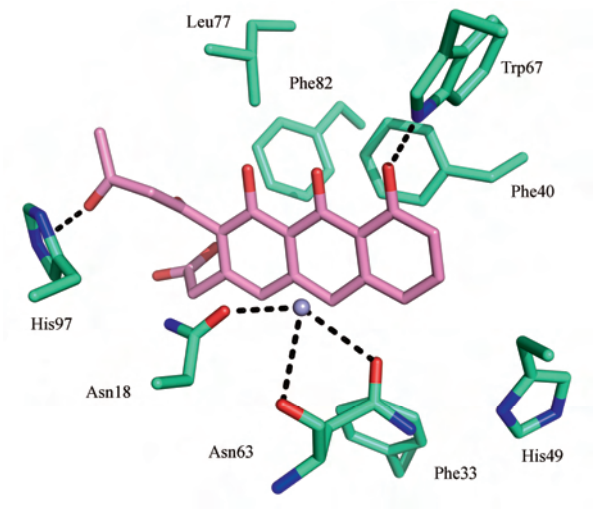


Figure 26. 12-deoxynogalonic acid, the natural substrate of SnoaB, modeled in the active site. The putative catalytic water is shown in light blue. The possible hydrogen bonding interactions are shown with dashed lines.

3.3.3.3 Structural comparison of SnoaB and related enzymes

The structure of SnoaB is very similar to that of ActVA-Orf6, the only homologous oxygenase in polyketide biosynthesis, for which a structure is available in the PDB. This explains why molecular replacement was successful despite the low sequence

identity. A search among protein structures deposited in the PDB with the program DaliLite (Holm *et al*., 2008) results in hundreds of hits of various bacterial and eukaryotic origins, reflecting the evolutionary conservation of the ferredoxin-fold. Most of the proteins are annotated with unknown function. Interestingly, the homologous proteins include the tetracenomycin F2 cyclase from *S. glaucescens*, another example of divergent evolution within polyketide biosynthesis (Thompson *et al.*, 2004). In general, the ferredoxin-like fold and the mode of dimerization *via* the β-sheets and strand exchange are conserved among the homologues.

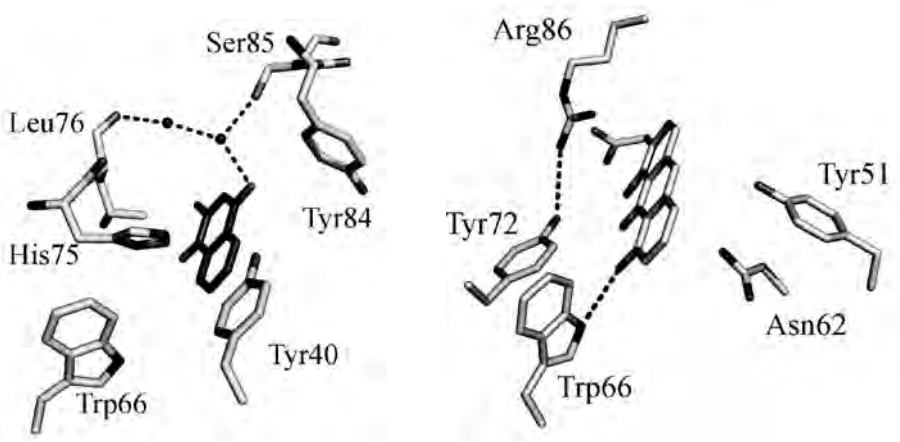


Figure 27. The active sites and putative catalytic residues in a) YgiN and b) ActVA-Orf6

Among the structural homologues, two monooxygenases have been characterized to be purely cofactor-independent: the above mentioned ActVA-Orf6 (Sciara *et al*., 2003) and the quinol monooxygenase YgiN from *E. coli* (Adams & Jia, 2005). Despite the overall similarity of the folds, the active sites are very different in SnoaB, ActVA-Orf6 and YgiN. In YgiN it is considerably smaller, whereas in ActVA-Orf6 it is of the same size range as in SnoaB, but the residues surrounding it are poorly conserved (paper V, figure 4a). Trp67 is the only active site residue conserved in all three enzymes. In addition, Asn63 is conserved between SnoaB and ActVA-Orf6. Thus, most of the proposed catalytic residues suggested for ActVA-Orf6 (Tyr51, Asn62, Trp66, Tyr72 and Arg86) (Sciara *et al*., 2003) (figure 27b) are absent in SnoaB. The active site residues suggested to possible have a catalytic role in YgiN (Tyr40, Tyr66, His75 and Tyr84) are also absent in SnoaB, with the above mentioned Trp67 being an exception (figure 27a) (Adams & Jia, 2005).

3.3.4 Biochemical characterization of SnoaB

3.3.4.1 SnoaB is a cofactor-independent monooxygenase

SnoaB is a monooxygenase utilizing molecular oxygen as substrate, as shown with the oxygen isotope $^{18}O_2$ (paper V, figure 2). As the native substrate, 12-deoxy nogalonic acid, was not available, a substrate analogue dithranol was used in activity measurements. Several substrate homologues were shown to be oxygenated by SnoaB (paper V, table 1), reflecting wide substrate tolerance. In addition, SnoaB is

catalytically active in up to 95% (v/v) acetonitrile, as well as after heating to 95 ˚C and cooling down to room temperature. In the electron density maps of SnoaB there is no density for metal ions or other cofactors. Addition of a common metal ion chelator, ethylene diamine tetraacetic acid (EDTA), to the reaction mixture did not reduce enzymatic activity, neither did addition of metal ions (paper V, table 4). These experiments showed that SnoaB truly is a cofactor-independent monooxygenase.

3.3.4.2 Active site mutations

At first sight, the active site of SnoaB was puzzling – the only plausible catalytic residues are Trp67, Asn18, Asn63 and His49. In order to investigate their catalytic roles, these residues were exchanged by site-directed mutagenesis into phenylalanine and alanines, respectively. In addition, Arg90 was exchanged into glutamine, as it was previously suggested to be conserved and important for catalysis in the family of enzymes (Sciara *et al*., 2003). All mutants were expressed in soluble form and their activities were investigated. The Michaelis-Menten kinetic parameters as well as relative activities are shown in table 5 of the paper V. The two asparagines are important for the reaction, as their mutation reduced the catalytic activity to 1-2%. The tryptophan has an important role in catalysis, but it is not vital, as shown by the residual activity of 1.0%. His49 and Arg90 appear not to be important for catalysis. All mutants were properly folded: the mutant N63A was crystallized and diffraction data was collected to 2.5 Å resolution. The data shows a well structured active site. The mutants W67F and N18A were crystallized but the crystals were of poor diffraction quality. However, all mutants produced identical spectra in circular dichroism (CD) –studies, proving that all were properly folded and that the reduction in enzymatic activity is due to a catalytic role rather than structural instability (figure 28), albeit smaller structural changes cannot be excluded.

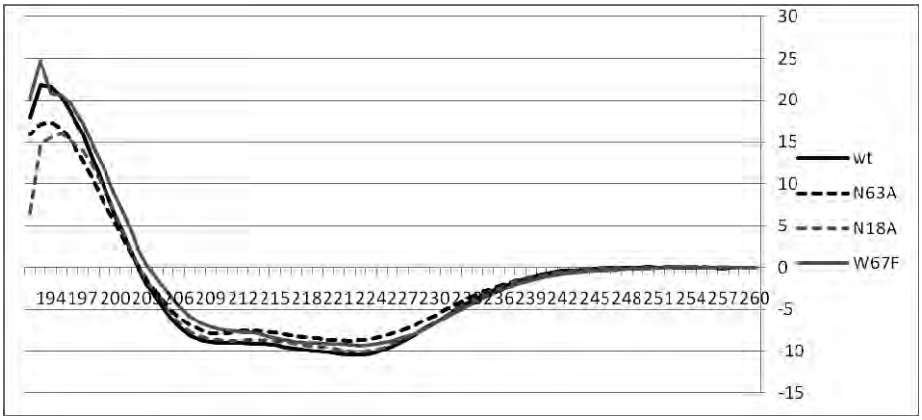


Figure 28. CD-spectra with SnoaB WT and the mutants N18A, N63A and W67F. On the X-axis λ (nm), on the y-axis ellipticity (mdeg).

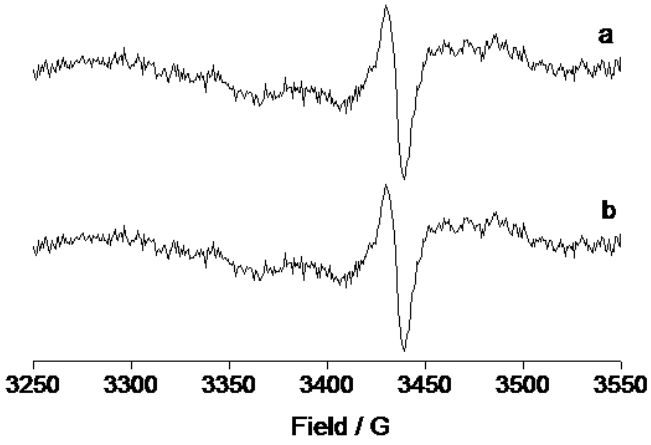


Figure 29: EPR spectra at 25 K. a) Dithranol alone b) dithranol + SnoaB. The spectras were kindly provided by Ana Popovic-Bijelic.

3.3.4.3 Electron paramagnetic resonance (EPR)

In order to determine whether the catalysis by SnoaB could proceed *via* an organic radical intermediate, we started electron paramagnetic resonance (EPR) investigations in collaboration with the group of Prof. Astrid Gräslund at Stockholm University. With EPR a radical species of the substrate or enzyme could be detected in case SnoaB does proceed *via* a radical mechanism. 9.5 GHz EPR spectra were recorded on a Bruker ESP 300 X-band spectrometer. Two different substrates (dithranol and emodin anthrone) were tested in the presence and in the absence of oxygen at various temperatures. The substrates produced a clear signal resulting from auto-oxidation, a known property of the 9-anthrones used as substrates for SnoaB (Motten *et al.*, 1994). However, the spectra produced of the substrate in presence of the enzyme could not be distinguished from the one obtained with the substrate only (figure 29). Although it would be tempting to conclude that no radical intermediate is involved in catalysis, it is possible that our experimental set-up was not appropriate for detecting the short-lived radicals.

3.3.5 Suggested reaction mechanism

The two putative mechanisms of action, one proceeding *via* a radical intermediate and one *via* a substrate carbanion, were considered for SnoaB.

3.3.5.1 Option 1: Mechanism involving radical intermediates

We could only think one possible way for generation of a substrate/enzyme radical. The reaction would be initiated by transfer of a hydrogen atom from the substrate to Trp67. This would yield a neutral radical pair of the substrate semiquinone and a reduced tryptophan radical, Trp67-H•. The substrate radical could then react with molecular oxygen to form a radical hydroperoxyl intermediate (figure 30). To obtain

again a non-radical substrate species, the enzyme would transfer an electron or a hydrogen atom to the substrate, most likely from the Trp67-H• which thereby returns to the neutral non-radical form. In addition to Trp67, the only candidate amino acid that could form an amino acid radical is His49, but replacement of this residue with alanine had little effect on the reaction rate.

We could not detect any enzyme radical species in our EPR studies, which does not necessary mean that such would not exist. However, the most likely radical mechanism would require oxidation of the substrate to form a reduced tryptophan radical. We could not find any example in the literature of an enzymatic system involving a reduced tryptophan radical: usually tryptophan residues in biological system form a radical *via* oxidation. To our knowledge the only example of a reduced tryptophan radical was described by Moan and Kaalhus in the 1970s, where L-tryptophan was x-ray irradiated in solution (Moan & Kaalhus, 1974). More importantly, the mutation W67F had a significant effect on the enzymatic activity but did not abolish it, as would be expected if the residue was performing a crucial catalytic step.

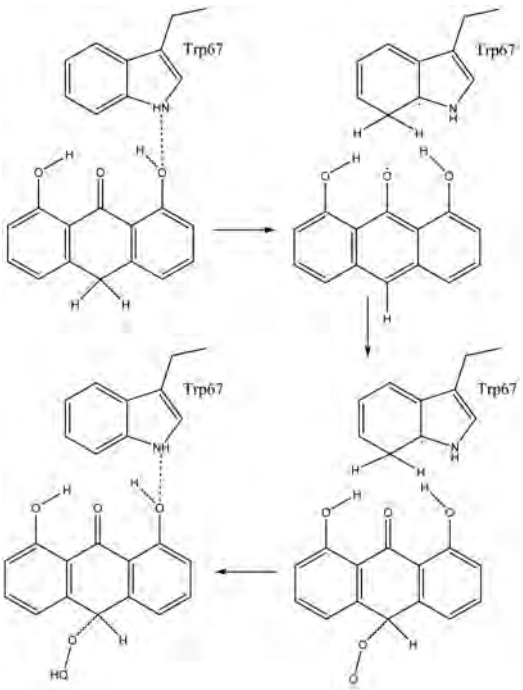


Figure 30. The possible mechanism proceeding *via* radical intermediates

3.3.5.2 Option 2: Mechanism involving carbanionic intermediate

The initial problem with the mechanism proceeding *via* a carbanion intermediate was that there are no obvious catalytic bases in the active site of SnoaB. However, in the active site of the subunit with the putative fatty-acid bound, there is a well-defined water molecule located between the two catalytically relevant asparagines, Asn18 and Asn63 (figures 25 and 26). This water molecule could serve as a catalytic base in the initial deprotonation step. We suggest that catalysis by SnoaB proceeds *via* a substrate-carbanion assisted mechanism, as outlined below and in figure 31.

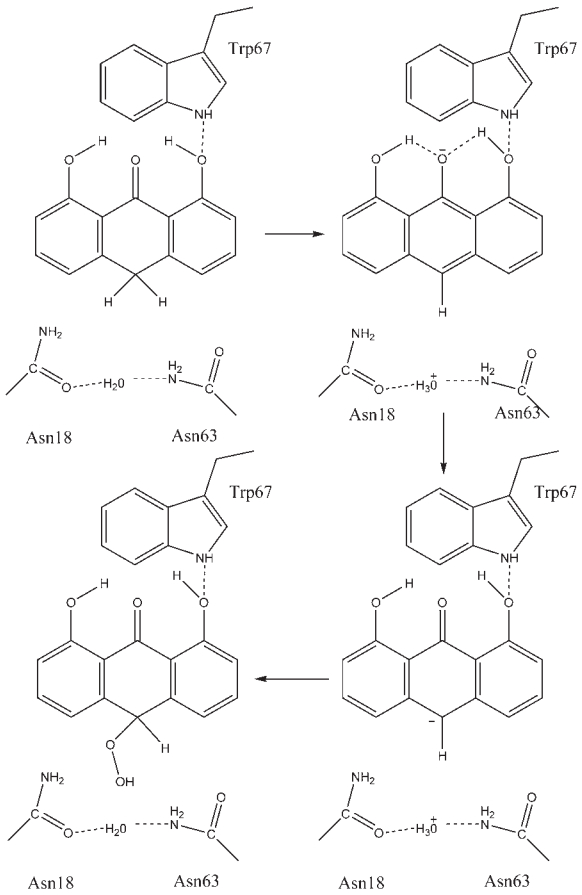


Figure 31. The putative mechanism proceeding *via* a carbanion intermediate

The substrate is first deprotonated by the water molecule bound between the two asparagines. This reaction is facilitated by the acidic nature of the substrate 9-anthrones. A requirement for the single-electron transfer is that the negative charge can be stabilized in the anionic species (Frerichs-Deeken *et al.*, 2004). This requirement is met in SnoaB: the polyaromatic substrate can delocalize the negative charge. The formation of the anion intermediate is followed by activation of oxygen: the anion transfers an electron to O_2, and a caged radical pair of a superoxide-anion and substrate-

semiquinone is formed. These radical species collapse with spin-inversion, as described above for flavins. This results in a peroxide-semiquinone intermediate. The peroxide is reprotonated to form a semiquinone-hydroperoxide-species, most likely by the same water molecule that was responsible for the initial deprotonation. The semiquinone-hydroperoxide is stabilized by hydrogen bonding with the two asparagines and the water molecule (paper V, figure 6b). The product is subsequently formed from this intermediate by dehydration.

3.3.6 SnoaB and other cofactor-free oxygenases

The catalytic triad of SnoaB (Asn18, Asn63 and a water molecule) differs considerably from the catalytic machinery of ActVA-Orf6 (Sciara *et al.*, 2003) (figure 27b), however, the proposed reaction mechanisms are very similar. The residues involved in substrate binding (Trp67) and catalysis (Asn18 and Asn63) are conserved in the more closely related enzymes, such as the putative oxygenases in *S. steffisburgensis* (CAJ42321.1) and in *S. peucetius* (AAA65205.1) and CosX from *S. olindensis* (ABC00737.1) (paper V, figure 4a). This may indicate that these enzymes catalyze the oxygenation reaction similarly to SnoaB. SnoaB, YgiN and ActVA-Orf6 appear to have diverged from each other long time ago; however, they have retained the overall structure and overall chemistry, although the catalytic machineries are different. Another interesting feature in their catalysis is that the substrate provides the reducing power for the reaction – no additional electron source is needed.

In addition to the cofactor-independent monooxygenases involved in biosynthesis of polyketide antibiotics, the enzyme fold is conserved in bacteria and also present in higher organisms. Most of these homologous enzymes are of unknown function. Most likely some of them are involved in the oxygenations of various cellular compounds, such as YgiN in synthesis of quinols (Adams & Jia, 2005). It appears that the fold provides the enzyme with exceptional stability and tolerance towards external stress: at least two homologues are described with similar heat-resistance as SnoaB: AknX (Chung *et al*., 2002) and the thermostable plant protein SP1 from *Populus tremula* (aspen) (Dgany *et al*. 2004). This protein forms naturally higher oligomers and because of its thermal stability, it has been experimentally used to form nanostructures (Medalsy *et al*., 2008).

In addition to SnoaB, ActVA-Orf6 and YgiN, three other cofactor-independent proteins with the same fold have been described as possessing oxygenase activity. The two heme-degrading enzymes IsdG and IsdI from *Staphylococcus aureus* have been characterized as oxygenases or hydroxylases. However, they possess a heme-iron center in the substrate, which may be used in activation of oxygen. The two enzymes thus form a special class of cofactor-independent oxygenases (Wu *et al*., 2005). In addition, a domain predicted to be structurally similar to SnoaB has been discovered fused with a truncated hemoglobin domain. This fusion protein was shown to possess cofactor-independent quinol-oxygenase activity. The natural function of this peculiar fusion protein is not known (Bonamore *et al*., 2007). As mentioned in section 1.1.2.5, three cofactor-independent dioxygenases have thus far been biochemically characterized: oxoquinaldine (Hod) and oxoquinoline dioxygenases (Qdo) (Fischer *et al*., 1999; Fischer & Fetzner, 2000) and DpgC from vancomycin biosynthesis route

(Fielding *et al*., 2007; Widboom *et al*., 2007). Although not a "natural" enzyme, cofactor-independent oxygenase activity was also described for a mutant form of the dihydroneopterin aldolase by Wang and coworkers (Wang *et al*., 2006). Furthermore, urate oxidase activates oxygen without cofactors (Kahn & Tipton, 1998; Colloc'h *et al*., 2008; Gabison *et al*., 2008). For all these enzymes, a mechanism relying on a substrate-carbanion and a caged radical pair has been proposed based on biochemical characterization (Fischer & Fetzner, 2000; Frerichs-Deeken *et al.*, 2004; Wang *et al*., 2006; Widboom *et al*., 2007; Colloc'h *et al*., 2008). These results lead Susanne Fetzner to propose that a substrate carbanion-dependent mechanism is a common feature among to the enzymes activating dioxygen without cofactors (Fetzner, 2007). Our results with SnoaB support her suggestion, and are in agreement with the results obtained with other cofactor-independent oxygenases and oxidases.

4 CONCLUSIONS

In this study, five oxygenases with three potentially different mechanisms for activation of oxygen were studied. Insights into oxygen-activation and catalytic mechanisms at molecular level were obtained for previously described enzyme-classes and additinoally a novel cofactor was discovered.

Studies with the FAD-dependent aromatic hydroxylases showed how enzymes with an identical fold and the same overall mechanism can show subtle variations in the mode of action at the molecular level: RdmE may require deprotonation of the substrate for the hydroxylation reaction to proceed, like pHBH, however, no hydrogen-bond chain to the bulk solvent was discovered. No catalytic bases were identified in the active site of PgaE. The oligomeric states and dimerization interactions as well as substrate entrance vary in the family of enzymes. Although the main steps in the catalysis of aromatic hydroxylases of pHBH type appear to be conserved and are rather well known, the details are different for the different enzymes. This is maybe not surprising, as the enzymes have evolved very different metabolic functions. There are yet a number of questions to be answered: What triggers the conformational shift of FAD and its reduction by NADPH? What is the role of the different oligomeric states? What is the function of the C-terminal domain - is it merely a left-over from evolution?

SnoaB is a cofactor-independent monooxygenase catalyzing a crucial step in biosynthesis of the anthracycline compound nogalamycin. Our data are most consistent with oxygenation *via* a substrate-carbanion assisted mechanism. It is the substrate itself that activates the molecular oxygen and provides the reducing power. SnoaB exhibits high structural similarity to two other characterized cofactor-independent monooxygenases. However, few active site residues are conserved and although it is likely that a carbanion mechanism is also employed by these enzymes, the catalytic machinery is different.

With RdmB, a novel function for S-adenosyl-L-methionine was discovered; participation in an oxygenation reaction. This is thus yet another role for this versatile and common cofactor. It does not have a direct role in catalysis or in activation of molecular oxygen, but it is required to stabilize the anionic substrate intermediate. Reducing power is obtained externally. RdmB exhibits high similarity at sequence and structural level to DnrK, a methyltransferase, yet the two enzymes catalyze very different reactions.

Thus some general conclusions can be made from these studies. It can be misleading to predict the catalytic activity of an enzyme based solely on sequence identity and even structural homology, as shown by RdmB and DnrK. At the present stage of our ability to predict function from sequence and/or structure, biochemical characterization is still required to verify functional annotations.

The most fascinating observation of the studies described here is, however, that despite very different structural architecture, molecular machineries and electron sources,

Nature has utilized the same basic mechanism of oxygen activation in all three enzyme families. All enzymes utilize a stabilized carbanion species to initially activate molecular oxygen, which subsequently results in a caged radical pair and a hydroperoxide-intermediate.

In addition to providing insights into the enzymatic activation of oxygen with three cofactors, the studies have contributed to the structural and functional characterization of three important tailoring steps in aromatic polyketide biosynthesis. Hopefully this information will be useful in future research and generation of novel polyketide antibiotics.

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