Enzymes involved in epoxide degradation in *Xanthobacter* Py2

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Enzymes involved in epoxide degradation in *Xanthobacter* Py2

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Proefschift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 10 juni 1998
des namiddags te vier uur in de Aula.

Voorkant: Xanthobacter geel.

ISBN: 90-5485-856-7
The European Community (BIOT-CT91-0269) financed the research described in this thesis.

Stellingen.

1. De afbraak van epoxides door de *Xanthobacter* Py2 is complexer dan voorheen aangenomen.

   Dit proefschrift.

2. Het component II eiwit van de *Xanthobacter* Py2 lijkt het gebrek aan kwaliteit door kwantiteit te hebben gecompenseerd.

   Dit proefschrift.

3. Racisme is vooral een economisch probleem.

4. Een kind uit een gemengd huwelijk is geen halfbloed maar een dubbelbloed.

   Ineke Soorani-Lunsing.

5. In tegenstelling tot het sportiviteits beginsel dient een topsporter in spé niet tegen zijn of haar verlies te kunnen.

6. Hoewel ouders veelal denken dat de ouder-kind relatie symbiotisch is, is deze in feite parasitair.

7. Er zou over een vaderinstinct gesproken worden als de vader evenveel tijd aan zijn kind zou besteden als de moeder nu.

8. Hoewel de partij programma's anders doen vermoeden, is het beter voor het milieu om de ministerpost voor VROM te laten bezetten door een VVD minister dan door een PvdA minister.

9. Afgaande op de reclames van Fa is het zeer opmerkelijk dat het product nog met verpakking geleverd wordt.

Stellingen behorende bij het proefschrift:

"Enzymes involved in epoxide degradation in Xanthobacter Py2".

Voorwoord

Allereerst wil ik alle mensen die voor het tot stand komen van dit proefschrift essentieel zijn geweest van harte bedanken:

Jan de Bont, je scherpzinnigheid en je "meedenkend vermogen", ook met microbiologische onderwerpen waar je niet zo in thuis bent, heeft me altijd aangenaam verrast. Ik heb dan ook met veel plezier bij je gewerkt.

Aart de Kok en Adri Westphal, ik heb erg van onze samenwerking genoten en ik ben dan ook erg blij dat Aart er in heeft toegestemd om als co-promotor op te treden. Jullie kennis over de flavine bevattende disulfide-oxidoreductasen heeft het onderzoek een flinke zet vooruit gegeven.

Natuurlijk zijn er ook de studenten geweest waarmee ik met veel plezier heb samengewerkt. Andor, Aline, Wilco en Linda, heel erg bedankt voor jullie inzet en enthousiasme.

Verder zijn, speciaal in de beginfase, Carel Weijers en Ab van Ooijen erg belangrijk geweest voor het onderzoek. Carel heeft veel voorbereidend werk gedaan en bij Ab kon ik altijd terecht voor vragen over de moleculaire genetica.

De mensen van het lab: Mariët, Frans, Martine, Michiel, Gert, Pauline, Sonja, Rogier, Herman(n), Marcel en vele anderen: allemaal heel erg bedankt voor de plezierige tijd. Speciaal wil ik echter de volgende mensen bedanken: Jan Wery en Jan Verdoes voor de prettige samenwerking en Edwin Kets voor het, vooral kritisch, bediscusseren van ongeveer alles.

Ook wil ik Maurice Franssen heel erg bedanken voor het enthousiast meedenken over een organisch-chemisch correct mechanisme voor de afbraak van epoxides door de *Xanthobacter* Py2.

Buiten IM om zijn er ook nog veel vrienden en kennissen die op de een of andere manier (een beetje) invloed hebben gehad op dit proefschrift, waarbij ik speciaal Vesna en de vele "nieuwe" collega's in Haren (Arnold, het is nu echt af!), waar ik nu alweer meer dan anderhalf jaar met veel plezier rondloop wil noemen.

Ach lieve familie, het lijkt er op dat de promotie nu eindelijk plaats zàl vinden. Heel erg bedankt voor jullie steun. Pa en Ma, ik vind het erg fijn dat jullie mij altijd de vrijheid en de mogelijkheden hebben gegeven om te doen wat ik wou doen.

En om te bewijzen dat ik toch echt wel meer dan een heel klein beetje Spaans kan: Gracias a los Argüello Zutta, mi familia Colombiana, por el interés que han demostrado tanto por mi, como por mi investigación. Espero volver a encontrarles muchas veces.

Lieve Monica, deze laatste alinea van het voorwoord is gereserveerd voor jou. Jij en onze lieve dochter zijn gewoon het allerbeste wat mij ooit is overkomen.

Moi

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1 Introduction

Epoxides are versatile intermediates in organic synthesis. Enantiopure epoxides are of greater interest because they can serve as high-value synthons in the production of chiral pharmaceuticals and agrochemicals.

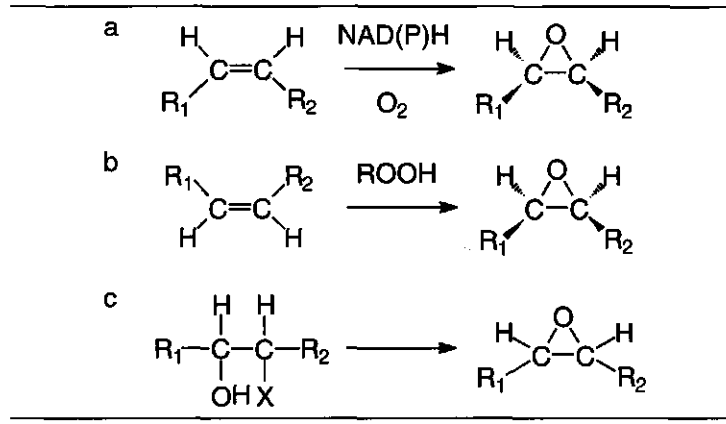

Fig. 1. Epoxidation reactions found in microorganisms. (a) Monooxygenases. (b) peroxidases, and (c) halohydrin epoxidases.

Several review articles describe both the chemical and the biological production of enantiopure epoxides (De Bont, 1992, Leak *et al.*, 1993, Besse & Veschambre, 1994). The biological research initially focused on the direct epoxidation of alkenes by several types of monooxygenases, or on the use of other enzyme systems like peroxidases, and halohydrin epoxidases (Fig. 1). More recently, enantioselective resolution of epoxides has attracted attention (Chapter 2 of this thesis). Sofar two types of enantioselective degradation by microorganisms have been investigated in more detail, the epoxide hydrolase found in several microorganisms and the enantioselective degradation of 2,3-epoxyalkanes found in *Xanthobacter* Py2. The enzymes involved in this latter type of enantioselective resolution are described in this thesis

Chapter 2 of this thesis presents an overview on the various types of epoxide degradation enzyme systems found in microorganisms to show the diversity of epoxide conversion.

The isolation and characterization of mutants of *Xanthobacter* Py2 not able to degrade epoxyalkanes, the (sub)cloning and restriction analysis of DNA fragments complementing this mutation and the heterologous expression is described in Chapter 3. Four open reading frames (ORFs) were found on the DNA fragment complementing a *Xanthobacter* Py2 mutant.

In Chapter 4 the involvement of one of these ORFs, the ORF3 protein in epoxyalkane degradation, is demonstrated. The amino acid sequence of this protein is analyzed and its relation with pyridine nucleotide-disulfide oxidoreductases is established. Furthermore, this chapter deals with the cofactors involved in the degradation of epoxyalkanes in crude extract.

Chapter 5 describes the purification and characterization of the ORF3 protein. The involvement of this protein in the degradation of epoxyalkanes in *Xanthobacter* Py2 is discussed.

Finally, Chapter 6 is of a speculative nature. Based on the information available, a mechanism is postulated to describe the degradation of epoxyalkanes in *Xanthobacter* Py2.

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2 Microbial transformation of epoxides

Enzyme & Microbial Technology (1998), 22, 19-26

Jelto Swaving and Jan A.M. de Bont

Enantioselective degradation of racemic epoxides is an interesting method to obtain optically pure epoxides. In this review, an overview is presented on the bioconversion of epoxides in microorganisms. Both the degradation and biosynthesis routes involving epoxides are discussed as well as their usefulness in enantioselective degradation.

Introduction

In recent years, the production of optically pure epoxides has received considerable attention because these compounds are valuable synthons in organic synthesis. As discussed in recent reviews (Leak *et al.*, 1992, De Bont, 1993, & Besse & Verschambre, 1994), biotechnological research initially focused on direct epoxidation of alkenes by action of monooxygenases. More recently, however, the enantioselective degradation of racemic mixtures of epoxides has been studied in more detail. This method for the production of optically pure epoxides suffers from the disadvantage that at least 50% of the substrate is not converted into product. But the method may find applications in obtaining optically pure epoxides because racemic mixtures of epoxides are cheaply prepared. Furthermore, the resulting degradative product may be an optically pure compound of interest for further applications. As a consequence, the interest in microbial epoxide degradation has increased. Several groups are now engaged in research on more fundamental aspects of epoxide degradation. In view of this development, the transformation of epoxides in microbial systems is reviewed. Distinct systems for epoxide conversion will be shown.

We first will touch on epoxides as intermediates in organic synthesis, and then epoxides degrading enzymes will be discussed by grouping them into

(i) enzymes having a detoxifying function, (ii) enzymes playing a role in biosynthetic routes, and (iii) enzymes involved in the conversion of epoxides as intermediates in degradation pathways.

EPOXIDES IN ORGANIC SYNTHESIS

Due to their electronic polarization and the strain of the three-ring structure, epoxides are highly reactive molecules that react readily with a number of reagents in particular nucleophiles. Commonly used nucleophiles reacting with epoxides are oxygen, nitrogen, sulfur, halide, and carbon compounds. The direction in which unsymmetrical epoxides can be opened depends on the reaction conditions used. A nucleophilic base will attack on the less hindered epoxide carbon. If, however, an acid is used predominantly, the more substituted carbon is attacked. Because of these properties, epoxides are very versatile intermediates in organic synthesis.

Optically pure epoxides are attracting more and more attention as high-value chiral intermediates especially in the synthesis of bioactive compounds. In a review by Furahashi (1986) the microbial production and use of different kinds of optically pure epoxides has been described. Among the optically pure epoxides produced by *Nocardia corallina* B-276 are aryl glycidyl ethers used for the synthesis of β-blokkers and the chiral part of ferroelectric liquid crystals. Some more recent examples on the use of optical pure epoxides in the production of chiral (bioactive) compounds are, e.g., leukotrienes (Neumann & Colson, 1991, Bellamy *et al.*, 1992), insect pheromones (Millar *et al.*, 1990, Rollin & Klaffe, 1991) steroids (Joannou & Reeder, 1996, Scherlitz Hoffmann *et al.*, 1996) and antibiotic compounds (Evans *et al.*, 1991, Gala & Dibenedetto, 1994) such as HIV protease inhibitors (Ng *et al.*, 1995). For this latter compound, an interesting example on the strength of enantioselective degradation of epoxides using microbial systems has been published recently. The optically pure intermediate of the HIV protease inhibitor 1(S), 2(R) indene oxide could be prepared with an e.e. of 100% from a racemic mixture using a fungal epoxide hydrolase (Zhang *et al.*, 1995).

Detoxification of epoxides

Epoxides are generally formed from olefinic and aromatic compounds using (cytochrome P-450) monooxygenases. The highly reactive epoxides

react readily with a great number of compounds including cell compounds such as proteins and DNA. It is therefore important for organisms to immediately eliminate these compounds, e.g., by direct attack on the epoxide group. Two types of enzymes have been described for the detoxification of epoxides: (i) glutathione transferases as a class of general detoxifying enzymes and (ii) epoxide hydrolases that are specific for the detoxification of epoxides.

Glutathione transferase

Glutathione transferases (GST) are found in most aerobic eukaryotes and prokaryotes. They protect cells against chemically induced toxicity by catalyzing the S-conjugation between the thiol group of glutathione and the electrophilic moiety of the (toxic) substrate (Fig. 1a). GSTs have been studied most extensively in mammalian systems (Armstrong, 1987, Dirr *et al.*, 1994) where five different classes of mammalian GSTs have been found. Four classes are soluble forms, named according to their primary structure and subunit composition (Mannervik *et al.*, 1992), and the fifth class is a microsomal form (DeJong *et al.*, 1988). 3D structures of several soluble GSTs are known and have been reviewed (Dirr *et al.*, 1994). The data obtained from these 3D structures suggest that in the active site, the thiol group of the glutathione is destabilized, thereby enhancing the nucleophilic reactivity. Furthermore, evidence suggests that the oxygen of the epoxide group forms a hydrogen bond with the hydroxyl group of a tyrosine present in the active site, thus facilitating the addition of GS^- to the carbon of the oxiran ring (Johnson *et al.*, 1993, Ji *et al.*, 1994).

GSTs are widely spread in fungi and yeast (Ando *et al.*, 1988, Kumagi *et al.*, 1988, Tamaki *et al.*, 1989, Datta & Samanta, 1992) as well as in bacteria (Di Ilio *et al.*, 1988, Iizuka *et al.*, 1989, Piccolomini *et al.*, 1989, Arca *et al.*, 1990, Di Ilio *et al.*, 1993, Orser *et al.*, 1993, Zablotowicz *et al.*, 1995). The size and composition of the GST proteins found in microorganisms resemble the mammalian GSTs in being dimers with molecular weights of approximately 50 kDa. The few amino acid sequences known from bacterial (LaRoche & Leisinger, 1990, Orser *et al.*, 1993, Mignogna *et al.*, 1993) and yeast (Tamaki *et al.*, 1991) GSTs show little homology with the mammalian sequences or each other. Antigenic characterization (Piccolomini *et al.*, 1989, Di Ilio *et al.*, 1991) as well as the substrate specificity studies of the purified GSTs indicate that there are

a large number of different GSTs. Only few bacterial GSTs have been reported to degrade the model epoxide compound 1,2-(p-nitrophenoxy)-propane (Di Ilio *et al.*, 1988, Di Ilio *et al.*, 1993). The activity of these GSTs toward this compound is very low. Other examples of GSTs involved in epoxide conversion are reported by Arca *et al.* (1990), where a GST from *Escherichia coli* is involved in epoxide fosfomycin conversion, and by Van Hylckama Vlieg *et al.* (1996), where a GST from a *Rhodococcus* sp. degrades a range of epoxides; e.g., isoprene monoxide and aliphatic epoxides. To our knowledge, no reports on the degradation of epoxides by fungal or yeast GSTs have been published.

At present, very limited information on the stereoselectivity of epoxide degradation by GSTs is available. The mammalian Class Mu GST isoenzyme 4-4 is very selective toward phenantrene 9,10-oxide (Zhang *et al.*, 1992). This shows that, in theory, this enzyme can be used for the production of optically pure epoxides. A distinct disadvantage of any GST-catalyzed reaction in the production of optically pure epoxides is the requirement for glutathione or possibly another sulfhydryl compound as reactant. This requirement may, in principle, make the use of GSTs more costly than the use of epoxide hydrolases.

Epoxide hydrolase

Another example of an enzyme involved in detoxification reactions in mammals is the epoxide hydrolase, but contrary to the glutathione transferases, epoxide-hydrating enzymes have more functions in biological systems. They are involved in several degradation pathways and in the biosynthesis of biologically important compounds. In mammals, two kinds of epoxide hydrolases have been described - the microsomal and the soluble epoxide hydrolases. These enzymes have been purified and are well characterized. In several instances, their nucleotide sequences have been determined (Beetham *et al.*, 1995).

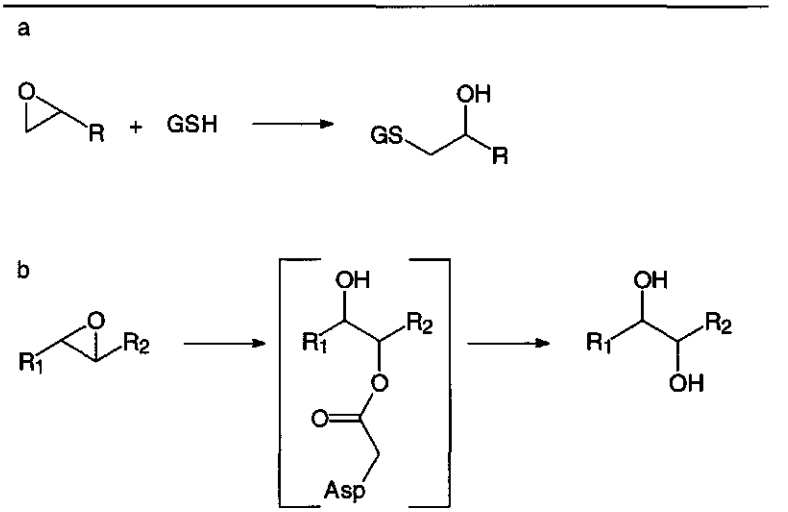

Fig. 1. Detoxification of epoxides by (a) glutathione transferases and (b) epoxide hydrolases including the covalent hydroxyacyl intermediate.

Both types of epoxide hydrolases show evolutionary relationships with each other and with other hydrolases (Arand *et al.*, 1994, Koonin & Tatusov, 1994, Beetham *et al.*, 1995). By analogy to the haloalkane dehalogenase of *Xanthobacter autotrophicus* GJ10, a putative catalytic scheme for epoxide hydrolases is thought to involve a hydroxyacyl enzyme intermediate (Arand *et al.*, 1994, Hammock *et al.*, 1994, Borhan *et al.*, 1995, Beetham *et al.*, 1995, Arand *et al.*, 1996) (Fig. 1b). The amino acids involved in the reaction have been identified (Arand *et al.*, 1996).

Recently, epoxide hydrolases from microorganisms have received considerable attention for use in biotransformations. The stereoselective transformation of epoxides by epoxide hydrolases is very interesting for a number of reasons. Firstly, no cofactors are involved. Secondly, the epoxide hydrolases studied sofar have broad substrate specificity. Furthermore, both the remaining optically pure epoxide and the optically pure diol product can be used as synthons in asymmetric synthesis.

The first examples of microbial epoxide hydrolases found in the literature describe a *Flavobacterium* (Martin & Foster, 1955) and a *Pseudomonas* (Allen & Jacoby, 1969) species involved in the conversion of 2,3-epoxysuccinate into tartaric acid and a *Flavobacterium* sp. converting

dibromopropanol into glycerol (Castro & Bartnicki, 1968). The latter bacterium contains a halohydrin epoxidase capable of converting a halohydrin into an epoxide. Due to increased interest in the degradation of environmentally important halogenated compounds, a number of bacterial epoxide hydrolases have been studied in bacteria containing halohydrin epoxidases (Van Den Wijngaard *et al.*, 1989, Kasai *et al.*, 1992, Nakamura *et al.*, 1994). Furthermore, epoxide hydrolases from other bacterial sources (Michaels *et al.*, 1980, De Bont *et al.*, 1982, Rustemov *et al.*, 1991, Hechtberger *et al.*, 1993, Madyasta & Gururaja, 1993, Carter & Leak, 1995) as well as from fungal (Chen *et al.*, 1993, Pedragosa-Moreau *et al.*, 1993, Zhang *et al.*, 1995, Nellaiah *et al.*, 1996) and yeast (Weijers, 1997) sources have been identified. In biotransformation studies, some of these epoxide hydrolases are very effective for the enantioselective hydrolysis of epoxides (Chen *et al.*, 1993, Hechtberger *et al.*, 1993, Pedragosa-Moreau *et al.*, 1993, Nakamura *et al.*, 1994, Carter & Leak, 1995, Mischitz *et al.*, 1995, Zhang *et al.*, 1995, Nellaiah *et al.*, 1996, Weijers, 1997).

In contrast to the epoxide hydrolases of mammals, the microbial epoxide hydrolases have received only limited attention from molecular biologists. Some bacterial epoxide hydrolases have been purified and characterized (Jacobs *et al.*, 1991, Nakamura *et al.*, 1994, Mischitz *et al.*, 1995). From a *Pseudomonas* sp. (Jacobs *et al.*, 1991) a *Corynebacterium* sp. (Yu *et al.*, 1994) and *Stigmatella* sp. (EMBL accession number: Q06816), (partial) amino acid and DNA sequences have been determined. Especially, the sequences of *Corynebacterium* and *Stigmatella* do show similarity to the known mammalian epoxide hydrolases. The two C-terminal amino acids of the three amino acids proposed to form the catalytic triad of soluble epoxide hydrolase (Arand *et al.*, 1996) are easily identified in these sequences, indicating that these bacterial epoxide hydrolases probably have the same reaction mechanism as proposed for mammalian epoxide hydrolases.

EPOXIDES AS INTERMEDIATES IN BIOSYNTHESIS

Epoxides are not only a toxicological problem for microorganisms but are also used for the synthesis of complex molecules. Here the formation of three of these molecules is discussed. This shows the broad diversity of reactions occurring with an epoxide intermediate.

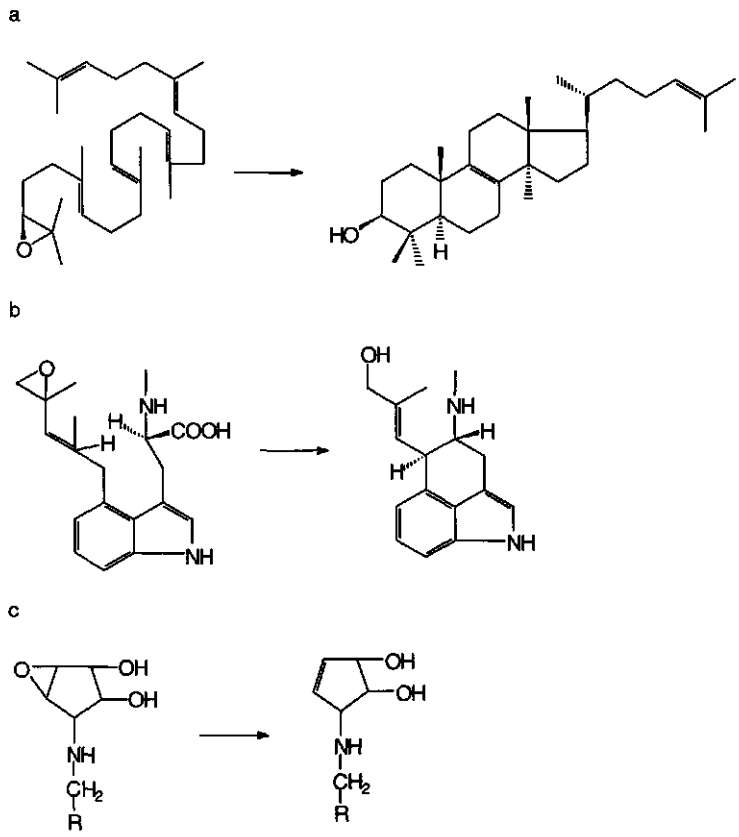

Fig. 2. Conversion of epoxides as intermediates in biosynthesis. (a) The epoxide opening by oxidosqualene cyclases leads to the formation of a four-ring structure. (b) Decarboxylation and reduction leads to the formation of the third ring (ring C) of chanoclavine-I, an intermediate in the formation of ergot alkaloid. (c) The oxygen of epoxyqueuosine is eliminated and reduced to the double bond found in queuosine.

The best-studied reaction is found in the biosynthesis of lanosterol in *Saccharomyces cerevisiae* and *Candida albicans*. In this reaction, opening of the epoxide initiates the formation of a four-ring structure (Fig. 2a). This remarkable reaction has been studied extensively and oxidosqualene cyclases from *C. albicans* (Buntel & Griffin, 1992) and *S. cerevisiae* (Corey *et al.*, 1994, Shi *et al.*, 1994) have been cloned and sequenced. The amino acid sequences deduced from the nucleotide sequences are unusually rich in tryptophan and tyrosine which have electron-rich side chains thought to be involved in the folding of the substrate in the active site and in the stabilization of the transition states or intermediates (Shi *et al.*, 1994).

In the fungus *Claviseps* strain SD 58, opening of an epoxide leads to the formation of the third ring of chanoclavine-I (Kozikowski *et al.*, 1993) (Fig. 2b). This is an intermediate in the formation of ergot alkaloid. No enzymatic confirmation on the reduction and decarboxylase steps has yet been obtained.

A third example of an epoxide as an intermediate in biosynthesis is epoxyqueuosine involved in the synthesis of queuosine (Slany & Kersten, 1994). Queuosine is a tRNA nucleoside usually replacing the guanosine in the anticodon of $tRNAs_{GUN}$ of eubacteria and cytoplasmic and mitochondrial tRNAs of eukaryotes other than yeast. Queuosine is produced *de novo* only in prokaryotes while eukaryotes get the free base queuine from their nutrients. The last step in the biosynthesis involves reduction and elimination of the oxygen of the epoxy group of epoxyqueuosine to a double bond of queuosine (Frey *et al.*, 1988) (Fig. 2c). Apart from the fact that this step requires vitamin B_{12}, nothing is known about the catalytic mechanism, proteins, or genes involved in this reaction.

THE CONVERSION OF EPOXIDE INTERMEDIATES IN DEGRADATION PATHWAYS

Examples are presented of three different degradation routes involving an epoxide as a reaction intermediate. In all of these cases, the epoxides to be degraded are formed by the action of monooxygenases.

The degradation of α-pinene by Nocardia *sp. and* Pseudomonas *sp.*

In *Nocardia* sp. strain P18.3 as well as in *Pseudomonas fluorescens* NCIMB 11671, α-pinene oxide is an intermediate of α-pinene degradation (Best *et al.*, 1987, Griffith *et al.*, 1987). An NADH-linked monooxygenase is responsible for the formation of the epoxide. An α-pinene oxide lyase catalyzing the cleavage of both the carbocyclic rings of α-pinene oxide (Griffith *et al.*, 1987) (Fig. 3a) has been purified. The 40 - 50 kDa protein is a heterodimer of 22 kDa and 17 kDa subunits. No exogenous cofactors are necessary for the reaction. The substrate specificity, however, is very limited. The only substrate known is α-pinene oxide. The inhibition of the protein by sulfhydryl active agents suggests the involvement of thiol groups in the rearrangement reaction. A mechanism has been proposed in which the epoxide group is destabilized

by addition of a proton to the epoxide group followed by a cascade of rearrangement reactions leading to opening of the ring structure of α-pinene oxide (Griffith *et al.*, 1987) (Fig. 3a).

Styrene oxide isomerase

Styrene oxide is an intermediate in the degradation pathway of styrene involving an attack on the side chain rather then on the aromatic ring (Warhurst & Fewson, 1994). Several bacteria and fungi are able to convert styrene oxide to phenylacetic acid (Shirai & Hisatsuka, 1979, Baggi *et al.*, 1983, Bestetti *et al.*, 1984, Hartmans *et al.*, 1989, Cox *et al.*, 1993). Two different degradation pathways have been postulated. Shirai and Hisatsuka (1979) and Bestetti *et al.* (1984) proposed a reduction of the styrene oxide by *Pseudomonas* to 2-phenylethanol while it was concluded that the conversion of the epoxide by *Xanthobacter* 124X yielded phenylacetaldehyde (Hartmans *et al.*, 1989). Other strains, including a fungus, *Exophilia jeanselmei* (Cox *et al.*, 1993) degraded styrene oxide using the same pathway (Hartmans *et al.*, 1990). According to Hartmans *et al.* (1995) the degradation product, 2-phenylethanol found

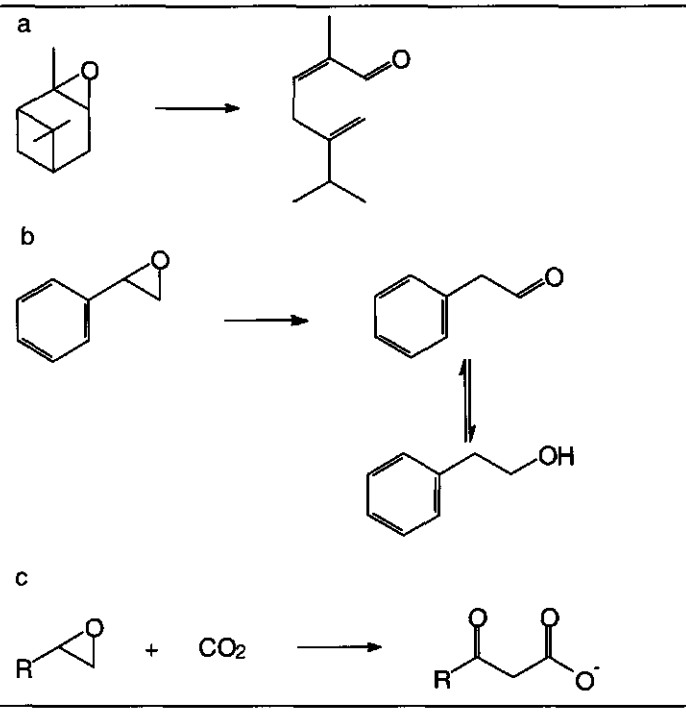

Fig. 3. Conversion of epoxide intermediates in degradation pathways.
(a) The α-pinene oxide lyase found in *Nocardia* and *Pseudomonas* strains.
(b) Styrene oxide isomerase.
(c) The NAD/NADPH dependent carboxylation of epoxyalkanes to ß-keto acids found in *Xanthobacter* Py2.

by Shirai and Hisatsuka (1979) and Bestetti *et al.* (1984) was the result of the reduction of 2-phenylacetaldehyde to 2-phenylethanol by an alcohol dehydrogenase. This suggests that these organisms also contained a styrene oxide isomerase (Hartmans, 1995) (Fig. 3b). Recently, Marconi *et al.* (1996) reported the cloning of the styrene catabolic genes from *P. fluorescens* ST.

Epoxyalkane degradation in Xanthobacter *Py2*

In recent years, a number of studies on alkene and epoxyalkane degradation by *Xanthobacter* Py2 have been published. The strain is examined not only for its monooxygenase involved in the epoxidation of the alkenes (Van Ginkel & De Bont, 1986, Van Ginkel *et al.*, 1986, Ensign *et al.*, 1992, Zhou *et al.*, 1996), and its cometabolic degradation of chlorinated compounds (Weijers *et al.*, 1988, Reij *et al.*, 1995, Small *et al.*, 1995), but also for its enantioselective degradation of epoxyalkanes.

Weijers *et al.* (1995) reported the degradation of epoxyalkanes (C_2-C_6) by *Xanthobacter* Py2. Both enantiomers of 1,2-epoxyalkanes are degraded, however, with racemic mixtures of 2,3-epoxyalkanes, the 2S forms are degraded. This leaves optically pure 2R-2,3-epoxyalkanes.

The epoxyalkane degradation has been studied in more detail by Weijers *et al.* (1995) in crude extracts of propene-grown *Xanthobacter* Py2. It was shown that the degradation of epoxyalkanes was completely dependent on NAD and a unidentified low molecular mass fraction. Dithiol compounds like DTT and 1,3-propanedithiol could replace this low molecular mass fraction while monothiols were unable to replace the fraction.

Both ketones (Weijers *et al.*, 1995) and ß-keto acids (Small & Ensign, 1995, Allen & Ensign, 1996) have been detected as the degradation product of epoxyalkane conversion. ß-Keto acids are formed by carboxylation of the epoxyalkane (Fig. 3c) while the ketones are thought to be formed only in the absence of carbonate and are not true intermediates in epoxyalkane degradation.

Swaving *et al.* (1995) isolated several mutants of *Xanthobacter* Py2 unable to grow on 1,2-epoxypropane. One of these mutants was used for complementation studies restoring growth on 1,2-epoxypropane. A 4.8 kb subclone complementing the mutation of *Xanthobacter* Py2M10 was isolated. Using this clone for heterologous expression experiments,

epoxide degradation was detected in *Xanthobacter autotrophicus* GJ10. The sequence of the 4.8 kb subclone revealed four open reading frames. The first two open reading frames showed little or no homology with known proteins. ORF3 resembled pyridine nucleotide disulfide oxidoreductases. ORF4 had homology with 3-oxoacyl reductases. Although it is not clear how these proteins are involved in epoxide degradation, Swaving *et al.* (1996) and Chan Kwo Chion & Leak (1996) showed that the ORF1 and ORF3 proteins play a role in this process. The ORF3 protein was purified and characterized (Chapter 5 of this thesis) and shown to be NADPH dependent. It was found that NADPH could replace the low molecular mass fraction and it was proposed to be the physiological cofactor (Swaving *et al.*, 1996, Chan Kwo Chion & Leak, 1996). The dithiols, which may replace the low molecular mass fraction, are able to reduce the redox active disulfide bridge of the ORF3 protein directly whereas NADPH donates electrons via FAD bound to the ORF3 protein (Swaving *et al.*, 1996)

Conclusions

The degradation of epoxides by microorganisms is extremely diverse. The reactivity of the epoxide group enables the organisms to use this epoxide for very different types of reactions, S_N2-types of reactions, reactions with or without cofactors, isomerization, and carboxylation. In some cases the opening of the epoxide ring changes the whole overall structure of the compound.

Surprisingly, only little information is available on one particular type of epoxyalkane conversion: the reduction of the epoxide group to an alcohol. In rat liver homogenate this type of reduction has been observed (Steckbeck *et al.*, 1982). In microorganisms, some (incomplete) data has been published on reduction of epoxides. For example, the degradation of limonene to α-terpeneol via a limonene oxide intermediate (Fig. 4a) has been suggested (Kieslich *et al.*, 1986). Recently, also the reduction of *p*-nitrophenyl-1,2-epoxyheptan-3-one to *p*-nitrophenyl-2,3-dihydroxyheptane by *S. cerevisiae* (Fig. 4b) has been reported, (Horak *et al.*, 1995) but both examples lack information on the enzymology of the reduction described.

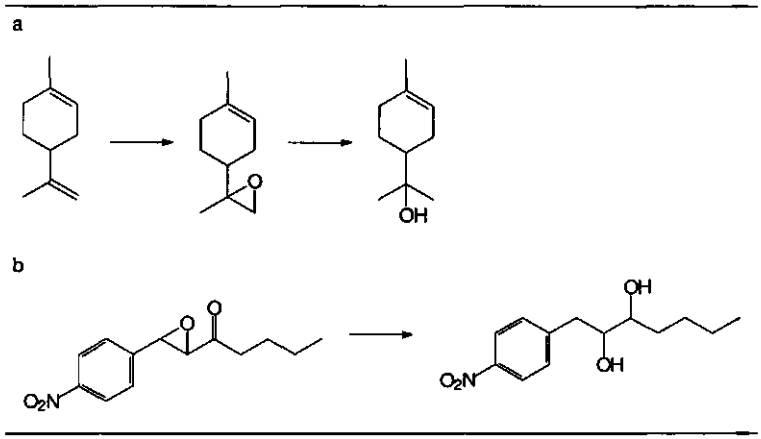

Fig. 4. Reduction of epoxides.
(a) α-Terpeneol is formed from limonene via reduction of the epoxide group of the limonene oxide intermediate.
(b) p-Nitrophenyl-1,2-epoxyheptan-3-one is reduced by *Saccharomyces cerevisiae* to p-nitrophenyl-2,3-dihydroxyheptane.

Curiously, bacteria degrading alkenes via an epoxide intermediate have developed an epoxide-degrading system different from the effective and relative simple epoxide hydrolases. As discussed before, *Xanthobacter* Py2 has developed a very complex enzyme system for the degradation of epoxyalkanes. Several other *Xanthobacter* and *Nocardia* species isolated on alkenes probably have the same type of epoxyalkane-degrading system. *Nocardia* A60 isolated on 1,2-epoxypropane used an epoxide hydrolase and was lacking an alkene monooxygenase (De Bont *et al.*, 1982). These observations indicate that the complex epoxyalkane-degrading system is linked to an alkene monooxygenase as has been suggested previously (Zhou *et al.*, 1996, Ensign, 1996).

Outlook for applying epoxide-degrading enzymes

Epoxide-degrading enzymes may be of practical use in the enantioselective degradation of cheaply available racemic mixtures of epoxides. The resulting enantiopure epoxide may be a very valuable compound in the further syntheses of biologically active compounds; however, not all of the enzymes discussed here are good candidates for applying them in a biotechnological process.

Glutathione transferases suffer from their inherent dependency on glutathione as cofactor. As a consequence, even if more than one enantioselective enzyme (Zhang *et al.*, 1995) is to be discovered, it remains questionable if the glutathione transferases will be applied.

Styrene oxide isomerases are not enantioselective. Furthermore, they do not yield an enantiopure product. These enzymes therefore are not good candidates for industrial applications.

The epoxyalkane-degrading enzyme as characterized in *Xanthobacter* Py2 has very interesting properties because it is able to degrade certain 2,3-epoxyalkanes in a completely enantioselective way. The problem here is that the enzyme system is very complex, and needs cofactors. As a consequence, it cannot be applied outside a metabolically active cell. Furthermore, the range of racemic epoxide mixtures degraded enantioselective is restricted to a few compounds only.

The most promising enzyme from a practical point of view beyond any doubt is the epoxide hydrolase. These enzymes have now been detected in many microorganisms. Depending on the microbial source, they have different substrate specificities and enantioselectivities. If a specific bioconversion is required, it then seems possible to screen for a suitable epoxide hydrolase for the specific bioconversion.

Some of the epoxide-degrading enzymes such as the α-pinene oxide lyase and epoxide reductases have not been studied in sufficient detail yet to allow an assessment of their practical applications.

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Published in
Microbiology (1995),
141, 477-484

Complementation of *Xanthobacter* Py2 mutants defective in epoxyalkane degradation, and expression and nucleotide sequence of the complementing DNA fragment

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Three *Xanthobacter* Py2 mutants (M3, M8 and M10) lacking epoxyalkane-degrading activity were isolated and characterized. All mutants were able to grow on acetone, the degradation product of 1,2-epoxypropane conversions. Furthermore, they contained the unidentified 'low molecular mass fraction' (LMF) necessary for epoxyalkane-degrading activity. Three cosmids from a gene bank complemented the mutation in M10 and M8 but not in mutant M3. Epoxyalkane-degrading activity in crude extracts of 1,2-epoxypropane-grown complemented mutants was similar to the wild-type activity. Surprisingly, M10 transformed with complementing cosmid pEP9 showed a constitutively expressed epoxyalkane-degrading activity, which was not observed in the wild-type strain. The cosmid pEP9 was conjugated into *Xanthobacter autotrophicus* GJ10, which is not able to degrade 1,2-epoxypropane. In crude extracts of *X. autotrophicus* GJ10(pEP9), epoxyalkane-degrading activity was demonstrated, but only after the addition of the LMF from *Xanthobacter* Py2. Hybridization experiments demonstrated an overlap on complementing cosmids pEP1, pEP3 and pEP9. Subcloning revealed a 4.8 kb *Eco*RI-*Hin*dIII fragment to be necessary for complementing the mutant M10. In the sequence of this fragment four different ORFs were found.

Introduction

Several methods have been described to produce optically pure epoxides by biological methods (Weijers *et al.*, 1988b, Leak *et al.*, 1992, De Bont, 1993). One such method is the enantioselective degradation of racemic epoxides; however, such a process yields a maximum of 50 % product. *Xanthobacter* Py2 was isolated on propene as sole carbon and energy source (Van Ginkel & De Bont, 1986). This strain is able to degrade the (2*S*)-forms of *trans*-2,3-epoxybutane and 2,3-epoxypentane, resulting in optically pure (2*R*)-2,3-epoxyalkanes; the C_3-C_6 1,2-epoxyalkanes are degraded completely (Weijers *et al.*, 1988a).

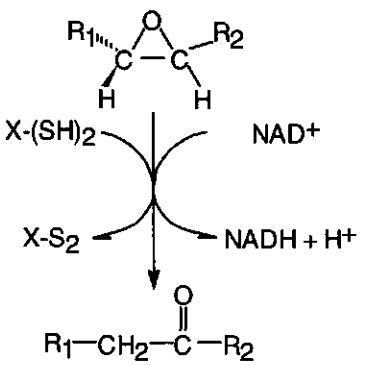

Fig. 1. The proposed degradation pathway of epoxyalkanes by *Xanthobacter* Py2. The X-$(SH)_2$ represents the LMF necessary for epoxyalkane degradation (Weijers *et al.*, 1995)

Ketones have been identified as degradation products of epoxyalkane metabolism in crude extracts of propene-grown cells (Fig. 1). It has been demonstrated that both NAD and an unidentified 'low molecular mass fraction' (LMF) are involved in the reaction. In a reaction mechanism proposed by Weijers *et al.* (1995) it was suggested that the LMF is involved in the reduction of the epoxyalkanes to secondary alcohols since the LMF can be replaced by reducing compounds like DTT and other dithiol compounds. The presence of NAD is necessary for the oxidation of the alcohols to the corresponding ketones.

Attempts to purify the epoxyalkane-degrading enzyme have not been successful. Consequently, we have now investigated the genetics of

epoxyalkane degradation in *Xanthobacter* Py2. Eventually, we hope to be able to obtain the enzyme to study further its reaction mechanism.

In this study we report the selection and characterization of mutants devoid of epoxyalkane-degrading activity, the complementation of these mutants and the expression of one of the complementing cosmids in *Xanthobacter autotrophicus* GJ10. In addition, a 4.8 kb fragment complementing the M10 mutation was sequenced and analyzed.

Methods and Materials

GENERAL METHODS

Strains and plasmids used in this study are listed in Table 1. Escherichia coli cells were grown on LB medium (Sambrook et al., 1989). Xanthobacter strains were grown at 30°C on yeast extract-glucose medium (10 g l^{-1} each) or in sealed flasks with mineral medium (Wiegant & De Bont, 1980) to which 5 mM volatile compound was added to the water phase or 5% (v/v) propene added to the gas-phase. For induction of the epoxyalkane-degrading activity, cells were grown in yeast extract-glucose medium (750 ml) in sealed 5 l flasks to which 1% propene was added to the gas phase as inducing agent. Cells cultivated on plates were placed in a desiccator when a volatile compound was used as a substrate; for each plate (20 ml) 0.1 mmol of the substrate was added to give a final concentration of 5 mM. The antibiotics used for the selection of plasmids were tetracycline (12.5 µg ml^{-1}), ampicillin and kanamycin (both 50 µg ml^{-1}). Preparation of crude extracts, and LMF, and the activity assay for epoxyalkane degradation in which the initial degradation rate of 1,2-epoxypropane in extracts is followed using head-space GC analysis have been described by Weijers et al., (1995).

MUTANT ISOLATION

Exponentially growing cultures (5 ml) of Xanthobacter Py2 grown on propene were UV-irradiated until less than 1% of the cells survived. The cells were placed in the dark in 20 ml liquid mineral medium with 5 mM acetone until growth was visible. The cells were harvested by centrifugation at 15,000 g for 5 min, washed and resuspended in the same volume of mineral media containing 1,2-epoxypropane. Glycine (2%,

Table 1. Bacterial strains and cosmids used.

Organism or cosmid	Relevant properties	Reference
E. coli		
LE392	RecA$^-$	Sambrook *et al.*, (1989)
TG1	RecA$^-$	Sambrook *et al.*, (1989)
HB101	RecA$^-$	Boyer & Roulland-Dussoix (1969)
Xanthobacter		
Py2	epoxyalkane using wild-type	Van Ginkel & De Bont (1985)
Py2M3	defective in epoxyalkane degradation	this study
Py2M8	defective in epoxyalkane degradation	this study
Py2M10	defective in epoxyalkane degradation	this study
GJ10	growth on acetone, not on epoxyalkanes	Janssen *et al.*, (1985)
Cosmids		
pLAFR5	Tc^r, RK2 replicon, *mob*$^+$	Keen *et al.*, (1988)
pEP1, -3, -9	pLAFR5 containing *X.* Py2 DNA	this study
pRK2013	*tra* (RK2), ColE1 replicon, Km^r	Figurski & Helinski (1979)
pT7/T3-19	Ap^r, pUC19 derivative	Life Technologies

w/v) was added after 8 h incubation to kill growing cells. After overnight incubation in the glycine-containing medium, the cells were harvested and washed in sterile demineralized water. Recovery, starvation and killing of the cells was repeated once more before spreading them on yeast extract-glucose plates.

GENE BANK PREPARATION

Total DNA was prepared from propene-grown wild-type Xanthobacter Py2 cells. Cells from 750 ml cultures were harvested, resuspended in 25 ml TEG (10 mM Tris, 1 mM EDTA, 50 mM glucose, pH 8.0) and lysed by the addition of SDS (2%, w/v, final concentration). Proteins were removed by extraction with phenol/chloroform, and DNA was precipitated with 2-propanol. Purified DNA was dissolved in TE buffer, partially digested with *Sau*3A and dephosphorylated. The two pLAFR5 cosmid arms were prepared by digestion with ScaI and BamHI (Keen et al., 1988). Ligated DNA was packaged using the Packagene in vitro packaging system supplied by Promega and used to transduce E. coli LE392; the transduced cells were spread on plates (100-200 colonies per plate). After overnight growth cells were resuspended and pooled in LB supplemented with 30% glycerol and stored at -80°C.

TRIPARENTAL MATING

This was carried out essentially as described by Janssen et al. (1989) but instead of NB agar, yeast extract-glucose agar was used. The conjugated recipient strains were selected on tetracycline-containing mineral medium agar plates with either 1,2-epoxypropane or 2-propanol as substrate.

DNA MANIPULATIONS, SOUTHERN-TRANSFER, HYBRIDIZATION AND NUCLEOTIDE SEQUENCE

Standard recombinant DNA techniques were performed as described by Sambrook et al. (1989). DNA was isolated from agarose gel using Geneclean II (Bio 101). For Southern transfer, digested cosmid DNA was separated on a 0.7% agarose gel, blotted onto a nylon membrane (Nytran NY 13N; Schleicher & Schuell) by capillary transfer and fixed on the membrane by UV-irradiation for 2 minutes. Probes used were prepared using the DIG DNA labeling and detection kit (Boehringer). Hybridization and detection were performed using the same kit, except that the hybridization temperature was 71°C. A 4.8 kb EcoRI-HindIII fragment was ligated into plasmid pT7/T3α-19. The insert was sequenced in both directions by Pharmacia Biotech Benelux using the primer walking method. Nucleotide sequencing was performed using the dideoxy chain-termination method (Sanger et al., 1977) using 5'-FITC-labelled primers. Computer analysis of the sequence was accomplished with the Staden program.

Results

ISOLATION OF XANTHOBACTER PY2 MUTANTS DEVOID OF EPOXYALKANE-DEGRADING ACTIVITY

The complementation method used for the isolation of *Xanthobacter* Py2 DNA sequences involved in epoxyalkane degradation by *Xanthobacter* Py2 requires mutants defective in the protein responsible for epoxyalkane degradation. To ensure that only this protein is mutated and not enzymes further down the degradation pathway, these mutants should still be able to grow on acetone, the first detectable degradation product of 1,2-epoxypropane. These mutants should also contain the unidentified 'low molecular mass fraction' (LMF) required for epoxide degradation.

Mutants were obtained as described in Methods. Single colonies were streaked on mineral medium plates and incubated for one week in a

desiccator containing 1,2-epoxypropane. Wild-type cells able to grow on 1,2-epoxypropane formed large slimy colonies while mutant cells formed very small colonies. Of the 450 putative mutants tested, 23 did not grow on 1,2-epoxypropane. These small colonies were sub-cultured twice on mineral medium plates with 1,2-epoxypropane as substrate. This procedure resulted in 11 mutants unable to grow on 1,2-epoxypropane. These 11 mutants were tested for growth in liquid containing 1,2-epoxypropane; three mutants did not grow. These mutants designated M3, M8 and M10, all grew on acetone with growth rates comparable to wild-type *Xanthobacter* Py2.

CHARACTERIZATION OF THE MUTANTS DEFECTIVE IN EPOXYALKANE DEGRADATION

The three mutants devoid of epoxyalkane-degrading activity were tested for the presence of the LMF. In wild-type *Xanthobacter* Py2 no epoxyalkane-degrading activity was found when cells were grown on yeast extract-glucose medium; however, when 1% propene was added to the head-space during growth on yeast extract-glucose, low activities were observed [approx. 2 nmol 1,2-epoxypropane degraded min^{-1} (mg protein)$^{-1}$]. Crude extracts of mutant cells grown on yeast extract-glucose in the presence of 1 % propene showed no epoxyalkane-degrading activity. These crude extracts were used for the preparation of the LMF and the protein fraction. By combining the protein fraction of the wild-type *Xanthobacter* Py2 grown on 1,2-epoxypropane with the LMF of a mutant, it is possible to detect whether the LMF is present in the mutant. The combination of the active protein fraction of the wild-type *Xanthobacter* Py2 with the LMF of the mutants resulted in epoxyalkane-degrading activity between 4 and 6 nmol 1,2-epoxyalkane degraded min^{-1} (mg protein)$^{-1}$. The specific activity of combined wild-type *Xanthobacter* Py2 LMF and protein fraction was 12 nmol 1,2-epoxyalkane degraded min^{-1} (mg protein)$^{-1}$. No activity was detected when the LMF of the wild-type *Xanthobacter* Py2 was combined with the protein fraction of the mutant cells. These results show that the LMF is still present in the three mutants and that the protein fractions of the mutants are inactive.

COMPLEMENTATION OF XANTHOBACTER PY2 MUTANTS

The broad-host-range cosmid pLAFR5 was used as a cloning vector for *Xanthobacter* Py2 because of its ability to replicate in *Xanthobacter* species

(Janssen *et al.*, 1989). The vector contains a double *cos* site, allowing the insertion of relatively large DNA fragments.

Screening of the gene bank with an average insert size of 17 kb was by individually transferring the recombinant cosmids from the transduced *E. coli* LE392 to the recipient mutant M10 using triparental mating as described in the Methods section. The conjugated recipients were selected for growth on mineral medium plates in the presence of tetracycline and using 1,2-epoxypropane as substrate. Nine tetracycline resistant colonies were able to grow on the substrate. To rule out reversion of the mutation and to eliminate unstable cosmids, the nine cosmids were isolated from these complemented M10 colonies, used to transformed to *E. coli* TG1 and again conjugated into M10. Five out of nine cosmids (pEP1, pEP3, pEP4, pEP5 and pEP9) restored the ability to grow on 1,2-epoxypropane both on plates and in liquid mineral medium.

Restriction enzyme analysis of the five different cosmids with *Bam*HI, *Xho*I, *Eco*RI and *Sal*I revealed that the restriction pattern of pEP3 was identical to pEP4; the same was observed for pEP5 and pEP9, leaving three different cosmids (pEP1, pEP3 and pEP9) complementing M10. The insert sizes of the three different cosmids were 24 kb, 26 kb and 22 kb for pEP1, pEP3 and pEP9, respectively.

The three cosmids were conjugated into the mutants M3 and M8. They complemented M8 as seen from good growth on plates in the presence of 1,2-epoxypropane. The M3 mutation, however, was not complemented by the cosmids. A subsequent conjugation of 2200 clones from the gene bank into the M3 did not yield any cosmid restoring its ability to grow on 1,2-epoxypropane.

EPOXYALKANE-DEGRADING ACTIVITY IN COMPLEMENTED MUTANT CELLS

Epoxyalkane-degrading activity was measured in crude extracts of mutants M8 and M10 grown under different conditions. The activities of cells grown on 1,2-epoxypropane was comparable to the wild-type *Xanthobacter* Py2 [13±2 nmol min^{-1} (mg protein)$^{-1}$]. Surprisingly, the M8 and M10 mutants complemented with pEP9 showed a rather high epoxyalkane-degrading activity of 22 nmol min^{-1} (mg protein)$^{-1}$ when grown on yeast extract-glucose medium, indicating constitutive expression. This was not observed

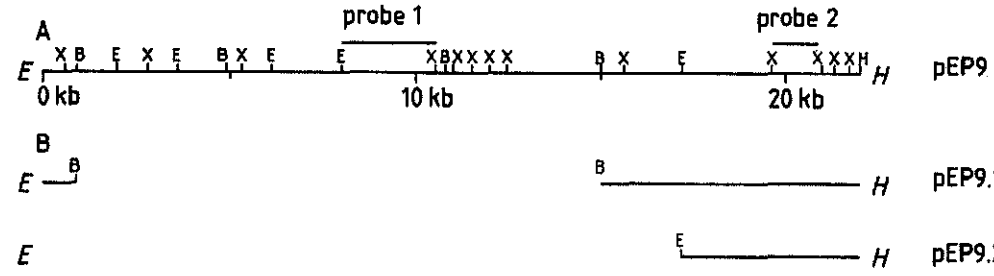

Fig. 2. (A) The restriction map of the 22 kb insert of pEP9. The lines on top represent the probes used for the Southern hybridization with pEP1, pEP3 and pEP9 (Fig. 3). Restriction enzymes used: B: *Bam*HI; E: *Eco*RI; X: *Xho*I and H: *Hin*dIII. The restriction enzymes in italics belong to the multiple cloning site of the vector. (B) The deletion clones of pEP9. pEP9.1 is a *Bam*HI and pEP9.2 is an *Eco*RI deletion clone.

in wild-type *Xanthobacter* Py2 cells nor in the mutants M8 and M10 complemented with pEP1 or pEP3.

COFACTOR DEPENDENT EPOXYALKANE-DEGRADING ACTIVITY IN XANTHOBACTER AUTOTROPHICUS *GJ10*

The plasmid pEP9, which constitutively expressed epoxyalkane-degrading activity in M8 and M10 was conjugated into *X. autotrophicus* GJ10. This *Xanthobacter* strain is not able to grow on epoxyalkanes. Conjugants were selected on mineral medium plates containing tetracycline and 2-propanol. The conjugated *X. autotrophicus* GJ10 was not able to grow on 1,2-epoxypropane, and no epoxyalkane-degrading activity was detected in crude extracts of cells grown on yeast extract-glucose grown cells. However, 1,2-epoxypropane was degraded by these extracts [2 nmol 1,2-epoxypropane degraded min^{-1} (mg protein)$^{-1}$] if the LMF prepared from propene-grown *Xanthobacter* Py2 cells was added. No such activity was found when the LMF was added to crude extracts of wild-type *X. autotrophicus* GJ10.

RESTRICTION MAP OF pEP9 AND HYBRIDIZATION STUDIES WITH pEP1 AND pEP3

Because pEP9 carries the information for epoxyalkane-degrading activity, as seen from *X. autotrophicus* GJ10 conjugated with pEP9, this cosmid was further characterized. A restriction map of pEP9 was constructed based on migration patterns of digested pEP9 and on Southern hybridization of digests of pEP9 with several probes from the pEP9 insert (Fig. 2).

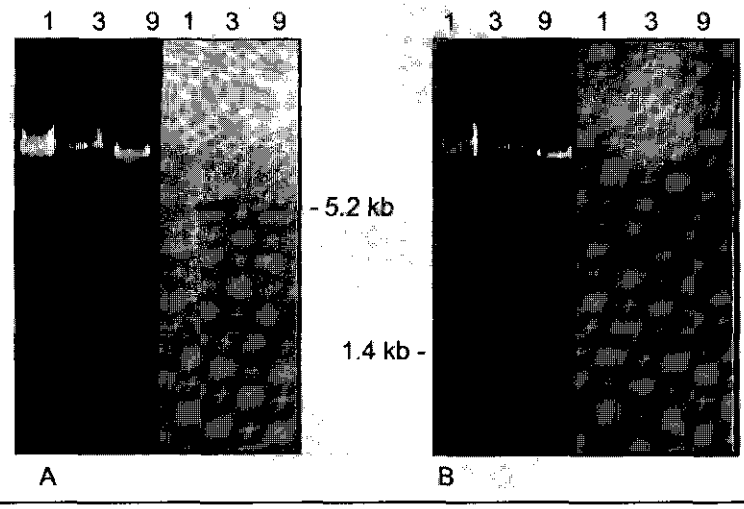

Fig. 3. Southern hybridization studies of *Xho*I-digested pEP1 (lane 1), pEP3 (lane 3), and pEP9 (lane 9). (A) is the digest and the blot hybridized with probe 1 (Fig. 2). (B) is the digest and probe hybridized with probe 2.

Southern hybridizations of *Xho*I digests of pEP1, pEP3 and pEP9 are shown in Fig. 3. The blots were hybridized to two different probes of pEP9 (Fig. 2). Probe 1 is a 2.5 kb *Eco*RI-*Xho*I fragment (from 8 kb to 10.5 kb on the map) and probe 2 is a 1.4 kb *Xho*I fragment (from 19.6 kb to 21 kb on the map). Probe 1 hybridized only to a 5.2 kb *Xho*I fragment of pEP3, probe 2 hybridized to both pEP1 and pEP3. Further restriction analysis with *Xho*I (Fig. 3), *Eco*RI, *Sal*I and *Bam*HI showed that pEP3 is homologous to pEP9 from approximately 5 kb to 22 kb on the pEP9 map. The cosmid pEP1 showed considerably less homology with both pEP3 and pEP9. Restriction homology with *Xho*I (Fig. 3) and *Sal*I was only found from the 18 kb point to approximately the 21.5 kb point of cosmid pEP9.

SUBCLONING OF PEP9

The homology studies with pEP1 in particular indicated that the complementing area was present on the right-hand end of pEP9 (Fig. 3). Therefore, this part of pEP9 was used to construct subclones. Cosmid pEP9 was digested with *Bam*HI or *Eco*RI and religated to delete the internal fragments. The resulting cosmids were checked by restriction analysis and assayed for complementation of the mutant M10. The *Bam*HI and the *Eco*RI deletion clones of pEP9, designated pEP9.1, and pEP9.2, respectively, had the ability to complement mutant M10 (Fig. 2). The nucleotide sequence of the complementing 4.8 kb *Eco*RI-*Hin*dIII fragment of pEP9.2 was determined.

NUCLEOTIDE SEQUENCE OF THE 4.8 KB COMPLEMENTING FRAGMENT OF PEP9.2

The nucleotide sequence of the 4.8 kb complementing part of pEP9 is presented in Fig. 4. The G + C content of the fragment was 65.4 mol %, which is lower than the 70 mol % found in *Xanthobacter* Py2 (Van Ginkel & De Bont, 1986). Four different ORFs were found, using a codon preference normally observed for *Xanthobacter* DNA. The four ORFs code for proteins of 41690, 7388, 57315 and 26111 Da, respectively. In the opposite strand no ORFs of significant length were found.

A computer search of the Swiss-Prot protein bank did not reveal homology for the first two ORFs. But interestingly, ORF3 showed significant homology with mercury (II) reductase (E.C. 1.16.1.1) from *Bacillus* strain RC607 (P16171), glutathione reductase (NADPH) (E.C. 1.6.4.2) from *Pseudomonas aeruginosa* (P23189) and with dihydrolipoamide dehydrogenase (E.C. 1.8.1.4) from pig (P09623). For ORF4, homologies were found with 3-oxoacyl reductase (E.C. 1.1.1.35) from *Cuphea lanceolata* (P28643) and glucose 1-dehydrogenase (E.C.1.1.1.47) from *Bacillus subtilis* (P12310).

Discussion

Three major problems are encountered concerning the isolation of the gene or genes responsible for the epoxyalkane degradation in *Xanthobacter* Py2. The first problem is the absence of a purified enzyme (Weijers *et al.*, 1994). The information gained from a purified enzyme is of great importance for both the understanding and the cloning of any enzyme. The second problem encountered is the low specific activity of the enzyme. The specific epoxyalkane-degrading activity measured in crude extracts with 1,2-epoxypropane is only 13 nmol per minute per mg protein. For *trans*-2,3-epoxybutane, which is degraded enantioselective, this activity is even more than 10 times lower (±1 nmol per minute per mg protein). Consequently, 1,2-epoxypropane was chosen as the model substrate to monitor epoxide-degrading activity. The third problem is the cofactor requirement in the epoxyalkane-degradation. Apart from NAD another yet unidentified factor is necessary for activity. This cofactor requirement makes the isolation of the epoxyalkane-degrading protein or expression of the cloned genes in other strains more difficult. Recently it has been demonstrated DTT replaces the unknown cofactor (Weijers *et al.*, 1995). In the present study the

physiological low molecular weight fraction rather than the artificial cofactor has been used to avoid any possible artifacts.

In view of the above difficulties, it was decided to isolate mutants lacking epoxyalkane-degrading activity which, subsequently, in complementation studies were used for the isolation of the gene or genes involved in the epoxyalkane degradation.

The procedure for creating and selecting mutant *Xanthobacter* strains used in this study was very successful. The three mutants selected met all the requirements to be of use in the complementation studies. They were unable to grow on 1,2-epoxypropane, but grew on acetone, the product of 1,2-epoxypropane degradation. Furthermore they contained the LMF.

Three different cosmids were found restoring the ability to grow on 1,2-epoxypropane using the randomly chosen mutant M10 for complementation experiments. Cosmids found with mutant M10 were also able to complement the M8 mutant. The epoxyalkane-degrading activities of the complemented mutants M8 and M10 were comparable to the wild-type *Xanthobacter* Py2. A special case, however, is the mutant M3. The finding that this mutant is not complemented by the cosmids suggests that this mutant belongs to a complementation group different from M8 and M10. However, the inability to complement this mutant with the *Xanthobacter* Py2 gene bank shows that the M3 mutation is more complex.

The complementation of mutants lacking epoxyalkane-degrading activity does not automatically imply that the genes responsible for the activity have been cloned. The mutants can be mutated in the structural genes but also in the regulatory regions. To ensure that the catalytic protein was cloned, the cosmid pEP9 was conjugated to *Xanthobacter autotrophicus* GJ10. Although the GJ10 harboring pEP9 did not grow on 1,2-epoxypropane, the protein required for epoxyalkane-degrading activity was present in extracts of yeast extract-glucose grown cells. This was demonstrated by combining LMF obtained from the wild-type *Xanthobacter* Py2 with the protein fraction of *Xanthobacter autotrophicus* GJ10 harboring pEP9, which resulted in 1,2-epoxypropane degradation and acetone formation.

At this point it remains open to further investigation which open reading frames code for the one or more proteins involved in epoxyalkane degradation. At present the epoxide-degrading enzyme has been characterized to a limited extend only, but a reaction mechanism has been

put forward (Weijers *et al.*, 1994). The suggested mechanism involves the reduction of a disulfide bridge in the catalytic center of the epoxide-degrading enzyme. Similarities were noted with vitamin K epoxide reductase and very recently the involvement of lipoamide reductase was implied in supplying reducing equivalents to the enzyme (Thyssen *et al.*, 1994). Obviously, in this respect it is very interesting that the ORF3 protein shows significant homology with lipoamide reductase.

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AAGCTTGGCTGCAGGTCGACGGATCAAGAAAATAGGTGCGGAAGCTTTCCCACTGCTCGGCGGGATTGCGGTAATGCAGG
        10        20        30        40        50        60        70        80

CAGATGTGGCGATGAAGATGCGCATGGGTGTGCGCGTGGGCCTGCCCCGCCAGGGGCGCGGCTCCGCCGCCGCGGCCGGG
        90       100       110       120       130       140       150       160

GGCGCGAGGGCAGGGGCGGCGACCTCGCCGACCTCCTCGCTGCGCCCGGCGAGGAAGGCGTCCAGCACCTTGCGATCGAA
       170       180       190       200       210       220       230       240

TCGCCGCTCGCCGCGCAGGCCGACGCGCAGACAGGCCAATTGGCCGTCGCTCGTCCAGCGGCGCAGGGTCGTCTTGCTAA
       250       260       270       280       290       300       310       320

ACCCTAGGTATTGGGCGGCTTCCTCGAGGGTAAGCAGGTTGTTCTCTTCATTCATTGCGTCCGTCGTCCTCGGAGCGAGA
       330       340       350       360       370       380       390       400

ATAGGTTTGACTAACTTCGGACATGTCCCATCGGCTCCTTGGGCATGGCCAATCCTCCCCTTTGGTTTCTTATCGAGCTG
       410       420       430       440       450       460       470       480

TCCGCCCTCTTAGCACCCTTCGACCCATAACGGAAATGTGCGACAGTCTGGTGCGGTACAGATATGTATGAATAAGATTG
       490       500       510       520       530       540       550       560

TAATCAAGTCTCGGTGAGCGGCGGGACGCGGCGGAGTGTCCCACGCCCGGGTTCGGCGCCCGGCCGTCCGGATTCGCCGT
       570       580       590       600       610       620       630       640

TCGGCGCCGGAAAACCCGCAAAAGCTCGAGAATCTCATGCTGGCCCGTGCGAGGCGCTTGACAGCGTCCATGGTCGGTGA
       650       660       670       680       690       700       710       720

CTAAGAATGATCAACTATAGATAATAATGGCGACTTCCGAGCAGGAATGGTGCTGGAATGTCGTCATCATTGAGAAGGCC
       730       740       750       760       770       780       790       800

                                                                  M   L
GACGACGCATCGTGGCCAGGCAGATTGGGTCTGCCGCGGCACGAGACAGTCAGGCGGCATCAGGGAGGATGGGAATGCTG
       810       820       830       840       850       860       870       880

 I  R  G  E  D  V  T  I  P  T  S  M  V  G  N  Y  P  N  P  R  W  W  D  A  Q  F  A
ATCCGAGGGGAAGACGTCACGATTCCGACCAGTATGGTCGGCAACTATCCCAATCCGCGGTGGTGGGACGCCCAGTTCGC
       890       900       910       920       930       940       950       960

  R  T  W  T  G  D  Q  E  P  P  D  A  L  I  Q  E  S  L  E  D  A  V  A  A  I  A
CCGCACCTGGACGGGTGATCAGGAGCCGCCGGACGCGCTGATCCAGGAATCCCTCGAGGATGCGGTGGCTGCCATCGCCC
       970       980       990      1000      1010      1020      1030      1040

R  D  Q  E  R  A  G  L  D  I  I  S  D  G  R  V  H  G  D  N  Y  A  E  Q  A  L  Y
GCGATCAGGAGCGCGCCGGCCTCGACATCATCAGCGACGGCCGCGTGCACGGCGACAATTACGCCGAACAGGCCCTTTAT
      1050      1060      1070      1080      1090      1100      1110      1120

 Y  Y  Y  R  R  L  G  Y  D  L  K  G  G  Y  L  G  F  P  I  Y  S  R  L  H  A  G  T
TATTACTACCGGCGCCTCGGCTACGACCTCAAGGGCGGCTATCTCGGCTTTCCGATCTACAGCCGGCTTCACGCGGGCAC
      1130      1140      1150      1160      1170      1180      1190      1200

  L  T  G  E  V  R  R  H  G  A  I  M  V  E  Q  A  K  A  L  K  K  A  T  G  K  P
CCTCACCGGCGAGGTCCGGCGCCACGGCGCGATCATGGTCGAGCAGGCCAAGGCGCTGAAGAAGGCCACTGGCAAGCCCA
      1210      1220      1230      1240      1250      1260      1270      1280

T  K  V  Q  Y  T  G  V  Q  A  L  T  Q  A  T  N  D  L  H  Y  K  S  S  R  D  R  A
CCAAGGTGCAGTATACGGGCGTCCAGGCGCTCACCCAGGCGACCAACGACCTGCACTACAAGTCGAGCCGCGATCGCGCC
      1290      1300      1310      1320      1330      1340      1350      1360

 M  A  I  A  K  A  I  N  E  D  I  R  E  V  D  A  L  G  V  D  F  I  Q  I  D  E  F
ATGGCCATCGCCAAGGCCATCAACGAGGACATCCGCGAGGTGGACGCGCTCGGCGTCGACTTCATTCAGATCGACGAGTT
      1370      1380      1390      1400      1410      1420      1430      1440

  T  W  P  Y  F  F  E  D  W  A  I  E  A  F  N  A  A  V  D  G  V  K  N  A  K  I
CACCTGGCCCTATTTCTTCGAGGACTGGGCCATCGAAGCCTTCAACGCGGCGGTGGACGGCGTGAAGAACGCCAAGATCA
      1450      1460      1470      1480      1490      1500      1510      1520

I  A  H  V  C  W  G  N  W  G  G  T  P  A  Y  Y  P  D  E  T  A  A  S  G  E  I  F
TCGCCCATGTGTGCTGGGGCAACTGGGGCGGCACGCCCGCTTACTATCCCGACGAGACGGCGGCCAGCGGCGAGATCTTC
      1530      1540      1550      1560      1570      1580      1590      1600

 D  L  T  K  R  K  A  E  A  T  K  A  T  A  T  G  S  I  V  P  K  A  Y  E  A  R  L
GATCTCACCAAGCGCAAGGCGGAAGCCACCAAGGCCACCGCCACCGGCTCCATCGTGCCGAAGGCCTACGAGGCCCGGCT
      1610      1620      1630      1640      1650      1660      1670      1680

  D  V  L  N  L  E  S  C  G  R  R  S  D  D  L  S  G  L  H  V  M  K  N  H  P  L
CGACGTGCTCAACCTGGAAAGCTGCGGGCGCCGCTCCGATGATCTCAGCGGCCTTCACGTGATGAAGAACCATCCCCTGC
      1690      1700      1710      1720      1730      1740      1750      1760
```

```
P  D  N  V  S  F  W  A  G  V  I  D  V  K  S  T  I  T  E  T  A  D  E  V  A  N  R
CGGACAATGTCTCGTTCTGGGCGGGCGTCATCGACGTGAAGAGCACCATCACGGAGACGGCCGACGAGGTGGCAAACCGC
      1770      1780      1790      1800      1810      1820      1830      1840

I  R  R  L  L  E  I  V  P  A  D  R  L  G  V  T  T  D  C  G  L  I  L  L  Q  R  Y
ATTCGCCGGCTGCTGGAGATCGTCCCGGCGGACCGCCTCGGCGTGACCACCGACTGCGGCCTCATTCTGCTCCAGCGCTA
      1850      1860      1870      1880      1890      1900      1910      1920

   I  A  Q  D  K  L  H  A  L  V  E  G  T  K  I  V  R  A  E  L  A  K  A  K  Q  A
TATCGCCCAGGACAAGCTGCACGCCCTCGTCGAGGGCACCAAGATTGTCCGAGCAGAGCTCGCCAAGGCCAAGCAGGCGG
      1930      1940      1950      1960      1970      1980      1990      2000

A                                                                       M  P
CCTGATCTAAAGACGTCAGTCATCTTTCAATCGTCCGGGCGAACACATGAGCTCGGGCGATGAGGGAGGAGCCATGCCTG
      2010      2020      2030      2040      2050      2060      2070      2080

V  Y  T  I  K  C  P  D  C  G  H  V  F  R  G  M  V  M  E  G  T  R  K  P  R  V  W
TCTATACCATCAAATGCCCGGACTGCGGCCATGTCTTCCGCGGGATGGTCATGGAGGGCACGCGCAAGCCCCGCGTCTGG
      2090      2100      2110      2120      2130      2140      2150      2160

V  C  S  Q  C  K  S  E  R  P  Q  I  M  A  D  R  P  A  E  P  H  P  F  E  C  T  E
GTGTGCTCCCAGTGCAAGAGCGAGCGGCCACAGATCATGGCCGACCGCCCCGCCGAGCCCCACCCGTTCGAATGCACGGA
      2170      2180      2190      2200      2210      2220      2230      2240

   N  G  G  G  C  L  C  C  G  R
AAACGGCGGCGGATGCCTGTGCTGCGGGCGCTAGCCCGCAGCATCCATCCGCCCCGCACGAACTCAACCTGATCGTCCCG
      2250      2260      2270      2280      2290      2300      2310      2320

      M  K  V  W  N  A  R  N  D  H  L  T  I  N  Q  W  A  T  R  I  D  E  I
AGGAGTAGACGGTGAAAGTCTGGAACGCCCGAAACGACCATCTCACCATCAATCAATGGGCCACGCGGATCGATGAGATC
      2330      2340      2350      2360      2370      2380      2390      2400

L  E  A  P  D  G  G  E  V  I  Y  N  V  D  E  N  D  P  R  E  Y  D  A  I  F  I  G
CTCGAGGCGCCCGATGGCGGAGAGGTCATCTACAACGTGGACGAGAACGATCCACGCGAATACGACGCCATCTTCATCGG
      2410      2420      2430      2440      2450      2460      2470      2480

   G  G  A  A  G  R  F  G  S  A  Y  L  R  A  M  G  G  R  Q  L  I  V  D  R  W  P
CGGCGGCGCCGCCGGGCGCTTCGGCTCGGCCTATCTGCGCGCCATGGGCGGCCGGCAGCTCATCGTCGACCGCTGGCCGT
      2490      2500      2510      2520      2530      2540      2550      2560

F  L  G  G  S  C  P  H  N  A  C  V  P  H  H  L  F  S  D  C  A  A  E  L  M  L  A
TCCTGGGCGGCTCGTGCCCGCACAATGCGTGCGTGCCGCACCATCTGTTCTCCGACTGCGCGGCCGAGCTGATGCTCGCG
      2570      2580      2590      2600      2610      2620      2630      2640

R  T  F  S  G  Q  Y  W  F  P  D  M  T  E  K  V  V  G  I  K  E  V  V  D  L  F  R
CGCACCTTCTCGGGCCAGTACTGGTTCCCGGACATGACCGAGAAGGTGGTCGGCATCAAGGAGGTGGTCGATCTGTTCCG
      2650      2660      2670      2680      2690      2700      2710      2720

   A  G  R  N  G  P  H  G  I  M  N  F  Q  S  K  E  Q  L  N  L  E  Y  I  L  N  C
CGCCGGGCGCAACGGCCCGCACGGCATCATGAACTTCCAGTCCAAGGAACAGCTCAACCTCGAATACATCCTCAACTGCC
      2730      2740      2750      2760      2770      2780      2790      2800

P  A  K  V  I  D  N  H  T  V  E  A  A  G  K  V  F  K  A  K  N  L  I  L  A  V  G
CGGCCAAGGTGATCGATAATCACACCGTCGAGGCGGCCGGCAAGGTGTTCAAGGCCAAGAACCTGATCCTCGCGGTGGGC
      2810      2820      2830      2840      2850      2860      2870      2880

A  G  P  G  T  L  D  V  P  G  V  N  A  K  G  V  F  D  H  A  T  L  V  E  E  L  D
GCGGGGCCGGGCACGCTCGACGTGCCGGGCGTCAACGCCAAGGGCGTCTTCGACCACGCGACGCTGGTGGAGGAGCTCGA
      2890      2900      2910      2920      2930      2940      2950      2960

   Y  E  P  G  S  T  V  V  V  V  G  G  S  K  T  A  V  E  Y  G  C  F  F  N  A  T
CTACGAGCCCGGCAGCACCGTGGTCGTGGTGGGCGGCTCGAAGACCGCGGTCGAATATGGCTGCTTCTTCAACGCCACCG
      2970      2980      2990      3000      3010      3020      3030      3040

G  R  R  T  V  M  L  V  R  T  E  P  L  K  L  I  K  D  N  E  T  R  A  Y  V  L  D
GCCGGCGCACCGTGATGCTGGTGCGCACCGAGCCGCTCAAGCTCATCAAGGACAACGAGACCCGCGCCTACGTGCTCGAC
      3050      3060      3070      3080      3090      3100      3110      3120

R  M  K  E  Q  G  M  E  I  I  S  G  S  N  V  T  R  I  E  E  D  A  N  G  R  V  Q
CGCATGAAGGAGCAGGGCATGGAGATCATCTCCGGCTCCAACGTCACGCGCATCGAGGAGGACGCCAACGGCCGCGTTCA
      3130      3140      3150      3160      3170      3180      3190      3200

   A  V  V  A  M  T  P  N  G  E  M  R  I  E  T  D  F  V  F  L  G  L  G  E  Q  P
GGCGGTGGTGGCCATGACGCCCAACGGCGAGATGCGCATCGAGACCGACTTCGTCTTCCTCGGCCTCGGCGAGCAGCCCC
      3210      3220      3230      3240      3250      3260      3270      3280

R  S  A  E  L  A  K  I  L  G  L  D  L  G  P  K  G  E  V  L  V  N  E  Y  L  Q  T
GCTCGGCGGAGCTGGCGAAGATCCTGGGCCTCGATCTCGGCCCCAAGGGCGAGGTGCTGGTCAACGAATATCTTCAGACC
      3290      3300      3310      3320      3330      3340      3350      3360

S  V  P  N  V  Y  A  V  G  D  L  I  G  G  P  M  E  M  F  K  A  R  K  S  G  C  Y
AGCGTGCCCAACGTCTACGCGGTGGGCGACCTCATCGGCGGGCCCATGGAGATGTTCAAGGCCCGCAAGTCCGGCTGCTA
      3370      3380      3390      3400      3410      3420      3430      3440

   A  A  R  N  V  M  G  E  K  I  S  Y  T  P  K  N  Y  P  D  F  L  H  T  H  Y  E
TGCCGCGCGCAACGTCATGGGCGAGAAGATCTCCTACACGCCCAAGAATTATCCCGACTTCCTGCACACCCATTACGAGG
      3450      3460      3470      3480      3490      3500      3510      3520

V  S  F  L  G  M  G  E  E  E  A  R  A  A  G  H  E  I  V  T  I  K  M  P  P  D  T
TCAGCTTCCTCGGCATGGGCGAGGAGGAAGCCCGCGCGGCGGGGCACGAGATCGTCACCATCAAGATGCCGCCGGACACG
      3530      3540      3550      3560      3570      3580      3590      3600

E  N  G  L  N  V  A  L  P  A  S  D  R  T  M  L  Y  A  F  G  K  G  T  A  H  M  S
GAGAACGGCCTCAACGTGGCGCTGCCGGCCTCCGACCGCACCATGCTCTACGCCTTCGGCAAGGGCACGGCCCACATGTC
      3610      3620      3630      3640      3650      3660      3670      3680

   G  F  Q  K  I  V  I  D  A  K  T  R  K  V  L  G  A  H  H  V  G  Y  G  A  K  D
GGGCTTCCAGAAGATCGTCATCGACGCCAAGACCCGCAAGGTGCTCGGCGCCCATCACGTGGGCTATGGCGCGAAGGACG
      3690      3700      3710      3720      3730      3740      3750      3760
```

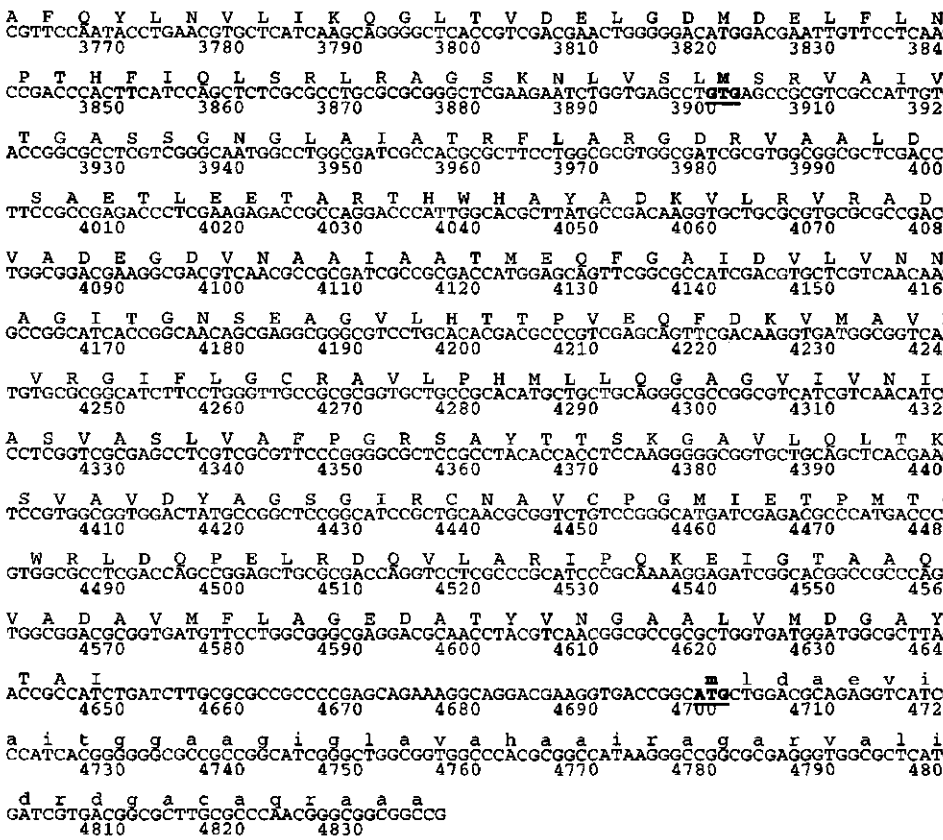

Fig. 4. Nucleotide sequence of the 4.8 kb *Eco*RI/*Hin*dIII fragment complementing *Xanthobacter* Py2M10 mutant deficient in epoxyalkane-degrading activity and the deduced amino acid sequence of the four open reading frames. The open reading frames are: ORF1: 875-2002; ORF2: 2074-2271; ORF3: 2332-3900; ORF4: 3900-4649. The start codon of each open reading frame is printed in bold. The nucleotide sequence is presented from the *Hin*dIII to the *Eco*RI site. The EMBL accession number for this sequence is X79863.

Acknowledgements

We thank Prof. Dr. D.B. Janssen for providing helpful advice and for supplying strains and plasmids. We would like to thank Dr. D. Leak and Prof. M. Fonseca and their coworkers for interesting discussions in the framework of our joint E.C.-project. This study was supported by a grant from the European Community (BIOT-CT91-0269).

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A novel type of pyridine nucleotide-disulfide oxidoreductase is essential for NAD^+- and NADPH-dependent degradation of epoxyalkanes by *Xanthobacter* strain Py2

Jelto Swaving, Jan A.M. de Bont, Adri Westphal and Aart de Kok

Epoxide degradation in cell extracts of *Xanthobacter* strain Py2 has been reported to be dependent on NAD^+ and dithiols. This multicomponent system has now been fractionated. A key protein encoded by a DNA fragment complementing a *Xanthobacter* strain Py2 mutant unable to degrade epoxides was purified and analyzed. This NADP dependent protein, a novel type of pyridine nucleotide-disulfide oxidoreductase, is essential for epoxide degradation. NADPH, acting as the physiological cofactor replaced the dithiols in epoxide conversion.

Introduction

Propene grown *Xanthobacter* strain Py2 (Van Ginkel & De Bont, 1986) contains an enzyme system capable of degrading epoxyalkanes, which are metabolites arising from alkenes by the action of alkene monooxygenase. Recently, both the monooxygenase (Zhou *et al.*, 1996) and the epoxide-degrading system of the organism have received considerable attention. Initially, this interest was based on applied aspects because the organism may be used in the degradation of chlorinated alkenes and epoxides (Ensign *et al.* 1992, Reij *et al.*, 1995, Small *et al.*, 1995, Ensign, 1996) and in the production of optically pure epoxides (Weijers *et al.*, 1988). As it turns out the epoxide-degrading enzyme system has very intriguing properties and was therefore investigated in detail.

Initially, epoxide degradation was studied at the whole cell level (Weijers *et al.*, 1988, Small & Ensign, 1995). Recently, Weijers, *et al.* (1995) were able to demonstrate enzyme activity in extracts if both NAD^+ and a low-molecular-mass fraction were included in the assay system. Furthermore, they showed that a range of artificial dithiol compounds, such as dithiothreitol (DTT) can replace the low-molecular-mass fraction. Ketones were the product formed under their assay conditions. Allen and Ensign (1996) also studied the fate of epoxides in extracts. They included carbonate in their assay system and proposed that the enzyme system of *Xanthobacter* strain Py2 carboxylated 1,2-epoxyalkanes to form ß-keto acids. In their view, ketones are dead end products, which are formed only when carbonate is limiting. The formation for either product from an epoxide is redox neutral. The requirement of both NAD^+ and a dithiol therefore suggests that reduction of the epoxide is followed by oxidation or vice versa.

The first report of a successful fractionation of the epoxide-degrading enzyme system was by Leak and coworkers (Imperial College, London, United Kingdom). They were able to devise a method resulting in two fractions, both of which were required to reconstitute an active epoxide-degrading system (Vassaroti *et al.*, 1995).

Swaving *et al.* (1995) reported the cloning of a 4.8 kb DNA fragment required for complementation of mutants of *Xanthobacter* strain Py2 defective in epoxide degradation. From the deduced amino acid sequences of the four open reading frames of this fragment no clear information on how the degradation of epoxides proceed could be gained. However, the protein encoded by ORF3 was of great interest because of its homology to the family of pyridine nucleotide-disulfide oxidoreductases (Williams, 1992). The homology was of special interest because most members of this family use dithiols as a substrate, whereas dithiols replaced low-molecular-mass fraction in the epoxide-degrading assay (Weijers *et al.*, 1995).

In the present paper we present a comparison of the amino acid sequence of the ORF3 protein with these of other important proteins from the family of pyridine nucleotide-disulfide oxidoreductases. This comparison led us to test for the involvement of NADPH in the epoxide-degrading reaction. This involvement was confirmed by using cell extracts as well as purified ORF3 protein.

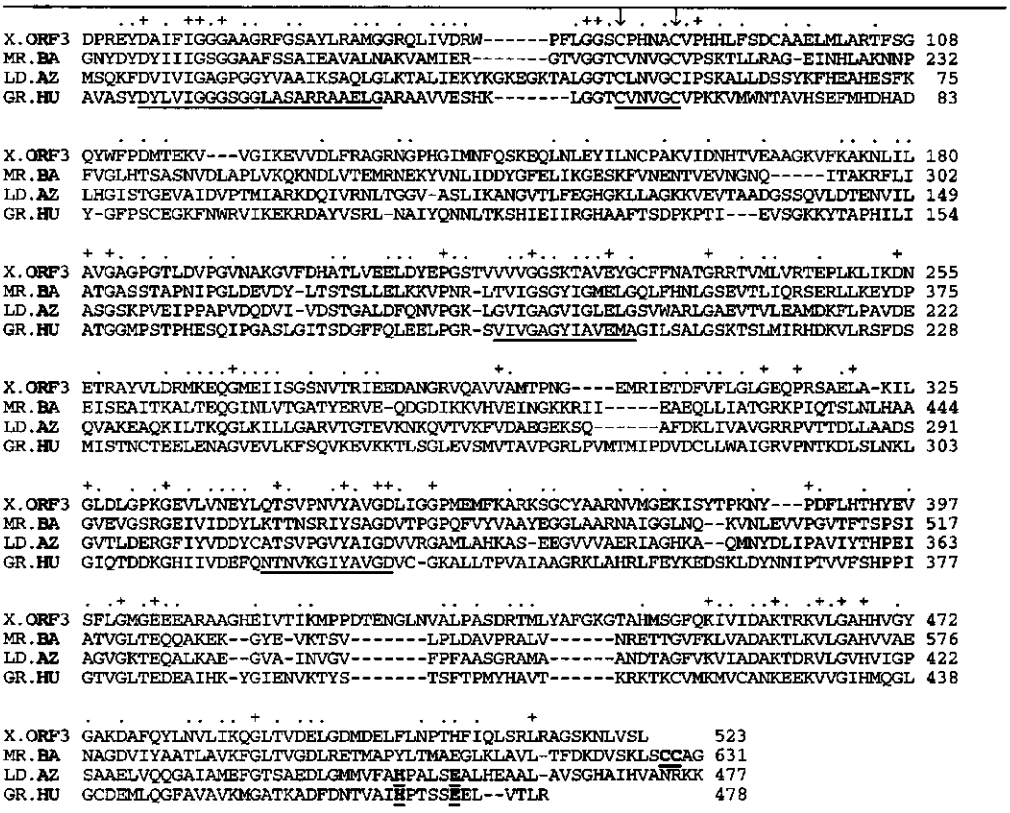

Fig. 1. Alignment of the *Xanthobacter* strain Py2 ORF3 protein amino acid sequence (X. ORF3) to mercuric reductase (MR.BA) of *Bacillus* sp. RC607 (Wang *et al.*, 1989), dihydrolipoamide dehydrogenase (LD.AZ) of *Azotobacter vinelandii* (Westphal & De Kok, 1988), and humane glutathione reductase (GR.HU) (Tutic *et al.*, 1990). For the latter three proteins, the three-dimensional structures are known (Schiering *et al.* 1991, Mattevi *et al.* 1991, Karplus & Schultz, 1989). Site of interest are underlined (see text). Amino acids identical in all the species are indicated with a plus; similar amino acids with a dot; and gaps in the sequences are indicated by dashes. The two cysteines of the redox-active disulfide bridge are indicated with arrows. The N terminal sequence of the ORF3 protein starts at position 39.

AMINO ACID SEQUENCE ANALYSIS OF THE ORF3 PROTEIN

The pyridine nucleotide-disulfide oxidoreductases are a class of enzymes with a consensus primary structure (Williams, 1992) consisting of the nucleotide binding site of flavin adenine dinucleotide (FAD) (Wierenga *et al.*, 1986) at the N terminus followed by the redox-active disulfide bridge (Williams, 1992), the nucleotide binding site of NAD(P) (Wierenga *et al.*, 1986), and the ribityl moiety of the FAD (Eggink *et al.*, 1990) followed by either an active site His-Glu dyad or a cysteine pair (mercuric reductase)

Enzyme	Amino acid sequence	Position
DLDH_ACHLA	VIV**G**G**G**VI**G**VEFATVFNSFGSKVTII**E**MMDGILPTMDDD	215
DLDH_AZOVI	GVI**G**A**G**VI**G**LELGSVWARLGAEVTVL**E**AMDKFLPAVDEQ	223
DLDH_BACSU	VVI**G**G**G**YI**G**TELGTAYANFGTELVIL**E**GGDEILPGFEKQ	218
DLDH_BACST	VVI**G**G**G**YI**G**IELGTAYANFGTKVTIL**E**GAGEILSGFEKQ	218
ORF3	VVV**G**G**S**KT**A**VEYGCFFNATGRRTVMLV**R**TEPL**K**LIKDNE	256
MERA_BACSR	TVI**G**S**G**YI**G**MELGQLFHNLGSEVTLIQ**R**SERLL**K**EYDPE	376
MERA_STAAU	AVI**G**S**G**YI**A**AELGQMFHNLGTEVTLMQ**R**SERLF**K**TYDPE	292
GSHR_PSEAE	LVV**G**G**G**YI**A**VEFASIFNGLGAETTLLY**R**RDLFL**R**GFDRS	208
GSHR_PEA	AIV**G**G**G**YI**A**LEFAGIFNGLK**S**EVHVFI**R**QKKVL**R**GFDEE	288

Fig. 2. Comparison of the binding sites of the ADP parts of the NAD- and NADP-dependent pyridine nucleotide-disulfide oxidoreductases. The dihydrolipoamide dehydrogenases (accession numbers with DLDH) are NAD dependent; the mercuric reductases (MERA) and glutathione reductases (GSHR) are NADP-dependent. For details, see text. E, conserved Glu residue; R, Arg forming a salt bridge with the 2' phosphate moiety. The other residues in bold represent the conserved βαβ-fold of NAD(P)-specific enzymes.

at the C terminus (Williams, 1992). In Fig. 1, the ORF3 protein sequence is aligned with those of a mercuric reductase, a glutathione reductase, and a dihydrolipoamide dehydrogenase with known three-dimensional structures. With the exception of the His-Glu dyad or the C terminal cysteine pair, all above-mentioned features are conserved, indicating that the ORF3 protein belongs to the class of pyridine nucleotide-disulfide oxidoreductases.

The pyridine nucleotide binding motifs for NAD and NADP specific enzymes show some characteristic differences (Fig. 2) next to the conserved βαβ-fold (Wierenga *et al.*, 1986). The motif for NAD (dihydrolipoamide dehydrogenases) has a conserved Glu (Fig. 2) that forms a H bridge with the 2' and 3' OH of ribose. In the NADP-dependent enzymes (glutathione reductase and mercuric reductase) this Glu is no longer conserved and an arginine forms a salt bridge with the 2' phosphate moiety (Karplus & Schultz, 1989). A nearby Arg or Lys is also involved in the interaction. On the basis of these differences, the sequence data strongly suggest that ORF3 encodes an NADP-dependent protein.

THE ORF3 PROTEIN IS INVOLVED IN EPOXIDE DEGRADATION

To establish whether the ORF3 protein is involved in epoxide degradation we decided to purify the protein by using the diaphorase activity assay (Straub, 1939) with NADPH as the electron donor and dichlorophenol-indophenol as the acceptor. The ORF3 protein was purified from wild-

Table 1. The specific 1,2-epoxypropane degrading activities in fractionated extracts of *Xanthobacter* strain Py2[a].

Protein fraction	Specific activity
crude extract	14
second peak from gel-filtration	6
HIC_{yellow} + HIC_{rest}	6
HIC_{yellow}	<1
HIC_{rest}	1
purified ORF3 protein	0
purified ORF3 + HIC_{rest}	10[b]

[a] The specific activities (in nanomols of 1,2-epoxypropane degraded per minute per milligram of protein) were determined by adding 2 mg of protein from each fraction to a 30-ml serum bottle in a total volume of 300µl. 1,2-Epoxypropane (1 mM), NAD^+ (2 mM), and $KHCO_3$ (50 mM) were added and the reaction was started by adding NADPH (2 mM). The assay was performed by measuring the consumption of 1,2-epoxypropane by headspace analysis using gas chromatography as described before (Weijers *et al.*, 1995).

[b] Although equal amounts of purified ORF3 protein and HIC_{yellow} were added, the purified ORF3 contained twice as much FAD-protein as the HIC_{yellow} fraction as judged from the A_{450} of FAD, which might explain the higher specific activity as observed in HIC_{yellow} + HIC_{rest}. Under the assay conditions used here the amount of ORF3 protein seems to be rate limiting for the epoxide degrading activity.

type *Xanthobacter* strain Py2 grown on propene, according to the procedure used for dihydrolipoamide dehydrogenase (Swaving, unpublished data, Westphal & De Kok, 1988). Ion-exchange chromatography on a HiLoad Q-Sepharose column (Pharmacia) was performed as an additional step in the purification. The specific activity with dichlorophenol-indophenol as the acceptor was 2.7 U mg^{-1}. The N-terminal sequence (performed by Eurosequence, Groningen, The Netherlands) of the ORF3 protein, Met-Lys-Val-Trp-Asn-Ala-Arg, matched the DNA-derived amino acid sequence exactly.

Next, an extract without the ORF3 protein was prepared. A crude extract of propene-grown *Xanthobacter* strain Py2 cells was prepared (Weijers *et al.*, 1995) and 5 ml (25 mg of protein per ml) was loaded onto a 75-cm Sephacryl S-300 (Pharmacia) gel filtration column and was eluted with a 20 mM EPPS (N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonic acid]) buffer, pH 8.0 (0.75 ml/min). The chromatogram showed two major peaks, and epoxide-degrading activity was found in the second peak (yellow). To this second fraction $(NH_4)_2SO_4$ was added to a final concentration of 1 M. The second fraction was then loaded onto a phenyl

Superose 5/5HR hydrophobic interaction column (Pharmacia) for fast protein liquid chromatography (FPLC) and the proteins were eluted with a 0.7 to 0 M $(NH_4)_2SO_4$ buffered (20 mM EPPS, pH 8.0) gradient (0.5 ml/min, 15-ml elution volume). One major yellow peak (the only one) was eluted at an early stage. A fraction containing this yellow peak, designated HIC_{yellow} was separated from the rest fractions (HIC_{rest}). Both fractions were concentrated to 1.0 ml with the Amicon Centriprep 3 system and were washed several times with EPPS buffer to remove the $(NH_4)_2SO_4$. Although the combined fractions did restore 1,2-epoxypropane-degrading activity completely, no activity was measured in either of the fractions. The purified ORF3 protein combined with the HIC_{rest} fraction could restore activity (Table 1), clearly demonstrating that the ORF3 protein is essential in the epoxide-degrading system of *Xanthobacter* strain Py2.

THE ROLE OF NADPH IN EPOXIDE DEGRADATION

From both the amino acid sequence analysis and the ORF3 protein purification, it was established that NADP is the cofactor for ORF3. Up to the time of present study, the influence of NADP on epoxide-degrading activity had been tested only by replacing NAD^+ in the reaction (Weijers *et al.*, 1995); little or no effect was found. The effect of NADP and other (co)factors on specific epoxide-degrading activity was therefore investigated in more detail (Table 2). A dialyzed crude extract from propene-grown cells was prepared and the activity assay was performed as described before (Weijers *et al.*, 1995). The addition of 50 mM $KHCO_3$ has a positive effect on epoxide-degrading activity, as was shown before (Allen & Ensign, 1996). NAD, NADPH, or DTT alone did not restore activity. The most interesting result from these experiments is, however, that the artificial cofactor DTT can be replaced by the physiologically relevant cofactor NADPH. These results strongly suggest that NADPH is the true physiological cofactor for epoxide degradation.

Table 2. Effects of NAD^+, NADPH, DTT, and CO_2 on the rate of degradation of 1,2-epoxypropane by dialyzed extract of *Xanthobacter* strain Py2[a].

(co)factors used	relative activity (%)[b]
none	3
NAD^+	5
NADPH	12
DTT	15
$KHCO_3$	6
NAD^+ + DTT	100
NAD^+ + $KHCO_3$ + DTT	180
NAD^+ + NADH	5
NAD^+ + NADPH	55
NAD^+ + $KHCO_3$ + NADPH	95

[a] The concentrations of the compounds used were as follows: NAD^+ and NADPH, 2 mM; DTT, 10 mM; and $KHCO_3$, 50 mM. Rates were determined by adding 1,2-epoxypropane (1 mM) to extracts; the compounds were subsequently added.

[b] The rate of 1,2-epoxypropane degradation after adding the combination of NAD^+ and DTT was taken as 100%.The observation that dithiols can be replaced by NADPH can be explained by the nature of the ORF3 protein. Both NADPH and DTT are able to reduce the redox-active disulfide bridge of the ORF3 protein. NADPH donates electrons via the FAD, whereas DTT directly reduces the active site disulfide bridge.

The ORF3 protein described here is the first protein identified as being involved in the complex degradation of epoxides by *Xanthobacter* strain Py2. At the same time the results show that at least one other protein is required for epoxide conversion. For now, we can only speculate on the true nature of the ORF3 protein. Whether it acts directly on the epoxide or passes on its reducing equivalents to other proteins remains a subject for future research.

Acknowledgment

This work was financially supported by the European Community (BIOT-CT91-0269) and by the Netherlands Organization for Scientific Research (NWO) under the auspices of the Netherlands Foundation for Chemical Research (SON). We are grateful to C.K. Chan Kwo Chion and D. J. Leak for communicating their results prior to publication and for useful discussions.

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5

Submitted for publication

Purification and characterization of a flavoprotein involved in the degradation of epoxyalkanes by *Xanthobacter* Py2

Adrie H. Westphal, Jelto Swaving, Linda Jacobs and Aart de Kok

Spectroscopic and stopped flow experiments involving reduction by NADPH show three phases: rapid reduction of the flavin and formation of the reduced flavin-$NADP^+$ charge-transfer complex, followed by the slow formation of the charge-transfer complex between flavin and disulfide and finally very slow further reduction and redistribution of reducing equivalents. Photoreduction in the presence of deazaflavin also results in rapid reduction of the FAD followed by a slow formation of a stable semiquinone. Full reduction could not be achieved by photoreduction or with NADPH and was incomplete even with dithionite or NADPH in the presence of arsenite. The results indicate component II to shuttle between the oxidized and the two electron reduced state.

Steady state kinetic studies using the artificial substrate 1,3-propanedithiol show component II to have a ternary complex mechanism in the direction of $NADP^+$ reduction.

The active site base, a histidine, which is normally present in lipoamide dehydrogenase and glutathione reductase, is replaced by an asparagine. The effects on catalysis, decreased activation of the dithiol substrate and a greatly diminished rate of electron transfer from FAD to the dithiol, are similar to mutants of lipoamide dehydrogenase in which the histidine is replaced by other residues.

Introduction

The *Xanthobacter* Py2 (Van Ginkel & De Bont, 1986) contains an intriguing enzyme system capable of degrading epoxyalkanes which are metabolites arising from alkenes by the action of alkene monooxygenase (Weijers *et al.*, 1988). In whole cells (Small & Ensign, 1995) and in crude extracts (Allen & Ensign, 1996) it was demonstrated that the epoxyalkanes are carboxylated to β-keto acids. For this bioconversion several (co)factors are necessary. It was first thought the reaction was dependent on both NAD^+ and a low molecular mass fraction, which could be replaced by dithiol compounds like 1,3-propanedithiol and dithiothreitol (Weijers *et al.*, 1995). Later it was shown that the reaction is dependent on both NADPH and NAD^+ (Swaving *et al.*, 1996).

Swaving et al. (1995) reported the cloning of a 4.8 kb DNA fragment required for complementation of mutants of *Xanthobacter* strain Py2 defective in epoxide degradation. This fragment contained four open reading frames. The protein encoded by *orf*1 shows weak similarity with cobalamin independent methionine synthase (EC 2.1.1.14). It contains the motif DCGL, which was implicated in Zn binding (Gonzalez et al, 1997). The protein, named component I, was shown to be involved in the degradation of epoxides (Chan Kwo Chion & Leak, 1996; Swaving *et al.*, 1996, Allen and Ensign, 1997b). *Orf*2 encodes a 7.4 kDa protein that shows similarity with rubredoxin 2 from *Pseudomonas oleovorans*. Interestingly, all 8 cysteines are conserved. This protein has as yet not been identified or implicated in epoxide degradation. The *orf3* encoded protein showed significant similarity with pyridine nucleotide-disulfide oxidoreductases (Swaving *et al.*, 1996). This *orf*3 encoded protein, named component II and shown, as is the case with component I, to be involved in epoxide degradation. Recently two other highly homologous proteins, component III and IV, were described as being required to functionally reconstitute the epoxide degradation system (Allen & Ensign, 1997a,b). These proteins are encoded respectively by *orf*4 on the original 4.8 kb DNA fragment and *orf*5 located on a additional 2.2 kb DNA fragment, also sequenced by Swaving et al (1995). Sofar no evidence has been obtained for a physical association of these enzymes into a multi enzyme complex. According to Allen and Ensign (1997a) the overall pathway might be visualized as indicated in Fig. 1. Component I has been

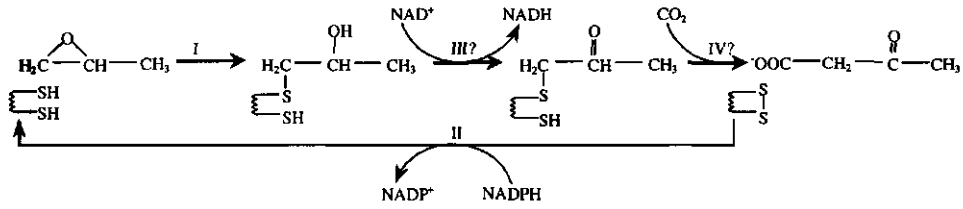

Fig. 1. A hypothetical scheme for the conversion of 1,2 propane epoxide to acetoacetate by *Xanthobacter* Py2, based on Allen and Ensign (1997a), indicating the role that component II might play in the conversion. The origin of the redox active disulfide moiety is unknown.

identified by Allen and Ensign (1997) as the epoxide binding protein by using the inhibitor methylepoxypropane. The identity of the disulfide substrate is at present unknown. It could be the *orf2-encoded* protein, component II or a disulfide located on any of the other enzymes.

Although the sequence identity of component II with known disulfide oxidoreductases is low (19% similarity with the mercuric reductase of *Bacillus*), similarity is found within all four domains. Interesting differences with existing disulfide reductases are found, most striking is the observation that the C-terminal active site His is replaced by Asn (Swaving *et al.*, 1996).

To obtain more insight in the complex degradation of epoxides by the *Xanthobacter* Py2 and to unravel the role of this new type disulfide oxidoreductase, component II was purified and characterized.

Materials and Methods

MATERIALS

NAD(P)H and $NAD(P)^+$ were from Boehringer. Lipoamide, 2,6-dichlorophenolindophenol (Cl_2Ind), 5,5'-dithio*bis*(2-nitrobenzoate) (Nbs_2), 1,3-propanedithiol, reduced glutathione and biological buffers were from Sigma Inc. Oxidized glutathione was from Janssen Chimica. Reduced lipoamide was prepared from lipoamide by reduction with borohydride (Reed *et al*, 1958). Deaza riboflavin was a gift from Dr S.G. Mayhew (Dublin, Ireland). HiLoad Q-Sepharose, Sephacryl S400 and Superdex 200 were from Pharmacia (Uppsala, Sweden). Lipoamide dehydrogenase was isolated from *Azotobacter vinelandii* as described

previously (Westphal and De Kok, 1988) and glutathione reductase from *Escherichia coli* was a gift from mrs. P.A.W van den Berg.

ANALYTICAL METHODS

All activity assays were performed in 50 mM sodium pyrophosphate , pH 8.0 containing 1 mM EDTA (buffer A) at 25 °C, except for the steady-state kinetics which were performed in 50 mM EPPS (N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonicacid]) buffer supplemented with 1 mM EDTA, pH 8.5. The concentration of NAD(P)H and $NAD(P)^+$ was 0.15 mM unless stated otherwise. The reaction with reduced or oxidized lipoamide, reduced or oxidized glutathione as well as the oxidation of aliphatic dithiols, 0.75 mM final concentration (e.g. 1,3-propanedithiol), was assayed spectrophotometrically following the NAD(P)H concentration at 340 nm. The reduction of Nbs_2 (0.75 mM) was followed at 412 nm and the reduction of Cl_2Ind (0.45 mM) at 600 nm. The final volume was 1 ml. The reaction was started by the addition of the enzyme. Each cuvette contained 41 pmol enzyme (monomer). With the physiological substrate, 4.1 pmol was used.

The FAD determination was done according to Wassink & Mayhew (1975). Routinely, the FAD concentration was determined spectrophotometrically by using an extinction coefficient at 465 nm of 11300.

To determine the pH optimum of component II, the Nbs_2 activity was assayed using different buffer systems at different pH's. The conductivity for all buffers used was adjusted to 5 mS with KCl. The molar extinction coefficient of 5-thio 2-nitrobenzoate is dependent on the pH (pH 5.5 - 11800 M^{-1} cm^{-1}; pH 6.0 - 13000 M^{-1} cm^{-1}; pH 6.5 - 13200 M^{-1} cm^{-1}; pH 7.0 to pH 10.5 - 13600 M^{-1} cm^{-1}).

PROTEIN PURIFICATION

Wild type *Xanthobacter* Py2 was cultivated in 6 l mineral medium with propene as sole carbon source to induce the epoxyalkane degrading enzymes as described before (Swaving *et al.*, 1995). Cells were harvested (10 min, 12,000 g, 4°C), washed in 50 mM potassium phosphate pH 7.0 and resuspended in 30 ml of the same buffer, containing 0.1mM phenylmethanesulfonylfluoride (buffer B). Crude extract was obtained

after passage of the cells through a French Press. The extract was clarified by centrifugation (45 min, 27,000 g, 4°C). The pellet was resupended in 20 ml buffer B and again disrupted using the French press and centrifuged at the same speed. Both supernatants were pooled and protamine sulfate was added to a concentration of 6 mg/ml and the solution was clarified by centrifugation at 27,000 g..

Ammonium sulfate was added to the supernatant to 40 % saturation and the solution was clarified by centrifugation at 27,000 g for 30 min. Ammonium sulfate was further added to 60 % saturation and the resulting precipitate was recovered by centrifugation at 27,000 g for 30 min. The pellet was dissolved in 35 ml 25 mM EPPS pH 8.0, 0.5 mM EDTA and 0.1 mM phenylmethanesulfonylfluoride.

The protein solution was applied to a 60 ml HiLoad Q-Sepharose column and eluted using a 0.0 - 0.5 M KCl gradient in the EPPS buffer. Active fractions were pooled, and concentrated to 5.4 ml using an Amicon YM 30 system. Finally the protein solution was applied to a Sephacryl S400 (2.5 x 100 cm) column and eluted using the EPPS-buffer containing 150 mM KCl. Active fractions were analyzed on SDS-PAGE and the purest fractions were pooled and concentrated to 4.5 ml.

ANALYTICAL METHODS

SDS-PAGE was performed as described by Laemmli (1970). Protein concentrations were estimated by the microbiuret method (Goa, 1953). Bovine serum albumin was used as a standard.

The native molecular weight was determined using a Superdex 200 column. The column was equilibrated using 20 mM potassium phosphate pH 7.0, containing 150 mM KCl and 0.5 mM EDTA. Marker proteins were run in parallel (catalase, 240 kDa, dihydrolipoamide dehydrogenase from *Azotobacter vinelandii*, 100 kDa, and ovalbumine, 45 kDa).

Eurosequence (Groningen, The Netherlands) carried out N-terminal sequence determination.

Absorption spectra were recorded with a Perkin Elmer 550S spectrophotometer or with a HP 8453 diode array spectrophotometer. Enzyme solutions were made anaerobic by alternate evacuation and flushing with oxygen free argon. The cuvette was equipped with a side

arm that contained 1.5 μM 5'-deaza riboflavin, 1 mM methylviologen and 15 mM EDTA. The methylviologen was photo-reduced by irradiation with a 150 W Fi-L151 projector (Fiberoptic Heim AG) equipped with a fiberglass cable.

Photo-reduction of component II was carried out by stepwise illumination using a 150 W slide projector. The cuvette contained 50 mM EPPS pH 8.5, 10% glycerol, 15 mM EDTA, 1.5 μM 5-deaza-riboflavin and 40 μM component II.

Photo-reduction of component II was carried out by stepwise illumination using a 150 W slide projector. The cuvette contained 50 mM EPPS pH 8.5, 10% glycerol, 15 mM EDTA, 1.5 μM 5-deaza-riboflavin and 40 μM component II.

Rapid reaction kinetics were carried out at 25° C using a temperature-controlled single wavelength stopped-flow spectrophotometer from High Tech Scientific Inc., type SF-51 with 1.3 ms dead time. The instrument was interfaced to an IBM computer for data acquisition and analysis. Data were analyzed with a program from High Tech Scientific Inc. One syringe contained 20 μM component II in buffer A, the other 40 μM NADPH in H_2O. Solutions were made anaerobic by repeated flushing with oxygen-free argon. In the spectral titrations with NADPH or dithionite, the stoichiometry is expressed as molar equivalents with respect to the flavin content.

Table 1. Purification procedure of component II. Contaminating activities of NAD-dependent enzymes (e.g. lipoamide dehydrogenase) were measured during purification of component II.

Purification step	**Volume**	**Protein**	**Specific activity (U/mg)**				**Total activity**	**Yield**
			NADPH/	**NADH/**	**NADH/**	**NAD/**	**(U) NADPH/**	
	(ml)	**(mg)**	**Nbs_2**	**Nbs_2**	**$LipS_2$**	**$Lip(SH)_2$**	**Nbs_2**	**(%)**
Crude extract	86	830	0.32	0.031	0.91	2.68	265	100
Protamine sulfate	133	321	0.74	0.012	0.46	1.86	238	90
$(NH_4)_2SO_4$	35.4	176	1.06	0.005	0.30	0.92	187	71
HiLoad Sepharose Q	5.4	72	2.04	0	0.02	0.08	146	55
Sephacryl S400	4.5	63	2.21	0	0	0	140	53

Results

ENZYME PURIFICATION

Component II was purified from wild type *Xanthobacter* Py2 grown on propene as sole carbon source to induce the epoxyalkane degrading proteins as described in the Materials and Methods section. The physiological disulfide substrate of the enzyme is not known, therefore, the protein was assayed using a general electron acceptor (Nbs_2) specific for the dithiol group of pyridine nucleotide-disulfide oxidoreductases (Ohnishi *et al*, 1995). In Table 1 the purification scheme is given. The purification factor of 6.9 is comparable to that of Allen and Ensign (1997a), 5.7. The difference may be due to differences in the assay procedure, in the latter case based on reconstitution of epoxide carboxylase activity. Harsh procedures, such as a heat treatment, were omitted in this procedure. The overall yield was 53%, compared to 32% as obtained by Allen and Ensign (1997a).

From Fig. 2 it is clear that component II is the most abundant protein present in crude extracts of propene grown *Xanthobacter* Py2. The other major band of around 41 kDa represents component I, which is proposed to be also involved in epoxyalkane degradation (Chan Kwo Chion and

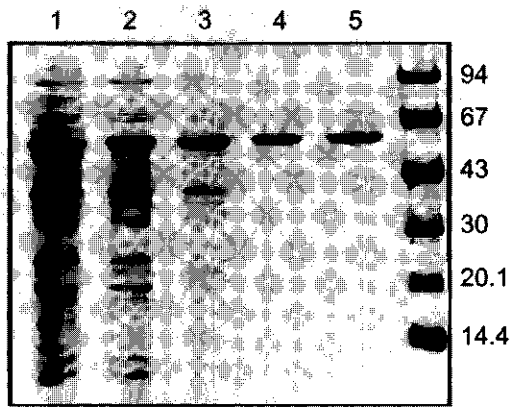

Fig. 2. Purification of component II from *Xanthobacter* Py2 as followed on a 10 % Tricine/SDS-PAGE. Lane 1, crude extract; lane 2, protamine sulfate precipitation pool; lane 3, $(NH_4)_2SO_4$ precipitation pool; lane 4 HiLoad Sepharose Q pool, lane 5 the Sephacryl S400 pool; last lane, marker proteins (kDa).

Leak, 1996, Allen and Ensign, 1997a,b). To ensure that the purified component II protein is not contaminated with dihydrolipoamide dehydrogenase, Table 1 shows the specific activities of dihydrolipoamide dehydrogenase and the NADH-dependent Nbs_2 activity. Table 1 also demonstrates that component II is completely dependent on NADPH.

The identification of the purified enzyme with component II was carried out by the determination of the N-terminal sequence. This sequence MKVWNAR matched the DNA-derived sequence exactly (Swaving *et al.*, 1995).

PROPERTIES OF THE COMPONENT II

The enzyme has a bell-shaped pH optimum at pH 8.5 when using Nbs_2 as the electron acceptor. This value is consistent with the observation of Weijers *et al.* (1995), who found the pH-optimum of epoxyalkane degradation in crude extracts to be around pH 8.2.

Based on the elution pattern of the Superdex 200 column the apparent native molecular mass of the protein is 116 kDa (data not shown). From the sequence it is known that the subunit molecular weight is 57.3 kDa, therefore component II is apparently a homodimeric protein, as are all other members of the family of pyridine nucleotide-disulfide oxidoreductases (Williams, 1992). The enzyme contains 1 non-covalently bound FAD per subunit, as concluded from the molar extinction coefficient and FAD content (Wassink & Mayhew, 1975).

Next to substrates used to measure the diaphorase activity (Cl_2Ind or Nbs_2) and oxidase activity of component II, several mono and dithiol compounds were tested as substrates. Swaving *et al.* (1996) suggested dithiol compounds like dithiothreitol and 1,2-propanedithiol as substrates for component II. In Table 2 the specific activities of component II with several of these compounds are presented and compared with that of lipoamide dehydrogenase and glutathione reductase. No NADPH oxidation activity was observed with 1,2-epoxypropane as substrate. Preincubation with this compound did not inhibit the activity. Therefore a direct interaction with epoxypropane is unlikely. As shown in Table 2 all activities tested are lower compared to lipoamide dehydrogenase and glutathione reductase with the exception of the NADPH/Nbs_2 activity.

Table 2. Substrate specificity of component II, compared to lipoamide dehydrogenase and glutathione reductase. All assays were carried out in buffer A at 25°C as described in Materials and Methods. NADP(H) was used as a substrate for component II and glutathione reductase, while NAD(H) was used as a substrate for lipoamide dehydrogenase.

Substrates	**Specific activity**		
	Component II	**Lipoamide dehydrogenase**	**Glutathione reductase**
	U/mg protein	**U/mg protein**	**U/mg protein**
NAD(P)H/Nbs_2	2.22	0.18	0.24
NAD(P)H/Cl_2Ind	1.04	38.2	1.07
NAD(P)H/O_2	0.16	1.57	0.034
NAD(P)H/$LipS_2$	0	61	0
$NAD(P)^+$/$Lip(SH)_2$	0.12	187	0.61
NAD(P)H/GSSG	0	nd	160.1
$NAD(P)^+$/GSH	0	0	0.48
$NAD(P)^+$/1,3-Propanedithiol	0.23	31.3	0.4
$NAD(P)^+$/Dithiothreitol	0.011	0	0
NAD(P)H/1,2-Epoxypropane	0	0	nd

The reaction with 1,3-propanedithiol was completely inhibited upon incubation of the enzyme for 30 min. with 1 mM N-ethylmaleimide in the presence of 0.1 mM NADPH. The NADPH / Nbs_2 reaction was also completely inhibited, while the NADPH / Cl_2Ind activity decreased 94%. No inhibition was observed in the absence of NADPH. This indicates that the enzyme contains a redox active disulfide group.

SPECTRAL PROPERTIES OF COMPONENT II

The purified enzyme has a characteristic flavin spectrum with optima at 380 and 465 nm and a shoulder at 490 nm.

Anaerobic stopped flow experiments were performed following the change in absorption at 465 nm and 700 nm with 1 molar equivalent NADPH as reductant. The decrease in 465 nm absorption and the increase in absorption at 700 nm were extremely fast and more than 85% completed in the dead time of the instrument (1.3 ms). The rate constant is estimated at >2000 s^{-1}. The observed rate is comparable with the rate of

the initial step, >300 s^{-1}, in the reduction of *A. vinelandii* lipoamide dehydrogenase by NADH (Benen *et al*, 1992) and shows that this step is not rate-limiting. From the spectral changes involved and in analogy with other disulfide reductases (Benen *et al*, 1991, Veeger and Massey, 1963, Lennon and Williams, 1997) it is concluded that this step represents the formation of a charge-transfer complex between the reduced flavin and $NADP^+$. A charge-transfer complex between oxidized flavin and NADPH with similar spectral characteristics (Huber and Brandt, 1980, Lennon and Williams, 1997) seems unlikely in view of the short lifetime of this intermediate and the absence of intermediate phases in the stopped-flow traces. However, we cannot exclude that the 700 nm absorption is due to a mixture of both species, which are in rapid equilibrium. This rapid phase was followed by a much slower phase in which the flavin absorption at 465 nm increased and the 700 nm absorption decreased. Presumably this step represents the transfer of electrons from the flavin to the redox-active disulfide. Similar, but faster changes in the absorption spectra have been observed by Benen et al (1992) with *A. vinelandii* lipoamide dehydrogenase in which the active site His has been replaced by a Ser.

The slower phase of the reaction could be followed more accurately with the diode array spectrophotometer (Fig. 3A). The enzyme was mixed with 4.2 molar equivalents of NADPH and spectra were immediately recorded. After 30 s the initially formed charge-transfer complex between reduced flavin and $NADP^+$ can still be observed absorbing at wavelengths beyond 800 nm, mixed with the charge-transfer complex between the oxidized flavin and a thiolate of the reduced disulfide bridge, as indicated by the blue shifted 450 nm absorption and the 540 nm absorption. The decrease in NADPH absorption (see inset) indicated the transfer of 1.57 electrons per FAD to the enzyme. After 38 min the 700 nm absorption band had almost disappeared and the flavin absorption started to decrease slowly over a period of hours, together with a further decrease in NADPH absorption. After 24 hr a small amount of free semiquinone was present in the mixture, indicated by an increase in absorption at 580 nm. Further reduction is very difficult to achieve. Even with a forty-fold molar excess of NADPH only a marginal further reduction is achieved. Only in the presence of arsenite the flavin is slowly further reduced, but not completely (Westphal *et al*, 1997).

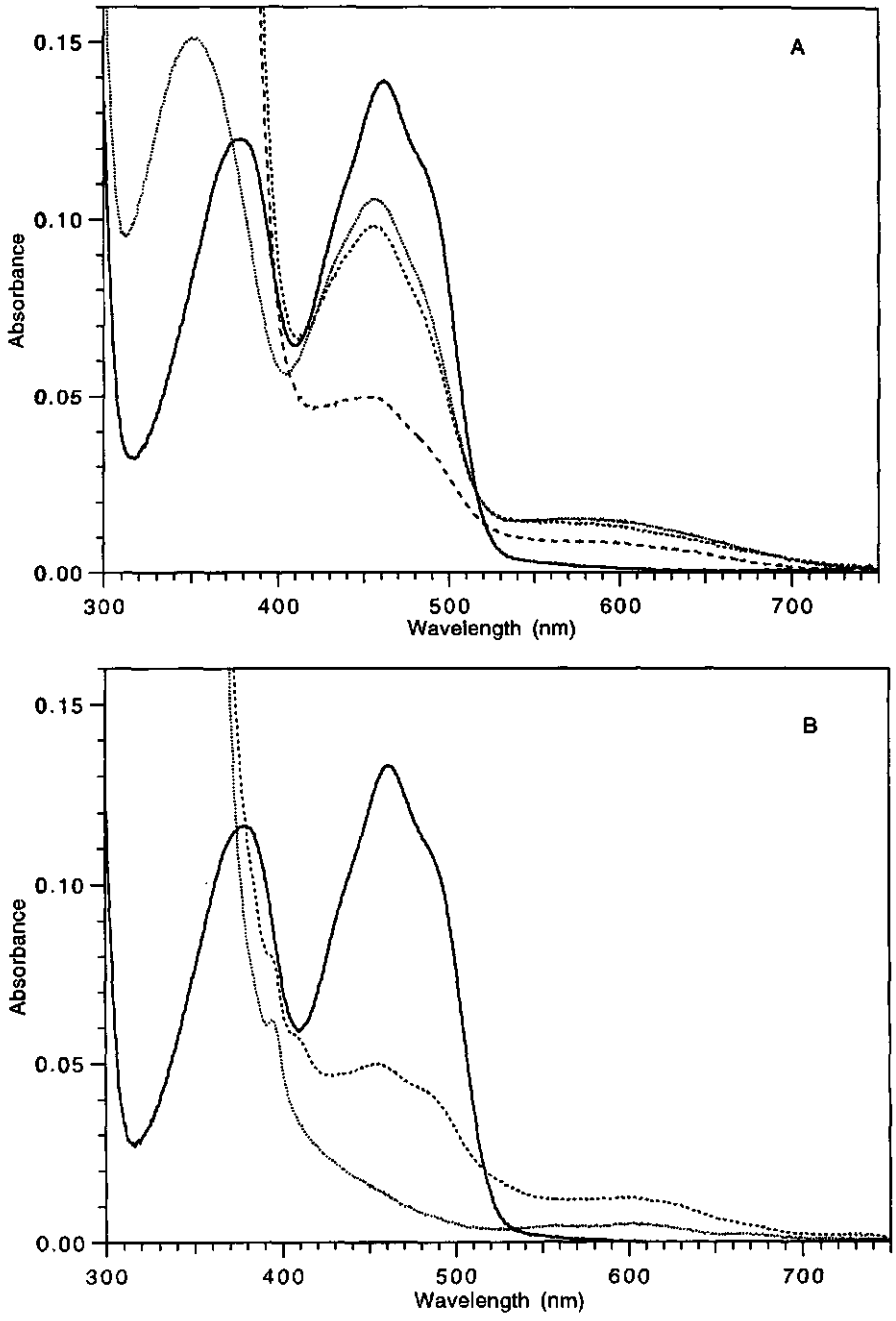

Fig. 3. Anaerobic reduction of component II. A, reduction with NADPH in buffer A. (———) The cuvette contained 19.8 nmoles (FAD) of oxidized component II in 1 ml., (.....) 30 s after addition of 42 nmoles NADPH, (-.-.-.-.-) after 38 min, (------) after 10 h. B, reduction with sodium dithionite. (———). The cuvette contained 12.3 nmoles (FAD) of oxidized component II in 1 ml, (.....) 5 min after addition of 200 nmoles sodium dithionite, (-----) immediately after addition of 7.5 nmoles $NADP^+$.

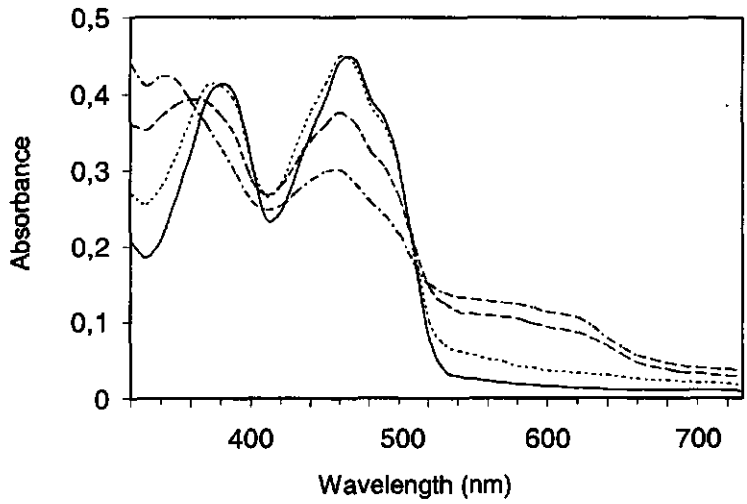

Fig. 4. Spectra of component II upon photoreduction with 5-deaza-riboflavin. The cuvette contained 50 mM EPPS pH 8.5, 10% glycerol, 15 mM EDTA, 1.5 μM 5-deaza-riboflavin and 40 μM component II. The lines represent stable spectra taken after different times of photoreduction. (———) No photoreduction; (- - - -) 20 s of photoreduction; (— — —) 40 s of photoreduction and (— · — ·) 80 s of photoreduction.

Anaerobic reduction by dithionite was also slow and incomplete. Even with a 32-fold molar excess a small amount of semiquinone remained present (Fig. 3B). The subsequent addition of 1.2 equivalent $NADP^+$ yielded a mixture of the oxidized enzyme and semiquinone.

In Fig. 4 photoreduction of the enzyme in the presence of deaza riboflavine and EDTA is shown. Directly after irradiation the flavin is reduced, rapidly forming the flavin semiquinone as indicated by a decrease in absorption at 465 nm and an increase at 570 nm. Directly after reduction the flavin slowly reoxidizes ($k = 0.016\ s^{-1}$) while passing the electrons to the disulfide forming a stable charge-transfer complex, characterized by absorption at 450 nm and 530 nm and a decrease of the 570 nm absorption. Each spectrum was taken after no further changes took place. Different stages of the reduction process of component II can be clearly seen in Fig. 4.

(i). The transfer of electrons from the flavin to the disulfide can be seen in the FAD spectrum after 20 seconds of photoreduction. There is no change in the absorption of the flavin (465 nm) but there is a small shift of the maximum to a lower wavelength, furthermore, in the range of 520-600 nm the absorption increases, indicating the formation of the flavin-thiolate charge-transfer complex (Williams, 1992).

(ii). After a longer period of photoreduction the semiquinone forms, characterized by a broad band centered at 610 nm (Müller *et al.*. 1972).

(iii). No further reduction to the EH_4 species could be achieved, even after prolonged irradiation.

STEADY-STATE KINETIC PROPERTIES OF COMPONENT II

Because the physiological substrate of the component II protein is not known the artificial substrate 1,3-propanedithiol was used to study the steady-state kinetics of the enzyme. Nbs_2 could not be used for this study because at higher concentrations of the compound substrate inhibition was observed (data not shown).

In Fig. 5 Lineweaver-Burk plots showing the enzyme activity with varying 1,3-propanedithiol and fixed $NADP^+$ concentrations (Fig. 5A) and varying $NADP^+$ at fixed 1,3-propanedithiol concentrations (Fig. 5B) respectively are shown. The convergent lines demonstrate that component II of Xanthobacter Py2 at least with the substrate used is not functioning according to a ping-pong mechanism but to a ternary complex mechanism in the direction of $NADP^+$ reduction. From secondary double reciprocal plots (see inset) the V and Km values were determined: V = 0.65 U/mg, kcat = 0.62 s^{-1}, Km 1,3-propanedithiol = 0.63 mM, Km $NADP^+$ = 11.6 μM.

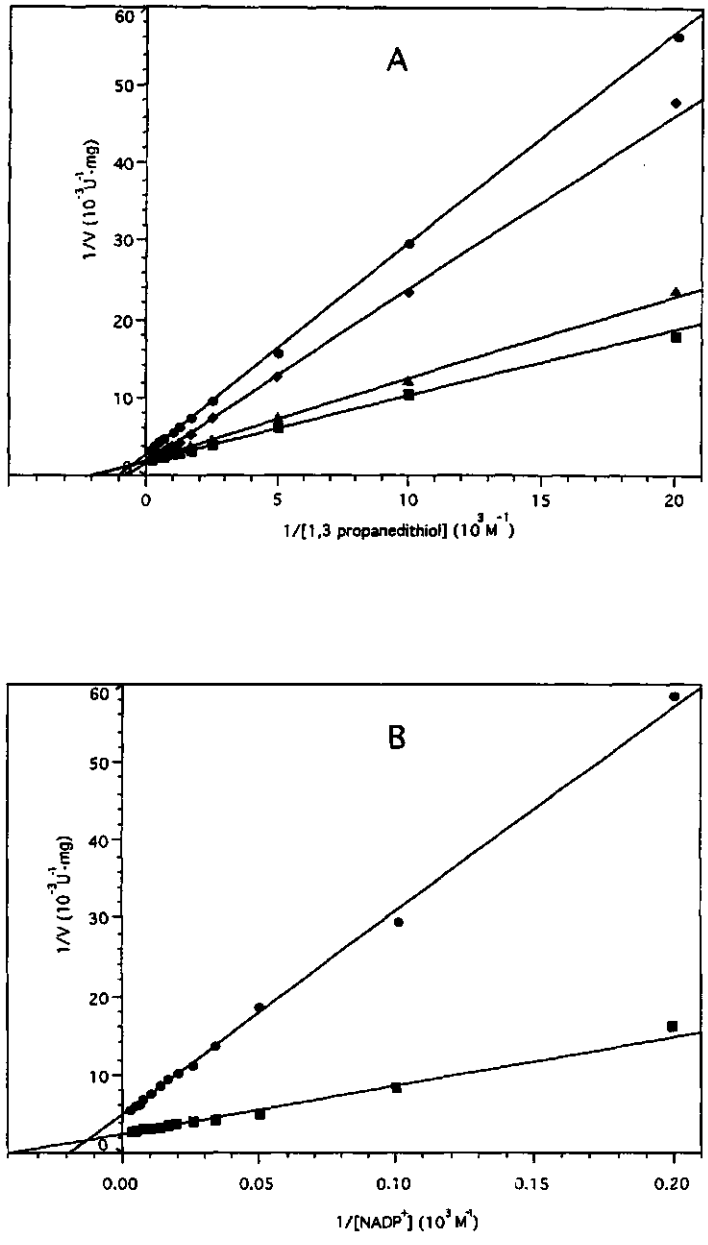

Fig. 5. Steady-state kinetics of the reaction of component II with 1,3-propanedithiol. The activities were measured in EPPS-buffer pH 8.5 (for details see the Materials and Methods section). A. Lineweaver-Burk plot of the reaction with [1,3-propanedithiol] varied and [$NADP^+$] fixed. (●) 30 μM; (◆) 150 μM; (▲) 300 μM and (■) 500 μM $NADP^+$. (B) Lineweaver-Burk plot of the reaction with [$NADP^+$] varied and [1,3-propanedithiol] fixed. (●) 0.4 mM and (■) 4 mM 1,3-propanedithiol.

Discussion

Component II of the epoxide carboxylase pathway has been purified and shown to be a homodimeric protein of about 117 kDa. The protein contains a tightly, but non-covalently, bound FAD and a redox active disulfide bridge and is NADP dependent.

The absorption spectra of the oxidized enzyme and some intermediates are typical for pyridine nucleotide disulfide oxidoreductases. The main difference with other disulfide oxidoreductases is the very slow transfer of electrons from the flavin to the disulfide bridge. This may be caused by the lack of the active site His-Glu diad, as inferred from comparison of the sequence with that of other disulfide reductases (Swaving *et al*, 1996). Mutants of dihydrolipoamide dehydrogenase from *A. vinelandii* (Benen *et al.*, 1991, 1992), *E. coli* (Williams *et al.*, 1989) and human (Kim and Patel, 1992), where the C-terminal active site histidine has been replaced by other residues, have an approximately 200-fold lower activity towards disulfides, comparable to component II in its activity to disulfides. The role of the active site His in lipoamide dehydrogenase is not only restricted to the activation of the dithiol substrate but also results in the stabilization of the nascent thiolates of the disulfide bridge. In the H450S mutant the rate of reduction of the flavin by NADH is unimpaired, but the rate of transfer of electrons from FAD to the disulfide bridge is very slow compared to the wild type enzyme (Benen *et al*, 1992). A similar situation seems to apply to component II. As this rate is independent of the type of electron acceptor used, this observation also shows that the low activity of component II is not caused by the use of artificial substrates such as dithiothreitol and 1,3-propanedithiol, but is an inherent property of this enzyme.

Another exceptional property of the enzyme is that full reduction to the EH_4 state is very difficult to achieve. Obviously the redox potential of the EH_2/EH_4 couple is very low and component II shuttles apparently between the oxidized form (Eox) and the two electron reduced (EH_2) state. Therefore, despite the common lack of the active site His, the enzyme differs in this respect from mercuric reductase (Distefano *et al.*, 1989).

The enzyme operates in the "reverse" reaction, dithiol $\rightarrow$ $NADP^+$, by a ternary complex mechanism. Such a mechanism is also observed in

glutathione reductase in the reverse direction, GSH → $NADP^+$ (Mannervik, 1973, Rakauskiené et al, 1989) or in *the E. coli* mutant Y177S in the physiological direction (Berry *et al*, 1989). Apparently in all these cases, the slow reduction of the flavin by one of the substrates allows the enzyme-substrate binary complex to bind the other substrate. Due to the lack of an oxidized dithiol substrate the reaction could not be studied in the forward reaction.

The study of component II is seriously hindered by the lack of knowledge on the true physiological substrate. At this moment we cannot exclude the possibility that the enzyme is activated by interaction with another protein. Of interest in this respect is the nature of the *orf*2 gene product which contains rubredoxin and thioredoxin like CxxC motifs (Swaving *et al.*, 1995). None of the other *orf*'s contains such motifs. On the other hand the component II activity towards disulfides and the amount of the protein present (at least 15% of the total soluble protein) is probably enough to keep up with the epoxyalkane degradation activity, approximately 0.02 U/mg, found in crude extracts of *Xanthobacter* Py2 (Weijers *et al.*, 1995).

Another possible substrate for component II is the epoxyalkanes. Based on inhibition by sulfhydryl blocking reagents (Weijers *et al*, 1995) it is suggested that epoxyalkanes are reduced by (binding to) a redox active disulfide (Fig. 1). We tested this option with component II, but no activity was found (Table 2). Furthermore, a possible interaction of component II with epoxypropane was studied, looking at changes in the FAD spectrum of component II (both oxidized or reduced) after addition of epoxypropane. No indication of such an interaction was found (data not shown). These data are in agreement with the findings of Allen & Ensign (1997a,b) who propose component I as the epoxide binding enzyme on the basis of inhibition by substrate analogs. Whether the product of the reaction catalyzed by component I is bound to a disulfide, e.g. the *orf*2-*encoded* protein or component II, as proposed by Allen and Ensign (1997a) or to another factor during the reaction cycle, is central to the role of component II in epoxide degradation and requires further investigation.

Acknowledgments

We like to thank Dr. S.G. Mayhew (Dublin, Ireland) for his hospitality and help with the photoreduction experiments. This work was financially supported by the European Community (BIOT-CT91-0269) and by the Dutch Foundation for Chemical research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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6 Speculations on the mechanism of epoxyalkane degradation in *Xanthobacter* Py2

Initially, only little information was available concerning the degradation of epoxyalkanes by the *Xanthobacter* Py2. Ketones were thought to be the degradation product of epoxyalkane conversion and NAD and an unknown low molecular mass fraction were found to be necessary for epoxyalkane conversion in crude extracts of propene grown *Xanthobacter* Py2.

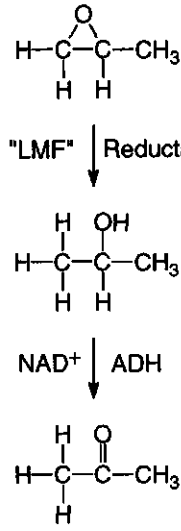

Fig. 1. The pathway as proposed initially for degradation of epoxyalkanes to ketones involving a reductase and a secondary alcohol dehydrogenase (ADH).

Based on these data a degradation pathway for epoxyalkanes was proposed. The epoxyalkane was thought to be reduced first to a secondary alcohol, which in turn was oxidized by an alcohol dehydrogenase to a ketone using NAD^+ as cofactor (Fig. 1). This view on the degradation implied that the key protein in the degradation of epoxyalkanes was the

reductase involved in the conversion of epoxyalkanes to an alcohol. Although later the degradation of epoxyalkanes by *Xanthobacter* Py2 showed to be more complex as described in this thesis, the basic thought of epoxides first being reduced and then oxidized however has not changed.

Weijers *et al.* (1995) proposed a more detailed, mechanism. Based on their finding that DTT could replace the unknown cofactor and the inhibiting effect of NEM (a sulfhydryl-binding compound) a mechanism involving binding of the epoxide substrate to a disulfide bridge (Fig. 2A) was proposed. In the literature several other attempts have been presented to include new findings in the degradation pathway of epoxides. These mechanisms are all based on the mechanism originally proposed by Weijers *et al.* (1995). The common theme in all of these models is that the substrate binds to a thiolate of a redoxactive disulfide bridge (Chan Kwo Chion & Leak, (1996), Fig.2B; and Allen & Ensign, (1997a), Fig. 2C).

Very recently, a paper was published by Allen and Ensign (1997b) concerning the purification of enzymes involved in epoxide degradation. This paper shows that four proteins are involved in the degradation of 1,2-epoxypropane. These proteins are encoded (partially) on the 4.8 kb subclone found to complement mutants defective in epoxide degradation (Chapter 3). The proteins are ORF1 (component I), ORF3 (component II), ORF4 (component III) and ORF5 (component IV). Allen and Ensign showed that for every epoxide carboxylated, one NADPH and one NAD^+ are needed.

In this chapter, these proteins together with the *orf2*-encoded protein and their possible role in the degradation epoxides by the *Xanthobacter* Py2 are discussed.

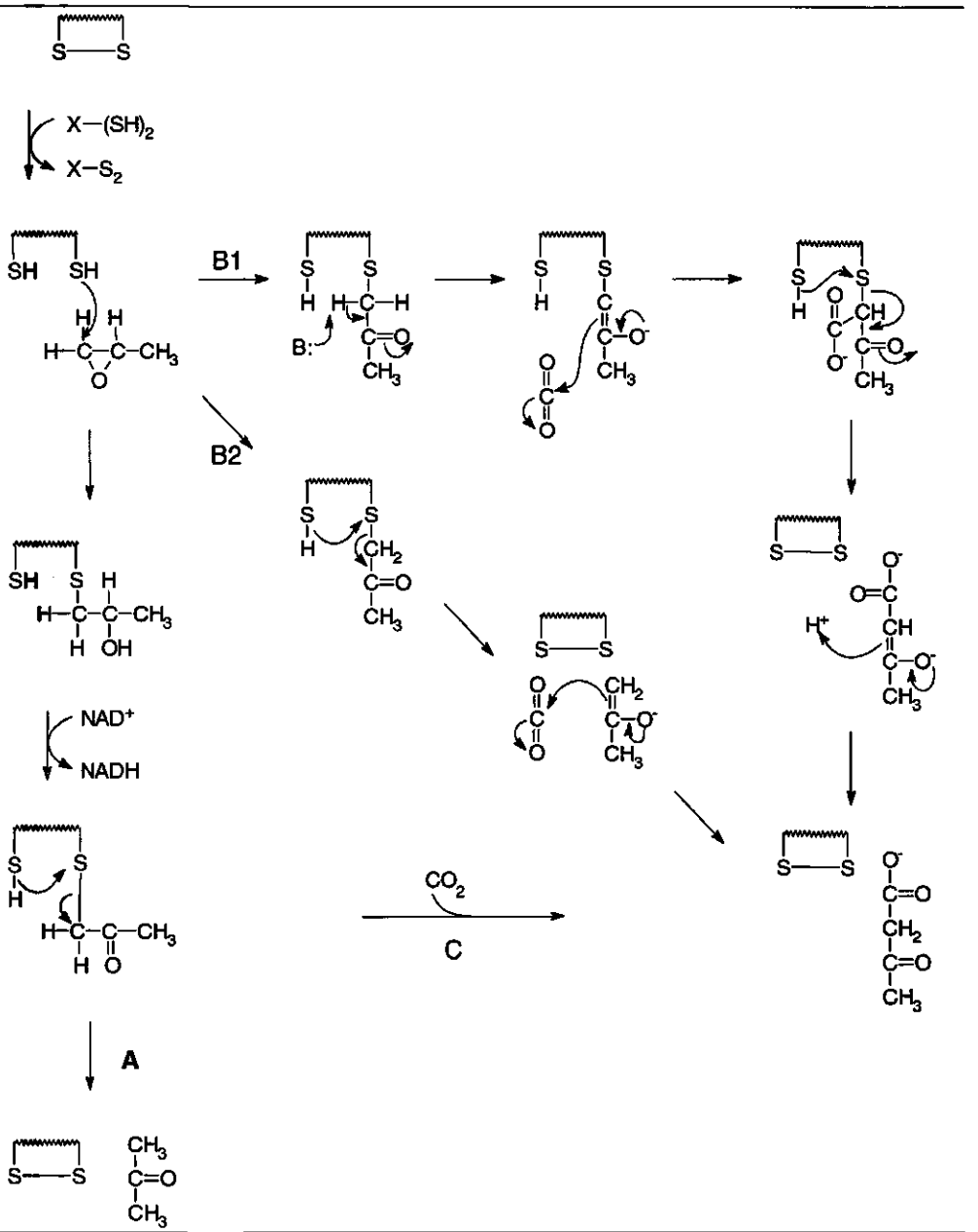

Fig. 2. Mechanisms of the degradation of 1,2-epoxypropane as proposed in literature. The first proposal came from Weijers et al. (1995). They proposed the conversion of 1,2-epoxypropane to acetone, involving opening of the epoxide ring by binding of the epoxide to a disulfide bridge (A). The product is released from the disulfide bridge after oxidation of the hydroxy-group. Allen and Ensign (1997) propose almost the same mechanism, but included a carboxylation step into the release of the product from the disulfide bridge (C). Chan Kwo Chion and Leak (1996) suggested a different approach. After binding of the epoxide to the disulfide bridge, the C1 is activated by either the action of a base B: (B1) of the release of the disulfide bridge (B2) so it can be carboxylated to a ß-keto acid.

THE PROTEINS

Component I is the substrate binding protein

Only little homology is found of the component I protein with other proteins listed in the SwissProt protein database. However, there is a small but significant homology with cobalamine-independent methionine synthase (METE, E.C. 2.1.1.14). This enzyme catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine forming methionine. Only recently it was shown that the methionine synthase is a zinc containing metalloprotein (Gonzalez *et al.*, 1996) Furthermore, it was shown that the conserved C-terminal cysteine is involved in binding the metal (Gonzalez *et al.*, 1996). In component I this cysteine and surrounding amino acids are very well conserved, which might indicate binding of a zinc atom to component I (Table 1). Zinc (a strong Lewis acid) is known to be an excellent cofactor for substrate binding and activation of a Lewis base (e.g. an oxygen group).

Table 1. The C-terminal part of component I is aligned to the C-terminal parts of METE proteins using the ClustalW program. Similar amino acids are shown in bold, identical amino acids are also underlined. The zinc-binding cysteine is double underlined.

Enzyme	Amino acid sequence
Component I	**I**RRLLEIV**P**A**D**RLG**V**T**T**D**C**G**L**ILLQRYI**A**QDK**L**HA**L**V**E**G**T**K**T**VR**R**AELAKAKQAA
METE_YEAST	**I**STILKSY**P**A**E**KFW**V**N**P**D**C**G**L**KTRGWEE**T**RLS**L**TH**M**V**E**AA**A**KYF**R**EQYKN
METE_ECOLI	**L**KKAAKRI**P**A**E**RLW**V**N**P**D**C**G**L**KTRGWPE**T**RAA**L**AN**M**V**Q**AA**Q**NL**R**RG
METE_HAEIN	**L**RKALNVI**P**K**E**RLW**V**N**P**D**C**G**L**KTRGWTE**T**IDQ**L**KV**M**V**D**VT**K**KL**R**AELA

Allen & Ensign (1997a) showed methylepoxypropane to be an inactivator of epoxide degradation, probably acting by binding irreversibly to a substrate-binding protein(s). Using this property they were able to purify component I by complementing methylepoxypropane treated extracts with component I containing fractions (Allen & Ensign, 1997b). Therefore, the protein seems to be the epoxide-binding component. Component I is a hexameric protein and was shown, as discussed before, to contain 0.83 mole of zinc per mole of subunit component I.

THE SUBSTRATE OF COMPONENT II IS UNKNOWN

The component II protein is discussed extensively in the preceding chapters. The homodimeric protein is essential for epoxide degradation. The protein is strictly NADP(H) dependent, however, it is not clear what the component II protein reduces.

In the family of pyridine nucleotide-disulfide oxidoreductases there are some examples of enzymes using a "charge transporter" for the transport of electrons from or to a recipient (e.g. thioredoxin, glutaredoxin, and rubredoxin reductases). In Chapter 5 it was speculated that another protein is the substrate for component II, and the protein encoded by *orf2* was proposed to be a possible candidate. Although, Allen and Ensign (1997b) did not find the *orf2*-encoded protein to be involved in epoxide carboxylation, it is a very interesting protein and will therefore be discussed here.

Orf2-encoded protein maybe a thioredoxin like protein

The *orf2*-encoded protein is a hypothetical protein of only 66 amino acids. Four of the eight cysteines found on the peptide are arranged in such a way that they form two Cys-X-X-Cys disulfide motives which seem to resemble the redox active disulfide found in thioredoxin, or the metal binding disulfide motive found in rubredoxin (Table 2). Both these small peptides are involved in electron transport from the corresponding reductase to the target protein, thioredoxin via the redox active disulfide, and rubredoxin using a Fe-S center. Rubredoxin and thioredoxin reductases are both members of the same family of pyridine nucleotide-disulfide reductases as the component II protein. It is therefore not unlikely if the component II passes on its electrons to this small peptide (Chapter 5), which in turn passes them on to either an epoxide degrading protein or to (a derivative of) the epoxide.

Table 2. Comparison of the two disulfide motives found in the hypothetical *orf2*-encoded protein of *Xanthobacter* Py2 to thioredoxins and rubredoxins.

	1st disulfide motive	**2nd disulfide motive**
ORF2	TIK**C**PD**C**GHV	VWV**C**SQ**C**KSE
RUBR_CLOST	DWV**C**PD**C**GVG	DWV**C**PD**C**GVG
RUB2_PSEOL	KWI**C**IT**C**GHI	KWI**C**IT**C**GHI
THIO_ECOLI		AEW**C**GP**C**KMI
THI1_YEAST		ATW**C**GP**C**KMI

```
Component III   -----MSRVAIVTGASSGNGLAIATRFLARGDRVAALDLSAETLEETARTHWHAYADKVL
Component IV    ---MLDAEVIAITGGAAGIGLAVAHAAIRAGARVALIDRDGACAQRAAAEFGAAAWG---
DHG2_BACME      MYTDLKDKVVVVTGGSKGLGRAMAVRFGQEQSKVVVNYRSNEEEALEVKKEIEEAGGQAI
FABG_BACSU      ---MLNDKTAIVTGASRGIGRSIALALAKSGANVVVNYSGNEAKANEVVDEIKSMGRKAI
                         .** . * * ..*         *

Component III   RVRADVADEGDVNAAIAATMEQFGAIDVLVNNAGITGNSEAGVLHTTPVEQFDKVMAVNV
Component IV    -VGADVTDEAAITAAMAGAQRALGPLTGLVNNAGIAG---FGSVHATEVETWSRIMAVNV
DHG2_BACME      IVRGDVTKEEDVVNLVETAVKEFGSLDVMINNAGVEN---PVPSHELSLENWNQVIDTNL
FABG_BACSU      AVKADVSNPEDVQNMIKETLSVFSTIDILVNNAGITR---DNLIMRMKEDEWDDVININL
                 *  **.    .   .  .      .  ..****.              .    ..  *.

Component III   RGIFLGCRAVLPHMLL-QGAGVIVNIASVASLVAFPGRSAYTTSKGAVLQLTKSVAVDYA
Component IV    TGTFLASKAALFGMLE-RGRGAIVNFGSVAGLVGIPTMAAYCAAKGAVVNLTRQMAADYS
DHG2_BACME      TGAFLGSREAIKYFVENDIKGNVINMSSVHEMIPWPLFVHYAASKGGMKLMTETLALEYA
FABG_BACSU      KGVFNCTKAVTRQMMK-QRSGRIINVSSIVGVSGNPGQANYVAAKAGVIGLTKSSAKELA
                 * *  ..      .     * ..*  *.  .   *    * ..*  .  .*   * . .

Component III   GSGIRCNAVCPGMIETPMTQWRLD----QPELRDQVLARIPQKEIGTAAQVADAVMFLAG
Component IV    GRGIRVNVVCPGTVAGTDMGRQLLGTDCDPELEARRLAKYPMGRFGTPEDIAEAAVFLLS
DHG2_BACME      PKGIRVNNIGPGAIDTPINAEKFA----DPEQRADVESMIPMGYIGKPEEIASVAAFLAS
FABG_BACSU      SRNITVNAIAPGFISTDMTDKLAK------DVQDEMLKQIPLARFGEPSDVSSVVTFLAS
                  .*  * . ** .                .         *    *    ..    **

Component III   EDATYVNGAALVMDGAYTAI--------
Component IV    TKAAFVTGSVLAVDGGMTAI--------
DHG2_BACME      SQASYVTGITLFADGGMTKYPSFQAGRG
FABG_BACSU      EGARYMTGQTLHIDGGMVM---------
                  * ...*  *  **
```

Fig. 3. Alignment of component III, component IV, the glucose 1-dehydrogenase of *Bacillus megaterium* (DHG2_BACME) and *Bacillus subtilis* 3-oxoacyl [acyl-carrier-protein] (FABG_BACSU) reductase, showing the high degree of identity (*) and homology (.).The conserved active site tyrosine (Y) is indicated in bold.

Component III and component IV are similar to short chain alcohol dehydrogenases

Fig. 3 clearly shows that both the components III and IV are member of the short chain alcohol dehydrogenases. The highest similarity is found with each other indicating that they may originate from gene duplication. Short chain alcohol dehydrogenases are non-metal proteins that act through base-acid catalysis.

Allen and Ensign (1997b) showed that both these proteins are involved in the carboxylation of epoxyalkanes. The proteins are dimers, but curiously, the two subunits of component III are different in size. From mass-spectrometry it was concluded the molecular weights of the subunits are 26124 Da and 26025 Da. It is not clear what causes this difference of 99 Da.

Different steps in epoxide carboxylation

For the conversion of epoxyalkanes into ß-keto acids at least three distinct steps can be identified, an oxidation and a reduction step which follows from the involvement of NAD^+ and NADPH, and a carboxylation step. However, there are three different oxidoreductases involved in epoxide carboxylation, component II and the two alcohol dehydrogenases, component III and IV. There are several explanations for this surplus of oxidoreductases: (i) one of the oxidoreductases is not doing a redox reaction, (ii) there is an additional reduction/oxidation step, (iii) one of the oxidoreductases is reducing/oxidizing an other oxidoreductase protein.

The data from Allen and Ensign (1997b) concerning the use of the suicide-substrate methylepoxypropane clearly demonstrates the epoxide ring is opened while it is in contact with component I. Probably, the zinc polarizes the epoxide ring, making the carbon more susceptible for attack.

The component II is strictly NADPH-dependent; therefore this protein is involved in the reduction step. As a consequence one of the alcohol dehydrogenases (component III or component IV) is involved in the oxidation step using NAD^+.

There is no clear understanding on which proteins are responsible for the carboxylation step.

A DEGRADATION MECHANISM OF 1,2-EPOXYPROPANE BY XANTHOBACTER PY2

There are now four different enzymes isolated from which it is known that they are involved in the conversion of epoxides to ß-keto acids. It is, however, not clear if these enzymes form a multi enzyme complex or if they act independently on the substrate. In favor of a multi enzyme complex is the fact that no intermediates of the conversion of epoxides to ß-keto acids have been detected. On the other hand, no multi enzyme complex has been detected sofar.

In the case of a multi enzyme complex the substrate is often bound to a flexible chain or carrier protein, so it is able to traverse to the different proteins to undergo specific reactions. Based on proteins involved in epoxide degradation and the carboxylation reaction there maybe some similarities to three different "enzyme carriers" from known multi enzyme complexes. First a flexible chain containing lipoamide used in the

pyruvate dehydrogenase complex. Second, an ACP (Acyl Carrier Protein) like protein used in the biosynthesis of fatty acids. And third a biotin linker domain; biotin is often found to be a carrier for CO_2 in ATP-dependent carboxylation reactions. However, no similarities to these carriers could be identified on the proteins involved in the epoxide degradation on the primary amino acid sequence level.

Alternatively, a protein that on basis of its amino acid sequence could be a candidate for a substrate carrier, is the *orf2*-encoded protein. Allen and Ensign (1997b) however, did not find this protein in their preparations. This protein however is very small (7.3 kDa) and consequently hard to detect. It can therefore not be excluded that this protein may still play a role in the degradation of epoxides. Especially, because this *orf2* is located in the same operon-like structure as the genes encoding component I-IV.

In Fig. 4 a possible degradation mechanism is presented on basis of the information available. The mechanism involves a combined action of zinc bound to component I, and an unidentified reduced redox active disulfide bridge, which can be the *orf2*-encoded protein. The zinc bound to component I polarizes the epoxide ring, making the carbon more susceptible for attack by one of the –SH groups of the disulfide bridge. From a chemical point of view, a ketone is more easily carboxylated than an alcohol, so the substrate is first oxidized to a ketone by one or both short-chain alcohol dehydrogenase (component III or IV). If no CO_2 is present, ketones are released from the protein upon oxidation of the ß-hydroxy group. If, however, CO_2 is present in the reaction the substrate is carboxylated to a ß-keto acid and is released from the disulfide bridge. Possibly, the other alcohol dehydrogenase (component III or IV) is involved in this carboxylation reaction.

Sofar, only parts of the puzzle have been studied in detail giving new insights on the mechanism and cofactor use of epoxide degradation in *Xanthobacter* Py2. The main problem for a detailed study on the enzymology of the epoxyalkane degradation was, and still is, the low specific activity. Using genetic and biochemical techniques to investigate the involvement of each of the proteins, is probably the most promising way to obtain more information on the mechanism of this new and interesting type of degradation of epoxyalkanes as found in *Xanthobacter* Py2.

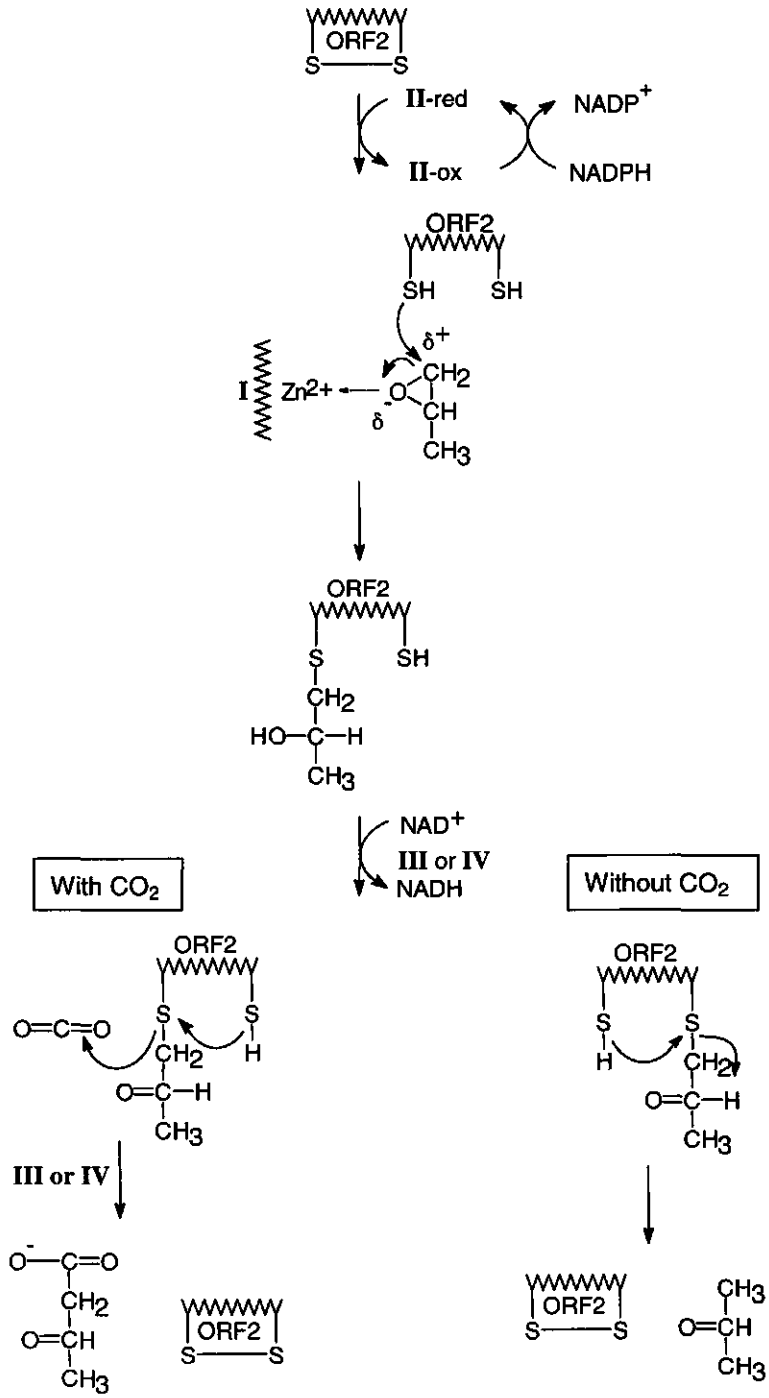

Fig. 4. Proposed degradation mechanism of 1,2-epoxypropane, for details see text.

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Summary

Due to the differences in biological activity of the enantiomers of racemic compounds, the use of enantiomerically pure drugs and agrochemicals is very much encouraged. The availability of optically pure synthons for the production of drugs is, therefor, of the utmost importance for the pharmaceutical industry.

A very versatile intermediate in organic synthesis is the epoxide group. Epoxides very easily undergo stereospecific ring-opening reactions and are, therefor, very useful to function when available in an enantiomerically pure form as synthons in the production of optically pure drugs.

In recent years, a great deal of research has been devoted to the development of a biocatalytic method to produces these optically pure epoxides. A very promising method for this is the enantioselective degradation of racemic epoxides (Chapter 2). Although, such a method has a yield of at most 50% it still can be an interesting option, because, racemic epoxides are relatively cheap. A very nice example of enantioselective degradation is found in the bacterium *Xanthobacter* Py2 which is able to enantioselectively degrade a racemic mixture of 2,3-epoxyalkanes. The 2S-enantiomers are degraded completely, resulting in optically pure 2R-epoxyalkanes.

At the start of the research done on the degradation of epoxyalkanes by *Xanthobacter* Py2 described in this thesis, only little information was available on how degradation proceeds. From experiments performed in crude extracts of propene grown *Xanthobacter* Py2 it was thought that ketones were the product of epoxyalkane degradation (later it was shown epoxyalkanes are carboxylated to ß-keto acids). Furthermore, it was concluded that the degradation was dependent on NAD and an unknown low molecular mass compound, which could be replaced by dithiol compounds such as dithiothreitol (DTT).

To study the degradation of epoxyalkanes by *Xanthobacter* Py2 in more detail it was decided to do complementation studies using *Xanthobacter* Py2 mutants devoid of epoxyalkane-degrading activity (Chapter 3). Several cosmids were found complementing restoring the ability of the

mutants to grow on epoxides. One of these cosmids, pEP9 was conjugated into *Xanthobacter autotrophicus* GJ10. This strain is not able to grow on 1,2-epoxypropane nor able to degrade the compound. In crude extracts of *Xanthobacter autotrophicus* GJ10 complemented with the pEP9, however, epoxyalkane-degrading activity was demonstrated, but only after addition of the LMF or DTT, indicating the right genes were cloned.

Subcloning revealed a 4.8 kb fragment able to complement the mutation. This fragment was sequenced and found to contain four open reading frames which code for proteins of 41690, 7388, 57315 and 26111 Da, respectively. A homology search using the Swiss-Prot protein bank did reveal little or no homology for the first two ORFs. For ORF4, homologies were found with short-chain alcohol dehydrogenases like 3-oxoacyl reductase and glucose 1-dehydrogenase. Interestingly, ORF3 showed significant homology with pyridine nucleotide-disulfide oxidoreductases like mercury (II) reductase, glutathione reductase and with dihydrolipoamide dehydrogenase.

In Chapter 4 and Chapter 5, the protein encoded by this third open reading frame was investigated in more detail. All consensus primary structures of pyridine nucleotide-disulfide oxidoreductase are present on the ORF3 amino acid sequence but the C-terminal active site. Furthermore, from the amino acid sequence it was deduced that the nucleotide binding site of NAD(P) showed more resemblance with NADP-dependent proteins then with NAD-dependent proteins, indicating that the ORF3 is NADP-dependent. Using this information the involvement of NADP was tested in the conversion of epoxyalkane in dialyzed crude extracts of propene grown *Xanthobacter* Py2. It was shown that NADPH and NAD^+ could restore the epoxyalkane degradation, indicating that NADPH is in fact the low molecular mass fraction. The dithiols replacing the low molecular mass fraction are probably able to directly reduce the redox active disulfide bridge on the ORF3 protein. NADPH also reduces this disulfide bridge by passing on the electrons via a FAD, which is bound to the protein.

The ORF3 protein was purified and shown to be involved in epoxyalkane degradation by fractionating crude extracts of propene grown *Xanthobacter* Py2 and complementing fractions without the ORF3 protein with the purified protein, thus restoring the epoxyalkane-degrading activity.

The purified ORF3 protein in this stage called component II, was characterized (Chapter 5). The protein was shown to be a homodimeric protein, each subunit containing a tightly bound FAD. The spectral properties of the FAD were investigated and kinetic studies were performed to characterize the protein.

Characterization on component II showed this protein to have common themes as well as distinct difference with other pyridine nucleotide-disulfide oxidoreductases. This comparison gave no clear understanding on the substrate of the component II and its action in the degradation of epoxides.

Because the component II protein is missing the C-terminal His-Glu dyad active site found in lipoamide dehydrogenases and glutathione reductases, the protein has a very low activity towards dithiols, therefore the substrate of component II may not even be a dithiol compound. 1,2-Epoxypropane is not a substrate for component II, nor is there any interaction with the compound. The involvement of an other (hypothetical) protein, ORF2 ENCODED, is suggested.

Finally, in Chapter 6, a possible mechanism for 1,2-epoxypropane degradation is discussed.

Samenvatting

De biologische activiteit van de verschillende enantiomeren van racemische mengsels van stoffen kan sterk verschillen. Om deze reden is het heel gunstig om van de optisch zuivere vorm van deze stoffen gebruik te maken voor b.v. geneesmiddelen of insecticiden. De beschikbaarheid van optisch zuivere grondstoffen (of intermediairen) is dan ook van het grootste belang voor de industrie.

Een veelzijdige intermediair die veel gebruikt wordt in de organsiche synthese is de epoxide. De functionele groep van epoxide bestaat uit een driering van één zuurstof en twee koolstof atomen en kan vrij gemakkelijk stereospecifiek geopend worden. Deze eigenschap maakt het mogelijk dat de epoxide, als het tenminste verkrijgbaar is in een optisch zuivere vorm, als intermediar wordt gebruikt voor de productie van optisch zuivere geneesmiddelen.

Omdat biokatalyse bij uitstek geschikt is voor de productie van optisch zuivere stoffen, is er de afgelopen jaren dan ook erg veel onderzoek gedaan naar de productie van optisch zuivere epoxiden. Een veel belovende methode hiervoor is de biologische enantiselectieve afbraak van racemische mengsels van epoxiden (hoofdstuk 2). Hoewel de opbrengst met deze methode maximaal 50% is, kan deze methode economisch gezien toch interessant zijn omdat de uitgangsstof, het racemisch mengsel van epoxiden, relatief goedkoop is ten opzichte van het product, de optisch zuivere epoxide.

Een interessant voorbeeld van enantioselectieve afbraak wordt gevonden in de *Xanthobacter* Py2. Deze bacterie is in staat om een racemisch mengsel van 2,3-epoxyalkanen enantioselectief af te breken. Alleen 2S-enantiomeren worden totaal afgebroken waardoor optisch zuivere 2R-epoxyalkanen overblijven.

In dit proefschrift wordt het onderzoek naar de afbraak van epoxyalkanen door de *Xanthobacter* Py2 beschreven. Tijdens de start van het onderzoek was er erg weinig bekend over hoe de *Xanthobacter* Py2 de epoxyalkanen afbreekt. Uit experimenten uitgevoerd met extracten van *Xanthobacter* Py2 werd in eerste instantie aangenomen dat ketonen het product waren van epoxyalkaan afbraak, later bleek echter dat epoxyalkanen

gecarboxyleerd werden tot ß-keto zuren. Verder was het bekend dat naast NAD nog een andere factor nodig was voor activiteit. Deze fractie kon vervangen worden door dithiolen zoals dithiothreitol (DTT).

Om de afbraak van epoxyalkanen door de *Xanthobacter* Py2 beter te kunnen onderzoeken werd besloten om de genen betrokken bij de afbraak op te sporen en te analyseren (hoofdstuk 3). Om dit te bewerkstelligen werden mutanten van de *Xantobacter* Py2 gemaakt die niet meer op 1,2-epoxypropaan konden groeien. Deze mutanten werden vervolgens gecomplementeerd met een genenbank van *Xanthobacter Py2*. Op deze manier werden verschillende plasmiden gevonden die in staat waren deze mutanten weer te laten groeien op 1,2-epoxypropaan. Eén van deze plasmiden, de pEP9, werd naar de *Xanthobacter autotrophicus* GJ10 overgebracht. Deze stam is niet in staat om op 1,2-epoxypropaan te groeien. In een extract van *Xanthobacter autotrophicus* GJ10(pEP9) werd epoxyalkaan afbraak waargenomen.

Van een 4.8 kb groot DNA-fragment waarop alle informatie ligt nodig om de mutanten te complementeren werd de basenvolgorde bepaald. Er bleken vier open reading frames (ORF's) op te liggen die coderen voor eitwitten van respectivelijk 41690, 7388, 57315 en 26111 Da. De eerste twee ORF's bleken geen homologie te hebben met eiwitten in de SwissProt-eiwit-databank. ORF4, daarentegen, vertoonde sterke homologie met 'short-chain'-alcoholdehydrogenases zoals 3-oxoacyl-reductase en glucose-1-dehydrogenase. Opvallend was de homologie van ORF3 (component II) met pryridine-nucleotide-dusulfide oxidoreductases zoals kwik- en glutathion-reductase en dihydrolipoamide dehydrogenase.

In de hoofdstukken 4 en 5 wordt het eiwit dat door het derde ORF (component II) codeerd in detail beschreven. Uit de aminozuurvolgorde blijkt dat alle consensus structuren van de pyridine-nucleotide-disulfide-oxidoreductases, op de C-terminale active-site na, aanwezig zijn. Verder bleek dat de nucleotide-bindingsite van de NAD(P) meer overeenkomsten vertoonde met NADP-afhankelijke-enzymen dan met NAD-afhankelijke-enzymen. Naar aanleiding van dit laatste werd de invloed van NADP op de epoxyalkaan afbraak onderzocht. Het bleek dat zowel NAD^+ als NADPH nodig zijn voor de activiteit; de onbekende fractie, die vervangen kon worden door DTT bleek niet nodig. In hoofdstuk 4 wordt uitgelegd dat dithiolen (DTT) in staat zijn om de disulfidebrug van de component II-

eiwit direct te reduceren, terwijl NADPH dat doet via een FAD groep die gebonden is aan het enzym.

Het component II-eiwit bleek essentieel voor de epoxyalkaan afbraak. Dit werd aangetoond door het eiwit te zuiveren en toe te voegen aan gefractioneerd extracten van *Xanthobacter* Py2. De fractie waar het component II-eiwit uit was verwijderd, was niet actief, maar als hier gezuiverd component II aan werd toegevoegd werd de activiteit hersteld (hoofdstuk 4).

Het component II-eiwit werd gezuiverd en nader onderzocht (hoofdstuk 5). Het enzym is een dimeer, met aan elke groep een sterk gebonden FAD. Hoofdstuk 5 beschrijft de spectrale eigenschappen en de kinetiek van het enzym. Het component II-eiwit bleek zowel overeenkomsten als verschillen te vertonen met de andere leden van de familie van pyridine-nucleotide-disulfide-oxidoreductases, maar het is niet duidelijk aan welke disulfide oxidoreductase het eitwit het meest verwant is. Ook werd het niet duidelijk wat nu precies het substraat van component II is en wat de rol is die het speelt bij de afbraak van epoxiden. Epoxypropaan is geen substraat voor het eiwit, en er is ook geen interactie van het eiwit met deze stof. In hoofdstuk 5 wordt gespeculeerd dat een ander eitwit het substraat van component II is.

In Hoofdstuk 6 tenslotte, wordt een speculatief mechanisme voor de afbraak van epoxiden door de *Xanthobacter* Py2 besproken.

Curriculum vitae

Jelto Swaving werd op 15 maart 1964 geboren te Groningen. Hij groeide op in Onnen (750 inwoners, drie kerken, één café) op ongeveer 10 km afstand van Groningen. In 1985 behaalde hij het Atheneum diploma aan het "Augustinus College" te Groningen. Direct hierna vervulde hij de dienstplicht, om in 1986 te beginnen met de studie Scheikunde aan de Rijksuniversiteit Groningen, afstudeerrichting Biochemie. De auteur rondde in oktober 1990 zijn studie af. In 1991 begon hij als AIO bij de sectie Industriële Microbiologie van de Landbouwuniversiteit Wageningen, waar hij aan de moleculaire biologie van de epoxide afbraak door de bacterie *Xanthobacter* Py2 werkte. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. In 1996 begon hij aan een postdoc aanstelling aan de Rijksuniversiteit Groningen, waar hij onderzoek doet naar eiwit translocatie in *Bacillus subtilis*.

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