will be the key to determine the enantioselectivity of the nitrile hydratase, although the precise mechanism is not clear at present.

**Hydrolysis of Malononitrile**

Is the nitrile hydratase always enantioselective if there are two cyano groups in a molecule? To answer this question, malononitriles were used as the substrate, only one representative example is shown below [5]. Benzylationmalononitrile (8) gave amide carboxylic acid 9, in an entirely different manner compared with the reaction of 6 (Scheme 5). First, both of two cyano groups were hydrolyzed, one to a carboxy and the other to an amide group. Control experiments revealed that the key intermediate was diamide 10, and this was proved to be also obtained starting from racemic cyano amide. Thus, nitrile hydratase showed no enantioselectivity. The chirality of the product was determined by recognition of the prochirality of 10 by the amidase.

In conclusion, while nitrile hydratase exhibits enantioselectivity only to a limited number of substrates, amidase catalyzes highly enantioselective reactions of a wide range of amides.

---

**Enantioselective Oxidation by Non-Heme Iron Monooxygenases from *Pseudomonas***

Marcel G. Wubbolts*, Sven Panke, Jan B. van Beilen, and Bernard Witholt

**Introduction**

*Pseudomonas oleovorans* alkane hydroxylase (EC 1.14.13.5) and xylene oxygenase (EC 1.14.13.-) from *P. putida* mt-2 are industrially relevant monooxygenases used for the production of optically active epoxides and (hetero)aromatic alcohols and acids [1]. Oxidation of aryl allyl ethers to (+)-aryl glycidyl ethers by alkane hydroxylase has thus provided a synthesis route towards the optically active β-blockers (-)-(5)-metoprolol and (-)-(5)-atenolol, developed by Shell and Gist Brodes (Pat. EP 256386, US 4956284, and [2]). Heterocyclic aromatic acids produced by *Lonza* using these monooxygenases and subsequent enzymes (Pat. US 5242816, US 5236832, and [1]) are of use for the synthesis of pharmaceuticals such as the anti-hyperglycemia drug Glipizide.

The industrial application of these monooxygenases from *P. oleovorans* and *P. putida* mt-2 is limited to the use of the wild-type organisms, which fortuitously accumulate the desired products as non-metabolizable intermediates. Synthesis of 'up-stream' intermediates, such as heterocyclic aromatic alcohols, or of metabolizable intermediates (e.g. derivatives of benzyl alcohol or benzoic acid) is not feasible with these strains. Furthermore, the wild-type biocatalysts is grown on alkanes (*P. oleovorans*) or xylenes (*P. putida* mt-2), which are substrates that can compete with the desired starting compounds, thus reducing productivity.

We have constructed, by genetic engineering, biocatalysts that contain alkane hydroxylase or xylene oxygenase, regulated by inducers that do not cause competitive inhibition, that are devoid of undesired 'down-stream' metabolic activities. Furthermore, we have introduced xyl- and alk-based 'biotransformation cassettes' into the chromosome of *E. coli* and *Pseudomonas* strains in order to obtain

---

*Correspondence: Dr. M.G. Wubbolts*  
Institut für Biotechnologie  
ETH-Hönggerberg, HPT  
CH-8093 Zürich

[1] 'Enzyme Catalysis in Organic Synthesis,' Ed. by K. Druzh and H. Waldmann, VCH, Weinheim, 1994, pp. 365-375.  
[2] Y. Asano, T. Yasuda, Y. Tani, H. Yamada, Agric. Biol. Chem. 1982, 46, 1183.  
[3] H. Kakeya, N. Sakai, T. Sugai, H. Ohta, Tetrahedron Lett. 1991, 32, 1343.  
[4] M. Yokoyama, N. Imai, T. Sugai, H. Ohta, J. Mol. Catol., B Enzymatic 1996, 1, 135.  
[5] H. Kakeya, N. Sakai, A. Sano, M. Yokoyama, T. Sugai, H. Ohta, Chem. Lett. 1991, 1823.  
[6] M. Yokoyama, T. Sugai, H. Ohta, Tetrahedron Asymmetry 1993, 4, 1081.
will be the key to determine the enantioselectivity of the nitrile hydratase, although the precise mechanism is not clear at present.

Hydrolysis of Malononitrile

Is the nitrile hydratase always enantioselective if there are two cyano groups in a molecule? To answer this question, malononitriles were used as the substrate, only one representative example is shown below [5]. Benzylmethylmalononitrile (8) gave amide carboxylic acid 9, in an entirely different manner compared with the reaction of 6 (Scheme 5). First, both of two cyano groups were hydrolyzed, one to a carboxyl and the other to an amide group. Control experiments revealed that the key intermediate was diamide 10, and this was proved to be also obtained starting from racemic cyano amide. Thus, nitrile hydratase showed no enantioselectivity. The chirality of the product was determined by recognition of the prochirality of 10 by the amidase.

In conclusion, while nitrile hydratase exhibits enantioselectivity only to a limited number of substrates, amidase catalyzes highly enantioselective reactions of a wide range of amides.

Enantioselective Oxidation by Non-Heme Iron Monoxygenases from Pseudomonas

Marcel G. Wubbolts*, Sven Panke, Jan B. van Beilen, and Bernard Witholt

Introduction

Pseudomonas oleovorans alkane hydroxylase (EC 1.14.13.5) and xylene oxygenase (EC 1.14.13.-) from P. putida mt-2 are industrially relevant monoxygenases used for the production of optically active epoxides and (hetero)aromatic alcohols and acids [1]. Oxidation of aryl allyl ethers to (+)-aryl glycidyl ethers by alkane hydroxylase has thus provided a synthesis route towards the optically active β-blockers (−)-(S)-metoprolol and (−)-(S)-atenolol, developed by Shell and Glaxo Brocades (Pat. EP 256586, US 49562843, and [2]). Heterocyclic aromatic acids produced by Lonza using these monoxygenases and subsequent enzymes (Pat. US 5242816, US 5236832, and [1]) are of use for the synthesis of pharmaceuticals such as the anti-hyperglycemia drug Glipizide.

The industrial application of these monoxygenases from P. oleovorans and P. putida mt-2 is limited to the use of the wild-type organisms, which fortuitously accumulate the desired products as non-metabolizable intermediates. Synthesis of ‘up-stream’ intermediates, such as heterocyclic aromatic alcohols, or of metabolizable intermediates (e.g., derivatives of benzyl alcohol or benzoic acid) is not feasible with these strains. Furthermore, the wild-type biocatalysts is grown on alkanes (P. oleovorans) or xylenes (P. putida mt-2), which are substrates that can compete with the desired starting compounds, thus reducing productivity.

We have constructed, by genetic engineering, biocatalysts that contain alkane hydroxylase or xylene oxygenase, regulated by inducers that do not cause competitive inhibition, that are devoid of undesired ‘down-stream’ metabolic activities. Furthermore, we have introduced xylene and alk-based ‘biotransformation casettes’ into the chromosome of E. coli and Pseudomonas strains in order to obtain

*Correspondence: Dr. M.G. Wubbolts
Institut für Biotechnologie
ETH-Hönggerberg, HPT
CH-8093 Zürich

Figure. Products of alkane-hydroxylase or xylene-oxygenase-mediated oxidation of aliphatic and (heterocyclic) aromatic compounds [1,2,7,9]. Optically active epoxides without an assigned absolute configuration are represented with a dashed line. Substituents (R) that are accepted by the enzymes are indicated below each molecule.

[1] Enzyme Catalysis in Organic Synthesis, Ed. by K. Drauz and H. Waldmann, VCH, Weinheim, 1994, pp. 365-375.
[2] Y. Asano, T. Yasuda, Y. Tani, H. Yamada, Agric. Biol. Chem. 1982, 46, 1183.
[3] H. Kakeya, N. Sakai, T. Sugai, H. Ohta, Tetrahedron Lett. 1991, 32, 1343.
[4] M. Yokoyama, N. Imai, T. Sugai, H. Ohta, J. Mol. Catal., B Enzymatic 1996, 1, 135.
[5] H. Kakeya, N. Sakai, A. Sano, M. Yokoyama, T. Sugai, H. Ohta, Chem. Lett. 1991, 1823.
[6] M. Yokoyama, T. Sugai, H. Ohta, Tetrahedron Asymmetry 1993, 4, 1081.
production organisms that can be stably maintained in the absence of antibiotic selection using a mini-Tn5 system developed by de Lorenzo and coworkers [3].

Construction of Specialized Biocatalysts

The OCT plasmid from *P. oleovorans* contains the genetic information for the degradation of alkanes. The molecular biology of alkane degradation by this organism has been evaluated in numerous studies (reviewed in [4]). *P. putida* mt-2 is capable of growth on xylenes by virtue of the TOL plasmid pWWO. As many as twenty structural and two regulatory genes have been reported to be involved in the metabolism of these aromatic hydrocarbons [5].

As a source of alkane hydroxylase, we made use of plasmid pGE47 that contains all of the *alk* genes [6] and its derivative pGE29, which is devoid of *alkJ* (alkanol dehydrogenase) [7]. Plasmid pBG63 (xyl-MA) was used to express xylene oxygenase in *E. coli* [8]. Using these plasmids, we have constructed *E. coli* and *P. putida* PpS81 recombinants. These strains, the latter by virtue of a chromosomal alcohol dehydrogenase mutation (*alca81*), are not able to consume alkanols or substituted benzyl alcohols, which makes them suitable hosts to harbour the genes for alkane hydroxylase and xylene oxygenase.

Substrate Range of Alkane Hydroxylase and Xylene Oxygenase

In the absence of product consumption, we have been able to assess the substrate range of both alkane hydroxylase and xylene oxygenase using the above-mentioned recombinant strains [9]. Both enzymes catalyze the oxidation of terminal Me groups to the corresponding alcohols and also epoxidize terminal double bonds (see Fig.). Alkane hydroxylase has a broad substrate spectrum and substrates ranging from n-alkanes, branched alkanes, cyclic alkanes, and alkyl-substituted aromatic hydrocarbons can be oxidized stereospecifically. The substrate range of xylene oxygenase is limited to aromatic hydrocarbons with a Me side group, with indole (oxidized to indoxyl) and styrenes (oxidized to styrene oxides) as exceptions. The enzyme is very tolerant towards additional *meta*- and *para*-substituents at the aromatic ring of tolune derivatives, but does not allow any substituents in *ortho*-position relative to the Me group. Both alkane hydroxylase and xylene monooxygenase have been reported to oxidize heterocyclic aromatic hydrocarbons carrying Et or Me substituents, respectively, to heterocyclic alcohols, which are further metabolized to the corresponding acids [1].

The oxidation of vinylic end groups by both alkane hydroxylase and xylene oxygenase produces optically active epoxides, which are useful synths in enantioselective chemical synthesis. Alkane hydroxylase has the wider substrate range. Alkenes and alkadienes, but also allylbenzenes and aryl phenyl ethers can be oxidized to the corresponding optically active epoxides. Xylene monooxygenase does not oxidize alkenes or allylbenzene, but its activity is restricted to *meta*- and *para*-substituted styrenes, which are oxidized to the corresponding epoxides [8].

Application of Engineered Biocatalysts in Two-liquid-Phase Media

The substrates of alkane hydroxylase and xylene monooxygenase are typically water-insoluble and many of the substrates such as the lower alkanes, toluene, and styrene, but also products such as most of the epoxides, are toxic to the microorganisms.

We have used whole-cell biocatalysts of *Pseudomonas* and recombinant *E. coli* based on alkane hydroxylase as well as xylene monooxygenase in two-liquid-phase media with *n*-octane and higher *n*-alkanes as a semi-immertioned liquid phase in addition to the aqueous phase. In such systems, product and substrate toxicity are diminished and product recovery is facilitated. The lab-scale production of epoxyl-alkanes, styrene oxides, octan-1-ol, and 1-octanoic acid has been realized in such two-liquid-phase systems in batch and continuous mode using specialized microorganisms [10].

Structure of Alkane Hydroxylase and Xylene Monooxygenases

The alkane hydroxylase system consists of the membrane monooxygenase AlkB, rubredoxin (AlkG), and rubredoxin reductase (AlkT), which channel electrons from NADH to the membrane monooxygenase. Xylene monooxygenase shows a similar architecture, although electron transfer takes place through only one protein (XyIA). Genetic analyses demonstrated that AlkB and XyIM are transmembrane polypeptides which span the membrane six times [11].

Both membrane monooxygenases require iron for activity and probably contain a heme-like Fe*4* center or a di-iron-oxo center at the catalytic site [12]. We have obtained evidence by site-directed mutagenesis that the iron may well be bound to several histidine clusters (typically HxxH) at the cytoplasmic membrane inner surface that occur at almost identical positions in both monooxygenases. Studying these enzymes in molecular detail may enable us to understand the mechanism of oxidation of non-heme iron monooxygenases and could provide and answer to the differences in substrate range of the enzymes.

[1] A. Kiener, *Chem. Tech.* 1995, Sept., 31; M.G. Wubbolts, R. Noordman, J.B. van Beilen, B. Witholt, *Recl. Trav. Chim. Pays-Bas* 1995, 114, 139.
[2] S.L. Johnstone, G.T. Phillips, B.W. Robertson, P.D. Watts, M.A. Bertola, H.S. Koger, A.F. Marx, in *Biocatalysis in Organic Media*, Ed. C. Laane, J. Tramper, and M.D. Lilly, Elsevier, Amsterdam, 1987, p. 387.
[3] V. de Lorenzo, K.N. Timmis, in *Methods in Enzymology*, Ed. V.L. Clark and P.M. Bavoil, Academic Press Inc., San Diego, 1994, Vol. 235, p. 386.
[4] J.B. van Beilen, M.G. Wubbolts, B. Witholt, *Biodegradation* 1994, 5, 161.
[5] S. Harayama, unpublished; S. Harayama, M. Rekik, *Mol. Gen. Genet.* 1993, 239, 81.
[6] G. Eggink, R.G. Lageveen, B. Altenburg, B. Witholt, *J. Biol. Chem.* 1987, 262, 17712.
[7] A. Bosetti, J.B. van Beilen, H. Preusting, R.G. Lageveen, B. Witholt, *Enzyme Microb. Technol.* 1992, 14, 702.
[8] M.G. Wubbolts, J. Heyens, B. Melgert, B. Witholt, *Enzyme Microb. Technol.* 1994, 16, 887.
[9] M.G. Wubbolts, P. Reuvekamp, B. Witholt, *Enzyme Microb. Technol.* 1994, 16, 608; J.B. van Beilen, J. Kingma, B. Witholt, ibid. 1994, 16, 904.
[10] M.J. de Smet, B. Witholt, H. Wijnberg, *J. Org. Chem.* 1981, 46, 3128; O. Favre Bulle, T. Schouen, J. Kingma, B. Witholt, *Bio/ Technology* 1991, 9, 367; O. Favre-Bulle, B. Witholt, *Enzyme Microb. Technol.* 1992, 14, 931; O. Favre Bulle, E. Weenink, T. Vos, H. Preusting, B. Witholt, *Biotecnol. Bioeng.* 1993, 41, 263; B. Witholt, M.J. de Smet, J. Kingma, J.B. van Beilen, M. Kok, R.G. Lageveen, G. Eggink, *Trends Bio/ technol.* 1990, 8, 46.
[11] J.B. van Beilen, D. Penninga, B. Witholt, *J. Biol. Chem. Vol.* 1992, 267, 9194; M.G. Wubbolts, manuscript in preparation.
[12] S. Harayama, M. Kok, E.L. Neidle, in *Annual Review of Microbiology*, Ed. L.N. Ornston, 1993, Vol. 46, p. 565; J. Shanklin, E. Whittle, B.G. Fox, *Biochemistry* 1994, 33, 12787.