Asymmetric Synthesis

Coupling of Biocatalytic Asymmetric Epoxidation with NADH Regeneration in Organic–Aqueous Emulsions**

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The pivotal role of chiral oxyfunctionalized hydrocarbons as building blocks for pharmaceuticals or agrochemicals has driven the research for effective methods to obtain them in enantiopure form. Over the past few decades, numerous chemical routes have been developed for catalytic asymmetric epoxidations.[1,2] Amongst them, chiral salen–Mn^{III} complexes and metalloporphyrins have emerged as efficient catalysts for syntheses of optically active epoxides. Despite their applicability in synthetically useful organic oxidations and related reactions, these catalysts are often limited by their low stability against autoxidation, resulting in low turnover numbers.[3]

Alternatively, enzymes can be applied, as they are promising catalysts owing to their regio- and enantiodiscrimination, high turnover numbers ($k_{cat} = 1–20\ s^{-1}$), broad substrate spectra,[4] and environmentally friendly reaction conditions.[5] Chiral epoxides can be obtained through various biocatalytic methods.[6] The most elegant approach is the asymmetric synthesis from the corresponding olefins by using oxidoreductases.[7,8] Oxygenases are particularly interesting biocatalysts because of their ability to activate molecular oxygen in situ. Reactive oxygen donors such as peracids, ozone, and iodosylbenzene are thus becoming obsolete.

The specific technical approach to a particular biocatalytic process must take into account the performance of the relevant enzyme under process conditions. For oxygenases, the best catalysts developed thus far are whole cell systems, which stabilize the often complex heteromultimeric enzyme (up to four individual enzyme subunits) and enable coenzyme regeneration based on cell metabolism. At the same time, the use of whole cells introduces further complexity in a biocatalytic process owing to the frequently observed overoxidation and the functional necessity of cellular and metabolic integrity of the biocatalyst. Products have to be separated from complex mixtures that contain not only substrate, products, and a single catalyst, but also whole cells and cell debris. Thus, as biocatalytic processes develop further, isolated oxygenases coupled with NAD(P)H regeneration may well become a viable alternative to whole-cell applications. Several challenges are identified and become an issue in cell-free oxygenations.[9] The supply of reducing equivalents can, in principle, be solved as several regeneration systems become available.[10] In contrast, oxygenase availability and stability under process conditions represent major obstacles towards preparative cell-free biocatalytic oxygenations and so far remain unsolved.

Herein we report the first example of the preparation and synthetic application of a bacterial monooxygenase as a reagent for asymmetric cell-free epoxidation. As catalyst, we chose the soluble flavin- and NADH-dependent styrene monooxygenase (StyAB) from *Pseudomonas* sp. VLB120. StyAB catalyzes the enantiospecific epoxidation of a broad range of styrene-type substrates, such as 1,2-dihydronaphthalene and indene.[11] This two-component enzyme consists of the actual oxygenase subunit (StyA) and a reductase (StyB).[12]

StyA was obtained in multigram amounts from a 30-L-scale fermentation of recombinant *Escherichia coli* JM101 (pSPZ10), yielding 1.2 kg of wet cells.[13] Overall, approximately 15 g of technical-grade StyA was obtained in one purification step from 280 g of wet cells (Table 1). This corresponds to more than 22000 U. Thus a total of 60 g of catalyst (almost 100000 U) can be obtained from a single 30-L fermentation within one week. Lyophilization was used to attain a stable biocatalyst preparation, and full specific enzyme activity was retained for at least three months.[14] This biocatalyst powder was used for the production of selected aromatic epoxides. Previous results (unpublished data) indicated that StyA is deactivated in the presence of high substrate and product concentrations, possibly caused by the covalent attachment of the epoxides to nucleophilic amino acid residues of proteins. To circumvent StyA deactivation, a biphasic reaction setup was applied in which the organic phase serves as substrate reservoir and product sink (Figure 1).

Dodecane was used as the organic phase so that the reactant concentrations in the aqueous, enzyme-containing phase could be maintained at values that did not significantly

Table 1: Parameters for StyA enrichment during expanded bed chromatography.

Sample	Protein [g]	StyA [g][a]	Specific activity [U mg^{-1}][b]	Total activity [kU]	Yield [%]
Crude cell extract	59.7	16.1	0.57	24.3	100
Fractions containing StyA[c]	25.3	15.2	0.89[d]	22.5	93

[a] Calculated from purities in the enzyme samples. [b] 1 U (international unit) corresponds to 1 μmol product formed per minute. [c] Upon stepwise elution with KCl two StyA-containing fractions were obtained (45% and 70% purity). [d] Average activity of the two different pools obtained after expanded bed chromatography.

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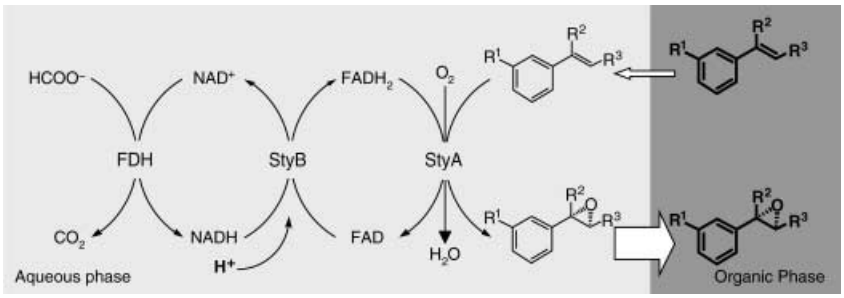


Figure 1. Reaction pathway during biocatalytic epoxidation in a biphasic system. The organic phase serves as a substrate reservoir and product sink. In the aqueous phase, FDH and formate were used for the regeneration of NADH. StyB transfers the reducing equivalents from NADH to FAD. $FADH_2$ and oxygen are cosubstrates for olefin epoxidation by StyA. $R^1 = H, Cl$; $R^2 = R^3 = H, CH_3$.

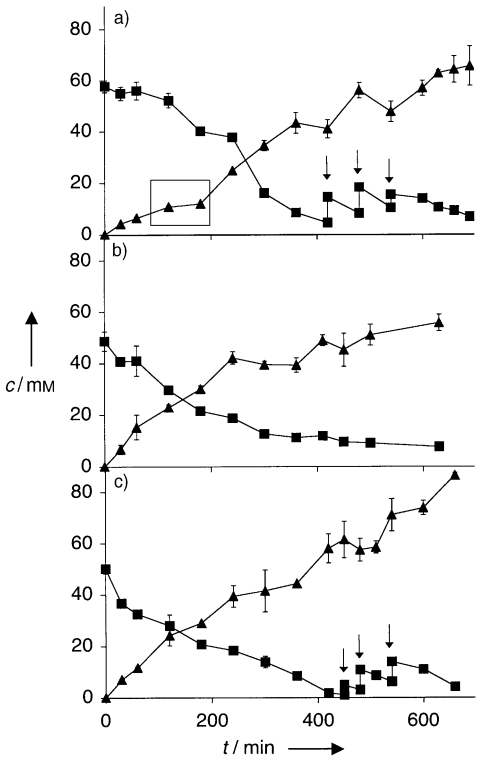
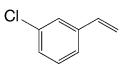
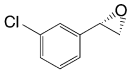
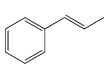
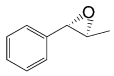


Figure 2. Biocatalytic epoxidation of different styrene derivatives by StyAB in a biphasic system. The time courses of substrate depletion (■) and product formation (▲) are shown for the epoxidation of 3-chlorostyrene (a), α-methylstyrene (b), and *trans*-β-methylstyrene (b). The substrate was pulsed at the time points indicated by arrows in reactions a (a total of 1.9 mmol) and c (2.5 mmol). In the beginning of reaction a (enclosed in box), oxygen was supplied at a low rate. Immediately after increasing aeration (180 min), the epoxide formation rate also increased.

affect StyA activity.[15] An emulsion was formed to avoid mass-transfer limitations across the phase boundary. Enzyme denaturation at the liquid–liquid interface was apparent from the formation of white protein aggregates, but could be almost completely avoided by the addition of bovine serum albumin (BSA). This prolongs StyA epoxidation activity from several minutes to hours (data not shown). StyA was supplied with the reducing equivalents needed for the activation of molecular oxygen by its native NADH:flavin reductase StyB.[16] Concomitant NADH regeneration was achieved by the formate/formate dehydrogenase (FDH) system of *Pseudomonas* sp. 101, which has a higher solvent tolerance and high substrate affinity than other FDHs.[17] Maximal StyA activity relative to epoxidation activity was achieved by adding a four–fivefold excess of FDH.[18] Figure 2 shows the time course of three preparative epoxidation reactions with 3-chlorostyrene (**1**), α-methylstyrene (**2**), and *trans*-β-methylstyrene (**3**) as substrates. Reaction parameters are given in Table 2.

Aeration was detrimental to enzyme activity, as protein denaturation was observed at the gas–liquid interface at high aeration rates. At the same time, oxygen limitation should be avoided, as O_2 is a cosubstrate in the epoxidation reaction. The influence of aeration on StyA epoxidation activity is shown in Figure 2a (box). A diminished activity was observed

Table 2: Data for the conversion of different vinyl aromatics into their enantiopure epoxides in a cell-free biphasic system with isolated styrene monooxygenase.

Substrate	Product	Substrate [mM]	Conversion [%]	Initial product-formation rate [U g^{-1}][a]	Average product-formation rate [U g^{-1}][b]	TN_{StyA}[c]	TN_{NAD}[d]	*ee* [%]	Yield [%][e]
 **1**	 **1a**	50 (+2.5)[f]	90.5	108	48	2171	66	> 99.9	73
 **2**	 **2a**	50	87.9	112	44	1844	56	98.1	75
 **3**	 **3a**	50 (+1.9)[f]	95.3	118	66	2867	87	99.7	87

[a] Activity measured for the first 30 min. [b] Average activity measured over the entire reaction period. [c] TN_{StyA}: Total turnover number for StyA = (moles of product formed)/(moles of StyA). The amount of StyA was calculated based on the purity (70%) of the lyophilized sample. [d] TN_{NAD}: Total turnover number for NAD^+ = (moles of product formed)/(moles of NAD^+ in the reaction). [e] Yields of isolated product after purification. [f] In parenthesis, the amount of substrate (in mmol) pulsed during the reaction is given.

at a low aeration rate at the beginning of the reaction. StyA activity could be recovered by increasing the air supply. A compromise with respect to the aeration rate was found by regulating the dissolved oxygen in the reaction mixture at 10% of oxygen saturation. Under these conditions, gram-scale production of each epoxide with *ee* values higher than 98% and yields up to 87% were achieved; no by-products were detected besides trace amounts of diols (from spontaneous, non-enzymatic hydrolysis),[19] phenethyl alcohols, and phenylacetaldehydes.

We showed the simple and fast preparation of an easily applicable oxygenase in multigram amounts. Despite the early development stage, we were able to use this enzyme to produce enantiopure epoxides in a simple and scalable reaction setup. Volumetric product formation rates ($\approx 1\ g L^{-1} h^{-1}$) are already in the range of those reported for whole-cell oxygenations.[11,20,21] For the first time, nearly complete conversion was achieved with high reactant concentrations, which was not possible so far in other applications with isolated oxygenases in a biphasic system.[22] For StyA, total catalyst turnover numbers and average reaction rates of 1800–2800 and 3–4.3 min^{-1}, respectively, were achieved under process conditions. These values are already promising and compare well with chemical catalysts for which total turnovers below 1000 and frequencies mostly below 1 min^{-1} have been reported.[23] Furthermore, the purification of the products from the reaction mixture is simple. Overall, StyA was shown to epoxidize sterically hindered C-C double bonds, especially *trans* olefins, complementing the product spectrum of commonly applied chemical catalysts.[1,24] We observed a significant decrease of StyA activity (eightfold lower than in biphasic short-term assays). Further investigations will therefore focus on increasing the turnover number of StyA.

Overall, the modular character of the presented biocatalytic reaction allows the substitution of single components to achieve not only epoxidations but also specific hydroxylations, as well as Baeyer–Villiger or heteroatom oxidations. This study has shown that cell-free oxygenase catalysis can be a versatile tool for the asymmetric synthesis of enantiopure oxyfunctionalized hydrocarbons.

Experimental Section

Chemicals: All substrates were obtained from Sigma-Aldrich Chemie GmbH, Germany. Epoxide standards were prepared and characterized as described in the literature.[11] Flavin adenine dinucleotide (FAD), sodium formate, β-nicotinamide adenine dinucleotide (NAD^+), buffer components, and solvents were obtained from Fluka AG, Switzerland.

Cell Cultures: *E. coli* JM101 containing the plasmid pSPZ10[8] (carrying the styrene monooxygenase genes *styAB*) were grown as described in the literature,[21] but in a single-phase rather than a biphasic medium. The expression of *styAB* was induced with dicyclopropylketone (0.05% v/v; Fluka AG, Switzerland).

Large-scale enrichment of StyA: Wet cells (280 g) were disrupted in a bead mill and the resulting crude extract was loaded onto 500 mL of a Streamline DEAE matrix in the expanded-bed mode through a Streamline^{50} column. After the flowthrough had become clear, the packed-bed mode was applied. A stepwise salt gradient at 0, 0.16, and 0.24 M KCl was used for elution. During elution 100-mL fractions were collected. The amount of StyA in every fraction was determined by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), and StyA activity was measured.[25] StyA-containing samples were supplemented with 1% (v/v) sucrose and 2% (v/v) mannitol and lyophilized in a Lyovac GT 3 Freeze-Drying Plant (Leybold-Heraeus GmbH, Germany).

Biphasic reaction: The preparative biphasic conversion of the different styrene derivatives was performed in 350-mL Sixfors reactors (Infors HT, Switzerland) with pH and temperature control (pH 7.5, 30 °C) at a stirrer speed of 400 rpm. The aqueous phase contained StyA ($2\ g L^{-1}$), StyB ($0.03\ g L^{-1}$), FAD (0.1 mM), sodium formate (50 mM), FDH ($8\ U mL^{-1}$; Jülich Fine Chemicals, Germany), Catalase ($250\ U mL^{-1}$; Fluka AG, Switzerland), BSA ($2\ g L^{-1}$; Sigma-Aldrich Chemie GmbH, Germany), and NAD^+ (1 mM) in a total volume of 100 mL in Tris buffer (50 mM; pH 7.6). The organic phase was composed of dodecane (100 mL) that contained the substrate (50 mM). During the reaction, formate (1 mmol) was added every 1 h. After reaction termination, the phases were separated by centrifugation and the enantiomeric excess was determined by normal-phase high-pressure liquid chromatography (HPLC).[26]

Product purification: A silica-gel column (Fluka AG, Switzerland) was used to separate the product from substrate and dodecane. The substrate was eluted with hexane containing 1% triethylamine, and the product was eluted with hexane containing 1% triethylamine and 10% diethyl ether to afford **1a** (0.78 g), **2a** (0.51 g), and **3a** (0.88 g).

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[25] Standard assay conditions are available as Supporting Information.
[26] Standard conditions for the determination of *ee* values are available as Supporting Information.

