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Stereospecific Biocatalytic Epoxidation: The First Example of Direct Regeneration of a FAD-Dependent Monooxygenase for Catalysis

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Abstract: Catalysis for chemical synthesis by cell-free monooxygenases necessitates an efficient and robust in situ regeneration system to supply the enzyme with reducing equivalents. We report on a novel approach to directly regenerate flavin-dependent monooxygenases. The organometallic complex $[Cp^*Rh(bpy)(H_2O)]^{2+}$ catalyzes the transhydrogenation reaction between formate and isoalloxazine-based cofactors such as FAD and FMN. Coupling this $FADH_2$ regeneration reaction to the $FADH_2$-dependent styrene monooxygenase (StyA) resulted in a chemoenzymatic epoxidation reaction where the organometallic compound substitutes for the native reductase (StyB), the nicotinamide coenzyme (NAD), and an artificial NADH regeneration system such as formate dehydrogenase. Various styrene derivatives were converted into the essentially optically pure *(S)*-epoxides (ee > 98%). In addition, StyA was shown to be capable of performing sulfoxidation reactions. The productivity of the chemoenzymatic epoxidation reaction using 6.5 μM StyA reached up to 6.4 mM/h, corresponding to approximately 70% of a comparable fully enzymatic reaction using StyB, NADH, and formate dehydrogenase for regeneration. The coupling efficiency of the nonenzymatic regeneration reaction to enzymatic epoxidation was examined in detail, leading to an optimized reaction setup with minimized quenching of the electron supply for the epoxidation reaction. Thus, up to 60% of the reducing equivalents provided via $[Cp^*Rh(bpy)(H_2O)]^{2+}$ could be channeled into epoxide rather than hydrogen peroxide formation, allowing selective synthesis with high yields.

Introduction

The versatility of the oxirane function as starting point for chemical synthesis has driven considerable research effort for the preparation of enantiopure epoxides. Diverse approaches such as the use of chiral matrixes,[1] chiral dioxiranes,[2] and metal oxoporphyrins as low-molecular weight mimics for P450 enzyme systems[3] have been reported. Since the breakthrough of the catalytic asymmetric epoxidation by the Katsuki−Sharpless method,[4] great advances were achieved, especially with the (salen) manganese(III) complexes reported by Jacobsen and co-workers.[5] Despite the increasing number of examples for highly specific catalysts,[3] selectivities achieved so far with terminal and *trans*-olefins are less satisfactory.[6] Other limitations of this method include the laborious design and synthesis of suitable chiral ligands. Furthermore, activated oxygen must be supplied stoichiometrically, generally in the form of hydrogen peroxide, hypochlorite, or iodosylbenzene, causing undesired side reactions.

In contrast, enzymatic routes offer excellent chemo-, regio-, and enantiospecificities; high catalytic performance under mild reaction conditions; and the in situ activation of molecular oxygen.[7,8] In principle, cell extracts containing a suitable biocatalyst (e.g. from recombinant microbial strains) can be used as chemical reagents. The major challenge in using cell-free monooxygenases as biocatalysts is to supply the monooxygenase with the reducing equivalents needed for the reductive activation of molecular oxygen.[9] In their natural environment, monooxygenases derive these reducing equivalents from reduced nicotinamide cofactors that are supplied by cellular metabolism. Therefore, one widespread approach is to mimic this natural regeneration and to use a dehydrogenase for the in situ regeneration of NAD(P)H from its oxidized form ($NAD(P)^+$). Most commonly, formate dehydrogenase is used for this purpose.[10−13] However, these cofactor regeneration processes are expensive and require considerable optimization efforts to function adequately.[14]

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An interesting alternative is to directly regenerate the catalytically active monooxygenase component. Direct regeneration of monooxygenases has mostly been described for cytochrome P450 enzymes. Direct reduction with dithionite[15] and indirect methods using cobalt(III) sepulchrate as mediator between the enzyme and an electrode[16,17] or elementary zinc[18] have been reported. By cathodic reduction of the iron-sulfur protein putidaredoxin, Vilker and co-workers were able to omit the reductase component and NADPH in cell-free hydroxylation and epoxidation reactions.[19,20] However, direct regeneration of a FAD (or FMN)-dependent monooxygenase has not been reported so far. Typically, such enzymes show turnover numbers of about 10−20 s^{-1} [21,22] for product formation and are therefore very interesting catalysts.

Organometallic complexes such as (2,2′-bipyridyl)(pentamethylcyclopentadienyl) rhodium ($[Cp^*Rh(bpy)(H_2O)]^{2+}$) have been described as potent redox catalysts for the specific regeneration of nicotinamide cofactors (NAD(P)H) using either formate or a cathode as source of reducing equivalents.[23−26] Recently, we reported that $[Cp^*Rh(bpy)(H_2O)]^{2+}$ can also reduce alloxazine derivatives such as FAD or FMN at rates comparable to those obtained for NAD(P)H regeneration.[27] Therefore, $[Cp^*Rh(bpy)(H_2O)]^{2+}$ appears to be an ideal candidate to catalyze the direct regeneration of FAD-dependent monooxygenases for synthetic purposes.

Styrene monooxygenase (StyAB) from *Pseudomonas* sp. VLB120[28] catalyzes the specific *(S)*-epoxidation of a broad range of *m*- and *p*-, as well as α- and *β*-, substituted styrene derivatives.[29] The enzyme is composed of a FAD-dependent monooxygenase component (StyA) that catalyzes the epoxidation reaction and a NADH-dependent reductase component (StyB) that delivers the reducing equivalents from NADH to StyA via $FADH_2$.[30] StyAB may be classified as a member of the "two-component flavin-diffusible monooxygenase" family.[30,31] Partially purified StyAB from recombinant *Escherichia coli* has been used for cell-free biocatalytic epoxidation reactions using formate dehydrogenase for the in situ regeneration of NADH (Scheme 1, upper panel).[32]

In the present study we show that a $FADH_2$-dependent monooxygenase (in this case StyA) can be regenerated directly by means of non-native redox catalysts such as $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (Scheme 1, lower panel). We show that this cell-free chemoenzymatic approach can be used for the production of enantiopure epoxides via asymmetric synthesis. In particular, we compare the non-native redox catalyst to the native reductase with respect to conversion rate, substrate range, and optical purity of the resulting products. Finally, we identify the factors that influence the extent to which the catalytic regeneration reaction is coupled to the biocatalytic epoxidation reaction, provide a quantitative measure for the coupling efficiency, and compare it with the "native", fully enzymatic approach.

Experimental Section

Chemicals. Unless indicated otherwise, all chemicals were purchased from Fluka (Buchs, Switzerland) in the highest quality available and used without further purification. Appropriate safety precautions were taken where suitable, especially when working with rhodium, 3-chloroperoxobenzoic acid, and organic solvents.

$[Cp^*Rh(bpy)(H_2O)]^{2+}$ was synthesized according to literature methods[33] by addition of 2 equiv of 2,2′-bipyridine to a suspension of $[\{Cp^*RhCl(\mu\text{-}Cl)_2\}]$ (Aldrich, Buchs, Switzerland) in methanol. $[Cp^*Rh(bpy)Cl]Cl$ was precipitated upon addition of diethyl ether. Aqueous stock solutions were stored at room temperature for several months without detectable loss of activity. Racemic epoxides were produced from the corresponding styrene derivatives with 3-chlorobenzoic acid according to literature procedures.[34]

StyA (95% pure) was obtained from recombinant *E. coli* JM101 (pSPZ10)[35] as described.[32] StyB was purified from recombinant *E. coli* JM109 (pTEZ302) as reported previously.[36]

Methods. Determination of the Catalytic FAD Reduction by $[Cp^*Rh(bpy)H]^+$ and StyB. The rates of the formate-driven, $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed reduction of FAD and FMN were measured in oxygen-free media by monitoring the depletion of oxidized FAD (FMN) at $\lambda = 450$ nm ($\epsilon \approx 10\,000\ M^{-1}\ cm^{-1}$). Alternatively, the fast reoxidation of reduced isoalloxazine in O_2-containing media leading to hydrogen peroxide was quantified on the basis of either the depletion of O_2 using a Clark electrode (Strathkelvin Instruments Ltd., Glasgow, UK) or the formation of hydrogen peroxide, determined using the colorimetric assay of Tanaka and co-workers.[37] Each method gave comparable values for the catalytic performance of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ [e.g. at 37 °C and *c*(formate) = 150 mM turnover frequencies for $[Cp^*Rh(bpy)(H_2O)]^{2+}$ of 74.3, 69.9, and 67.6 h^{-1} were determined using FAD depletion, H_2O_2 formation, or O_2 consumption, respectively]. The activity of StyB was determined on the basis of the hydrogen peroxide formation rate.

General Reaction Conditions for the Chemoenzymatic Epoxidation Reactions. Unless indicated otherwise, the chemoenzymatic epoxidations were performed in 2.0-mL Eppendorf tubes placed in an Eppendorf 5436 thermomixer. The reactions were performed in a 50 mM potassium phosphate buffer containing 150 mM sodium formate, pH 7.6. After supplementation with StyA and FAD, the reaction mixture was maintained at the indicated reaction temperature. The reactions were started by the simultaneous addition of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and

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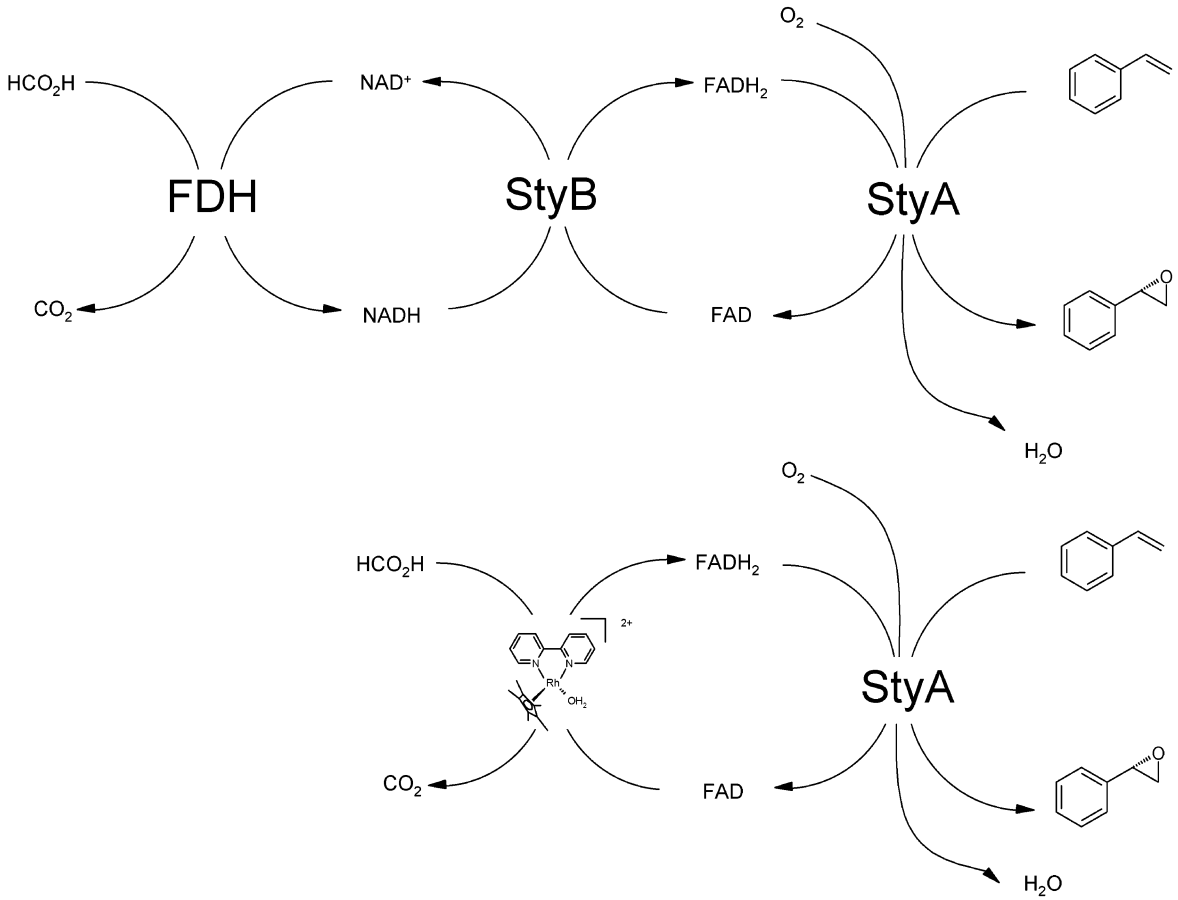
Scheme 1. Comparison of the "Traditional" Cell-Free Biocatalytic Epoxidation Reaction Using the Reductase Unit StyB and Formate Dehydrogenase (FDH)-Catalyzed NADH Regeneration (upper) and the Novel Chemoenzymatic Approach with $[Cp^*Rh(bpy)(H_2O)]^{2+}$ Replacing FDH, NAD, and StyB (lower)



reactant. Unless indicated otherwise, the reactions were terminated after 15 min by the addition of 500 μL of ice-cold acetonitrile and centrifugation of the precipitated proteins at 10300*g* for 15 min at 4 °C. The supernatant was analyzed by high performance liquid chromatography (HPLC).

HPLC Analysis. HPLC analyses were performed on a Merck/Hitachi system (LaChrom series) consisting of a L-7200 autosampler, a D-7100 gradient-pump, a L-7360 column oven, and a L-7455 diode array detector. Chromatograms were recorded at 210 nm. For achiral HPLC, a CC250/4 Nucleosil 100-5 C18HD column with acetonitrile/water (60:40 isocratic, 1 mL/min) was used. Product identification was done by comparison of the retention times with authentic samples. Optical purities of the prepared epoxides were determined by HPLC using a CC 200/4 column with Nucleodex α-PM as chiral stationary phase and methanol/0.1% TEAA buffer (40:60 isocratic, 0.7 mL/min) as eluent. Commercially available enantiopure epoxides (for styrene oxide and 3-chlorostyrene oxide) or the chemically prepared racemic epoxides were used as standards. Optical purities of indene oxide, *trans*-β-methylstyrene oxide, and the sulfoxides were determined on a DIACEL OB-H chiral column using hexane/2-propanol (98:2 isocratic at 0.5 mL/min).

Results

Characterization of the Regeneration System. The catalytic performance of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ for the formate-driven regeneration of $FADH_2$ was examined as a function of pH, temperature, and concentration of reactants. In accordance with our prior findings for the $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed regeneration of NAD(P)H,[27] we found no influence of the pH on the rate of FAD reduction between at least pH 6 and 8 (data not shown).

The temperature dependence of the $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed reduction of FAD followed an Arrhenius-like curve. The turnover rate (number of catalytic cycles per time) at 30 and 37 °C was 28.9 ± 0.5 and 69.9 ± 2.5 h^{-1}, respectively.

The FAD reduction rate was linearly dependent on the concentration of $[Cp^*Rh(bpy)(H_2O)]^{2+}$, whereas the concentration of FAD had no significant influence on the initial reduction rates. However, the total number of catalytic cycles of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ showed a distinct dependency on the molar ratio FAD/$[Cp^*Rh(bpy)(H_2O)]^{2+}$. Figure 1 shows the catalytic performance of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ over time at varying FAD/$[Cp^*Rh(bpy)(H_2O)]^{2+}$ ratios (measured via the formation of hydrogen peroxide). Above a 2.5-fold molar excess of FAD over $[Cp^*Rh(bpy)(H_2O)]^{2+}$, no decrease of H_2O_2 formation was observed throughout the experiment. However, below this ratio, the catalytic activity ceased with time. In the absence of FAD, (when hydrogen peroxide is generated directly by the oxidation of the reduced hydrido rhodium complex)[26] $[Cp^*Rh(bpy)(H_2O)]^{2+}$ performs only about 11 catalytic cycles.

Thus, given appropriate reaction conditions (especially an optimized ratio of FAD to reduction catalyst), $[Cp^*Rh(bpy)(H_2O)]^{2+}$ appears to be a suitable catalyst for the continuous regeneration of reduced flavin cofactors.

The Influence of StyB on the Chemoenzymatic Reaction. With the aim of elucidating whether the reductase component (StyB) is crucial for the epoxidation mechanism (e.g. by exerting an activating influence on StyA or by facilitating product release), chemoenzymatic epoxidation reactions in the presence

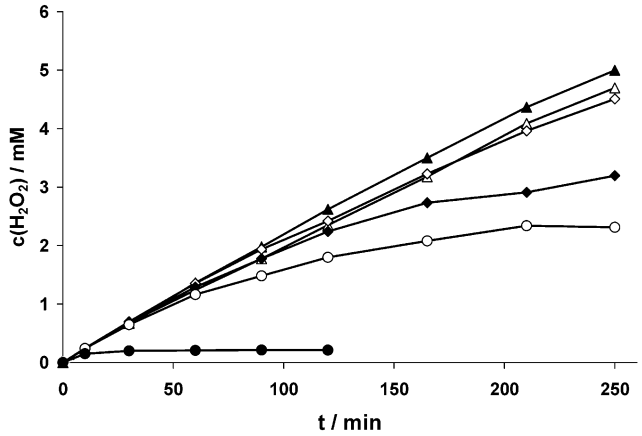


Figure 1. Time course of hydrogen peroxide formation at different FAD/$[Cp^*Rh(bpy)(H_2O)]^{2+}$ ratios. $c([Cp^*Rh(bpy)(H_2O)]^{2+}) = 19\ \mu M$, $c(NaHCO_2) = 0.15$ M, $T = 37$ °C, $c(FAD) = 0\ \mu M$ (●), 10 μM (○), 20 μM (◆), 50 μM (◇), 100 μM (▲), 200 μM (△).

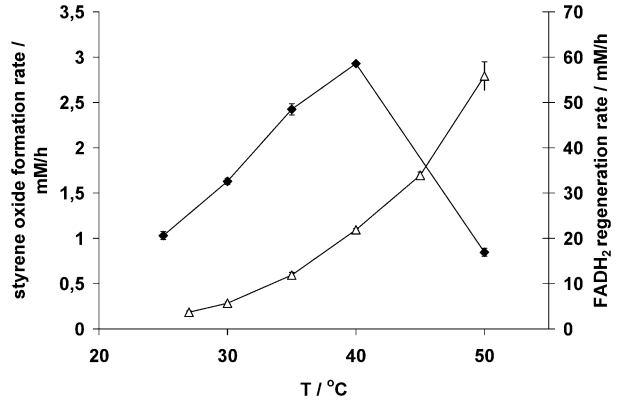


Figure 2. Temperature dependence of the chemoenzymatic epoxidation reaction (◆, left ordinate) and of the $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed regeneration reaction (△, right ordinate). Conditions for the styrene oxide formation rate: 50 mM potassium phosphate buffer (pH 7.6), $c([Cp^*Rh(bpy)(H_2O)])^{2+} = 0.2$ mM, $c(NaHCO_2) = 150$ mM, $c(FAD) = 50\ \mu M$, $c(StyA) = 1.97\ \mu M$, $c(catalase) = 1.3$ kU mL^{-1}, $c(styrene) = 4$ mM, $u = 1500$ rpm. Data for $FADH_2$ regeneration are based on previous results [$c([Cp^*Rh(bpy)(H_2O)]^{2+}) = 25\ \mu M$, $c(NaHCO_2) = 0.5$ M, $c(FAD) = 0.25$ mM][27] and were confirmed experimentally.

and absence of StyB were performed. No significant difference in the styrene oxide productivity was observed if 0.42 equiv of StyB (relative to StyA) was applied under otherwise identical conditions. Furthermore, optical purities of the products obtained from the chemoenzymatic reaction were as high as reported for whole-cell biotransformations.[29] Apparently, the reductase component does not influence the catalytic efficiency of the monooxygenase component (StyA) and is not necessary if $FADH_2$ is regenerated by $[Cp^*Rh(bpy)H]^+$, the catalytically active reduced complex. Therefore, all subsequent chemoenzymatic reactions were performed in the absence of StyB.

Optimal pH and Temperature Conditions. The effect of temperature on the activity of the chemoenzymatic epoxidation setup was examined over the range of 25−50 °C. The styrene oxide productivity increased linearly from 25 to 40 °C. Above this value, a distinct decrease was observed with only 28% of the maximum activity at 50 °C (Figure 2).

Since $[Cp^*Rh(bpy)(H_2O)]^{2+}$ was shown to exhibit increasing catalytic performance toward the reduction of FAD within this temperature range,[27] the stability of the biocatalyst with increasing temperature was examined in more detail. At 37 °C, a half-life of approximately 45 min was found for StyA. The inactivation rate is increased about 2-fold when the monooxygenase was exposed to shear stress (caused by rotation of the reaction vessel). However, at 30 °C, StyA retained more than 90% of its initial activity after 45 min, even under shear stress conditions.

Table 1. Comparison of the Fully Enzymatic and Chemoenzymatic Epoxidation of Indene at Different FAD Concentrations[a]

	(1*S*,2*R*)-indene oxide formation rate (mM/h)		
[FAD] (μM)	enzymatic[b]	chemoenzymatic[c]	relative activity (%)[d]
50	4.48	3.13	70
100	3.46	2.43	70
300	1.65	0.81	49
600	0.90	0.34	38

[a] General conditions: 50 mM sodium phosphate buffer (pH 7.6), $c(StyA) = 167\ \mu g\ mL^{-1}$ (3.6 μM), $c(catalase) = 650$ U mL^{-1}, $c(NaHCO_2) = 150$ mM, $c(indene) = 2$ mM, $T = 30$ °C, 800 rpm, reaction time = 15 min. [b] $c(StyB) = 1.6\ \mu g\ ml^{-1}$ (89 nM, 0.16 U mL^{-1}); c(FDH) = 2.6 U mL^{-1}; $c(NADH) = 2$ mM. [c] $c([Cp^*Rh(bpy)(H_2O)]^{2+}) = 0.2$ mM. [d] ratio of productivities.

A maximal epoxidation rate was observed at pH 7.6 with more than 80% of maximum activity between pH 7.4 and 7.8. Considering the pH independence of the mediator activity within this pH range, the optimum observed can be attributed entirely to the monooxygenase.

Comparison of $[Cp^*Rh(bpy)(H_2O)]^{2+}$- and StyB-Catalyzed Regeneration of $FADH_2$. One measure for the efficiency of the chemoenzymatic process is its productivity (in terms of product formation rate) compared to the fully enzymatic (native) regeneration. To obtain comparable reaction conditions, equivalent "reductase activities" of both StyB and $[Cp^*Rh(bpy)(H_2O)]^{2+}$ were coupled with StyA. Thus, a reaction mixture containing 0.26 U/mL formate dehydrogenase, 2 mM NADH, and 0.16 U/mL StyB (approximately 100 U/mg) under otherwise identical conditions (Table 1) was found to exhibit the same FAD reduction rate as 0.2 mM $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (one unit is defined as the catalyst amount that produces one μmol of product per minute). Under these conditions, the rate of the chemoenzymatic epoxidation of styrene to (*S*)-styrene oxide was up to 76 ± 1.6% of the styrene epoxidation rate of the fully enzymatic reaction (data not shown). Table 1 compares the initial productivities for the epoxidation of indene to (1*S*,2*R*)-indene oxide catalyzed by StyA using the chemoenzymatic approach with the initial productivities of the fully enzymatic regeneration at variable FAD concentrations.

Interestingly, the epoxidation rate in both setups depends inversely on the FAD concentration in the reaction medium. The effect however is less pronounced for the fully enzymatic approach. This effect was examined in more detail.

Uncoupling of the Regeneration Reaction from the Epoxidation Reaction. Styrene oxide was not formed in control experiments without StyA [50 mM potassium phosphate buffer (pH 7.6), $c(NaHCO_2) = 150$ mM, $c([Cp^*Rh(bpy)(H_2O)]^{2+}) = 0.2$ mM, $c(styrene) = 2$ mM, $T = 30$ °C, 800 rpm, $t = 15$ min].

We therefore conclude that the enantiospecific epoxidation reaction strictly depends on the presence of StyA and a putative 4α-hydroperoxoflavin in the active site of StyA.

Figure 3 shows that significant molar surpluses of FAD over StyA are necessary in order to obtain high productivities. As a

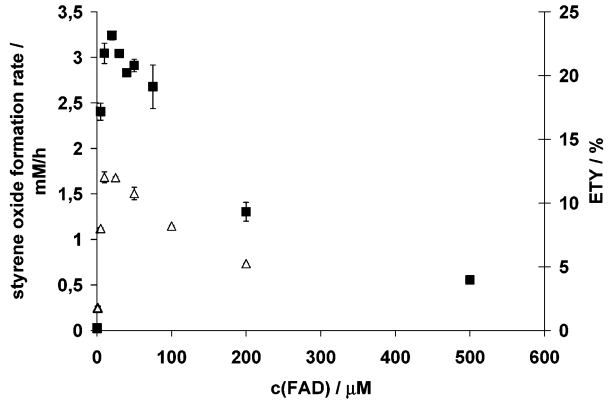


Figure 3. Styrene oxide formation rates (left ordinate) and the corresponding electron-transfer yields (ETYs, right ordinate) at varying FAD concentrations. General conditions: 50 mM potassium phosphate buffer (pH 7.6), $c([Cp^*Rh(bpy)(H_2O)]^{2+}) = 0.2$ mM, $c(NaHCO_2) = 150$ mM, c(catalase) $= 1.3$ kU mL^{-1}, c(styrene) $= 4$ mM, reaction time $= 15$ min, $T = 37$ °C, $u = 1400$ rpm. (△) c(StyA) $= 1.25$ μM, (■) c(StyA) $= 2.23$ μM.

consequence, large amounts of freely diffusible FAD are present in the reaction medium. Reduction of these FAD molecules results in an uncoupling of the $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed regeneration reaction from the StyA-catalyzed epoxidation reaction, because $FADH_2$ is rapidly reoxidized in the presence of molecular oxygen.[38,39] As a quantitative measure for the uncoupling, also including uncoupling at the active site of StyA, we defined an electron-transfer yield (ETY) as the ratio between the styrene oxide formation rate and the FAD reduction rate. The latter is based on the hydrogen peroxide formation rate determined in the absence of the vinyl aromatic substrate and catalase under otherwise identical conditions. The ETY corresponds to the coupling efficiency of the regeneration reaction and the epoxidation reaction.

To determine the influence of the total FAD concentration on the ETY, reactions at varying FAD and fixed catalyst (StyA and $[Cp^*Rh(bpy)(H_2O)]^{2+}$) concentrations were performed for two biocatalyst concentrations (Figure 3).

We found a strong dependence of the overall styrene oxide formation rate on the amount of FAD applied. For both enzyme concentrations maximum styrene oxide formation rates were found at FAD concentrations between 20 and 50 μM with maximum specific StyA activities of 0.49 ± 0.02 U mg^{-1}, whereas the optimal ETYs were 13% and 25% at StyA concentrations of 1.25 and 2.23 μM, respectively (Figure 3). The sharp increase of product formation rate and ETY with increasing FAD concentration up to approximately 20 μM can partially be attributed to a decreasing inactivation of the regeneration catalyst over time with increasing FAD concentration. Exceeding this FAD concentration, the $FADH_2$-regeneration rate remains unchanged (Figure 1), indicating that the decreasing styrene oxide productivity is either due to inhibition of StyA or due to the increasing uncoupling of the regeneration reaction from the epoxidation reaction.

To further investigate the chemoenzymatic reaction, a series of experiments at fixed FAD and $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and varying StyA concentrations were performed (Figure 4).

Similar to the findings given in Figure 3, a maximum for the specific enzyme activity of 0.48 ± 0.02 U mg^{-1} was obtained.

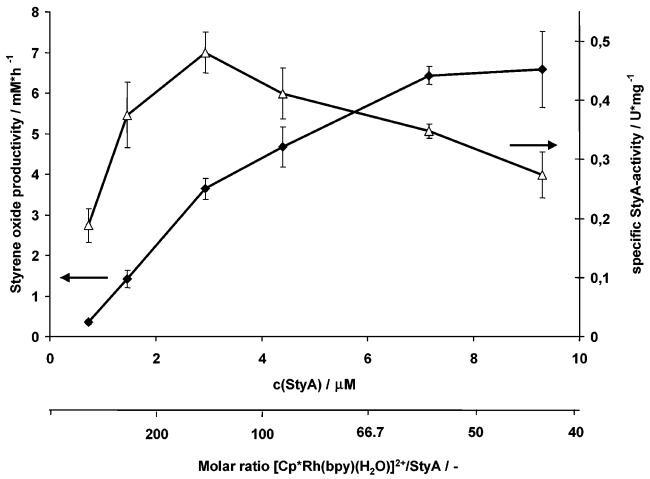


Figure 4. Styrene oxide productivity (◆) and specific StyA activity (△) at varying StyA concentrations. General conditions: 50 mM potassium phosphate buffer (pH 7.0, $T = 37$ °C, $t = 15$ min, $u = 1400$ rpm), $c([Cp^*Rh(bpy)(H_2O)]^{2+}) = 0.4$ mM, c(FAD)$= 50$ μM, $c(NaHCO_2) = 150$ mM, c(catalase) $= 1.3$ kU mL^{-1}, c(styrene) $= 4$ mM.

The increasing specific StyA activity with increasing StyA concentration can be explained by the more complete utilization of nascent $FADH_2$, which leads to lower uncoupling. The styrene oxide formation rate showed saturation-like behavior with respect to StyA, and a maximum ETY of 23% was calculated. This suggests that even high concentrations of StyA cannot compete with the fast uncoupling reaction and that an ETY around 23% is maximal under these reaction conditions.

Overall, the uncoupling of the regeneration reaction from the epoxidation reaction can be identified as the factor limiting the efficiency of the chemoenzymatic reaction.

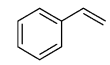
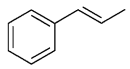
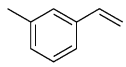
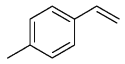
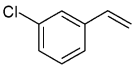
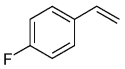
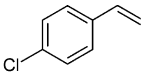
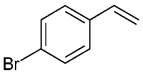
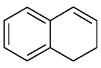
Product Spectrum of the StyA/$[Cp^*Rh(bpy)(H_2O)]^{2+}$ System. From a mechanistic point-of-view, the isoalloxazine-based cofactors FAD and FMN (as well as their reduced forms) should be rather similar. The specificity of StyA for FAD as cofactor was examined by substituting FAD with FMN. The in situ FMN concentration was varied from 50 to 600 μM; however, no formation of styrene oxide or indene oxide was detectable. Since $[Cp^*Rh(bpy)(H_2O)]^{2+}$ exhibits identical reducing activity for FAD and FMN,[27] this lack of epoxidation activity is attributed to a high specificity of StyA for FAD.

During in vivo biotransformations, styrene monooxygenase has been shown to catalyze to greater than 98% enantiospecific epoxidation of a broad range of vinyl aromatic compounds.[29] To elucidate the synthetic potential of the chemoenzymatic approach, various substrates were tested (Table 2).

Apart from trace amounts of diols (products from spontaneous degradation of the corresponding epoxides), only epoxides were detected as products formed from the vinyl aromatic substrates. The enantiomeric purities of the products were generally greater than 98% (with the exception of *p*-bromostyrene oxide, ee $=$ 95.6%) and thereby comparable to the ee values obtained in the in vivo reactions.[29] For styrene oxide and 3-chlorostyrene oxide the (*S*)-configuration was confirmed using commercially available enantiopure standards. For (1*S*,2*R*)-indene oxide the absolute stereochemistry was confirmed by its chromatographic behavior.[40] In contrast to whole-cell reactions,[29] all substrates tested (with the exception of 3-chlorostyrene) were converted at higher initial rates than styrene. No obvious correlation between the initial epoxidation rate and electron-donating or

(38) Kemal, C.; Chan, T. W.; Bruice, T. C. *J. Am. Chem. Soc.* **1977**, *99*, 7272−7286.
(39) Massey, V. *J. Biol. Chem.* **1994**, *269*, 22459−22462.

Table 2. Chemoenzymatic Conversion of Various Vinyl Aromatic Compounds[a,d]

Substrate	Initial epoxidation rate [b] [mM×h^{-1}]	Final epoxide [mM]/ Yield [%][c]	StyA	[Cp*Rh(bpy)(H$_2$O)]$^{2+}$			e.e value [%]	Product ratio [g×g^{-1}] [c]
			TTN[c]	TTN[c]	TF [b] [h^{-1}]	ETY [b] [%]		
	1.9	2.2/ 76	662	10.9	9.4	32.5	98.0 (99.5)	1.1 (1.8)
	2.6	2.3/ 66	701	11.5	13.2	45.7	97.6 (99.8)	1.3 (0.9)
	2.7	2.3/ 73	698	11.5	13.8	47.7	99.4 (n.d.)	1.3 (n.d.)
	2.9	1.6/ 66	482	7.9	14.4	49.8	n.d. (99.9)	0.9 (1.0)
	1.9	0.9/ 27	277	4.6	9.4	32.5	98.7 (99.4)	1.2 (1.5)
	2.0	1.7/ 72	506	8.3	10.2	35.3	99.4 (n.d.)	1.0 (n.d.)
	2.9	2.2/ 35	664	10.9	14.4	49.8	98.1 (n.d.)	1.9 (n.d.)
	2.7	2.0/ 27	598	9.6	13.6	47.0	95.6 (n.d.)	1.7 (n.d.)
	3.0	2.5/ 73	771	12.7	15.2	52.6	97.9 (98.0)	1.4 (1.3)
	3.5	2.5/ 61	752	12.4	17.6	60.9	98.9 (98.5)	1.5 (0.4)

[a] Values in parentheses were taken from whole-cell biotransformations;[29] n.d. = not determined. [b] Determined after 15 min: TF (turnover frequency) = c(product)$_{15min}$/(*c*([Cp*Rh(bpy)(H$_2$O)]$^{2+}$) × reaction time). [c] Determined after 2 h: Yield = 100 × (*c*(product)$_{final}$/(*c*(product)$_{final}$ + *c*(substrate)$_{final}$). Product ratio = *m*(Product)/*m*(StyA + [Cp*Rh(bpy)(H$_2$O)]$^{2+}$). TTN (total turnover number of catalyst) = *c*(product)$_{final}$/*c*(catalyst). [d] General conditions: 50 mM potassium phosphate buffer (pH 7.6), *c*(NaHCO$_2$) = 150 mM, *c*(catalase) = 650 U mL^{-1}, *c*(StyA) = 3.3 μM, *c*([Cp*Rh(bpy)(H$_2$O)]$^{2+}$) = 0.2 mM, *c*(FAD) = 50 μM, *T* = 30 °C, *u* = 800 rpm, substrate was added above its solubility (approximately 2 mM).

-withdrawing effects of the substituents could be detected. Qualitatively, substitution of the double bond increased the initial reaction rate; this effect was more distinct for cis- than for trans-substitution (*trans*-β-methylstyrene vs indene and dihydronaphthalene). Methyl substitution at the aromatic ring increased the reactivity, irrespective of the position of the methyl group, whereas only *p*-halogenation at the aromatic ring increased the reaction rate as compared to styrene (*m*- vs *p*-chlorostyrene). Obviously, steric effects influencing substrate binding interfere with electronic effects activating the double bond.

Among the vinyl aromatic compounds that were not converted by the chemoenzymatic reaction, two groups can be distinguished. The first group contains bulky substrates such as *p-tert*-butylstyrene as well as *cis*- and *trans*-stilbene or vinylferrocene. These substrates probably exhibit too much steric hindrance to efficiently bind to the active site of StyA. The second group of nonsubstrates such as 4-aminostyrene and indole as well as pyridines inhibits the formate driven formation of the catalyti-

(40) Jinyoua, Z.; Reddya, J.; Robergea, C.; Senanayakea, C.; Greashama, R.; Chartrain, M. *J. Ferm. Bioeng.* **1995**, *80*, 244−246.

cally active hydridorhodium complex (data not shown). Here, the only exception was 2-vinylpyridine, which was converted to a yet undefined more polar compound. Overall, a broad range of chiral epoxides is accessible via the chemoenzymatic epoxidation procedure.

Most flavoprotein monooxygenases utilize 4α-hydroperoxoflavin as oxygenating reagent.[22] This reactive intermediate is chemically similar to peracids that are of broad utility for synthetic chemistry, not only for epoxidation reactions but also for Baeyer−Villiger oxidations or the oxygenation of heteroatoms. To elucidate the applicability of StyA for such reactions, we chose structural analogues of styrene such as benzyl alcohol (oxidation of alcohols), benzaldehyde (oxidation of aldehydes and/or Baeyer−Villiger oxidation), and acetophenone (Baeyer−Villiger oxidation), as well as thiophenol ethers (sulfoxidation) as test substrates. However, only organic sulfides such as methyl and ethyl phenyl sulfide (but not the bulky diphenyl sulfide) were converted into the corresponding sulfoxides (data not shown). Sulfoxides were not formed in the absence of StyA under otherwise identical conditions (H_2O_2, which may oxidize the S-atom, is dismutated by catalase). The sulfoxidation rates were in the same range as the epoxidation rates of styrene (e.g. thioanisol was converted at 2.1 mM h^{-1} under the conditions indicated in Table 2). Further oxidation of the sulfoxide group to sulfones was not observed. Quite interestingly, the enantiospecificity of the sulfoxidation reaction was rather poor with only 26% ee for methyl phenyl sulfoxide and 13% ee for the ethyl phenyl sulfoxide.

Therefore, we conclude that the putative 4α-hydroperoxoflavin in the active site of StyA is activated to oxygenate at least sulfur atoms in addition to double bonds.

Discussion

One of the major limitations for cell-free biocatalysis using monooxygenases hitherto has been the complexity of the regeneration chain supplying the enzymes with the reducing equivalents that are needed for catalysis. Most approaches focus on the regeneration of costly nicotinamide cofactors (NAD(P)H). In the present study, we report on the simplification of such electron transport chains while preserving the benefits of the native cycles such as high reaction rates and enantiospecificity. By means of the organometallic complex $[Cp^*Rh(bpy)(H_2O)]^{2+}$, we directly regenerated the flavin-dependent styrene monooxygenase and thereby circumvented the need for a reductase, the nicotinamide coenzyme, and an enzymatic regeneration system. In these experiments we used formate as a source of reducing equivalents for the in situ regeneration of the catalytically active hydridorhodium complex. However, the use of electrodes as a source of reducing equivalents is possible as well.[26]

$[Cp^*Rh(bpy)(H_2O)]^{2+}$ was found to be active for the reduction of FAD over a broad range of pH and temperature, exhibiting a catalytic performance with formate as electron donor of approximately 1.2 turnovers per minute at 37 °C, corresponding to a specific activity of 2.8 U mg^{-1}. The apparent zero-order kinetics of the regeneration reaction with respect to FAD suggests that under the conditions applied, the formation of the catalytically active hydridorhodium complex is the rate-limiting step. This becomes evident considering the Michaelis−Menten-like kinetics of its formation[41] and the apparent K_M value of 78 mM for formate.[27] Thus, at a formate concentration of 0.15 M, $[Cp^*Rh(bpy)H]^+$ is not regenerated at maximal rates, limiting the rate of the overall reaction.

The extent to which the reducing equivalents provided by the regeneration system are coupled to the epoxidation reaction influences the efficiency of the presented chemoenzymatic approach. The correct amount of FAD present in the reaction medium is crucial for high efficiency. Excess of FAD over $[Cp^*Rh(bpy)(H_2O)]^{2+}$ is desirable in order to minimize the direct oxidation of the hydridorhodium complex to a so far undefined complex species that is catalytically inactive under the reaction conditions used. Maximizing the specific biocatalyst activity necessitates a defined surplus of FAD with respect to StyA of approximately 10−20-fold. Exceeding this value leads to increasing redirection of the reducing equivalents into unproductive and hazardous formation of hydrogen peroxide. The occurrence of this uncoupling reaction is independent of the regeneration catalyst ($[Cp^*Rh(bpy)(H_2O)]^{2+}$ or StyB) used (Table 1). The nature of this uncoupling at high FAD concentrations is not fully understood yet. One possible explanation is based on the assumption that only the reduction of StyA-bound FAD can productively be channeled into epoxide formation (this assumption is supported by the low stability of reduced $FADH_2$ in the presence of O_2). Above a certain FAD concentration, StyA would be saturated with FAD and further increase of *c*(FAD) would exclusively increase the concentration of free FAD. As a consequence, the (constant) reducing activity of $[Cp^*Rh(bpy)(H_2O)]^{2+}$ would then increasingly be channeled into unproductive reduction of free FAD at the expense of the reduction rate of StyA-bound FAD, leading to a decreased epoxidation rate. Another explanation is the assumption of a competition of FAD and $FADH_2$ (irrespective, whether it was formed at the active site of StyA, or not) for the flavin binding site on StyA. In this case, high overall concentrations of FAD would shift the binding equilibrium toward StyA-bound FAD and freely diffusing $FADH_2$, which in the presence of O_2 is reoxidized to FAD and H_2O_2. Alternatively, a combination of both mechanisms is possible. Interestingly, the fully enzymatic reaction appears to be less sensitive to high FAD concentrations (Table 1). Further investigations to clarify this phenomenon are underway.

The maximization of the ETY necessitates high biocatalyst concentrations relative to regeneration catalyst and FAD concentration. Under the conditions outlined in Figure 4, a maximal ETY of 23% for the epoxidation of styrene was found at a molar ratio of StyA to $[Cp^*Rh(bpy)(H_2O)]^{2+}$ above 1:40. This can be explained by the more complete utilization of nascent $FADH_2$, which leads to lower uncoupling.

Table 2 indicates that the ETY also strongly depends on the substrate used. For example, the ETY obtained with dihydronaphthalene as substrate is almost twice as high as for styrene. These results may be explained by assuming that substrate-binding (or electronic activation of the double bond) limits the rate of the chemoenzymatic epoxidation. Furthermore, the rate-limiting step seems to be less temperature sensitive than the substrate-independent formation of hydrogen peroxide. The optimal ETY obtained for the epoxidation of styrene at 37 °C is 23%, whereas it is more than 30% at 30 °C (Table 2). Apparently, high temperatures accelerate the unproductive formation of H_2O_2 more than the epoxidation reaction and thus

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Table 3. Comparison of Chemoenzymatic Reactions with Respect to Total Turnover Number and Activity

enzyme	substrate/product	source of reducing equivalents/mediator	mediator		relative activity[a]
			c (mM)	TTN	
P450 BM3[f]	12-pNPD[b]	Zn/Co(sep)[c]	0.5	0.15	21.8
P450rFP4504A1[g]	decanoic acid	cathode/Co(sep)	1	0.015	59.1
P450cam[h]	camphor	cathode/Pdx[d]	1.03	0.51	3.5
	styrene	cathode/Pdx[d]	0.5	0.06	2.9[e]
StyA	styrene	formate/$[Cp^*Rh(bpy)(H_2O)]^{2+}$	0.2	10.9	76

[a] Ratio of initial productivities of the mediated setup and the "native" reaction cycle. [b] 12-pNCA = *p*-nitrophenoxydodecanoic acid. [c] Co(sep) = cobalt(III) sepulchrate. [d] Pdx = putidaredoxin. [e] The regeneration of reduced Pdx was achieved by Pdx reductase. [f] see ref 18. [g] see ref 17. [h] see refs 9, 19, 20. Values were recalculated from data in the references.

result in lower ETYs. An alternative approach for the optimization of the ETY might be to control the concentration of dissolved oxygen. Provided the K_M of StyA for O_2 is suitably low, a lower O_2 concentration would minimize direct reaction of $FADH_2$ with O_2.

The relative epoxidation rates of StyA for different substrates observed in the cell-free chemoenzymatic reactions differ significantly from the results obtained from whole-cell biotransformations,[29] where the highest epoxide formation rate was achieved for styrene. This is most likely due to the lack of the transport limitations across microbial membranes and other influences of complex whole cell systems.

Overall, a broad range of synthetically interesting enantiopure epoxides is accessible with the presented chemoenzymatic approach. The unsatisfying low optical purities of the sulfoxides might be due to structural reasons. So far, only thiophenol derivatives were tested, which might not present the sulfur in the most suitable position for the oxygenation reaction. Benzyl thioethers might deliver the sulfur closer to the putative reactive 4α-hydroperoxoflavin, which could enhance the stereospecificity. This structural assumption might also explain why no Baeyer−Villiger oxidation was observed with the substrates investigated so far (carbonyl carbon atom adjacent to the benzene ring). Therefore, *β*-keto aromatics might be the substrates of choice for StyA-catalyzed Baeyer−Villiger oxidations.

Table 3 lists different direct regeneration systems that were used in combination with monooxygenases in order to replace NAD(P)H. Cobalt(III) sepulchrate and the redox protein putidaredoxin were used as shuttle systems to transfer electrons from the source of reducing equivalents (cathode or elementary zinc) to the monooxygenase. Multiple turnovers (up to 4000) were reported for the monooxygenases. However, the mediators were applied in concentrations up to 60 times higher than the maximal product concentration with the result that the TTNs of the mediators were between 0.015 and 0.51. The authors state that the uncoupling of the regeneration reaction from the enzymatic reaction is a limiting factor in the mediated reaction setups. However, no quantification for the impact of quenching of the electron supply chain by molecular oxygen is given. The high excesses of mediators used suggest that there is a major impact on the overall reaction, necessitating high concentrations of the reduced mediators. We achieve excellent catalytic performance of the chemoenzymatic reaction with up to 60% coupling of the redox equivalents provided by the regeneration reaction to the enzymatic epoxidation reaction. The epoxidation rates of the chemoenzymatic reactions are in the range of 70% of the comparable fully enzymatic reaction. Thus, the described $[Cp^*Rh(bpy)(H_2O)]^{2+}$-based regeneration setup represents substantial progress in the direct regeneration of monooxygenases and emphasizes the potential of this concept for preparative application. This concept has now been proven for StyA, which seems to use FAD as a substrate rather than as prosthetic group. Scaling up for preparative purposes is straightforward, since the process setup may consist of an aerated stirred tank reactor. On the basis of the numbers given in Table 2, it can be estimated that the production of 1g of any epoxide using the present setup requires a biocatalyst amount of approximately 500 mg. This amount of enzyme can be easily produced by simple fed-batch fermentation followed by chromatographic purification on a technical scale.[32] The present reaction setup has so far not been optimized for preparative synthesis. It may be possible to improve the StyA catalyst by common reaction engineering procedures such as immobilization onto a solid support to enhance thermal and mechanical stability,[42,43] and/or the use of a second organic phase to withdraw the highly reactive products from the enzyme,[44] and by protein engineering procedures.[45−47]

Compared to chemical catalysis, e.g. using (salen) manganese(III) complexes (Jacobsen catalysts), the biocatalytic, chemoenzymatic approach features some important advantages. The enantiospecificity of the enzymatic epoxidation reaction resulted in product ee's of more than 98%. For the Jacobsen catalysts, comparable stereospecificities have been reported for various substrates, but specificity and activity tend to be low for terminal and trans-configured double bonds.[6] The catalytic performances of such transition metal catalysts (that are used in one to several mole percent) are typically in the range of a few hundred catalytic cycles at rates in the range of several turnovers per hour.[3] These chemical catalyst values are poorer than those for the biocatalyst, whereas they correspond to the numbers calculated for the regeneration catalyst $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

In recent years the preparative usefulness of synthetic flavin hydroperoxides has been recognized and exploited for catalytic oxidation reactions. In addition, asymmetric variants are in the development stage.[48] However, since these reactions do not imply in situ regeneration of the reduced flavins, they depend on the presence of stoichiometric amounts of peroxides to drive the oxidation reaction.[49,50]

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(43) Katchalski-Katzir, E.; Kraemer, D. M. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 157−176.
(44) Held, M.; Schmid, A.; Kohler, H.-P. E.; Suske, W. A.; Witholt, B.; Wubbolts, M. G. *Biotechnol. Bioeng.* **1999**, *62*, 641−648.
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In this context, the polypeptide StyA can be considered as a chiral, macromolecular ligand for 4α-hydroperoxoflavin. This ligand not only provides an asymmetric matrix for the reaction but also exerts an activating effect on the 4α-hydroperoxoflavin to perform epoxidation reactions. Numerous other chiral ligands (flavin-dependent monooxygenases) supporting asymmetric epoxidation, hydroxylation, Baeyer−Villiger oxidation, heteroatom oxygenation, and halogenation reactions have been described.[22,51] The concept of the $[Cp^*Rh(bpy)(H_2O)]^{2+}$-catalyzed regeneration of $FADH_2$ ($FMNH_2$) can now be extended to these interesting catalysts.

Thus, the chemoenzymatic reaction may be considered as complementary to "traditional" homogeneous catalysts, extending the toolbox of chemical oxidation catalysts.

Conclusion

In the present study we have demonstrated the feasibility of the cell-free biocatalytic assymmetric synthesis of enantiopure epoxides and have addressed the major limitation of the in vitro application of monooxygenases. By using $[Cp^*Rh(bpy)(H_2O)]^{2+}$ as a regeneration catalyst, we eliminated the need for a complicated enzymatic electron delivery system for the monooxygenase and the nicotinamide cofactor. Factors influencing the coupling efficiency of the regeneration reaction to the epoxidation reaction were identified and partially optimized. Despite the early stage of development, the chemoenzymatic epoxidation reaction is complementing traditional chemical routes. Overall, we have shown that biocatalytic epoxidation reactions using an isolated oxygenase can easily be applied as tools for chemical synthesis.

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