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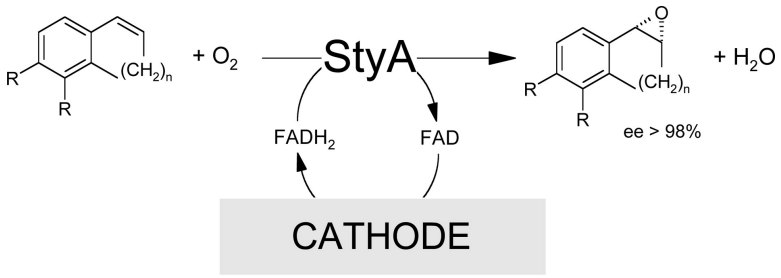


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# Direct Electrochemical Regeneration of Monooxygenase Subunits for Biocatalytic Asymmetric Epoxidation

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Enzymes are increasingly recognized as valuable tools for asymmetric synthesis. The scope of enzyme catalysis remains largely unexploited as the vast majority of synthetic biocatalytic applications has been confined to simple, hydrolysis-based kinetic resolution reactions.[1] Monooxygenases catalyze the reductive activation of molecular oxygen and its incorporation into a variety of organic substrates, frequently with high regio- and stereoselectivity and under environmentally benign reaction conditions. However, monooxygenases are cofactor-dependent enzymes, which have to be supplied with reducing equivalents derived from costly and instable nicotinamide cofactors (NAD(P)H). The reducing equivalents are often transferred to the terminal oxygenase via complex multienzyme electron transport chains. Besides, NAD(P)H-to-product stoichiometry is impaired by undesired oxidative side-reactions.[2] These impediments restrict the practical value of monooxygenases as chiral catalysts.

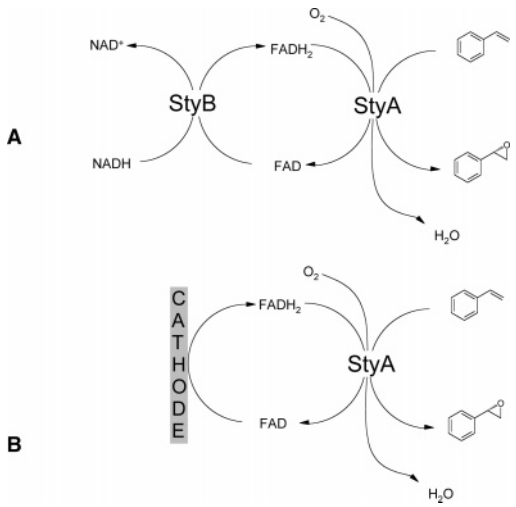
A potential remedy for the mentioned challenges is the direct introduction of reducing power into the catalytic cycle by cathodic reduction. Research on electroenzymatic oxidations has been mainly confined to P450−monooxygenase-catalyzed oxyfunctionalizations[3,4] and flavin-dependent oxidases,[5] whereas flavin-dependent monooxygenases have been largely neglected. However, these enzymes are versatile catalysts performing specific oxygenation reactions, including hydroxylations, epoxidations, halogenations, and heteroatom and Baeyer−Villiger oxidations.[1]

For example, styrene monooxygenase (StyAB) from *Pseudomonas* sp. VLB120 catalyzes the specific *S*-epoxidation of styrene derivatives.[6] The enzyme is composed of an $FADH_2$-dependent oxygenase component (StyA), accomplishing the epoxidation reaction and an NADH-dependent reductase component (StyB), delivering reducing equivalents from NADH to StyA. Both components possess their own catalytically active centers, and the epoxidation capability of StyA is exclusively dependent on $FADH_2$.[6a]

In the present study, we establish direct electrochemical regeneration of $FADH_2$ to substitute for the complex native regeneration cycle including StyB and NADH (Scheme 1).

Electrolyses were conducted potentiostatically in a temperature-controlled stirred tank reactor (10 mL volume) with external aeration. Cylindrical carbon felt served as cathode (working electrode) and a Pt wire as anode (counter electrode).[7] Application of a cathode potential of −550 mV vs $Ag/AgCl_{sat.}$ to the reaction containing StyA, FAD, and catalase in phosphate buffer allowed the conversion of *trans*-$\beta$-methyl styrene into practically enantiopure (1*S*,2*S*)-1-phenylpropylene oxide. No product formation was detectable when either StyA or FAD was omitted from the reaction medium. Selected vinyl aromatic compounds could be transformed into corresponding (*S*)-epoxides with high enantiomeric purity (>98%) (Table 1). Apparently, the substrate spectrum, as well as the stereodiscrimination of StyA, was not impaired under the unnatural, highly simplified reaction conditions.

***Scheme 1.*** StyA-Catalyzed Epoxidation of Styrenes Following the Native Cycle (A) and the Simplified Electroenzymatic Approach (B)[a]




[a] Holo-StyA binds reduced FAD for in situ activation of $O_2$ followed by stereospecific *S*-epoxidation of styrenes.[6a]

The rate of the electroenzymatic reaction did not meet the values obtained with the native cycle. For example, a specific StyA activity of 35.5 ± 2.1 U $g^{-1}$ was calculated for the electroenzymatic epoxidation of *trans*-$\beta$-methyl styrene,[8] while specific StyA activities of up to 2100 U $g^{-1}$ (corresponding to more than 1.6 catalytic turnovers per second) have been observed in kinetic studies using the native system.[6] Apparently, the full catalytic potential of StyA was by far not exploited yet.

We assumed substrate limitation by $FADH_2$ to account for the low activity of the biocatalyst. Slow electrochemical reduction of the flavin and fast aerobic reoxidation may explain the putatively low in situ concentration of $FADH_2$. No obvious change in the spectroscopic properties of the reaction medium could be observed throughout aerobic electrolyses ($\lambda$ = 450 nm), indicating a very low steady-state concentration of reduced flavin. We therefore investigated the influence of electrochemical parameters on the rate of the epoxidation reaction. A shift of cathode potential to more anodic values resulted in essentially identical reaction profiles, indicating that electron transfer to FAD was not rate-limiting.[7] However, the ratio of cathode surface to reaction volume had a significant influence on the rate of the electroenzymatic epoxidation

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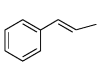
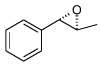
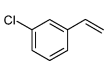
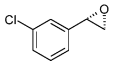
***Table 1.*** Electroenzymatic Epoxidation of Substituted Styrene Derivatives[a]

| Substrate | Product | Rate [mM $h^{-1}$] | ee-value [%] [b] |
|---|---|---|---|
|  |  | 0.143 | 98.5 (99.5) |
|  |  | 0.074 | 99.5 (96.7) |
|  |  | 0.191 | > 99.9 (99.8) |
|  |  | 0.222 | 99.2 (98.5) |
|  |  | 0.136 | 98.1 (99.4) |

[a] General conditions: 10 mL of potassium phosphate buffer (50 mM, pH 7.5), $T$ = 30 °C, $c$(StyA) = 2.13 $\mu$M, $c$(FAD) = 300 $\mu$M, $c$(catalase) = 480 U $mL^{-1}$, $c$(substrate) = 2 mM, cathode area = 14 $cm^2$. Due to the volatility of the reactants, no yields are given. [b] Values in parentheses originate from whole-cell biotransformations.[6b]

***Table 2.*** StyA Activity Depending on Cathode Surface and Aeration[a]

| area/volume quotient ($cm^{-1}$)[b] | | aeration ($cm^3$ $min^{-1}$) | StyA activity [U $g^{-1}$] ($min^{-1}$)[c] |
|---|---|---|---|
| 1 | 0.21 | 7 | 15.1 (0.71) |
| 2 | 0.74 | 7 | 34.5 (1.62) |
| 3 | 2.12 | 7 | 74.5 (3.50) |
| 4 | 2.12 | 0 | 26.6 (1.25) |
| 5 | 2.12 | 6 | 64.5 (3.03) |
| 6 | 0.94 | 40 | 178.7 (8.40) |

[a] General conditions: phosphate buffer (50 mM, pH 7.5), $T$ = 30 °C, $c$(StyA) = 4.9−5.1 $\mu$M, $c$(FAD) = 300 $\mu$M, $c$(catalase) = 480 U $mL^{-1}$, $c$(substrate) = 2 mM. [b] Macroscopic cathode surface ($cm^2$) divided by medium volume ($cm^3$). [b] Product formation rates may be normalized to the biocatalyst concentration since this linearly correlates to the product formation rate.[7]

reaction (Table 2, entries 1−3), which can be ascribed to the heterogeneous character of the electrochemical regeneration reaction.

Since reduced flavins are subject to aerobic reoxidation,[9] $O_2$ may further reduce the in situ concentration of $FADH_2$ and negatively influence the enzymatic epoxidation rate ($K_{M,StyA,FADH_2}$ > 10 $\mu$M[10]). Similarly, electroenzymatic reactions with P450−monooxygenases are hampered by undesired oxidative quenching of the regeneration reaction.[3,4] We found a positive correlation between aeration rate and epoxide formation (Table 2, entries 4−6). The specific StyA activity could be increased more than 5-fold compared to initial values, corresponding to over 170 U g(StyA)$^{-1}$ by increasing the rate of external aeration. Apparently, the enzymatic epoxidation rate is more oxygen sensitive than the oxidative quenching reaction rate. The proposed autocatalytic $FADH_2$ oxidation mechanism might explain this observation.[7,9] According to the mechanism, formation of a flavin−semiquinone would be rate-limiting, and aerobic $FADH_2$ reoxidation should be largely independent from the concentration of oxygen. Adversely, formation of the catalytically active 4a-peroxoflavin, the rate-limiting step of the enzymatic reaction,[6] depends directly on the oxygen concentration. Increased supply of $O_2$ increases the rate of the enzymatic reaction, while the rate of the uncoupling reaction remains largely unaltered.

Stripping out of the reactants and StyA inactivation correlate with the vigorousness of aeration under the experimental conditions applied in this study. The low stability of StyA currently represents the major impediment to preparative applications of the electroenzymatic epoxidation reaction. We are evaluating bubble-free aeration techniques, such as hollow-fiber modules or anodic water oxidation, that will allow efficient aeration under mild reaction conditions.[7]

In conclusion, we have demonstrated the feasibility of direct electrochemical regeneration of a flavin-dependent monooxygenase for catalysis. Driven only by electrical power, optically pure epoxides were synthesized from corresponding vinyl aromatic compounds. The complicated native enzyme system consisting of three enzymes (StyA, StyB, and an NADH regenerating enzyme) and two cofactors (NADH and FAD) was minimized to the oxygenase component and its flavin prosthetic group. In principle, this approach is applicable to any enzymatic reaction involving reduced flavins within the catalytic cycle.[4,11] Optimization of the bottlenecks identified in this study is underway and is expected to result in a practical route to enantiopure epoxides, thus adding electroenzymatic oxyfunctionalization to the toolbox of asymmetric synthesis.

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**Supporting Information Available:** Further experimental data and materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) One international unit (U) is defined as the biocatalyst amount forming one micromole of product per minute. In the case of StyA (MW = 47 × $10^3$ g $mol^{-1}$), a specific enzyme activity of 1 U $g^{-1}$ translates into a catalytic performance (turnover frequency, TF) of 0.047 $min^{-1}$.
(10) $K_M$ value = Michaelis−Menten constant, the concentration of (co)substrate at which the catalyst exhibits half-maximal activity: Otto, K.; Schmid, A. Unpublished results.
(11) Various P450−monooxygenases rely on FAD and/or FMN as electron shuttles.

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