Photoenzymatic epoxidation of styrenes†

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Two-component-diffusible-flavomonooxygenases are versatile bio-
catalysts for selective epoxidation-, hydroxylation- or halogenation
reactions. Their complicated molecular architecture can be simpli-
fied using photochemical regeneration of the catalytically active,
reduced FADH₂ prosthetic group. In this contribution we provide
the proof-of-concept and characterization for the direct regenera-
tion of the styrene monooxygenase from Pseudomonas.

So-called two-component, diffusible flavin monooxygenases
(2CDFMOs) are a diverse and preparatively highly interesting
class of enzymes. For example, 2CDFMs catalyse regioselective
aromatic hydroxylations and halogenation reactions as well as
stereoselective epoxidation reactions.1–4

For this, 2CDFMOs rely on the reductive activation of molecu-
lar oxygen mediated by an enzyme-bound, reduced flavin
cofactor (generally the reduced form of flavin adenine dinucleo-
tide, FADH₂). FADH₂ itself is regenerated by a NAD(P)H-dependent
reductase. There is an ongoing debate on the mechanism on how
FADH₂ reaches the monooxygenase subunit. Some studies suggest
a freely diffusible FADH₂5 while others found indications for a
complex between the reductase- and monooxygenase subunits
thereby channelling the reduced flavin and protecting it from
spontaneous aerobic reoxidation.6–8 The complicated molecular
architecture of 2CDFMOs poses a challenge for their preparative
application, which is mostly addressed by whole-cell systems.9–12

More recently, also fusion proteins combining the reductase- and
monooxygenase subunits in one polypeptide chain are moving
into the research focus.13,14 Reactions utilising isolated enzymes
require the entire cascade outlined in Scheme 1.15–17 Hence, it is
no surprise that alternative, more direct and simple regeneration
systems for the reduced flavin cofactor have been evaluated.
Examples include, transition metal-catalysed reduction of
FADH₂,18,19 direct electrochemical regeneration20,21 or using
chemical reductants.22

All these methods, however, despite significantly simplifying
the reaction scheme, exhibited drawbacks such as reliance on
specialized equipment or dependency on costly and enzyme-
inactivating transition metal complexes. Instead, direct photo-
chemical regeneration of reduced enzyme prosthetic groups
is gaining increasing attention.23,24 We therefore set out to
explore the possibility of direct, photocatalytic regeneration of
FADH₂ to promote StyA-catalysed epoxidations of styrene and
its derivatives (Scheme 1, bottom). Very recently, Kottke and
coworkers reported the successful application of this approach
to promote a 2CDFM-driven halogenation reaction.25

The biocatalyst used in our study was styrene monooxygenase
from Pseudomonas sp. VLB120 (StyA).5,26 The enzyme was recom-
binantly expressed in Escherichia coli and purified in one step

† Electronic supplementary information (ESI) available: For more details on the
catalyst preparation, reaction procedure and GC chromatograms, etc. See DOI:
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Scheme 1 Comparison of the traditional regeneration system for StyA
involving two additional enzymes (A) and the nicotinamide cofactor with
the simplified, direct photochemical regeneration of FADH₂ (B).

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Table 1  Product scope of the photoenzymatic epoxidation system

| Product | R₁ | R₂ | R₃ | [Product] [mM] | ee [%] |
|---------|----|----|----|---------------|-------|
| 2a      | H  | H  | H  | 0.60 ± 0.04   | >99   |
| 2b      | m-CH₃ | H | H | 2.12 ± 0.11 | 98   |
| 2c      | p-F | H | H | 0.86 ± 0.13 | >99   |
| 2d      | m-Cl | H | H | 3.10 ± 0.19 | 95   |
| 2e      | p-F | H | H | 1.06 ± 0.14 | 97   |
| 2f      | p-Br | H | H | 0.65 ± 0.15 | >99   |
| 2g      | H  | H | CH₃ | 1.45 ± 0.09 | >99   |
| 2h      | H  | H | CH₃ | 0.40 ± 0.12 | >99   |

Conditions: [substrate]₀ = 5 mM, [StyA] = 5.3 μM, [FAD] = 200 μM, [EDTA] = 20 mM, [catalase] = 600 U ml⁻¹, [DMSO] = 1.25% (v/v), 100 mM KPi buffer pH 7, 35 °C, stirring at 300 rpm, light intensity of 40% for 1 h. The standard deviations represent those for three independent experiments.

yielding approximately 0.11 g of technically pure StyA per gram of cell free extract. Crude cell free extracts (CFE) were used for the first experiments (Fig. S1, ESI†). Even though the CFE contained significant catalase activity (Fig. S12, ESI†), we routinely added catalase externally to circumvent any possible negative effect of stemming from the spontaneous aerobic reoxidation of FADH₂.²⁷ Pleasingly, already in a first experiment under arbitrary reaction conditions, catalytic turnover and production of enantio-pure (S)-styrene oxide was observed (Fig. S3, ESI†). It is worth mentioning here that all negative control experiments (i.e. performing the reaction under identical conditions while leaving out one of the reaction components StyA, FAD, EDTA or light) did not yield detectable product formation [data not shown].

Encouraged by these results, the substrate scope of this photoenzymatic reaction system was investigated. As shown in Table 1, both the relative reaction rates and the enantioselectivity of the photoenzymatic epoxidation reaction are comparable to results reported previously.¹¹

Indeed, excellent enantiomeric excess was achieved (95 to >99%). In order to further understand this system, we further characterised the influence of the single reaction components on the efficiency of the overall reaction using the technically pure StyA (Fig. 1). The product formation rate correlated linearly with the biocatalyst concentration applied (Fig. 1A), indicating that the biocatalytic step was overall rate-limiting.

Also increasing the concentration of the photocatalyst increased the overall product formation reaction (Fig. 1B) indicating that the concentration of the photoexcited FAD was rate-limiting as well. Below an FAD concentration of 100 μM no product formation was detectable, which may be attributed to the corresponding low concentration of FADH₂ and inefficient utilisation by StyA.²⁸,²⁹ Above approximately 200 μM FAD, no further acceleration of the reaction rate was observed. Possibly this can be attributed to the decreasing optical transparency of the reaction mixture at elevated FAD concentration resulting in a complete utilization of all photons offered to the reaction system. Alternatively, elevated FAD concentrations may also favour the (undesired) futile oxidation of FADH₂ to FAD and H₂O₂ as observed in previous experiments.²⁷ Similar observations have been made previously.¹⁹,²⁰,³⁰ Variation of the concentration of the sacrificial electron donor (EDTA) had a similar effect (Fig. 1C). Additionally, the light intensity significantly influenced the overall reaction (Fig. 1D). To a certain extent, brighter reaction conditions favoured increased product formation. However, at very high light intensities also a dramatic reduction of the reaction rate was observed. We attribute this to an increased photobleaching of FAD leading to flavin degradation products,³¹ which are not accepted by StyA as prosthetic group. Finally, it is worth mentioning that an apparent optimal temperature of ca. 35 °C was observed (Fig. 1E). This is perfectly in line with the mesophilic character of the original host of StyA.

One shortcoming of the current photoenzymatic reaction setup, however, is the comparably poor robustness of the reactions. In general, after reaction times of ca. 1 h, no further conversion could be detected. In order to shed light on the reason for this limitation, a range of control experiments was conducted (Fig. S13, ESI†). The biocatalyst (StyA) itself was stable under the reaction conditions (Fig. S13, ESI† column 2).
However, when illuminating the photocatalyst (FAD) for 30 min prior start of the reaction (Fig. S13, ESI,† columns 3 and 4) significantly reduced styrene oxide accumulation was observed. This inactivation was almost complete illuminating FAD alone and was somewhat less pronounced in the presence of EDTA (serving as reducing agent for photoexcited FAD; the resulting reduced flavin being less photoactive). Clearly, the photo-instability of FAD represents the major limitation of the current reaction setup. This is also supported by the changes of the FAD spectrum upon illumination (Fig. S14, ESI†) and is in line with the well-known photodegradation of FAD to lumichrome.32 While the latter still possesses the desired photochemical properties it is not accepted by StyA as a prosthetic group, thereby explaining the poor robustness of the current reaction setup.

Overall, in the present study, we have provided a proof-of-concept for the direct, photochemical regeneration of styrene monooxygenase for the generation of enantiomerically pure epoxides. The photostability of the photocatalyst/prosthetic group FAD was identified as the major limitation of the current setup. Even though at the present stage of development this system is not suitable for preparative application we are convinced that it’s conceptual simplicity will convince others to further improve the system.

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Conflicts of interest

There are no conflicts to declare.

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