VIP Biocatalysis

DOI: 10.1002/ange.200605169

A Light-Driven Stereoselective Biocatalytic Oxidation**

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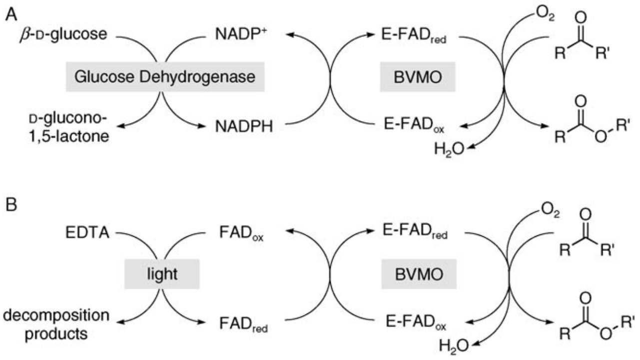
During the last decades, numerous flavin-dependent monooxygenases have been discovered, characterized, and studied mechanistically.[1] They catalyze the efficient and selective insertion of one oxygen atom from O_2 into organic substrates, as in Baeyer–Villiger (BV) reactions, aromatic hydroxylations, epoxidations, sulfoxidations, or even halogenations.[1,2] As some of these reactions are difficult to perform selectively using chemical catalysts,[3] this class of enzymes has gained considerable interest as catalysts in synthetic organic chemistry.[2] Mechanistically, the reduced enzyme-bound flavin (FAD or FMN) reacts with dioxygen from air to form a flavin-peroxide species, which functions as the oxidant in the desired oxygenation reaction.[1,2] To close the biocatalytic cycle, the reduced form of the flavin is then regenerated by a nicotinamide cofactor (NADH or NADPH). As these cofactors are expensive, a number of chemical, electrochemical, and enzymatic regeneration methods have been developed to enable their use in only catalytic amounts.[4] However, owing to the necessity of an additional catalytic system and the lack of generality, these approaches are limited to specialized applications. Thus, the quest for new approaches to regeneration systems continues. Herein, we demonstrate the utility of light as the driving force for the regeneration of the reduced flavo-enzyme for catalysis. It is known that flavins can be photochemically reduced using simple sacrificial electron donors such as ethylenediaminetetraacetate (EDTA).[5] Even though this finding opens up a fascinating new way for the direct regeneration of reduced flavin cofactors, thus circumventing the need for reduced nicotinamides as electron donors, it has never been exploited for biocatalytic purposes.

We therefore devised a catalytic cycle in which NADPH is replaced by light in combination with the sacrificial electron donor EDTA, with the model reaction being the BV oxidation of ketones. Thus, we introduce a new drastically simplified system (Scheme 1B) compared to the traditional multicomponent regeneration approach (Scheme 1A). As the flavin-dependent enzyme, we chose a mutant of phenylacetone monooxygenase[6] (PAMO-P3), which we had previously engineered to catalyze the enantioselective BV reaction of ketones such as **1a**, **1b**, and **3**. These substrates are either not accepted by the wild-type enzyme or they are oxidized with poor stereoselectivity.[7] In that study, we showed that the kinetic resolution of **1a** and **1b** catalyzed by mutant P3 is highly *R*-selective (> 97% *ee* of products **2a** and **2b**) and that in the case of *rac*-**3** a 1:1 mixture of regioisomers (−)-**4** (92% *ee*) and (−)-**5** (99% *ee*) is formed.[7]



Scheme 1. Comparison of the conventional regeneration of a Baeyer–Villiger monooxygenase (BVMO, e.g. PAMO) for catalysis through enzymatic regeneration of NADPH (A) and the novel simplified light-driven pathway using a flavin (B). Part (A) shows an enzyme-coupled regeneration system employing the common glucose dehydrogenase to catalyze the regeneration of the reduced nicotinamide cofactor. The use of this coupled enzyme can be replaced by visible light, which reduces the complexity of the setup by eliminating the need for a coupled enzyme. (E-FAD: enzyme bound flavin adenine dinucleotide.)

Upon attempting the BV reaction of *rac*-**1a** in the presence of EDTA, FAD, a white light bulb, and catalytic amounts of the mutant monooxygenase P3, but in the absence of $NADP^+$, no conversion of **1a** occurred. However, this finding can be explained by the fact that the nicotinamide cofactor stays bound to a Baeyer–Villiger monooxygenase during the active catalytic cycle, suggesting a pivotal role of the bound nicotinamide cofactor in sustaining a catalytically active conformation of the enzyme.[1h,8]

We therefore repeated the reaction, this time in the presence of catalytic amounts of $NADP^+$. Gratifyingly, this protocol was successful, giving almost the maximum theoretical conversion of 50% in the kinetic resolution of ketone **1a** to lactone (*R*)-**2a** with excellent enantioselectivity (48% conversion, 97% *ee*). The optical purity of the product is essentially identical to that obtained from previous experiments utilizing whole cells[7a] or an in vitro system based on conventional cofactor regeneration.[7b] Apparently, the inherent stereoselectivity of PAMO-P3 is not impaired by the unnatural regeneration conditions (Table 1), suggesting a non-altered mechanism of the actual oxidation reaction. Moreover the decomposition products of EDTA have no detrimental effect on the course of the reaction (Figure 1).

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[**] This work was supported by the Fonds der Chemischen Industrie and the Deutsche Forschungsgemeinschaft (RE 359/13-1). We thank S. Ruthe and F. Kohler for GC analyses.

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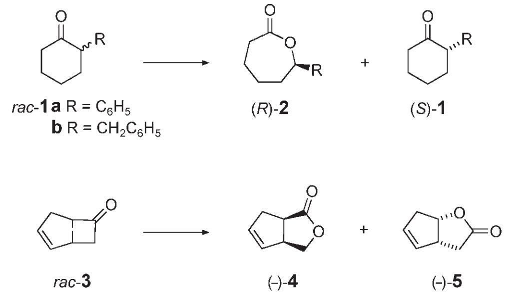
Table 1: Stereoselective Baeyer–Villiger reactions.[a]



Substrate	Conversion [%]	*ee* [%]
1a	48 (≤50)	97 (96)
1b	30 (40)	97 (99)
3	93 (95)	92 (**4**), ≥95 (**5**) (92 (**4**), >99 (**5**))

[a] General conditions for light-driven reactions: 10 μM PAMO P3, 25 mM EDTA, 100 μM FAD, 250 μM NADP$^+$, 1 or 2 mM substrate, 50 mM Tris (pH 7.4); $T = 30$ °C, 100 W Osram white light bulb. Values given in parentheses refer to data obtained in the conventional NADPH-based process[7] and are included here for comparison.

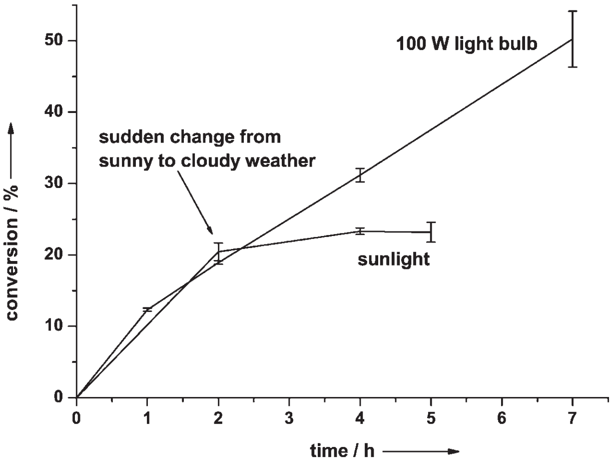


Figure 1. Time-resolved conversion of the light-driven BV reaction of *rac*-**1a**. Conditions for sunlight-driven reactions: 10 μM PAMO P3, 25 mM EDTA, 100 μM FAD, 250 μM NADP$^+$, 1 mM substrate, 50 mM Tris (pH 7.4). Reaction vessels were placed on aluminum foil and exposed to sunlight. For comparison, the reaction employing a lamp as light source is also shown.

Control experiments corroborated the mechanistic postulate of Scheme 1B, including reactions in the absence of either one of the components shown in Scheme 1B (i.e. light, EDTA, FAD, or PAMO), with each experiment showing no conversion of **1a**. It is therefore clear that the tris(hydroxymethyl)aminomethane (Tris) base used as buffer is not the source of electrons under these reaction conditions. Moreover, a putative flavo-H_2O_2-shunt pathway[4d] was excluded, as in the presence of H_2O_2, FAD, and PAMO no BV oxidation was found to occur (see below). Control experiments also showed that FAD can be replaced by riboflavin or FMN, which proves that no significant exchange of reduced FAD with protein-bound cofactor occurs. In further experiments, we found no evidence for the photoreduction of NADP$^+$. The apparent necessity for non-enzyme-bound FAD is in accord with previous findings, suggesting a catalytic role of free flavin in the reduction of the enzyme-bound cofactor, which is otherwise inefficient (see Supporting Information).[5b]

In the case of ketone **1b**, the reaction was likewise successful, delivering essentially enantiomerically pure lactone (*R*)-**2b** (97% *ee*) in 30% yield. Finally, the BV reaction of *rac*-**3** afforded as expected a 1:1 mixture of (−)-**4** (92% *ee*) and (−)-**5** (95% *ee*) in 90% total yield. Thus, all of these light-driven BV oxidations show essentially the same stereo- and regioselectivity as the corresponding reaction using the mutant P3 with the native NADPH as reductant.[7]

Inspired by the encouraging results obtained using an artificial source of light, we decided to utilize the most abundant source of energy available, namely sunlight. This "green" regeneration system provided results comparable to those obtained using a light bulb. For example, in the case of ketone **1a** two hours of bright sunshine in central Europe induced the expected stereoselective transformation, with a conversion of 20% reached under such conditions. As the weather turned cloudy, the conversion began to level off (Figure 1). Compared to the use of a light bulb, the rates were similar, whereas the conversion was limited as a result of the sudden weather change.

Note that under the current conditions, the catalytic performance of PAMO is significantly lower than in cases where a conventional regeneration system is used (Table 2).[7b]

Table 2: Performance of the light-driven BVMO regeneration in comparison with a native regeneration system and an electrochemical system of comparable simplicity.

Enzyme	Mediator	Souce of reducing equivalents	TOF[a] [h^{-1}]	TON[b]	
				Enzyme	Mediator
PAMO[c]	FAD	EDTA, *hν*	10	96	9.6
StyA[9b]	FAD	cathode	104	26	0.2
PAMO[7b]	NADPH[d]	2-propanol	394	9471	400

[a] TOF: catalyst turnover frequency (initial rate). [b] TON: total turnover number. [c] This study. [d] NADPH regeneration by a coupled enzyme.[7b]

Several reasons may account for this reduced performance of the novel light-driven approach. Decoupling of the regeneration from the enzymatic oxidation reaction to result in formation of H_2O_2 can in principle severely impair enzyme activity and stability. Therefore, we investigated the influence of H_2O_2 on PAMO activity (Figure 2). PAMO proved to be remarkably resistant to elevated concentrations of H_2O_2. Thus, any inactivation of the enzyme caused by H_2O_2 accounts only partially for the reduced performance. Therefore, we suspect slow electron-transfer kinetics to be rate-limiting under the present conditions, which is also in accord with the linear course of the reaction (Figure 1). Further experiments are underway to elucidate this hypothesis.

In summary, we have devised and implemented experimentally for the first time a catalytic scheme for a light-driven flavin-dependent enzymatic reaction. Using light as a source of energy and EDTA as a reservoir of electrons,

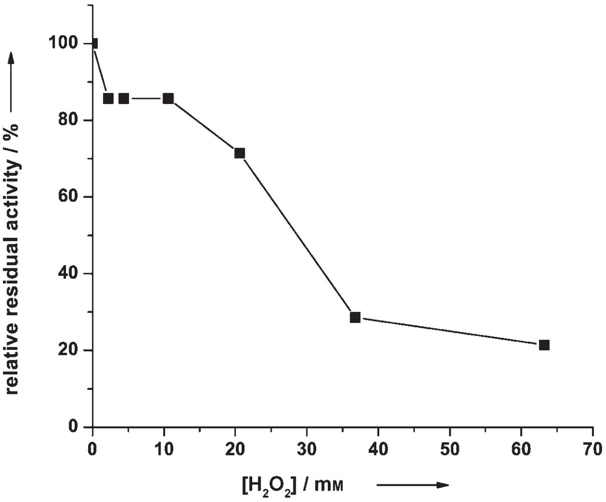


Figure 2. Tolerance of hydrogen peroxide by PAMO-P3. Preparations of PAMO P3 were incubated in the presence of various concentrations of H_2O_2 for 20 h. Subsequently, residual activities were determined using a NADPH-depletion assay.[7] The graph shows the residual activities relative to a sample incubated in the absence of H_2O_2.

expensive stoichiometric amounts of NADPH or a complicated regeneration system can be avoided. Thus, the overall complexity of the system is greatly reduced. Despite the early developmental stage of the novel photoenzymatic BV oxidation (optimization was not strived for in this study), multiple catalytic turnover for all reaction components was achieved. Other simplified systems have also been reported in the literature.[2m,9] The system with the highest degree of simplification is based on a cathode that was used as the source of reducing equivalents (Table 2). Under optimized conditions, significantly higher rates were obtained at the expense of a noncatalytic performance of the mediator FAD, which suggests an even higher degree of decoupling.[9b] In addition, the rather harsh reaction conditions led to a rapid deactivation of the enzyme. In contrast, our system was stable for at least seven hours with no indication of decomposition of the enzyme (Figure 1). So far, the process has been performed using small amounts of substrate and catalyst. Upscaling requires additional work utilizing the two-liquid phase system described earlier for PAMO and its mutants.[7] The present light-driven BV reaction can be expected to work just as well using other flavin-dependent Baeyer–Villigerases such as cyclohexanone monooxygenase (CHMO), which accepts a broader range of substrates.[2g]

Possible effects of the nature and power of the light source, the flavin cocatalyst, and the sacrificial electron donor will be investigated in more detail. Furthermore, we are on the way to expand the scope of the photoenzymatic reaction system to include other flavin-dependent enzymes to perform other oxidation and reduction reactions.

Experimental Section

A typical light-driven reaction was set up as follows: A mixture (final reaction volume 250 μL) containing 10 μM PAMO-P3, 25 mM EDTA, 100 μM FAD, 250 μM NADP$^+$, 1 mM or 2 mM substrate, and 50 mM Tris-HCl (pH 7.4) was incubated under aerobic conditions at 30 °C in a water bath exposed to the light of a 100-W OSRAM white light bulb (CLASSIC A, CLAS A CL 100, 230 V, E27/ES). The light was filtered through 1 cm water and approximately 0.5 cm DURAN glass. The approximate distance between the light source and the reaction vessels was 6 cm. The reaction mixture was extracted with ethyl acetate (275 μL; containing 40 μM *n*-hexadecane GC standard for 2-phenyl cyclohexanone) and analyzed by GC and GC/MS using authentic standards. For all experiments described here, PAMO-P3 was produced and purified by affinity chromatography as described previously[7a] to allow for accurate quantification of the results. However, the reaction works as well using crude enzyme as obtained after bacterial lysis.[7b]

Received: December 21, 2006
Revised: January 29, 2007

Keywords: asymmetric catalysis · cofactors · enzyme catalysis · kinetic resolution · photochemistry

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