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Deazaflavins as mediators in light-driven cytochrome P450 catalyzed hydroxylations†

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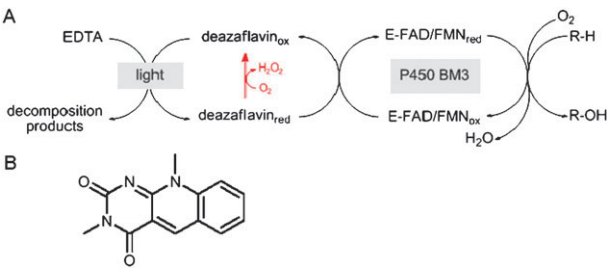
A light-driven deazaflavin-dependent direct enzyme regeneration system has been developed for a P450-BM3 catalyzed CH-activating hydroxylation, thereby avoiding the need for the expensive NADPH cofactor.

The P450 monooxygenase BM3 from *Bacillus megaterium*,[1,2] like other members of the P450 family, catalyzes the monooxygenation of non-activated substrates with concomitant consumption of NADPH and molecular oxygen. Unlike other protein family members, it represents a fusion protein comprising a monooxygenase and a reductase domain. The diflavin-reductase domain is reduced by NADPH. Subsequent electron transfer to the heme domain of the monooxygenase domain allows for initiation of a catalytic cycle. This domain fusion ensures a highly concerted catalytic activity (up to 17 100 min^{-1} for hydroxylation of arachidonic acid)[3] and makes the protein an interesting candidate for industrial applications.[4,5] The requirement of the unstable and prohibitively expensive cofactor NADPH, however, hinders broad application. Hence, enzyme coupled cofactor recycling systems[6–9] or whole cell catalysis[10–12] are used to achieve regeneration of NADPH. Both approaches have their respective drawbacks such as undesired substrate/product oxidation and limited substrate accessibility due to membrane impermeability.[13,14] Other approaches utilize artificial electron mediators and aim to directly regenerate P450-BM3.[15–17] Herein, we report an alternative approach based on the use of deazaflavins in a light-driven process.

Recently, we described a light-driven direct cofactor regeneration system for the flavin-dependent Baeyer–Villiger monooxygenase PAMO and the enoate-reductase YqjM, deriving reducing equivalents from the inexpensive sacrificial electron donor EDTA.[18,19] In this system flavin mediators such as FAD, FMN or riboflavin are excited by irradiation with visible light which allows for reaction with the sacrificial electron donor to generate a reduced flavin mediator. Subsequent electron transfer to the enzyme-bound flavin cofactor initiates enzyme catalysis (Scheme 1). In this process EDTA decomposition leads to formation of side products.[20] The presence of oxygen, which is obviously required for a monooxygenase, was suspected to be a major limitation of this system,[18] leading to the decoupling of the regeneration reaction from the enzymatic reaction due to the rapid reaction of reduced flavin species with molecular oxygen (Scheme 1).[21] Such a decoupling results in a waste of reduction equivalents, inefficient electron transfer and the generation of reactive oxygen species such as hydrogen peroxide which can be detrimental to enzyme activity.

In order to avoid the decoupling reaction, we recently proposed the use of deazaflavins,[18] since reduced deazaflavins were reported to react very slowly with molecular oxygen.[22] Furthermore, it has been demonstrated in mechanistic studies and sensor applications that rat liver P450 reductase[23] and rabbit liver P450-2B4 monooxygenase[24,25] can undergo reduction in the presence of soluble flavins, sacrificial electron donors and light in an oxygen-free system. However, to the best of our knowledge this type of electron transfer has never been used to achieve catalysis, *i.e.*, product formation with P450 monooxygenases. By choosing deazaflavins as oxygen-stable electron mediators, we hoped to achieve robust and efficient light-driven direct regeneration of a P450 monooxygenase for the first time.

As the model system, we chose the P450-BM3 catalyzed oxidation of lauric acid. Reduction of the heme domain represents the first step of the catalytic cycle in P450 monooxygenase catalysis. Therefore, this step was studied in exploratory experiments by shining light from a 100 Watt lamp onto aerobic samples comprising purified BM3-WT, EDTA and various types of soluble flavins. Selected samples



Scheme 1 Mechanism of the light-driven P450-BM3 catalyzed hydroxylation (A) and structure of the deazaflavin mediator, 3,10-dimethyl-5-deazaflavin (B). The decoupling of the regeneration reaction from the enzymatic reaction can be largely prevented by using a deazaflavin mediator and therefore the formation of reactive oxygen species such as H_2O_2 (red pathway) is essentially suppressed.[21]

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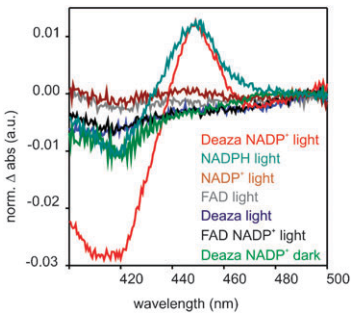

Fig. 1 Formation of the characteristic 450 nm absorption band in the CO difference spectra obtained by light-driven reduction of the P450-BM3 heme domain.

were further spiked with $NADP^+$. The successful reduction of the heme domain was monitored by the characteristic Fe^{II}(heme)(CO) absorption band at 450 nm in the CO difference spectra. A reduction of the heme can be monitored by the appearance of the 450 nm absorption band after purging the sample with CO.[26] CO difference spectra show that under aerobic conditions a pronounced reduction of the P450-BM3 heme domain is achieved only in the presence of deazaflavin with additional $NADP^+$ or under positive control conditions (NADPH). In contrast to this, FAD as a mediator failed to yield notable reduction of the heme domain.

This result corroborates our hypothesis of superior electron-mediator properties of deazaflavins compared to flavins of the isoalloxazine-type (FAD, FMN, riboflavin) under aerobic conditions (Fig. 1).

To study if the light-driven reduction of P450-BM3 can drive catalytic oxyfunctionalizations, we performed the reaction in the presence of lauric acid, a typical BM3-WT substrate,[27] and analyzed the amount of product formed after 17 h (Fig. 2). As a control, the deazaflavin was omitted or replaced by FAD under otherwise identical conditions. Some samples were supplemented with $NADP^+$, since this has previously been shown to improve PAMO catalyzed light-driven reactions.[18,19] As expected, in the absence of light NADPH leads to almost complete conversion of lauric acid.

Deazaflavin shows in the corresponding experiment a strong and light-dependent conversion if $NADP^+$ is present as well. No substantial hydroxylation is observed in the presence of deazaflavin or $NADP^+$ alone or in the absence of flavins. Furthermore, FAD as an electron mediator does not lead to hydroxylation, even if supplemented with $NADP^+$. In order to test whether the rather unstable P450-BM3 reductase domain is required for the light-driven reaction, we performed corresponding experiments with the isolated P450-BM3 heme domain. Under our set of conditions no product formation was observed. In another system based on photoexcitation reduction of the heme was achieved under anaerobic conditions.[28]

Analysis of the product distribution of hydroxylated lauric acid, achieved when using full-length P450-BM3, was found to be essentially the same as in the normal reaction. This shows that regioselectivity is not altered under the conditions of the light-driven recycling system (see ESI† Table S1).

Next, the kinetics of the light-driven hydroxylation of lauric acid were investigated (Fig. 3). The use of NADPH in a conventional process leads to a quick and complete hydroxylation of lauric acid within one hour. The light-driven reaction proceeds at a significantly lower rate (only 12% conversion after one hour). Enzyme stability was apparently not impaired since product formation steadily continued for 17 h, reaching 74% conversion. Increasing the biocatalyst concentration did not significantly alter the course of the reaction (data not shown), indicating that the light-driven regeneration is rate limiting.

The turnover frequency (TOF) was estimated to be 117 h^{-1} (Table 1). This rate is about 2000 times lower than that observed for P450-BM3 using NADPH as a cofactor ($\sim$5000 min^{-1}).[3] However, it is in the same range as the previously described catalytic rate of eukaryotic P450s.[35]

Among the light-driven regeneration systems the deazaflavin system shows the highest turnover number (TON) and is in the range of the best reported direct regeneration systems for cytochrome P450 enzymes (Table 1). The TOF is in the same order of magnitude as the oxygen independent YqjM system and about 10 fold higher than for using sodium dithionite as reducing agent. Although the present deazaflavin-based system is a very efficient direct regeneration system, so far it cannot compete with well-studied enzymatic NADPH-recycling systems. In contrast to the light-driven regeneration, in many other cases of direct regeneration of monooxygenases, the turnover frequency observed for the enzyme exceeds the total turnover number. This suggests a low biocatalyst stability in these systems due to the unnatural reaction conditions. The light-driven regeneration approach also has the advantage that it only uses inexpensive and environmentally benign

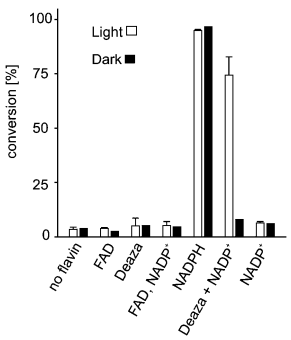

Fig. 2 Light-driven hydroxylation of lauric acid by P450-BM3 under aerobic conditions for 17 h. Light-driven reactions: $n = 3$, error bars correspond to the standard deviation; dark reactions: $n = 1$.

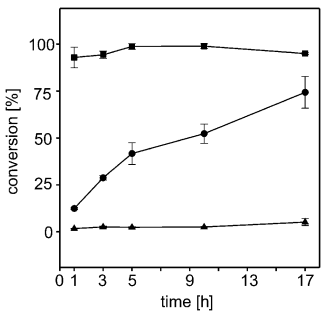

Fig. 3 Time-course of light-driven hydroxylation of lauric acid by P450-BM3. The experiment was performed as described in Fig. 2. ■: NADPH, ●: Deazaflavin + $NADP^+$, ▲: FAD + $NADP^+$. All $n = 3$. The error bars correspond to the standard deviation.

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Table 1 Comparison of direct flavo-enzyme regeneration systems

Enzyme, reference	Mediator	Source of reducing equivalents	TOF [h^{-1}]	TON	
				Enzyme	Mediator
PAMO-P3[18]	FMN, *hν*	EDTA	10	96	9.6
YqjM[18,29]	FMN, *hν*	EDTA	194	383	10
P450-BM3 (this study)	Deazaflavin, *hν*	EDTA	117	698	7.4
StyA[30]	FAD	Cathode	104	26	0.2
P450-BM3[15]	Cobaltocene cation	Cathode	984	224	n/a[a]
P450-BM3[15]	Cobalt (III) sepulchrate	Cathode	2268	835	n/a[a]
P450-BM3[31]	Dithionite	Dithionite	17.4	n/a[a]	n/a[a]
P450-BM3[32]	NADPH	NADPH	4866	n/a[a]	n/a[a]

[a] n/a = not available. Further direct regeneration systems have been reported for other P450 monooxygenases.[33,34]

components in a simple setup. It should be noted that the present system was intended for a proof-of-principle and that an optimization of the reaction conditions could lead to an even more efficient setup.

Our experiments show that only the deazaflavin mediator in the presence of catalytic amounts of $NADP^+$ with respect to the substrate is capable of mediating productively the light-driven recycling pathway. The fact that the presence of $NADP^+$ is essential might be explained by NADP-induced structural alterations necessary for catalysis, as previously suspected for the Baeyer–Villiger monooxygenase PAMO.[18] A putative indirect pathway *via* the reduced nicotinamide cofactor was previously excluded since the presence of $NADP^+$ was not necessary for the light-driven regeneration of the reductase YqjM.[18] Furthermore, control experiments using an alcohol dehydrogenase-based detection for NADPH exclude this possibility as well (see ESI† Fig. S1).

In summary, we report the first light-driven cytochrome P450 catalyzed monoxygenation using a soluble deazaflavin mediator in combination with an inexpensive sacrificial electron donor. By using deazaflavins as electron mediators, instead of normal flavins, a light-driven regeneration of flavin-dependent enzymes can also proceed efficiently under aerobic conditions, possibly due to suppression of the decoupling of the regeneration reaction from the enzymatic reaction (Scheme 1). Furthermore, the unnatural reaction conditions do not alter the regioselectivity of the P450 catalyzed hydroxylation. The light-driven regeneration system presented here requires only a simple setup without additional regeneration enzyme and appears to be generally applicable to flavin-dependent enzymes.

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Notes and references

1 L. O. Narhi and A. J. Fulco, *J. Biol. Chem.*, 1982, **257**, 2147–2150.
2 L. P. Wen and A. J. Fulco, *J. Biol. Chem.*, 1987, **262**, 6676–6682.
3 M. A. Noble, C. S. Miles, S. K. Chapman, D. A. Lysek, A. C. MacKay, G. A. Reid, R. P. Hanzlik and A. W. Munro, *Biochem. J.*, 1999, **339**(Pt 2), 371–379.
4 V. B. Urlacher and S. Eiben, *Trends Biotechnol.*, 2006, **24**, 324–330.
5 M. K. Julsing, S. Cornelissen, B. Buhler and A. Schmid, *Curr. Opin. Chem. Biol.*, 2008, **12**, 177–186.
6 S. C. Maurer, H. Schulze, R. D. Schmid and V. B. Urlacher, *Adv. Synth. Catal.*, 2003, **345**, 802–810.
7 R. Wichmann and D. Vasic-Racki, *Adv. Biochem. Eng. Biotechnol.*, 2005, **92**, 225–260.
8 H. Zhao and W. A. van der Donk, *Curr. Opin. Biotechnol.*, 2003, **14**, 583–589.
9 D. Holtmann and J. Schrader, in *Modern Biooxidation*, ed. R. Schmid and V. B. Urlacher, Wiley-VCH, Weinheim, 2007.
10 H. Schewe, B. A. Kaup and J. Schrader, *Appl. Microbiol. Biotechnol.*, 2008, **78**, 55–65.
11 S. Schneider, M. G. Wubbolts, D. Sanglard and B. Witholt, *Appl. Environ. Microbiol.*, 1998, **64**, 3784–3790.
12 U. Schwaneberg, C. Otey, P. C. Cirino, E. Farinas and F. H. Arnold, *J. Biomol. Screening*, 2001, **6**, 111–117.
13 R. R. Chen, *Appl. Microbiol. Biotechnol.*, 2007, **74**, 730–738.
14 Y. Ni and R. R. Chen, *Biotechnol. Bioeng.*, 2004, **87**, 804–811.
15 A. K. Udit, F. H. Arnold and H. B. Gray, *J. Inorg. Biochem.*, 2004, **98**, 1547–1550.
16 F. Hollmann and A. Schmid, *J. Inorg. Biochem.*, 2009, **103**, 313–315.
17 U. Schwaneberg, D. Appel, J. Schmitt and R. D. Schmid, *J. Biotechnol.*, 2000, **84**, 249–257.
18 A. Taglieber, F. Schulz, F. Hollmann, M. Rusek and M. T. Reetz, *ChemBioChem*, 2008, **9**, 565–572.
19 F. Hollmann, A. Taglieber, F. Schulz and M. T. Reetz, *Angew. Chem., Int. Ed.*, 2007, **46**, 2903–2906.
20 W. R. Frisell, C. W. Chung and C. G. Mackenzie, *J. Biol. Chem.*, 1959, **234**, 1297–1302.
21 V. Massey, *J. Biol. Chem.*, 1994, **269**, 22459–22462.
22 V. Massey and P. Hemmerich, *Biochemistry*, 1978, **17**, 9–16.
23 J. L. Vermilion and M. J. Coon, *J. Biol. Chem.*, 1978, **253**, 8812–8819.
24 J. A. Peterson, R. E. White, Y. Yasukochi, M. L. Coomes, D. H. O'Keeffe, R. E. Ebel, B. S. Masters, D. P. Ballou and M. J. Coon, *J. Biol. Chem.*, 1977, **252**, 4431–4434.
25 V. V. Shumyantseva, T. V. Bulko, R. D. Schmid and A. I. Archakov, *Biosens. Bioelectron.*, 2002, **17**, 233–238.
26 T. Omura and R. Sato, *J. Biol. Chem.*, 1964, **239**, 2370–2378.
27 S. S. Boddupalli, R. W. Estabrook and J. A. Peterson, *J. Biol. Chem.*, 1990, **265**, 4233–4239.
28 H. M. Girvan, D. J. Heyes, N. S. Scrutton and A. W. Munro, *J. Am. Chem. Soc.*, 2007, **129**, 6647–6653.
29 A. Taglieber, *PhD thesis*, Ruhr-Universität Bochum, Bochum, 2007.
30 F. Hollmann, K. Hofstetter, T. Habicher, B. Hauer and A. Schmid, *J. Am. Chem. Soc.*, 2005, **127**, 6540–6541.
31 X. Fang and J. R. Halpert, *Drug Metab. Dispos.*, 1996, **24**, 1282–1285.
32 L. A. Cowart, J. R. Falck and J. H. Capdevila, *Arch. Biochem. Biophys.*, 2001, **387**, 117–124.
33 K. M. Faulkner, M. S. Shet, C. W. Fisher and R. W. Estabrook, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7705–7709.
34 M. S. Shet, K. M. Faulkner, P. L. Holmans, C. W. Fisher and R. W. Estabrook, *Arch. Biochem. Biophys.*, 1995, **318**, 314–321.
35 C. Helvig and J. H. Capdevila, *Biochemistry*, 2000, **39**, 5196–5205.

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