

Supporting Information for:

Deazaflavins as mediators in light-driven cytochrome P450 catalyzed hydroxylations

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1) Product distribution of P450-BM3 catalysed hydroxylation

Table S1 Comparison of the product distribution for P450-BM3 catalyzed hydroxylation of lauric acid.

Data from this study shows that the regioselectivity of the P450-BM3 catalyzed hydroxylation does not depend on the enzyme regeneration system used. The light-driven recycling system does not alter the product distribution. Moreover the data presented here are in line with previously published results. N = 3.

	Product distribution %			
	ω -1	ω -2	ω -3	reference
Whole cell catalysis	40 ± 10–15%	27 ± 10–15%	33 ± 10–15%	1
NADPH	36	30	34	2
NADPH	38	28	34	3
NADPH	34 ± <5%	29 ± <5%	37 ± <5%	4
NADPH	35 ± ~5%	29 ± ~5%	36 ± ~5%	this study
hv, EDTA, deazaflavin	33 ± ~5%	29 ± ~5%	38 ± ~5%	this study

2) Analysis for the direct NADP⁺ reduction

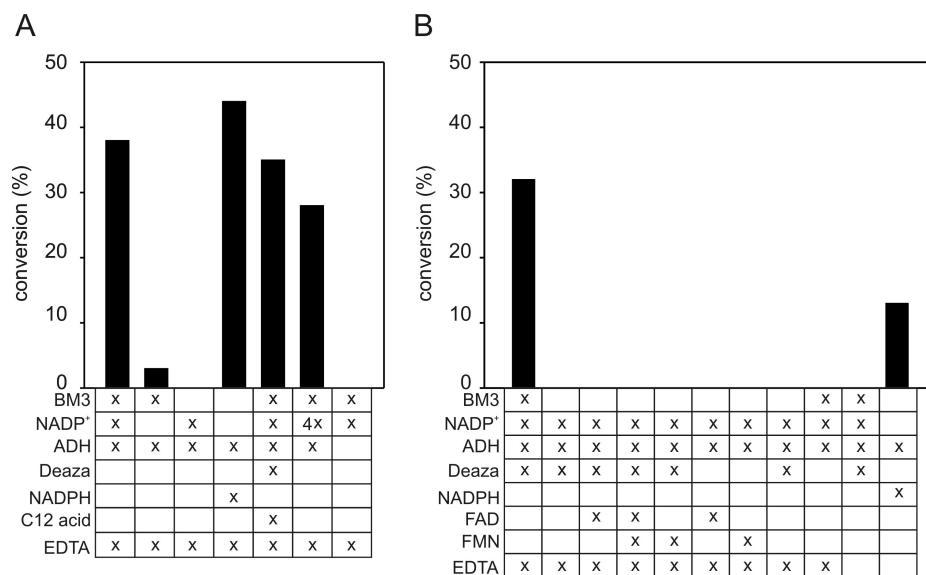


Figure S1 The Deazaflavin does not directly reduce NADP⁺ in light-driven reactions.

In order to investigate whether NADP⁺ is directly reduced by the deazaflavin during the light-driven reaction, we performed an enzyme assay which allows monitoring of the NADPH formation. In this assay, any formed or directly added NADPH is used by an alcoholdehydrogenase (ADH) to convert 2-octanon into 2-octanol. The formation of octanol indicates the formation of NADPH in the reaction mix using the standard setup.

Our results show that deazaflavin is not capable to directly reduce NADP^+ during the light-driven reaction. This finding indicates a transfer of electrons from the reduced deazaflavin directly to P450-BM3. For BM3-catalyzed product formation (hydroxylated lauric acid) the presence of NADP^+ is necessary, although NADP^+ apparently does not change its oxidation state. NADP^+ probably induces conformational changes allowing a more efficient electron transfer from deazaflavin to BM3 or within BM3 from the diflavin-reductase to the heme domain. Analogous observations were previously discussed for Baeyer-Villiger monooxygenases.⁵⁻⁶

Furthermore, we could show that if P450-BM3 is also present, NADPH is formed in the light-driven reaction. The addition of lauric acid or an increased NADP^+ concentration did essentially not alter the NADPH formation. This indicates that any conformational change initiated by substrate binding to BM3 has no influence on NADPH formation. Moreover, excess of NADP^+ seems not to compete with the deazaflavin for the BM3 binding site, leaving the NADPH formation at a similar level (Figure S1A).

In order to study whether NADPH is formed in the light-driven reaction in presence of P450-BM3 due to a specific effect or by presence of very small amounts of soluble flavin cofactors (FAD and FMN) in the protein preparation, we investigated the ability of mixtures of flavins, deazaflavin and NADP^+ to form NADPH. None of the tested condition lead to NADPH formation (Figure S1B). Hence, the deazaflavin mediated NADPH formation in presence of P450-BM3 might be explained in two ways. Firstly, the flavins bound to enzymes possess different reduction potentials than their soluble correspondents allowing different redox-reactions.⁷ Secondly, a transhydrogenase activity has been described for P450-BM3 and other flavin-dependent P450 reductases.^{8,9} In our system this secondary activity could allow the reduced enzyme to transfer electrons to NADP^+ as well (Figure S1B).

3) Experimental Section

Plasmid

The gene *p450-bm3/ cyp102a1* encoding P450-BM3 from *Bacillus megaterium* (ATTC 14581, DSM32) was cloned into the pETM11 vector (EMBL, Germany) carrying an N-terminal His-tag, as described previously,¹⁰ to give the vector pETM11-P450-BM3-WT.

Protein expression and purification

E. coli BL21 DE3 gold (Novagen) was transformed with the vector pETM11-P450-BM3-WT. An aliquot of an overnight culture in LB medium, supplemented with kanamycin (20 mg/l) and tetracycline (12,5 mg/l) for selection, was used to inoculate TB medium supplemented with 50 mg/l kanamycin, 0,1 g/l glutamate, 0,4% (v/v) glycerin, trace metals (50 μ M FeCl₃, 20 μ M CaCl₂, 10 μ M MnCl₂, 10 μ M ZnSO₄, 2 μ M CoCl₂, 2 μ M CuCl₂, 2 μ M NiCl₂, 2 μ M Na₂MoO₄, 2 μ M H₃BO₃) and 1 mM MgCl₂. After cultivation at 37 °C and reaching an OD_{600nm} of ~0.6–0.8, IPTG was added to a final concentration of 100 μ M, the temperature reduced to 25 °C and the shaking rate reduced from 250 rpm to 130 rpm. The expression was performed for 24 h. Afterwards the bacteria were harvested by centrifugation. The pellet was stored at -80 °C until further processing. For lysis of the bacterial cells, the pellet was thawed by adding lysis buffer (25 mM Tris, 20% glycerin, 0.1% Tween-20 and 20 mg /l DNase I (Applichem, Germany)) and resuspended. The cells were broken by passing the suspension through a French press (American Instruments Company, Silverspring, USA) at a pressure of 1200 psi. The collected lysate was centrifuged for 1 h at 9000 g. Subsequently, the supernatant was passed through a 0.4 μ m filter.

For analysis of the light-driven heme-domain reduction, the purification was continued as follows: The lysate was loaded on a His-Trap column (GE Healthcare, USA) using an ÄKTA purifier (GE Healthcare, USA) and the recombinant protein bound with 25 mM Tris/HCl, 15 mM imidazole at pH

8.0. By mixing the previous buffer with 25 mM Tris/HCl, 500 mM NaCl, 500 mM imidazole, a step-gradient was achieved to specifically elute P450-BM3. The enzyme containing fractions were pooled and liberated from imidazole using a PD-10 desalting column (GE Healthcare, USA).

For analysis of light-driven conversion the purification was continued as follows: The lysate was
5 desalted using 100 mM Tris/HCl pH 7.8 buffer on 4 in-line coupled HiTrap desalting columns (5 ml
bed volume each, GE Healthcare, USA) connected to an ÄKTA purifier (GE Healthcare, USA). The
eluate was fractionized and the most prominent red fractions pooled for anion exchange
chromatography. Anion exchange chromatography was performed similar to the previously described
protocol¹¹ using 21 ml Tosoh DEAE-5PW resin (Tosoh Bioscience, Japan) as stationary phase. The
10 desalted lysate was purified by a two step salt gradient (100 mM Tris/HCl pH 7.8; 1M NaCl, 100 mM
Tris/HCl pH 7.8). The red colored, enzyme containing fractions were analyzed by SDS-gel
electrophoresis for purity. The most concentrated and pure fractions were pooled and the enzyme
concentration determined by CO difference spectrum analysis.¹² The total protein content was
determined using the BioRad protein assay with a BSA dilution series as reference (BioRAD, USA).

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Analysis of the light-driven reduction of the P450-BM3 heme domain

In order to estimate the efficiency of the light-driven P450-BM3 reduction, a CO difference spectrum
analysis was used. This analysis leads to formation of the characteristic 450 nm absorption band upon
binding of CO to the reduced heme.¹² A mix containing 1,02 μ M enzyme, 25 mM EDTA, 100 μ M
20 flavins and 250 μ M nicotinamide cofactor (all final concentrations) was prepared and split into four
equal aliquots. Two were exposed to a 100 Watt white light bulb^{6,5} for 20 min at 30 °C. The other pair
was kept in the dark in the meantime. From each pair the difference spectrum was recorded using a
spectrometer (UV-2401PC, Shimadzu, Japan) after purging one aliquot from each pair with CO for 30
s.

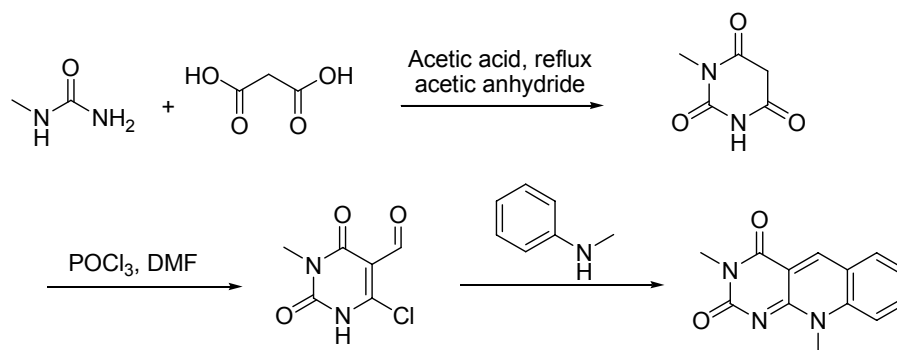
Analysis of the light-driven P450-BM3 catalyzed hydroxylation

A mix of 1.06 μM purified P450-BM3, 100 μM soluble flavin, 250 μM NADP^+ , 25 mM EDTA and 1 mM lauric acid was prepared, in order to study the light-driven biocatalysis (all final concentrations). As described earlier,⁵ the mix was exposed to white light from a 100 Watt light-bulb at 30 °C in a water bath. Dark controls were wrapped in aluminum foil. Light-driven reactions were performed in triplicates, for dark reactions only single reaction performed. Due to the buffered reaction medium the pH value of the sample essentially did not change during the course of reaction. After the indicated time the reactions were stopped. Afterwards 0.1 volumes 10% hydrochloric acid were added. As a GC standard 1 mM decanoic acid was added to the reaction mix before it was extracted twice with 1 volume of MTBE. The pooled organic phases were evaporated using a speed vac and the samples were recovered with 0.2 volume MTBE and analyzed by quantitative GC: 6890N (Agilent, USA); column: 15m DB-Free Fatty Acid Phase, inner diameter of 0.25 mm, film thickness of 0.25 μm , J&W, Germany; pressure: 0.5 bar H_2 ; injector: 240 °C; temperature gradient: 80 °C – 240 °C with 8 °C/min, 15 min isothermal. The conversion of lauric acid was calculated by comparison of the peak areas from lauric acid with the reaction standard decanoic acid, taking the fact of a 1.22 times bigger signal area for lauric acid than decanoic acid at the same concentration into account. The product distribution was calculated by setting the product peak areas directly into relation to each other. The TOF was calculated from the conversion (12%) after 1 h of light-driven reaction (mol product/mol enzyme / time of reaction). The TON are calculated for the enzyme and mediator after completion of 17 h reaction (mol product / mol enzyme or mol product / mol mediator).

ADH-experiments

Reaction mixtures having final concentrations of 0.6 μM P450-BM3, 1 U/ml ADH (# 002, Codexis, Jülich, Germany), 1 mM 2-octanon, 250 μM / 1mM NADP^+ (Codexis, Jülich, Germany), 300 μM (Figure S1A)/ 100 μM (Figure S1B) NADPH (Codexis, Jülich, Germany), 100 μM deazaflavin, 100 μM FAD (>90%, Fluka), 100 μM FMN (~85% HPLC, Fluka), 1 mM lauric acid and 25 mM EDTA were prepared. All samples were illuminated under aerobic conditions for 1 h at 30 °C. For analysis, the samples were acidified with 0.1 volumes 10% HCl and extracted with MTBE. The conversion of 2-octanon to 2-octanol was analyzed by GC for indirect detection of NADPH-formation (6890N, Agilent, USA); column: 30m Rtx-1 (Restek, USA), 100% dimethylpolysiloxane; inner diameter of 0.25 mm, film thickness of 0.25 μm ; 0.6 bar H_2 ; injector: 220 °C; temperature gradient: 60 °C – 200 °C with 6 °C/min, 200 °C–340 °C with 12 °C/min, 340 °C 5min isothermal; 350 °C FID).

Synthesis of 3,10-dimethyl-5-deazaflavin



The 3,10-dimethyl-5-deazaflavin was prepared according to the above scheme using previously reported procedures. *N*-methyl barbituric acid was prepared from malonic acid and methyl urea according to a procedure of M. T. Shamin *et al.*¹³ *N*-methyl barbituric acid was converted to 6-chloro-5-formyl-3-methyluracil under Vilsmeier-Haack conditions and subsequently converted to the 5-deazaflavin by reaction with *N*-methyl aniline according to a modified procedure of F. Yoneda.¹⁴

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