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Engineering Cytochrome P450 Enzymes for Improved Activity towards Biomimetic 1,4-NADH Cofactors

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Cytochrome P450s are heme-containing enzymes that can oxidize a broad range of compounds, often at unreactive carbon–hydrogen bonds, with high regio- and enantioselectivity. Two bacterial P450s, the "self-sufficient" fusion protein, P450 BM-3 from *Bacillus megaterium*, and the multicomponent P450, P450cam from *Pseudomonas putida*, are well-studied and serve as model P450s. These prokaryotic P450s also have potential as industrial catalysts because they are water soluble, easily expressed in a bacterial host, such as *Escherichia coli*, and are relatively stable in the presence of organic cosolvents. However, the application of isolated P450 enzymes for large-scale synthesis has been hindered by many obstacles, including the expense of the required cofactor, NAD(P)H[1] (Figure 1). The search for alternative cofactors and/or regeneration systems has thus been met with great interest. Several alternative electron sources have been evaluated for their ability to support P450 catalysis, including sodium dithionite,[2] ascorbic acid,[3] and electrochemical means;[4,5] however, substrate conversion and initial rates for such systems are significantly lower compared to those that use NAD(P)H.[6] An ideal alternative cofactor should be inexpensive to synthesize, should not inhibit the P450 catalyst, and should support a rate of substrate oxidation that is at least comparable to that obtained with the natural cofactor.

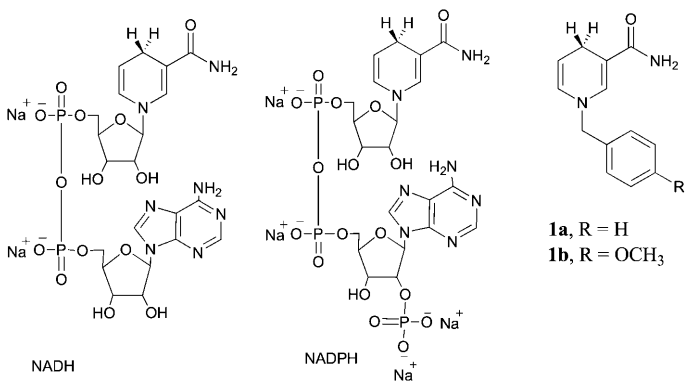

Figure 1. Structures of natural and biomimetic cofactors.

N-Benzyl-1,4-dihydronicotinamide (**1a**, Figure 1) is a biomimetic NADH analogue that has been used previously with two NADH-dependent oxidoreductases. Lo and Fish showed that **1a** (generated in situ with $[Cp^*Rh(bpy)H]^+$) can be used by horse liver alcohol dehydrogenase (HLADH) to catalyze the reduction of achiral ketones to chiral alcohols,[7] while Lutz et al. reported that **1a** can supply the electrons necessary for phenol oxidation by 2-hydroxybiphenyl 3-monooxygenase, HbpA.[8] Surprisingly, to date there have been no reports of **1a** or any other NADH biomimic being used with a cytochrome P450. Therefore, the objectives of the present study were to evaluate the ability of **1a** and *N*-4-methoxybenzyl-1,4-dihydronicotinamide, **1b** (Figure 1), to support oxidation reactions catalyzed by cytochrome P450s, and to determine whether the activity of P450s towards biomimetic cofactors could be improved by protein engineering.

In a previous study directed toward improving P450 BM-3 for the industrially-relevant oxidation of cyclohexane, Maurer et al. created the mutants W1064A, W1064S, and R966D/W1064S, all of which enabled the substitution of NADPH with NADH, and thereby reduced the overall process cost. The K_M and k_{cat} values for the NAD(P)H-dependent reduction of cytochrome c by the mutant P450s were compared, and the double mutant, R966D/W1064S, had the greatest catalytic efficiency (k_{cat}/K_M) for reactions with NADH. The double mutant also had a lower preference for NADPH over NADH than the wild-type enzyme;[9] therefore, we generated the same mutant and assayed its ability to reduce cytochrome c with NADH or biomimics **1a** and **1b** (Table 1).

The catalytic constants reported in Table 1 for NADH exhibit the same trend, but differ somewhat from those reported by Maurer and co-workers. This is most likely because the two variables known to influence K_M and k_{cat}—the ionic strength of the assay buffer and the concentration of cytochrome c—

Table 1. K_M and k_{cat} values for cytochrome c reduction by wild-type and double mutant P450 BM-3 by using NADH, **1a**, or **1b** as the cofactor.

P450 BM-3	Cofactor	K_M[a] [μM]	k_{cat}[a] [min^{-1}]	k_{cat}/K_M[a] [min^{-1} μM^{-1}]
wild-type	NADH	810	346	0.43
	1a	no reaction	–	–
	1b	no reaction	–	–
W1064S/R966D	NADH	10.5	4260	405
	1a	29.5	1220	41.3
	1b	96.0	2500	26.0

[a] The reported values are the averages of three measurements for which the standard deviations were less than 10%.

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could have differed between our assays and those of Maurer et al. More importantly, the R966D/W1064S double mutant was capable of using **1a** and **1b** to reduce cytochrome c, whereas the wild-type enzyme had no activity with either biomimic. Furthermore, the catalytic efficiencies for the double mutant that used both **1a** and **1b** were much greater than that of the wild-type enzyme with NADH.

p-Nitrophenoxydecanoic acid (*p*-NCA) is a model substrate for P450 BM-3, which forms ω-oxydecanocarboxylic acid and releases *p*-nitrophenol upon hydroxylation.[10] The wild-type enzyme does not hydroxylate *p*-NCA with either **1a** or **1b**, as evidenced by the lack of *p*-nitrophenol detection after 1 h. However, both NADH biomimics support *p*-NCA hydroxylation by the R966D/W1064S double mutant. Moreover, the double mutant achieved the same conversion for *p*-NCA oxidation (>95%) with NADPH, NADH, or **1a** as did the wild-type enzyme with its preferred cofactor, NADPH. However, reactions with the double mutant and **1b**—a biomimetic cofactor with a *p*-methoxy group for potential enhanced molecular recognition at the cofactor binding site—provided only a 42% conversion based on the initial concentration of *p*-NCA.

The initial rates of *p*-NCA hydroxylation with wild-type and W1064S/R966D P450 BM-3 are shown in Table 2. The reactions with the wild-type enzyme and NADPH had the highest initial rate (43 nmol s^{-1} per mg BM-3); however, the initial rate of the double mutant reaction with **1a** was only 1.9-fold slower. The initial rate was 1.6-fold slower with **1b** than with **1a**; this indicates that the *p*-methoxy group might not be available for noncovalent interactions at the cofactor binding site. These results clearly demonstrate that protein engineering can be used to generate a mutant of P450 BM-3 that has good activity with at least two NADH biomimics toward which the wild-type enzyme was inactive.

Table 2. Initial rates of *p*-NCA hydroxylation by wild-type and double mutant P450 BM-3 by using NADPH, NADH, **1a**, or **1b** as the cofactor.

Cofactor	Rate [nmol s^{-1} per mg BM-3]	
	WT[a]	W1064S, R966D[a]
NADPH	43.6	30.4
NADH	1.6	34.5
1a	no reaction	23.3
1b	no reaction	14.7

[a] Reactions contained 60 μM *p*-NCA, 200 μM cofactor, and 0.13 μM wild-type or 0.15 μM W1064S/R966D P450 BM-3. The reported values are averages of three measurements for which the standard deviations were less than 10%.

Although P450 BM-3 is a good model system for reductase proteins that have both an FAD and FMN ligand, such as human P450 reductase,[11] the reductase of P450cam is a more appropriate model for other oxygenase-coupled NADH-dependent ferredoxin reductases (ONFRs) that are necessary for electron transport to a number of hydroxylases, including alkane and benzene/diphenyl hydroxylases.[12] Unlike wild-type (WT) P450 BM-3, WT P450cam was able to catalyze substrate oxidation by using both **1a** and **1b** (Table 3). In contrast to the results for P450 BM-3, the initial rate of WT P450cam was nearly the same with both **1a** and **1b**, but these initial rates were much less than the rate with the natural cofactor, NADH. Cytochrome P450cam requires two electron transfer proteins for catalytic activity: putidaredoxin reductase (PdR) and putidaredoxin (PdX). Previous work showed that the initial rate of WT P450cam varied with changing PdX and P450cam concentrations when NADH was the cofactor.[13,14] However, when either **1a** or **1b** was used, the rate of camphor oxidation was not influenced by the concentration of either PdX (range: 0.5–7.5 μM) or P450cam (range: 0.5–1.5 μM). In contrast, the initial rate increased 1.3-fold when the concentration of PdR was increased from 0.75 to 2.8 μM; this suggests that the reduction of the FAD ligand within PdR was rate-limiting when **1a** or **1b** was used, and that the rate could be further improved by using a mutant of PdR that is more active towards the NADH biomimics.

Table 3. Initial rates of P450cam-mediated camphor oxidation to hydroxyl camphor supported by **1a**, **1b**, or NADH.

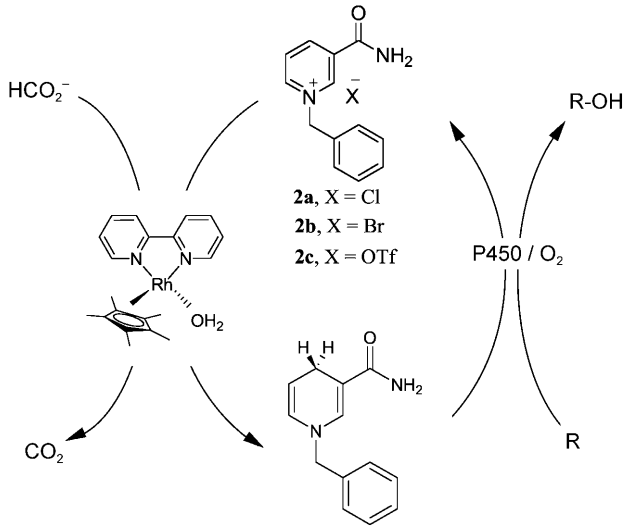
Cofactor	Reductase	Initial rate [nmol s^{-1} per mg P450cam]
1a[a]	WT	0.72
	E300A	1.80
	E300L	0.92
1b[a]	WT	0.82
	E300A	1.93
	E300L	1.11
NADH[b]	WT	130
	E300A	78
	E300L	125

[a] Reactions contained 5 mM of either **1a** or **1b**, 1.0 mM camphor, 1.5 μM P450cam, 7.5 μM PdX, and 2.8 μM PdR. [b] Reactions contained stoichiometric amount of NADH and 0.5 μM P450cam, 2.5 μM PdX, and 0.25 μM PdR. The reported values are averages of three measurements for which the standard deviations were less than 10%.

There is no tryptophan residue corresponding to W1064 of P450 BM-3 in the NADH binding pocket of PdR. Instead, the corresponding residue is a tyrosine (Y159), which has a different conformation relative to the FAD than W1064 of P450 BM-3.[15] Therefore, we chose to mutate E300, which is proximal to Y159 and participates in hydrogen bonding with the ribose ring closest to the nicotinamide portion of NADH.[15] Two single point mutations, E300A and E300L, were introduced, and the mutants were assayed for camphor oxidation with both **1a** or **1b** (Table 3). Both mutations improved the initial rate of camphor oxidation with the NADH analogues, and E300A afforded a greater increase than E300L for both **1a** and **1b**.

Pertinently, NADH biomimics will be less expensive to prepare than NAD(P)H, and regeneration of these analogues could further improve the cost efficiency of P450 reactions. Steckhan and co-workers demonstrated that NAD^+ could be reduced to NADH using an organorhodium catalyst.[16] Therefore, as before,[7] we used the same non-enzymatic strategy with P450

reactions to regenerate the cofactor in situ. Reactions included 5 mM **2a**, **2b**, or **2c**, [Cp*Rh(bpy)(H_2O)](OTf)$_2$ as the catalyst precursor, and sodium formate as the hydride source for cofactor regeneration (Scheme 1). As shown in Table 4, the initial rate of substrate oxidation depends on the anion associated with the oxidized analogue (**2a**–**c**). In general, reactions containing triflate anions (**2c**) were faster than those with chloride (**2a**) or bromide anions (**2b**). Moreover, the initial rates of substrate oxidation by both P450 s are nearly the same; this suggests that the rate of biomimic reduction by the organorhodium catalyst is rate limiting.



Scheme 1. In situ cofactor regeneration system in tandem with P450 oxidation by using [Cp*Rh(bpy)(H_2O)](OTf)$_2$ as the catalyst precursor and sodium formate as the hydride source.

Table 4. Initial rates and TTN of camphor oxidation by P450cam and *p*-NCA oxidation by W1064S/R966D P450 BM-3 with cofactor regeneration of **1a** from **2a**, **2b**, or **2c** by using [Cp*Rh(bpy)(H_2O)](OTf)$_2$ and sodium formate.

P450	Cofactor	Initial rate[a] [pmol s^{-1} per mg P450]	TTN[a]
P450cam	**2a**	118	33
	2b	90	24
	2c	150	38
W1064/R966D P450 BM-3	**2a**	125	264
	2b	83	180
	2c	144	312

[a] The reported values are the averages of three measurements for which the standard deviations were less than 10%.

When assayed after incubation with 50 μM [Cp*Rh(bpy)(H_2O)](OTf)$_2$ for 1 h, P450cam retained only 10% of its activity, whereas P450 BM-3 was relatively stable in the presence of the organorhodium complex and maintained 70% of its activity under the same conditions. It is possible that the organorhodium precatalyst binds in the substrate binding domain of P450cam more so than P450 BM-3, and the structural differences between the active sites of P450cam and P450 BM-3 account for the difference in observed inhibition. Table 4 also reports the total turnover numbers (TTN), defined as the concentration of product formed divided by the enzyme concentration. The TTN were much larger for P450 BM-3 than for P450cam, owing to the greater stability of P450 BM-3 than P450cam under the reaction conditions.

We also measured the rate of NAD^+ reduction by the organorhodium hydride complex after incubation for 1 h with P450 BM-3, P450cam, or bovine serum albumin. None of these proteins inhibited the activity of the organorhodium hydride complex. Thus, protein lysine residues did not appear to interact with and inhibit the rhodium complex, as suggested by Lutz et al.[8] Although the organorhodium precatalyst could inhibit the enzyme, the enzyme does not appear to inhibit cofactor regeneration.

In conclusion, WT P450cam oxidation reactions can be supported by **1a** and **1b**, but WT P450 BM-3 was not active with these 1,4-NADH biomimetic analogues. While mutations of a key residue in the reductase (PdR) for P450cam afforded only a small increase in initial rate, mutations in the P450 BM-3 reductase domain enabled the use of NADH analogues at rates comparable to that of the natural cofactor. Presumably, other P450 and oxidoreductase mutants with comparable or even greater activity than the wild-type enzymes with the biomimics can be generated with protein engineering, although the utility of such systems in practice will also depend on their operational stability and susceptibility to uncoupling. Furthermore, P450 BM-3 was stable in the presence of [Cp*Rh(bpy)(H_2O)](OTf)$_2$, and non-enzymatic cofactor regeneration is promising for select enzymatic reactions under optimized buffer conditions. Protein engineering in combination with chemically-catalyzed cofactor regeneration is thus an important strategy for utilizing economically advantageous NADH biomimics in P450-catalyzed oxidation reactions.

Experimental Section

Synthesis of NADH biomimics and [Cp*Rh(bpy)(H_2O)](OTf)$_2$: Methods described by Lutz et al.[8] were used to synthesize *N*-benzylnicotinamide salts (**2a**–**c** and *N*-4-methoxybenzylnicotinamde) and **1a** from **2b**. Compounds **1b** and [Cp*Rh(bpy)(H_2O)](OTf)$_2$ were synthesized according to Lo et al.[17]

P450cam activity assays with 1a and 1b: The activity of P450cam with the NADH analogues was measured in phosphate buffer (20 mM), with KCl (50 mM), at pH 7.4. Reactions (250 μL) contained camphor (1.0 mM). Unless otherwise specified, the reactions contained PdR (2.8 μM), PdX (7.5 μM) and P450cam (1.5 μM). A stock solution (100 mM) of each analogue was prepared in DMSO immediately prior to the assay, and was added (2.5 μL) to initiate the reaction. To terminate the reactions, acetone was added (100 μL), and the aqueous phase was extracted with hexane (250 μL) containing decane (1.0 mM) as an internal standard. The concentrations of hydroxycamphor and camphor in the extract were analyzed by gas chromatography.

P450cam activity assays with [Cp*Rh(bpy)(H_2O)](OTf)$_2$: Assays were conducted in phosphate buffer (20 mM) prepared by combining KH_2PO_4 (20 mM) and K_2HPO_4 (20 mM) to achieve a pH of 7.4. The reaction (250 μL) contained P450cam (1.5 μM), PdX (7.5 μM),

PdR (2.8 μM), **2a**, **2b**, or **2c** (5.0 mM), [Cp*Rh(bpy)(H_2O)]OTf_2 (50 μM), sodium formate (150 mM), and camphor (1.0 mM). Reactions were stopped and analyzed as stated above.

P450 BM-3 activity assays with 1a and 1b: Tris buffer (0.1 M, pH 8.0) was used for P450 BM-3 assays with **1a** and **1b**. Reactions were carried out in 96-well plates. The final reaction volume was 250 μL and the final DMSO concentration was 1.6%. A *p*-nitrophenoxydecanoic (*p*-NCA) stock solution (6.0 mM) was made in DMSO, and that solution was added to the reaction mixture (2.5 μL) to give a final substrate concentration of 60 μM. The final enzyme concentration was 0.15 μM for the double mutant and 0.13 μM for WT P450 BM-3. A stock solution of **1a** or **1b** (33 mM) in DMSO was used to prepare a solution in buffer (1.0 mM), and that solutions was added (50 μL) to initiate the reaction and to give an initial cofactor concentration of 200 μM. The absorbance of *p*-nitrophenol was measured at 410 nm and the concentration determined from a calibration curve.

P450 BM-3 activity assays with [Cp*Rh(bpy)(H_2O)]$(OTf)_2$: Assays were conducted in 96-well plates by using KH_2PO_4/K_2HPO_4 (20 mM), pH 7.4. The reactions (250 μL) contained P450 BM-3 (0.15 μM), *p*-NCA (60 μM), sodium formate (150 mM) and either **2a**, **2b**, or **2c** (5 mM). A stock solution of [Cp*Rh(bpy)(H_2O)]$(OTf)_2$ (25 μL; 0.5 mM) was added to initiate the reaction. The concentration of *p*-nitrophenol was determined as stated above.

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