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Authors
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Hollman, Frank
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Jochen Lutz $^a$, Frank Hollmann $^a$, The Vinh Ho $^a$, Adrian Schnyder $^a$, Richard H. Fish $^{b,*}$, Andreas Schmid $^{a,*}$

$^a$ Institute of Biotechnology, Swiss Federal Institute of Technology, ETH Hoenggerberg, HPT, CH-8093 Zurich, Switzerland.

$^b$ Lawrence Berkeley National Laboratory, 70-108B, University of California, Berkeley, California 94720, USA.
Bioorganometallic Chemistry: Biocatalytic Oxidation Reactions with Biomimetic NAD⁺/NADH Co-factors and [Cp*Rh(bpy)H]⁺ for Selective Organic Synthesis

Jochen Lutz ⁵, Frank Hollmann ⁵, The Vinh Ho ⁵, Adrian Schnyder ⁵, Richard H. Fish ⁶,*,
Andreas Schmid ⁵,*

⁵ Institute of Biotechnology, Swiss Federal Institute of Technology, ETH Hoenggerberg, HPT, CH-8093 Zurich, Switzerland.
⁶ Lawrence Berkeley National Laboratory, 70-108B, University of California, Berkeley, California 94720, USA.

* Corresponding authors. Tel.: +1-510-486-4850; fax: +1-510-486-7303. E-mail address: rhfish@lbl.gov (R. H. Fish). Tel.: +41-1-633-3691; fax: +41-1-633-1051. E-mail address: andreas.schmid@biotech.biol.ethz.ch (A. Schmid).
Abstract

The biocatalytic, regioselective hydroxylation of 2-hydroxybiphenyl to the corresponding catechol was accomplished utilizing the monooxygenase 2-hydroxybiphenyl 3-monooxygenase (HbpA). The necessary natural nicotinamide adenine dinucleotide (NAD$^+$) co-factor for this biocatalytic process was replaced by a biomimetic co-factor, N-benzylnicotinamide bromide, 1a. The interaction between the flavin (FAD) containing HbpA enzyme and the corresponding biomimetic NADH compound, N-benzyl-1,4-dihdronicotinamide, 1b, for hydride transfers, was shown to readily occur. The in situ recycling of the reduced NADH biomimic 1b from 1a was accomplished with [Cp*Rh(bpy)H](Cl); however, productive coupling of this regeneration reaction to the enzymatic hydroxylation reaction was not totally successful, due to a deactivation process concerning the HbpA enzyme peripheral groups; i.e., -SH or -NH$_2$ possibly reacting with the precatalyst, [Cp*Rh(bpy)(H$_2$O)](Cl)$_2$, and thus inhibiting the co-factor regeneration process. The deactivation mechanism was studied, and a promising strategy of derivatizing these peripheral -SH or -NH$_2$ groups with a polymer containing epoxide was successful in circumventing the undesired interaction between HbpA and the precatalyst. This latter strategy allowed tandem co-factor regeneration using 1a or 2a, [Cp*Rh(bpy)(H$_2$O)](Cl)$_2$, and formate ion, in conjunction with the polymer bound, FAD containing HbpA enzyme to provide the catechol product.

Keywords: Biocatalysis, Co-factor regeneration, Chemoenzymatic reactions, Monoxygenase enzymes, NAD$^+$/NADH biomimics, Organorhodium Hydride, [Cp*Rh(bpy)(H)]$,^+$, Epoxide Polymer

Introduction

Despite recent advances in catalytic oxyfunctionalization reactions, this class of reactions still represents one of the major challenges for synthetic organic chemistry [1]. The design of efficient catalysts has often been inspired by examples from nature.
However, most biomimics do not meet the performances of their natural precedents. In particular, the regio-, chemo-, and enantiospecificity of the catalyzed reactions are not as efficient as the natural enzymes, and include low turnover frequencies (TF), as well as turnover numbers (TN), which are also several orders of magnitude lower. This above-mentioned situation might also be attributed to the dependency on activated oxygen species, such as peroxides, dioxiranes, or high valent halogen compounds, which are incorporated into the substrate with low selectivity, and frequently at unsatisfactory rates.

Monooxygenase enzymes, on the other hand, utilize molecular oxygen for the specific incorporation of one oxygen into unactivated C-H, C-C, and C=C bonds [2], while minimizing the number of undesired side-reactions. Furthermore, monooxygenase enzymes have been reported to exhibit turnover frequencies of 10-20 s\(^{-1}\) [3], making them highly important catalysts for synthetic organic chemistry purposes. The availability of monooxygenase enzymes, and other aspects associated with preparative scale reactions, as well as stability under process conditions, are now thought to be considered standard practices, while the requirements of reducing equivalents appears to be a continuing challenge [4-6].

In general, monooxygenase enzymes are 1,4-dihydronicotinamide adenine dinucleotide co-factor dependent enzymes; i.e., NADH or NAD(P)H. These co-factors are costly and hydrolytically unstable, and therefore, represent a major barrier for preparative applications utilizing monooxygenases [4,7]. The ribose, pyrophosphate, and adenosine groups (Figure 1) entails the complex structure of NADH, \(2b\), and NAD(P)H, \(3b\), thus making chemical synthesis or isolation from biological sources tedious, and creates the high costs of these biocatalytic processes. It has recently been
shown by Fish and co-workers [8] that apparently only the nicotinamide ring was required as the redox active site for co-factor regeneration during the enzymatic transfer hydrogenation reaction catalyzed by horse liver alcohol dehydrogenase (HLADH) enzyme, with the finding that N-benzynicotinamide triflate, 1a (in this paper bromide as the counter ion instead of triflate), and N-benzyl-1,4-dihyronicotinamide, 1b, could be biomimics for natural NAD$^+$ /NADH and provide chiral alcohols from achiral ketones with high enantioselectivity.
To further explore these initial findings on HLADH enzyme recognition of 1b as an NADH biomimic for reductions of achiral ketones to chiral alcohols, we decided to expand the focus of the NADH model, 1b, with a monooxygenase enzyme, 2-hydroxybiphenyl 3-monooxygenase (E.C. 1.14.13.44, HbpA), from Pseudomonas azelaica HBP1. The HbpA monooxygenase enzyme catalyzes the specific ortho-hydroxylation of a broad range of α-substituted phenols to corresponding catechols (4b) (Figure 2) [9]. The oxidizing species of the enzyme, designated above, is a
4α-hydroperoxo flavin [10]. The latter species was formed in a reaction sequence consisting of hydride transfer from 2b to the oxidized flavin (FAD), followed by reaction with O₂, and proton abstraction to provide the resulting hydroperoxide. Thus, we will report on the substitution of 2b by the simple model compound, 1b, to sustain the HbpA’s catalytic cycle. Furthermore, we evaluated the *in situ* regeneration of 1b from 1a using the precatalyst, [Cp*Rh(bpy)(H₂O)](Cl)₂ and sodium formate to catalytically generate, [Cp*Rh(bpy)(H)](Cl), for the regioselective hydrogenation of 1a to 1b [14].

![Enzymatic reaction and substrates](image)

**R = Ph, 2'-OH-Ph, 2',2'-(OH)₂-Ph, F, Cl, Br, Me, Et, Pr, i-Pr, Bu**

4a, R= Ph  
4b, R= Ph

Figure 2: Enzymatic reaction and substrates that are utilized by 2-hydroxybiphenyl 3-monooxygenase (HbpA).
Results and Discussion

Preliminary experiments on the specific ortho-hydroxylation of 4a (2 mM) using HbpA as the enzyme catalyst (substrate/catalyst ratio (S/C): 8300), with 2 mM 1b as the stoichiometric source of reducing equivalents, resulted in approximately a 6.3 % yield of 4b after 3 hours. Thus, the HbpA monooxygenase enzyme performed more than 500 cycles at an average turnover frequency (TF) of 2.9 min⁻¹. Interestingly, and in contrast to the results of Fish and co-workers with horse liver alcohol dehydrogenase, only a fraction of the potential enzymatic activity of HbpA, (896 min⁻¹), was obtained [11].

To clarify this phenomenon, we further investigated the kinetic properties of HbpA with the reduced nicotinamide model, 1b. According to the Michaelis-Menten theory, the enzymatic rate is dependent on the binding affinity of HbpA for the substrate 1b, as well as on the maximum rate for substrate conversion \( (v = \frac{v_{\text{max}} \cdot S}{K_m + S}) \). Both parameters \( (K_m, v_{\text{max}}) \) were expected to differ between the natural and the biomimetic co-factor due to the apparent structural differences. Thus, we examined the influence of varying the concentration of 1b on the conversion rate of 4a to 4b (Figure 3).
Figure 3: Dependence of HbpA activity and stability on the concentration of reduced NADH model 1b. General conditions: 50 mM KP$_2$-buffer (pH 7.5), T = 30 °C; [HbpA] = 0.66 $\times$ 10$^{-6}$ M, [2-hydroxybiphenyl] = 2 $\times$ 10$^{-3}$ M, 4a, reaction time = 15 min.

As shown in Figure 3, a Michaelis-Menten like activity profile was observed with 1b concentrations up to 4 mM. Thus, a K$_m$ value of 3.77 mM (0.25 mM $\leq$ [1b] $\leq$ 4 mM) was estimated. This corresponds to an approximately 130 fold decrease in affinity of HbpA towards the NADH model, 1b, as compared to 27 µM for natural NADH, and readily explains the lower activity observed for HbpA in presence of 1b. Therefore, at very high concentrations of 1b, maximum catalytic activity might be expected. Surprisingly, the opposite effect was observed, as further increases of 1b concentration decreased the initial HbpA activity. Furthermore, we observed that the 1b oxidation rate was far higher than catechol formation. Similar effects, though to a far lesser extent, had also been observed with 2b as the reductant, which was explained by the
uncoupling of \( 2b \) oxidation from substrate hydroxylation, leading to the formation of hydrogen peroxide. \([10,11]\).

Figure 4 shows a typical reaction profile of the HbpA-catalyzed hydroxylation of \( 4a \) with \( 1b \) as reductant. Less than 20% of the reducing equivalents provided to HbpA by \( 1b \) were used for productive \( \text{O}_2 \)-activation; i.e., hydroxylation of \( 4a \). In the case of NADH, \( 2b \), this value was greater than 85%.

![Figure 4: Reaction profile of a typical enzymatic hydroxylation of 2-hydroxybiphenyl leading to the corresponding catechol (\( \bullet \)), with HbpA promoted by stoichiometric amounts of 1b, \( \square \). General conditions: 50 mM TRIS-buffer (pH 7.5), \( T = 30 \degree \text{C} \), \([\text{HbpA}] = 1 \cdot 10^{-6} \text{ M}, [2\text{-hydroxybiphenyl}] = 2 \cdot 10^{-3} \text{ M}\).](image-url)

Therefore, we are suggesting that an increased interaction of \( 1b \) with FAD, resulted in hydrogen peroxide formation, rather than in catechol product formation, \( 4b \).

Upon reaction with FAD alone, \( 1b \) was consumed approximately 40 times faster than native NADH, \( 2b \). Therefore, we concluded that hydride transfer from the reduced pyridine ring to the oxidized FAD alloxazine moiety occurred more efficiently with \( 1b \) than with \( 2b \).
The dramatically increased rate of 1b oxidation in comparison to 2b may be explained by steric, electronic, and conformational effects that facilitate a hydride transfer between 1b and FAD. Further investigations to clarify this mechanistic point will be accomplished in the future. Based on these results, the interesting dependency of HbpA activity and the concentration of 1b may be explained by two opposing effects. Firstly, the formation of reduced FAD at the active site was rate limiting in the catalytic mechanism of HbpA. Thus, according to the Michaelis-Menten model, increasing the concentration of 1b should have increased the rate of the hydroxylation reaction. Moreover, the increased rate of formation of FADH$_2$ also supports the nonproductive generation of hydrogen peroxide (H$_2$O$_2$). We suggest that catalytically relevant residues of HbpA; e.g., cysteine SH groups, at the active site of HbpA, were directly affected by in situ generated H$_2$O$_2$.

Apparently, the use of 1b as a reductant does not yield the high correlation between consumption of reducing equivalents and substrate hydroxylation that was observed with natural NADH, 2a [11]. One plausible approach to rationalising these results assumes that the NADH backbone, presumably via multiple H-bond and other non-covalent interactions, induces major structural changes of the active site geometry, which positions the phenolic substrate in close proximity to the activated 4α-hydroperoxoflavin, thereby minimizing undesired side reactions [12]. Since 1b lacks the backbone of NADH, none of the aforementioned effects presumably occurs, and the 4α-hydroperoxoflavin becomes susceptible to hydrolysis to H$_2$O$_2$ instead of ortho hydroxylation of substrate. Further experiments are currently underway to establish this hypothesis.
We next envisioned *in-situ* regeneration of the biomimetic co-factor, 1b, using [Cp*Rh(bpy)(H$_2$O)](Cl)$_2$ as the precatalyst, with formate as the hydrogen source, to form 1b from 1a [14a]. The usual reduction procedure used by enzymologists entails the role of formate dehydrogenase (FDH) to regenerate native NADH, 2b. However, even after several hours in the presence of 1 mM 1a, no formation of 1b was detectable, with FDH as the reducing catalyst. This apparent lack of reactivity of FDH compared to HbpA might be explained by the different enzymatic mechanisms of the FDH reaction for the regeneration of 1b by formate. For example, Labrou and co-workers described Lys360 to be involved in molecular recognition of 2a via the ribose ring [13]. FDH brings 2a and formate in close proximity via specific non-covalent binding of both substrates, which stabilizes the transition state. Thus, the reaction of formate and 2a was found to be accelerated by several orders of magnitude by FDH. In the case of HbpA, as shown above, the chemical regeneration of FADH$_2$, which is the reactive species for the monooxygenase enzyme, proceeds very rapidly even in the absence of enzyme. Therefore, in the case of 1b, HbpA was not necessary for the reductive half reaction; i.e., the transfer of hydride to FAD.

The active site of HbpA controls the regiospecific hydroxylation reaction of the substrate via its proximity to the FADH hydroperoxide. Recently, Lo and Fish proposed a possible role for the zinc metal ion center at the active site of HLADH, which was thought to facilitate the stereospecific hydride transfer from 1b to the achiral ketone substrate for chiral alcohol synthesis[8]. Moreover, in contrast to HLADH, FDH possesses no metal centers to which 1a might bind, and therefore, be in position in proximity to the formate ion to form 1b. More importantly, the formate driven regeneration of reduced nicotinamide models, 1a to 1b or 2a to 2b, has been reported to
be catalyzed by the organorhodium hydride, [Cp*Rh(bpy)(H)]+ [14], with catalytic activities in the range of 11 h⁻¹ being observed (Figure 5).

Figure 5: Tandem co-factor regeneration, using [Cp*Rh(bpy)(H₂O)](Cl)$_2$ and formate with 1a (bromide counter ion) to provide 1b, with a flavin containing and NAD$^+$ dependent, monooxygenase enzyme, 2-hydroxybiphenyl 3-monooxygenase (HbpA), for selective hydroxylation of 2-hydroxybiphenyl, 4a, to its catechol derivative, 2,3-dihydroxybiphenyl, 4b.

Thus, we decided to couple the 1b regeneration as shown in Figure 5 with the HbpA catalyzed hydroxylation of 2-hydroxybiphenyl. We found only small amounts of product (in the µM range) when combining biomimetic co-factor regeneration with the enzymatic hydroxylation reaction. Further analysis revealed that this lack of catechol product could be explained by the loss of activity of both the organorhodium precatalyst, [Cp*Rh(bpy)(H₂O)](Cl)$_2$, and the HbpA monooxygenase enzyme under the reaction conditions. Since the general reaction conditions show that each catalytic species was comparatively stable [15, 16], we propose an inhibitory interaction between both reactants. In fact, we observed a deactivation of HbpA in the presence of the
precatalyst \([\text{Cp}^*\text{Rh(bpy)(H}_2\text{O})]\)(\text{Cl})_2\), accompanied by the formation of a yellowish precipitate (Table 1).

Table 1: Deactivation of HbpA and the precatalyst, \([\text{Cp}^*\text{Rh(bpy)(H}_2\text{O})]\)(\text{Cl})_2\), at different concentrations

<table>
<thead>
<tr>
<th>c([\text{Cp}^*\text{Rh(bpy)(H}_2\text{O})])(\text{Cl})_2) [mM]</th>
<th>c(HbpA) [U ml(^{-1})]</th>
<th>Residual HbpA-activity [%] (^{[a]})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.11</td>
<td>100</td>
</tr>
<tr>
<td>0.02</td>
<td>0.11</td>
<td>28</td>
</tr>
<tr>
<td>0.04</td>
<td>0.11</td>
<td>8</td>
</tr>
<tr>
<td>0.02</td>
<td>0.22</td>
<td>16</td>
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<tr>
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<td>0.44</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>1.52</td>
<td>68</td>
</tr>
</tbody>
</table>

General conditions: KPi buffer (50 mM, pH 7.5), \(T = 30^\circ\)C.\(^{[a]}\) Determined after a 60 min incubation followed by a spectroscopic assay described previously\(^{[16]}\).

As shown in Table 1, the extent of decreasing HbpA activity depended on both the HbpA and the \([\text{Cp}^*\text{Rh(bpy)(H}_2\text{O})]\)(\text{Cl})_2\) concentrations, and suggested a stoichiometric interaction of both components causing this inhibition reaction. In a similar situation to the inhibition of HbpA, the \([\text{Cp}^*\text{Rh(bpy)(H}_2\text{O})]\)(\text{Cl})_2\) activity for the reduction of N-benzylnicotinamide bromide, \(1a\), in the presence of formate ion, was also decreased. Therefore, we suggest that the potential nucleophilic residues on HbpA, such as lysines (-NH\(_2\)) or cysteines (-SH), may possibly coordinate to the \text{Cp}^*\text{Rh metal ion center. A strongly binding amino acid probably will not be displaced by formate ion, therefore, inhibiting the rate of the organorhodium hydride regeneration reaction,
and may also explain the deactivation of HbpA. This was demonstrated via the influence of ammonium ions on the HbpA activity in the presence of 
\([\text{Cp}^*\text{Rh(bpy)}(\text{H}_2\text{O})](\text{Cl})_2\), and is shown in Figure 6; a clear decrease in activity was noted with increasing ammonium ion concentrations. Therefore, the peripheral groups on HbpA could bind to the 
\([\text{Cp}^*\text{Rh(bpy)}(\text{H}_2\text{O})](\text{Cl})_2\) dication, via aqua ligand displacement, and possibly affect the ternary structure of HbpA, while decreasing its water solubility.

![Figure 6: Influence of various NH$_4^+$ concentrations on the residual HbpA activity in the presence of \([\text{Cp}^*\text{Rh(bpy)}(\text{H}_2\text{O})](\text{Cl})_2\). General conditions: 50 mM phosphate buffer (pH 7.5, T = 30 °C); [HbpA] = 0.39 μM, \([\text{Cp}^*\text{Rh(bpy)}(\text{H}_2\text{O})](\text{Cl})_2] = 0.04 mM, [FAD] = 20 μM, \([\text{NH}_4^+] = 0 (\bigstar), 10 (\Box), 20 (\blacksquare), 50 (\triangle),\) and 100 (•) mM. At intervals, samples were withdrawn, supplemented with NADH (final 0.2 mM) and 2-hydroxybiphenyl (final 1 mM) and analyzed at λ = 340 nm.]

Alternatively, we experimented to ascertain if the nucleophilic functional groups on HbpA could be derivatized, and thus prevent the putative 
\([\text{Cp}^*\text{Rh(bpy)}(\text{H}_2\text{O})](\text{Cl})_2\)
deactivation reaction by covalently immobilizing HbpA with Eupergit C, a polymer with epoxide groups that would react with the –NH₂ or -SH groups on the HbpA enzyme. Using natural NAD⁺, 2a, as the co-factor, with in situ regeneration catalyzed by [Cp*Rh(bpy)H]⁺, resulted in the expected reactivity of HbpA and hydroxylation of 4a to 4b with a TF of 4 h⁻¹ (Figure 7); the low TF being a consequence of presumable mass transfer effects associated with the polymer supported enzyme. Furthermore, in similar experiments with 1a, only trace amounts of 4b were observed, and we are now conducting experiments to understand the reasons why there was such low product formation.

Figure 7: Chemoenzymatic hydroxylation of 2-hydroxybiphenyl with immobilized HbpA and [Cp*Rh(bpy)(H₂O)](Cl)₂ using formate as the source of reducing equivalents, and natural NAD⁺, 2a, as the co-factor. General conditions: potassium phosphate buffer (50 mM, pH 7.5, T=25°C), c(NaHCO₃)=150 mM, c(HbpA)=0.175 mM (immobilized on 150 mg Eupergit C), c(NAD⁺) = 0.2 mM, c(catalase)=50 U ml⁻¹, c([Cp*Rh(bpy)(H₂O)](Cl)₂) = 0.04 mM. (□: 2-hydroxy biphenyl, ◆: 2,3-dihydroxy biphenyl)
Conclusions

In the present study, we have extended the use of the biomimetic
NAD\(^{+}\)/NADH models, \(1a\) and \(1b\), from previously described horse liver alcohol
dehydrogenase to the FAD containing monooxygenase, HbpA [15]. Although
the activity of HbpA with the biomimetic NADH co-factor, \(1b\), was in the 6 %
range compared to 85% for natural NADH, the ease of chemical synthesis of this
biomimic, as well as the increased rates of hydride transfer from the reduced
biomimetic co-factor, \(1b\), directly to FAD provides a simple process for in–vitro
applications of this and other FAD containing monooxygenases (e.g. styrene
monooxygenase, cyclohexanone monooxygenase).

While formate dehydrogenase, FDH, from \textit{Candida boidinii} did not
reduce the oxidized biomimic, \(1a\), the \([\text{Cp*Rh(bpy)(H}_2\text{O)}]\)(Cl)\(_2\)/formate system
appears to deactivate the HbpA monooxygenase enzyme via binding to
functional groups, such as -NH\(_2\) and -SH. This deactivation process was
somewhat circumvented via derivatization of the enzyme with an epoxide
containing polymer to provide, with co-factor \(2b\), the hydroxylation product
catechol, \(4b\), while with biomimic co-factor, \(1a\), minor product formation was
observed.

In future studies, various strategies will be tested, such as site directed
mutagenesis of nucleophilic amino acids, protection of exposed amino acid
functional groups by other modifications (acylation, etc.), or directed evolution
approaches, to reduce enzyme deactivation by \([\text{Cp*Rh(bpy)(H}_2\text{O)}]\)(Cl)\(_2\), while
increasing the activity towards the reduced biomimic, \(1b\), should also allow
satisfactory product formation with other chemoenzymatic cell-free hydroxylation systems.

Acknowledgements

We gratefully acknowledge Martin Neuenschwander for the initial synthesis of N-benzyl-1,4-dihydronicotinamide, and Philipp Angerer for support with the $^1$H NMR experimental measurements. RHF gratefully acknowledges Department of Energy funding to LBNL from the Advanced Energy Projects and Technology Research Division, Office of Computational and Technology Research, under Contract No. DE AC03-76SF00098.
Experimental Section

Chemicals and Enzymes

All chemicals, benzyl bromide, nicotinamide, sodium dithionite, NADH, FAD, sodium formate, 2-hydroxypiphenyl and 2,3-dihydroxybiphenyl, dioxane, methanol, acetone and buffers, were obtained from Fluka, as well as the enzymes Candida boidinii formate dehydrogenase (EC 1.2.1.2) and bovine liver catalase (EC 1.11.1.6) in the highest available quality.

The HbpA (EC 1.14.13.44) was partially purified from recombinant E. coli JM101 (pHBP461\cite{17, 18}, via anionic expanded bed adsorption chromatography and subsequent desalting, saturation with FAD and dialyzed against 50 mM sodium phosphate buffer of pH 7.5 using a VariPerm L hollow fiber module from Stagroma (Reinach, Switzerland), and stored at –20 °C. The specific activity was calculated to be 0.78 U/mg corresponding to approximately 22 % purity. UV activity assays were accomplished as described in the following reference\cite{19}.

Synthesis of 1a, 1b, and [Cp*Rh(bpy)(H₂O)](Cl)_2

The N-benzylnicotinamide bromide (1a) was synthesized by dissolving 10 g nicotinamide in 200 ml 1,4-dioxane/50 ml methanol, followed by the addition of 11.6 ml benzyl bromide, and heating for 5 hours at 80 °C under reflux. The precipitate was filtered and washed three times with dioxane, recrystallized from methanol, and filtered and dried under vacuum and stored at –20 °C. The bromide anion was not exchanged by precipitaiton with silver triflate (Yield 64 % based on nicotinamide), as was described by Fish et al\cite{14a}. The reduced N-benzyl-1,4-dihydronicotinamide (1b) was obtained by dithionite reduction\cite{20}. Therefore, 2.5 g of 1a, in presence of 4.6 g sodium carbonate, was dissolved in 60 ml water and reduced by slow addition of sodium dithionite in a 4
fold molar excess for 2 hours. The precipitate was filtered, thoroughly washed with water, dried (Yield 82 %), stored at −20 °C, and checked by $^1$H NMR. The $^1$H NMR spectrum of 1b corresponds to the published spectral data [14a]. The 100 mM stock solutions of 1a and 1b in methanol were prepared fresh. The [Cp*Rh(bpy)(H$_2$O)](Cl)$_2$ complex was synthesized as published elsewhere [8, 14].

**Biotransformation, co-factor regeneration by [Cp*Rh(bpy)(H$_2$O)](Cl)$_2$**

In typical biotransformations, either 50 mM potassium phosphate or 50 mM TRIS buffer of pH 7.5, was used. The 2 mM 2-hydroxybiphenyl (4a) (100 mM stock solution in methanol) and typically 2-4 mM 1b (without in-situ co-factor regeneration) or 2-4 mM 1a in presence of 10-50 µM [Cp*Rh(bpy)(H$_2$O)](Cl)$_2$ and 150 mM sodium formate (with in-situ co-factor regeneration) were used at 30 °C and 200-250 rpm. Enzyme concentrations varied from 1/1000 to 1/10 dilutions of an enzyme stock solution of 6.1 U/ml and 7.7 mg/ml protein, but mostly HbpA was diluted 1/15.

**Eupergit Immobilisation**

The 2 ml HbpA stock solution and 1ml of potassium phosphate buffer (pH 7.5) aqueous buffer were incubated at 4 °C for 2 days with 0.4 g Eupergit® C. The immobilisation efficiency was calculated from UV enzyme assays[17] and Bradford protein assays [22] of the supernatant, before and after the immobilisation. Immobilisation efficiencies were in the range of 20 % of activity and protein. After washing the immobilisates, they were used in biotransformations on a 3 ml scale (see above).

**Analytical Techniques**

To determine product formation in aqueous solutions, the enzymatic reaction was stopped by the addition of 0.5 % (v/v) perchloric acid and the resulting precipitate
was removed by centrifugation (20000 g, 5 min). The samples were analyzed on a LiChroCART® column (125-4 RP-18) using a Merck LaChrom D-7000 high pressure liquid chromatography system (VWR international AG, Switzerland) running under isocratic conditions at 40 % acetonitrile and 60 % water (0.1 % perchloric acid) and 0.75 ml/min flow rate.

**Spectroscopy:**

The \(1b\) concentrations were measured at 350 nm, and \(2b\) concentrations at 340 nm using a Cary 1E (Varian, Switzerland) spectrophotometer. The extinction coefficient of \(1b\) was determined to be 5.76 l/(mmol cm)^{-1}. Spectra were recorded in quartz cuvettes from 200-600 nm in 15 min intervals.

**Interaction of \(1b\) or \(2b\) with FAD**

The 0.2 mM FAD, in the presence of either 0.3 mM \(1b\) or \(2b\), were incubated at 30 °C in TRIS buffer and the spectra over time were recorded at 15 min intervals. As a control experiment, FAD, \(1b\), and \(2b\) were incubated separately. The time course at 350 nm for \(1b\) and 340 nm for \(2b\), demonstrated the decreases in concentrations of \(1b\) and \(2b\) in the presence of FAD.

**Enzymatic regeneration of \(1b\) by formate dehydrogenase**

The 2.5 U formate dehydrogenase was incubated in the presence of 1 mM \(1a\) and 160 mM sodium formate in 50 mM Tris buffer, pH 7.5 at 30 °C, in a 1 ml cuvette. The UV/VIS spectra were recorded at 15 min intervals. No peak formation at 350 nm (characteristic for \(1b\)) could be detected during 2 hours of incubation, while directly after addition of either 0.5 mM \(2a\) or 10 µM \([\text{Cp}^*\text{Rh(bpy)}(\text{H}_2\text{O})]^2\) to the above assay, the 350 nm peak was observed.
References


