aza-RGD mimetics showed a clear preference for the $\alpha_v\beta_3$ receptor.

Lead structure **5** was found to exhibit a relatively high degree of polarity; an unfavorable pharmacokinetic profile can thus be expected for this compound. However, it has been shown in the past that a hydrophobic residue in the β-position to the carboxy group is tolerated by the $\alpha_v\beta_3$ receptor.[4d] For this reason, we replaced the terminal carboxamide group with a phenyl residue. The less polar RGD mimetic **10** exhibited similar selectivity and considerably increased activity on the $\alpha_v\beta_3$ receptor compared to the polar mimetic **5**.

The results obtained would appear to support our concept for identifying new, low molecular weight integrin ligands through application of combinatorial solid-phase synthesis, biological on-bead evaluation, and mass spectrometry to the selected compounds.

Received: June 30, 2000
Revised: October 9, 2000 [Z15368]

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[12] The guanylation was performed as per: Y. Wu, G. R. Matsueda, M. Bernatowicz, *Synth. Commun.* **1993**, *23*, 2055 – 3060; the pyrimidylation was performed as per: C. Gibson, H. Kessler, *Tetrahedron Lett.* **2000**, *41*, 1825 – 1728.

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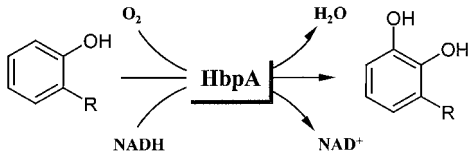
The First Synthetic Application of a Monooxygenase Employing Indirect Electrochemical NADH Regeneration**

Frank Hollmann, Andreas Schmid,* and Eberhard Steckhan†

In memory of Eberhard Steckhan

One of the most important challenges in applying monooxygenase reactions in vitro is to find an effective regeneration system for the necessary co-enzyme (mostly NAD(P)H). The well-established methods for the regeneration of the nicotinamide co-enzyme mainly consist of an enzyme-coupled approach utilizing formate dehydrogenase[1, 4c] (for NAD(P)H) or glucose-6-phosphate dehydrogenase (for NADPH).[2] Additionally, non-enzymatic redox catalysts have been developed and successfully applied to NAD(P)H-dependent dehydrogenases.[3] Thus only the producing enzyme and a mediator together with the electrode, as a source of reducing equivalents, are needed.

Here we report on the first application of an isolated monooxygenase with an indirect electrochemical regeneration of NADH. The enzyme employed is the 2-hydroxybiphenyl-3-monooxygenase (HbpA, E.C. 1.14.13.44), a member the class of flavine-dependent monooxygenases, from *P. azelaica*.[4] The homotetramer with a total mass of 256 kDa catalyzes the specific *ortho*-hydroxylation of several α-substituted phenol derivatives (Scheme 1). To the best of our knowledge no chemical counterpart with comparable specificity is known.



Scheme 1. Specific *ortho*-hydroxylation of α-substituted phenols catalyzed by 2-hydroxybiphenyl-3-monooxygenase. R = alkyl (Et, Pr, *i*Pr), aryl (Ph, 2-HOC_6H_4), Hal (F, Cl, Br).

For the regeneration of NADH we applied the [Cp*Rh(bpy)Cl]Cl complex which had been developed in our group ($Cp^* = C_5Me_5$; bpy = 2,2′-bipyridine). The corresponding hydridorhodium complexes, which can be generated either electrochemically by cathodic reduction at -750 mV (versus $Ag/AgCl_{sat.}$) or chemically with formate, transform $NAD(P)^+$ efficiently into the enzymatically active 1,4-NAD(P)H form[3, 5] (Scheme 2). The conversion rates

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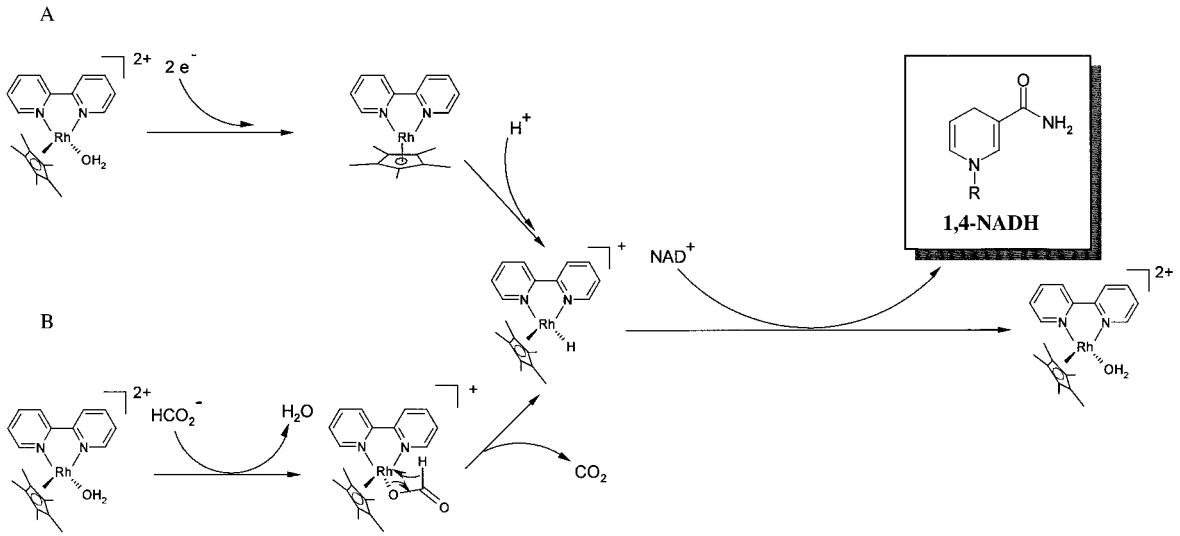
[**] This work was supported by BASF AG. We thank DEGUSSA AG for the gift of chemicals.



Scheme 2. Indirect electrochemical (A) and chemical regeneration of NADH (B).

(Table 1) of the enzymatic reaction with reduced coenzyme and under indirect chemical regeneration of NADH (Scheme 2B) prove the applicability of the proposed regeneration system. Hence we advanced the enzymatic reaction to the indirect electrochemical regeneration of NADH (Scheme 2A).

Table 1. Comparison of the productivity rates (dc/dt) of 2,3-dihydroxybiphenyl with reduced coenzyme (NADH), under indirect chemical and indirect electrochemical NADH regeneration.[a]

Regeneration	HbpA [U]	dc/dt [μmol min^{-1}]	Rel. activity[b] [%]
none[c]	2.24	2.09	93.3
chemically[d]	2.60	2.33	89.6
electrochemically[e]	19[f]	1.83	9.65[f]

[a] General conditions: phosphate buffer (50 mM, pH 7.5, saturated with air); $T = 25\,^{\circ}C$; [Cp*Rh(bpy)] = 0.5 mM; [2-hydroxybiphenyl] = 0.8 mM. [b] Productivity relative to the amount of HbpA applied. [c] [NADH] = 1.0 mM. [d] [NAD$^+$] = 1.09 mM; [HCOONa] = 150 mM. [e] [NAD$^+$] = 1.0 mM ; air intake = 10 cm^3 min^{-1}; [2-hydroxybiphenyl] = 2 mM; [FAD] = 20 μM; $E = -750$ mV (versus Ag/AgCl$_{sat.}$). [f] On account of the fast HbpA inactivation (see text) large amounts of enzyme had to be applied; the air intake is rate limiting.

This approach was examined by cyclic voltammetry. Even at proton concentrations less than 10^{-8} M (pH 8) the uptake of a proton into the ligand sphere of the reduced rhodium species proceeds quite quickly so that no oxidation peak is visible. Strong increases in the peak current (catalytic effects) are detected, even at high sweep rates in the presence of NAD$^+$ (Figure 1). The reduction peak potential lies at -750 mV (versus Ag/AgCl$_{sat.}$), which is more positive by far than the first reduction potential of NAD$^+$ (-920 mV versus the saturated calomel electrode (SCE)).[6] Thus, coenzyme inactivation by direct electron transfer becomes negligible.

The role of molecular oxygen was examined in detail. We found that it inhibits the transfer of hydride from the reduced rhodium complex to NAD$^+$ both in the chemical and the electrochemical regeneration. As can be clearly seen in Figure 2 the regeneration rate of NADH strictly depends on the rate of air input. For example, the rate of NADH production decreases from 1.1 (without air input) to 0.27 mmol L^{-1} h^{-1} with an air input of 10 cm^3 min^{-1}. No generation of NADH is detectable at a flow rate of 15 cm^3 min^{-1}. This inhibition however is reversible: after ceasing the air input both the rate of NADH production and the concentration of NADH recover to their maximum values.

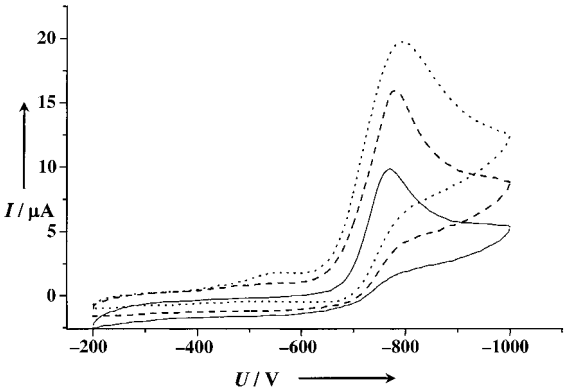


Figure 1. Catalytic effects of [Cp*Rh(bpy)Cl]Cl (5×10^{-4} M in 0.1 M TRIS/HCl buffer pH 7.5; TRIS = tris(hydroxymethyl)aminomethane) in the presence of various concentrations of NAD$^+$ (0 (——), 5×10^{-4} (– – –), and 1×10^{-3} M (- - - -)); $v = 81$ mV s^{-1}; degassed buffers.

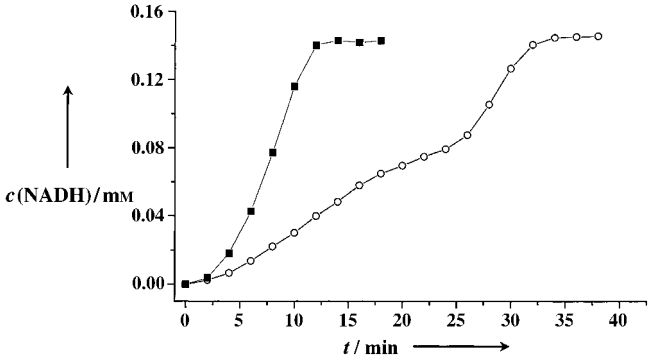


Figure 2. Influence of O_2 on the indirect electrochemical regeneration of NADH; [Cp*Rh(bpy)Cl]Cl (0.1 mM in 50 mM potassium phosphate buffer pH 7.5) (■ no air insertion; ○ air input 10 cm^3 min^{-1} until $t = 25$ min).

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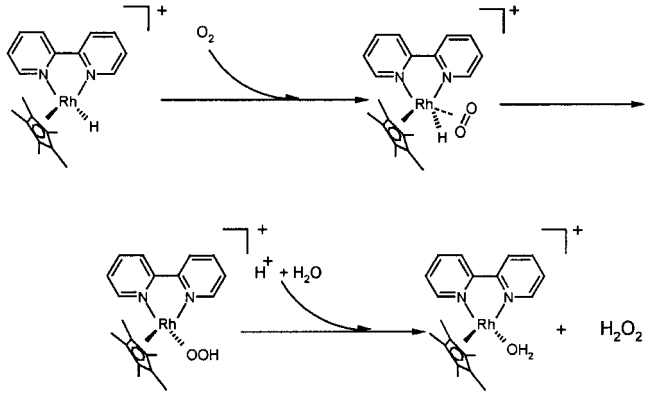
We characterized the product from the reaction of the hydridorhodium complex and molecular oxygen as hydrogen peroxide. We postulate its formation as depicted in Scheme 3.[7] In addition, molecular oxygen is reduced at the applied cathode potential and also yields hydrogen peroxide. In the course of the electro-enzymatic conversion it is dismutated into O_2 and H_2O by catalase.



Scheme 3. Postulated mechanism of the reaction between the hydridorhodium complex and molecular oxygen.

During the enzymatic reaction without external input of oxygen the catechol production stops on account of a lack of molecular oxygen in the reaction medium (typically at product concentrations between 0.2 and 0.4 mM). With the active input of air into the reaction medium new product is formed. Thus productivity rates of up to 1.1 mmol $L^{-1}h^{-1}$ (204 mg $L^{-1}h^{-1}$) are achieved. The mediator's turnover frequency can be calculated to be 11 h^{-1}.

The rate-determining step is the inhibition of the mediator by molecular oxygen rather than the heterogeneous charge transfer at the cathode. Control experiments with homogeneous chemical regeneration of the hydridorhodium complex under otherwise comparable conditions show that the productivity rates are in the same range (for example 0.673 and 0.668 mmol $L^{-1}h^{-1}$ at an air intake of 8 $cm^3 min^{-1}$ with total conversions of 100 and 95% under electrochemical and chemical regeneration, respectively).

The poor long-term stability of the previous electro-enzymatic conversions is the result of the experimental setup: the input of heterogeneous air results in enzyme inactivation. In addition HbpA adsorbes onto the electrode surface and thus is exposed to locally high H_2O_2 concentrations that cannot be sufficiently dismutated by catalase. The half-time values for HbpA activity in phosphate buffer under the conditions of the indirect chemical regeneration are in the range of 30–40 h (depending on the speed of air input), whereas during electrolysis (depending on the amount of HbpA applied and the mass of the electrode) no enzymatic activity is detectable after about 2 h.

To overcome this stability problem we are evaluating possibilities of immobilizing the enzyme onto a solid matrix thus protecting it from the electrode. Using this division of the whole process setup into an electrochemical and an enzymatic part, the ambivalent role of molecular oxygen (substrate of the enzymatic reaction and inhibitor of the NADH regeneration process) can be handled better. In preliminary studies the actual production time could be enhanced by more than 2.5 fold to over 8 h.

In conclusion, we could for the first time couple a flavine-dependent monooxygenase reaction to an indirect electrochemical regeneration of NADH. The productivity rates of this not-optimized process (204 mg $L^{-1}h^{-1}$) are already at about 50% of the in vivo process (390 mg $L^{-1}h^{-1}$)[8] and the in vitro process with enzymatic regeneration of the coenzyme (405 mg $L^{-1}h^{-1}$).[4c].

Experimental Section

The rhodium catalyst was prepared according to the method of Kölle and Grätzel.[9]

HbpA was enriched from the recombinant *E. coli* JM101 [pHBP461].[8] The raw extract was purified and enriched by expanded-bed chromatography.[4c] Enzymatic activity was determined by a UV-spectrometric assay.[4a]

Cyclovoltammogramms were taken with a BAS-100B/W analyzer (Bioanalytical Systems) in an undivided cell employing a glassy carbon working electrode (i.d. = 3 mm), a Pt-wire counter electrode, and an $Ag/AgCl_{sat.}$ reference electrode (Cypress Systems). Electroenzymatic conversions were performed in a thermostated electrolysis cell. A cylindrical carbon-felt electrode ($V = 27$ cm^3) served as the cathode and the potential was adjusted versus a $Ag/AgCl_{sat.}$ reference electrode. A divided cell was achieved by placing the platinum counter electrode within a dialysis membrane. The following components were dissolved in 100 mL of potassium phosphate buffer (50 mM, pH 7.5): 2-hydroxybiphenyl (2 mM), NAD^+ (0.2 mM), [Cp*Rh(bpy)Cl]Cl (0.1 mM), FAD (20 μM), catalase (250000 U), and HbpA (19 U). Oxygen was supplied by a heterogeneous input of air. The input rate was adjusted before each enzymatic conversion in such a way that the NAD^+ reduction rate reached its maximum value.

The reaction was followed by HPLC analysis on a RP-18 column eluated with methanol/water (0.1% H_3PO_4) (60/40). No by-products were detected. The product was sublimated and characterized by NMR and MS analysis.

Received: June 27, 1999 [Z15343]

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