An NAD(P)H-dependent Artificial Transfer Hydrogenase for Multi-enzymatic Cascades

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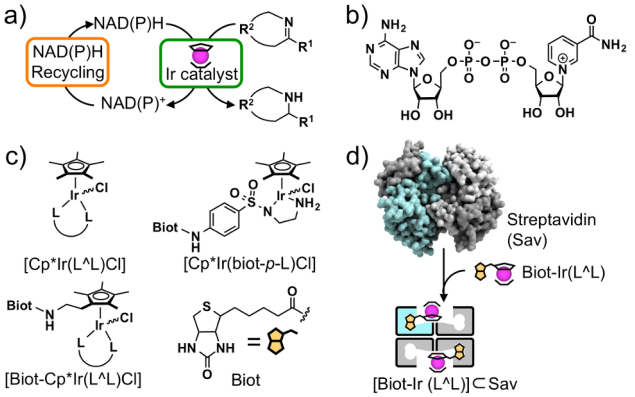
Supporting Information Placeholder

ABSTRACT: Enzymes typically depend on either NAD(P)H or $FADH_2$ as hydride source for reduction purposes. In contrast, organometallic catalysts most often rely on isopropanol or formate to generate the reactive hydride moiety. Here we show that incorporation of a Cp*Ir cofactor possessing a biotin moiety and 4,7-dihydroxy-1,10-phenanthroline into streptavidin (Sav) yields an NAD(P)H-dependent artificial transfer hydrogenase (ATHase). This ATHase (0.1 mol%) catalyzes imine reduction with 1 mM NADPH (2 mol%), which can be concurrently regenerated by a glucose dehydrogenase (GDH) using only 1.2 equivalents glucose. A four enzyme cascade consisting of the ATHase, the GDH, a monoamine oxidase and a catalase leads to the production of enantiopure amines.

The introduction of synthetic catalysts into a biological context is at the focus of current efforts in both synthetic- and chemical biology.[1,2] Goals include i) the supplementation of existing- or engineered metabolic pathways, ii) decaging inactive forms of enzymes to trigger enzymatic cascades,[2,3] iii) shifting the redox equilibrium in cancer cells to induce apoptosis[4] or iv) produce fuels with the help of biological redox equivalents.[3-5] Achieving high productivity of synthetic organometallic catalysts inside a living system remains challenging and progress is likely to be incremental.[2,6] In contrast, the combination of isolated enzymes and transition metal catalysts in carefully designed *in vitro* systems of modest complexity experiences increasing success.[7-9]

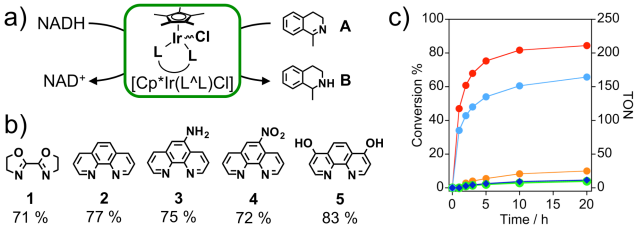
Transition metal-mediated formal hydride transfer occupies a prominent role in many of these initiatives.[9] Remarkably, synthetic catalysts and enzymes have gained common ground for the conversion of ketones to alcohols, imines to amines (and vice versa), the reduction of activated double bonds and the racemisation of secondary alcohols and amines.[10] A few isolated studies have shown that transition metal complexes can accept NAD(P)H as a hydride source.[5,11,12] To the best of our knowledge however, their *concurrent* use coupled with enzymatic processes, where the hydride is utilized in a productive fashion, have not been disclosed yet (Scheme 1a). We hypothesize that this may be traced back to the mutual deactivation of both transition metal catalysts and natural enzymes. To overcome this challenge, spatial separation of both catalytic partners has proven most effective.[13-15] In this context, artificial metalloenzymes (AMEs) have received increasing attention as alternative to both homogeneous catalysts and enzymes.[16] To test the versatility of AMEs for the implementation of enzymatic cascades, we reported on the compartmentalization of a biotinylated Cp*Ir-based transfer hydrogenation catalyst into streptavidin (Sav) variants (Scheme 1d).[15] To drive the hydride transfer reactions at reasonable rates however, concentrations of formate in the *molar range* were required.[7,15,17] Such concentrations of formate may lead to inactivation of natural enzymes and are thus incompatible with *in vivo* applications. Here, we show that the biological reducing agent NAD(P)H can serve as an efficient hydride source for an Cp*Ir-catalyzed hydride transfer in multienzymatic cascade reactions at millimolar loadings and near perfect stoichiometric fidelity under concurrent enzymatic NAD(P)H regeneration.

Scheme 1. (a) NAD(P)H as a hydride source for imine hydrogenation. Structure of (b) NAD^+, (c) pianostool complexes (L^L is a bidentate ligand) and (d) schematic representation of a Sav-based ATHase.



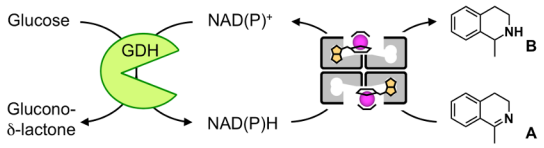
In previous designs of artificial transfer hydrogenases (ATHase) based on d^6-pianostool complexes, we relied on aminosulfonamide or aminoamide ligands to catalyze the transfer of hydride from formate to ketones, imines, enones[18] and *N*-alkylated nicotinamides (Scheme 1c).[15,19] Inspired by Sadler's work on the NADH oxidation catalysts,[11] we screened a range of commercial bidentate ligands (L^L **1**-**27**) for their ability to act as ligands for Cp*Ir in the oxidation of dihydronicotinamides (NADH) in the presence of an acceptor substrate (Scheme 2a). The amount of consumed NADH was determined by absorbance spectroscopy in the presence of 1 eq. 1-methyl-3,4-dihydroisoquinoline (MDQ **A** hereafter), $[Cp^*IrCl_2]_2$ (12.5 mol%) and L^L (27.5 mol%) possessing various donor and acceptor properties (Scheme 2b and S1). Among the ligands displaying catalytic oxidation of NADH (> 25 % = 1 turnover), bisoxazoline **1** and phenanthroline ligands **2**-**5** gave the highest activities (Scheme 2b and Scheme S1). To distinguish competitive hydrogen evolution from substrate reduction, these ligands were employed in reactions with $[Cp^*IrCl_2]_2$ (0.2 mol%), ligand L^L (0.44 mol%) and stoichiometric amount of NADH (25 mM). Formation of 1-methyl-1,2,3,4-tetrahydroisoquinoline (MTQ **B** hereafter) was monitored by HPLC (Scheme 2c). The electron rich 4,7-dihydroxy-1,10-phenanthroline **5**,[20] and to a lesser extent the bioxazoline **1**, led to significantly improved rates with respect to the product formation.

Scheme 2. (a) NADH as hydride source for the reduction of MDQ A catalyzed by [Cp*Ir(L^L 1-27)Cl]. (b) Selected ligands and corresponding conversion of NADH into NAD^+. (c) Time-course plot of MDQ A hydrogenation by $[Cp^*IrCl_2]_2$ with ligands 1 (blue), 2 (orange), 3 (green), 4 (red) and 5 (cyan).



Having identified the most promising bidentate ligand L^L **5**, the corresponding ATHase [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav was assembled (Scheme 1c, d).[21-23] Next, we combined this ATHase with glucose dehydrogenase (GDH) to regenerate the consumed NAD(P)H. This enables the use of glucose as reductant, yielding NAD(P)H and glucono-δ-lactone (Scheme 3). The performance of the concurrent enzymatic cascade was optimized by screening Sav variants focusing on close-lying positions S112 and K121.[22] The reactions contained GDH (0.1 mg/ml), NAD^+ (1 mM, 2 mol%), glucose (60 mM, 120 mol%), Biot-Cp*Ir(L^L **5**)Cl (50 μM, 0.1 mol%), Sav free binding sites (100 μM) and MDQ **A** (50 mM). Compared to [Biot-Cp*Ir(L^L **5**)Cl], the conversion by [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav doubled, highlighting the shielding effect of the host protein (Scheme 3, entries 1 and 2). Mutation at position S112 did not improve the performance of the ATHase and no activity was observed in the presence of coordinating aminoacids at either S112 or K121 (Scheme 3, entries 3, 4 and Table S7, entries 1-6). The conservative mutation K121R afforded the most active ATHase [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav K121R (Scheme 3, entry 5). Raising the temperature from 25 to 37°C and replacing NAD^+ by $NADP^+$ led to further improvements (Scheme 3, entries 5-7). Finally, extending the reaction time from 14 to 16 hours yielded full conversion, corresponding to TON_{Ir} = 1000 and to TON_{NADP^+} = 50 (Scheme 3, entry 8). Under these conditions, only 1.2 equivalents of glucose are required to drive the imine reduction to completion. Even under physiological NADP(H) concentrations (i.e. 120 μM),[24] a TON_{Ir} of 164 was obtained (Scheme 3, entry 9). The previously reported HCOOH-dependent ATHase [Cp*Ir(biot-*p*-L)Cl]⊂Sav S112A-K121A (Scheme 1c),[25] afforded a modest TON_{Ir} of 69 (6.9 % conversion) in the presence of 60 mM sodium formate (i.e. 120 mol%) (Scheme 3, entry 10, compare Table S7).

Scheme 3. Transfer-hydrogenation coupled with NAD(P)H regeneration by a GDH and glucose (Glu).



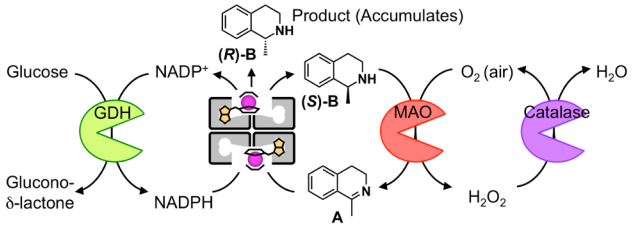
entry	Sav isoform	Hydride source	TON_{Ir}	*e.e.* [%]
1	No Sav	NAD^+/Glu	75	1 (*R*)
2	WT	NAD^+/Glu	202	17 (*S*)
3	S112A	NAD^+/Glu	214	8 (*S*)
4	K121A	NAD^+/Glu	101	6 (*S*)
5	K121R	NAD^+/Glu	270	13 (*R*)
6 [a]	K121R	NAD^+/Glu	679	13 (*R*)
7 [a]	K121R	$NADP^+$/Glu	961	13 (*R*)
8 [a, b]	K121R	$NADP^+$/Glu	>999	14 (*R*)

9[a,c,d]	K121R	$NADP^+$/Glu[e]	164	13 (*R*)
10[a,f]	S112A-K121A	HCOONa[g]	69	6 (*R*)

Experiments were performed at 25 or [a] 37 °C for 14, [b] 16 or [c] 24 h; 50 μM [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav variant, 0.1 mg/ml GDH, 1 mM NAD(P)$^+$, 50 mM MDQ **A** and 60 mM glucose in 0.3 M MOPS (pH 7.9). [d]100 μM [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav. [e] 120 μM $NADP^+$. [f] [Cp*Ir(biot-*p*-L)Cl] was used instead of [Biot-Cp*Ir(L^L **5**)]Cl. [g] 60 mM HCOONa. Average values for duplicate reactions are displayed; standard deviations were ≤ 4.0 %.

However, the enantiomeric excess obtained via the above cascade was modest (Scheme 3 and Scheme 4, entry 4). To overcome this drawback, we integrated a monoamine oxidase (MAO) and a catalase, into the GDH/ATHase cascade for the production of MTQ **B** (Scheme 4).[15,26] Combining the highly enantioselective MAO-catalyzed oxidation of (*S*)-MTQ **B** with simultaneous ATHase-driven reduction resulted in perfect enantioselectivity to yield (*R*)-MTQ **B** in full conversion with only 2 equivalents of glucose required (Scheme 4, entry 3). In this multienzymatic cascade reaction, the benefit of compartmentalizing the cofactor [Biot-Cp*Ir(L^L **5**)Cl] within Sav K121R is most prominent: the conversion improved from a mere TON_{Ir} of at least 30 (6% yield) for the free cofactor to a TON_{Ir} of greater than 445 (89% yield) with the ATHase (Scheme 4, entries 1, 2).

Scheme 4. Enzyme cascade for the deracemization of cyclic amines.[a]

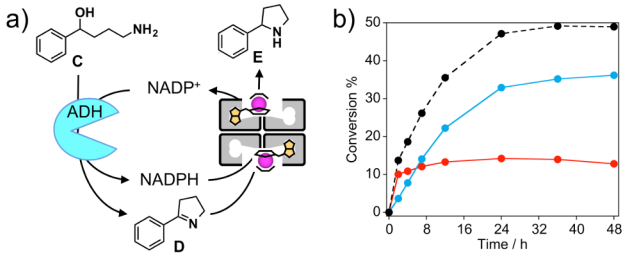


entry	Sav isoform	MAO [mg/ml]	time [h]	conv. [%]	*e.e.* [%]
1	No Sav	0.2	12	6	>99 (*R*)
2	K121R	0.2	12	89	75 (*R*)
3	K121R	0.2	24	>99	>99 (*R*)
4	K121R	–	24	>99	13 (*R*)

[a] Experiments were performed under the following conditions; 100 μM [Biot-Cp*Ir(L^L **5**)Cl], 0.1 mg/ml GDH, 0.2 mg/ml MAO, 500U catalase, 1 mM $NADP^+$, 50 mM MDQ **A** and 100 mM glucose in 0.3 M MOPS (pH 7.9) at 37 °C. Average values for duplicate reactions are displayed and standard deviations were ≤ 1.0 %.

Instead of GDH, we also coupled an alcohol dehydrogenase (ADH), another representative NAD(P)H regeneration system, with the ATHase [Biot-Cp*Ir(L^L **5**)Cl] ⊂Sav K121R. Relying on a hydrogen-borrowing strategy,[27] we designed a cascade reaction to enable a two step transformation of a linear aminoalcohol into a cyclic amine via an imine. For this purpose, 4-amino-1-phenyl-1-butanol **C** was selected as a substrate (Scheme 5a). In view of the exquisite enantioselectivity of ADH, one enantiomer of the aminoalcohol **C** is thought to be transformed into the corresponding dihydropyrrole **D** and pyrrolidine **E** in 12. 8% and 36.1 % yield respectively after 48 h (Scheme 5b and Table S11).[28]

Scheme 5. (a) A hydrogen-borrowing cascade reaction comprised of an ADH with the NAD(P)H-dependent ATHase and (b) its reaction progress: of 2-phenyl-3,4-dihydro-5H-pyrrole D (red), 2-phenylpyrrolidine E (cyan), and their sum (black).[a]



[a] Experiments were performed at 37 °C; 100 μM [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav(K121R), 0.25 mg/ml ADH, 1 mM $NADP^+$, 1.25 mM $MgSO_4$, 10 mM substrate **C** in 0.3 M MOPS (pH 7.9). Average values for duplicate reactions are plotted; standard deviations were ≤ 0.23%.

Finally, we investigated the transhydrogenase activity of the ATHase [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav K121R. Transhydrogenases rapidly equilibrate mixtures of NAD(H) and NADP(H) in a situation where one of NAD(H) and NADP(H) is more rapidly consumed than the other.[29] In the absence of ATHase, the redox equilibration between NADPH and NAD^+ or NADH and $NADP^+$ was slow (Table 1, entries 2, 4). In contrast, [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav K121R significantly accelerates the reaction to reach its equilibrium within 30 min (Table 1, entries 1, 3 and Table S13). This transhydrogenase activity may find applications to equilibrate the NAD(P)H levels in reaction cascades which combine both NADH- and NADPH-dependent enzymes. Examples include the biocatalytic synthesis of the potent drug hydromorphone[30] or oxidation reactions catalyzed by P450 enzymes.[31]

Table 1. Transhydrogenase activity of ATHase.[a]

entry	catalyst	reductant /substrate	NADPH/NADH (mM)
1	ATHase	NADPH/NAD^+	1.9/2.4
2	–	NADPH/NAD^+	4.6/0.1
3	ATHase	NADH/$NADP^+$	1.9/2.4
4	–	NADH/$NADP^+$	0.1/4.7

[a] Experiments were performed at 37 °C; 50 μM [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav K121R, 5 mM NAD(P)H and 5 mM NAD(P)$^+$ in 0.3 M MOPS (pH 7.9) for 30 min. Average

values for duplicate reactions are displayed and standard deviations were ≤ 1.0 %.

In summary, NAD(P)H-dependent ATHases were successfully developed that allow the use of NAD(P)H as hydride source for transfer-hydrogenation under physiological conditions. Two representative enzymatic NAD(P)H regeneration systems were shown to be compatible with this ATHase. A chemogenetic optimization strategy allowed to rapidly identify [Biot-Cp*Ir(L^L **5**)Cl]⊂Sav K121R as a versatile ATHase for the reduction of imines. Addition of a monoamine oxidase coupled with a catalase allows to produce enantiopure amines as a result of a four enzyme cascade. Further researches to exploit this ATHase *in vivo* are in progress.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website. The supporting information includes materials, instruments, experimental procedures and additional data of Scheme 2-4 and Table 1. (PDF)

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