Artificial Metalloenzymes Hot Paper

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Upregulation of an Artificial Zymogen by Proteolysis

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***Abstract:** Regulation of enzymatic activity is vital to living organisms. Here, we report the development and the genetic optimization of an artificial zymogen requiring the action of a natural protease to upregulate its latent asymmetric transfer hydrogenase activity.*

Complex biochemical cascades, which are essential to sustain life, rely on tightly cross-regulated enzymatic processes. Several complementary mechanisms are used by the cell to achieve such exquisite regulation including: 1) tuning of their expression (e.g. transcription or translation), 2) reversible chemical modification (e.g. phosphorylation), 3) substrate or product inhibition, 4) reversible binding of a small molecule distant from its active site (i.e. allosteric regulation), 5) selective proteolysis by a protease.[1–3] The latter strategy enables organisms to spatially and temporally control the activity of toxic enzymes that are expressed as pro-enzymes, also called zymogens. The unraveling of their catalytic activity requires the selective hydrolysis by a cognate protease to convert them into their corresponding active state.

In recent years, artificial metalloenzymes (ArMs hereafter), which result from anchoring an organometallic catalyst within a macromolecular scaffold, have emerged as an attractive alternative to organometallic catalysts. Thanks to their genetic encoding, well defined second coordination sphere, and non-natural organometallic cofactor, these hybrid catalysts combine attractive features of both homogeneous catalysts and enzymes.[4–8] Our group recently demonstrated that such ArMs improve the bio-compatibility of the organometallic cofactor, the protein host providing protection against deactivation, allowing their integration into cascades with natural enzymes.[10–12]

In a biomimetic spirit, we surmised that the activity of an anchored catalyst precursor might be upregulated by the action of an external trigger, ideally a natural enzyme, at a remote position in the host protein. Such systems could form the basis of more elaborate cross-regulated enzyme cascades whereby the product of the ArM-catalyzed reaction inhibits the activating enzyme.

Herein, we report the optimization of an artificial zymogen based on the biotin-streptavidin technology (Figure 1).

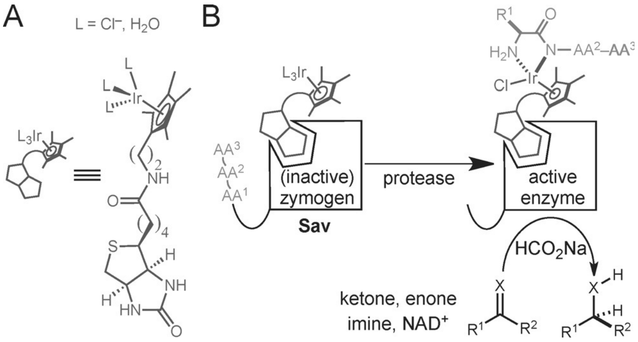

Figure 1. Upregulation of an artificial metalloenzyme is achieved by proteolytic cleavage of a tripeptide. A) Structure of the biotinylated cofactor. B) The biotinylated cofactor is activated upon coordination of a tripeptide that is released from the C-terminus of an engineered streptavidin (Sav = streptavidin, only one monomer depicted for clarity; AA = amino acid).

Inspired by a publication by Whitesides, we and others exploit the biotin–streptavidin technology for the creation of ArMs.[13–18] In the context of upregulation, we hypothesized that a biotinylated Cp*M moiety ($M = Rh^{III}$, Ir^{III}) might prove versatile as 1) the $[Cp^{*biot}MCl_2]_2$ precursor (or in combination with wild-type streptavidin, WT Sav hereafter) shows limited catalytic activity, both for C–H activation ($M = Rh^{III}$) and for imine reduction ($M = Rh^{III}$ or Ir^{III});[19–21] 2) addition of a suitable ligand (e.g. amidoamines, derived from natural amino acids) leads to significant rate acceleration for the transfer hydrogenation of imines and ketones;[19–22] and 3) embedding a Cp*Ir moiety within Sav shields the cofactor from inhibition by other proteins, enabling enzyme cascades.[10–12]

In the context of ligand-accelerated catalysis,[23] Hilvert et al. screened a library of tripeptides to identify ligands that accelerate the $[Cp^{*}IrL_3]$-catalyzed transfer hydrogenation ($L = Cl^-$ or H_2O). They identified glycine–glycine–phenylalanine (GGF hereafter) as the tripeptide yielding the most active complex in combination with $[Cp^{*}IrCl_2]_2$.[19] The tripeptide is thought to coordinate in a bidentate fashion to

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the iridium via the nitrogen atoms of the first two residues (i.e. a primary amine and an amide).[19,22] Installing the tripeptide ($AA^1AA^2AA^3$ hereafter) at the C-terminus of a protein impedes its coordination to the iridium by masking the primary amine as an amide. It may be possible to upregulate the ATHase activity (ATHase) of $[Cp^{*biot}IrL_3]\subset$Sav, by encoding a cleavable tripeptide at the C-terminus of Sav. In the presence of a site-specific protease, the tripeptide is cleaved, yielding a primary amine with increased affinity towards the iridium cofactor. The resulting Sav-embedded pianostool complex $[Cp^{*biot}Ir(AA^1AA^2AA^3)L]\subset$Sav may display enhanced ATHase compared to $[Cp^{*biot}IrL_3]\subset$Sav (Figure 1).

Prior to engineering the zymogen, both the tripeptide and Sav can be optimized independently. For this purpose, a tripeptide $AA^1AA^2AA^3$ library was combined with the zymogen consisting of $[Cp^{*biot}IrL_3]\subset$K121A Sav. This single-point mutant was selected for initial screening as it was previously shown to afford superior ATHase activity compared to WT Sav.[10,20,24] The biotin-binding site/$[Cp^{*biot}IrL_3]$/$AA^1AA^2AA^3$ ratio was set to 2:1:2.2 to drive the equilibria towards formation of $[Cp^{*biot}Ir(AA^1AA^2AA^3)L]\subset$K121A Sav.[25] To identify the best $AA^1AA^2AA^3$ and Sav isoform combination, the following procedure was implemented (Figure 2): 1) mix $[Cp^{*biot}IrCl_2]_2$ and Sav in MOPS buffer (MOPS = 3-(*N*-morpholino)propanesulfonic acid); 2) incubate for 0.5 hours at 37 °C; 3) add the tripeptide $AA^1AA^2AA^3$; 4) incubate for 12 hours; 5) add sodium formate and substrate; 6) stir the reaction for 24 hours at 37 °C; and 7) analyze the reaction mixture by HPLC.

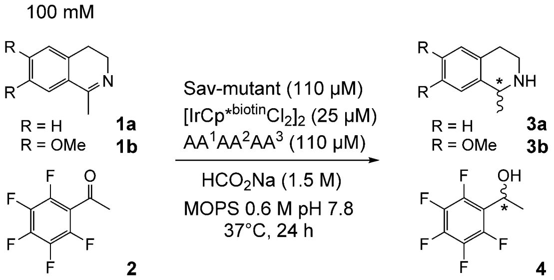

Figure 2. Asymmetric transfer hydrogenation of imines and ketones catalyzed by $[Cp^{*biot}Ir(AA^1AA^2AA^3)L]\subset$Sav. Varying the streptavidin isoform and the tripeptide allows to optimize the performance of the artificial ATHase.

Selected results, collected in Table 1, lead to the following conclusions:

1) In the absence of the tripeptide, the biotinylated cofactor displays limited catalytic activity, both in the absence and in the presence of Sav (Table 1, entries 1 and 2).

2) Addition of glycineamide or a di- or tripeptide leads to significant ATHase activity (Table S1 in the Supporting Information (SI)), confirming their ligand-accelerating effect on the ArMs.[19,20,22]

3) Although the activity of all peptide–iridium complexes $[Cp^{*biot}Ir(AA^1AA^2AA^3)L]$ was reduced upon incorporation within Sav, the enantioselectivity significantly increased (Table S2, SI), highlighting the critical influence of the second coordination sphere around the iridium cofactor provided by Sav.

4) Although the GGF sequence reported by Hilvert performs very well in K121A (Table 1, entry 3), the first amino acid of the $AA^1AA^2AA^3$ sequence could be further fine-tuned (Table S2, SI). The SGF tripeptide proved superior in the presence of Sav K121A: up to 829 TON were obtained after 24 hours with imine **1b**, 1773 TON with imine **1a** and 1431 TON with ketone **2** (Table 1, entry 4). For ketone **2**, the addition of SGF to $[Cp^{*biot}IrL_3]\subset$K121A increases activity by a factor 49. Prolonging the reaction time leads to full conversion (i.e. 2000 TONs) with imine **1b** within a week (Figure S2, SI), TONs > 3500 can be achieved with ketone **2** within 48 hours using 300 mM substrate, highlighting the robustness of the ArM.

5) Next, Sav mutants were screened in the presence of SGF. As previously observed,[20,26] K121A outperformed WT Sav for transfer hydrogenation. Introduction of an additional mutation $[Cp^{*biot}Ir(SGF)L]\subset$S112A K121A Sav afforded 1956 and 1754 TON for imine **1a** and ketone **2** respectively. Up to 57% *ee* (*R*)-**4** was obtained (Table 1, entry 8).

6) Taking advantage of this dual-component feature of the optimization scheme, we set out to improve the enantioselectivity by combining promising first and second coordination sphere components: $[Cp^{*biot}Ir(YGF)L]\subset$K121E Sav and

Table 1: Selected results for the reduction of substrates **1a**, **1b** and **2** catalyzed by $[Cp^{*biot}Ir(AA^1AA^2AA^3)L]\subset$Sav.[a]

			1a		**1b**		**2**	
Entry	Sav mutant	Ligand	TON	*ee* [%]	TON	*ee* [%]	TON	*ee* [%]
1	–	–	50	–	4	–	8	–
2	K121A	–	199	10 *(R)*	46	–	29	–
3	K121A	GGF	1151	10 *(R)*	444	41 *(R)*	734	33 *(R)*
4	K121A	SGF	1773	17 *(R)*	829	2 *(R)*	1431	38 *(R)*
5	K121A	YGF	1161	25 *(R)*	444	46 *(R)*	1014	31 *(R)*
6	WT	SGF	551	11 *(S)*	123	4 *(R)*	484	17 *(R)*
7	K121E	SGF	626	4 *(R)*	359	35 *(R)*	411	38 *(S)*
8	S112A K121A	SGF	1956	10 *(R)*	741	10 *(S)*	1754	57 *(R)*
9	K121E	YGF	888	21 *(R)*	457	73 *(R)*	290	7 *(R)*
10	S112A K121A	EGF	1406	7 *(R)*	231	81 *(R)*	449	24 *(R)*

[a] Reactions were carried out at 37 °C with 100 mM substrate, 50 µM $[Cp^{*biot}Ir(AA^1AA^2AA^3)L]$, 100 µM biotin binding sites of Sav mutant, 1.5 M sodium formate in 0.6 M MOPS buffer pH 7.8 (see the Supporting Information). All listed experiments were performed in triplicate: TON ± 10%, *ee* ± 1%; *ee* values are omitted for results with TON < 50).

Table 2: Factor Xa activation of the artificial zymogen.[a]

			1a		1b		2	
Entry	Sav mutant	Factor Xa	TON	*ee* [%]	TON	*ee* [%]	TON	*ee* [%]
1	K121A–SGF	–	195	9 *(R)*	70	23 *(R)*	23	–
2	K121E–YGF	–	130	1 *(R)*	36	–	13	–
3	K121A	+	203	9 *(R)*	72	19 *(R)*	32	–
4	K121E	+	149	6 *(R)*	34	–	13	–
5	K121A–SGF	+	2000	17 *(R)*	855	1 *(R)*	1778	39 *(R)*
6	K121E–YGF	+	815	20 *(R)*	331	72 *(R)*	220	8 *(R)*

[a] Reactions were carried out at 37 °C with 100 mM substrate, 50 μM $[Cp^{*biot}Ir(AA^1AA^2AA^3)L]$, 100 μM biotin binding sites of Sav mutant, with or without 5 μg Factor Xa, 1.5 M sodium formate in 0.6 M MOPS buffer pH 7.8 (see the Supporting Information). All listed experiments were performed in triplicate: TON ±10%, *ee* ±1%; *ee* values are omitted for results with TON < 50).

$[Cp^{*biot}Ir(EGF)L]\subset$S112A K121A Sav afford (*R*)-**3b** in 73% *ee* and 81% *ee*, respectively (Table 1 entries 9 and 10).

Having identified suitable combinations of Sav variants and tripeptides, the genetic encoding of Sav variants that include a protease cleavage site flanked by the tripeptide ligand was undertaken: Factor Xa was selected as the activating protease.[27] Correspondingly, its recognition site (e.g. IDGR\) followed by the XGF-activating peptide (X = S, Y) was genetically encoded at the C-terminus of Sav, starting at position A156. The C-terminal sequence of the mature Sav (A156-V-Q-Q159)[28] was replaced by I156-D-G-R**S**-**G**-**F**162 and I156-D-G-R**Y**-**G**-**F**162, for both K121A Sav (abbreviated K121A–SGF) and K121E Sav (K121E–YGF).

UPLC-MS analysis revealed that both K121A–SGF and K121E–YGF are cleaved within a few hours by Factor Xa (Figure S1, SI). The following protocol was implemented: 1) mix $[Cp^{*biot}IrCl_2]_2$ and K121A–SGF (or K121E–YGF) in MOPS buffer; 2) add Factor Xa; 3) incubate 12 hours; 4) add formate and substrate; 5) stir for 24 hours at 37 °C; and 6) analyze by HPLC. Selected results illustrate the following features (Table 2):

1) In the absence of Factor Xa, low conversion is observed, suggesting that the C-terminal tripeptide XGF (X = S, G) cannot bind the cofactor to activate the artificial zymogens $[Cp^{*biot}IrL_3]\subset$K121A–SGF and $[Cp^{*biot}IrL_3]\subset$K121E–YGF (Table 2, entries 1 and 2). The background activity corresponds to that observed with $[Cp^{*biot}IrL_3]\subset$K121A Sav and $[Cp^{*biot}IrL_3]\subset$K121E Sav in the presence of Factor Xa and the absence of peptide (Table 2, entries 3 and 4). Together, these results suggest that the presence of the activating tripeptides at the C-terminus of Sav have no effect in the absence of Factor Xa, and the protease itself does not activate the cofactor by direct complexation.

2) Incubation with Factor Xa restores the ATHase activity and enantioselectivity of both zymogens (Table 2, entries 5 and 6). This confirms that the ArMs resulting from proteolytic cleavage are essentially identical to those obtained by combining the synthetic tripeptides with Sav mutants. This feature is reminiscent of natural zymogens. In contrast to $[Cp^{*biot}IrL_3]\subset$K121A–SGF, the ATHase derived from $[Cp^{*biot}IrL_3]\subset$K121E–YGF is slower (up to ca. 25%) than its synthetic counterpart, possibly due to the slower cleavage by Factor Xa of K121E–YGF compared to K121A–SGF (Figure S1, SI).

In conclusion, an artificial zymogen with latent ATHase activity was designed and its upregulation by a natural enzyme demonstrated. Various creative strategies have been pursued in the past to engineer switchable homogeneous catalysts.[21,29,30] To the best of our knowledge, however, this constitutes the first report of an artificial zymogen upregulated by a natural protease. Both the host protein and the activating peptide ligand can be genetically encoded and orthogonally optimized to improve the catalytic activity and fine-tune enantioselectivity. This proof of concept presents a biocompatible strategy for the upregulation of an ArM, paving the way to implement and regulate bioorthogonal catalytic activity within a living organism.

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Keywords: artificial metalloenzyme · artificial zymogen · asymmetric catalysis · transfer hydrogenation · upregulation

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