Upregulation of an Artificial Zymogen by Proteolysis

Zhe Liu,* Vincent Lebrun,* Taku Kitanosono, Hendrik Mallin, Valentin Köhler, Daniel Häussinger, Donald Hilvert, Shu Kobayashi and Thomas R. Ward*

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: (i) tuning of their expression (e.g. transcription or translation), (ii) reversible chemical modification (e.g. phosphorylation), (iii) substrate or product inhibition, (iv) reversible binding of a small molecule distant from its active site (i.e. allosteric regulation), (v) 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 calledzymogens. The unraveling of their catalytic activity requires the selective hydrolysis of 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.[9–12]

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). Inspired by a publication by Whitesides, we and others exploit the biotin-streptavidin technology for the creation of ArMs.[13–16] In the context of upregulation, we hypothesized that a biotinylated Cp*M moiety (M = Rh(III), Ir(III)) might prove versatile as:

(i) The [Cp*IrL2Cl2] 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)).[17,18] (ii) 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] (iii) Embedding a Cp*Ir moiety within Sav shields the cofactor from inhibition by other proteins, enabling enzyme cascades.[16–19]

In the context of ligand-accelerated catalysis,[20] Hilvert et al. screened a library of tripeptides to identify ligands that accelerate the [Cp*IrL2]2-catalyzed transfer hydrogenation (L = Cl− or H2O). They identified glycine-glycine-phenylalanine (GGF hereafter) as the tripeptide yielding the most active complex in combination with [Cp*IrCl2]2.[19] The tripeptide is thought to coordinate in a bidentate fashion to the iridium via the nitrogen atoms of the first two residues (i.e. a primary amine and an amide).[19,20] Installing the tripeptide (AA2A′AA3A′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*IrL2]⊂Sav, by encoding a cleavable tripeptide at the C-terminus of Sav. In the presence of a site-specific protease, the tripeptide is cleaved,

\[ \text{Figure 1. Upregulation of an artificial metalloenzyme is achieved by proteolytic cleavage of a tripeptide. A) Structure of the biotinylated cofactor. B) The biotinylated cofactor is activated upon coordination of a tripeptide that is released from the C-terminus of an engineered streptavidin (Sav = streptavidin, only one monomer depicted for clarity; AA = aminocacid).} \]
yielding a primary amine with increased affinity towards the iridium cofactor. The resulting Sav-embedded pianostool complex \([\text{[Cp}^*\text{Ir}(\text{AA}\text{AA}^3\text{AA})\text{]}\subset\text{Sav}\] is may display enhanced ATHase compared to \([\text{[Cp}^*\text{Ir}(\text{L})\subset\text{Sav}\] (Figure 1).

Prior to engineering thezymogen, both the tripeptide and Sav can be optimized independently. For this purpose, a tripeptide AA\text{AA}^3 library was combined with the zymogen consisting of \([\text{[Cp}^*\text{Ir}(\text{L})\subset\text{K121A Sav}\] single point mutant was selected for initial screening as it was previously shown to afford superior ATHase activity compared to WT Sav.\cite{10,20,24} The biotin-binding site/\([\text{[Cp}^*\text{Ir}(\text{AA}\text{AA}^3\text{AA})\text{]}\subset\text{K121A Sav}\] \cite{25} To identify the best AA\text{AA}^3 and Sav isoform combination, the following procedure was implemented (Figure 2): i) mix \([\text{[Cp}^*\text{Ir}(\text{L})]\subset\text{AA}\text{AA}^3\text{AA}\] and Sav in MOPS buffer (MOPS = 3-(N-morpholino)propanesulfonic acid); ii) incubate for 0.5 hours at 37 °C; iii) add the tripeptide AA\text{AA}^3\text{AA}; iv) incubate for 12 hours; v) add sodium formate and substrate; vi) stir the reaction for 24 hours at 37 °C and vii) analyze the reaction mixture by HPLC.

**Figure 2.** Asymmetric transfer hydrogenation of imines and ketones catalyzed by \([\text{[Cp}^*\text{Ir}(\text{AA}\text{AA}^3\text{AA})\text{]}\subset\text{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:

i) 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, 2).

ii) Addition of glycineamide or a di- or tripeptide leads to significant ATHase activity (Table S1, SI), confirming their ligand-accelerating effect on the ArMs.\cite{10,20,22}

iii) Although the activity of all peptide-iridium complexes \([\text{[Cp}^*\text{Ir}(\text{AA}\text{AA}^3\text{AA})\text{]}\subset\text{Sav}\] was reduced upon incorporation within Sav, the enantioselectivity significantly increased (Table S1, SI), highlighting the critical influence of the second coordination sphere around the iridium cofactor provided by Sav.

iv) Although the GGF sequence reported by Hilvert performs very well in K121A (Table 1, Entry 3), the first amino acid of the AA\text{AA}^3 AA sequence could be further fine-tuned (Table S1, 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 \([\text{[Cp}^*\text{Ir}(\text{L})\subset\text{K121A Sav}\] 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.

v) Next, Sav mutants were screened in the presence of SGF. As previously observed\cite{20,26} K121A outperformed WT Sav for transfer hydrogenation. Introduction of an additional mutation \([\text{[Cp}^*\text{Ir}(\text{SGF})\text{]}\subset\text{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).

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

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

[a] Reactions were carried out at 37°C with 100 mM substrate, 50 mM \([\text{[Cp}^*\text{Ir}(\text{AA}\text{AA}^3\text{AA})\text{]}\subset\text{Sav}\].]
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*\text{IrCl}][\text{Cl}]] \subset K_{121}E \text{Sav and } [Cp*\text{IrCl}][\text{L}]] \subset S_{112}A K_{121}A \text{Sav afford } (R)-3b \text{ in } 73\% \text{ ee and } 81\% \text{ ee respectively (Table 1 entries 9, 10).}

Having identified suitable combinations of Sav variants and tripetides, 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. Correspondingly, its recognition site (e.g. IDGR) was 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 \((A_{156}-V_{Q_{159}})\) was replaced by 156-D-G-R-S-G-F162 and 156-D-G-R-Y-G-F162, 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, S2). The following protocol was implemented: i) mix [Cp*\text{IrL}][\text{Cl}]] and K121A-SGF (or K121E-YGF) in MOPS buffer, ii) add Factor Xa, iii) incubate 12 hours, iv) add formate and substrate, v) stir for 24 hours at 37 °C and vi) analyze by HPLC. Selected results illustrate the following features (Table 2): i) 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 \([\text{Cp*}^{\text{IrL}]}][\text{Cl}]] \subset K_{121}A-\text{SGF and } [\text{Cp*}^{\text{IrL}]}][\text{Cl}]] \subset K_{121}E-\text{YGF} \text{ (Table 2, entries 1 and 2). The background activity corresponds to that observed with } [\text{Cp*}^{\text{IrL}]}][\text{Cl}]] \subset K_{121}A \text{ Sav and } [\text{Cp*}^{\text{IrL}]}][\text{Cl}]] \subset K_{121}E \text{ 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 XGF in the absence of Factor Xa, and the protease itself does not activate the cofactor by direct complexation. ii} Incubation with Factor Xa restores the ATHase activity and enantioselectivity of bothzymogens (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 \([\text{Cp*}^{\text{IrL}]}][\text{Cl}]] \subset K_{121}A-\text{SGF, the ATHase derived from } [\text{Cp*}^{\text{IrL}]}][\text{Cl}]] \subset K_{121}E-\text{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-\text{SGF} \text{ (Figure S1, S2).}

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,[21,26,30] 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.

Table 2. Factor Xa activation of the artificial zymogen.\(^{[a]}\)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sav mutant</th>
<th>Factor Xa</th>
<th>TON</th>
<th>ee (%)</th>
<th>TON</th>
<th>ee (%)</th>
<th>TON</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K121A−SGF</td>
<td>-</td>
<td>195</td>
<td>9 ((R))</td>
<td>70</td>
<td>23 ((R))</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>K121E−YGF</td>
<td>-</td>
<td>130</td>
<td>1 ((R))</td>
<td>36</td>
<td>-</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>K121A</td>
<td>+</td>
<td>203</td>
<td>9 ((R))</td>
<td>72</td>
<td>19 ((R))</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>K121E</td>
<td>+</td>
<td>149</td>
<td>6 ((R))</td>
<td>34</td>
<td>-</td>
<td>13</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>K121A−SGF</td>
<td>+</td>
<td>2000</td>
<td>17 ((R))</td>
<td>855</td>
<td>1 ((R))</td>
<td>1778</td>
<td>39 ((R))</td>
</tr>
<tr>
<td>6</td>
<td>K121E−YGF</td>
<td>+</td>
<td>815</td>
<td>20 ((R))</td>
<td>331</td>
<td>72 ((R))</td>
<td>220</td>
<td>8 ((R))</td>
</tr>
</tbody>
</table>

[a] Reactions were carried out at 37°C with 100 mM substrate, 50 μM \([\text{Cp*}^{\text{IrL}]}][\text{Cl}]]\), 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 Supporting Informations). All listed experiments were performed in triplicate: TON ± 10%, ee ± 1%; ee values are omitted for results with TON<50).

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


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.

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