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**E. coli Nickel-Iron Hydrogenase 1 Catalyses Non-native Reduction of Flavins: Demonstration for Alkene Hydrogenation by Old Yellow Enzyme Ene-reductases**

Shiny Joseph Srinivasan, Sarah E. Cleary, Miguel A. Ramirez, Holly A. Reeve, Caroline E. Paul, and Kylie A. Vincent*

Abstract: A new activity for the [NiFe] uptake hydrogenase 1 of Escherichia coli (Hyd1) is presented. Direct reduction of biological flavin cofactors FMN and FAD is achieved using H$_2$ as a simple, completely atom-economical reductant. The robust nature of Hyd1 is exploited for flavin reduction across a broad range of temperatures (25–70°C) and extended reaction times. The utility of this system as a simple, easy to implement FMNH$_2$ or FADH$_2$ regenerating system is then demonstrated by supplying reduced flavin to Old Yellow Enzyme “ene-reductases” to support asymmetric alkene reductions with up to 100% conversion. Hyd1 turnover frequencies up to 20.4 min$^{-1}$ and total turnover numbers up to 20 200 were recorded during flavin recycling.

Academic and industrial fields are increasingly looking to biotechnology to make chemical manufacturing more sustainable.[1] Enzymes provide many advantages: they are renewable, biodegradable, nonhazardous, and provide high selectivity. Furthermore, the once-limited scope of known enzyme reactions is rapidly expanding, aided by enzyme engineering and ongoing discovery and characterization of new enzymatic functions.[2,3]

Old Yellow Enzyme (OYE) ene-reductases are gaining prominence in industrial biotechnology for catalysis of asymmetric alkene reductions. OYES contain a tightly bound FMN prosthetic group which transfers electrons from an external reductant to an activated alkene (Supporting Information, Figure S2). Most commonly, OYES are supplied with reducing equivalents via the expensive cofactors NADPH or NADH, and hence they are typically operated with a cofactor recycling system for the reduced nicotinamide cofactors such as glucose/glucose dehydrogenase (GDH). OYE ene-reductases can also accept reducing equivalents from synthetic analogues of NADH,[4] although work is still needed on effective recycling systems for these artificial cofactors. There are also reports[5,6] of electron uptake from reduced flavins, FMNH$_2$ or FADH$_2$ (oxidized and reduced forms are shown in Scheme 1). Presumably the tightly bound prosthetic flavin in OYES is sufficiently exposed to allow this promiscuity in terms of reductant. Supply of a catalytic quantity of oxidized FMN or FAD, together with a recycling system for reduced flavin is preferable to stoichiometric addition of FMNH$_2$ or FADH$_2$, both in terms of lowering cost and minimizing waste. Reduced flavins have been recycled in situ by means of photochemistry, electrochemistry or metal catalysis,[6] which can suffer from biocompatibility challenges (such as mutual inactivation, mismatched ideal solvent, pH, or temperature).[6,10] Milder biocatalytic approaches to flavin recycling are cumbersome (Supporting Information, Figure S3), requiring both an NAD(P)H-dependent reduc- tase to produce FMNH$_2$ or FADH$_2$ at the expense of NAD(P)H and GDH/glucose for recycling the NAD(P)H.

Use of H$_2$ for cleaner enzymatic NAD(P)H cofactor recycling has been demonstrated.[10–14] The soluble hydrogenase from Cupriavidus necator (formerly Ralstonia eutropha) natively uses H$_2$ to provide electrons for NAD$^+$ reduction at a prosthetic flavin cofactor.[15] Reduction of external flavin substrates by this enzyme under H$_2$ has long been known,[15] and presumably occurs at the NAD$^+$ binding

![Scheme 1. Oxidized (left) and reduced (right) FMN or FAD cofactors.](https://example.com/scheme1)
Hyp1 is natively expressed in *E. coli* and, unlike many hydrogenases, it is O₂-tolerant and active over a wide pH range. Like other uptake hydrogenases, the basic unit of Hyp1 is a heterodimer of the large subunit (HyaB) housing the [NiFe] active site, and the small subunit (HyaA) housing the iron-sulfur cluster electron transfer relay. Natively, Hyp1 exists as a homodimer, (HyaAB)₂ and is coupled to a cytochrome electron acceptor. Our isolated enzyme comprises predominantly the dimeric HyaAB and our preparation lacks the cytochrome (Supporting Information, Figure S1).

The H₂ oxidation activity of Hyp1 is typically measured using the artificial electron acceptor benzyl viologen in colourimetric assays. Electrons from H₂ oxidation at the [NiFe] active site (green, red, blue) are transferred to 2 electrons that are transferred to the protein surface via FeS clusters (yellow, orange). The figure, showing the homodimer of HyaAB units, was prepared using PyMOL 2.3.4 (PDB: 6FPW).

The H₂ oxidation activity of Hyp1 is known to be robust which inspired us to test H₂-driven flavin reduction. Hyp1 is known to be robust which inspired us to test H₂-driven flavin reduction at different temperatures after from reactions performed at different temperatures after

**Figure 1.** Flavin reduction by Hyp1. H₂ oxidation at the [NiFe] active site (green, red, blue) provides 2 electrons that are transferred to the protein surface via FeS clusters (yellow, orange).

**Figure 2.** Activity assay for H₂-driven Hyp1 reduction of flavin measured by in situ UV/Vis spectroscopy. A) Hyp1 reducing FMN. B) Hyp1 reducing FAD. C) Calculated [FMN] based on \( \lambda_{max} = 445 \text{ nm} \) (\( e = 12.50 \text{ mM}^{-1} \text{ cm}^{-1} \)). D) Calculated [FAD] based on \( \lambda_{max} = 450 \text{ nm} \) (\( e = 11.10 \text{ mM}^{-1} \text{ cm}^{-1} \)). Reaction conditions: General Procedure A in Tris-HCl buffer (50 mM, pH 8.0, 25 °C).

**Figure 2** shows the results of in situ UV/Vis spectrophotometric assays to explore H₂-driven FMN and FAD reduction by Hyp1 (produced and isolated in accord with the Supporting Information, Methods Section S1.2; reaction follows General Procedure A). The flavin moiety of FMN gives \( \lambda_{max} \) at 445 nm and FAD at 450 nm, both of which bleach upon two-electron reduction (Figure 2 A,B; see the Supporting Information, Figure S6 for spectra of fully reduced FMN). The decrease in oxidized flavin concentration over time was used to calculate initial enzyme activity (Figure 2 C,D). Control experiments indicated that omission of Hyp1 or H₂ led to negligible flavin reduction (Supporting Information, Figures S7–S9).

Upon addition of Hyp1, a lag phase was observed during FMN and FAD reduction, which is attributed to the well-characterized H₂-dependent activation phase for aerobically purified Hyp1. Later experiments (when indicated) used Hyp1 that was first activated under a H₂ atmosphere. The lag phase was followed by a decrease in absorbance consistent with FADH₂/FMNHH₂ formation, and clear isosbestic points at 330 nm corroborate a lack of side products. Specific initial activities for FMN and FAD reduction (76 and 32 nmol \text{ min}^{-1} \text{ mg}^{-1} Hyp1, respectively) were determined during the linear reaction phase. The higher activity for reduction of FMN compared with FAD cannot be attributed to thermodynamic driving force since both cofactors have similar reduction potentials but could relate to the cofactors’ ability to interact at the protein surface.

Hyp1 is known to be robust which inspired us to test H₂-driven flavin reduction activity at different temperatures (25–70 °C, General Procedure A). Figure 3 shows the conversions from reactions performed at different temperatures after
30 minutes relative to a standard reaction performed at 25°C. This standard temperature and stop time were selected to leave room for improvement in conversions of FMN and FAD at the higher temperatures. Reactions at 25–50°C using FMN were performed twice, and the corresponding bars indicate the average relative conversion with the range of results represented with error bars (±3–12%). This level of reproducibility is likely to extend to FAD owing to an identical reaction set up. Results for FMN and FAD may not be directly comparable due to different purity levels of the cofactors which were obtained from different suppliers. Conversion of FAD and FMN to the reduced forms after 30 min reaction time increased with temperature (Figure 3), suggesting that Hyd1 is likely to open new doors to cofactor recycling for flavoenzymes with optimal activity at higher temperatures.

To demonstrate the utility of Hyd1 in biotechnologically-relevant flavin recycling, we first coupled Hyd1-catalysed flavin reduction with the OYE-type ene-reductase from Thermus scotoductus, TsOYE,[32,33] to catalyze enantioselective reduction of ketoisophorone (1) to (R)-levodione (2, Table 1). Reactions were conducted according to General Procedure B (Supporting Information) and monitored using chiral-phase GC-FID after extraction of the reaction mixture in phosphate buffer (50 mM, pH 8.0). Conversion was calculated after 30 min using UV-visible spectroscopy.

![Figure 3](image)

**Figure 3.** Hyd1-catalysed flavin reduction at different temperatures (left: FMN; right: FAD). Conversion relative to standard = Conversion at temp × 100%. The FMN 25–50°C bars represent the average of relative conversions calculated from duplicate experiments, with the range represented as error bars. Reaction conditions: General Procedure B (Supporting Information) and monitored using GC conversion to [%][b]

| Entry | [TM] (mM) | [FMN] (mM) | Conv. to [%][b] | Hyd1 TOF [min]–1[¢][f] | Hyd1 TTN[÷][d] | FMN TN[‡][d] |
|-------|-----------|-----------|----------------|------------------------|----------------|-------------|
| 1     | 1         | 0.5       | 100            | 2100                   | 4              |             |
| 2     | 2         | 0.1       | 100            | 2100                   | 20             |             |
| 3     | 5         | 0.1       | 95 (100)       | 5200                   | 50             |             |
| 4     | 10        | 0.1       | 62 (97)        | 10200                  | 97             |             |
| 5     | 20        | 0.1       | 24 (37)        | 7800                   | 74             |             |
| 6[¢]  | 20        | 0.1       | [44]           | 9300                   | 88             |             |
| 7[¢]  | 10        | 0.1       | [94]           | 9900                   | 94             |             |
| 8[‡]  | 20 then   | 0.1       | [29] then      | 20200                  | 240[‡]         | 240[‡]      |

[a] Reaction conditions: In accord with General procedure B using 57 μg Hyd1, 72 μg TsOYE in Tris-HEC (50 mM, pH 8), 1 vol% DMSO at room temperature (20°C–22°C). [b] GC conversion to [%] at 15 h (and 24 h).
[c] Hyd 1 turnover frequency (mol per mol Hyd1 per min) was calculated after 60 minutes.
[d] Hyd1 total turnover number (mol per mol Hyd1) and FMN turnover number (mol per mol FMN) were determined at the end of the reaction.
[e] 4 bar H2; [f] 35°C; some evaporation of 1 and 2 was observed from GC-FID. [g] 71 μg Hyd1 was used.
[h] Reaction was fed with additional 72 μg TsOYE and 4.2 mM 1 at 66 h and 71 h, respectively.
[i] Conversion, Hyd 1 TTN and FMN TN were determined at 134 hours, additional time point data in the Supporting Information, Figure S12.

To test stability over time, entry 5 was replicated using 71 μg Hyd1, and as the reaction neared full conversion an additional 72 μg TsOYE then 4.2 mM 1 was added (66 h and 71 h, respectively, see entry 8). Though the reaction likely still had active enzymes (Supporting Information, Figure S12), the reaction was stopped for analysis at 134 h (5.5 days) after which Hyd1 TTN reached 20200 and FMN TN 240. This represents an improvement in stability over *R. eutropha* SH (TTN 8400) for flavin recycling with TsOYE.[34] The 20200 TTN is of an appropriate order of magnitude for use as a catalyst in the pharmaceutical and fine chemicals industries,[35] approaches values measured from commercial grade enzymatic processes,[36] and there remains room for further optimization to that end. The demonstrated continuous Hyd1 stability over time (Supporting Information, Figure S12) is an important performance benchmark for potential commercial applications, particularly in flow.[37] Furthermore, this appli-

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**Table 1: H2-driven enzymatic reduction of 1 under various conditions.[i]**

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These are not the final page numbers!
cation is likely to extend to TsOYE variants, which have demonstrated broad substrate acceptance, are robust in harsh conditions, and can switch enantioselectivity.\textsuperscript{[130]}

We extended this system to two commercially available ene-reductases, ENE-103 and ENE-107 (Johnson Matthey), which are typically sold as a kit with GDH and formate dehydrogenase for NAD(P)H recycling. The alkene reductions demonstrated were dimethyl itaconate (3) reduction to dimethyl (R)-methyl succinate (4) by ENE-103 and 4-phenyl-3-buten-2-one (5) reduction to 4-phenyl-2-butanone (6) by ENE-107 (Table 2), using the same protocols established for TsOYE. Control experiments to show that each component is required for substrate conversion are summarized in the Supporting Information, Tables S3,S4.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Entry & Substrate & [FMN] (mM) & Ene-reductase & t [h] & Conv. [%]\textsuperscript{[b]} & \textit{ee} [%]\textsuperscript{[c]}
\hline
1 & 3 & 0.1 & ENE-103 & 42 & 81 & > 99
2 & 3 & 0.5 & ENE-103 & 42 & 98 & > 99
3\textsuperscript{[a]} & 5 & 0.1 & ENE-107 & 24 & 20 ± 1 & n.a.\textsuperscript{[d]}
4\textsuperscript{[a]} & 5 & 0.5 & ENE-107 & 24 & 33 ± 3 & n.a.\textsuperscript{[d]}
5 & 5 & 0.1 & ENE-107 & 40 & 35 & n.a.\textsuperscript{[d]}
6 & 5 & 0.5 & ENE-107 & 40 & 100 & n.a.\textsuperscript{[d]}
\hline
\end{tabular}
\caption{H$_2$-driven enzymatic alkene reductions using commercial ene-reductases.\textsuperscript{[a]}}
\end{table}

\textsuperscript{[a]} Reaction conditions: In accord with General procedure B using 142 \mu g Hyd1, 3 mg ene-reductase and 5 mM substrate in Tris-HCl (50 mM, pH 8), 1 vol% DMSO at room temperature (20°C-30°C). \textsuperscript{[b]} GC conversions to 4 or 6. \textsuperscript{[c]} Entries 3 and 4 were performed in triplicate and are shown ± 1 standard deviation, and were separate experiments from entries 5 and 6. \textsuperscript{[d]} Not applicable.

With ENE-103, enantioselective (> 99 \% \textit{ee}) reduction to (R)-4 improved from 81 \% to 98 \% conversion as FMN concentration was increased from 0.1 mM to 0.5 mM (entries 1,2). Conversion of 5 to 6 using ENE-107 was drastically improved when FMN concentration increased from 0.1 mM to 0.5 mM (compare entries 3 and 4, and entries 5 and 6), increasing from 35 \% to 100 \% conversion in the 40 hour experiment. These results highlight the straightforward application of different ene-reductases with Hyd1-catalysed flavin recycling, suggesting that this simplified H$_2$-driven system could be valuable in applications that require low waste, high catalyst stability and temperature tolerance.

Our work has shown a clear, atom-efficient way of driving commercial ene-reductase enzymes with flavin recycling in place of nicotinamide cofactor recycling. Further modifications to Hyd1, which is tolerant of mutagenesis,\textsuperscript{[29,31]} might enhance its non-native flavin reduction activity. Other promising synthetically interesting flavin-dependent enzymes, including halogenases (chlorination, bromination, iodination)\textsuperscript{[7]} and flavoprotein monooxygenases (epoxidation, hydroxylation, Baeyer–Villiger oxidation)\textsuperscript{[39,40]} are currently under-utilized in industrial biotechnology, perhaps due to the lack of available simplified flavin recycling systems. This proof-of-concept work shows that the robust Hyd1, tolerant to a range of conditions, is a promising catalyst to develop for clean flavin recycling in biotechnology.

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\section*{Conflict of interest}

The authors declare no conflict of interest.

\textbf{Keywords:} asymmetric catalysis · biocatalysis · cofactor recycling · ene-reductase · hydrogenation

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Robust [NiFe] hydrogenase 1 (Hyd1) from *Escherichia coli* is shown to have non-native, \( \text{H}_2 \)-dependent activity for FMN and FAD reduction. It is a promising recycling system for FMNH\(_2\) or FADH\(_2\) supply to flavoenzymes for chemical synthesis when coupled with an Old Yellow Enzyme ene-reductase.