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H_2 as a fuel for flavin- and H_2O_2-dependent biocatalytic reactions†

Ammar Al-Shameri,[a] Sébastien J.-P. Willot,[b] Caroline E. Paul,*[b] Frank Hollmann*[b] and Lars Lauterbach*[a]

The soluble hydrogenase from *Ralstonia eutropha* provides an atom efficient regeneration system for reduced flavin cofactors using H_2 as an electron source. We demonstrated this system for highly selective ene-reductase-catalyzed C=C-double bond reductions and monooxygenase-catalyzed epoxidation. Reactions were expanded to aerobic conditions to supply H_2O_2 for peroxygenase-catalyzed hydroxylations.

Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are key versatile cofactors for electron transfer and substrate conversion. Flavins are found as prosthetic groups in many biocatalysts including ene-reductases, monooxygenases, hydrogenases and oxidases. These flavoenzymes catalyze various industrially relevant reactions such as asymmetric hydrogenations and sulfoxidations, selective amine oxidations, and Baeyer–Villiger oxidations.[1]

In their reduced form ($FNMH_2$ and $FADH_2$, respectively), flavins serve as electron donors for a broad range of biological redox reactions many of which are relevant in the organic synthesis.[2] In the case of preparative chemical applications (*i.e.*, biocatalysis), the stoichiometric reductant plays a crucial role in envisioning the economic feasibility and environmental impact of the reaction system. Mimicries of the natural, NAD(P)H-dependent regeneration pathways are currently the most widely used approach, but suffer from complex reaction schemes, the need for the nicotinamide cofactor and the formation of significant wastes originating from stoichiometric reductants such as glucose.[3] In addition, some photo[4] and electrochemical[5,6] approaches to regenerate reduced flavins have been reported.

Hydrogen would be an ideal reductant, especially from an environmental point of view as it does not yield any waste-products and is sustainable when generated by electrolysis using renewable energy sources. The NAD^+-reducing hydrogenase from *Ralstonia eutropha* (soluble hydrogenase, SH) uses molecular hydrogen (H_2) to reduce NAD^+ to NADH and consists of a hydrogenase module (HoxHY) with a H_2 converting active site and a diaphorase module ($HoxFUI_2$) with a NAD^+ binding site (Fig. 1).[7–9] H_2 is oxidized at the NiFe active site with three biologically untypical CN^- and CO ligands. The electrons are transferred *via* the FMN-a close to the active site within the hydrogenase module and the Fe–S cluster chain to the second FMN-b at the NAD^+ binding site (Fig. 1). FMN-b serves here as a mediator between the one electron centers (Fe–S clusters) and the two-electron acceptor NAD^+.[10] The flavins of the SH are non-covalently bound. During SH purification, FMN-a is easily lost and can be reconstituted by adding free FMN to the purified enzyme, which significantly improves the NAD^+ reduction activity.[11,12] Experiments with separately produced hydrogenase and diaphorase modules showed that the FMN-a of the hydrogenase module can be reconstituted under reducing conditions and the FMN-b of the diaphorase module is released under reducing conditions.[8,9] Purified SH shows a lag phase during activity, since most of the enzyme population is inactive and requires reverse electron transfer from NADH generated from an active enzyme population for reactivation.[13,14]

In contrast to most other hydrogenases, the SH is O_2 tolerant; *i.e.* its catalytic activity is not impaired by ambient O_2.[15] SH has been employed for the recycling of NAD(P)H in various enzymatic reactions *in vitro* and *vivo*.[16–18] Compared with common NAD(P)H-regeneration systems such as formate dehydrogenase (generating CO_2 as a stoichiometric by-product) and glucose-6-phosphate dehydrogenase (yielding the corresponding gluconolactone), H_2-driven cofactor recycling has the advantage of being 100% atom efficient, by-product-free and cheap since it only relies on H_2 as reductant.[19,20] Besides NAD^+, SH is also known to reduce other electron acceptors such as ferricyanide, methylene blue and methyl viologen.[13] This catalytic

[a] *Institute of Chemistry, Technische Universität Berlin, Strasse des 17, Juni 135, 10623 Berlin, Germany. E-mail: lars.lauterbach@tu-berlin.de*
[b] *Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629HZ Delft, The Netherlands. E-mail: f.hollmann@tudelft.nl, c.e.paul@tudelft.nl*

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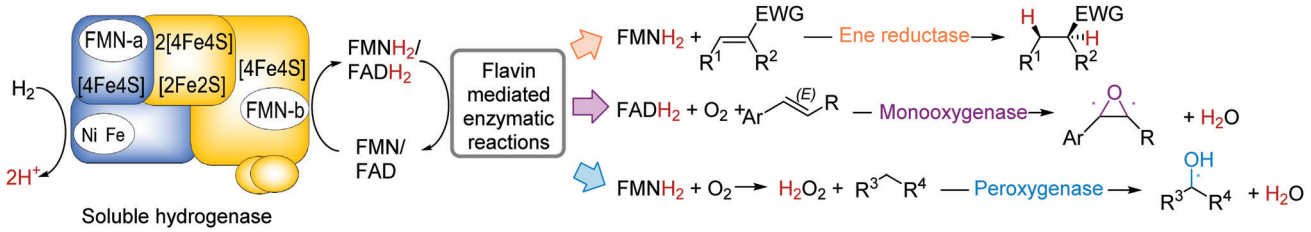


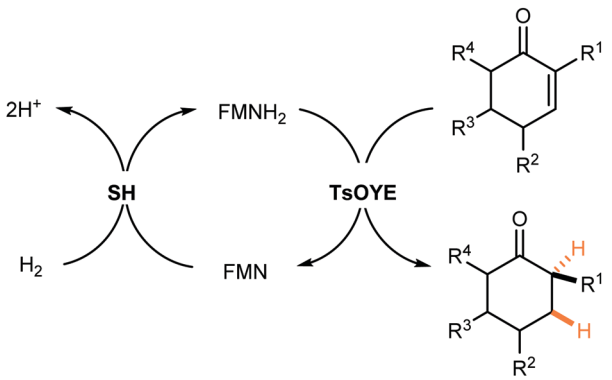
Fig. 1 H_2-driven reduced flavin regeneration by soluble hydrogenase (SH) coupled with three biocatalysts to catalyze asymmetric hydrogenation, epoxidation and selective hydroxylation with high atomic efficiency. SH consists of a hydrogenase module (blue) with the NiFe active site responsible for H_2 conversion and a diaphorase module (yellow) with the NAD^+ binding site near FMN-b. Red H atoms show the fate of H atoms after H_2 oxidation and illustrating the atom efficiency of H_2-driven flavin reduction. The resulted protons from H_2 splitting are released to the solution and electrons are transferred *via* FMN-a, Fe–S clusters and FMN-b to the NAD^+ binding site for FMN or FAD conversion to $FMNH_2$ and $FADH_2$, respectively. Protons come from solution for flavin reduction. EWG = electron withdrawing group.

promiscuity in terms of electron acceptors may open up new avenues of H_2-driven biocatalysis while avoiding costly nicotinamide cofactors. We therefore evaluated SH-driven reduction of flavins to promote a range of biocatalytic redox reactions of synthetic interest.

First, we investigated the kinetic parameters for the SH-catalyzed reduction of FMN and FAD ($FADH_2$/FAD and $FMNH_2$/FMN $E^{\circ\prime}$ = −190 mV) driven by H_2 oxidation ($H_2/2H^+$ $E^{\circ\prime}$ = −412 mV), which is thermodynamically favoured. The specific activity of SH for reducing FMN was 5.8 U mg^{-1} (20.3 s^{-1}), with a $K_{m,FMN}$ of 680 μM (Fig. S1, ESI†). Adding catalytic amounts of NADH increased the FMN specific activity by 100% and led to the disappearance of the lag phase. The disappearance of the lag phase is explained by faster reactivation under reducing conditions and reverse electron transfer (see above). Surprisingly, SH exhibited a significantly lower activity on FAD (0.14 U mg^{-1}, 0.49 s^{-1}), which prohibited the precise determination of its K_m value. The higher activity towards the FMN compared with the longer FAD, as well as the increased FMN activity in the presence of NADH, support the reaction model, that the reduction occurs by temporarily substituting the prosthetic FMN-b by free flavin. After reduction of bound flavin, it is released again (Fig. 1). The FMN-b in the Rossmann fold is deeper submerged than the binding site of NAD^+, as seen in the crystal structure of the related SH from *Hydrogenophilus thermoluteolus*,[21] explaining the 17 times lower specific activities for FMN in comparison to NAD^+ as the substrate.[15] The Fe–S clusters of the SH are buried in the protein matrix,[21] which may explain only a low contribution of Fe–S cluster in flavin reduction. The kinetic experiments demonstrated that SH-catalysed reduction of flavins is indeed feasible. Recently, the hydrogenase Hyd1 from *Escherichia coli* was described to catalyse the H_2-driven reduction of free flavins.[22] However, Hyd1 shows 180 times lower specific activity for FMN in comparison to SH and contains only additional Fe–S clusters and no flavins besides the NiFe active center. This supports the hypothesis that FMN-b of SH plays an important role for improved H_2-driven FMN reduction.

Encouraged by the high H_2-driven reduction activity of the SH, we explored the H_2-driven regeneration of reduced flavins to drive biocatalytic redox reactions (Fig. 1). As a first model reaction, we chose the stereoselective reduction of activated C=C-double bonds catalyzed by the ene reductase Old Yellow Enzyme from *Thermus scotoductus* (*Ts*OYE).[23] In accordance with the substrate spectrum of *Ts*OYE, a range of cyclic enones was reduced (Table 1).[24] Control reactions in the absence of either SH or H_2 under otherwise identical conditions gave no detectable conversion. In particular, ketoisophorone was fully converted (99% conversion, Table 1, entry 1), albeit yielding a product with a significantly reduced optical purity (37%). Similar observations were made with this product and are generally associated with racemization under the reaction conditions (Table S3, ESI†). Interestingly, the control reaction without FMN

Table 1 H_2-driven asymmetric reduction of alkenes



R^1	R^2	R^3	R^4	Product/mM	ee/%
Me	=O	H	$(CH_3)_2$	21.8 ± 0.4	37 (*R*)
H	H	H	H	9.7 ± 0.2	n.a.
Me	H	(*S*) isopropenyl	H	1.9 ± 0.2	>99, 98.1 de (2*R*,5*S*)
Me	H	(*R*) isopropenyl	H	1.1 ± 0.1	>99, 93.2 de (2*R*,5*R*)
Me	H	H	H	17.8 ± 0.5	>94 (*R*)

Total turnover numbers[a]

*Ts*OYE	SH	FMN
2625	8400	105

Reaction conditions: 2 mL of KPi buffer pH 7.5 containing: 200 μM FMN, 22 mM substrate. The buffer was purged with H_2 prior to adding the substrate, SH (2.5 μM) and *Ts*OYE (8 μM). The reaction was performed at 30 °C for 4–16 hours. Maximum products yields are indicated. Conversions and ee values were determined by GC-FID analysis. n.a. = not applicable. SH = NAD^+-reducing hydrogenase, *Ts*OYE = ene reductase. [a] Total turnover numbers of mol 2-methylcyclohexanone per mol enzyme or cofactor were determined at end of experiment.

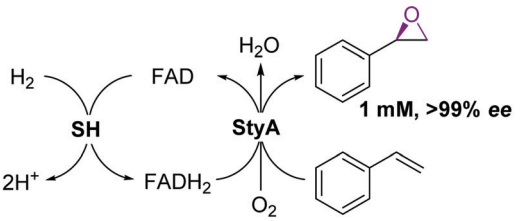
gave a low but detectable conversion, which may be indicative of a slow, direct electron transfer from SH to the flavin prosthetic group in *Ts*OYE.[25] Pleasingly, already under non-optimized conditions, significant catalytic performances for both SH and FMN with TTN up to 8400 and 105, respectively, were observed (Table 1).

In order to demonstrate the O_2 tolerance of the SH for flavin regeneration and the extension to the FAD cofactor, we used our system to drive the epoxidation of styrenes catalyzed by styrene monooxygenase from *Pseudomonas* sp. VLB120.[26] Styrene monooxygenase is a two-component, flavin monooxygenase composed of StyA, the $FADH_2$-dependent monooxygenase subunit and StyB (a flavin reductase catalyzing the *in situ* generation of $FADH_2$ from FAD and NADH).[27] The $FADH_2$ regeneration system proposed here circumvents the nicotinamide cofactor together with the reductase subunit (StyB). Up to 1 mM of enantiopure (*S*)-styrene oxide was obtained upon incubation of StyA with SH and FAD under O_2/H_2 atmosphere for the reduction of styrene with the co-substrate O_2 (Scheme 1). The TTN of SH for FAD reduction was up to 333.

One major side reaction of the H_2-driven styrene epoxidation reaction was the oxidative uncoupling of the $FADH_2$ regeneration from the StyA-catalyzed epoxidation reaction. Due to the high reactivity of reduced flavin species with O_2, the StyA-related formation of 4a-hydroperoxoflavin yielding the desired epoxidation product competes with the spontaneous aerobic reoxidation of $FADH_2$ yielding H_2O_2.[28] Although this 'Oxygen Dilemma' severely impedes the StyA-catalyzed epoxidation reaction,[29] it is a promising approach to promote selective oxyfunctionalization reactions catalyzed by peroxygenases (UPOs). While UPOs rely on H_2O_2 as a stoichiometric oxidant, they are also rapidly inactivated in the presence of excess amounts of H_2O_2, thereby necessitating precise control over the *in situ* H_2O_2 concentration.[30] We therefore coupled the proposed SH-based flavin reduction system to the recombinant peroxygenase from *Agrocybe aegerita* (r*Aae*UPO) for selective hydroxylation reactions (Scheme 2 and Fig. S2, ESI†).[31,32]

To minimize evaporative loss of the volatile reagents under the non-optimized reaction conditions for the proof-of-concept, we utilized the two liquid phase approach (*i.e.*, applying the substrate as a neat organic layer to the aqueous reaction mixture). Under these conditions, 1.5 mM of enantiopure (*R*)-1-phenylethanol was obtained from ethylbenzene. The overall robustness of the reaction process needed to be improved, as the reaction typically ceased after 1 h. We attributed this short reaction time to the poor stability of SH in the presence of hydrophobic solvents,[33] which could be alleviated by immobilization of the biocatalyst as demonstrated previously.[34] Hence, we were pleased to observe that using 95 nM of the Amberlite FPA54 immobilized SH under identical conditions resulted in an accumulation of 4.4 mM of the enantiomerically pure product. Under these conditions (substituting ethylbenzene with cyclohexane), up to 3.7 mM of cyclohexanol product were obtained (Scheme 2 and Table S4, ESI†). Notably, the TTN for SH increased from 750 to approximately 46 000.

In air, H_2 has a flammability range of 4–74% and can be explosive at concentrations of 19–57%.[35] An undesired combustion

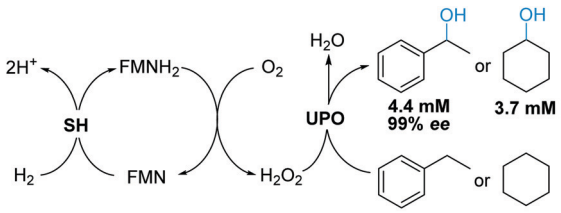


Total turnover numbers[a]		
StyA	SH	FAD
200	333	3.3

Scheme 1 H_2-driven asymmetric epoxidation of styrene. Reaction conditions: 1 mL of KPi buffer pH 7.5 containing 300 μM FAD, 5 mM substrate. The gas mixture was 10% O_2 30% N_2 and 60% H_2. SH (3 μM) and styrene monooxygenase (5 μM) was added to start the reaction. The reaction was performed at 30 °C for 16 hours. Conversions and ee values were determined by GC-FID analysis. SH = NAD^+-reducing hydrogenase, StyA = styrene monooxygenase. [a]Total turnover numbers of mol styrene epoxide per mol enzyme or cofactor.

during H_2 driven catalysis can be prevented with mixtures that are very dilute in either H_2 or O_2. As the SH has a K_M for H_2 in the lower μM range,[36] this usually does not present a problem. Recently we developed a scalable platform that employs electrolysis to power biotransformations.[37] For H_2 driven flavin reactions, a similar setup could be used in future studies. The supply of the right amount of H_2 by electrolysis, which is continuously oxidized by the SH, would ensure a safe H_2 handling by avoiding the formation of explosive gas mixtures.[37]

In conclusion, we have demonstrated as a proof of concept the H_2-driven reduction of flavins to promote selective biocatalytic reduction and oxidation reactions. The presented flavin recycling by H_2 oxidation enabled the efficient reduction of activated alkenes, selective hydroxylation of alkanes and asymmetric epoxidation of styrene. The cofactor regeneration by a



Total turnover numbers[a]		
UPO	SH	FMN
11250	750	225
	46000 (immobilized)	

Scheme 2 H_2-driven hydroxylation of ethylbenzene and cyclohexane Reaction conditions: 750 μL of KPi buffer pH 7.5 containing 200 μM FMN was added to 750 μl of pure substrate in a two-phase system. The reaction was performed in pressure secured closed vials with 7 mL excess of headspace filled with gas composed of 40% O_2 and 60% H_2. The reaction was performed at 30 °C for 4 hours. Sampling was taken from the organic phase. 95 nM of immobilized SH on 210 mg Amberlite™ FPA54 and 4 μM UPO were added to start the reaction. Conversions and ee values were determined by GC-FID analysis. SH = NAD^+-reducing hydrogenase, UPO = unspecific peroxygenase. [a]Total turnover numbers of mol ethylbenzene per mol enzyme or cofactor.

hydrogenase resulted in 100% atom efficiency without by-products, with a high turnover rate of up to 20.3 s^{-1} and a total turnover number of up to 46 000. The electron delivery strategy circumvents NAD(P)H as the reductant and shortens previous multi-protein systems with reductases to a two-enzyme system. The O_2 tolerance of the hydrogenase allowed aerobic reactions with a monooxygenase, whereas the spontaneous aerobic reoxidation of $FMNH_2$ to H_2O_2 fueled peroxygenase-catalyzed hydroxylation reactions. The general applicability of the NAD(P)H-free, H_2-driven flavin regeneration system was demonstrated using different oxidoreductases (*Ts*OYE, StyA and UPO) and substrates (*e.g.*, 2-methylcyclohexenone, styrene, ethylbenzene). The ability to use H_2 as a fuel for flavin recycling opens up opportunities for highly atom-efficient biocatalysis, providing a versatile platform for future biotransformations with flavin- and H_2O_2-dependent enzymes.

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Conflicts of interest

There are no conflicts to declare.

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