Supplementary Information

A hydrogen-driven biocatalytic approach to recycling synthetic analogues of NAD(P)H

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Contents

1. General information

All reagents were purchased with the highest purity available and used as received.

Formate dehydrogenase (FDH) was obtained from Evocatal. Glucose dehydrogenase (GDH) was provided by Johnson Matthey. These enzymes were used as supplied, with no purification step.

*Re*SH was prepared according to: J. Preissler, S. Wahlefeld, C. Lorent, C. Teutloff, M. Horch, L. Lauterbach, S. P. Cramer, I. Zebger and O. Lenz, *Biochim. Biophys. Acta Bioenerg.*, 2018, 1859, 8-18.

*Ht*SH was prepared according to: E. Schwartz, U. Gerischer and B. Friedrich, *J. Bacteriol.*, 1998, 180, 3197-3204.

Mo-FDH was prepared according to: T. Hartmann and S. Leimkuhler, *FEBS J.*, 2013, 280, 6083-6096.

GC traces were measured on a ThermoScientific Trace 1310 GC equipped with a flame ionization detector (FID).

2. Determination of GDH and FDH activity on cofactors

Raw data used to determine activity of glucose dehydrogenase and formate dehydrogenase for reduction of a range of cofactors. Experiments were performed in a plate reader (BMG, NanoStar) such that the activity of each enzyme could be analysed for multiple cofactors in parallel. Spectra were recorded every 10 min over the time periods used. For clarity, only the first and last scans are shown in Figure S1. Subtle changes in absorbance reflect evaporation of the small reaction volumes from the plate reader over the long time frames used.

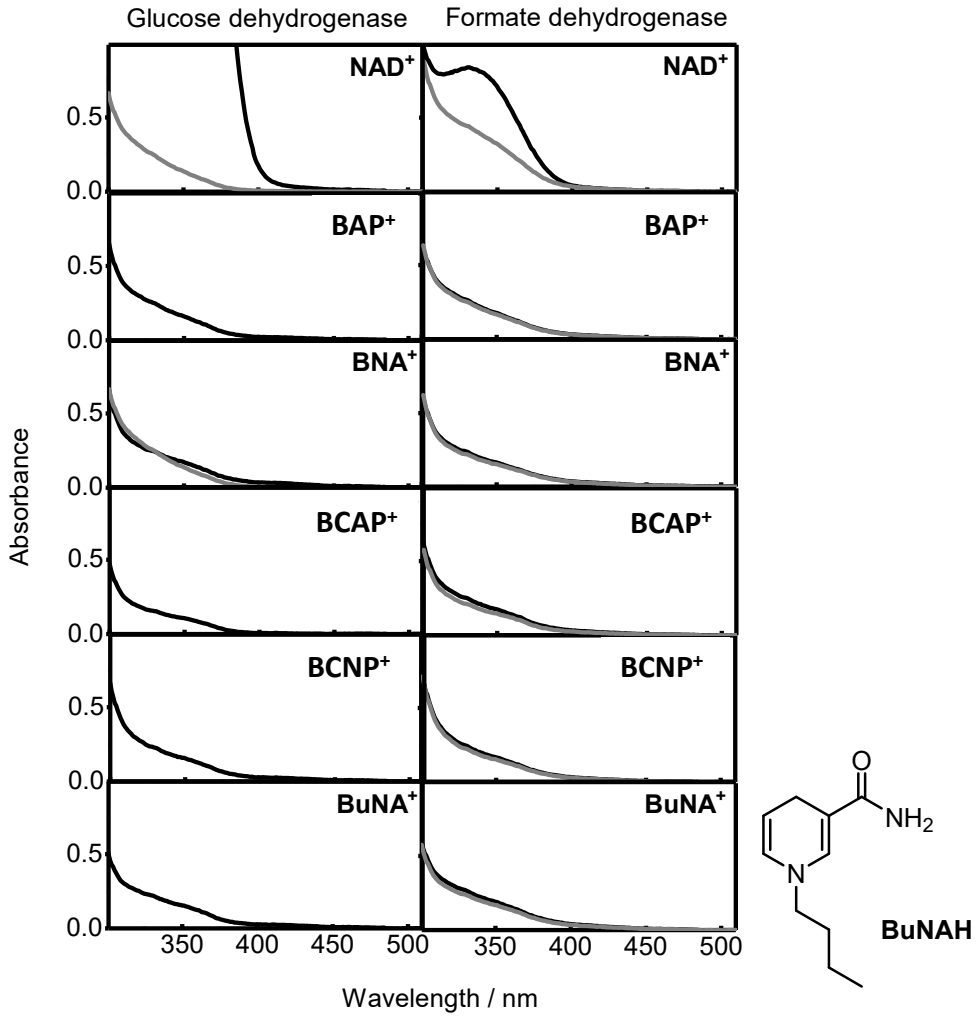

Figure S1. UV-visible spectra recorded after addition of either glucose dehydrogenase (0.2 mg) or formate dehydrogenase (0.2 mg) to a solution containing oxidised cofactor (5 mM), and glucose (10 mM) or formate (10 mM), respectively. Other conditions: pH 8.0, 50 mM Tris-HCl buffer containing 2% v/v DMSO, 30 °C, 100 µL scale. The first spectrum was recorded after *ca* 2 min (grey) and the final spectrum (black) were recorded after 1 h (glucose dehydrogenase) or 6.5 h (with formate dehydrogenase). The structure of the reduced form of $BuNA^+$, BuNAH is shown on the right. Structures of the other reduced cofactors are shown in the main text, Figure 1: BNAH: 1-benzyl-1,4-dihydronicotinamide; BAPH: 1-benzyl-3-acetyl-1,4-dihydropyridine; BCNPH: 1-benzyl-3-cyano-1,4-dihydropyridine; AmNAH: 1-carbamoylmethyl-1,4-dihydronicotinamide, BCAPH: 1-benzyl-1,4-dihydropyridine-3-carboxylic acid). Due to limited stability of BuNAH in solution it was not taken forward in further experiments in this study.

3. Experimental conditions for H_2-driven artificial cofactor recycling

3.1. *In situ* UV-vis spectroscopy for H_2-driven reactions

Buffer (either 50 mM MOPS-NaOH pH 7.0 or 50 mM Tris-HCl pH 8.0) was saturated with H_2 gas for at least 1 h prior to use for H_2-driven reactions. To initiate the reaction 40 µg of hydrogenase was added to 1 mL of H_2 saturated buffer containing 2 mM of oxidised cofactor in a sealed UV-Vis cuvette under a flow of H_2. The temperature of the reaction was controlled by a Peltier cell holder. The reaction was monitored by UV-Vis spectrometry (Agilent Cary 60) by taking scans every minute. The rate of reaction was determined by monitoring the change at the λ_{max} of the cofactor (A min^{-1}) and converting this to the change in concentration of reduced cofactor over time (µmol min^{-1}) using literature extinction coefficients for each cofactor. The specific activity (µmol min^{-1} mg^{-1}) of the soluble hydrogenase was then determined.

4. Activity of soluble hydrogenases towards synthetic cofactors

The results in Figure S2 show UV-vis spectra recorded over time for reactions containing BAP^+ in the presence H_2 gas, with either (a) *Ht*SH or (b) *Re*SH. The increase in absorbance at 375 nm is diagnostic for generation of BAPH, demonstrating that the soluble hydrogenases are capable of H_2-driven BAPH generation. Similar spectra were recorded for generation of AmNAH and BCNPH by *Ht*SH.

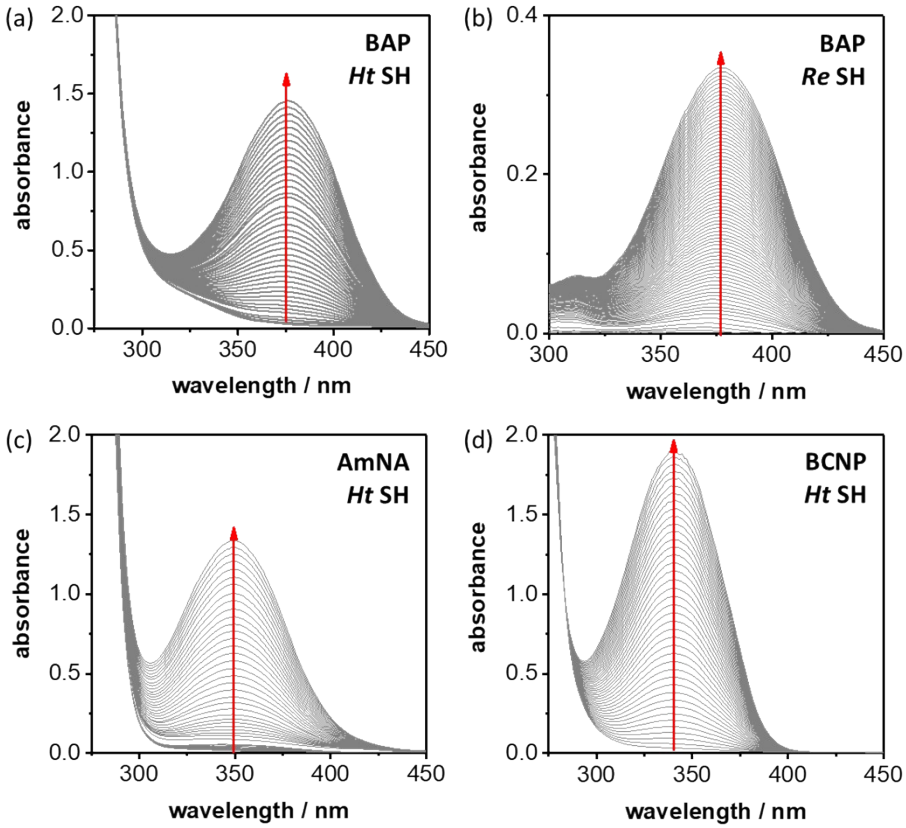

Figure S2. UV-Vis spectra over time showing the formation of reduced artificial cofactors by soluble hydrogenase over time. All reactions were performed in a 1 mL UV-vis cuvette under a flow of H_2 gas. Reactions with *Ht*SH were performed in 50 mM MOPS-NaOH buffer, pH 7.0 at 50 °C and reactions with *Re*SH were performed in 50 mM Tris-HCl, buffer pH 8.0 at 25 °C. Scans were taken every minute for 40 min. Other reaction conditions: oxidised artificial cofactor (2 mM); SH (40 µg); DMSO (2% v/v). Negative controls without enzyme or cofactor showed no change in absorbance.

5. Activity of *Rc* Mo-FDH towards artificial cofactors

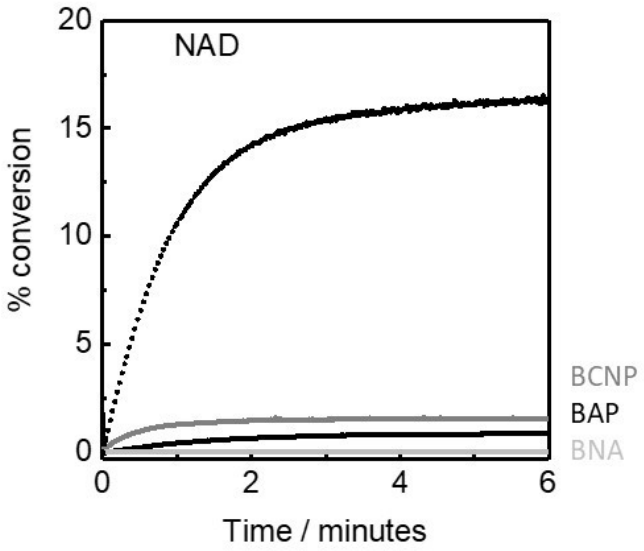

Figure S3. Time courses for the conversion of oxidised to reduced cofactor by *R. capsulatus* Mo-FDH (7.2 µg) in a solution of 50 mM Tris-HCl, pH 8.0, 30 °C, 2% v/v DMSO, 10 mM formate (with a total volume of 200 µL), with 1 mM oxidised cofactor. The reactions were monitored *in situ* using UV-visible spectroscopy kinetics at λmax for each reduced cofactor. Synthetic cofactors BNA (light grey), BCNP (dark grey) and BAP (black) are compared to the biological cofactor, NAD (black, dotted).

6. Supplementary methods for H_2-driven C=C-bond reductions

6.1. H_2-driven reduction of carvone

Buffer (50 mM MOPS-NaOH pH 7.0 containing 2% v/v DMSO) was saturated with H_2 gas for 1 h. To initiate the reaction, 200 µg of *Ht*SH was added to 1 mL of buffer containing 2 mM of $BCNP^+$ in a sealed UV-Vis cuvette under a flow of H_2. The reaction was allowed to proceed until approximately 1 mM of BCNPH had been produced, at this point 1 mM of carvone was added followed by the addition of 2 µM of *Ts*OYE. After all of the reduced cofactor had been consumed the reaction mixture was extracted by an equal volume of EtOAc and analysed by GC-FID.

6.2. Stability of *N*-ethylmaleimide and *N*-ethylsuccinimide

N-ethylmaleimide is known to be susceptible to hydrolysis to *N*-ethylmaleamic acid (Gregory, J.D., *J. Am. Chem. Soc.* 1955, 77, 3922–3923), and evaporation can also be a problem over a longer reaction timecourse. The stability of *N*-ethylmaleimide and *N*-ethylsuccinimide was analysed before and after a 2 h incubation under reaction conditions (in the presence of H_2 flow, 50 °C). The GC traces in Figure S5 show that (a) only 58% of the substrate (*N*-ethylmaleimide) remains in solution after 2 h, but that (b) the product (*N*-ethylsuccinimide) is stable over this time frame. For this reason, reactions were performed as fed-batch experiments in order to minimise the time that the substrate spent in solution.

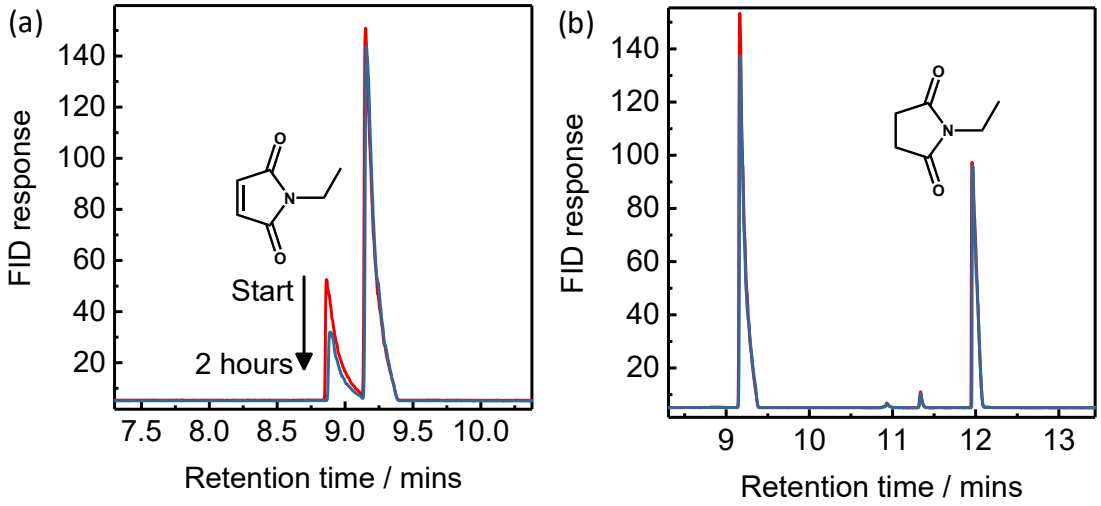

Figure S4. Stability of *N*-ethylmaleimide and *N*-ethylsuccinimide under reaction conditions.

6.3. H_2-driven reduction of N-ethylmaleimide

Buffer (50 mM MOPS-NaOH pH 7.0 containing 2% v/v DMSO) was saturated with H_2 gas for 1 h. To initiate the reaction 200 µg of *Ht*SH was added to 1 mL of buffer containing BAP^+ (0.5 mM) and *Ts*OYE (2 µM) in a sealed UV-Vis cuvette under a flow of H_2. The reaction was allowed to proceed until approximately 0.2 mM of BAPH had been produced, at this point 0.22 mM of *N*-ethylmaleimide was added. This was repeated 8 times. After all of the reduced cofactor had been consumed the reaction mixture was extracted by an equal volume of EtOAc and analysed by GC-FID by the method shown below.

6.4. GC-FID method

GC method used for determining conversion of carvone to dihydrocarvone, enantiomeric excess of (+)-dihydrocarvone and conversion of *N*-ethylmaleimide and *N*-ethylsuccinimide.
Column: CP-Chirasil-Dex CB (Agilent), 25 m length, 0.25 mm diameter, 0.25 μm (film thickness), fitted with a guard of 10 m deactivated fused silica of the same diameter.
Carrier: He (CP grade), 2 mL/min (constant flow)
Inlet temperature: 200 ˚C
Injection conditions: Splitless with split flow 60 mL/min, splitless time 0.8 min, purge 5 mL/min.
Injection volume = 0.2 μL
Detection: FID
Detector temperature: 200 ˚C
Detection gases: H_2 (35 mL/min), air (350 mL/min), makeup N_2 (40 mL/min)
Oven heating programme:

Time (min)	**Temperature**
0 ✂ 5	Hold at 70 ˚C
5 ✂ 20	Ramp to 165 ˚C at 10 ˚C / min
20 ✂ 21	Ramp to 200 ˚C at 50 ˚C / min
21 ✂ 26	Hold at 200 ˚C for 5 min