ChemComm

COMMUNICATION

View Article Online
View Journal

Cite this: DOI: 10.1039/c5cc10316a

Received 11th January 2016,
Accepted 15th March 2016

DOI: 10.1039/c5cc10316a

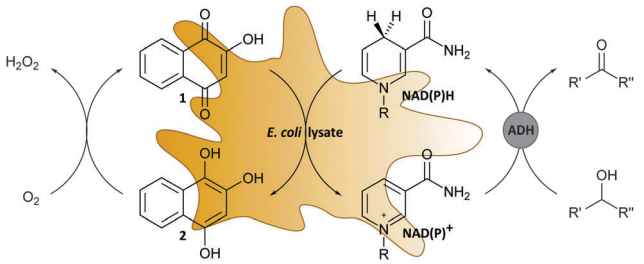
www.rsc.org/chemcomm

A quinone mediator drives oxidations catalysed by alcohol dehydrogenase-containing cell lysates†

Julian Haas,[a] Michael A. Schätzle,[a] Syed M. Husain,‡[a] Johannes Schulz-Fincke,[abc] Manfred Jung,[a] Werner Hummel,[d] Michael Müller[a] and Steffen Lüdeke*[a]

Spontaneous electron transport to molecular oxygen led to regeneration of oxidised nicotinamide cofactor in cell lysates that contain an alcohol dehydrogenase, a quinone reductase and a quinone mediator. This concept allows the efficient oxidation of alcohols in the presence of alcohol dehydrogenase-containing *E. coli* lysates and catalytic amounts of the quinone lawsone.

In living cells, the generation of alcohol and carbonyl metabolites frequently involves reductions and oxidations catalysed by alcohol dehydrogenases (ADHs). These reactions mostly depend on nicotinamide adenine dinucleotide (phosphate) cofactor [NAD(P)] with reductions using NAD(P)H and oxidations consuming $NAD(P)^+$, each in stoichiometric amounts. Therefore, when ADHs are used as biocatalysts in organic synthesis, cofactor regeneration systems are required to decrease costs, to avoid problems from cofactor or by-product inhibition and to generate a thermodynamic driving force in either direction.[1] The regeneration of the oxidised cofactor $NAD(P)^+$ is particularly challenging. This is reflected in the high number of different approaches made in the past.[2–4] While many of these approaches show good performance in a number of individual reactions, none can be considered as broadly applicable for $NAD(P)^+$ regeneration. Living cells on the other hand, have strategies to ensure permanent nicotinamide cofactor supply, which is exploited in whole-cell transformations.[5,6] These intrinsic redox cycling mechanisms involve NAD(P)-dependent enzymes and reducible or oxidisable substrates for electron shuttling. For example in mammalian cells, the NAD^+/NADH ratio can be increased in the presence of the *ortho*-quinone β-lapachone.[7] Recently, we made an observation that could be explained by a similar mechanism in *Escherichia coli* (*E. coli*): the naphthoquinone lawsone (**1**) was reduced under consumption of NAD(P)H in the presence of *E. coli* lysate with the resulting naphthohydroquinone, 1,2,4-trihydroxynaphthalene (**2**), being reoxidised spontaneously by molecular oxygen from ambient air (Scheme 1).[8]



Scheme 1 ADH-catalysed alcohol oxidation in the presence of lawsone (**1**) and *E. coli* lysate.

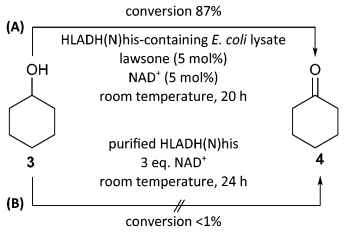
These findings suggest that quinones, due to their redox properties, interfere with enzymes that are involved in maintaining the $NAD(P)^+$/NAD(P)H ratio in living cells. The spontaneous reoxidation of the hydroquinones that have been generated by the cellular quinone reductases (QRs) establishes virtually an unlimited supply of $NAD(P)^+$. Therefore, oxidation of an alcohol to a carbonyl group by an $NAD(P)^+$-dependent ADH may only require the addition of a reoxidisable quinone to ADH-containing cell extracts (Scheme 1).

We verified this hypothesis through the oxidation of cyclohexanol (**3**) by *E. coli* lysate containing heterologously produced His-tagged horse liver ADH (HLADH(N)his) in the presence of catalytic amounts (5 mol%) of NAD^+ and **1** (Scheme 2A). Lawsone (**1**) is a highly abundant naphthoquinone from the henna tree, *Lawsonia inermis*, and has been used for millennia as a dye for skin and hair. The *E. coli* lysate was obtained from the supernatant after cell disruption and removal of cell debris by centrifugation. After incubating for 20 h with catalytic amounts of cofactor and **1** we observed 87% conversion of **3** into cyclohexanone (**4**) (Scheme 2A).

[a] *Institute of Pharmaceutical Sciences, University of Freiburg, Albertstraße 25, 79104 Freiburg, Germany. E-mail: steffen.luedeke@pharmazie.uni-freiburg.de*
[b] *German Cancer Consortium (DKTK), Freiburg, Germany*
[c] *German Cancer Research Centre (DKFZ), Heidelberg, Germany*
[d] *Institute of Molecular Enzyme Technology, Heinrich-Heine-University of Düsseldorf, Research Centre Jülich, Stetternicher Forst, 52426 Jülich, Germany*

† Electronic supplementary information (ESI) available: General methods, molecular cloning and protein purification, enzymatic oxidation, calibration curves, analytical data. See DOI: 10.1039/c5cc10316a

‡ Present address: Centre of Biomedical Research, SGPGI Campus, Raebarelli Road, Lucknow 226014, India.



Scheme 2 Oxidation of cyclohexanol (**3**, 20 mM) by HLADH(N)his-containing *E. coli* lysate (4 U mL^{-1}) with lawsone (**1**)-dependent cofactor regeneration (A) or by purified HLADH(N)his (4 U mL^{-1}) in the presence of excessive NAD$^+$ (3 eq.) (B).

The yield exceeds by far the amount of added NAD$^+$ (5 mol%) indicating cofactor regeneration. As expected, in absence of naphthoquinone **1** the reaction catalysed by purified HLADH(N)his showed only negligible conversion, even in the presence of three equivalents of NAD$^+$ (Scheme 2B). This fact emphasises the benefit of cofactor regeneration established by naphthoquinone **1** and *E. coli* lysate.§

According to Scheme 1, enzymes from *E. coli* lysate catalyse the NAD(P)H-dependent reduction of naphthoquinone **1**. Most probably, these enzymes are NAD(P)H- and flavin-dependent QRs that are widespread among most organisms and known to be capable of catalysing two electron reductions of quinones.[9] The QRs NfsA and NfsB from *E. coli* were already identified as reductases of **1**.[10] While NfsA shows a strong preference for the phosphorylated cofactor NADPH, NfsB tolerates both NADPH and NADH with moderate preference for the latter.[10] Hence, we used purified, His-tagged NfsB (NfsB(N)his) together with purified HLADH(N)his for further characterisation of the regeneration process.

The oxidation of alcohol **3** was monitored over time in the presence of all putatively involved components [purified HLADH(N)his, purified NfsB(N)his, substrate **3** (20 mM), NAD$^+$ (5 mol%) and **1** (5 mol%)] and in the absence of either the quinone mediator **1** or NfsB(N)his.§ The role of the individual components for the enzymatic oxidation and NAD$^+$ regeneration was deduced from the evaluation of the respective reaction progress (Fig. 1). As expected, high conversions (90%) only occur in the presence of both NfsB(N)his and quinone **1**. In the absence of NfsB(N)his, the conversion of **3** into ketone **4** was approximately 5%, which suggests stoichiometric consumption of the initially added 0.05 equivalents of NAD$^+$. In the absence of naphthoquinone **1**, HLADH(N)his-catalysed oxidation still took place, albeit with less than 20% conversion after 24 h. This indicates lawsone-independent cofactor regeneration in the presence of NfsB(N)his to some extent, which suggests a low background NADH oxidase activity of this enzyme that is independent from quinone reduction.

We hypothesised the redox cycling of **1** depends on the oxidation of hydroquinone **2** by molecular oxygen. Therefore we tested the effect of oxygen supply by maintaining the oxygen pressure at 2 bar during the HLADH(N)his-catalysed oxidation of substrate **3** (Fig. 1).§ We observed an initial increase in the conversion rate reducing the time until maximal conversion nearly by half (4 h, instead of 8 h), which suggests that oxygen supply is rate-limiting. Although an increase in oxygen supply

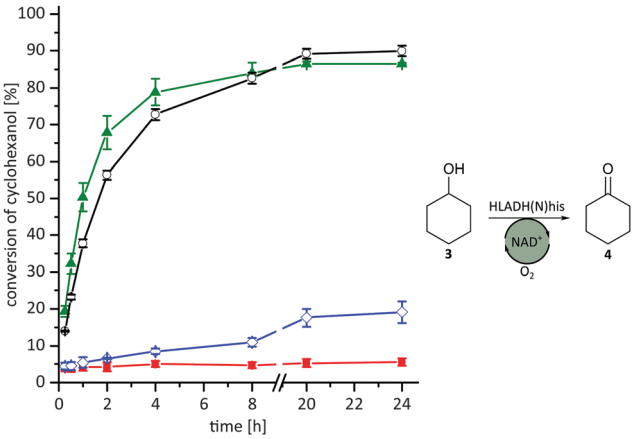


Fig. 1 Monitoring of the HLADH(N)his-catalysed oxidation of cyclohexanol (**3**) under different conditions: —○— standard conditions: cyclohexanol (**3**, 20 mM), lawsone (**1**, 1 mM), NAD$^+$ (1 mM), NfsB(N)his (10 U mL^{-1}), HLADH(N)his (4 U mL^{-1}); —■— w/o NfsB(N)his; —◇— w/o lawsone (**1**); —▲— + 2 bar O_2 gauge pressure. Experiments were performed in triplicate (the error bars represent the standard deviation).

should shift the equilibrium in favour of the oxidised product **4**, we did not observe an improvement in the overall conversion after 24 h. We assume that other parameters, such as enzyme inhibition by putative H_2O_2-formation, are limiting the overall conversion. For instance, it is known that Zn^{2+}-containing ADHs, such as HLADH(N)his, are deactivated over time through oxidation by H_2O_2.[11,12] According to the suggested mechanism in Scheme 1, H_2O_2 is formed during the spontaneous oxygen-dependent oxidation of the naphthohydroquinone **2** into **1**.[13] Therefore, during cofactor regeneration, the amount of generated H_2O_2 should be equivalent to the amount of recycled NAD$^+$.

For validation of this hypothesis, we assayed the time-dependent depletion of NADPH and NADH photometrically, either in the presence of purified NfsB(N)His, in the presence of **1**, or by adding a combination of both (Fig. 2A).¶ A significant decrease in NAD(P)H content over time was only observed in presence of both **1** and NfsB(N)His. The oxidation proceeds slightly slower for NADPH compared to NADH, which is in agreement with the previously reported preference of NfsB for NADH.[10] As **1** was added in sub-stoichiometric amounts (10 mol%), this experiment confirms the catalytic role of the naphthoquinone in the NAD(P)H oxidation process leading to approximately ten reduction/oxidation cycles until maximum conversion (95%) after ten minutes. At maximum conversion, the H_2O_2 concentration should be proportional to the concentration of NAD(P)H that was added.

We quantified H_2O_2 formation during naphthoquinone-dependent NfsB-catalysed oxidation by the use of a horseradish peroxidase-based fluorometric assay at different concentrations of NADH.¶[14] An almost linear relationship between the formation of H_2O_2 and the NADH concentration used in each corresponding experiment was observed (Fig. 2B). This is consistent with stoichiometric H_2O_2 formation in each regeneration cycle and agrees with the proposed mechanism illustrated in Scheme 1.

The maximum amount of formed H_2O_2 is equivalent to the substrate starting concentration, which was 20 mM for the

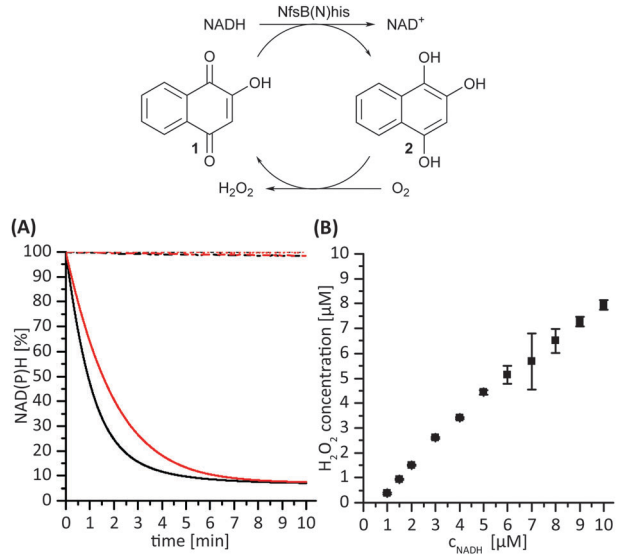


Fig. 2 (A) Depletion of NAD(P)H (0.2 mM) in the presence of NfsB(N)his and/or lawsone (**1**, 0.02 mM), determined photometrically by monitoring the absorbance at λ = 340 nm. — NADH/lawsone (**1**)/NfsB(N)his; — NADPH/lawsone (**1**)/NfsB(N)his; ---- NADH/NfsB(N)his; ---- NADPH/NfsB(N)his; ···· NADH/lawsone (**1**); ···· NADPH/lawsone (**1**). (B) NADH concentration-dependent formation of H_2O_2 in the presence of NfsB(N)his and catalytic amounts of lawsone (2 μM). Experiments were performed in triplicate (the error bars in (B) represent the standard deviation).

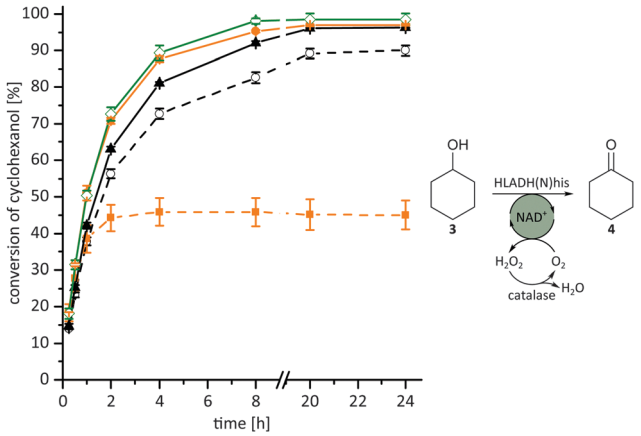


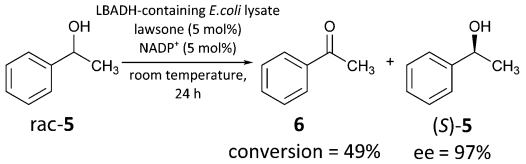
Fig. 3 The influence of H_2O_2 and/or catalase on the HLADH(N)his-catalysed oxidation of cyclohexanol (**3**): ---○--- standard conditions: cyclohexanol (**3**, 20 mM), lawsone (**1**, 1 mM), NAD^+ (1 mM), NfsB(N)his (10 U mL^{-1}), HLADH(N)his (4 U mL^{-1}); ---■--- + H_2O_2 20 mM; —▲— + catalase; —●— + catalase, + H_2O_2 20 mM; —◇— + catalase, + 2 bar O_2 gauge pressure. Experiments were performed in triplicate (the error bars represent the standard deviation).

HLADH(N)his-catalysed oxidation of alcohol **3**. We performed the reaction in the presence of H_2O_2 (20 mM) to assess its role in enzyme inhibition.§ As expected, the HLADH(N)his-catalysed oxidation of substrate **3** showed significantly lower performance with the conversion slowing down after 1 h and coming to a complete standstill (45% conversion) after 4 h (Fig. 3). The addition of catalase, an enzyme that dismutates H_2O_2 into H_2O and O_2, completely rescued and even slightly improved the catalytic performance (Fig. 3). In the presence of catalase nearly complete conversion (>95%) was achieved after 20 h, irrespective of whether O_2 gauge pressure was applied, H_2O_2 was added, or neither of the two.§ As expected, the O_2 gauge pressure accelerates the initial conversion rate compared to conditions where ambient air is the sole source of O_2. Surprisingly, this is also true for the sample where H_2O_2 and catalase were added, which suggests that fast dismutation of H_2O_2 by catalase contributes to an improved supply with molecular oxygen.

As NfsB has comparable activity with both NADH and NADPH as a cofactor,[10] we hypothesised that quinone-driven cofactor regeneration could also be applied in NADP-dependent oxidations. This was tested for the oxidation of racemic 1-phenylethanol (**5**) catalysed by NADP-dependent ADH from *Lactobacillus brevis* (LBADH). Highly stereoselective reductases such as LBADH have been used for kinetic resolution of racemic alcohols to give access to chiral chemicals.[6,15,16] LBADH is known for the highly enantioselective reduction of acetophenone (**6**) to alcohol (*R*)-**5**.[17,18] Therefore, the LBADH-catalysed oxidation of the racemic alcohol **5** should yield unreacted enantiopure (*S*)-**5** (50%) and acetophenone (**6**, 50%). Indeed, the oxidation reaction, driven by addition of 5 mol% of **1** and LBADH-containing *E. coli* lysate, led to a mixture of oxidation product **6** and remaining substrate (*S*)-**5** with a high enantiomeric excess (ee) of 97% (Scheme 3).§

In summary, our results show that the addition of a quinone redox mediator to reactions catalysed by ADH-containing *E. coli* lysates is sufficient to generate a driving force for highly selective oxidations of alcohols under aerobic conditions. Our studies employing purified enzymes are in good agreement with quinone reduction by an NAD(P)H-dependent QR such as NfsB, which supplies the ADH reaction with the oxidised cofactor. As the hydroquinone is reoxidised by molecular oxygen, H_2O_2 is formed as a by-product. This requires the choice of a quinone redox mediator with sufficiently low Nernst potential, such as the naphthoquinone lawsone (**1**).[19,20] For obvious reasons, such as the ease of preparation and storage stability, in most cases non-purified enzymes in host cell lysates are particularly attractive for applications in preparative scale reactions. For $NAD(P)^+$-regeneration mediated by **1** the *E. coli* lysate provides the QRs required for the recycling process without further need for QR overexpression or addition. Although host cell lysates may inherently provide enzymes for detoxification of H_2O_2, it is recommendable to add catalase in order to avoid potential inhibitory effects from this by-product.

QRs are generally assumed to be involved in the detoxification of semiquinone radicals that are formed by single electron transfer processes by catalysing a two-electron reduction to



Scheme 3 LBADH-catalysed kinetic resolution of *rac*-1-phenylethanol (*rac*-**5**, 20 mM) by LBADH in *E. coli* lysate (3 U mL^{-1}).

usually more stable hydroquinones.[9] Nevertheless, some hydroquinones are spontaneously reoxidised in the presence of molecular oxygen, thereby leading to formation of H_2O_2 and non-physiological ratios of $NAD(P)^+$/NAD(P)H.[7] Besides biological activity as enzyme inhibitors,[21] high H_2O_2 levels and redox cycling may contribute to the cytotoxicity and antibiotic activity of quinones. In this study, we exploit redox-cycling for a naphthoquinone-mediated cofactor regeneration system to drive highly specific ADH-catalysed oxidations.

Financial support of this work by the DFG (IRTG 1038, RTG 1976) is gratefully acknowledged. We thank Prof. A. Stolz, Stuttgart, for providing NfsB.

Notes and references

§ For enzymatic conversions with HLADH(N)his, the substrate **3** was dissolved in Tris-HCl buffer (50 mM, pH = 8.5) to give a 20 mM solution. For the quinone driven reaction lawsone (1 mM), NAD^+ (1 mM) and HLADH(N)his-containing *E. coli* lysate (4 U mL^{-1}) were added to start the reaction. For oxidations without cofactor regeneration, 3 eq. NAD^+ (60 mM) and purified HLADH(N)his were added instead. For the monitored reactions with purified enzymes standard cofactor-regeneration conditions correspond to addition of 5 mol% of both NAD^+ and lawsone (**1**) and 10 U mL^{-1} of purified NfsB(N)his. Modified conditions were identical except for the absence of either **1** or NfsB(N)his, the presence of either H_2O_2 (20 mM) and/or catalase (295 U mL^{-1}) or the injection of O_2 (2 bar). For the latter, a laboratory pressure reactor system (Miniclave, Büchi AG, Uster, Switzerland) was used. The reaction was started by the addition of 4 U mL^{-1} of purified HLADH(N)his. The typical total volume of the reaction mixture was 1 mL. After defined periods of time (15 and 30 min, and 1, 2, 4, 8, 20 and 24 h), samples of 50 μL were removed and immediately extracted with 100 μL of ethyl acetate. Subsequently, the conversion was determined by GC-MS analysis of the organic phase. Conditions for the enzymatic conversion of *rac*-**5** with LBADH-containing *E. coli* lysate (4 U mL^{-1}) corresponded to those given above, except that Tris-HCl (50 mM, pH = 8.0) containing 1 mM $MgCl_2$ was used as the buffer in a total volume of 50 mL. After the solution was stirred at room temperature for 24 h, it was extracted three times with 50 mL ethyl acetate. The combined organic phase was washed twice with 100 mL 0.01 M NaOH solution to remove residual lawsone, then dried over $MgSO_4$, and concentrated. The resulting yellow oil was analysed by ^1H NMR spectroscopy and chiral-phase HPLC.

¶ For photometric monitoring of NAD(P)H depletion 1 mL of a solution of either NADH or NADPH (0.2 mM) and lawsone (0.02 mM) in KPi buffer (50 mM, pH = 7.5) was placed in a cuvette (path length 1 cm). Purified NfsB(N)his was added to start the reaction. The change in absorbance at λ = 340 nm was monitored for 10 min. Control reactions were prepared without addition of either lawsone or NfsB(N)his. H_2O_2 formation was determined in a 384 well-plate with a total volume of 20 μl and a lawsone concentration of 2 μM in each well. NADH concentration was varied between 0 and 10 μM. The reaction was started by addition of NfsB(N)his (8 U mL^{-1}). After 10 min of incubation at room temperature the assay buffer (Ampliflu Red, 50 μM; horseradish peroxidase, 1 U mL^{-1}) was added. The fluorescence intensity was measured at an excitation wavelength of λ_{ex} = 510 nm and an emission wavelength of λ_{em} = 615 nm. For the calibration H_2O_2 standard solutions instead of the reaction mixtures were used.

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