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Novel Chemo-Enzymatic Mimic of Hydrogen Peroxide-Forming NAD(P)H Oxidase for Efficient Regeneration of NAD^+ and $NADP^+$

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Abstract: The current methods for regeneration of NAD^+ and $NADP^+$ in dehydrogenase-catalyzed organic synthesis are not technologically mature due to problems with operational stability, kinetic efficiency or thermodynamic equilibrium. We show here that *Candida tenuis* xylose reductase converts 9,10-phenanthrenequinone with good turnover frequency when using NADH ($12\ s^{-1}$) or NADPH ($1.6\ s^{-1}$) as the coenzyme and exhibits high binding affinity for this quinone substrate ($K_m \leq 13\ \mu M$). Because chemical reaction of the hydroquinone product with dissolved molecular oxygen regenerates the quinone at a rate comparable to that of the enzymatic reduction, a chemo-enzymatic process is established where the reductase, a catalytic concentration of 9,10-phenanthrenequinone (25 μM) and molecular oxygen promote efficiently the production of $NAD(P)^+$ from NAD(P)H. Oxidation of the hydroquinone occurs *via* a radical chain reaction that involves superoxide as the propagating species and yields a molar equivalent of hydrogen peroxide for each 9,10-phenanthrenequinone recycled. Using the NAD^+- or $NADP^+$-dependent transformation of D-mannitol (25 mM) into D-fructose as a model transformation, the total turnover numbers for 9,10-phenanthrenequinone and the coenzyme obtained in a single batchwise conversion were 1000 and ≤125, respectively. The yields of ketose product were quantitative, indicating that molecular oxygen reduction drives the thermodynamically unfavourable synthetic reaction. Oxygen transfer to the liquid phase was shown to be rate-limiting for the overall process under conditions of surface aeration and bubble-free molecular oxygen supply. Xylose reductase was fully stable during the reaction (25 °C, pH 8.0). The novel chemo-enzymatic system should therefore be broadly applicable to biocatalytic synthesis with isolated dehydrogenases utilizing NAD^+ or $NADP^+$.

Abbreviations: *Ct*XR: xylose reductase from *Candida tenuis* (E.C. 1.1.1.21); M2DH: D-mannitol 2-dehydrogenase (E.C. 1.1.1.67); OTR: O_2 transfer rate; PQ: 9,10-phenanthrenequinone; PQH_2: 9,10-phenanthrene hydroquinone; SOD: superoxide dismutase; *STY*: space-time yield; TTN: total turnover number.

Keywords: biocatalytic alcohol oxidation; *Candida tenuis* xylose reductase; coenzyme regeneration; NAD^+; $NADP^+$; quinone reduction

Introduction

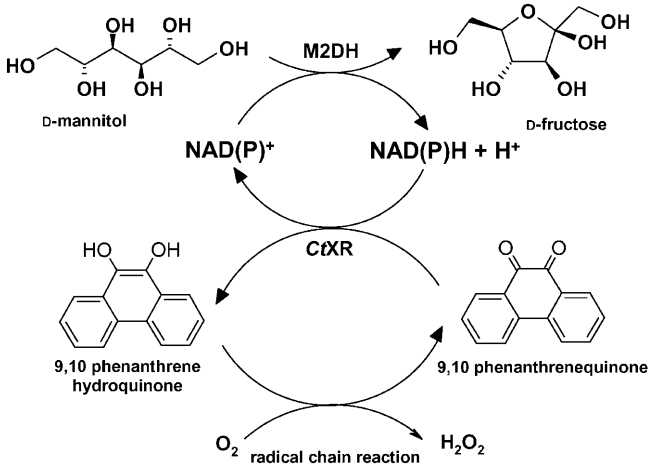
$NAD(P)^+$-dependent dehydrogenases are powerful catalysts for organic synthesis.[1–3] Depending on the direction of the reaction utilized, they promote the stereoselective reduction of a prochiral precursor substrate or the regio- and enantioselective oxidation of diverse chemical groups such as OH, NH_2, and others. Economic considerations dictate that the biotransformation of interest be coupled with another reaction that regenerates the reduced or oxidized form of the coenzyme required in the synthetic step.[4–9] Recycling of NADH and NADPH for reductive conversions by isolated enzymes has been established at the manufacturing scale and is considered solved.[10–12] By contrast, methods proposed for the regeneration of NAD^+ and $NADP^+$ are not technologically mature.[6–8]

Kinetic limitations, insufficient thermodynamic driving force, and low operational stability are problems oftentimes encountered with $NAD(P)^+$ regeneration systems.[4,5,7] Multi-step electrochemical and photochemical reactions suffer from low recycling rates and total turnover numbers for NAD(P)H.[7,8] Among the reported enzymatic approaches, the reductive amination of α-ketoglutarate by L-glutamate

dehydrogenase faces problems of substrate chemical stability and generation of the organic product glutamate complicating work-up.[7] Carbonyl group reductions usually provide little "pull" to biosynthetic oxidations that normally proceed thermodynamically uphill.[7,13] A compelling method therefore is the use of NAD(P)H oxidases, which couple the catalytic oxidation of NAD(P)H to the thermodynamically highly favourable reduction of O_2.[14–21] The NAD(P)H oxidases are flavoenzymes that can be categorized according to whether their reaction yields H_2O[14–18,20–22] or H_2O_2[19,23,24] as the terminal product of O_2 chemistry. However, members of either group have been described as oftentimes unstable under the conditions of coenzyme recycling and as being easily inactivated by oxidants.[15,20] Here, we report a novel strategy of coenzyme regeneration that addresses many of the shortcomings of existing methodology and can be flexibly applied to produce NAD^+ and $NADP^+$.

Results and Discussion

Scheme 1 displays the salient features of the chemo-enzymatic system in which 9,10-phenanthrenequinone (PQ) has a pivotal role. *Candida tenuis* xylose reductase (*Ct*XR) utilizes NADH or NADPH for the reduction of PQ. Chemical oxidation of the resulting hydroquinone (PQH_2) by O_2 recycles the PQ. We show application of the novel coenzyme regeneration strategy in the conversion of D-mannitol to D-fructose catalyzed by NAD^+- and $NAD(P)^+$-dependent forms of D-mannitol 2-dehydrogenase (M2DH) from *Pseudomonas fluorescens.*



Scheme 1. Chemo-enzymatic system for the regeneration of NAD^+ or $NADP^+$ during biocatalytic synthesis with isolated dehydrogenases. The oxidation of D-mannitol is used here as a model reaction.

Enzymatic Reduction of PQ

Results of a steady-state kinetic analysis of the NADH- or NADPH-dependent reduction of PQ catalyzed by *Ct*XR are summarized in Table 1. PQ is an outstanding substrate for NAD(P)H reduction by *Ct*XR: its reactivity measured as k_{cat}/K_m is by far the highest among a series of known non-natural aldehyde and carbonyl substrates of the enzyme whose conversion by *Ct*XR is characterized by k_{cat}/K_m values in the range of 10^1–$10^4\,M^{-1}s^{-1}$.[25,26] At saturating coenzyme concentrations (300 μM), k_{cat}/K_{PQ} for the NADH-dependent reaction was 20 times that for the corresponding NADPH-dependent conversion. Contrary to the reaction with the physiological substrate xylose, in which *Ct*XR prefers NADPH about 30-fold over NADH,[27] the enzyme shows a lower K_m value for PQ when NADH is utilized for the catalytic reduction as compared to the same reaction in the presence of NADPH. Values of K_{PQ} in the low micromolar range imply that it will be straightforward to saturate *Ct*XR with the PQ substrate under conditions of biocatalytic synthesis (see later). k_{cat} values of about $12\,s^{-1}$ (NADH) and $1.6\,s^{-1}$ (NADPH) are expected to ensure a useful turnover frequency of PQ in the process.

Table 1. Kinetic parameters of *Ct*XR for reduction of PQ at 25 °C and pH 7.0.[a]

Parameter	NADH	NADPH
k_{cat} [s^{-1}]	12	1.6
k_{cat}/K_{PQ} [$M^{-1}s^{-1}$]	2.2×10^6	1.3×10^5
K_{PQ} [μM]	5.3	12.6
$K_{NAD(P)H}$ [μM]	24.7	4.5

[a] From triplicate experiments at 25 °C and pH 7.0; parameters have S.E. of < 20%.

Chemical Reoxidation of PQH_2 by Molecular Oxygen

Figure 1A displays a time course of PQ reduction by NADH under conditions in which the concentration of PQ (90 μM) was saturating in respect of K_m and limiting in respect of the conversion. The results reveal that consumption of NADH continued beyond the point of apparent exhaustion of PQ (indicated by an arrow in Figure 1A) until all of the initial NADH (275 μM) had become oxidized. This result implies that the substrate for enzymatic reduction by NADH was regenerated 3 times under the reaction conditions. Early studies on the chemical interconversion of PQ and PQH_2 have shown that PQH_2 is rapidly reoxidized by O_2.[28] We therefore monitored the level

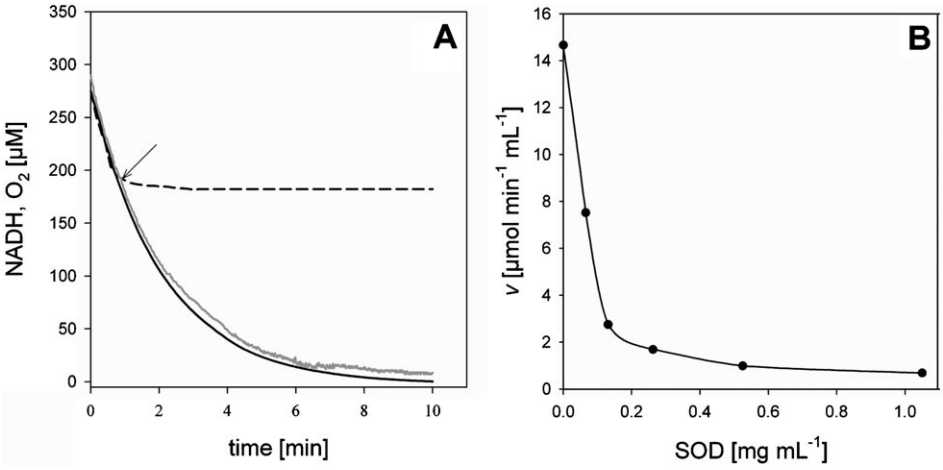


Figure 1. O_2-dependent recycling of PQ in the course of its enzymatic reduction (**A**) and inhibition of this process by added SOD (**B**). The black and grey lines indicate NADH and O_2, respectively. The dashed line shows NADH under anaerobic conditions. The arrow indicates the point of exhaustion of the initial concentration of PQ (90 μM). **B:** The initial rate of oxidation of NADH (300 μM) was measured in the presence of 3 μM PQ and 280 μM O_2. The concentration of *Ct*XR was 5 μM.

of dissolved O_2 during the *Ct*XR-catalyzed reduction of PQ. Figure 1A (grey line) shows that O_2 was consumed in the course of enzymatic reaction at a rate identical within experimental error to that for the oxidation of NADH. A time course of NADH consumption during enzymatic conversion of PQ under the exclusion of O_2 is also shown in Figure 1A. Once an amount of NADH corresponding to the initial concentration of PQ (90 μM) had been oxidized, the reaction did not progress further, suggesting that recycling of PQ for NADH-dependent reduction by *Ct*XR was completely suppressed under the anaerobic reaction conditions. We performed experiments at different concentrations of O_2 in the range 50–280 μM and found that the rate of oxidation of NADH decreased at O_2 levels ≤ 80 μM.

Previous works report chemical processes involved in the oxidation of PQH_2 by O_2.[29–33] The deprotonated hydroquinone PQH^- is thought to react with O_2 to yield the semiquinone ($Q^{\bullet-}$) and the superoxide radical ($O_2^{\bullet-}$). By producing the $O_2^{\bullet-}$, a free radical chain reaction [Eq. (1) and Eq. (2)] is initiated that regenerates PQ.

We therefore analyzed the effect of added superoxide dismutase (SOD) on the rate of NADH consumption by *Ct*XR under conditions where the overall conversion of NADH in the absence of redox cycling would be limited by the concentration of PQ (3 μM). The degree of quenching of the reaction rate increased dramatically as the amount of SOD was raised (Figure 1B), suggesting that the superoxide anion radical serves an important role in promoting the recycling of PQ under the conditions used. We also determined that about 0.98 (± 0.02; $N = 12$) equivalents of H_2O_2 were produced per each NAD(P)H consumed in the conversion of PQ by *Ct*XR. The molar product ratio of H_2O_2 and $NAD(P)^+$ did not change in response to variation of the initial concentrations of NADP(H) (34–134 μM). These results suggest a net reaction for reoxidation of PQ where $PQH_2 + O_2 \longrightarrow PQ + H_2O_2$, which is in good agreement with previously proposed routes of hydroquinone oxidation by radical chain reaction.[31–33]

Transient Kinetic Analysis

To determine the kinetic correlation between chemical oxidation of PQH_2 and enzymatic reduction of PQ in greater detail, we performed rapid mixing stopped-flow experiments in which, for a varied concentration of enzyme in the range 0.31–36 μM, time courses of absorbance at 340 nm and 420 nm resulting from the conversion of NADH (200 μM) and PQ (125 μM), respectively, were recorded. At high enzyme concentration as shown in Figure 2A, the absorbance trace at 420 nm revealed gradual depletion of PQ over time,

$$H^+ + PQH^- + O_2^{-\bullet} \rightleftharpoons Q^{-\bullet} + H_2O_2 \quad (1)$$

$$Q^{-\bullet} + O_2 \rightleftharpoons Q + O_2^{-\bullet} \quad (2)$$

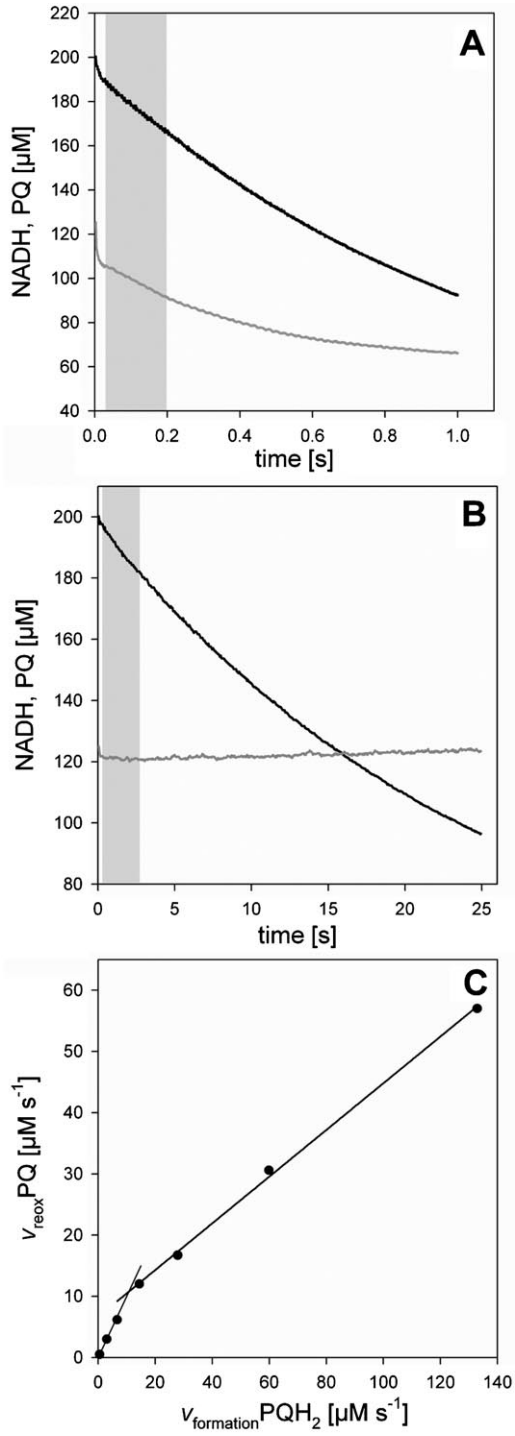


Figure 2. A–C Stopped flow analysis of NADH-dependent reduction of PQ catalyzed by *Ct*XR and O_2-dependent chemical oxidation of PQH_2. Representative time courses of oxidation of NADH (black lines) and reduction of PQ (grey lines) are shown in panels **A** and **B** for conditions of high (36 μM) and low (0.83 μM) enzyme concentrations, respectively. The concentrations of PQ (125 μM) and NADH (200 μM) were saturating in the steady state. The grey areas indicate the steady-state phase of the reaction from where the rates of consumption of NADH (V_{NADH}) and PQ (V_{PQ}) were obtained. Panel **C** compares the rates of formation of PQH_2 ($V_{formation}PQH_2 = V_{NADH}$) and the PQ recycling rate ($V_{reox}PQ = V_{NADH} - V_{PQ}$). Recycling of PQ becomes partly rate-limiting when $V_{formation}PQH_2$ exceeds a value of about 10 μM s^{-1}.

indicating that the rate of PQ reduction by *Ct*XR was larger than that of oxidation of PQH_2. At 0.83 μM enzyme, however, the concentration of PQ was almost invariant with time (Figure 2B, grey line), implying that PQH_2 was oxidized much faster under these conditions than it was formed by the enzyme *via* NADH-dependent PQ reduction.

Panel C of Figure 2 uses the stopped flow data obtained at different enzyme concentrations to compare the observed rates of recycling of PQ (chemical reaction; $V_{reox}PQ$) with the corresponding rates of formation of PQH_2 (enzymatic reaction; $V_{formation}PQH_2$). This plot delineates clearly the two kinetic regimes in which the enzymatic reduction of PQ may be performed. Oxidation of PQH_2 was partly rate-determining under conditions where the enzymatic rate exceeded a value of about 10 μM s^{-1}. In this region, the slope of the linear relationship between $V_{reox}PQ$ and $V_{formation}PQH_2$ had a value of 0.41 ± 0.02, significantly smaller than unity. For reaction rates at or below 7 μM s^{-1}, however, the recycling of PQ was considerably faster than the consumption thereof. The linear dependence of $V_{reox}PQ$ on $V_{formation}PQH_2$ was therefore characterized by a slope of 1. These results imply that for a given O_2 concentration of 280 μM, a catalyst loading producing a reaction rate higher than 7 μM s^{-1} ($\approx$1 μM *Ct*XR) will lead to a situation where chemical recycling of PQ contributes to the overall conversion rate. However, the data in Figure 2C also suggest that rate limitation by oxidation of PQH_2 will not occur to an extent that would be prohibitive for NAD(P)$^+$-dependent synthesis.

Application of Chemo-Enzymatic Regeneration of NAD$^+$ or NADP$^+$ to Biocatalytic Oxidation of D-Mannitol

Figure 3 shows time courses of D-fructose production by NAD$^+$- and NADP$^+$-dependent enzymatic oxidation of D-mannitol (25 mM) catalyzed by wild-type M2DH from *Pseudomonas fluorescens* and an engineered variant thereof, in which Glu^{68} and Asp^{69} had been substituted by Lys and Ala, respectively. Unlike the wild-type enzyme, which is specific for NAD$^+$, the doubly mutated enzyme utilizes NADP$^+$ for D-mannitol oxidation with a catalytic efficiency ($k_{cat}/K_m = 1.7 \times 10^3$ s^{-1} M^{-1}) and turnover number ($k_{cat} = 12$ s^{-1}) similar to those of the native M2DH for the NAD$^+$-dependent reaction.[34] A pH of 8.0 was chosen for good activity and stability of the M2DHs during biocatalytic

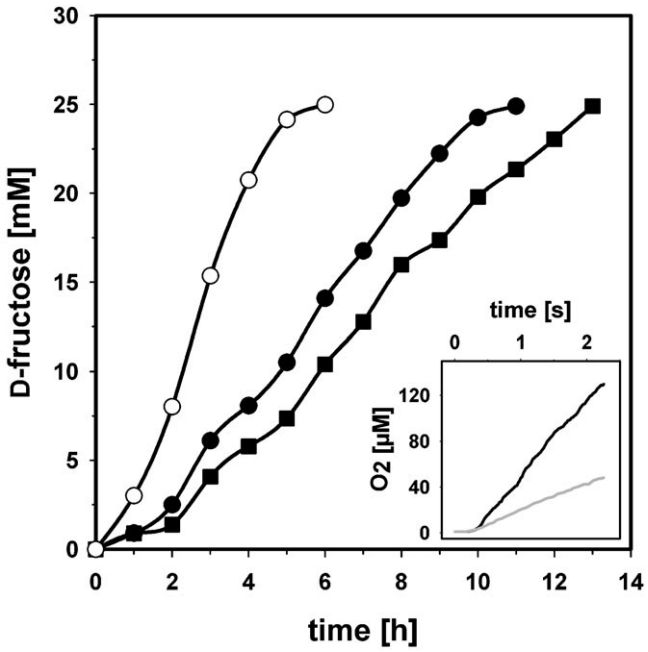


Figure 3. Biocatalytic synthesis of D-fructose using chemo-enzymatic regeneration of NAD^+ or $NADP^+$. Closed symbols show reactions employing surface aeration for O_2 supply (NAD^+, circles; $NADP^+$, squares). Open circles show NAD^+-dependent synthesis using membrane gassing. The inset depicts time courses of oxygen transfer to the liquid phase using membrane gassing (black line) or surface aeration (grey line). See the Experimental Section for details.

synthesis. It was proven that the autoxidation rate of PQH_2 was not pH-dependent in the range 7.0–8.0, hence the conclusions from Figure 2C remain valid.

In each reaction depicted in Figure 3, complete conversion of D-mannitol into D-fructose was achieved. The results imply a total turnover number (TTN) of 1000 for PQ under the conditions used. They also indicate that the chemo-enzymatic system for regeneration of $NAD(P)^+$ effectively drives the biosynthetic reaction, considering that the equilibrium constant for $NAD(P)^+$-dependent oxidation of D-mannitol in the absence of an $NAD(P)^+$ recycling system at pH 8.0 and 25 °C has a value of 0.4.[34] The TTN values for NAD^+ and $NADP^+$ were 125 and 40, respectively. However, the TTN for coenzyme and PQ could of course be increased further by raising D-mannitol start concentration. The results summarized in Table 2 show that the addition of catalase and BSA was required to prevent inactivation of M2DH in the course of the reaction, while *Ct*XR turned out to be fully stable even in the absence of the stabilizing agents. We measured the enzymatic activities of M2DH and *Ct*XR in samples taken from the reaction mixture at certain times until the end of the conversion and observed that both enzymes were fully stable under the optimized conditions reported in the experimental section.

Many dehydrogenases that have been employed for $NAD(P)^+$-dependent synthesis of chiral compounds are compatible with the oxidizing reaction conditions occurring during O_2-dependent cofactor regeneration. They include alcohol dehydrogenases from horse liver[35–37] and various bacterial sources[3,5,17,18,37], mandelate dehydrogenase[19], L-phenylalanine dehydrogenase[15,19], L-*tert*-leucine dehydrogenase[21], and L-glutamate dehydrogenase[16]. Yeast alcohol dehydrogenase which was reported to be unstable in the presence of O_2 appears to be an exception.[37] The literature therefore indicates scope for the application of the new $NAD(P)^+$ recycling system. However, the possibility remains that chemical oxidation and denaturation at the gas-liquid interface (*vide infra*) promotes inactivation of a dehydrogenase, like M2DH that is less robust than *Ct*XR and the enzymes mentioned above. Considering the general requirement for optimization during biocatalytic synthesis by two

Table 2. The effects of different modes of O_2 supply and addition of stabilizers on the performance of the coupled chemo-enzymatic system during oxidation of D-mannitol.

Reaction conditions	Half-life M2DH [h]	Half-life *Ct*XR [h]	$TTN_{NAD(P)}$	TTN_{PQ}	Product yield [%]
surface aeration[a]	1.0	stable	25	100	5
surface aeration, BSA[a,c]	2.0	stable	100	400	20
surface aeration, BSA, catalase[a,c]	stable	stable	125	1000	100
surface aeration, BSA, catalase[b,c]	stable	stable	40	1000	100
bubble aeration[a]	1.0	5	25	100	5
bubble aeration, BSA[a,c]	1.5	5	100	400	20
membrane gassing, BSA, catalase[a,c]	stable	stable	125	1000	100

[a] NAD^+ regeneration.
[b] $NADP^+$ regeneration; the doubly mutated M2DH was used.
[c] The concentrations of BSA and catalase were 1 $mg\,mL^{-1}$ and 2.5 $U\,mL^{-1}$, respectively. Half-life times of the enzymes were estimated from the time courses of loss of activity measured during biocatalytic synthesis. "Stable" means that more than 90% of the original enzyme activity was measured in the reactor after exhaustive conversion of the substrate (25 mM mannitol). The analytical product yield is reported.

coupled dehydrogenases, it is therefore recommended that prior knowledge about the operational stability of the synthetic dehydrogenase is collected early during the process development. The case of M2DH shows clearly how stability problems might be remedied.

Except for a short lag phase that was not further pursued, formation of D-fructose in Figure 3 took place in a roughly linear dependence on the reaction time irrespective of whether NAD^+ or $NADP^+$ was used as cosubstrate for enzymatic oxidation of D-mannitol. If oxidation of D-mannitol was truly rate-limiting for the overall conversion catalyzed by the coupled enzyme system, one would expect from the substrate K_m values of the M2DHs (≈ 7 mM)[34] that the progress curves level out in response to a gradual depletion of D-mannitol. We determined that the concentration of dissolved O_2 was below the limit of detection by the used analytical method (≤ 0.5 μM) during the entire course of the biotransformations. Therefore, this result implies that all O_2 transferred to the liquid phase was immediately consumed by autoxidation of PQH_2 and because the rate of O_2 supply was rate-limiting overall, the observed rate of D-fructose production was only about 1/8 (NAD^+) and 1/24 ($NADP^+$) that expected from the amount of M2DH activity present. Enhancement of the O_2 transfer rate (OTR) should thus increase the space-time yield (*STY*) of the reaction. Considering that sparging O_2 into the solution has a strong negative effect on enzyme stability (Table 2), we used a module of spirally wound silicon tubing for bubble-free O_2 supply and show the results in Figure 3 (open circles). The *STY* was doubled under these conditions as compared to the reaction performed with simple surface aeration. The inset of Figure 3 shows the results of measurements of the OTR using the dynamic gassing-out method. From the slopes of the shown time courses, values of 63 μM min^{-1} and 26 μM min^{-1} were calculated for OTR by oxygenation *via* the silicon tubing and surface aeration, respectively. These data are in good agreement with the corresponding transformation rates of 69 μM min^{-1} and 34 μM min^{-1}.

Conclusions

NAD(P)H-dependent reduction of PQ by *Ct*XR in the presence of dissolved O_2 presents a synthetically useful chemo-enzymatic system for the regeneration of NAD^+ as well as $NADP^+$. The enzymatic step proceeds with a good turnover frequency and because the K_m of *Ct*XR for PQ is lower than the typical K_m values (≥ 20 μM) of many dehydrogenases for $NAD(P)^+$, the catalytic concentration of PQ required in the reaction will usually be smaller than that of $NAD(P)^+$. The achievable TTN for PQ will therefore surpass that of $NAD(P)^+$ in batchwise operated processes. The indirect coupling of NAD(P)H oxidation to a two-electron reduction of O_2 provides a very strong driving force for $NAD(P)^+$-dependent biosynthetic reactions that proceed thermodynamically uphill. The components of the $NAD(P)^+$ recycling system are stable under the conditions of enzyme reactor operation and appear to be resistant against the oxidants present. The reported method should thus be generally applicable for biocatalytic synthesis using $NAD(P)^+$-dependent oxidation with isolated dehydrogenases.

Experimental Section

Chemicals and Enzymes

PQ was (≥ 99%) from Sigma–Aldrich (St. Louis, MO, U.S.A.). Coenzymes [$NAD(P)^+$, NAD(P)H] (purity ≥ 98%) were obtained as sodium salts from Roth (Karlsruhe, Germany). All other chemicals were of reagent grade. Recombinant M2DH from *Pseudomonas fluorescens* in wild-type form and E68K-D69A doubly mutated variant of the enzyme were produced and isolated as described elsewhere.[34,38] Superoxide dismutase (SOD) from bovine erythrocytes, horseradish peroxidase and catalase from bovine liver were obtained from Sigma–Aldrich (St.Louis, MO, U.S.A.) and had declared specific activities of 4470 U/mg, 180 U/mg and 4190 U/mg, respectively. These enzymes were used without further purification.

The gene encoding xylose reductase from *Candida tenuis* was sub-cloned into the plasmid vector pQE-30 from Qiagen (Hilden, Germany) to fuse a His-tag to the N-terminal Ser-1 of the enzyme. The His-tagged enzyme was produced in *E. coli* JM 109 using conditions described previously[39] and purified to apparent electrophoretic homogeneity by using the same protocol as described for the M2DH.[38]

Determination of Enzyme Activities and Characterization of *Ct*XR and M2DH

Enzyme activities of the M2DHs and *Ct*XR were determined in potassium phosphate buffer (50 mM, pH 8.0) at 25 °C by recording initial rates of formation or consumption of NAD(P)H at 340 nm with a Beckman Coulter DU-800 UV-VIS spectrophotometer using plastic cuvettes with 1 cm optical pathlength. Assays for measuring M2DH activity were performed in the presence of saturating concentrations of D-mannitol (100 mM) and NAD^+ (2 mM) for the wild-type enzyme and $NADP^+$ (2 mM) in the case of the doubly mutated form. Saturating concentrations of PQ (50 μM) and NAD(P)H (300 μM) were applied for *Ct*XR.

Kinetic characterization of *Ct*XR was carried out by measuring initial rates of NAD(P)H-dependent reduction of PQ at 25 °C in potassium phosphate buffer (50 mM, pH 7.0). Concentrations of PQ (1–50 μM) or of NAD(P)H (8–80 μM) were varied while the concentration of NAD(P)H (300 μM) or PQ (50 μM) was saturating and constant. Solu-

tions containing PQ were prepared by diluting a DMSO solution containing PQ (1 mM) with the assay buffer to a defined concentration. Initial concentrations of PQ and NAD(P)H in the reaction mixture were verified by determining absorbances at 420 nm ($\varepsilon^{PQ}_{420}=1.5\ mM^{-1}cm^{-1}$)[40] and 340 nm ($\varepsilon^{NAD(P)H}_{340}=6.22\ mM^{-1}cm^{-1}$), respectively. Assays contained *Ct*XR (0.04 μM) and were started by addition of NAD(P)H. Control measurements lacking the enzyme were performed, and blank readings were used to correct the measured reaction rates.

Apparent kinetic parameters V_{max} and K_m were obtained from unweighted non-linear least-square fits using SigmaPlot 2004 of the Michaelis–Menten equation to the initial rates. One unit of enzyme activity corresponds to formation or consumption of 1 μmol NAD(P)H/min. Turnover numbers (k_{cat}) were obtained by using the relationship $k_{cat}=V_{max}/[E]$, where [E] is the molar enzyme concentration which was calculated from the protein concentration and the molecular mass of *Ct*XR (36 kDa). Protein concentrations were determined using the Pierce BCA assay referenced against bovine serum albumin BSA.

Analysis of Enzymatic Reduction of PQ and O_2 Dependent Chemical Oxidation of PQH_2

Consumption of NADH and O_2 during enzymatic PQ reduction was measured simultaneously using a Cary 50 BIO UV-VIS spectrophotometer (Varian, Palo Alto, CA, USA) for the detection of NADH at 340 nm and a fluorescence microsensor (Microtox TX3-AOT, Presens, Regensburg, Germany) coupled to an online data acquisition system for detection of O_2.[41] The O_2-sensor had a detection limit of ≤ 0.5 μM O_2. It was placed into the cuvette containing the reaction mixture (1 mL) such that absorbance and oxygen measurements were not disturbed. The reaction solution was oxygen saturated at atmospheric pressure and 25 °C (280 μM) and contained potassium phosphate (50 mM, pH 7.0), PQ (90 μM), NADH (275 μM) and *Ct*XR (0.2 μM).

Enzymatic reduction of PQ was carried out under the conditions described above using concentrations of dissolved oxygen that varied at six different levels between ≤ 2 μM (anaerobic) and 280 μM. Anaerobic buffer, substrate and enzyme solutions (< 0.5 μM O_2) were prepared in a glove box (Plas-Labs, Lansing, MI, USA). Enzymatic reactions were performed in an air-tight quartz cuvette, adding coenzyme through a septum.

Formation of H_2O_2 during the chemical oxidation of PQH_2 by O_2 was determined after all NAD(P)H present in the reaction mixture for enzymatic PQ reduction (0.3 μM *Ct*XR) had been exhaustively converted. The original solution contained PQ (27 μM) and NAD(P)H in the range 34–134 μM. H_2O_2 was measured using the peroxidase catalyzed oxidation of *o*-dianisidine.[42] The assay for H_2O_2 was blanked against the relevant initial concentration of PQ.

Rapid Mixing Kinetic Experiments

An Applied Photophysics SX.18MV Stopped-Flow Reaction Analyzer equipped with a 20 μL flow cell of 1 cm path length was used. Measurements were performed at 25 °C using air-saturated buffer (50 mM potassium phosphate, pH 7.0). The PQ solution (250 μM) was mixed with an equal volume of the enzyme solution (0.62–72 μM *Ct*XR) containing 400 μM NADH. Absorbance traces were recorded at 340 nm and 420 nm for up to 100 s. There is no significant spectral overlap between PQ ($\varepsilon_{420}=1.5\pm0.1\ mM^{-1}cm^{-1}$) and NADH at 420 nm. PQH_2 produced through NADH-dependent reduction of PQ under anaerobic conditions showed no absorbance at 420 nm. Therefore, formation and consumption of PQ could be inferred directly from changes in absorbance at 420 nm. We determined that PQ and PQH_2 have the same molar extinction coefficients at 340 nm ($\varepsilon_{340}=4.1\pm0.2$). Changes in absorbance at 340 nm therefore provide direct measure of changes in the NADH concentration.

Biocatalytic Synthesis

Reactions were performed at 25 °C in glass beakers with a diameter of 4.5 cm. The working volume was 20 mL, yielding a reactor height to diameter ratio of 1.6. A magnetic stirrer (length: 2.7 cm; diameter: 5 mm) was used for mixing at 200 rpm. Solutions contained D-mannitol (25 mM), PQ (25 μM), and NAD^+ (200 μM) or $NADP^+$ (600 μM) dissolved in 50 mM potassium phosphate buffer, pH 8.0. NAD^+-dependent synthesis was performed using *P. fluorescens* M2DH (25 U) and *Ct*XR (7 U; NADH). $NADP^+$-dependent synthesis used the doubly mutated variant of M2DH (50 U) and *Ct*XR (14 U; NADPH). Note that reported enzyme activities were determined with the standard assays. Catalase (50 U) and BSA (1 $mg\,mL^{-1}$) were added to the reactions when indicated.

Oxygen was supplied *via* surface aeration, bubble aeration or bubble-free membrane gassing. Bubble aeration was performed by moderate bubbling of oxygen (0.1 bar pressure) into the reaction mixture that was constantly stirred at 200 rpm using a magnetic stirrer (see above). For membrane gassing, a 30 cm spirally-wound module of air-permeable silicon tubing (Rotilabo, Roth, Karlsruhe, Germany) having internal and external diameters of 3 mm and 5 mm, respectively, was used. Oxygen was passed through the module at 0.5 bar pressure with a flow rate of 0.06 $L\,min^{-1}$, measured with a mass-flow meter from Kobold Industries (Vienna, Austria). The volumetric oxygen transfer rate was determined for each reactor set-up using the dynamic gassing out method.[43] Oxygen uptake was recorded with the fluorescence microsensor. Samples (500 μL) were withdrawn in regular time intervals, incubated for 5 min at 99 °C, and centrifuged (5000 rpm, 25 °C, 5 min). Supernatants were used for analysis of D-fructose and D-mannitol.

A colorimetric assay for D-fructose used the Seliwanoff reagent (25 mg resorcin dissolved in 50 mL 50% HCl).[44] The sample (150 μL) was incubated with 350 μL reagent in an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) at 99 °C for 5 min. The absorbance at 487 nm was measured at room temperature. The assay was calibrated with D-fructose standards in the range of 0.1–0.8 mM.

D-Fructose and D-mannitol concentrations were determined by HPLC analysis using a Merck-Hitachi La Chrom system (Darmstadt, Germany) equipped with an Aminex HPX-87C column (Biorad, Hercules, CA, USA) and a refractive index detector. The column temperature was at 85 °C, and H_2O was used as eluent at a flow rate of 0.6 $mL\,min^{-1}$.

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