

Cofactor Regeneration of both NAD⁺ from NADH and NADP⁺ from NADPH:NADH Oxidase from *Lactobacillus sanfranciscensis*

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Abstract: A possible solution for the regeneration of NAD⁺ from NADH is the oxidation of NADH with concomitant reduction of oxygen catalyzed by NADH oxidase (E. C. 1.6.-.-). We employ NADH oxidase from *Lactobacillus sanfranciscensis*, which reduces O₂ to innocuous H₂O, and (*R*)-alcohol dehydrogenase [(*R*)-ADH] from *Lactobacillus brevis* to perform enantioselective oxidation of racemic phenylethanol to acetophenone and (*S*)-phenylethanol with regeneration of either NADH or NADPH to their respective oxidized precursors. NADH oxidase from *L. sanfranciscensis* accepts both NADH and NADPH; in contrast, the wild-type (*R*)-ADH only accepts NADP(+)(H) whereas its G37D mutant strongly prefers NAD(+)(H). Highly purified. NADH oxidase (221 U/mg, two-step protocol) was coupled with wild-type ADH from *L. brevis* on NADP(H) and mutant ADH from *L. brevis* on NAD(H) to achieve 50% conversion of racemic phenylethanol to (*S*)-phenylethanol and acetophenone. Depending on the relative concentration of alcohol to cofactor, up to more than 100 turnovers were observed. We believe that this is the first demonstration of a regeneration scheme for both NAD⁺ from NADH and NADP⁺ from NADPH with the same enzyme.

Keywords: (*R*)-alcohol dehydrogenase; cofactor; cofactor regeneration; enzymes; NADH oxidase

Introduction and Motivation

For the pharmaceutical or crop protection industries, enantiomerically pure alcohols and derivatives represent versatile and thus valuable precursors or inter-

mediates.^[1,2] While a variety of NAD(P)-dependent dehydrogenases have been described for enzymatic synthesis of alcohols and derivatives,^[3,4] only very few alcohol dehydrogenases are commercially available. Although enantioselectivity and stability do not seem to pose a problem,^[4] some alcohol dehydrogenases feature limited substrate specificity. The ADH from *Lactobacillus brevis*^[5,8] has a broad substrate specificity and converts even bulky aromatic ketones with high activity.^[4–7] In addition, the enzyme is the only known completely (*R*)-specific ADH.

As dehydrogenases require pyridine dinucleotide cofactors such as NAD(H) or NADP(H), cofactor costs [\$ 90 per gram for NAD⁺ (Aldrich)] have to be considered and cofactors regenerated^[9] which cut costs by the turnover number for such cofactors, between 100 and up to 600,000.^[10] Cofactor regeneration with alcohol dehydrogenases can be performed by using the same enzyme for *in-situ* substrate conversion and cofactor regeneration, usually employing 2-propanol as co-substrate, as demonstrated with (*S*)-ADH from *Thermoaerobium Brockii* for both NADH and NADPH^[11] and with (*R*)-ADH from *L. brevis*^[12] for NADPH; this coupled-substrate approach, however, suffers from equilibrium limitations. The more common coupled-system approach, employing a separate second enzyme for regeneration, has been developed for reducing oxidized cofactors, NAD⁺ or NADP⁺, to NADH or NADPH. By far the most successful regeneration enzyme is formate dehydrogenase (FDH) for regeneration to either NADPH^[16,17] or NADH, the latter even up to an industrial scale.^[12–15] Other options include the use of glucose 6-phosphate dehydrogenase^[18] (to NADPH only) or of glucose dehydrogenase, GDH.^[19–21] For the opposite direction of regeneration, however, from NAD(P)H to oxidized cofactors NAD⁺ or NADP⁺, no universally accepted system exists.

As of recent years, NADH oxidases have emerged which are able to oxidize NADH to NAD⁺ with simultaneous reduction of O₂ to either H₂O₂ or

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Table 1. Purification of the two isoforms of *R*-ADH.^[a]

Step	Activity [U/mL]	Protein [mg/mL]	Specific activity [U/mg]	Yield [%]	Σ mg	Σ U	Purification factor
Wild-type ADH							
Crude extract	852	16	53.2	100	5.5	294	1
Phenylsepharose	13.66	0.18	77.9	84	3.6	247	1.46
Octylsepharose	13	0.158	82	75	2.7	221	1.54
G37D mutant							
Crude extract	6.6	3.01	2.2	100	18	40	1
Phenylsepharose	1.12	0.1	11.2	100	3.6	40	5
Octylsepharose	13	0.77	16.88	52	1.24	21	7.7

^[a] Activity data: wild-type ADH data measured with NADPH, G37D mutant data measured with NADH.

Table 2. Purification of NADH oxidase from *L. sanfranciscensis*.

Step	Activity [U/mL]	Protein [mg/mL]	Specific Activity [U/mg]	Yield [%]	Σ mg	Σ U	Purification Factor
Lysate (pH 5.0)	768.6	21.7	35.4	100	661.9	23,443	1.0
Dialysis (60 kDa MW cutoff membrane)/Acid precip pH 5.0	582.2	9.0	65.0	79.5	286.7	18,629	1.8
Displacement Source 30Q	136.2	0.6	220.9	26.2	27.75	6,131	6.2

H₂O.^[1,22–25] Four-electron reduction to benign H₂O is preferred over two-electron reduction to H₂O₂, which, even in small amounts, can deactivate either enzyme of the production-regeneration cycle. [Addition of catalase as a possible remedy increases complexity of the system to the point where three enzymes have to be coupled and adjusted as to their activity over time.] Recently, we published the isolation and characterization of a novel water-forming NADH oxidase from *Lactobacillus sanfranciscensis* which is able to utilize not only NADH but also NADPH as a substrate with an activity ratio of about 3:1.^[1] The ability of NAD(P)H oxidase to oxidize both cofactors renders the enzyme an extremely useful catalyst for coupled enzymatically-catalyzed oxidations.

To demonstrate feasibility of regeneration to either NAD⁺ or NADP⁺ by NADH oxidase from *L. sanfranciscensis*, the enzyme was combined with (*R*)-ADH from *L. brevis* to produce acetophenone and (*S*)-phenylethanol from racemic (*RS*)-phenylethanol. (*R*)-ADH from *L. brevis*^[3] was picked for the following advantages: i) (*R*)-1-phenylethanol is a very good substrate, on a par with the best substrates of the enzyme, ii) whereas the wild-type is mainly NADPH-dependent, the G37D mutant strongly prefers NADH over NADPH,^[2] albeit at reduced specific activity; iii) lastly, (*R*)-ADH from *L. brevis* has been explored extensively for the enzymatic generation of several pharmaceutically interesting chiral alcohols.^[4–7,17]

Results

High-Yield Purification of ADH from *L. brevis*

Purification of the two isoforms of the *R*-specific alcohol dehydrogenase from *L. brevis* has been demonstrated before^[5] and has been modified in this work with the result of much higher yields as listed in Table 1. A two-step purification yielded greater than 95% pure protein in both wild-type and G37D mutant cases, with yields of 52% and 75%, respectively. Verification of purity of (*R*)-ADH after the octylsepharose step is demonstrated by SDS-PAGE in Figure 1.

Purification of NADH Oxidase

A modification of the previously reported purification strategy^[1] was employed to obtain highly purified NADH oxidase. Instead of the sequence acid precipitation – Q-sepharose – 45% ammonium sulfate cut procedure described previously,^[1] we employed displacement chromatography after dialysis and acid precipitation. The displacer was naphthalene-1,3,6-trisulfonic acid, which opens the perspective of scale-up of this technique. As we wanted to make sure to employ NADH oxidase fractions free of interfering activities for cofactor regeneration in tandem with ADH, we aimed at the highest possible level of purity rather than high yield. As the results in Table 2 and the gel in Figure 1 reveal, we achieved purity in excess of 95% at 26% yield and found a specific activity of 221 U/

mg protein. The highly pure and active fractions were pooled and stored in 45% ammonium sulfate solution at 4 °C to preserve the enzyme's activity.

Alcohol-Ketone Conversion with Cofactor Regeneration

The results of coupled reactions after 12 hours, as analyzed by selective ion monitoring (SIM) mass spectrometry, are shown in Table 3. The standard curves

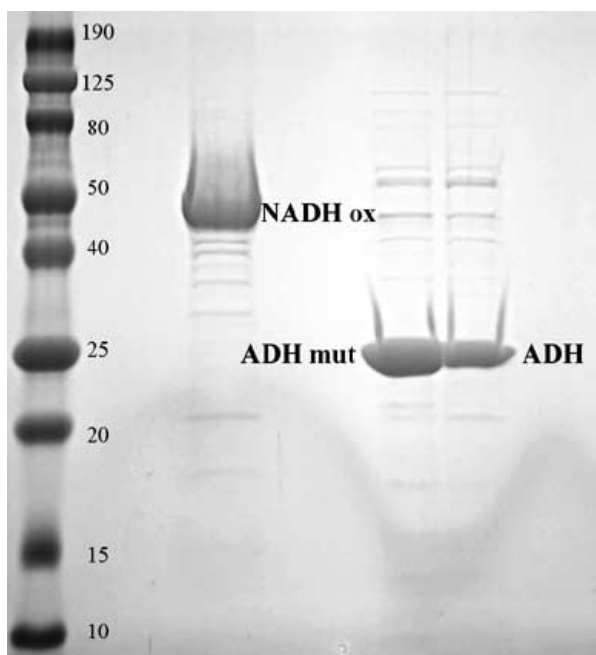


Figure 1. SDS-PAGE of the three enzymes employed in this work: NADH oxidase from *L. sanfranciscensis* and (*R*)-ADH from *L. brevis* (wild-type and G37D mutant). Molecular weight standards were Prosieve® Color Protein Markers from Cambrex.

used for SIM mass spectrometry are shown in Figure 2. Measured degrees of conversion values were normalized using the mass balance of acetophenone and phenylethanol to correct for manual injection error. Satisfactory linearity was obtained for phenylethanol up to 100 mM and for acetophenone up to 50 mM concentration.

The coupled reaction results shown in Table 3 are consistent with expected results from successfully coupled reactions. The comparison of reduced versus oxidized cofactor (runs 1 and 2 as well as 3 and 4) indicate that the starting oxidation state of the cofactor does not significantly impact the results. Given the higher stability and lower cost, the oxidized cofactor would be the reagent of choice for typical coupled reactions. The controls (runs 5–8) demonstrate that no conversion occurs without ADH (runs 5 and 7) and that slightly less than stoichiometric conversion was observed in the absence of NADH oxidase (runs 6 and 8) to regenerate the cofactor. Conversions in excess of stoichiometry would have indicated a potential NAD(P)H-oxidizing impurity in the ADH preparations. Reducing the cofactor concentration to 0.4 mM

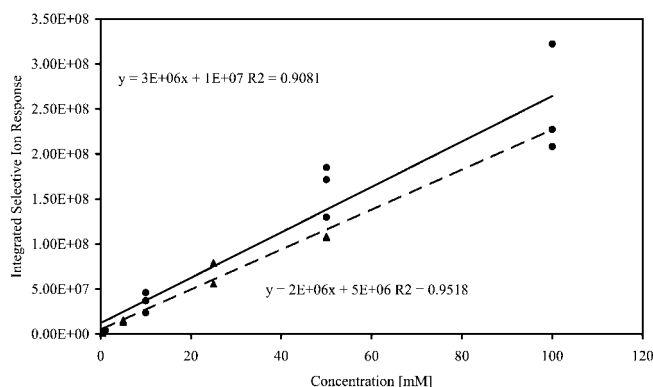


Figure 2. Standard curve for selective ion monitoring of phenylethanol (● mass 122) acetophenone (▲ mass 120).

Table 3. Coupled alcohol-ketone conversion with cofactor regeneration.

Sample#	Cofactor [4 mM]	ADH [U/mL]	ADH mut [U/mL]	NADH ox [U/mL]	Normalized Conversion [%]	Turnovers
1	NAD ⁺		2.0	8.0	43.6	10.9
2	NADH		2.0	8.0	35.0	8.7
3	NADP ⁺	2.0		8.0	38.2	9.5
4	NADPH	2.0		8.0	40.1	10.0
5	NAD ⁺			8.0	−2.3	−0.6
6	NAD ⁺		2.0		1.7	0.4
7	NADP ⁺			8.0	−0.7	−0.2
8	NADP ⁺	2.0			2.3	0.6
9	NAD ⁺ [a]		2.0	8.0	43.6	109.0
10	NADP ⁺ [a]	2.0		8.0	40.2	100.5
11	NAD ⁺ [a]		2.0	4.0	27.9	69.8
12	NADP ⁺ [a]	2.0		4.0	41.7	104.1

[a] These samples utilized 0.4 mM concentrations of cofactor. Standard conditions: 30 °C, pH 7.0 (50 mM HEPES), 5 mM DTT, 1 mM MgCl₂, 150 mM total ionic strength (addition of 138 mM NaCl), and 100 mM racemic phenylethanol.

(runs 9–12) still indicated effective conversion with concomitant higher number of turnovers of cofactor; however, a lower degree of conversion is observed for the mutant ADH in the presence of 4 U/mL instead of 8 U/mL NADH oxidase. After 12 h, nearly complete conversion (maximally 50% of racemic phenylethanol) was achieved in all but the case of the mutant ADH with NAD⁺.

Discussion and Conclusion

High-level purification of both (*R*)-ADH and NADH oxidase is deemed especially important for the task of this paper, regeneration of NAD(P)⁺ from NAD(P)H, for the purpose of avoiding false activities from contaminants in either enzyme preparation. Such redox-active contaminants would very likely interfere with the oxidation-regeneration cycle under investigation. The SDS-PAGE gel shown in Figure 1 indicates a high level of purity for all three enzymes used in this study. While some minor bands are apparent in the gel, due to the high loading of sample (~5 µg protein), the assumed purity for all of the enzymes is greater than 95%. In addition, the modifications to the previously published NADH oxidase purification shown in Table 2 using displacement chromatography are very amenable to further improvement upon scale-up.^[26] Displacement chromatography generally improves at higher loadings^[27] and the novel displacer, naphthalene-1,3,6-trisulfonic acid, is an inexpensive reagent in contrast to many other reported displacers.^[28]

After 12 h, nearly complete conversion (maximally 50% of racemic phenylethanol) was achieved in all but the case of the mutant ADH with NAD⁺. Currently, there is no explanation for the lower rate (less than $v_{\max}$) and thus lower conversion after 12 h. The number of turnovers ([acetophenone]/[cofactor]) of up to more than 100 clearly demonstrates catalysis by both enzymes involved. Thus, decay of NADH oxidase activity cannot be important over the time scale of our experiments. Further work will clarify this issue.

In conclusion, we have demonstrated the feasibility of enantiospecific oxidation of racemic phenylethanol as a model alcohol to acetophenone and remaining (*S*)-phenylethanol with regeneration of both NADH and NADPH using the same regeneration enzyme.

Experimental Section

Purification of the *R*-Specific Alcohol Dehydrogenase (recADH) and its NAD Mutant G37D

The enzyme purification was performed as described in ref.^[5] with slight modifications. In brief, after 16 h expression with 1 mM IPTG, *E. coli* HB101 cells were harvested and sonicated

in pulses for 10 min at 4 °C (Branson Sonifier, 40% output). Crude extract was clarified through centrifugation (10000 rpm, J2–21M rotor, Beckman centrifuge) and adjusted to 0.6 M (NH₄)₂SO₄. This solution was applied onto a Phenylsepharose 4FF (high sub) and eluted with decreasing (NH₄)₂SO₄ concentration from 0.6 to 0 M using a 3-step gradient (0.6 M, 0.48 M, 0.36 M, 0 M). Active fractions eluting at 0.48 M (NH₄)₂SO₄ were pooled, the (NH₄)₂SO₄ concentration was adjusted to 1.2 M and applied onto an Octylsepharose FF. Elution was performed through decreasing (NH₄)₂SO₄ concentration from 1.2 to 0 M using a 5-step gradient (1.2 M, 0.96 M, 0.72 M, 0.48 M, 0.24 M, 0 M.), active fractions were found at 0.72 M. After two chromatographic steps the enzyme was > 95% pure according to SDS-PAGE (Figure 1) and used in the cofactor regeneration assay.

Purification of NADH Oxidase

A modification of the previously reported purification strategy^[1] was employed to obtain highly purified NADH oxidase. Frozen cell pellets, 13 g WCP, were thawed and resuspended in 30 mL of 100 mM 1-methylpiperazine buffer pH 5.0 + 1 mM EDTA + 5 mM DTT + 5 mM spermine. The resulting cell slurry was sonicated with a Fisher Scientific 60 Sonic dismembrator for 6 × 2 minutes while floating the tube in ice/water for cooling. The resulting lysate was centrifuged at 20,000 rpm in a Beckman J2–21M for 45 minutes at 4 °C. The clarified lysate was then loaded into Specto/Por® regenerated cellulose dialysis membrane tubing (60 kDa MW cutoff) and dialyzed with 1.5 L of 20 mM 1-methylpiperazine pH 5.0 at 30 °C + 1 mM EDTA + 10 mM β-mercaptoethanol. The sample was dialyzed versus 1.5 L of buffer for two hours at 30 °C and 200 rpm stirring before exchanging the dialysis buffer and dialyzing for two more hours under the same conditions. Temperature and stirring conditions were maintained by a digital stir plate with an external temperature probe. The sample was then transferred into centrifuge tubes and centrifuged at 20,000 rpm for 45 minutes at 4 °C. The resulting clarified solution was then loaded onto an Amersham Pharmacia HiPrep 16/10 Source™ 30Q column on an AKTAexplorer system at 4 °C. The protein was then eluted with displacement chromatography utilizing 5 mM naphthalene-1,3,6-trisulfonic acid. After sample loading the column was washed with 10 column volumes of 20 mM 1-methylpiperazine pH 5.0 at 4 °C + 5 mM DTT. The protein elution phase was then started by switching to 20 mM 1-methylpiperazine pH 5.0 at 4 °C + 5 mM DTT + 5 mM naphthalene-1,3,6-trisulfonic acid. 5 mL fractions were collected at a flow rate of 5 mL/min. Fractions with a tested specific activity of over 200 were pooled and dialyzed at 4 °C against 2 L of 45% ammonium sulfate + 50 mM potassium phosphate buffer pH 6.8 + 1 mM EDTA + 10 mM β-mercaptoethanol using Specto/Por® regenerated cellulose dialysis membrane tubing (14 kDa MW cutoff). The total dialysis time was 12 hours with one buffer exchange after 6 hours. The resulting concentrated preparation of 23 mL total volume and 1.3 mg/mL was stored at 4 °C. No additional purification or loss of activity was apparent in the 45% ammonium sulfate preparation. The preparation was measured to have an activity of 137 U/mL or 221 U/mg protein on NADH on the day the coupled experiments were started.

Cofactor Regenerating Assay

Application of NADH oxidase in cofactor regeneration is performed using a batch conversion with R-ADH as the production enzyme. All reactions were run at 30 °C with standard buffer composed: 50 mM HEPES pH 7.0 at 30 °C and 150 mM total ionic strength by addition of 138 mM NaCl, 5 mM DTT, 1 mM MgCl₂, and 100 mM racemic phenylethanol. Cofactors and enzymes were then added to 100 µL of buffer as outlined in Table 3 and vortexed. 30 µL of the mixed solution were then added to 0.65 mL polypropylene PCR reaction tubes, capped, and floated in a water bath. Three identical vials were prepared for each condition. Time point samples were taken by centrifuging for 1 min at 14,000 rpm in a Microfuge and adding 270 µL methanol to the reaction vial.

GC/MS Analysis

Samples and a prepared standard curve were submitted to the IBB central mass spectroscopy facility for GC/selective ion analysis. The separate standard curves were prepared for the (±)-phenylethanol and acetophenone. The (±)-phenylethanol curve consisted of 100 mM, 10 mM, and 1 mM in the coupled reaction base buffer, diluted 1:10 in methanol. The acetophenone curve consisted of 50 mM, 10 mM, and 1 mM in the coupled reaction base buffer, diluted 1:10 in methanol. Total mass areas were reported for ions of mass 120 (acetophenone) and 122 [(±)-phenylethanol]. Sample concentrations of the coupled reaction were estimated by interpolation on these standard curves (R² for both curves > 0.90).

SDS-PAGE Gel Analysis

Samples of the purified enzyme preparations were run on a 12% tris-glycine SDS-PAGE gel (PAGeR[®] gold precast gel). The running buffer and samples were prepared according to the manufacturer's protocol. The NADH oxidase sample was diluted 1:10 in DI water prior to mixing with sample loading buffer. 20 µL of the wild-type ADH, G37D ADH mutant, and NADH oxidase (dil) samples were mixed with an equal volume of 2× sample loading buffer, vortexed, and then incubated in a water bath at 95 °C for fifteen minutes. Due to the presence of 50% glycerol in the purified wild-type ADH and G37D ADH mutant samples, sample-loading buffer without glycerol was utilized. Samples were then centrifuged at 14,000 g for 5 minutes and placed on ice prior to loading on the gel. 15 to 30 µL of each sample were loaded into the wells with blank sample buffer added to the empty wells. The gel was run on a Hoefer Mighty Small[™] (SE260) chamber with circulated cooling water at 4 °C. The gel was run under constant voltage (125 V) for 2.5 hours. At the completion of the electrophoresis run, the gel was washed with three changes of DI water. The gel was then stained with Pierce Gelcode blue for 1 hour and then transferred to DI water to destain for an additional hour. Images were taken in an Alpha Innotech AlphaImager 3300 for gel documentation.

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