Hydrogen peroxide-producing NADH oxidase (nox-1) from *Lactococcus lactis*

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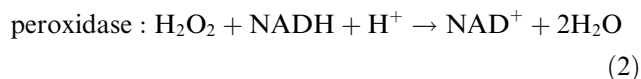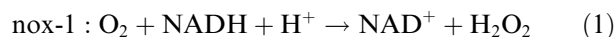
Abstract—We have successfully applied the sequence comparison-based approach to develop a novel hydrogen peroxide-forming NADH oxidase (nox-1) from *Lactococcus lactis* (*L. lactis*) that reduces oxygen to hydrogen peroxide. The nox-1 gene (AhpF) was isolated from genomic *L. lactis* DNA by PCR and cloned into the expression vector pET32. The His-tagged protein was over-expressed at 20 °C after induction with 167 μM IPTG, and purified by Co^{2+}-IMAC. After purification, nox-1 was found to be an apo-protein, so we reconstituted the holo-flavoenzyme with FAD cofactor, finding a 1:1 stoichiometry of FAD and nox-1 subunit and a K_M value of 54 μM. The maximum specific activity of 15 U/mg protein compares favorably to other nox-1 enzymes in the literature. While both products, NAD^+ and H_2O_2, inhibit nox-1, the enzyme seems rather robust in presence of moderate H_2O_2 concentrations. Titration of H_2O_2 formed with Amplex Red demonstrates that only half of the electrons from NADH go to H_2O_2.
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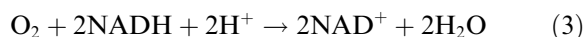
1. Introduction

For several oxidations of potential large-scale relevance, such as the transformation of monosodium L-glutamate to α-ketoglutarate,[7] regeneration of the cofactor NADH to NAD^+ is required, not only to recycle the valuable cofactor but also to drive the production reaction, that is, the oxidative biotransformation, to completion. NADH oxidases accomplish this task with ease: they oxidize NADH to NAD^+ via a coupled reaction of molecular oxygen to either water in a four-electron reduction (nox-2 enzymes) or to hydrogen peroxide in a two-electron reduction (nox-1 enzymes).[3] Recently, we published the characterization of a novel water-forming NADH oxidase from *Lactobacillus sanfranciscensis*, which accomplishes this task.[11,12] Herein, we report the reduction of oxygen to hydrogen peroxide with the help of NADH oxidase (nox-1) from *Lactococcus lactis* (*L. lactis*).

L. lactis, a member of the lactic acid bacteria family, has no catalase or functional electron-transfer chain and its aerotolerance is related to its ability to induce superoxide dismutase and NADH oxidase.[10] In *L. lactis*, a nox-2

protein is already known,[1] whereas up to now the nox-1 enzyme only existed as an annotated gene within the genome. Nox-1 in *L. lactis* is actually part of the alkyl hydroperoxide reductase system (AhpR), composed of two enzymes: nox-1 (gene name AhpF) and peroxidase (gene name AhpC). The full sequence of AhpC is just upstream of AhpF in *L. lactis*. Reactions catalyzed by AhpR are as follows:

$$\text{nox-1}: O_2 + NADH + H^+ \rightarrow NAD^+ + H_2O_2 \quad (1)$$

$$\text{peroxidase}: H_2O_2 + NADH + H^+ \rightarrow NAD^+ + 2H_2O \quad (2)$$

Overall reaction:

$$O_2 + 2NADH + 2H^+ \rightarrow 2NAD^+ + 2H_2O \quad (3)$$

Thus, the overall AhpR reaction is exactly the same as for the nox-2 enzymes.[11,12]

Streptococcus mutans (*S. mutans*), *Amphibacillus xylanus* (*A. xylanus*) and *Salmonella typhymurium* (*S. typhymurium*) feature AhpC and AhpF genes as well and each has been demonstrated to produce a functional alkyl hydroperoxide reductase.[6,8,10] Sequence alignment between nox-1 from *L. lactis* and other organisms shows identity as high as 34.1% and strong similarity up to

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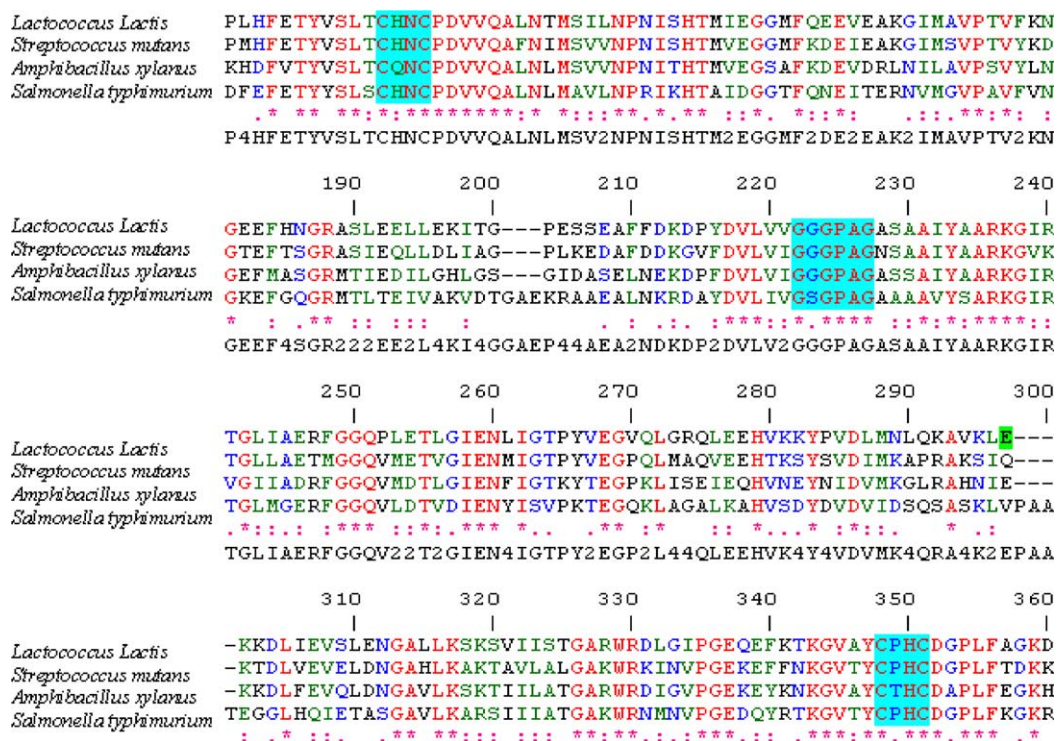
Figure 1. Part of the *L. lactis* nox-1 amino acid alignment with other known nox-1 proteins. Identity: 34.1%; strong similarity: 55.8%. CxxC: Catalytic Center; GxGxxG: flavin and NAD(P)H binding site.

55.8% at the amino acid level (Fig. 1). There is one catalytic center (CXXC) each near the N- and C-terminus, respectively.

A mechanism was proposed for alkyl hydroperoxide reductase from *S. mutans* and *S. typhymurium*[9,10] demonstrating the importance of the disulfide units close to the termini for electron transport. The crystal structure of *S. typhimurium* nox-1, a homodimer, features one disulfide unit in the N-terminal domain and another in the NADH/SS domain.[14] The peroxidase crystal structure from *S. typhymurium* shows an inter-subunit disulfide as the active center.[13] Electrons from the N-terminal disulfide center of nox-1 are presumed to transfer to the inter-subunit disulfide bond when AhpF and AhpC temporarily interact.

2. Materials and methods

2.1. Cloning and overexpression

The nox-1 coding gene from *L. lactis* was isolated from the genomic DNA using gene specific primers derived from the coding sequence. AhpF was successfully amplified by the polymerase chain reaction (PCR) using the Failsafe Kit (Epicenter, Madison, WI). The primers for PCR were ATG ATTTTAGATGAAACTCTTC and TTAGTTTCTTATTAAATAATCA. The AhpF PCR product was then purified through gel electrophoresis and subsequent elution (Gel Extraction Kit, Qiagen, Valencia, CA). AhpF was then cloned into pDrive vector that encoded blue/white screening feature (Qiagen PCR Cloning kit, Qiagen, Valencia, CA), and transformed into

Qiagen EZ competent cells. Positive clones were identified as white colonies. The recombinant pDnox1 was obtained through miniprep (Qiagen Spin Miniprep kit, Qiagen, Valencia, CA), analyzed by PCR and sequenced.

The pDnox1 construct was used as the template for the PCR of the desired gene AhpF and the PCR product was subcloned into the expression vector pET32 (EK/LIC cloning kit, Novagen, Madison, WI). Primers were designed as required: GACGACGACAAGATGATT TTAGATGAAACTCTTC and GAGGAGAAGCCC GGTTTAGTTTCTTATTAAATAATCA. The pET32 vector has a thioredoxin tag, a His tag, and an S tag on its N-terminus. Subcloning was successful and the recombinant was first transformed into NovaBlue cells for positive/negative test and then recombinant pET32-nox1 was transformed to expressing strain BL21(DE3)-RIL (Stratagene, La Jolla, CA).

The successful clone pET32nox1 was grown in LB broth with 50 μg/mL ampicillin and 50 μg/mL chloramphenical at 30 °C for 4h. Nox-1 expression was induced with 167 μM isopropyl β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.5–0.6, overexpression conducted at 20 °C, and cells harvested after 2.5h. At 20 °C, pET32-nox1 expressed a higher amount of soluble protein than at 30 and 37 °C. A cell pellet from 400 mL culture was then ultrasonificated in 15 mL 50 mM sodium phosphate buffer at pH 7 containing 300 mM NaCl.

2.2. Purification and dialysis of nox-1

By taking advantage of the N-terminal 6xHis tag on nox-1, the protein was purified by using immobilized

metal affinity chromatography (IMAC; BD TALON Co2+ Metal Affinity CellTru Resin, BD Biosciences, Palo Alto, CA). The soluble fraction of the culture was loaded to the Co^{2+} resin, previously equilibrated with a pH 8 50 mM sodium phosphate buffer with 300 mM NaCl. After the binding step, the resin was washed four times with a pH 7 50 mM sodium phosphate containing 300 mM NaCl and 15 mM imidazole. Buffer with 150 mM imidazole was used to elute the protein off the column. The protein was then dialyzed overnight against pH 7 50 mM HEPES, 1 mM EDTA buffer, using a UF membrane with 12–14 kDa MWCO (Spectrum Laboratories, Rancho Dominguez, CA); the buffer was changed twice. Protein concentration was measured by the Bradford assay after 5 min incubation with Coomassie Protein Assay Reagent (Pierce, Rockford, IL) using the Biophotometer (Eppendorf, Westbury, NY).

Dialysis during reconstitution experiments of apo-enzyme nox-1 with FAD was conducted at pH 7.0, in 50 mM HEPES buffer, with 1 mM EDTA and either 0 or 2 M NaBr added.

2.3. Wavelength-scan of nox-1 holoenzyme

After purification, nox-1 is colorless owing to its presence as an apo-enzyme. To reconstitute the holoenzyme, 9.1 μM of nox-1 apo-enzyme were incubated with a 30-fold molar excess of FAD at 30 °C for 10 min. Excess FAD was removed from the protein solution gel permeation chromatography over a PD-10 column (Amersham, Sweden). The eluent fraction with the highest volumetric activity was scanned from 250 to 700 nm in a quartz cuvette.

2.4. Amplex Red assay for H_2O_2

We employed the horseradish peroxidase (HRP)-catalyzed oxidation of 9-acetylresorufin ('Amplex Red', Molecular Probes, Eugene, OR) to fluorescent resorufin as our H_2O_2 assay. Amplex Red reacts with H_2O_2 according to a strict 1:1 stoichiometry, large extinction coefficient [ε: 54,000 L/(mol·cm), λ_{max}: 587 nm (emission)], and an extremely low detection limit of 100 nM resorufin product. A 10 μL sample was pipetted into a 96-well microplate after the reaction with 800 μM NADH was completed. Working solution (50 μL), which included HRP and Amplex Red, was subsequently added and the reaction finished after 30 min in the dark. A Fluorostar (BMG Labtechnologies, Durham, NC) platereader was used to detect resorufin fluorescence. 75 U of superoxide dismutase from bovine erythrocytes (Sigma-Aldrich, Milwaukee, WI) were employed to test for formation of the superoxide ion during the nox-1 reaction. Samples from the reaction were tested for H_2O_2 with Amplex Red as well.

2.5. Nox-1 kinetics

A DU-800 spectrophotometer (Beckman Coulter) was used to detect nox-1 activity by following the decrease of the substrate NADH absorbance at 340 nm (ε: 6220 M^{-1} cm^{-1}). Both disposable 1.75 mL UV transpar-

ent cuvettes (light path 1 cm, VWR) and 1.7 mL quartz cuvettes (light path 5 mm) were used. The measurement of kinetic parameters with substrate NADH and cofactor FAD was performed with nox-1 in air-saturated pH 7 50 mM HEPES solution at 30 °C. Nox-1 was first incubated with FAD of a given concentration at 30 °C for 5–7 min before adding NADH.

The K_M value of the cofactor FAD was determined at varying FAD concentrations from 3.33 to 500 μM. Standard conditions were then set at 30 °C, 53 μM FAD, incubation of nox-1 for 5–7 min, 0.24 mM NADH, pH 7 50 mM HEPES buffer. The K_M value of NADH was determined by 0.01–0.48 mM NADH at an FAD concentration of 53 μM ($=K_M$ of FAD).

Inhibition by NAD^+ was investigated at [NAD^+] of 0, 0.24, 0.48, and 0.72 mM at different NADH concentrations. Product inhibition by H_2O_2 was investigated by adding 0, 5.87, 11.73, or 17.61 mM of H_2O_2. K_I can be calculated from the intercepts on the Lineweaver–Burk plot: $K_I = [I]/(v_{max}*intercept - 1)$.

Catalase (0.4–7.2 U) from *Aspergillus niger* (SAF, Milwaukee, WI) was added to 4.5 μM nox-1 and NADH solution to check for any improvement in activity.

3. Results

3.1. DNA sequencing

DNA sequencing data of pET32nox1 revealed five silent mutations and an E290R mutation (GAG to AGG), compared to the annotated gene sequence in the NCBI database (1560 bp). We never found Glu at position 290 in any of our constructs or our PCR products. PCR product of the AhpF gene is shown in Figure 2.

3.2. Purity and yield

Nox-1 was overexpressed to 6.5% of cell protein at a level of 25.8 mg/L under the conditions mentioned above. After purification over IMAC, the overall activity
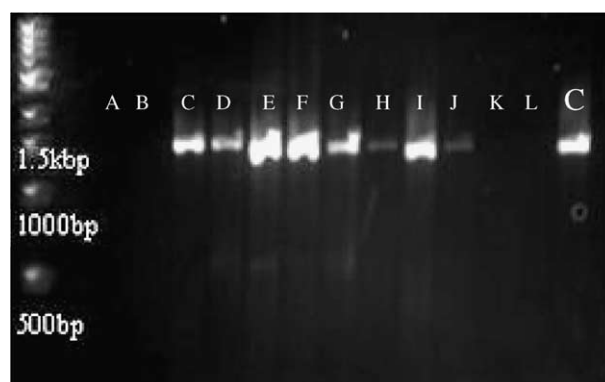
Figure 2. PCR of AhpF using Failsafe kit (buffers A–L). Buffers E and F are the best buffers for AhpF. PCR product is 1560 bp.
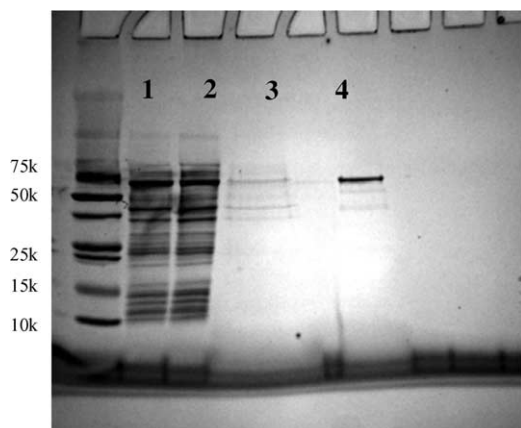
Figure 3. Purification of nox-1. 12% Tris Glycine SDS-PAGE gel. Lanes 1 and 2: lysate; Lane 3: run-off after first wash; Lane 4: run-off after second wash; Lane 5: protein purified (~67 kDa). Nox-1 is around 55 kDa and N-terminal tags are 12 kDa.

yield was 35% (at a protein concentration of 0.6 g/L). The results of the electrophoresis (12% SDS-PAGE) of lysate and purified protein are featured in Figure 3. As evidenced by the ratio of absorbances A_{450}/A_{280} (see below), the protein was judged to be >95% pure.

3.3. Reconstitution of nox-1 apo-protein with FAD

The nox-1-containing solution eluting off the Co^{2+} resin was colorless, thus being consistent with the presence of apo-protein. To reconstruct nox-1 holo-enzyme, we proceeded to incubate the apo-protein with different amounts of FAD while varying the temperature (4 vs 30 °C), incubation time (5 min vs overnight), and dialysis in the absence versus presence of 2 M NaBr to render its conformation more flexible, thus possibly easing FAD incorporation into the apo-protein.[5]

The incubation temperature turned out to be the crucial factor: incubation of apo-protein nox-1 with FAD at 30 °C for 5 min yielded the highest level of activity, considerably higher than incubation at 4 °C. The lower level of activity after overnight incubation at 30 °C, might be attributed to the onset of degradation of the apo-protein. Dialysis in the presence of 2 M NaBr did not yield any improvement in activity.

3.4. FAD/subunit

From the scan of the nox-1 holo-enzyme (Fig. 4), we found an absorbance ratio A_{450}/A_{280} of 0.108, a ratio characteristic of a pure flavoprotein with one FAD molecule per subunit of nox-1. The absorbance peaks at 378 and 445 nm corroborate nox-1 to be a flavoprotein with flavin in its oxidized form (the solution was air-saturated). Nox-1 holoenzyme was found to feature a specific activity of 2.04 U/mg at 0.24 mM NADH, 30 °C, and pH 7.

3.5. Kinetic parameters of nox-1

The K_M value of the cofactor FAD was found to be 54 μM, measured in the presence of 0.24 mM NADH; the apparent maximum velocity $v_{max,app}${$[O_2]$ =
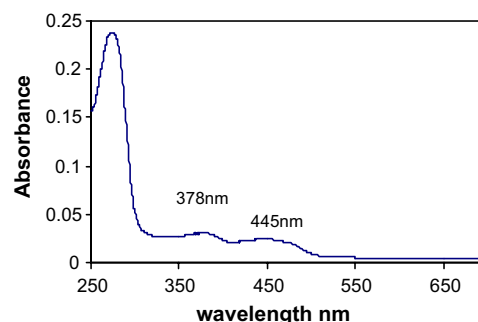

Figure 4. UV–vis absorbance spectrum of reconstituted holo-hydrogen peroxide forming NADH oxidase. Nox-1 was first incubated with 30-fold FAD before PD-10 column treatment. 50 mM HEPES, 1 mM EDTA, pH 7, 30° C. Absorbance peaks: 378 and 445 nm.

0.25 mM = const. in the air-saturated solution was 0.98 μmol/(L·s), resulting in an apparent maximum specific activity of 14.7 U/mg, as evidenced by the Hanes plot (Fig. 5). The K_M value of NADH, measured at 53 μM FAD, was found to be 76 μM, resulting in a maximum specific activity of 15.3 U/mg (Fig. 6). After adding catalase, no increase in nox-1 activity was observed.

3.6. Product inhibition

We found that both products, NAD^+ and hydrogen peroxide, inhibit nox-1. Hydrogen peroxide inhibits the enzyme noncompetitively (Fig. 7), with an inhibition con-
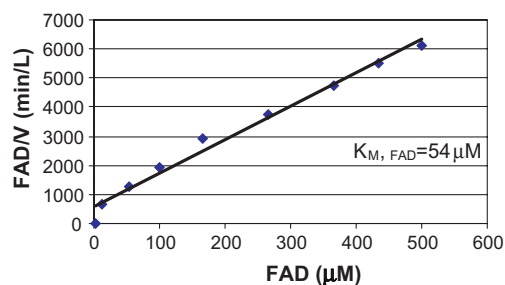

Figure 5. Kinetics of nox-1 with cofactor FAD in air-saturated solution, pH 7, 50 mM HEPES, 0.24 mM NADH, 30 °C. Hanes plot: K_M of FAD is 53 μM and maximum specific activity is 14.6 U/mg.
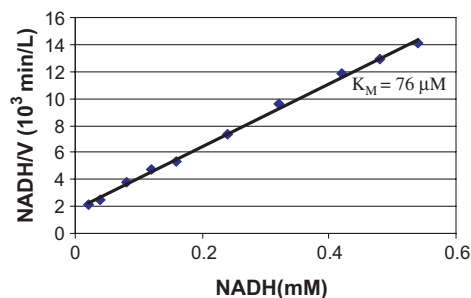
Figure 6. Kinetics of nox-1 with substrate NADH. pH 7 50 mM HEPES, 53 μM FAD, 30 °C. Hanes Plot. K_M of NADH is 76 μM and maximum specific activity is 15.3 U/mg.

stant K_I of about 12 mM. This constant is comparatively large: even at 17.6 mM, our highest H_2O_2 concentration investigated, the enzyme was still rather active. On the other hand, NAD^+ is a strong inhibitor. While we could not establish a definite inhibition constant due to scatter, we estimate the K_I to be around 0.2 mM.
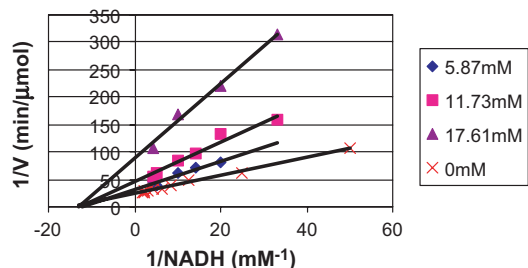
Figure 7. Non-competitive inhibition from H_2O_2. Lineweaver–Burk plot. K_I is around 12 mM.

3.7. Total turnover number (TTN)

The total turnover number (TTN) (total product produced/amount of enzyme [mol/mol]) of nox-1 during our experiments at 30 °C and pH 7 was found to be 44,000, based on conversion of NADH. This result indicates the good stability of our enzyme against turnover-based inactivation.

3.8. Coupled reactions with catalase and superoxide dismutase

As H_2O_2 inhibits and possibly even deactivates nox-1, we coupled nox-1 with an excess of catalase to investigate the influence on the rate of nox-1, as measured by NADH consumption. We did not observe a change in rate upon the addition of catalase. As expected, we did not find any H_2O_2 when checking with Amplex Red after joint incubation of nox-1 and catalase, indicting that catalase indeed converted all the generated H_2O_2 to O_2 and water. This finding also demonstrates the stability of nox-1 against stoichiometric levels of H_2O_2 formed when converting NADH.

To check the stoichiometry of NADH consumption and H_2O_2 generation according to Eq. 1, we measured H_2O_2 generation by detecting resorufin with the Amplex Red assay (see Section 2.4). We consistently detected 40 μM resorufin, equivalent to formation of 40 μM H_2O_2, upon conversion of 80 μM NADH with nox-1 holo-enzyme. Thus, we found only half of the electrons from NADH transferred to H_2O_2. We surmised that the other half might have been reacted to superoxide in a one-electron reduction. Consequently, we added superoxide dismutase (SOD) to aerated solutions of nox-1 and NADH. SOD would cause any present superoxide to dismutate to O_2 and H_2O_2, so the level of resorufin measured in the Amplex Red assay would have to be increased, thus reflecting the action of SOD. However, we did not find any increase in the level of H_2O_2 produced, so the superoxide was not formed in significant amounts. We are currently investigating the cause of the discrepancy between the NADH consumed and H_2O_2 formed.

4. Discussion and conclusion

We have successfully applied the sequence comparison-based approach to find a novel hydrogen peroxide forming NADH oxidase, which reduces oxygen directly to hydrogen peroxide, from *L. lactis*. The amino acid sequence alignment of nox-1 from *L. lactis* shows high identity with nox-1 from *Streptococcus mutans*, *Salmonella typhymurium* and *Amphibacillus xylanus*. There were discrepancies of the sequences in the database with those found experimentally in this work, an E290R and five silent mutations. Apparently they do not impair the enzyme activity.

Whereas most currently accessible nox-2 enzymes feature covalently bound FAD coenzymes, nox-1 enzymes apparently bind FAD noncovalently.[1,2,4,11,12] We measured an apparent maximum specific activity of *L. lactis* nox-1 of 15 U/mg, compared to 9.8 U/mg for the *S. mutans* and 1.96 U/mg for the *S. typhymurium* enzymes.[10] Interestingly, our specific holo-nox-1 activity of 2.04 U/mg is very close to that number. No information (including K_M values) regarding exogenous addition of FAD is provided for the nox-1 from *S. mutans* and *S. typhymurium*. The maximum specific activity of 15 U/mg found for our enzyme is about an order of magnitude smaller than values found for nox-2 enzymes of *Lactobacillus sanfranciscensis* (221 U/mg) or of *Lactobacillus brevis* (116 U/mg).[7,4] While our nox-1 enzyme from *L. lactis* is inhibited by both products, NAD^+ and H_2O_2, both the large value of K_I for H_2O_2 as well as the results with the coupled catalase reaction suggest that nox-1 is surprisingly stable against H_2O_2.

In conclusion, we have successfully applied the sequence comparison-based approach to find a novel H_2O_2-forming NADH oxidase functionality in *L. lactis* from an annotated part of the genome. A very sensitive assay for hydrogen peroxide, based on fluorescence of resorufin, has been employed successfully to demonstrate that only half of the electrons from NADH were transferred to hydrogen peroxide in experiments with isolated nox-1. The two most important future tasks concern the fate of the other half of the electrons donated by NADH not currently found as H_2O_2 as well as the influence of the peroxidase (AhpC) found upstream on the genome of *L. lactis*.

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