Comparison of Alkyl Hydroperoxide Reductase (AhpR) and Water-Forming NADH Oxidase from *Lactococcus lactis* ATCC 19435

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Abstract: We have successfully applied the sequence comparison-based approach to develop a peroxidase (gene AhpC) and a water-forming NADH oxidase from *Lactococcus lactis* (*L. lactis*). We found a considerably lower maximum specific activity of nox-1 (AhpF) (15 U/mg) from *L. lactis* compared to its nox-2 counterpart (95 U/mg). Both nox-1 and nox-2 are turnover-limited, as expected for enzymes with labile, redox-active thiols in the active site. In the absence of exogenously added thiols, both nox-1 and nox-1/peroxidase are considerably more stable against overoxidation than nox-2: the total turnover number TTN is 82,000 for nox-1 and nox-1/peroxidase *vs.* 39,000 for nox-2. Addition of exogenous thiols, however, increases nox-2 stability by a factor of two, up to the level of nox-1. Kinetic and stability analysis does not reveal any clear advantage for oxygen scavenging *via* the nox-1 or the nox-2 routes in lactic acid bacteria. Expression levels in lactic acid bacteria upon exposure to oxidative stress rather than kinetic performance more likely account for the previously observed superiority of nox-2 effectiveness over nox-1.

Keywords: cofactor; cofactor regeneration; NADH oxidase; single-sulfhydryl enzymes; total turnover number

Introduction

NADH Oxidases as Part of the Oxygen Defense System

Most *Lactobacilli* and *Lactococci*, members of the lactic acid bacteria family, have no functional electron-transfer chain and do not possess any heme-containing enzymes, such as catalases. Their aerotolerance is related to their ability to induce NADH oxidase (against molecular oxygen) and superoxide dismutase (against superoxide).[1] Thus, NADH oxidase is part of the oxygen defense system of facultative anaerobes such as *Lactobacilli* and *Lactococci*. *Streptococcus mutans* (*S. mutans*), also a member of the lactic acid bacteria family, has been shown to have both hydrogen peroxide-forming NADH oxidase (nox-1) and water-forming NADH oxidase (nox-2).[2,3] Nox-2 from *S. mutans* was reported to play an important role in regenerating NAD^+, whereas nox-1 was found to contribute only negligibly.[4]

Regeneration of NADH Cofactor to NAD^+

Many oxidative biological redox reactions require nicotinamide cofactors, such as the dehydrogenase-catalyzed transformation of monosodium L-glutamate (MSG) to α-ketoglutarate.[5] The regeneration of the co-produced cofactor NADH to NAD^+ is essential for efficient transformation, especially on large-scale – not only for cofactor economics but also to drive the production reaction towards completion. Both kinds of NADH oxidase, nox-1 and nox-2, can catalyze the irreversible reduction of oxygen.[6] Nox-1 is actually part of the alkyl hydroperoxide reductase system (AhpR), consisting of two enzymes: nox-1 (AhpF gene) and peroxidase (AhpC gene). Reactions catalyzed by AhpR are shown in Eqs. (1)–(3).

Thus, the overall AhpR reaction is identical to the nox-2 enzyme. Several organisms, among them *Lactococccus lactis* (*L. lactis*), important in the dairy industry, possess an alkyl hydroperoxide reductase (AhpR) system as well as a nox-2 enzyme. Owing to its importance,

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

$$\text{peroxidase (AhpC)} \quad H_2O_2 + NADH + H^+ \longrightarrow NAD^+ + 2\,H_2O \quad (2)$$

Overall reaction (identical to nox-2 system):

$$\text{Reductase (AhpR)} \quad O_2 + 2\,NADH + 2\,H^+ \longrightarrow 2\,NAD^+ + 2\,H_2O \quad (3)$$

the genome of *L. lactis* IL1403 was published recently.[7] The *L. lactis* genome contains several annotated nox-2 genes as well as both the AhpF and the AhpC genes.

Recently, we published the characterization of the nox-1 from *L. lactis*.[8] In this work, we report cloning, expression, purification, and characterization of both the peroxidase (AhpC) and the water-forming NADH oxidase from *L. lactis* ATCC 19435. We will focus on the comparison between nox-2 and the combined nox-1/peroxidase system with respect to their kinetic behavior and stability against overoxidation.

Background

Peroxidases (AhpCs) share strong similarity: sequence alignment between proteins from *L. lactis*, *Salmonella typhimurium* (*S. typhimurium*),[9] and *Amphibacillus xylanus* (*A. xylanus*) reveals identity at the amino acid level of 52% and additional strong similarity of 20% (Figure 1).[10]

Previous work with the peroxidase AhpC showed that it is not an independent enzyme, i.e., its activity is coupled to interaction with AhpF-like enzymes. The anaerobic assay of AhpC from *Salmonella typhimurium* was done in the stopped-flow UV spectrophotometer: AhpF-dependent peroxidase assay (5 nmol AhpC, 10–75 pmol AhpF) and AhpC-dependent peroxidase assay (5 nmol AhpF and 100–200 pmol AhpC).[9] Here, we monitored production of hydrogen peroxide to verify the activity of our AhpC from *L. lactis* with different AhpF/AhpC ratios and characterized its stability together with AhpF for the biocatalytic process.

Alignment of the *L. lactis* nox-2 shows a conserved Cys42 active site, as in many other water-forming NADH oxidases from *Lactobacillis sanfransciscensis* (*L. sanfransciscensis*),[11,12] *Enterococcus faecalis*,[13] *S. mutans*,[3,14] or *Lactobacillis brevis*.[15] The nox-2 enzyme from *L. lactis* MG1363 has been obtained previously *via* protein purification from cell lysate.[14] We report here nox-2 from ATCC 19435 obtained *via* cloning the gene and overexpressing the protein in *E. coli* (BL21(DE3)-RIL).

Results and Discussion

DNA Sequencing of AhpC and nox-2

DNA sequencing data of AhpC PCR product (Figure 2) and pET30ahpC revealed one silent mutation (TCG to

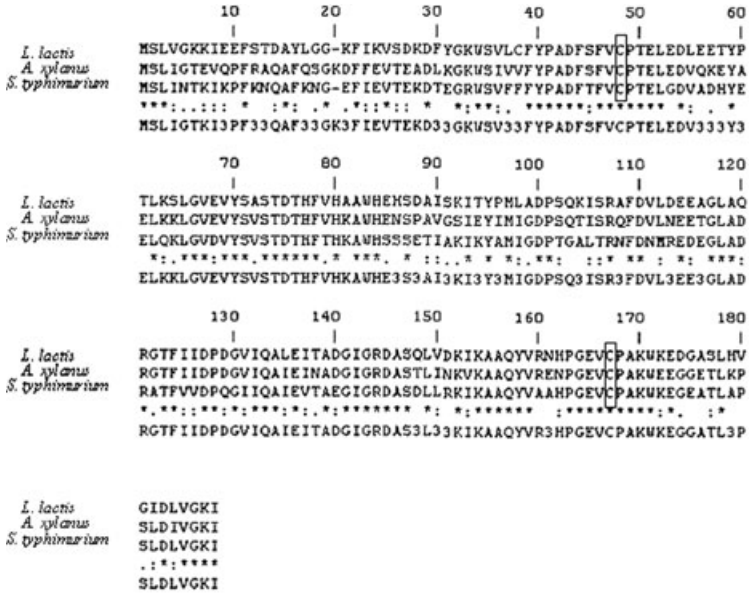

Figure 1. *L. lactis* peroxidase amino acid alignment with other known AhpC proteins. Identity: 52.7%; strong similarity: 20.7%. The highlighted C46 and C165 are catalytic Cys residues of AhpC (*L. lactis*).

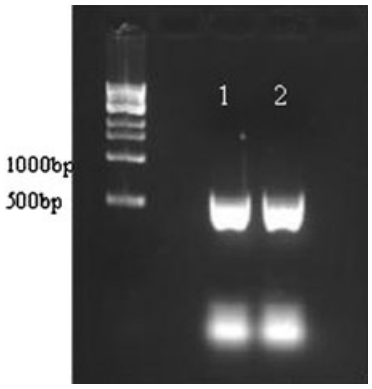

Figure 2. PCR of AhpC using *Taq* polymerase. Lane 1 and lane 2 show AhpC PCR products on the gel and the size is around 564 bp.

IL 1403	MKIVVIGTNHA--GIATANTL**ID**R**Y**PG
ATCC19435	MKIVVIGTNHA--GIATANTL**ID**Q**Y**PG
MG1363	MKIVVIGTNHAAGIATANTL**LE**Q**Y**PG

Figure 3. Amino acid alignment among different *L. lactis* strains IL1403, ATCC19435 and MG1363. Amino acids in bold are the differences among them.

TCA) at Ser63, compared to the annotated gene sequence in the NCBI database (564 bp). The sequencing results of pET30noxE and pET27noxE show five silent mutations and four amino acid discrepancies (R22Q, L212S, S278Y, N385S) when compared to the annotated noxE gene sequence from *L. lactis* IL1403 in the NCBI database.

If the N-terminal amino acid sequences of *L. lactis* IL1403 from the NCBI database, of the strain *L. lactis* MG1363,[14] and of the ATCC19435 strain in this work are compared, one finds four differences among the first 25 or 26 N-terminal amino acids (Figure 3). Thus, we expect that there are more differences in the overall sequence and possibly also in the properties between the *L. lactis* noxes-2 from the MG1363 and ATCC19435 strains.

Protein Expression and Purity

The open reading frame for nox-1, AhpC, and nox-2 is capable of encoding a protein with a molecular mass of 55 kDa for nox-1, 22 kDa for AhpC and 49 kDa for nox-2, respectively, which is in good agreement with previously published similar alkyl hydroperoxide reductases and water-forming NADH oxidases. SDS-PAGE of the proteins derived from the expressed genes exhibited prominent bands at the right size (Figures 4 and 5).

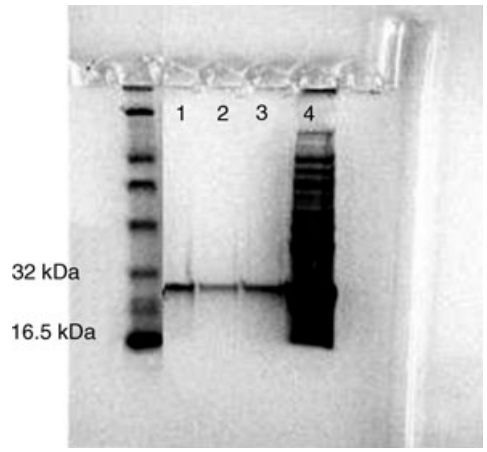

Figure 4. Purification of AhpC. 12% Tris-glycine SDS-PAGE gel. Lanes 1–3: protein purified (~21 kDa); Lane 4: lysate.

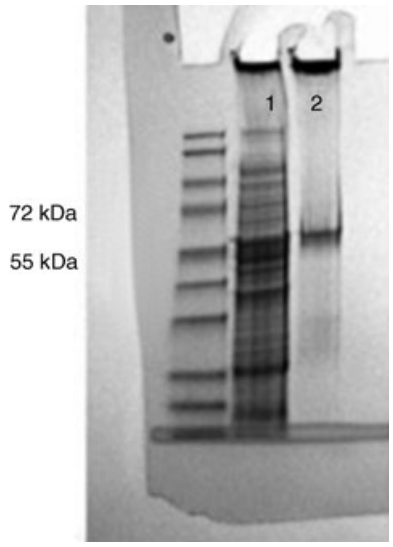

Figure 5. Purification of C-nox-2. Lane 1: lysate; Lane 2: protein purified.

All of the protein samples collected after IMAC showed no other bands other than the protein of interest on the SDS-PAGE gels (N-terminally His-tagged nox-2 is not shown).

FAD/Subunit

The native nox-2 in air-saturated buffer reveals a typical spectrum of a flavoprotein with absorption peaks at 380 nm and 441 nm. From the scan of the nox-2 (Figure 6), we found an absorbance ratio A_{450}/A_{280} of 0.126. The ratio of FAD associated per subunit of enzyme was calculated from the result of the Bradford test for the amount of enzyme, the assumption of dimeric association of *L. lactis* nox-2,[14] and the molar absorption coefficient ε for FAD of 11,300 $(M \cdot cm)^{-1}$.[16] The

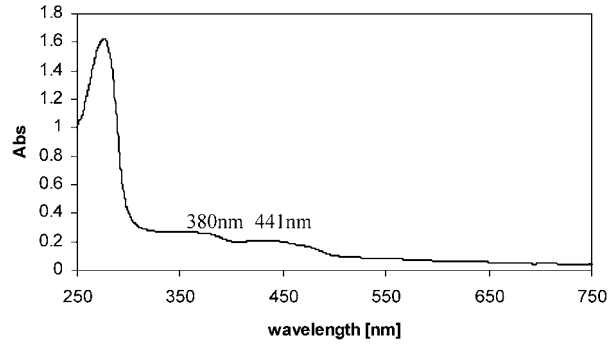

Figure 6. UV-Vis absorbance spectrum of nox-2 from *L. lactis.* 50 mM HEPES, 1 mM EDTA, pH 7, 30 °C. Absorbance peaks: 380 and 441 nm.

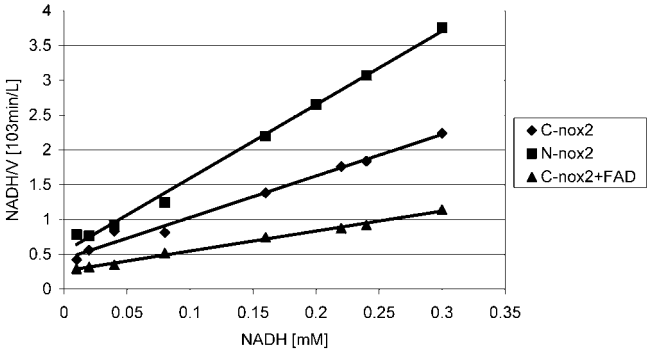

Figure 7. Hanes plot of kinetics of N-terminally and C-terminally His-tagged nox-2 from *L. lactis* with air-saturated substrate NADH (0.24 mM). Other conditions: pH 7.0, 50 mM HEPES, 30 °C, with or without 53 μM of FAD.

calculated value of 1.86 mole of subunit per mole of FAD indicates the presence of one FAD molecule per dimer, in contrast to the findings of one FAD molecule per subunit for nox-1 from *L. lactis* and for other nox-2 enzymes.[8] As we reported before, nox-1 from *L. lactis* needs to be reconstituted with FAD to obtain the holo-enzyme after purification because it loses its FAD during the purification step. While nox-2 from *L. lactis* appears yellow after applying the same purification method, we still proceeded to add exogenous FAD to our nox-2 preparation (results see below).

Kinetic Parameters of nox-1 and nox-2

The K_M and v_{max} values as well as the maximum specific activity of C-His nox-2 and N-His nox-2 were calculated from the Hanes plot (Figure 7). FAD stimulates the activity of both nox-1 and nox-2. In the case of nox-1, the activity of holo nox-1 is 2.04 U/mg (0.24 mM NADH), but with 53 μM externally added FAD, the activity becomes seven times higher. In the case of nox-2, the externally added 53 μM FAD doubled the activity of nox-2. The comparison of kinetic parameters between nox-1 and nox-2 is shown in Table 1.

pH Effect on nox-2

The optimum pH for nox-2 was observed to lie between pH 6.5 and pH 7.5. Rates at low pH (pH 4 to 5) are reported as net rates, with the chemical decomposition rate at low pH subtracted. Below pH 5.0, the activity of nox-2 is very low compared to its optimum value. The sample instantly loses its activity in both acetate and MES buffer at pH 5. At pH values above 5.0, the activity of nox-2 increases quickly, reaches its optimum and then falls off gradually.

Both nox-2 from *L. lactis* and nox-2 from *L. sanfranciscensis* (Figure 8) feature gaps in the activity-pH profile at pH 5.0 and pH 5.5, respectively. The calculated pI for nox-2 from *L. lactis* is 5.66 and the pI value for

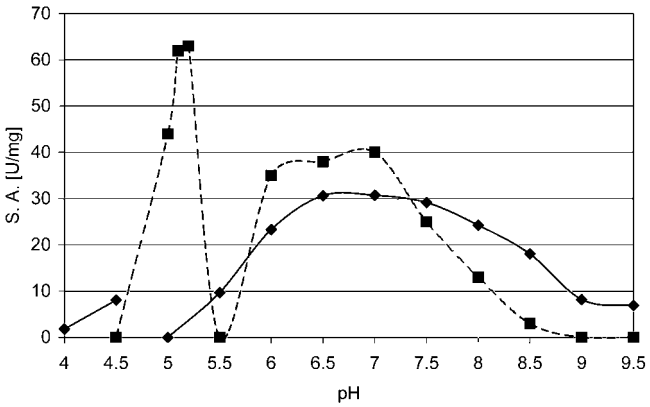

Figure 8. Activity-pH profile of *L. lactis* nox-2 ; comparison with profile of *L. sanfranciscensis* (dashed line).[11]

Table 1. Comparison of kinetic data of nox-1 and tagged nox-2 from *L. lactis*

Enzyme	K_M [μM], NADH	v_{max} [μmol/(L·s)]	v_{max}/[E] [U/mg]	K_M (μM), NADH (53 μM FAD)	v_{max} [μmol/(L·s)]	v_{max}/[E] [U/mg] (53 μM FAD)
Nox-1	n.d.	n.d.	2.04	76	0.98	14.7
C-nox2	73	1.88	45.1	90	3.92	94.1
N-nox2	53	0.12	23.9	n.d.	n.d.	n.d.

n.d. = not determined; conditions: air-saturated solution, 0.24 mM NADH, pH 7.0, 50 mM HEPES, 30 °C, with or without 53μM of FAD.

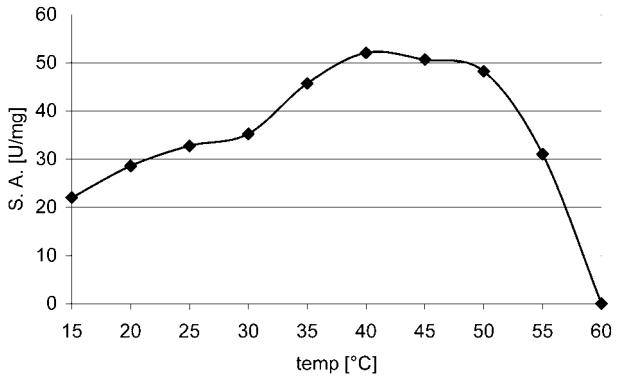

Figure 9. Activity-temperature profile of *L. lactis* nox-2. Optimum temperature: 40 °C.

nox-2 from *L. lactis* MG1363 was shown to be 4.8 on isoelectric focusing gels.[14] The calculated pI for nox-2 from *L. sanfranciscensis* is pH 5.4. Whether or not the gap is related to the proximity of the pH to the pI is under further investigation.

Activity Profile with Respect to Temperature

The temperature profile shows that nox-2 reaches its optimum at 40 °C in 50 mM HEPES buffer at pH 7.0. Above 15 °C, the activity of nox-2 gradually increases until it hits the optimum plateau between 40 °C and 50 °C, and drops quickly to zero when it reaches 60 °C. At 60 °C, the enzyme was completely inactive after the first 10–20 sec reaction with substrate NADH. The activation energy E_a was calculated to be 24.8 kJ/mol from the Arrhenius plot between 15 and 40 °C.

Hydrogen Peroxide Generation

To check H_2O_2 generation of alkyl hydroperoxide reductase and nox-2 from *L. lactis*, we measured the H_2O_2 generation by detecting resorufin with the Amplex Red assay.[11] We found that, at an AhpF/AhpC-ratio of 1:1, the H_2O_2 generated is the same as the control of AhpF alone. When the AhpF/AhpC-ratio is 1:20, 30% less H_2O_2 was generated comparing with the control. Only when the AhpF/AhpC-ratio reached 1:200, was no H_2O_2 detected. Therefore, AhpC needs to be present in large excess to be an effective H_2O_2 scavenger. When analyzing nox-2, although nox-2 is a water-forming NADH oxidase, we found 0.4–0.7% H_2O_2 generated after its reaction with NADH.

Total Turnover Number (TTN)

The operational stability of alkyl hydroperoxide reductase and NADH oxidase is not limited by temperature but rather by catalytic turnover. When testing the degree of conversion, as calculated from the decrease of absorption of NADH at 340 nm, at low enzyme concentration (<500 pM), we observed that the reaction came to a standstill with incomplete conversion of NADH. We verified inactivation of the enzyme by spiking again with NADH, but no further conversion of NADH occurred. The total turnover number of AhpF is not affected by the amount of added AhpC. For the C-terminally His-tagged nox-2, the TTN almost doubled upon addition of 5 mM DTT to the buffer (Table 2).

Discussion

We have successfully applied the sequence comparison-based approach to find AhpC and water-forming NADH oxidase (nox-2) genes in *L. lactis*. Upon sequencing both enzymes, we found just one silent mutation at Ser63 in the AhpC (188 amino acids) but five silent mutations and four amino acid changes in our nox-2 protein from ATCC19435 (448 amino acids) in comparison with the enzyme from the IL 1403 strain in the NCBI database.[7] Moreover, the nox-2 protein purified before from the lysate of the MG1363 strain differed from ours in four amino acid positions of the 26 positions sequenced N-terminally in the former (the complete nox-2 sequence from MG1363 still does not exist).[14] However, none of these discrepancies is in a conserved region and the differences do not seem to impair enzyme activity significantly.

We found that externally added FAD enhances the activity in both nox-1 and nox-2 *L. lactis* enzymes to apparent maximal specific activities of 15 and 94 U/mg, respectively. In *S. mutans*, which also features both AhpF/AhpC and nox-2,[2,3] the oxidase activity of nox-1 from *S. mutans* was found to be stimulated on addition of FAD, while that of nox-2 was not. Furthermore, the activities of nox-1 from *Bacilllus megaterium* and *Thermus thermophilus*,[17,18] and nox-2 from *Streptococcus faecalis* 10C1 are enhanced by FAD as well.[19] On the

Table 2. Total turnover numbers for nox-1 and nox-2 enzymes from *L. lactis*.

TTN	AhpF/AhpC (1000:1)	AhpF/AhpC (1:1)	AhpF/AhpC (1:20)	AhpF	N-nox2	C-nox2
HEPES	82,000	82,000	82,000	82,000	n.d.	38,740
HEPES, 5 mM DTT	82,000	82,000	82,000	82,000	52,000	78,480

Conditions: [AhpF] = 22 nM; [C-nox-2] = 30 nM; [N-nox2] = 51 nM; n.d. = not determined.

other hand, work on nox-2 from *L. sanfranciscensis* and *Leuconostoc mesenteroides* found that free FAD has no effect on activity of those enzymes.[4,11,12,20] Our calculated occupancy of nearly 1.0 upon the addition of exogenous FAD, in comparison with about 0.5 without added FAD, points to the possibility of free FAD diffusing to an apo-flavin site of *L. lactis* nox-1 and nox-2 variants and consequently enhancing specific activity of the fully occupied holo-enzyme. In several other nox-2 variants, FAD seems to be neither lost nor taken up, thus is probably tightly (though often non-covalently) bound; in those cases, addition of exogenous FAD predictably has no effect.

Although alkyl hydroperoxide reductase is active as a 1:1 protein complex between one molecule each of AhpF and AhpC, we found that only a vast excess of AhpC over AhpF leads to complete reduction of intermediately generated hydrogen peroxide to water. This finding points to mostly unproductive collisions between these two enzymes in solution resulting in incomplete turnover of hydrogen peroxide, although the N-terminal His tag, in contrast to a C-terminal tag, should not interfere with interactions with AhpC.

N-Terminally His-tagged nox-2 has a considerably lower specific activity than its C-terminally His-tagged counterpart. The possible reason is that the N-terminal domain is the NADH binding domain, so that an N-terminal his tag obstructs binding of NADH to the enzyme. Our C-terminally His-tagged nox-2 shows similar maximum specific activity as the untagged *L. lactis* nox-2 from MG1363 reported before,[14] 94 compared to 83 U/mg, but with a much higher K_M value of 90 μM compared to 4.1 μM on the latter.

It is remarkable that we found around the same percentage of released hydrogen peroxide generated by nox-2 from *L. lactis* and *L. sanfranciscensis*, 0.4 to 0.7% of the stoichiometrically expected value, as measured by generation of resorufin from Amplex Red. This finding might point to a possible common mechanism of nox-2 enzymes to not release intermittently formed hydrogen peroxide but rather convert it into water with electrons from a second molecule of NADH. The intermittently formed hydrogen peroxide is thought to be captured by the active-site cysteine42 as a sulfenic acid (cys42-SOH) intermediate, only a negligible amount (0.4–0.7%) is released. Electrons from a second molecule of NADH reduce the sulfenic acid to water and thiolate (cys42-S^-). We are currently investigating the feasibility of such a mechanism.

The results on total turnover for nox-1 (AhpF) indicate that the presence of AhpC at any stoichiometric ratio does not seem to enhance nox-1 operational lifetime. This points to deactivation not from hydrogen peroxide released from the nox-1 molecule but from hydrogen peroxide (or a subsequent product) generated by turnover even before release. Exogeneously added DTT has no effect on total turnover of nox-1, as expected, as the second thiol acts as a stabilizing nucleophile. While alkyl hydroperoxide reductase from *L. lactis* has a lower maximum specific activity than nox-2 under the same reaction conditions, its total turnover number was higher than that for the *L. lactis* nox-2. However, total turnover of nox-2 from *L. lactis* is doubled in the presence of DTT, which is in agreement with results on nox-2 from *L. sanfranciscensis*. For nox-2 from *L. sanfranciscensis*, TTN is increased dramatically from 3500 to above 112,000 upon the addition of 5 mM DTT in the reaction buffer.[5]

Conclusions

In conclusion, we found that nox-1 (AhpF) from *L. lactis* is considerably slower than its nox-2 counterpart. Both nox-1 and nox-2 are turnover-limited, as expected for enzymes with labile, redox-active thiols in the active site. However, as evidenced by the higher total turnover numbers, nox-1 is considerably more stable against overoxidation (TTN of 82,000) than nox-2 in the absence of exogenously added thiols, such as DTT (TTN of 39,000).

Addition of exogenous thiols, such as DTT, increases nox-2 stability by a factor of two, up to the level of nox-1. In comparison, nox-2 from *L. sanfranciscensis* is much less stable than its *L. lactis* homologue without exogenous thiols but is much more stabilized in their presence. As sequence identity between nox-2 of *L. lactis* and *L. sanfranciscensis* is only 33%, amino acid composition seems to influence stability behavior.

Kinetic and stability analyses do not reveal any clear advantage for oxygen scavenging *via* the nox-1 or the nox-2 routes in lactic acid bacteria. Differences in effectiveness therefore are likely to be caused by different expression levels rather than performance per protein molecule.

Experimental Section

Cloning

The AhpC and noxE genes from *L. lactis* ATCC19435 were isolated from the genomic DNA using the following gene-specific primers derived from the coding sequence: 5′ AATGTCATTAGTAGG TAAAAAAATAGAAG 3′ (AhpC) and 5′ TCTATATTTTACCTACAAGGTCAATACC 3′ (AhpC); 5′ ATGAAAATCGTAGTTATCGGTACG 3′ (noxE) and 5′ TTATTTTGCATTTAAAGCTGCAAC 3′ (noxE).

Both genes were successfully amplified by the polymerase chain reaction (PCR) using *Taq* polymerase (Fermentas, Hanover, MD). The PCR products were then purified through gel electrophoresis and subsequent elution (Gel Extraction Kit, Qiagen, Valencia, CA). AhpC and noxE were then cloned into pDrive vector that encoded blue/white screening feature (Qiagen PCR Cloning kit, Qiagen, Valencia, CA), and transformed into XL10-Gold competent cells. Positive clones were

identified as white colonies. The recombinant pDahpC and pDnoxE were obtained through miniprep (Qiagen Spin Miniprep kit, Qiagen, Valencia, CA), analyzed by PCR and sequenced.

pDnox1,[8] pDahpC and pDnoxE constructs were used as templates for the PCR of the desired genes and the PCR products were subcloned into the expression vector pET30 (EK/LIC cloning kit, Novagen, Madison, WI). The pET30 vector has a 6xHistidine tag and an S tag on its N-terminus. Plasmid pET27noxE was constructed by subcloning a *NdeI-HindIII* fragment engineered by PCR from the original pDnoxE into pET-27b(+) (Novagen, Madison, WI) that encodes a C-terminal His tag. All of the clones were successful and the recombinants were first transformed into XL10-Gold cells for positive/negative test and then recombinants pET30nox1, pET30ahpC, pET30noxE and pET27noxE were transformed to the expression strain BL21(DE3)-RIL (Stratagene, La Jolla, CA).

Induction and Overexpression

The successful clones pET30nox1, pET30ahpC, pET30noxE and pET27noxE were grown in LB broth with 30 μg/mL kanamycin and 50 μg/mL chloramphenicol at 37 °C for 3–4 h. Protein overexpression was induced with 200–500 μM isopropyl β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.3–0.7, overexpression was conducted at 37 °C, and cells were harvested after 3–4 h. Cell pellet was then ultrasonicated in 15 mL 50 mM sodium phosphate buffer at pH 7 containing 300 mM NaCl.

Protein Purification

By taking advantage of the N- or C-terminal 6xHis tag, all of the proteins were purified by using immobilized metal affinity chromatography as described before (IMAC; BD TALON Co^{2+} Metal Affinity CellTru Resin, BD Biosciences, Palo Alto, CA).[8] Nox-1 and AhpC were then dialyzed overnight against pH 7.0 50 mM HEPES, 1 mM EDTA buffer, using a UF membrane with 12–14 kDa MWCO (Spectrum Laboratories, Rancho Dominguez, CA) and Slide-A-Lyzer MWCO 3,500 Dialysis Cassette (Pierce, Rockford, IL), respectively; buffer was changed twice. Nox-2 was dialyzed in the same manner as nox-1 except using pH 7 50 mM HEPES, 1 mM EDTA, 5 mM dithiothreitol (DTT) buffer instead. Protein concentration was measured by the Bradford assay after 5 min incubation with Coomassie Protein Assay Reagent (Pierce, Rockford, IL) using a Biophotometer (Eppendorf, Westbury, NY).

NADH-Oxidase Activity Assay

Nox-1 was assayed as described before.[8] In brief, nox-1 activity was measured by the decrease in absorbance at 340 nm, corresponding to the decrease in concentration of the substrate NADH at 30 °C in a DU-800 spectrophotometer. It was reconstructed with FAD and scanned over the spectra of 250–750 nm. Its kinetic parameters were determined with 53 μM externally added FAD.

Nox-2 activity was assayed with a DU-800 spectrophotometer (Beckman Coulter) at 30 °C in a total volume of 1.5 mL air-saturated 50 mM HEPES buffer at pH 7.0 and 0.24 mM NADH. The reaction was initiated by the addition of a suitable amount of enzyme and the rate of NADH oxidase was monitored by the decrease in absorbance at 340 nm [$\varepsilon = 6220$ $(M\ cm)^{-1}$]. The measurement of kinetic parameters with substrate NADH and cofactor FAD was performed in a similar manner. Nox-2 was first incubated with 53 μM FAD at 30 °C for 10 min before adding NADH.

Wavelength Scan

Nox-2 was scanned in the air-saturated pH 7, 50 mM HEPES at 30 °C in a 1.5-mL quartz cuvette. The absorption spectrum (250–750 nm) was recorded in the DU-800 spectrophotometer.

Activity-pH Profile of nox-2

The following buffer systems were used to investigate the pH dependence of nox-2 at 30 °C with 0.24 mM NADH: Sodium acetate from pH 4.0 to pH 5.0; MES from pH 5.0 to pH 6.5; HEPES from pH 6.5 to 9.5. At pH 5 and 6.5, activity was measured in both sodium acetate and MES buffer at pH 5 and in MES and HEPES buffer at pH 6.5.

Effect of Temperature on nox-2

The temperature range of 15 to 60 °C was chosen to measure the activity of nox-2 in pH 7, 50 mM HEPES buffer with 0.24 mM NADH. Both the buffer and the substrate NADH were incubated at a chosen temperature for 10 to 20 min before adding nox-2 to the reaction solution.

Amplex Red Assay for H_2O_2 on Alkyl Hydroperoxide Reductase and nox-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.[8] Samples from the reactions of nox-1 (control), alkyl hydroperoxide reductase (AhpF/AhpC 1:1, 1:20, 1:200) were pipetted into a 96-well microplate after the reaction with 0.1 mM NADH. 50 μL sample from the reactions of nox-2 with 0.26 mM NADH was also tested for the hydrogen peroxide formation.

Total Turnover Number (TTN)

A specific amount of alkyl hydroperoxide reductase with different AhpF/AhpC ratios (1000:1, 1:20, 1:0, 1:1) and nox-2 were added to the 1.5 mL air-saturated 50 mM HEPES buffer ± 5 mM DTT at pH 7.0, 30 °C. NADH was added until the enzymes could no longer react.

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