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Studies on NADH oxidase and alkyl hydroperoxide reductase produced by *Porphyromonas gingivalis*

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Enzymes that detoxify oxygen or oxygen radicals are important to anaerobic microorganisms that inhabit oxygenated environments. In previous studies we have determined that *Porphyromonas gingivalis* W50 cell extracts possess NADH oxidase-like activity, which increases slightly under oxygenated conditions. The aim of this study was to characterize the protein responsible for this activity in order to establish whether it protects the microorganism from oxidative stress. Protein purification based on NADH oxidase activity did not isolate a conventional NADH oxidase. Instead, the NADH oxidase activity was found to be associated with a FAD-dependent enzyme identified as 4-hydroxybutyryl-CoA dehydratase (AbfD). The biological significance of this activity with respect to protection against oxidative stress is not clear; hydrogen peroxide (H_2O_2) was present after completion of the NADH oxidase assay with the purified protein. Northern blot analysis, examining the expression of other proteins likely to function as NADH oxidases/peroxidases in *P. gingivalis*, revealed the transcription of a protein similar to alkyl-hydroperoxide reductase (AhpF-C), which could serve as an NADH oxidase and H_2O_2-detoxification system. *AhpF* is transcribed in a polycystronic way with its neighboring gene, which encodes for the coupling protein AhpC. No transcript could be detected for the closest match to an NADH oxidase identified in the *P. gingivalis* genome sequence. In conclusion, *P. gingivalis* seems to lack a protective NADH oxidase but AhpF-C could contribute to its moderate tolerance to reactive oxygen species by metabolizing H_2O_2.

Key words: alkyl hydroperoxide reductase [AhpF-C]; 4-hydroxybutyryl-CoA dehydratase [AbfD]; NADH oxidase activity; oxidative stress; *Porphyromonas gingivalis*

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Oxidative stress has been defined as a disturbance in the prooxidant-antioxidant balance in favor of prooxidants (35) and has been reviewed, along with oxygen metabolism, in relation to dental plaque biofilms (18). Reactive oxygen species (ROS) such as $O_2^{\cdot -}$, $HO^{\cdot}$ and H_2O_2 are produced in aerobic environments, mediating cell damage (27). Of these species, H_2O_2 is extremely harmful as it easily penetrates membranes and diffuses through cells. It possesses the ability to form adducts (hydrogen-bonded chelate structures) with various cell constituents such as amino acids (e.g. histidine, alanine, glycine, aspartic acid), succinic acid and DNA bases, which act as H_2O_2 carriers (32). These characteristics allow H_2O_2 to act at sites distinct from the site of its production, enhancing its damaging potential. ROS are toxic to the cells as they are highly reactive and can cleave nucleic acids and oxidize essential proteins and lipids (2, 9). Oxidative damage to DNA involves single strand breaks and base alterations which can induce mutations (36), while protein oxidation converts several amino acid residues into their carbonyl derivatives and induces S-S and Tyr–Tyr cross-linking (37). Modifications or splitting of protein molecules dramatically changes protein conformation, inactivates enzymes and often leads to their denaturation (36).

The metabolism of anaerobes usually relies on metabolic schemes built around enzymes that react easily with oxygen (13). Hydrogen peroxide formation could therefore be one of the main sources of toxicity for anaerobic microorgansims. Indeed,

studies on the obligate anaerobe *Prevotella melaninogenica* demonstrated that, after exposure to O_2, the cells generated and accumulated O_2^- and H_2O_2, and that a crypto-OH radical generated through H_2O_2 was the active species mediating DNA damage (39). Moreover, obligate anaerobes utilize several dioxygen-sensitive enzymes for processes that assist in the maintenance of redox-balance during anaerobic fermentations (13).

The susceptibility of anaerobes to oxygen varies even among closely related microorganisms and possibly correlates with the levels of anti-oxidant enzymes present (4, 27). Therefore, O_2^- or H_2O_2-scavenging enzymes might be important in increasing the tolerance of anaerobic microorganisms to aerated environments. NADH oxidase is an enzyme involved in molecular oxygen detoxification and in maintaining a low redox potential inside cells. These enzymes catalyze the four-electron reduction of O_2 to H_2O ($2NADH + 2H^+ + 2O_2 \rightarrow 2NAD^+ + 2H_2O$) (24) and are exemplified by the NADH oxidase from *Enterococcus faecalis* (19), which contains a stabilized cysteine sulfenic acid (Cys-SOH) serving as a non-flavin redox center. On the other hand, alkyl hydroperoxide reductases (AhpF-C) belong to the peroxiredoxin oxidoreductase family. Apart from catalyzing the reduction of oxygen by NADH to form H_2O_2, they show extremely high peroxide reductase activity for H_2O_2 and alkyl hydroperoxides in the presence of the small disulfide redox protein AhpC (26). Examples of AhpF-C are found in many bacterial species (e.g. 25, 28).

Porphyromonas gingivalis is a gram-negative anaerobic cocco-bacillus strongly implicated in the etiology of adult periodontitis (8, 11). During the colonization of the oral cavity it is likely that *P. gingivalis* encounters different sources of oxidative stress. Adaptation to this challenge is necessary for the microorganism to survive and establish in the periodontal environment and anti-oxidant enzymes are likely to be a key factor contributing to this process. *P. gingivalis* possesses moderately high levels of superoxide dismutase (SOD) (4, 17), an enzyme that catalyzes the dismutation of superoxide radicals but disadvantageously produces hydrogen peroxide ($O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$) (1, 20, 22, 34). We have also reported that *P. gingivalis* W50 cell extracts possess NADH oxidase and NADH peroxidase activities (4). These activities are present, constitutively, in anaerobically grown cells, and increase slightly when the cells are exposed to oxygen. Moreover, open reading frames (ORF) encoding for a putative NADH oxidase/peroxidase (Nox) and for the two subunits of the AhpF-C system appear in the *P. gingivalis* W83 genome sequence (http://oralgen.lanl.gov). The aim of this study was, therefore, to purify and characterize the NADH oxidase activity seen in *P. gingivalis* cell extracts.

Materials and methods

Microorganism and maintenance of the strain

P. gingivalis W50 (ATCC 53978) was maintained short-term on anaerobic blood agar plates, incubated at 37°C in an atmosphere of 5% H_2, 5% CO_2 and 90% N_2.

Continuous culture growth conditions

To obtain cells for the purification of NADH oxidase activity and for RNA isolation, *P. gingivalis* was grown under continuous culture conditions in BM medium (33) supplemented with 5 mg/l of hemin, to achieve hemin-excess conditions, and 0.5 g/l of cysteine. Growth in a 365 ml working-volume chemostat was initiated by inoculating the growth chamber with a 24-h batch culture of the microorganism grown in the same medium. After 16–24 h of batch culture growth in the chemostat vessel, the growth medium reservoir pump was turned on and the medium flow adjusted to give a dilution rate of 0.069/h ($t_d = 10$ h), which was kept constant in all the experiments. The temperature was maintained at 36°C and the pH controlled at 7.4 by the automatic addition of 2 N KOH. The culture was sparged with the appropriate gas mixture at a flow rate of 300 cm^3/min. After about seven generations, under all conditions, and based upon lack of change in the optical density (OD_{560nm}), the culture was considered to have achieved steady-state. The redox potential (E_h) of the culture at each gassing stage was monitored with a redox electrode (model Pt 4805-DPAS-SC-K85/120, Mettler Toledo, Switzerland). Culture purity was checked daily by gram-staining. Initially, *P. gingivalis* was grown with an incoming gaseous atmosphere of N_2/CO_2 (90 : 5) which was then adjusted to contain 10% oxygen $N_2/CO_2/O_2$ (85 : 5 : 10).

Preparation of cell extracts and enzyme assays

At steady-state the chemostat was sampled and bacterial cells were harvested by centrifugation (6000 × *g*, 4°C for 30 min) and washed twice with cold 50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. The cells were then resuspended in an aliquot of the same buffer and lyzed by ten, 15-s sonications on ice. The disrupted cell suspensions were centrifuged (6000 × *g*, 4°C for 30 min) and the protein content of the supernatants was determined with a Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL), using bovine serum albumin as a standard. NADH oxidase activity was assayed at 25°C following the methods of Higuchi (10) by monitoring, spectrophotometrically, the oxidation of β-NADH at A_{340nm}. The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM β-NADH and cell extract (0.125 mg protein). One unit of activity was defined as the amount of extract that catalyzed the oxidation of 1 nmol of NADH/min. The effect of flavin adenine dinucleotide (FAD), as a possible cofactor for NADH oxidase was analyzed by adding 0.02 mM FAD to the NADH oxidase reaction mixture. To determine whether NADPH could be used as an alternate substrate for the oxidase, 0.1 mM of β-NADPH, instead of NADH, was added to the reaction mixture.

NADH oxidase activity was also assayed in 12% polyacrylamide gels after native gel electrophoresis of cell extracts or fractions from different enzyme purification steps (see below). Immediately after electrophoresis, gels were incubated for 5 min in 20 ml of ice-cold 0.1 M KPO_4 buffer (pH 7.0) and for a further 5 min in the same buffer at room temperature. After incubation, a freshly prepared NADH solution was mixed into the gel buffer to a final concentration of 50 μg/ml. After approximately 5–15 min, the gel was removed and placed on an ultraviolet (UV) light box to detect bands of NADH oxidase activity, appearing as dark bands in contrast to the fluorescent gel.

Purification of NADH oxidase activity

As the purification of any protein from *P. gingivalis* might be facilitated if its cysteine proteinases are inactivated, the effect of the cysteine proteinase inhibitor tosyl-L-lysine chloromethyl ketone (TLCK) on the activity of NADH oxidase was evaluated to determine whether the inhibitor could be utilized during the purification process. Unfortunately, TLCK at the required concentration to inhibit proteinase activity (1–5 mM) reduced the NADH oxidase activity in cell extracts by more than half. It was therefore decided to purify the enzyme in the absence of the inhibitor

to avoid any interference with its catalytic properties.

To obtain cells for the purification of NADH oxidase, the chemostat was sampled at steady-state under the oxygenated atmosphere of $N_2/CO_2/O_2$ (85 : 5 : 10). This gas phase was chosen to grow the cells for the enzyme purification in order to maximize the activity present. Cells were collected on ice, harvested by centrifugation (6000 × *g*, 4°C for 30 min) and washed with 50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. The cells were resuspended in an aliquot of the same buffer and lyzed by double passage through a French pressure cell at 1200 kg/cm^2. Remaining cells and debris were collected after centrifugation (6000 × *g*, 4°C for 30 min). The cell extract was treated with deoxyribonuclease I and ribonuclease A to hydrolyze nucleic acids. An aliquot of extract was stored at –80°C for further analysis. This sample was designated ''S1''.

The cell extract was then fractionated by stepwise $(NH_4)_2SO_4$ precipitation (20–75% saturation). The dissolved precipitate containing the majority of enzyme activity was desalted against distilled water using a 10 kDa cut-off Centriprep concentrator (Amicon Inc., Beverly, MA). An aliquot of this sample, designated ''S2'', was stored at –80°C for SDS–PAGE analysis.

This fraction was then subjected to Ion Exchange Chromatography (IEC) using an Econo-Pac Q ion exchange cartridge (Bio-Rad, Hercules, CA), bed volume 5 ml, equilibrated with 25 mM Tris, pH 8.0. Elution of protein from the column was achieved by the application of a linear gradient to a final concentration of 1 M NaCl (in 25 mM Tris, pH 8.0) at a flow rate of 1 ml/min. Fractions of 1 ml were then collected and tested for NADH oxidase activity. Fractions exhibiting the highest activity were pooled, desalted and concentrated. An aliquot of this sample, designated ''S3'', was stored at –80°C for further analysis.

Further separation was carried out by Hydrophobic Interaction Chromatography (HIC) with an Econo-Pac Methyl HIC cartridge, bed volume 5 ml (Bio-Rad) equilibrated with 1.5 M ammonium sulfate in 0.1 M sodium phosphate buffer, pH 6.8. The column was eluted with a linear concentration gradient of ammonium sulfate decreasing from 1.5 M to 0 M in the same buffer for 60 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected and tested for NADH oxidase activity. Fractions exhibiting the highest activity were pooled, desalted and concentrated. This final sample was designated ''S4''.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was used to monitor the purification of NADH oxidase and to estimate the enzyme's molecular mass. Electrophoresis was conducted in 12% polyacrylamide gels using the standard Tris-glycine buffer system of Laemmli (16). Gels were stained with Coomassie brilliant blue or with the SilverXpress™ Kit (Novex, San Diego, CA), according to the manufacturer's instructions.

Oxidase activity analysis of sample S4 (partially purified NADH oxidase)

Sample S4 was analyzed for NADH oxidase activity in the presence and absence of 0.2 mM FAD and for NADPH oxidase activity and production of H_2O_2 during the NADH oxidase assay. Hydrogen peroxide was assayed by a modification of the method described by Gibson et al. (6). Briefly, NADH oxidase reactions (total volume 800 µl) containing 28 nmol NADH, 0.1 M sodium phosphate buffer, pH 7.0, and 93 µg of sample S4 were allowed to proceed until NADH was completely oxidized ($OD_{340} \geq 0.03$). Then, 200 µl of a solution consisting of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) at 3 mg/ml and horseradish peroxidase at 0.2 mg/ml, in the same buffer, were added to the reaction mixture. The reaction was allowed to proceed for 20 min at room temperature and then the absorbance (A_{560nm}) was measured. Samples were compared to a standard curve generated by known concentrations of H_2O_2. As a control, a known concentration of H_2O_2 was added to the reaction mixture after completion of the NADH oxidase reaction and before adding the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and the horseradish peroxidase.

Identification of the protein with NADH oxidase activity in sample S4

SDS–PAGE separation of the final fraction (S4) showed that it contained more than one band. In order to identify which one was responsible for the NADH oxidase activity, sample S4 was loaded onto a non-denaturing PAGE gel (12% acrylamide) and assayed for NADH oxidase activity. Analysis of this gel under UV light revealed one active band. The R_f of this band was measured and the gel was then blotted onto a nitrocellulose membrane. The membrane was stained with Coomassie blue and the NADH oxidase-active band identified according to the R_f value. The protein was eluted from the membrane piece by placement overnight in a 50% acetonitrile solution. The supernatant, containing the eluted protein from the gel piece, was then concentrated in a Jouan RC10.10 rotary evaporator, redissolved and denatured in 2× SDS sample buffer, and re-run in an SDS–PAGE gel (12% T). The original S4 sample was also loaded onto another track of this gel. The band containing the NADH oxidase activity was then compared with the bands in the S4 fraction to identify the putative NADH oxidase-active band.

Mass spectrometry (MS) identification of purified protein

Proteins were identified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) coupled to an in-line microcapillary liquid chromatography system (µLC), running at ~200 nl flowrate. Briefly, proteins were digested with trypsin and peptides were then extracted, concentrated and submitted to µLC/ESI-MS/MS by loading 2–4 µl from 5 µl of peptide samples (<10 pmol) onto an 8-cm, 75-µm inner diameter, (µLC) column consisting of 300 Å, 5 µm C18 packing material (Michrom BioResources, Inc., Auburn, CA). The reverse phase (µLC) columns were prepared in-house using a pressure vessel (Biomedical Proteomics Research Group, http://www.expasy.org/bprg). Peptides were eluted over a 30-min gradient (acetonitrile (CH_3CN) 80%, acetic acid (CH_3COOH) 2% and HFBA 0.004%) using an HP1100 (Agilent Technologies Australia, Forest Hills, Victoria, Australia) with T-splitter (Upchurch Scientific, Oak Harbor, WA) to give a flow rate of approximately 300 nl/min. MS/MS was performed on a TSQ7000 (Thermo Finnigan, San Jose, CA).

The amino-acid sequence of the different peptides was derived from the fragmentation pattern after correlation with the *P. gingivalis* genome database (http://www.tigr.org) translated into protein. This method allows one to identify the ORF corresponding to the protein from which each peptide was generated.

Database searches and sequence alignments

P. gingivalis W83 nucleotide database translated into protein was searched using Blastp (7) at The Institute for Genomic Research Comprehensive Microbial Resource (tigr cmr) (http://tigrblast.tigr.org). *P. gingivalis* annotated genome sequence

was also accessed at http://www.oralgen.lanl.gov. Other sequences were obtained from the NCBI Entrez database (http://www.ncbi.nlm.nih.gov). Sequences were aligned using the program T-coffee (http://www.ch.embnet.orf/software/tcoffee.html).

Expression of Nox and AhpF-C under anaerobic and oxygenated environments

The RNA expression level of a putative NADH oxidase (*nox*) and of the alkyl-hydroperoxide reductase (*ahpF* and *ahpC*) genes, from cells grown under anaerobic and oxygenated environments, was analyzed by Northern blot hybridization. *P. gingivalis* RNA was isolated from cells grown at steady-state under the anaerobic atmosphere N_2/CO_2 (95 : 5) and under the oxygenated environment $N_2/CO_2/O_2$ (85 : 5 : 10). Total RNA was isolated by a modification of the method described by Sambrook et al. (30). RNA was separated by electrophoresis in MOPS buffer and transferred to a Hybond N^+ nylon membrane by capillary elution with 20× SSC buffer. Internal fragments of *Nox* and *AhpF* were used as specific probes. Densitometry analysis of the autoradiograph was normalized to the relative intensity of total 23S and 16S rRNA detected on the ethidium bromide-stained agarose gel to correct for any loading differences.

Results

Analysis of NADH oxidase activity in cell extracts

Extracts of *P. gingivalis* were able to catalyze the aerobic reduction of NADH without the requirement of added flavin. However, as seen in Fig. 1, addition of 0.2 mM FAD enhanced the NADH oxidase activity of cell extracts, indicating that FAD serves as a cofactor for NADH oxidase activity. NADPH could not serve as a source of reducing equivalents for oxidase activity.

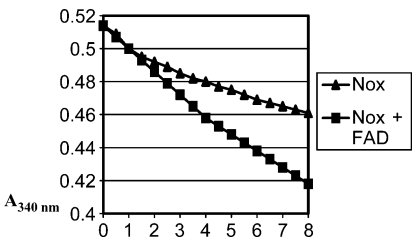

Fig. 1. NADH oxidase (Nox) activity (Units·mg/protein) of *P. gingivalis* cell extracts in the absences and presence of 0.2 mM FAD.

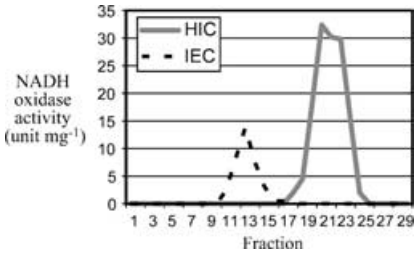

Fig. 2. Purification of NADH oxidase activity from ammonium sulfate fractionated cell extracts by ion exchange chromatography (IEC) and hydrophobic interaction chromatography (HIC).

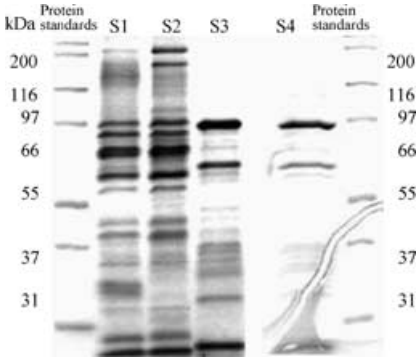

Fig. 3. SDS polyacrylamide gel of fractions from each purification step.

Isolation of NADH oxidase activity

Ammonium sulfate fractionation of sample S1 (extract of broken cells) resulted in the majority of the activity concentrated in the 60% ammonium sulfate precipitate. Figure 2 shows the results of a typical separation of sample S2 in the IEC and HIC systems. Figure 3 shows an SDS–PAGE gel of the samples from each purification step. The two proteins in fraction S4 can also be seen in sample S3. There is an approximately 10-fold increase in activity in Fraction S4 compared to S1 and S2, and a 2-fold increase compared to S3. Table 1 shows the NADH oxidase activity in the different fractions.

Analysis of NADH oxidase activity in sample S4

FAD (0.2 mM) increased the NADH oxidase activity in sample S4 by 13%, thus appearing to act as a cofactor for the protein responsible for the NADH oxidase activity in the purified sample. It was also observed that, in the presence of NADPH, the purified protein had no oxidase activity. These results agreed with our previous observations with the cell extracts.

Table 3 shows that the purified protein produces H_2O_2 during NADH oxidation. On average, 1 mol of H_2O_2 was detected per 2.8 mol of NADH added to the reaction mixture.

Identification of the protein with NADH oxidase activity in sample S4

As shown in Fig. 3, the SDS–PAGE analysis of the purified fraction (S4) revealed two bands, 54 kDa and 45 kDa, constituting most of the protein in the sample. Therefore, to identify the band in the SDS polyacrylamide gel responsible for the NADH oxidase activity, sample S4 was run in a native gel and assayed for NADH oxidase. Analysis of this gel under UV light showed one band displaying activity (gel not shown). Figure 4 shows the SDS gel where the band was re-run after being excised from the native gel and compared to the S4 fraction. The NADH oxidase activity corresponded to the two major bands in the sample. These two bands were then identified by mass spectrometry sequencing, both as PG0625 (accession number according to http://www.oralgen.lanl.gov); an ORF corresponding to the enzyme 4-hydroxybutyryl-CoA dehydratase (AbfD).

Identification of other candidate genes responsible for NADH oxidase activity in the *P. gingivalis* genome sequence

The results presented previously indicated that the main NADH oxidase activity

Table 1. NADH oxidase activity in the different purification fractions

Fraction*	Amount protein (mg)	Total activity (U)	Specific (units·mg/protein)	Yield (%)	Purification (fold)
S1 (crude cell lysate)	948.2	14981.6	15.8	100.0	1.0
S2 (60% $(NH_4)_2SO_4$ precipitate)	243.0	4544.1	18.7	30.3	1.2
S3 (IEC fraction)	22.3	1944.6	87.2	13.0	5.5
S4 (HIC fraction)	4.8	716.2	149.2	4.8	9.4

*For details, see text.

Table 2. Mass spectrometry identification data

Sample	MW observed[a] (kDa)	MW expected[b] (kDa)	Peptides sequenced	Protein ID[c]
Band A_1	54	54	(R)HPEIGPIVK (R)IENPVDHPMIR (R)LAEMDEYK	PG 0625
Band A_2	54	54	(R)IENPVDHPMIR (R)LAEMDEYK	PG 0625
Band B_1	45	54	(R)YLAGATGK (R)HPEIGPIVK	PG 0625
Band B_2	45	54	(R)YLAGATGK (R)HPEIGPIVK	PG 0625

[a]Observed molecular weight calculated relative to protein standards.
[b]Expected molecular weight as calculated from the *P. gingivalis* W83 nucleotide sequence (http://www.oralgen.lanl.gov).
[c]Accession numbers correspond to the numbers from the Oral Pathogen Sequence Database (http://www.oralgen.lanl.gov).

Table 3. H_2O_2 produced by the NADH oxidase reaction with the purified protein (S4). NADH oxidase assay mixture contained 93 μg of protein from sample S4 and 28 nmol NADH

Reaction mixture (1 ml)[a]	H_2O_2 detected (nmol)
NADH oxidase assay	11.81 ± 1.2
NADH oxidase assay + 5 nmol H_2O_2	14.01 ± 0.8
NADH oxidase assay + 20 nmol H_2O_2	28.20 ± 1.6

[a]H_2O_2 was detected after NADH consumption was completed. H_2O_2 5 nmol and 20 nmol was added to completed NADH oxidase reaction mixtures as a control. Data represent the mean of samples assayed in triplicate in two independent experiments.

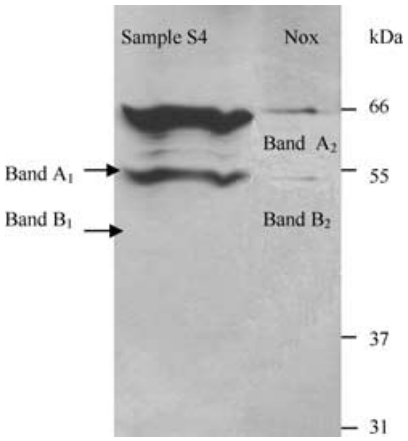

Fig. 4. Silver-stained SDS-PAGE gel of sample S4 and band presenting NADH oxidase activity eluted from a native gel (Nox).

present in *P. gingivalis* cell extracts might be the result of the NADH oxidase-like activity displayed by 4-hydroxybutyryl-CoA dehydratase (AbfD). However, as the purification of the enzyme was carried out under conditions where *P. gingivalis* cysteine proteinases had not been inactivated, we considered the possibility that other proteins, missed during the purification, contained NADH oxidase activity. Therefore, the *P. gingivalis* genome database was searched for other proteins likely to possess NADH oxidase activity.

The Blast search indicated that the most likely ORF matching a *nox* in *P. gingivalis* was PG0160 (accession number according to http://www.oralgen.lanl.gov). The alignment of this ORF, translated into protein (alignment not shown), with other characterized NADH oxidases from different microorganisms showed that PG0160 was composed of 938 amino acids, while the other NADH oxidases consisted of about 450 amino acids. Homology of PG0160 with NADH oxidase proteins only occurred, therefore, in the central region of the ORF (e.g. residues 127–668 are 48% similar to the enzyme from *Vibrio cholerae*, gene bank accession number AAF96545). Nevertheless, important identities and similarities between this central region of PG0160 and other NADH oxidases were found. For example, the cysteine in position 42 is conserved in all the sequences. This cysteine residue is an important feature of NADH oxidase proteins; it is known to exist as a stabilized cysteine-sulfenic acid (cys-SOH) serving as a non-flavin redox center (28). Other homologies existed in regions containing previously-identified sequence fingerprints for FAD-containing, pyridine nucleotide dependent, oxidoreductases.

When the *P. gingivalis* genome sequence was searched against AhpF, an ORF with high homology was identified (54% similar to alkyl hydroperoxide reductase subunit F of *Bacteroides fragilis*, gene bank accession number AAD52148). The alignment of this ORF, accession number PG0557 (according to http://www.oralgen.lanl.gov) and other AhpFs showed conservation in all the sequences of the two cysteine residues which have been shown to be involved in the catalytic activity of the enzyme against hydrogen peroxide or cumene hydroperoxide (25). Conserved regions for FAD and NADH binding were also found. Moreover, an ORF encoding for the second component of this system, the small subunit AhpC, exists immediately upstream of the *AhpF* gene, a genetic arrangement seen in other microorganisms (28).

Expression of Nox and AhpF-C under anaerobic and oxygenated environments

Northern analysis of *P. gingivalis* total RNA from the two conditions, anaerobic and oxygenated growth, with a DIG-labeled *ahpF*-specific probe is shown in Fig. 5. A transcript of approximately 2.2 kb appears in cells grown anaerobically as well as those grown in oxygen. The size of this transcript indicates that *ahpF* is transcribed in a polycystronic way with its upstream neighboring gene *ahpC*. *AhpF* is 1545 bp, *ahpC* is 564 bp and there is an intergenic region between the two of 167 bp, for a total transcript size of 2276 bp. The transcription of this fragment appears to increase slightly under oxygenated conditions, as judged by the intensity of the bands. However, the significance of the increase in this band is questionable due the small difference detected. On the other hand, although different probe concentrations and different hybridization temperatures were utilized for the detection of mRNA corresponding to the putative Nox, no signal was detected on the membranes under any of the conditions tested.

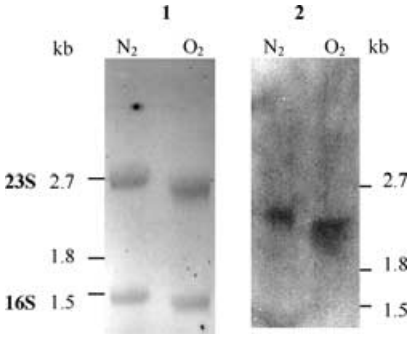

Fig. 5. Northern blot analysis of AhpF mRNA in *P. gingivalis* steady state cells grown in anaerobic (N_2) and oxygenated (O_2) environments.

Discussion

After purification, it was found that the NADH oxidase activity displayed by *P. gingivalis* cell extracts was associated with 4-hydroxybutyryl-CoA dehydratase, an enzyme having a role in the organism's glutamate metabolism. Indeed, Takahashi et al. (38) showed that *P. gingivalis* cell extracts possessed most of the activities involved in this pathway. AbfD, in particular, is involved in the dehydration of hydroxybutyryl-CoA to crotonyl-CoA, and although this step does not appear in their proposed pathway, other studies (3, 5, 31) of *Clostridium aminobutyricum* have shown that this is an essential step in the conversion of 4-hydroxybutyrate to crotonyl-CoA. Moreover, Gerhardt et al. (5) found that the *AbfD* gene of *C. aminobutyricum* forms part of a genetic region containing other genes involved in the same fermentation pathway. The arrangement of these genes is surprisingly similar in *P. gingivalis*. Although the dehydratase activity was not assayed in this study, all these observations, together with the high degree of homology of *P. gingivalis* AbfD with other similar proteins [e.g 75% similar to the AbfD from *C. aminobutyricum* (gene bank number CAB60035)], provide enough evidence to support the identity of PG 0625 as an AbfD.

The mechanism by which 4-hydroxybutyryl-CoA dehydratase could display NADH oxidase activity is unclear but a possible explanation is as follows. The dehydratase is known to bind FAD and so if it also reacts with NADH, it could reduce the FAD to $FADH_2$. This, in turn, would react with oxygen to produce H_2O_2. This last reaction could occur spontaneously and would not necessarily be enzyme-mediated. However, this activity would be contrary to the main FAD-dependent activity of the dehydratase – that is, on 4-hydroxybutyryl-CoA. The activity of *C. aminobutyricum* AbfD towards its main substrate, 4-hydroxybutyryl-CoA, is inhibited by oxygen (23). However, a second activity catalyzed by the enzyme, the isomerization of vinylacetyl-CoA to crotonyl-CoA is only partially inactivated by oxygen. Hence, the oxidase activity could also be resistant to oxygen and still be present in oxygen-stressed cells. This partial inactivation of AbfD activity by oxygen could also explain why *P. gingivalis* cell extracts present increased NADH oxidase-like activity when the cells are exposed to an aerobic environment. In such a situation, FAD, required for the main AbfD activity, would be ''free'' to react with NADH, producing $FADH_2$, which would subsequently react with oxygen to generate H_2O_2.

Therefore, it seems likely that the NADH oxidase-like activity displayed by the dehydratase would be a source of toxicity for *P. gingivalis*, *via* the generation of ROS, rather than an aid in the protection from oxygen. Indeed, the present study shows that H_2O_2 is generated after the consumption of NADH by the enzyme. Enzymes with similar activities, members of electron-transport chains that utilize FAD for univalent electron transport reactions, have been reported in the literature to be a main source of toxicity for microorgansims when exposed to oxygen. Examples are NADH dehydrogenase II, succinate dehydrogenase, fumarate reductase and sulfite reductase, which generate O_2^- and H_2O_2 inside cells because of the reaction of oxygen with the solvent-exposed flavin (12, 21). A Blast search of the *P. gingivalis* genome sequence for other enzymes with the potential to generate high levels of ROS revealed that this organism possesses one of the enzymes mentioned; namely, a putative fumarate reductase. This enzyme is involved in the reduction of fumarate to succinate under anaerobic conditions (14) and has been shown to be a primary source of ROS in *Escherichia coli* after exposure to air (12). This putative fumarate reductase could thus be serving as another source of ROS when *P. gingivalis* is exposed to air.

The presence of an NADH oxidase, similar to those of *E. faecalis* and *Streptococcus mutans* (10, 19), was also investigated in *P. gingivalis* genome sequence. Although an ORF with homology to a Nox protein was identified, no signal could be detected in the Northern blot analysis, even when different hybridization and washing conditions were tried. Failure to detect a signal in the Northern blot, although not conclusive, might suggest either that no RNA molecule for this protein is transcribed or that the transcribed RNA molecule is not stable. In this respect, it is interesting to note that the ORF encoding for the putative Nox in *P. gingivalis* does not correspond to the size of other NADH oxidases. The region from the 2814-nucleotides-ORF (PG0160) with homology to a Nox protein starts at nucleotide 375 and extends to nucleotide 1767, corresponding to the normal length of an NADH oxidase. An analysis (not shown) of the region upstream of nucleotide 375, but still inside PG0160, showed what appeared to be a promoter sequence with similarity to a consensus for different *P. gingivalis* genes reported by Jackson et al. (15), with the putative transcription start point located at nt –55. This type of arrangement, with translation start-sites located some distance from the transcription start point, is a common feature in *P. gingivalis* and other members of the *Bacteroidaceae* (15). However, no stop codons or a transcription termination site are found downstream from the nucleotides encoding for the last residue (Ala) in the putative NADH oxidase. Therefore, it seems that although the arrangement of nucleotides might favor transcription, the RNA molecule would be at least 1000 nucleotides longer than the size of the RNA required for translation into the NADH oxidase.

On the other hand, *P. gingivalis* appears to possess an alkyl hydroperoxide reductase system, formed by AhpF and AhpC, which could contribute to H_2O_2 detoxification. The sequence alignment of AhpF of *P. gingivalis* and that of *Amphibacillus xylanus* (gene bank accession number Q06369) shows a high degree of homology (alignment not shown) between these two proteins. Therefore, it is likely that the well-characterized AhpF from this microorganism (25) and the AhpF from *P. gingivalis* function in a similar manner. Further characterization of this enzymic activity in *P. gingivalis* would, however, be necessary.

In this respect, it is worth noting that preliminary results with mutants of *P. gingivalis* with interrupted transcription of *ahpF-C* possess NADH oxidase activity decreased by approximately 25% compared with the wild-type. Therefore, it seems that it is the dehydratase (AbfD) that utilizes most of the NADH in the NADH oxidase reaction with *P. gingivalis* cell extracts, generating H_2O_2. AhpF-C, however, seems to utilize some NADH as well, probably metabolizing some of the H_2O_2 and compensating the toxic effect of the dehydratase.

In summary, these experiments suggest that the majority of the NADH oxidase activity measured in *P. gingivalis* cell extracts might be due to an NADH oxidase-like activity displayed by 4-hydroxybutyryl-CoA dehydratase. The biological significance of this activity, with respect to a protection under oxygen stress, is not clear as it seems that the activity of the dehydratase for its natural substrate, 4-hydroxybutyryl-CoA, would compete with its NADH oxidase activity since both require the involvement of FAD. Rather, the dehydratase could be contributing to

the generation of H_2O_2 in *P. gingivalis* cells exposed to oxygen. An analysis of the expression of other proteins likely to function as NADH oxidases/peroxidases in *P. gingivalis* revealed that transcription occurs for a protein similar to alkyl-hydroperoxide reductase, which could have a role as an NADH oxidase-peroxidase system. AhpF-C could contribute to the moderate oxygen tolerance displayed by *P. gingivalis,* metabolizing H_2O_2. On the other hand, this study failed to identify a conventional and functional NADH oxidase.

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