Identification of two distinct NADH oxidases corresponding to H₂O₂-forming oxidase and H₂O-forming oxidase induced in *Streptococcus mutans*

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Two distinct NADH oxidases, corresponding to H₂O₂-forming and H₂O-forming enzymes were purified to homogeneity from *Streptococcus mutans* and their basic properties determined. The H₂O₂-forming enzyme was a tetramer with a subunit molecular mass of about 56 kDa and required flavin adenine dinucleotide (FAD) for full activity. The enzyme had an isoelectric point of 6.6 and exhibited optimal activity at pH 6.0. The H₂O-forming enzyme was a monomer with a molecular mass of 50 kDa and activity independent of exogenously added flavin. The enzyme had an isoelectric point of 4.8 and exhibited optimal activity between pH 7.0 and 7.5. Both enzymes oxidized NADH (*K_m* 0.05 and 0.025 mM for the H₂O₂- and H₂O-forming enzyme, respectively) but not NADPH and contained 1 mol of FAD per monomer. Spectra of the oxidized enzymes exhibited maxima at 271, 383 and 449 nm for the H₂O₂-forming enzyme and 271, 375 and 447 nm for the H₂O-forming enzyme. Antibodies raised against the H₂O₂-forming enzyme or the H₂O-forming enzyme reacted with their corresponding antigen, but did not cross-react. The amino-terminal regions of the two enzymes had completely different amino acid sequences.

Introduction

The *Streptococcus mutans* group, a causative agent of dental caries, lacks some major enzymes of O₂ metabolism and catalase (Hamada & Slade, 1980). Important characteristics distinguishing the group from other oral streptococci are the ability to synthesize an adhesive and highly branched glucan from sucrose and to utilize mannitol as a primary carbon source (Hardie, 1986).

Even though the *S. mutans* group is considered to be facultatively anaerobic, we previously demonstrated that O₂ affected both mannitol catabolism and growth on mannitol of various members of the group, including *S. mutans*, *S. cricetus*, *S. rattus* and *S. sobrinus* (Higuchi, 1984). The growth response to O₂ correlated with the ability of strains to induce NADH oxidase and superoxide dismutase (SOD) under aerobic conditions (Higuchi, 1984). These findings suggested that NADH oxidase played an important role in the regulation of

aerobic metabolism, through the regeneration of NAD from the additional NADH derived from the oxidation of mannitol 1-phosphate to fructose 6-phosphate.

However, the study of Higuchi (1992) suggested another possible function of NADH oxidase in defence against O₂ toxicity. The sensitivity to O₂ of strains grown on glucose as well as on mannitol varied and was inversely correlated with the level of induced NADH oxidase activity in cell-free extracts (Higuchi, 1992). Interestingly, the NADH oxidase induced by O₂ in an O₂-tolerant strain of *S. mutans* was shown to contain two types of enzyme activity, one forming water and the other H₂O₂ (Higuchi, 1992).

O₂ toxicity is caused by partially reduced intermediates of O₂, active oxygen species, such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (·OH) (McCord *et al.*, 1971; Repine *et al.*, 1981). Superoxide and H₂O₂ inhibited the growth of *S. sanguis* (Dugisepine & Fridovich, 1982) and caused DNA damage (Bedway & Karnovsky, 1980). Therefore, the induced synthesis of H₂O₂-forming NADH oxidase in the O₂-tolerant *S. mutans* seems to be a dilemma for a bacterium grown under air.

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Abbreviations: PCMB, *p*-hydroxymercuribenzoate; PPB, potassium phosphate buffer; SOD, superoxide dismutase.

The coexistence of two types of NADH oxidase activity in *O*₂-tolerant *S. mutans*, one antagonistic to *O*₂ toxicity and the other producing H₂O₂, prompted us to clarify their molecular properties and mechanisms of regulation. We present here the first step in these studies, i.e. the identification of induced H₂O₂- and H₂O-forming NADH oxidases in *S. mutans* NCIB11723 (JC2), and evidence that the purified enzymes differ from each other in their enzymic characteristics, N-terminal amino acid sequences and antigenic structure.

Methods

Bacterial strain and growth conditions. An *O*₂-tolerant *S. mutans* strain NCIB11723 (Carlsson, 1968; Higuchi, 1992) was grown in an anaerobic glove box (Hirasawa Works, Tokyo) under an atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon dioxide at 37 °C overnight in 300 ml of modified D87 medium (Carlsson, 1970), composed of glucose (10 g), NH₄HCO₃ (2 g), sodium L-glutamate (1 g), dried yeast extract (Difco; 2 g), L-cysteine/HCl (0.1 g), MgSO₄·7H₂O (0.2 g), NaCl (0.01 g), MnSO₄·4H₂O (0.01 g) and FeSO₄·7H₂O (0.01 g) in 1 litre 100 mM-potassium phosphate buffer (PPB; pH 7.0). The culture was transferred under air to a 5 litre fermenter (Sankiseiki) containing 3 litres D87 medium, and was kept at 37 °C. After culture optical density reached approximately 0.300 (OD₆₆₀), enzyme induction was started by sparging air into the culture at a flow rate of 3 l min⁻¹, with a stirrer speed of 300 r.p.m. Cells were harvested in the late-exponential phase (OD₆₆₀ 1.5) by centrifugation at 21 000 g, for 10 min at 4 °C. After washing the cell deposits 3 times with 50 mM-PPB (pH 7.0) containing 0.2 mM-EDTA, the cell deposits were stored at -80 °C.

Enzyme purification

All isolation steps were performed at 4 °C with buffers containing 0.2 mM-EDTA and 20% (v/v) glycerol, unless otherwise specified. Each fraction was analysed for protein (*A*₂₈₀) and NADH oxidase activity.

(i) **Preparation of a cell extract.** The cell deposits (130 g) were suspended in 50 mM-PPB containing 1 mM-phenylmethylsulphonyl fluoride (PMSF), and were twice passed through a French pressure cell at 1200 kg cm⁻². The cell debris was centrifuged at 150 000 g for 5 h to obtain a crude cell extract. PPB (50 mM) containing 50% (v/v) glycerol was added to the extract to give a glycerol concentration of 20% (v/v), before storage at -80 °C.

(ii) **Concentration of the cell extract.** The crude extract from (i) was concentrated to about one-tenth of its volume by ultrafiltration (Minitan System M-30, Millipore).

(iii) **Ammonium sulphate fractionation.** The extract from (ii) was taken to 55% saturation with a saturated solution of (NH₄)₂SO₄. After equilibration, the mixture was centrifuged (25 000 g, 1 h) and the resulting precipitate resolved in 60 ml 50 mM-PPB containing 1 M-(NH₄)₂SO₄ (AMS preparation 1). The supernatant freed from the precipitate was taken to 80% saturation with a saturated solution of (NH₄)₂SO₄. After equilibration, the mixture was centrifuged again, redissolved in a minimum volume of 50 mM-PPB and desalted by passing through a column of Sephadex G-25 (AMS preparation 2).

(iv) **Purification of AMS preparation 1. Step 1. First hydrophobic affinity chromatography separation.** The AMS preparation 1 from (iii) was applied directly to a column of Butyl-Sepharose (1.8 × 35 cm) equilibrated previously with 50 mM-PPB containing 1 M-(NH₄)₂SO₄. The column was washed with one bed volume of the same buffer and

then eluted with a linear gradient of 1–0 M-(NH₄)₂SO₄ in 50 mM-PPB. Fractions (9.3 ml) containing activity were pooled (88.7 ml) and concentrated by precipitation with (NH₄)₂SO₄. The resulting precipitate was redissolved in 50 mM-PPB containing 1 M-(NH₄)₂SO₄.

Step 2. Second hydrophobic affinity chromatography separation. The pooled fractions from step 1 were applied to a Phenyl-Superose (HR10/10) FPLC column equilibrated previously with 1 M-(NH₄)₂SO₄ in 50 mM-PPB. The column was washed with 3 bed volumes of 1 M-(NH₄)₂SO₄ in 50 mM-PPB followed by a solution with a linear gradient of 1–0 M-(NH₄)₂SO₄ in 50 mM-PPB. Fractions (2 ml) containing activity were pooled, concentrated and desalted, and the buffer exchanged with 20 mM-Tris/HCl buffer (pH 7.3) with an Amicon centriprep 30 (Diaflow Corp.).

Step 3. Ion-exchange chromatography separation. The concentrated enzyme solution from step 2 was applied to a MonoQ (HR5/5) FPLC column equilibrated with 20 mM-Tris/HCl buffer (pH 7.3). The column was washed with 20 mM-Tris/HCl (pH 7.3) followed by solution with a linear gradient of 0.25–0.35 M-KCl in 20 mM-Tris/HCl (pH 7.3). Fractions (0.5 ml) containing activity were pooled.

(v) **Purification of AMS preparation 2. Step 1. First ion-exchange chromatography separation.** The AMS preparation 2 from (iii) was applied to a column (5 × 10 cm) of DEAE-Sepharose CL-6B equilibrated with 50 mM-PPB (pH 7.3). The column was washed with approximately 3 bed volumes of the starting buffer and with 1 bed volume of 50 mM-PPB (pH 6.5) and then eluted with a linear gradient of 0–0.5 M-KCl in 50 mM-PPB (pH 6.5). Fractions (9.3 ml) containing activity were pooled and concentrated, and the buffer exchanged with 20 mM-Tris/HCl (pH 7.3).

Step 2. Second ion-exchange chromatography separation. The concentrated enzyme solution from step 1 was applied to a MonoQ (HR10/10) FPLC column equilibrated with 20 mM-Tris/HCl buffer (pH 7.3). The column was washed with 20 mM-Tris/HCl (pH 7.3) followed by solution with a linear gradient of 0.15–0.25 M-KCl in 20 mM-Tris/HCl (pH 7.3). Fractions (2.0 ml) containing activity were pooled and the buffer was exchanged with 50 mM-PPB containing 1 M-(NH₄)₂SO₄.

Step 3. Hydrophobic affinity chromatography separation. The pooled fractions from step 2 were applied to a Phenyl-Superose (HR10/10) FPLC column equilibrated with 1 M-(NH₄)₂SO₄ in 50 mM-PPB. The column was washed with 3 bed volumes of 1 M-(NH₄)₂SO₄ in 50 mM-PPB followed by a solution with a linear gradient of 1–0 M-(NH₄)₂SO₄ in 50 mM-PPB. Fractions (2 ml) containing activity were pooled.

NAD(P)H oxidase and protein assay. NAD(P)H oxidase activity was assayed at 30 °C by monitoring the oxidation of NAD(P)H in the reaction mixture (1 ml) at *A*₃₄₀. The reaction mixture contained 40 mM-PPB, 0.2 mM-EDTA, 0.17 mM-NADH with or without 0.02 mM-FAD and extracts (0.01–50 µg protein ml⁻¹) containing enzyme. The reaction was initiated by adding enzyme. One unit of NADH oxidase was defined as the amount of enzyme (mg protein) which catalysed the oxidation of 1.0 µmol NADH min⁻¹. Protein concentration was measured by the dye-binding method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Assay for O₂⁻ produced on oxidation of NADH. O₂⁻ was assayed by monitoring the reduction of ferricytochrome *c* (Green & Hill, 1984) at 550 nm and 30 °C. Both sample and reference cuvettes contained 50 mM-PPB (without glycerol, pH 7.5), and 75 µM-horse heart cytochrome *c*. The reference cuvette additionally contained 75 µl SOD (1 mg ml⁻¹). The reaction was initiated by the addition of a 100 µl sample derived from the NADH-dependent O₂ reduction mediated by NADH oxidase.

Assay for NADH peroxidase (EC 1.11.1.1). The activity was measured anaerobically at 30 °C by monitoring the oxidation of NADH using anaerobic cells in a medium identical to that used for assaying NADH oxidase, except for adding 0.3 mM-H₂O₂ and preparing

the reaction media in an anaerobic glove box. The difference in absorption between the reaction with and without H_2O_2 was monitored.

Estimation of O_2 uptake and H_2O_2 production. O_2 and H_2O_2 were estimated polarographically at 30 °C with an oxygen monitor (Yellow Springs Instrument Company), as described previously (Higuchi, 1984).

Electrophoresis and isoelectrofocusing (IEF). SDS-PAGE was performed in 7.5% (w/v) polyacrylamide, using a Tris/glycine system (Laemmli, 1970). Native PAGE was performed with a 4–15% polyacrylamide gel (Davis, 1964). Electrophoresis was done at 20 mA for 2–3 h with 25 mM-Tris/192 mM-glycine buffer (pH 8.3). The gels were stained with Coomassie brilliant blue (Weber & Osborn, 1969).

IEF of the pure enzyme preparations was done using a Bio-Rad model 111 mini IEF cell according to the manufacturer's recommendations. Gels were stained with Coomassie brilliant blue. Bio-Rad IEF standards were used for determination of the pI of the enzymes.

Molecular mass determinations. The mass of the subunit of the enzymes was determined using SDS-PAGE and by comparison with the mobilities of standard proteins (low range; Bio-Rad). The native molecular mass was determined by gel permeation chromatography on a Superose 12 FPLC column (Pharmacia) using marker proteins (Pharmacia LKB high and low molecular mass standards) for calibration.

Flavin identification. The identification and determination of enzyme-bound flavin was basically as previously described (Schmidt *et al.*, 1986). The enzyme-bound flavin was liberated by denaturation of the purified enzyme with trichloroacetic acid (final concn 3%, v/v) followed by centrifugation for 10 min at 10000 *g*. An absorption spectrum of the supernatant solution was taken and the amount of flavin determined using the known extinction coefficient for FAD (ϵ_{450} 11.3 $\text{mm}^{-1} \text{cm}^{-1}$; Whitby, 1953). After extraction of the trichloroacetic acid by diethyl ether, the supernatant was analysed by thin-layer chromatography (TLC) on TLC-silica gel plates (10 × 10 cm; Merck) with FAD and FMN as references, using a solvent system of *n*-butanol/acetic acid/water (12:3:5, by vol.).

Immunological techniques. Antisera were raised in Japanese white rabbits against the purified NADH oxidases by intramuscular injection of 300–400 µg protein emulsified with Freund's complete adjuvant. A booster injection with 300–400 µg protein, together with Freund's incomplete adjuvant, was given 4 weeks later and the rabbits were bled 1 week later. The determination of antibody titre against NADH oxidases was determined by double immunodiffusion tests (Ouchterlony, 1949).

Western analysis. Cell free extracts (about 10 µg protein) from an anaerobically grown culture (before exposure to O_2) and from an aerobically grown culture (after exposure to O_2) were applied to SDS-PAGE. Following separation on SDS-polyacrylamide gels, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in a Bio-Rad transblot cell apparatus (at 1 mA cm^{-2} , for 1 h, using a Tris/glycine buffer system). The membranes were treated with a TN buffer (25 mM-Tris/HCl, 0.5 M-NaCl, pH 7.5) containing 5% (w/v) skim milk. They were then immersed in a 1:2000 dilution of the NADH oxidases antisera in TN buffer containing 5% skim milk and agitated for 1 h at room temperature. Horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories), specifically bound to the PVDF membrane on subsequent incubation was visualized by oxidation of 3,3'-diaminobenzidine tetrahydrochloride with hydrogen peroxide.

N-terminal amino acid sequence determination. The amino acid sequences of the N-terminal region of purified enzymes were analysed with an Applied Biosystems model 477A/1120 gas-phase sequencer

operated by Dr Toda, Research Centre for Protein Engineering, Institute for Protein Research, Osaka University.

Chemicals. Ferricytochrome *c*, FAD, catalase, SOD and phenazine methosulphate (PMS) were purchased from Sigma. β -NADH, β -NADPH, nitroblue tetrazolium (NBT), PMSF and EDTA were purchased from Wako.

Results

Separation of active fractions corresponding to H_2O_2 - and H_2O -forming enzymes

The critical concentration of saturated ammonium sulphate for separating the major fraction of H_2O_2 -forming NADH oxidase activity from the major H_2O -forming NADH oxidase activity was determined as 55%, since AMS precipitation 1 contained 96% of H_2O_2 -NADH oxidase activity while 87% of H_2O -NADH oxidase activity was detected in AMS precipitation 2. The total activity of AMS precipitation 1 increased 2.4-fold with FAD. In contrast, the activity of AMS precipitation 2 was independent of FAD (increase in activity < 5%). This, H_2O_2 -forming oxidase required FAD for full activity, but H_2O -forming oxidase did not. Accordingly, the routine assay for H_2O_2 -forming oxidase and for H_2O -forming oxidase was carried out with and without FAD, respectively.

Purification of H_2O_2 -forming NADH oxidase and H_2O -forming NADH oxidase

The purification of both types of enzymes to homogeneity was achieved by additional affinity and ion-exchange chromatography (Fig. 1); in each case, only a single protein band was revealed by SDS-PAGE (Fig. 2). The H_2O_2 -forming NADH oxidase from AMS precipitate 1 was obtained as a yellow enzyme solution with a 46-fold increase in specific activity and 9% recovery (Table 1), and purification of the H_2O -forming NADH oxidase from AMS precipitate 2 resulted in a yellow enzyme solution with a 176-fold increase in specific activity and 19% recovery (Table 2).

Molecular mass and subunit structure

The molecular mass of native enzymes estimated by gel filtration chromatography with standard proteins was 220 kDa for H_2O_2 -forming oxidase and 50 kDa for H_2O -forming oxidase. The subunit molecular mass was estimated to be 56 kDa for H_2O_2 -forming oxidase and 50 kDa for H_2O -forming oxidase on the basis of mobility in SDS-polyacrylamide gels (Fig. 2). These data suggest that H_2O_2 -forming NADH oxidase from *S. mutans* consists of four identical subunits, while H_2O -forming NADH oxidase is a monomer.

Fig. 1. Purification of the H_2O_2 -forming and H_2O -forming NADH oxidases by the three chromatography steps. (a), (b) and (c) show the chromatographies of the H_2O_2 -forming enzyme on Butyl-Sepharose, Phenyl-Superose and MonoQ column, respectively. (d), (e) and (f) show the chromatographies of the H_2O -forming enzyme on DEAE-Sepharose CL6B, MonoQ and Phenyl-Superose. Absorbance at 280 nm (.....); KCl and $(\text{NH}_4)_2\text{SO}_4$ concn (—); activity of the NADH oxidases (—).

Isoelectric point

The isoelectric points of H_2O_2 - and H_2O -forming enzymes were about 6.6 and 4.8 respectively, on the basis of their mobilities in the Bio-Rad mini IEF cell compared with reference proteins.

Light absorption spectra and FAD content

Spectra of the two NADH oxidases showed absorption maxima at 271, 383 and 449 nm for the H_2O_2 -forming enzyme and at 269, 384 and 446 nm for the H_2O -forming

enzyme (Fig. 3); these maxima are typical of flavins. After reducing the enzymes with dithionite, the maxima at 449 and 446 nm decreased. The A_{280}/A_{450} ratios of the native enzymes were 4.9 and 4.7 for the H_2O_2 - and H_2O -forming oxidases, respectively. After liberation of the flavin moiety by denaturing the enzymes using trichloroacetic acid, the flavin component was identified as FAD by thin-layer chromatography. The FAD content per subunit was calculated to be 0.93 mol for the H_2O_2 -forming enzyme and 0.85 mol for the H_2O -forming enzyme. These data suggest that the native H_2O_2 -forming NADH oxidase contains 4 FAD per molecule, while the

Fig. 2. SDS-PAGE of the purified NADH oxidases. M, molecular mass standards; A, H_2O_2 -forming enzyme; B, H_2O -forming enzyme.

Fig. 3. Light absorption spectra of the purified NADH oxidases. a, H_2O_2 -forming enzyme (1.25 mg ml^{-1}); b, H_2O -forming enzyme (0.95 mg ml^{-1}).

H_2O -forming NADH oxidase has one FAD per molecule.

Effects of pH and temperature on enzyme stability

Fig. 4(a) shows enzyme activity remaining after treatment at the indicated pH at 37°C for 1 h in 50 mM-PPB (pH 5.0–8.0), 50 mM-acetate buffer (at pH 4.0) and 50 mM-Tris/HCl buffer (pH 8.0–10.0). Both enzymes

retained full activity at pH 7.0, but activity declined following incubation at either acidic or alkaline pH.

Both enzymes retained full activity when stored at -80°C for 6 months, but activity decreased by 80% within one week at 4°C in 50 mM-PPB (pH 7.0). After incubation for 1 h, in an identical buffer, both enzymes retained full activities at up to about 40°C , at 55°C activity markedly decreased, particularly for the H_2O -

Table 1. Purification of H_2O_2 -forming NADH oxidase of *S. mutans*

Purification step	Total protein (mg)	Total activity (U)*	Specific activity (U mg^{-1})*	Purification (fold)	Yield (%)
Crude extract	1036	832	0.81	1.0	100
55% $(\text{NH}_4)_2\text{SO}_4$ precipitate	145	223	1.54	1.9	27
Butyl-Sepharose	109	188	1.72	2.2	23
Phenyl-Superose	15	111	7.40	9.1	13
MonoQ	2	74	37.00	45.7	9

* FAD-dependent NADH oxidase activity was assayed.

Table 2. Purification of H_2O -forming NADH oxidase of *S. mutans*

Purification step	Total protein (mg)	Total activity (U)*	Specific activity (U mg^{-1})*	Purification (fold)	Yield (%)
Crude extract	1036	586	0.57	1.0	100
55–80% $(\text{NH}_4)_2\text{SO}_4$ precipitate	476	609	1.28	2.2	103
DEAE-Sepharose	54	423	7.83	13.7	72
MonoQ	6.6	204	30.95	54.2	35
Phenyl-Superose	1.2	115	100.28	175.9	19

* FAD-independent NADH oxidase activity was assayed.

Fig. 4. Effects of pH and temperature on the stability of the purified NADH oxidases. (a), pH (incubation at 30 °C); (b) temperature (incubation at pH 7.0); ●, H₂O₂-forming enzyme; ○, H₂O-forming enzyme. Enzyme activities were determined after 1 h at 30 °C, pH 7.0. See text for other experimental details.

forming enzyme (Fig. 4b). At 60 °C, 95% of the H₂O-forming enzyme activity was lost within 10 min, whereas the H₂O₂-forming NADH oxidase retained 5% activity for 30 min.

Optimum pH and temperature

The apparent optimum pH for H₂O₂-forming enzyme activity was at about pH 6.0. In contrast, the optimum pH for H₂O-forming enzyme activity was pH 7.0–7.5 (Fig. 5a). The maximum observed activity of both enzymes occurred at about 45 °C in 50 mM-PPB/0.2 mM-EDTA (Fig. 5b).

Substrate specificity and stoichiometry of the reaction

The purified enzymes were highly specific for NADH. The K_m value for NADH was 0.05 mM for the H₂O₂-forming enzyme and 0.025 mM for the H₂O-forming enzyme. No reaction was observed with NADPH. The oxidation of NADH gave stoichiometrically 1 mol NADH consumed (mol O₂)⁻¹ with the H₂O-forming enzyme, and 2 mol NADH consumed (mol O₂)⁻¹ with the H₂O₂-forming enzyme. The reaction with the H₂O₂-forming oxidase was also shown to give 1 mol H₂O₂ produced (mol O₂)⁻¹, by addition of catalase after complete oxidation of NADH. The reaction with the H₂O-forming oxidase was not changed by addition of catalase. O₂⁻ was not produced by either enzyme and NADH-dependent H₂O₂ oxidation under anaerobic conditions was not observed. The activity of the H₂O₂-

Fig. 5. Effects of pH and temperature on the apparent activity of the purified NADH oxidases. (a), pH; (b), temperature; ●, H₂O₂-forming enzyme; ○, H₂O-forming enzyme. In (a), activity was assayed at 30 °C in 40 mM-PPB (pH 5.0–8.0) or 40 mM-Tris/HCl (pH 7.5–10). In (b), activity was assayed in 40 mM-PPB (pH 7.0). Reaction mixtures excluding enzyme were equilibrated at the appropriate temperature for 3 min. The reaction was then initiated by adding enzyme and activity was determined from the rate of decrease in A_{340} during the first min after addition. Rates were constant during this period.

forming enzyme depended on FAD but not on FMN, and increased linearly as the FAD concentration was increased from zero to 30 μ M. At this concentration, FAD stimulated enzyme activity approximately five-fold. In contrast, FAD or FMN at concentrations up to 30 μ M did not affect the activity of the H₂O-forming enzyme.

Enzyme inhibition

The inhibitory effects of divalent cations (added as chloride salts except CuSO₄ and FeSO₄) and thiol reagents (reagents reacting with thiol groups) were investigated under standard assay conditions (see Methods). For both enzymes activity was completely inhibited by 0.1 mM-Hg²⁺, Cu²⁺ and Sn²⁺, slightly inhibited (up to 20%) by Mn²⁺ and Ca²⁺, but stimulated (130%) by Mg²⁺. Ba²⁺, Ni²⁺, Fe²⁺ and Zn²⁺ (0.1 mM) inhibited the activity of the H₂O₂-forming enzyme by 86, 73, 56 and 53% respectively, whereas no significant inhibition of the H₂O-forming enzyme was observed. Co²⁺ and Cr²⁺ gave moderate inhibition (31 and 49%, respectively) of the activity of the H₂O₂-forming enzyme, but severe inhibition of the H₂O-forming enzyme activity (89 and 92%, respectively). Cysteine and ascorbate (1 mM) inhibited the activity of the H₂O₂-forming enzyme by 54 and 81%, but of the H₂O-forming enzyme by only 1 and 45%, respectively. Dithiothreitol (DTT; 1 mM) slightly stimulated activity in both enzymes (to 103%).

Fig. 6. Effect of PCMB on the activity of the purified NADH oxidases. Assays were carried out in 40 mM-PPB (pH 7.0), 0.17 mM-NADH, 0.02 mM-EDTA and 0, 5, 10, 20, 50 or 100 μM-PCMB. Reactions were initiated by adding enzyme. ○, H₂O₂-forming enzyme (2.0 units ml⁻¹); ●, H₂O-forming enzyme (1.9 units ml⁻¹).

The severe inhibition by Hg²⁺ and stimulation by DTT, suggested that thiol (-SH) groups are involved in the catalytic active site of both H₂O₂-forming and H₂O-forming enzymes. However, a SH-blocking reagent, *p*-hydroxymercuribenzoate (PCMB), inhibited the activity of the two enzymes at different concentrations (Fig. 6). The H₂O-forming enzyme was completely inhibited by 50 μM-PCMB, but over 60% of the H₂O₂-forming oxidase activity was retained at 100 μM-PCMB. These data suggest that different redox-active disulphides are involved in the active sites of the enzymes.

Enzyme activation

The activating effect of DTT and cysteine was determined by incubating the enzymes (50 μg) for 5 min at 37 °C with 100 mM-DTT or cysteine, with or without FAD (150 μM), in 100 mM-PPB (pH 7.0). The subsequent activity of the H₂O₂-forming enzyme was increased only slightly (to 106%) by DTT with FAD, whereas the H₂O-forming enzyme was inhibited by both DTT (62% with FAD and 48% without FAD, respectively) and cysteine (42% with FAD and 38% without FAD, respectively).

Immunological reactions

The purified enzymes were tested for cross-reactivity in a double immunodiffusion test. With antisera obtained using H₂O₂-forming NADH oxidase, a precipitation reaction could be detected with H₂O₂-forming but not with H₂O-forming oxidase. Similarly, antisera obtained using H₂O-forming NADH oxidase only reacted with H₂O-forming NADH oxidase. Also, in simple Western type experiments (Fig. 7), the 56 kDa subunit of the

Fig. 7. Western blotting analysis of cell extracts using anti-NADH oxidase antisera. M, Molecular mass standard proteins. Lanes 1, 3 and 5: extracts of anaerobically-grown JC2. Lanes 2, 4 and 6: extracts of aerobically grown JC2. Lanes 1 and 2: proteins stained with Coomassie blue. Lanes 3 and 4: enzyme reacted with antiserum derived from H₂O₂-forming NADH oxidase. Lanes 5 and 6: enzyme reacted with antiserum derived from H₂O-forming NADH oxidase.

H₂O₂-forming NADH oxidase in aerobically grown cell extracts was detected only with the antiserum obtained using the H₂O₂-forming oxidase. Similarly, H₂O-forming oxidase (50 kDa) was revealed only with the antiserum obtained using the H₂O-forming oxidase. Neither oxidase was detected in extracts from anaerobically grown cells.

These data indicate that H₂O₂-forming and H₂O-forming NADH oxidases are present only in aerobically grown cells and differ in their primary structures.

Amino acid sequences

The amino acid sequences of the N-terminal region were A-L-D-A-E-I-K-E-Q-L-G-Q-Y-L-Q-L-L- for the H₂O₂-forming enzyme, and S-K-I-V-I-V-G-A-N-I-A-G-T-A- for the H₂O-forming enzyme. No significant overlaps in sequences between the two enzymes were found.

Discussion

The present study was initiated following the discovery that in crude extracts of aerobically-grown cells of an O₂-tolerant strain of *S. mutans*, NADH-dependent O₂ reduction yielded both H₂O and H₂O₂, and H₂O₂ production was accelerated by adding FAD (Higuchi, 1992). We initially supposed two possibilities to account for both H₂O and H₂O₂ formation by NADH oxidase. One was the involvement of a single enzyme controlled by FAD, a versatile coenzyme (Massey & Hemmerich, 1980) for O₂ reduction by NADH. Another possibility was the involvement of two structurally similar enzymes with different functions. Unexpectedly, in the present study, the two NADH oxidases corresponding to H₂O-forming and H₂O₂-forming oxidases, were purified as two distinct enzymes, as revealed by both N-terminal

Table 3. Comparison of NADH oxidase enzymes from bacteria

Sources	Ref.*	O ₂ reduced	Mol. mass (kDa)	Subunits	pI	Optimum pH	K _m (µM)	Substrate specificity	Electron acceptor	Activated with FAD	N-terminal amino acid sequences
<i>Streptococcus mutans</i> JC2	1	H ₂ O ₂	220	4 (56)	6.6	6.0	50	NADH	O ₂ , DCIP	Yes	A-L-D-A-E-I-K-E-Q-L-G-Q-Y-L-Q-L-L-
	1	H ₂ O	50	1 (50)	4.8	7.2	25	NADH	O ₂ , DCIP	No	S-K-I-V-I-V-G-A-N-I-A-G-T-A-
<i>Streptococcus faecalis</i> 10C1	2	H ₂ O				6.8		NADH	O ₂ , DCIP	Yes	
	3	H ₂ O	51	1 (51)			41	NADH	O ₂	Yes	
	4	H ₂ O	103	2 (50)				NADH	O ₂	Yes	V-V-V-V-G-C-T-H-A-G-T-S-A-V-K-
<i>Leuconostoc mesenteroides</i> KSM-1101	5	H ₂ O	104	2 (53)	4.0	6.5	120	NADH	O ₂ , DCIP, MB	No	
<i>Bacillus megaterium</i>	6	H ₂ O ₂	92	2 (52)		6.8	61	NAD(P)H	O ₂ , DCIP, MB	Yes	
<i>Thermus aquaticus</i> YT-1	7	H ₂ O ₂	110	2 (50)				NAD(P)H			
<i>Thermus thermophilus</i> HB8	7	H ₂ O ₂	25	1 (25)		5.0	4	NAD(P)H	O ₂ , DCIP, MB, cyt.c,	Yes	M-E-A-T-L-P-V-L-D-A-K-T-A-A-L-K-

* References: 1, Higuchi *et al.* (this paper); 2, Hoskins *et al.* (1962); 3, Schmidt *et al.* (1986); 4, Ahmed & Claiborne (1989); 5, Koike *et al.* (1985); 6, Saeki *et al.* (1985); 7, Park *et al.* (1992).

amino acid sequence analysis and the absence of immunological cross-reactions.

Flavin-containing NADH oxidases have been purified and characterized from several bacteria (Ahmed & Claiborn, 1989; Hoskins *et al.*, 1962; Koike *et al.*, 1985; Saeki *et al.*, 1985; Schmidt *et al.*, 1986; Park *et al.*, 1992) and mycoplasmas (Klomkes *et al.*, 1985; Reinard *et al.*, 1981). With the exception of the NADH oxidase from *Acholeplasma laidlawii*, which is a metallo-flavoprotein containing FMN and localized in the plasma membrane, all enzymes purified so far are flavoproteins containing FAD and are localized in the cytoplasm. The FAD-containing NADH oxidases isolated from bacteria may be divided into two groups by their specificity for NADH or NADPH (Table 3). One group yielding H₂O₂ from NAD(P)H were derived from aerobic bacteria possessing cytochromes and other haem-containing proteins generally associated with oxidative metabolism; these bacteria included *Bacillus megaterium*, *Thermus aquaticus*, and *T. thermophilus*. In contrast, the second group produced H₂O from NADH oxidation and were derived from lactic acid bacteria, including *Streptococcus faecalis* and *Leuconostoc mesenteroides* but excluding *S. mutans*.

The molecular mass of the purified enzymes varied according to their source, but their subunits were of about 50 kDa with the exception of *T. thermophilus* (25 kDa). The H₂O-forming NADH oxidase derived from aerobically-grown *S. faecalis* has been purified by Hoskins *et al.* (1962), Schmidt *et al.* (1986) and Ahmed & Claiborne (1989). However, even though the same

strain (10C1) was used, the determined molecular mass differed in each report. Interestingly, the H₂O₂-forming NADH oxidase derived from *S. mutans* was distinguished from both H₂O- and other H₂O₂-forming enzymes in possessing four identical monomer molecules. However, the requirement for FAD for full activity, was common to all previously characterized enzymes except that from *L. mesenteroides*.

The H₂O-forming NADH oxidase from *S. faecalis* consists of a monomer with molecular mass of 50 kDa (Schmidt *et al.*, 1986) or a dimer with molecular mass of 103 kDa (Ahmed & Claiborne, 1989). However, the enzyme is not similar to the H₂O-forming enzyme from *S. mutans* (about 50 kDa), since the latter enzyme showed no significant activation by pre-incubation with FAD and cysteine, as described for the *S. faecalis* enzyme by Hoskins *et al.* (1962) and Schmidt *et al.* (1986). Moreover, the amino acid sequence of the N-terminal region of the H₂O-forming NADH oxidase from *S. mutans* was distinct from that of the *S. faecalis* enzyme (Table 3). Similarly, the sequence of the N-terminal region of the H₂O₂-forming enzyme from *S. mutans* differed completely from that of *T. thermophilus*.

Only one type of NADH oxidase (H₂O-forming) from *S. faecalis* has been purified and characterized, although the presence of H₂O₂-forming NADH oxidase in anaerobically-grown cells of the same 10C1 strain was demonstrated (Dolin, 1955). Whether the aerobically grown *S. faecalis* 10C1 cells contain the H₂O-forming NADH oxidase but not the H₂O₂-forming enzyme, is

uncertain. In any case, the present results show that the aerobically-induced H_2O_2 - and H_2O -forming NADH oxidases in *S. mutans* differed from the *S. faecalis* enzyme.

It is noteworthy that the N-terminal amino acid sequences of the bacterial NADH oxidases purified so far are completely different from each other. In order to clarify the structural relationship between these enzymes, we are now investigating their genetic structure.

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