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Characterization of an Exceedingly Active NADH Oxidase from the Anaerobic Hyperthermophilic Bacterium *Thermotoga maritima*▽

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An NADH oxidase from the anaerobic hyperthermophilic bacterium *Thermotoga maritima* was purified. The enzyme was very active in catalyzing the reduction of oxygen to hydrogen peroxide with an optimal pH value of 7 at 80°C. The V_{max} was 230 ± 14 μmol/min/mg (k_{cat}/K_m = 548,000 min^{-1} mM^{-1}), and the K_m values for NADH and oxygen were 42 ± 3 and 43 ± 4 μM, respectively. The NADH oxidase was a heterodimeric flavoprotein with two subunits with molecular masses of 54 kDa and 46 kDa. Its gene sequences were identified, and the enzyme might represent a new type of NADH oxidase in anaerobes. An NADH-dependent peroxidase with a specific activity of 0.1 U/mg was also present in the cell extract of *T. maritima*.

Thermotoga maritima is a hyperthermophilic anaerobic bacterium capable of growing at 90°C. It utilizes carbohydrates and cell extracts such as yeast extract as energy and carbon sources, and it produces H_2, CO_2, acetate, and lactate (15). Although oxygen is toxic and sparse in the natural habitat for anaerobes, it has been reported that some strictly anaerobic microbes, including a few *Thermotogales* species, could grow in the presence of a micromolar level of oxygen (45, 46). However, the nature of the cellular system facilitating this oxygen tolerance has not been determined. In addition to enzymes such as superoxide dismutase (12), superoxide reductase (16), and peroxidase and catalase (10, 12, 49), NADH oxidase is considered to be an important enzyme involved in oxygen scavenging systems because of its potential to reduce the oxygen that is transiently encountered by anaerobes (14, 19, 20).

NADH oxidases are flavoproteins, which react with oxygen to produce either water in a four-electron transfer process or hydrogen peroxide (H_2O_2) in a two-electron transfer process (39). An H_2O_2-forming NADH oxidase from *Thermotoga hypogea* has been purified and characterized, and its catalytic properties are similar to other anaerobic hyperthermophilic NADH oxidases (51). The *T. hypogea* enzyme is a typical NADH oxidase that has the structure of a homodimer of 50 kDa and contains one flavin adenine dinucleotide (FAD) per subunit. However, NADH oxidase activity in *T. maritima* cell extract was about six times higher than that in *T. hypogea*, indicating that a much more active NADH oxidase in *T. maritima* may have a greater ability to remove accidentally encountered oxygen. The present paper reports that a highly active NADH oxidase from *T. maritima* was purified and characterized. We propose that the purified enzyme is part of an oxygen-scavenging system in the *T. maritima* cell.

Growth and NADH oxidase activities in the presence of oxygen. *T. maritima* was cultured in a medium modified from that of Huber (15) at 80°C, but without the addition of reducing agent and sulfur or sodium thiosulfate unless specified. As previously reported (46), this organism could grow in up to 1% (vol/vol) oxygen, corresponding to a dissolved oxygen concentration of 5.2 μM in the medium, which was estimated based on the partial pressure of oxygen as described previously (20). No growth occurred when the oxygen concentration was 1.5% (vol/vol) in the gas phase. NADH oxidase activity was determined in a glass cuvette by monitoring O_2-dependent oxidation of NADH spectrophotometrically at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}) at 80°C (48). One unit of NADH oxidase activity was defined as 1 μmol NADH oxidized per minute. NADH oxidase activities from cells that were grown anaerobically at their late log phase and then exposed to oxygen at concentrations of 0, 2.5, and 5% (vol/vol) for 30 min at 80°C were determined to be 1.0, 1.4, and 1.5 U/mg, respectively. When chloramphenicol (100 mg per liter) (15, 17) was added, there was no increase in the NADH oxidase activity if exposed to oxygen (5%, vol/vol), and there was no change in the NADH oxidase activity if there was no exposure to oxygen. Therefore, the slight increase of the NADH oxidase activities likely resulted from the enzyme production in response to oxygen in the growth media. There was a fairly constant NADH oxidase activity of 1.0 ± 0.1 U/mg from *T. maritima* cells grown in the absence of oxygen, even with the addition of cysteine HCl (0.4 g/liter) and sodium thiosulfate (3.2 g/liter) to the growth medium. This activity is still the highest compared to 0.13 U/mg in *T. hypogea* (51), 0.55 U/mg in *Thermotoga neapolitana* (our unpublished data), 0.29 U/mg in *Thermococcus guaymasensis* (our unpublished data), 0.042 U/mg in *Clostridium aminovalericum* (19), and 0.073 U/mg in *Amphibacillus xylanus* (31).

Purification of *T. maritima* NADH oxidase. To obtain sufficient *T. maritima* cell mass for the purification of NADH oxidase, large-scale culture (15 liters) was grown routinely at 80°C. Cell extract was prepared anaerobically from 50 g of *T. maritima* cells using procedures similar to those described previously (51), and the protein concentration was determined using the Bradford method with bovine serum albumin as the standard protein (4). The cell extract was applied to a DEAE-Sepharose Fast Flow column (5 by 10 cm; Amersham Biotech, Quebec, Canada) that was preequilibrated using buffer A (50 mM Tris-HCl, pH 7.8, 5% [vol/vol] glycerol, 1 mM sodium

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TABLE 1. Purification of NADH oxidase from *T. maritima*

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Cell-free extract	1,689	1,858	1.1	1	100
DEAE-Sepharose	401	1,502	3.8	3.5	80
Hydroxyapatite	138	1,003	7.2	6.5	54
Phenyl-Sepharose	13.8	678	49	45	37
Superdex 200	1.9	212	112	102	11
Q-Sepharose	0.75	108	144	131	6

dithionite, and 1 mM dithiothreitol) at a flow rate of 4 ml/min. The column was eluted with a linear gradient of 0 to 0.3 M NaCl in buffer A. The NADH oxidase started to elute out as 0.10 M NaCl was applied to the DEAE-Sepharose column. The fractions with high NADH oxidase specific activity (>4-fold of purification) were pooled and applied to a hydroxyapatite (Bio-Rad) column (2.6 by 10 cm) equilibrated with buffer A. The column was eluted with a linear gradient of 0 to 0.15 M KH_2PO_4 in buffer A at a flow rate of 2 ml/min. The NADH oxidase started to elute out as 0.065 M KH_2PO_4 was applied to the column. Fractions containing NADH oxidase activity were pooled and applied to a phenyl-Sepharose HP column (2.6 by 8 cm) equilibrated with 0.8 M $(NH_4)_2SO_4$ in buffer A. The column was eluted with a linear gradient of 0.8 to 0 M $(NH_4)_2SO_4$ at a flow rate of 2 ml/min. The NADH oxidase was eluted out as 0.52 M $(NH_4)_2SO_4$ was applied to the column. Fractions containing high NADH oxidase activity were pooled and concentrated by ultrafiltration (Amicon ultrafiltration membrane PM 30). The concentrated fraction (3.0 ml) was applied to a Superdex 200 column (2.6 by 60 cm; Amersham Biotech, Quebec, Canada) equilibrated with buffer A containing 100 mM KCl at a flow rate of 2 ml/min. Fractions containing high NADH oxidase activity were combined and applied to a Q-Sepharose HP column (1.6 by 10 cm) equilibrated with buffer A. The column was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A at a flow rate of 1.0 ml/min. NADH oxidase was eluted out as 0.25 M NaCl applied to the column. Fractions containing pure NADH oxidase as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22) were stored at −20°C until use.

The purification results are presented in Table 1. NADH oxidase was eluted out as a predominant single peak from each column, and the enzyme was purified approximately 130-fold, indicating that this enzyme is present in the cell in a quantity slightly less than 1% (Table 1).

Biophysical properties of the NADH oxidase. The purified NADH oxidase had two types of subunits with molecular masses of 54 and 46 kDa, respectively (Fig. 1). The native molecular masses of the purified enzymes were estimated to be 90 ± 10 kDa, calculated by using a calibrated Superdex 200 column (2.6 by 60 cm) equilibrated with buffer A containing 100 mM KCl at a flow rate of 2 ml/min. These results suggested that the purified NADH oxidase was a heterodimer, which is different from typical NADH oxidases previously characterized (48) but similar to a small number of such enzymes (36). However, it is the first of this type of NADH oxidase found in an anaerobic hyperthermophile.

The solution containing the purified NADH oxidase was yellowish, which was an indication of the presence of flavin. The oxidized enzyme solution (0.25 mg in 1 ml of 50 mM Tris-HCl buffer, pH 7.8) in a quartz cuvette was scanned to obtain an absorption spectrum from 190 nm to 600 nm (Varian Bio 50 UV-visible spectrophotometer). Absorbance peaks at 274, 366, and 445 nm were observed as characteristics of oxidized flavoprotein (data not shown). The flavin cofactor was extracted as a yellowish compound that was released after the enzyme was mixed with methanol and boiled for 10 min in the dark (51). Using thin-layer chromatography, the released flavin was further identified to be FAD, since it comigrated with commercially available FAD, which did not comigrate with flavin mononucleotide (FMN) and riboflavin (51). The NADH oxidase contained 1.9 ± 0.1 mol of FAD per mol native enzyme based on the absorbance value at 450 nm (ε_{450} = 11.3 mM^{-1} cm^{-1}) (50) and the protein amount from which the FAD was extracted.

T. maritima NADH oxidase was purified under strictly anaerobic conditions. However, during the purification, it was found that enzyme samples exposed to air exhibited a decrease in enzyme activity. Therefore, oxygen sensitivity of the NADH oxidase was further determined for both cell extract and the purified enzyme. The results showed that the inactivation rate of NADH oxidase activity was dependent on oxygen concentration. The times required for the loss of 50% of the enzyme activity from the purified enzyme were about 20 min and 40 min for oxygen concentrations of 20% (vol/vol) and 1% (vol/vol), respectively. However, the times required for the loss of 50% of the enzyme activity from the cell extract were about 60 min and 360 min for oxygen concentration of 20% (vol/vol) and 1% (vol/vol), respectively. Apparently, the enzyme in the cell extract was more resistant to oxygen inactivation than the purified enzyme. There might be unknown factors present in the cell extract, which protected the NADH oxidase from inacti-

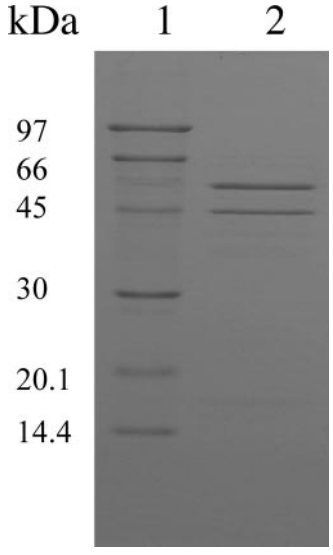

FIG. 1. SDS-PAGE of the purified NADH oxidase from *T. maritima*. The purified NADH oxidase (lane 2, 1.7 μg) and low molecular weight standards (lane 1) are indicated along with their corresponding molecular masses. The gel was stained using Coomassie blue R-250 (EMD Chemicals Inc., NJ).

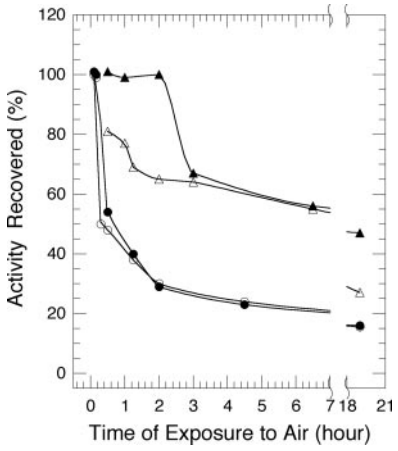

FIG. 2. Recoverability of inactivated NADH oxidase from *T. maritima*. Open circles, recovered activity from purified NADH oxidase exposed to air with an incubation time of 20 min in the presence of sodium dithionite and dithiothreitol; filled circles, recovered activity from purified NADH oxidase exposed to air with an incubation time of about 20 h in the presence of sodium dithionite and dithiothreitol; open triangles, recovered activity from cell extract exposed to air with an incubation time of 20 min in the presence of sodium dithionite and dithiothreitol; filled triangles, recovered activity from cell extract exposed to air with an incubation time of about 20 h in the presence of sodium dithionite and dithiothreitol. One hundred percent reactivation represents a full recovery of the lost NADH oxidase activity.

vation by exposure to oxygen. These results lead to a conclusion that the NADH oxidase in *T. maritima* was highly active and oxygen sensitive.

It is known that some inactivated oxygen-sensitive enzymes can be recovered by incubation with reducing reagents (33). Therefore, recoverability of the inactivated *T. maritima* NADH oxidase was tested in the presence of sodium dithionite and dithiothreitol under anaerobic conditions. Incubation with either sodium dithionite (2 mM) or dithiothreitol (2 mM) could achieve only partial recovery of the inactivated enzyme (~54%). A full reactivation (100%) of the enzyme activity was achieved only in the presence of both sodium dithionite (2 mM) and dithiothreitol (2 mM). Moreover, the recoverability for NADH oxidase activity from both purified enzyme and cell extract was also dependent on both the time periods of exposure to oxygen and incubation with sodium dithionite and dithiothreitol (Fig. 2). There was a quick recovery of approximately 65 to 80% of the activity in the cell extract within 10 min of incubation with sodium dithionite and dithiothreitol, while a 100% recovery of the activity required a longer incubation time (>10 h) when the cell extracts were exposed to air from 0.5 to 2 h. For the purified enzyme, it appeared that there was only a quick recovery process (approximately 50 to 100% of the activity within a half hour), and no significant further reactivation was observed after a longer period of incubation with sodium dithionite and dithiothreitol (>10 h) when the purified enzyme was exposed to air within one hour. Nevertheless, full activity recovery for the cell extract and the purified NADH oxidase was achieved only if the amount of time exposed to air was less than 2 h and 0.3 h, respectively (Fig. 2). It is concluded that the purified enzyme is much more subject to oxygen damage than the cell extract, and it is not clear which mechanism is involved in both the inactivation and reactivation processes.

Gene identification and sequence analysis. Protein samples from each of the two subunits from the SDS-PAGE were excised and treated and digested with trypsin, and the resulting peptides were extracted and cleaned using procedures similar to those described previously (40). The cleaned peptide samples were used for mass spectrometry analyses, which were carried out by the Mass Spectroscopy Facility at the University of Waterloo on a Waters Micromass Q-TOF Ultima spectrometer using nanospray injection as the sample delivery method. Tandem mass spectrometry profiling using PEAK software (BSI, Waterloo, ON) revealed that the large subunit (54 kDa) and the small subunit (46 kDa) were the products of *T. maritima* genes TM1432 and TM1433, respectively (30). Both genes were annotated as conserved hypothetical proteins with calculated molecular masses of 53,628 and 44,861 Da, respectively. A conserved flavin-binding site ($GXGX_2GX_3A$) was present at the N terminus of each protein encoded by the genes, which is consistent with the experimental value of ~2 FAD moieties per native enzyme. In addition, a bacterioferritin-associated ferredoxin-like binding region ($CXCX_{32}CX_4C$) was found to be present near the C terminus of the large subunit (TM1432), which presumably binds a [2Fe-2S] cluster that is not present in any other NADH oxidases known. Acid-labile sulfur content and iron content were measured using methylene blue formation (3) and an inductively coupled plasma-mass spectrometer (VG Elemental PlasmQuad 3 ICP-MS at the Chemical Analysis Laboratory, University of Georgia), respectively. The purified enzyme contained 2.0 ± 0.1 g atoms of acid-labile sulfur and 2.2 ± 0.2 g atoms of iron per mol, which confirmed the prediction of a [2Fe-2S] cluster based on the sequence analysis. This type of [2Fe-2S] cluster can have a low redox potential of −254 mV (35), which may make it subject to damage by exposure to oxygen. In fact, the acid-labile sulfur and iron contents of the enzyme that was exposed to air and had a loss of more than 60% of its activity were determined to be only 1.6 ± 0.1 and 1.4 ± 0.2 g atoms per mol, respectively, indicating oxygen indeed caused the damage of the [2Fe-2S] cluster present in the purified NADH oxidase from *T. maritima*. A Blast search was performed (1), and the results showed that the *T. maritima* NADH oxidase had 40 to 47% protein sequence identity and 59 to 67% protein sequence similarity to genes (locus tag numbers) present in anaerobes, which are TK1393 and TK1392 in *Thermococcus kodakaraensis* (13), PF2005 and PF2006 in *Pyrococcus furiosus* (37), PAB0183 and PAB0184 in *Pyrococcus abyssi* (9), TTE2001 and TTE2000 in *Thermoanaerobacter tengcongensis* (2), Teth39DRAFT_0751 and Teth39DRAFT_0752 in *Thermoanaerobacter ethanolicus* (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=AAKQ00000000), CsacDRAFT_2355 and CsacDRAFT_2356 in *Caldicellulosiruptor saccharolyticus* (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=AALW00000000), CA_C1322 and CA_C1323 in *Clostridium acetobutylicum* (32), CbeiDRAFT_3784 and CbeiDRAFT_3783 in *Clostridium beijerinckii* (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=AALO00000000), CPE2551 and CPE2550 in *Clostridium perfringens* (41), and CTC_02436 and CTC_02435 in *Clostridium tetani* (5). These pairs of genes were found to be organized as adjacent open reading frames that are likely within the same operon in the genome, and each of the gene products is generally annotated as either hypothetical protein,

predicted oxidoreductase, probable dehydrogenase, glycerol-3-phosphate dehydrogenase, or NADH oxidase, but none of them has been characterized. Although the big subunit showed higher sequence similarity to predicted dehydrogenases such as glycerol-3-phosphate dehydrogenase and the small subunit showed higher sequence homology to NADH oxidases, the purified *T. maritima* enzyme showed only NADH oxidase activity (see the next section), indicating that it may represent a new type of NADH oxidase in anaerobes. However, further investigation is required to understand why the big subunit is needed and what the function of the likely oxygen-sensitive [2Fe-2S] cluster is.

Catalytic properties of the NADH oxidase. The product of oxygen reduction is an important factor for evaluating the physiological function of NADH oxidases. Production of H_2O_2 by the purified *T. maritima* NADH oxidase was determined using the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) method described previously (52). It was found that more than 94% ± 3% of the NADH oxidized was used to produce a stoichiometric amount of H_2O_2. Therefore, it was concluded that the purified NADH oxidase from *T. maritima* catalyzed the reduction of O_2 to H_2O_2 exclusively using NADH as an electron donor. The optimal pH for the enzyme activity was determined to be 7.0 (data not shown). Maximal activity of the NADH oxidase from *T. maritima* was found to be at 80°C (data not shown), which is the optimal growth temperature for the organism. Tests for the thermal stability of the enzyme were carried out in 50 mM Tris/HCl buffer (pH 7.8) containing no substrate but 5% (vol/vol) glycerol at 80°C, and the estimated time for the loss of 50% activity ($t_{1/2}$) was about 100 min.

K_m values for NADH and oxygen were determined by measuring the initial rate (within 10 to 20 seconds) at different concentrations of NADH (0, 0.025, 0.05, 0.1, and 0.2 mM) and oxygen (0, 7.8, 16, 24, 38, 55, and 103 μM) in 100 mM phosphate buffer (pH 7.0) at 80°C. The buffer used for determining K_m for oxygen was prepared by adding different amounts of oxygen to stoppered glass cuvettes containing anoxic 100 mM phosphate buffer (pH 7.0) followed by vigorous shaking of the cuvettes. The dissolved oxygen concentration in 100 mM phosphate buffer was estimated to be 0.086 mM at 80°C when the partial pressure of oxygen was 0.2×10^5 Pa as reported previously (20), and this value was used for other calculations when the partial pressure of oxygen varied. NADH oxidase activity was dependent on concentrations of both NADH and O_2, and the catalysis followed Michaelis-Menten kinetics (data not shown). Both Lineweaver-Burk plots of the NADH-dependent oxidase activities at different oxygen concentrations (7.8, 16, 24, 38, 55, and 103 μM) and oxygen-dependent oxidase activities at different concentrations of NADH (0.025, 0.05, 0.1, and 0.2 mM) showed parallel lines, indicating a ping-pong catalytic mechanism (data not shown). Apparent V_{max} values corresponding to different concentrations of either NADH or oxygen obtained from the plots also followed Michaelis-Menten kinetics. These data were used for obtaining secondary Lineweaver-Burk plots, from which K_m values for NADH and O_2 and a V_{max} value were calculated to be 42 ± 3 μM, 43 ± 4 μM, and 230 ± 14 μmol min^{-1} mg^{-1}, respectively. The K_m value for O_2 (43 ± 4 μM) is the lowest among all known NADH oxidases from hyperthermophilic anaerobes (60 to 2,900 μM) (20, 48, 51). Its low K_m value for O_2 and high specificity constant (k_{cat}/K_m) of 548,000 min^{-1} mM^{-1} may suggest that the purified NADH oxidase is very efficient at removing oxygen that is transiently encountered by *T. maritima*.

TABLE 2. Relative activities of the purified *T. maritima* NADH oxidase

Substrate (mM)	Redox potential (mV)[a]	Activity (%)[b]
O_2 (0.1)	+830	100
Ferricyanide (0.5)	+360	98
Cytochrome *c* (0.05)	+250	5[c]
DCPIP (0.1)	+220	94
DTNB (0.1)	−40[d]	7
FMN (0.12)	−190	0
FAD (0.15)	−220	0
Riboflavin (0.05)	−222[e]	0
Benzyl viologen (1.0)	−350	20
Methyl viologen (1.0)	−440	10

[a] Values at 25°C from reference 25 unless specified.
[b] One hundred percent activity is 140 μmol of NADH oxidized per min. All assays but O_2 were carried out under anaerobic conditions (in the absence of O_2), and 0.7 μg purified enzyme was added to each assay mixture.
[c] Assay was performed at 50°C.
[d] Data from reference 7.
[e] Data from reference 28.

The purified NADH oxidase could not use NADPH as an electron donor for the reduction of oxygen. It catalyzed electron transfer from NADH to other electron acceptors under anaerobic conditions (Table 2). This enzyme exhibited the highest activity using O_2 as an electron acceptor (140 U/mg, 100%) compared to lower activities for benzyl viologen (20%) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 7%), while no activity was observed when FAD, FMN, or riboflavin was used as the electron acceptor. Its incapability to catalyze the reduction of flavins is similar to that of a water-forming NADH oxidase from *Lactococcus lactis* (23). Although 2,6-dichlorophenolindophenol (DCPIP) had a comparable activity with O_2 (94%), the apparent K_m value was six times higher (0.26 mM) and the apparent k_{cat}/K_m value was seven times lower (77,000 min^{-1} mM^{-1}) than those of O_2. It can be concluded that O_2 is the best substrate among all tested.

To determine if the purified NADH oxidase had functions other than NADH oxidation with oxygen as the electron acceptor, various enzyme activities were determined. The purified enzyme could not reduce H_2O_2 (NADH peroxidase; 11), α-ketoglutaric acid plus glutamine (glutamate synthase; 43), oxidized glutathione (glutathione reductase; 34), H^+ (hydrogenase; 26), sodium nitrate (nitrate reductase; 29), and sulfite (sulfite reductase; 42) when NADH was used as the electron donor under anaerobic conditions. Neither could this enzyme oxidize H_2 (hydrogenase; 24) or dihydrolipoamide (dihydrolipoamide dehydrogenase; 44) when NAD^+ was used as the electron acceptor. No activities of methyl viologen-dependent hydrogenase (24), NAD- or *T. maritima* ferredoxin-dependent glycerol-3-phosphate dehydrogenase (8, 21), sarcosine dehydrogenase (18), or sarcosine oxidase (47, 52) were detectable. Therefore, oxygen was found to be the only relevant electron acceptor identified for the purified *T. maritima* NADH oxidase.

The following chemicals were tested for their effects on the

activity of NADH oxidase: $HgCl_2$, $CuCl_2$, quinine, quinacrine, hydrocortisone, and NaCN. After the enzyme was preincubated anaerobically with each of the chemicals (3 mM) on ice for 1 h, the NADH oxidase activity assay was performed. The enzyme sample without incubation with any of the compounds served as a control (100%). The residual activities of *T. maritima* NADH oxidase were 1.7%, 40%, 54%, 77%, 95%, and 100% when incubated with $HgCl_2$, $CuCl_2$, quinacrine, quinine, hydrocortisone, and NaCN, respectively. Such sensitivity to the inhibitors quinacrine and quinine is similar to that observed for other NADH oxidases (39).

Physiological role of the NADH oxidase. In contrast to most of the mesophilic enzymes that catalyze the formation of H_2O (19), the purified NADH oxidase from *T. maritima* catalyzed the electron transfer from NADH to molecular oxygen and produced hydrogen peroxide exclusively, which is a distinctive property of NADH oxidases from hyperthermophilic microorganisms (51). No H_2O-forming NADH oxidase from this group of microbes has been reported, though a recombinant NADH oxidase from *P. furiosus* could produce both hydrogen peroxide (77%) and water (23%) (48). The production of hydrogen peroxide remains a puzzle for all NADH oxidases from hyperthermophilic microorganisms because hydrogen peroxide, a reactive oxygen species, is obviously more toxic than molecular oxygen. If the accumulated hydrogen peroxide cannot be removed fast enough, it will produce hydroxyl radicals and cause injury of cells in the presence of redox-active metal ions such as Fe^{2+} (38). The purified enzyme did not have either NADH- or NADPH-dependent peroxidase activity. However, it was found that an NADH-dependent, not NADPH-dependent, peroxidase activity was present in the cell extract of *T. maritima*. The peroxidase activity was dependent on concentrations of both NADH (0 to 0.2 mM) and H_2O_2 (0 to 0.36 mM), and the catalysis followed Michaelis-Menten kinetics (data not shown). Apparent K_m values for NADH and H_2O_2 were determined to be 6.5 μM and 0.25 mM, respectively. The apparent V_{max} was determined to be 0.1 U/mg, which is much lower than that of NADH oxidase activity (1 U/mg) in the cell extract. There was no H_2O_2 produced with a consumption of NADH up to 130 ± 10 μmol in the presence of oxygen up to 5% (vol/vol) in the gas phase and 186 μg *T. maritima* cell extract within 5 min at 80°C. However, the value of H_2O_2 produced over NADH consumed increased from 0.2% ± 0.1% to 8.3% ± 0.6% when the oxygen concentration increased from 8 to 20% (vol/vol) with a consumption of NADH from 186 ± 12 to 286 ± 18 μmol, respectively, under the same assay conditions. These results showed that *T. maritima* was in principle capable of reducing oxygen completely to H_2O with no accumulation of H_2O_2 when the concentration of exposed oxygen was low. This may provide an explanation as to why anaerobic *T. maritima* can only tolerate a low oxygen concentration.

No NADH peroxidase homolog was found to be present in the genome sequence of *T. maritima* (30). Although a possibility that a new type of NADH peroxidase may not show homology to any known ones cannot be excluded, the reduction of peroxide to H_2O may be carried out alternatively by an NADH-independent peroxidase, such as rubrerythrin (49). In fact, there are two such peroxidase homologs: TM0657 (rubrerythrin) which may function similar to peroxidase in *Desulfovibrio vulgaris* (10) and *P. furiosus* (49), and TM0807 (alkyl hydroperoxide reductase AhpC) which might be the catalytic subunit for reducing alkyl hydroperoxide or hydrogen peroxide to H_2O when other components (NADH and AhpF) are supplemented (6). Therefore, an oxygen-removing system present in *T. maritima* is proposed to work in two steps: firstly by converting O_2 to hydrogen peroxide by the NADH oxidase, and secondly by reducing the hydrogen peroxide to water by an NADH peroxidase or rubrerythrin or alkyl hydroperoxide reductase.

It was thought that NADH oxidase from *Thermoanaerobium brockii* might play other roles under physiological conditions (27). Such NADH-utilizing flavoproteins may transfer electrons to acceptors other than molecular oxygen. Therefore, it is probably reasonable to speculate that the purified NADH oxidase in *T. maritima* might play other roles in vivo.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Bao, Q., Y. Tian, W. Li, Z. Xu, Z. Xuan, S. Hu, W. Dong, J. Yang, Y. Chen, Y. Xue, Y. Xu, X. Lai, L. Huang, X. Dong, Y. Ma, L. Ling, H. Tan, R. Chen, J. Wang, J. Yu, and H. Yang.** 2002. A complete sequence of the *T. tengcongensis* genome. Genome Res. **5:**689–700.
3. **Beinert, H.** 1983. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. Anal. Biochem. **131:**373–378.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Brüggemann, H., S. Bäumer, W. F. Fricke, A. Wiezer, H. Liesegang, I. Decker, C. Herzberg, R. Martinez-Arias, R. Merkl, A. Henne, and G. Gottschalk.** 2003. The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. Proc. Natl. Acad. Sci. USA **100:**1316–1321.
6. **Bryk, R., P. Griffin, and C. Nathan.** 2000. Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature **407:**211–215.
7. **Casero, E., M. Darder, K. Takada, H. D. Abruna, F. Pariente, and E. Lorenzo.** 1999. Electrochemically triggered reaction of a surface-confined reagent: mechanistic and EQCM characterization of redox-active self-assembling monolayers derived from 5,5′-dithiobis-(2-nitrobenzoic acid) and related materials. Langmuir **15:**127–134.
8. **Chen, J., and D. K. Blanchard.** 1979. A simple hydrogenase-linked assay for ferredoxin and flavodoxin. Anal. Biochem. **93:**216–222.
9. **Cohen, G. N., V. Barbe, D. Flament, M. Galperin, R. Heilig, O. Lecompte, O. Poch, D. Prieur, J. Quérellou, R. Ripp, J. Thierry, J. V. der Oost, J. Weissenbach, Y. Zivanovic, and P. Forterre.** 2003. An integrated analysis of the genome of the hyperthermophilic archaeon *Pyrococcus abyssi*. Mol. Microbiol. **47:**1495–1512.
10. **Coulter, E. D., N. V. Shenvi, and D. M. Kurtz, Jr.** 1999. NADH peroxidase activity of rubrerythrin. Biochem. Biophys. Res. Commun. **255:**317–323.
11. **Diaz, P. I., P. S. Zilm, and A. H. Rogers.** 2002. *Fusobacterium nucleatum* supports the growth of *Porphyromonas gingivalis* in oxygenated and carbon-dioxide-depleted environments. Microbiology **148:**467–472.
12. **Dos Santos, W. G., I. Pacheco, M. Liu, M. Teixeira, A. V. Xavier, and J. LeGall.** 2000. Purification and characterization of an iron superoxide dismutase and a catalase from the sulfate-reducing bacterium *Desulfovibrio gigas*. J. Bacteriol. **182:**796–804.
13. **Fukui, T., H. Atomi, T. Kanai, R. Matsumi, S. Fujiwara, and T. Imanaka.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. Genome Res. **15:**352–363.
14. **Herles, C., A. Braune, and M. Blaut.** 2002. Purification and characterization of an NADH oxidase from *Eubacterium ramulus*. Arch. Microbiol. **178:**71–74.
15. **Huber, R., T. A. Langworthy, H. König, M. Thomm, C. R. Woese, U. B. Sleytr, and K. O. Stetter.** 1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch. Microbiol. **144:**324–333.

16. **Jenney, Jr., F. E., J. M. Verhagen, X. Cui, and M. W. W. Adams.** 1999. Anaerobic microbes: oxygen detoxification without superoxide dismutase. Science **286:**306–309.
17. **Jiang, Y., Q. Zhou, K. Wu, X.-Q. Li, and W.-L. Shao.** 2006. A highly efficient method for liquid and solid cultivation of the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*. FEMS Microbiol. Lett. **259:**254–259.
18. **Kato, K., P. Walde, H. Mitsui, and N. Higashi.** 2003. Enzymatic activity and stability of D-fructose dehydrogenase and sarcosine dehydrogenase immobilized onto giant vesicles. Biotech. Bioeng. **84:**415–423.
19. **Kawasaki, S., J. Ishikura, D. Chiba, T. Nishino, and Y. Niimura.** 2004. Purification and characterization of an H_2O-forming NADH oxidase from *Clostridium aminovalericum*: existence of an oxygen-detoxifying enzyme in an obligate anaerobic bacteria. Arch. Microbiol. **181:**324–330.
20. **Kengen, S. W. M., J. van der Oost, and W. M. de Vos.** 2003. Molecular characterization of H_2O_2-forming NADH oxidases from *Archaeoglobus fulgidus*. Eur. J. Biochem. **270:**2885–2894.
21. **Kito, M., and L. I. Pizer.** 1969. Purification and regulatory properties of the biosynthetic L-glycerol 3-phosphate dehydrogenase from *Escherichia coli*. J. Biol. Chem. **244:**3316–3323.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
23. **Lopez de Felipe, F., and J. Hugenholtz.** 2001. Purification and characterisation of the water forming NADH-oxidase from *Lactococcus lactis*. Int. Dairy J. **11:**37–44.
24. **Ma, K., and M. W. W. Adams.** 2001. Hydrogenase I and II from *Pyrococcus furiosus*. Methods Enzymol. **331:**208–216.
25. **Ma, K., and M. W. W. Adams.** 1994. Sulfide dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus*: a new multifunctional enzyme involved in the reduction of elemental sulfur. J. Bacteriol. **176:**6509–6517.
26. **Ma, K., Z. H. Zhou, and M. W. W. Adams.** 1994. Hydrogen production from pyruvate by enzymes purified from the hyperthermophilic archaeon, *Pyrococcus furiosus*: a key role for NADPH. FEMS Microbiol. Lett. **122:**245–250.
27. **Maeda, K., K. Truscott, X. Liu, and R. K. Scopes.** 1992. A thermostable NADH oxidase from anaerobic extreme thermophiles. Biochem. J. **284:**551–555.
28. **Malinauskas, A., T. Ruzgas, and L. Gorton.** 1999. Tuning the redox potential of riboflavin by zirconium phosphate in carbon paste electrodes. Bioelectrochem. Bioenerg. **49:**21–27.
29. **Moorhead, G. B. G., S. E. M. Meek, P. Douglas, D. Bridges, C. S. Smith, N. Morrice, and C. Mackintosh.** 2003. Purification of a plant nucleotide pyrophosphatase as a protein that interferes with nitrate reductase and glutamine synthetase assays. Eur. J. Biochem. **270:**1356–1362.
30. **Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, J. A. Eisen, O. White, S. L. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser.** 1999. Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. Nature **399:**323–329.
31. **Niimura, Y., K. Ohnishi, Y. Yarita, M. Hidaka, H. Masaki, T. Uchimura, H. Suzuki, M. Kozaki, and T. Uozumi.** 1993. A flavoprotein functional as NADH oxidase from *Amphibacillus xylanus* Ep01: purification and characterization of the enzyme and structural analysis of its gene. J. Bacteriol. **175:**7945–7950.
32. **Nölling, J., G. Breton, M. V. Omelchenko, K. S. Makarova, Q. Zeng, R. Gibson, H. Lee, J. Dubois, D. Qiu, J. Hitti, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, E. V. Koonin, and D. R. Smith.** 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J. Bacteriol. **183:**4823–4838.
33. **Pan, N., and J. A. Imlay.** 2001. How does oxygen inhibit central metabolism in the obligate anaerobe *Bacteroides thetaiotaomicron*. Mol. Microbiol. **39:**1562–1571.
34. **Patel, M. P., J. Marcinkeviciene, and J. S. Blanchard.** 1998. *Enterococcus faecalis* glutathione reductase: purification, characterization and expression under normal and hyperbaric O_2 conditions. FEMS Microbiol. Lett. **166:**155–163.
35. **Quail, M. A., P. Jordan, J. M. Grogan, J. N. Butt, M. Lutz, A. J. Thomson, S. C. Adrews, and J. R. Guest.** 1996. Spectroscopic and voltammetric characterization of the bacterioferritin-associated ferredoxin of *Escherichia coli*. Biochem. Biophys. Res. Commun. **229:**635–642.
36. **Reinards, R., J. Kubicki, and H. D. Ohlenbusch.** 1981. Purification and characterization of NADH oxdase from membranes of *Acholeplasma laidlawii*, a copper-containing iron-sulfur flavoprotein. Eur. J. Biochem. **120:**329–337.
37. **Robb, F. T., D. L. Maeder, J. R. Brown, J. DiRuggiero, M. D. Stump, R. K. Yeh, R. B. Weiss, and D. M. Dunn.** 2001. Genomic sequence of hyperthermophile, Pyrococcus furiosus: implications for physiology and enzymology. Methods Enzymol. **330:**134–157.
38. **Rosen, G. M., S. Pou, C. L. Ramos, M. S. Cohen, and B. E. Britigan.** 1995. Free radicals and phagocytic cells. FASEB J. **9:**200–209.
39. **Sakamoto, M., T. Uchimura, and K. Komagata.** 1996. Comparison of H_2O-forming NADH oxidase from *Leuconostoc mesenteroides* subsp. *mesenteroides* NRIC 1541^T and *Sporolactobacillus inulinus* NRIC 1133^T. J. Ferment. Bioeng. **82:**531–537.
40. **Shevchenko, A., M. Wilm, O. Vorm, and M. Mann.** 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal. Chem. **68:**850–858.
41. **Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi.** 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. USA **99:**996–1001.
42. **Siegel, L. M., P. S. Davis, and H. Kamin.** 1974. Reduced nicotinamide adenine dinucleotide phosphate-sulfite reductase of enterobacteria. III. The *Escherichia coli* hemoflavoprotein: catalytic parameters and the sequence of electron flow. J. Biol. Chem. **249:**1572–1586.
43. **Sodek, L., and W. J. da Silva.** 1977. Glutamate synthase: a possible role in nitrogen metabolism of the developing maize endosperm. Plant Physiol. **60:**602–605.
44. **Tsai, C. S.** 1980. Kinetic studies of multifunctional reactions catalyzed by lipoamide dehydrogenase. Int. J. Biochem. **11:**407–413.
45. **van Ooteghem, S. A., S. K. Beer, and P. C. Yue.** 2002. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Appl. Biochem. Biotechnol. **98-100:**177–189.
46. **van Ooteghem, S. A., A. Jones, D. van der Lelie, B. Dong, and D. Mahajan.** 2004. H_2 production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microaerobic growth conditions. Biotechnol. Lett. **26:**1223–1232.
47. **Wagner, M. A., and M. S. Jorns.** 2000. Monomeric sarcosine oxidase. II. Kinetic studies with sarcosine, alternate substrates, and a substrate analogue. Biochemistry **39:**8825–8829.
48. **Ward, D. E., C. J. Donnelly, M. E. Mullendore, J. van der Oost, W. M. de Vos, and E. J. Crane III.** 2001. The NADH oxidase from *Pyrococcus furiosus*. Implications for the protection of anaerobic hyperthermophiles against oxidative stress. Eur. J. Biochem. **268:**5816–5823.
49. **Weinberg, M. V., F. E. Jenney, Jr., X. Cui, and M. W. W. Adams.** 2004. Rubrerythrin from the hyperthermophilic archaeon *Pyrococcus furiosus* is a rubredoxin-dependent, iron-containing peroxidase. J. Bacteriol. **186:**7888–7895.
50. **Whitby, L. G.** 1953. A new method for preparing flavin-adenine dinucleotide. Biochem. J. **54:**437–442.
51. **Yang, X., and K. Ma.** 2005. Purification and characterization of an NADH oxidase from extremely thermophilic anaerobic bacterium *Thermotoga hypogea*. Arch. Microbiol. **183:**331–337.
52. **Yang, X., and K. Ma.** 2005. Determination of hydrogen peroxide generated by reduced nicotinamide adenine dinucleotide oxidase. Anal. Biochem. **344:**130–134.