Characterization of an NADH oxidase of the flavin-dependent disulfide reductase family from *Methanocaldococcus jannaschii*

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Methanocaldococcus jannaschii, a deeply rooted hyperthermophilic anaerobic methanarchaeon from a deep-sea hydrothermal vent, carries an NADH oxidase (Nox) homologue (MJ0649).

According to the characteristics described here, MJ0649 represents an unusual member within group 3 of the flavin-dependent disulfide reductase (FDR) family. This FDR group comprises Nox, NADH peroxidases (Npx) and coenzyme A disulfide reductases (CoADRs); each carries a Cys residue that forms Cys-sulfenic acid during catalysis. A sequence analysis identified MJ0649 as a CoADR homologue. However, recombinant MJ0649 (rMJNox), expressed in *Escherichia coli* and purified to homogeneity an 86 kDa homodimer with 0.27 mol FAD (mol subunit)⁻¹, showed Nox but not CoADR activity. Incubation with FAD increased FAD content to 1 mol (mol subunit)⁻¹ and improved NADH oxidase activity 3.4-fold. The FAD-incubated enzyme was characterized further. The optimum pH and temperature were ≥ 10 and ≥ 95 °C, respectively. At pH 7 and 83 °C, apparent K_m values for NADH and O₂ were 3 μ M and 1.9 mM, respectively, and the specific activity at 1.4 mM O₂ was 60 μ mol min⁻¹ mg⁻¹; 62% of NADH-derived reducing equivalents were recovered as H₂O₂ and the rest probably generated H₂O. rMJNox had poor NADPH oxidase, NADH peroxidase and superoxide formation activities. It reduced ferricyanide, plumbagin and 5,5'-dithiobis(2-nitrobenzoic acid), but not disulfide coenzyme A and disulfide coenzyme M. Due to a high K_m , O₂ is not a physiologically relevant substrate for MJ0649; its true substrate remains unknown.

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INTRODUCTION

Methanocaldococcus jannaschii is a deeply rooted hyperthermophilic methanogenic archaeon (Boone *et al.*, 1993; Jones *et al.*, 1983). This strictly autotrophic anaerobe

was isolated from a deep-sea hydrothermal vent (Jones *et al.*, 1983). The genome of *M. jannaschii* carries an ORF (MJ0649) that has been annotated as an NADH oxidase (Nox) homologue (Bult *et al.*, 1996) (Fig. 1), and such ORFs are present in most methanogens (Bult *et al.*, 1996; Hendrickson *et al.*, 2004; Smith *et al.*, 1997). Methanogens are strict anaerobes (Boone *et al.*, 1993). All anaerobic non-methanogenic archaea examined thus far also carry Nox homologues (Klenk *et al.*, 1997; Maeder *et al.*, 1999; Robb *et al.*, 2001). Since the Noxs of bacteria and eukaryotes catalyse the reduction of molecular oxygen (O₂) by either a two-electron transfer to produce hydrogen peroxide (H₂O₂) or a four-electron transfer to produce H₂O (Claiborne *et al.*, 1993; Toomey & Mayhew, 1998), it has been a puzzle why strictly anaerobic archaea carry Nox homologues. For this reason the Nox homologues of several non-methanogenic archaea have been investigated

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Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CoADR, coenzyme A disulfide reductase; CoMDR, coenzyme M disulfide reductase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); FDR, flavin-dependent disulfide reductase; NBT, nitro blue tetrazolium; Ni-NTA, nickel-nitrilotriacetic acid; Nox, NADH oxidase; Npx, NADH peroxidase; rMJNox, recombinant MJ0649 Nox; rPfRd, recombinant *P. furiosus* rubredoxin.

A supplementary table describing the purification of rMJNox is available with the online version of this paper.

Fig. 1. Multiple sequence alignment of MJ0649 (MjNox). Alignment was performed with CLUSTAL W (Thompson *et al.*, 1994). The sources for the enzymes analysed are as follows (accession or ORF number; reference). MjNox, *M. jannaschii* Nox (MJ0649; Bult *et al.*, 1996); PfNoxA-3, *P. furiosus* NoxA-3 (AAL81656; Maeder *et al.*, 1999; Robb *et al.*, 2001); AfNoxA-1, *Archaeoglobus fulgidus* NoxA-1 (AAB90976; Klenk *et al.*, 1997); AfNoxA-2, *A. fulgidus* NoxA-2 (AAB90838; Klenk *et al.*, 1997); EfNpx, *Ent. faecalis* NADH peroxidase (P37062; Ross & Claiborne, 1991); EfNox, *Ent. faecalis* NADH oxidase (CAA48728; Ross & Claiborne, 1992); SaCoADR, *S. aureus* coenzyme A disulfide reductase (AF041467; delCardayre & Davies, 1998); MmpRbo, *Methanococcus maripaludis* S2 NAD(P)H: rubredoxin oxidoreductase (MMP1259; Hendrickson *et al.*, 2004); MthNox, *Methanothermobacter thermautotrophicus* ΔH Nox (MTH1354; Smith *et al.*, 1997). The fully conserved residues are shown in white and with shading of selected colours. The residues showing functional conservation are shown in type of the appropriate colour. For both forms of presentation (coloured shading or coloured type), the following colour codes have been used: red, Asp and Glu; royal blue, Arg, Lys and His; green, Asn and Gln; olive, Ala, Gly, Ser and Thr; grey, Ile, Leu and Val; yellow, Phe, Tyr, Trp and His; navy blue, Pro; black, Cys; purple, Met. The marked sequence features are discussed in the text.

(Kengen *et al.*, 2003; Reed *et al.*, 2001; Ward *et al.*, 2001). These investigations considered the fact that some of the Nox proteins transfer electrons to peroxides, disulfides and a variety of membrane-associated acceptors (Kengen *et al.*, 2003; Reed *et al.*, 2001; Ward *et al.*, 2001). Some of these non-methanogenic archaea might use Nox homologues to counter the toxic effect of the molecular oxygen that they could be exposed to in their natural habitats (Kengen *et al.*, 2003; Ward *et al.*, 2001). Also, in *Pyrococcus horikoshii* and

Pyrococcus furiosus, a Nox homologue has been demonstrated to act as a coenzyme A disulfide reductase (CoADR) (Harris *et al.*, 2005) as well as sulfur reductase (Schut *et al.*, 2007). None of the methanogen Nox homologues have been investigated for their catalytic or *in vivo* functions. A related enzyme, NADPH-dependent disulfide coenzyme M reductase (CoMDR), has been isolated from *Methanothermobacter thermautotrophicus* (Smith & Rouviere, 1990); however, the gene for this

enzyme is unknown. As a result it is unclear whether the *nox* homologue of *M. jannaschii* (*mj0649*) represents a disulfide reductase. Also, in contrast to non-methanogenic anaerobic archaea, which carry three to nine *nox* homologues (Klenk *et al.*, 1997; Maeder *et al.*, 1999; Robb *et al.*, 2001), *M. jannaschii* carries only one such ORF (Pagala *et al.*, 2002). *Methanothermobacter thermautotrophicus*, *Methanococcus maripaludis*, *Methanosarcina acetivorans* and *Methanosarcina barkeri*, which are also methanogens, carry one, one, two, and one *Nox* homologues, respectively (Galagan *et al.*, 2002; Hendrickson *et al.*, 2004; <http://genome.ornl.gov/microbial/mbar/>; Smith *et al.*, 1997). Therefore, we are interested in elucidating the role of *mj0649*, the *nox* homologue of *M. jannaschii*. To begin in this direction, we have expressed this ORF in *Escherichia coli* and determined the molecular and kinetic characteristics of the recombinant protein.

METHODS

Bacterial strains, growth media and culture conditions. *E. coli* DH10B was used as a cloning host (Hanahan, 1983), and *E. coli* C41(DE3) (Miroux & Walker, 1996) carrying pRIL (Novagen) was used for the expression of recombinant Nox. These strains were grown in Luria-Bertani (LB) medium. During the cloning experiments, *E. coli* transformants were selected on plates or grown in liquid medium containing 100 µg ampicillin ml⁻¹. For the expression of recombinant Nox, *E. coli* transformants were grown in liquid medium containing 100 µg ampicillin ml⁻¹ and 25 µg chloramphenicol ml⁻¹.

Construction of an overexpression plasmid. Generally, all DNA manipulations were performed according to standard methods (Sambrook *et al.*, 1989). *mj0649* coding sequence was amplified from *M. jannaschii* genomic DNA with DeepVent_R DNA polymerase (New England Biolabs) using the oligonucleotide primers MJ0649xp/1F (5'-GGGGATCCAATGAGAGCAATAA TAATAGGAAGTGGAGCTG-3') and MJ0649xp/2R (5'-CATAACTGCAGCAATTATTTA TTAATC-AACTTTTCAAAGC-3'); the underlined sequences indicate engineered *Bam*HI and *Pst*II restriction sites, respectively. The resulting PCR product was cloned into the *Eco*RV site of pBluescript II SK(+) (Stratagene), to obtain the plasmid pMJ0649-1, and the cloned *mj0649* gene was determined to be free of mutation through sequencing of both of its strands. The *mj0649* insert of pMJ0649-1 was excised by restriction digestion with *Bam*HI and *Pst*II and ligated to the similarly digested pETDuet1 vector (Novagen) to obtain pMJ0649-2. In pMJ0649-2, the *mj0649* coding sequence was fused to an NH₂-terminal His₆ sequence and placed under the control of the T7 promoter. *E. coli* C41(DE3)(pRIL) was transformed with pMJ0649-2 for the expression of recombinant Nox (rMjNox).

Expression and purification of rMjNox. *E. coli* C41(DE3) (pRIL)(pMJ0649-2) was grown to an optical density of 0.6–0.8 (as measured using a model Lambda 25 UV-visible spectrophotometer, PerkinElmer instruments), followed by induction with 1 mM IPTG. After 3 h of induction, cells were harvested by centrifugation at 7000 g for 10 min at 4 °C. The resulting 3 g cell pellet was resuspended in 3 ml 100 mM potassium phosphate buffer, pH 7.0. The cell suspension was passed three times through a French pressure cell at a pressure of 1.28 × 10⁸ Pa. The resulting cell lysate was centrifuged at 18 000 g for 1 h at 4 °C to remove cell debris. The supernatant from this step was heated to 80 °C for 15 min and then centrifuged at 12 000 g for 10 min to remove the denatured proteins. The resulting 3.5 ml of supernatant was diluted to 7.0 ml with a solution

containing 600 mM NaCl and 20 mM imidazole, pH 7.0, to give the following composition: 50 mM potassium phosphate buffer, pH 7.0, 300 mM NaCl, 10 mM imidazole, and cell extract. This solution was loaded onto a 3 ml column bed (13 mm diameter) of nickel-nitrilotriacetic acid (Ni-NTA) Superflow agarose (Qiagen) that was pre-equilibrated with a 15 ml solution containing 50 mM potassium phosphate buffer, pH 7.0, 300 mM NaCl and 10 mM imidazole (buffer A). The column was washed with 15 ml buffer A, and then, to remove weakly bound contaminants, the column was further washed with 3 ml 0.5 M NaCl, 3 ml 1 M NaCl and 6 ml 2 M NaCl, sequentially; each wash contained 50 mM potassium phosphate buffer, pH 7.0, and 10 mM imidazole. To lower the salt concentration in the column, a final wash with 3 ml buffer A was performed. rMjNox was eluted from the column with a 60 ml linear gradient of 10–300 mM imidazole containing 50 mM potassium phosphate buffer, pH 7.0, and 300 mM NaCl. Fractions at about 250 mM imidazole contained most of the Nox activity and were pooled and concentrated on a YM-30 membrane (Amicon). The protein retained on the membrane was washed three times, each time with 5 ml 25 mM potassium phosphate buffer, pH 7.0, and recovered in 1 ml of the same buffer. The concentrated and desalted protein was loaded onto a 4 ml column bed (13 mm diameter) of Cibacron Blue 3GA agarose (Sigma-Aldrich) pre-equilibrated with 20 ml 25 mM potassium phosphate buffer, pH 7.0. The column was washed with 20 ml 25 mM potassium phosphate buffer, pH 7.0, and 50 mM NaCl, and rMjNox was eluted by sequential addition of 4 ml each of 100, 200, 300, 400, 500, 750 and 1000 mM NaCl containing 25 mM potassium phosphate buffer, pH 7.0. rMjNox activity was found in fractions containing 750 and 1000 mM NaCl. These fractions were pooled, concentrated, desalted and placed in 1 ml 25 mM potassium phosphate buffer, pH 7.0 as described above. This concentrate contained 1 mg homogeneous rMjNox.

Expression and purification of recombinant form of *P. furiosus* rubredoxin (rPfRd). *E. coli* NCM533 carrying pPfRd1 (Jenney & Adams, 2001), a plasmid allowing the expression of *P. furiosus* rubredoxin (PfRd) under the control of a T7 promoter, was obtained from Drs Francis E. Jenney, Jr, and Michael W. W. Adams, University of Georgia. The strain was transformed with pRIL. The cultivation of *E. coli* NCM533 (pPfRd1)(pRIL), the induction of the expression of PfRd, and the harvesting of the cells were carried out as described in the preceding section. *E. coli* cells carrying overexpressed PfRd from a 1 l culture were resuspended in 4 ml of a solution containing 150 mM NaCl and 25 mM potassium phosphate buffer, pH 7 (Solution B). The cells in the suspension were lysed and the resulting extract was heat-treated as described above for the purification of rMjNox. The heat-treated cell extract supernatant was then loaded onto a 50 ml 1.5 × 27 cm column bed of Sephadex G-200 (Pharmacia), which was pre-equilibrated with solution B. The column was then developed with solution B. The elution of rPfRd was followed by its characteristic deep burgundy colour. SDS-PAGE analysis showed that a pool of the coloured fractions was a homogeneous preparation of a protein with denatured molecular mass of 6 kDa, which corresponded well to PfRd (Jenney & Adams, 2001).

SDS-PAGE, size-exclusion chromatography, and protein assay. SDS-PAGE was performed according to Laemmli (1970) and protein assays were performed according to Bradford (1976) using reagents from Bio-Rad Laboratories. Size-exclusion chromatography was performed as described previously (Patel *et al.*, 2004) using molecular mass and size standards from Bio-Rad Laboratories with the following characteristics (standard, mass, Stokes radius; Mukhopadhyay & Purwantini, 2000): bovine thyroglobulin, 670 kDa, 85 Å (8.5 nm); bovine gamma globulin, 158 kDa, 52.5 Å (5.25 nm); chicken ovalbumin, 44 kDa, 30.5 Å (3.05 nm); horse myoglobin, 7 kDa, 19 Å (1.9 nm); vitamin B₁₂, 1.357 kDa.

Preparation of disulfide coenzyme M and coenzyme A. Disulfide coenzyme M (CoM-S-S-CoM) was prepared by dissolving 1 g 2-mercaptoethanesulfonate (HS-CoM) in 10 ml aqueous 30% NH₄OH and bubbling air through this solution for 72 h (Smith & Rouviere, 1990). The resulting disulfide coenzyme M solution was evaporated at 70 °C to dryness under vacuum in a rotary evaporator (model R-205, Büchi Labortechnik). This product was determined to be free of coenzyme M via an assay for free thiols (Ellman, 1958; Smith & Rouviere, 1990). Disulfide coenzyme A (CoA-S-S-CoA) was purchased from Sigma-Aldrich.

Nox assays. Assays were conducted in round glass cuvettes sealed with a cut-off butyl rubber stopper (Daniels & Wessels, 1984). The gas atmospheres in the tubes were manipulated via one of the following three methods. (I) For a moderate level (0.5–0.72 mM) of dissolved oxygen, a tube containing assay mixture was sealed under air and then pressurized with 100% O₂ to a desired total pressure. (II) For a low level (0.066–0.33 mM) of dissolved O₂, a sealed tube was made anaerobic via evacuation and pressurization with N₂ as described previously (Daniels & Wessels, 1984; Mukhopadhyay & Daniels, 1989). The final pressure was 1.2×10^2 kPa. Then, O₂ was added to this tube to the desired pressure. (III) To provide a high level of dissolved O₂ (0.96–1.4 mM), the assay tubes were evacuated and pressurized with O₂ to the desired partial pressure. The concentration of dissolved O₂ was calculated from Henry's relationship: $pO_2 = H_{O_2} \times x_{O_2}$, where pO_2 is the partial pressure of O₂ in atm, x_{O_2} is the mole fraction of dissolved O₂, and H_{O_2} is Henry's constant. From the available data in the 0–100 °C range (Liley *et al.*, 1984), the value of H_{O_2} at 83 °C was calculated to be 69 000 atm.

In most cases, prior to assays, the enzyme was incubated for 30 min at room temperature with FAD. The composition of this incubation mixture was 0.1 mg enzyme ml⁻¹, 25 mM potassium phosphate buffer, pH 7.0, and 0.1 mM FAD. Standard Nox assays were performed at 83 °C with oxygen as the electron acceptor in a 1 ml reaction mixture containing 50 mM HEPES-NaOH buffer, pH 7.0, 0.15 mM NADH, 1.4 mM dissolved O₂ [supplied by method (III), as described above] and 1 µM FAD (carried over from the enzyme stock). For pH studies the HEPES-NaOH buffer was replaced with constant ionic strength buffers containing 100 mM Tris, 50 mM glacial acetic acid and 50 mM MES (Mukhopadhyay *et al.*, 2000). The progress of the NADH oxidase reaction was followed spectrophotometrically at 340 nm. The initial velocity rates were calculated using a value of 6.22 mM⁻¹ cm⁻¹ for the absorption coefficient of NADH at 340 nm and were corrected for the chemical oxidation of NADH as measured in the absence of enzyme.

Other electron acceptors. For experiments involving electron acceptors other than oxygen, all assay tubes and assay components were made anaerobic by evacuating and pressurizing with nitrogen (1.4×10^2 kPa) three times. Additions of substrates were made from aqueous anaerobic stocks using an anaerobic syringe fitted with a needle (Daniels & Wessels, 1984; Mukhopadhyay & Daniels, 1989). For some of the electron acceptors, the progress of the reaction was followed at wavelengths other than 340 nm, and the initial rates were calculated by using the following absorption coefficient values (compound, assay wavelength, absorption coefficient): plumbagin, 419 nm, 3.95 mM⁻¹ cm⁻¹ (Rothery *et al.*, 1998); ferricyanide, 420 nm, 1.00 mM⁻¹ cm⁻¹ (Kengen *et al.*, 2003); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 412 nm, 13.6 mM⁻¹ cm⁻¹ (Kengen *et al.*, 2003); recombinant *P. furiosus* rubredoxin (rPfrd), 494 nm, 9.22 mM⁻¹ cm⁻¹ (Ma & Adams, 2001).

Determination of H₂O₂. Hydrogen peroxide produced in an NADH oxidase assay was assayed as outlined in Ward *et al.* (2001) but with some modifications as detailed here. An NADH oxidase assay mixture containing 110 µM NADH and 1.4 mM dissolved O₂ was incubated at

83 °C until the A₃₄₀ of the solution was <0.01, indicating that almost all of the NADH had been oxidized. Two-hundred microlitres of this solution was combined with 800 µl 125 mM potassium phosphate buffer, pH 7. The mixture was allowed to cool to room temperature and was then combined with 150 µl of a solution containing 2-2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich; 22 mg ml⁻¹) and 100 units horseradish peroxidase ml⁻¹ (Sigma-Aldrich). After incubation at room temperature for 30 min, the A₇₂₅ was measured. From this absorbance value and a standard curve, the amount of H₂O₂ produced in the assay was determined. The standard curve was generated by assaying 0–30 µM H₂O₂ solutions that were prepared by diluting a 3% (v/v) stock (Cumberland Swan Holdings) in 100 mM potassium phosphate buffer, pH 7. A control NADH oxidase assay mixture containing added H₂O₂ to 5.4 µM, but lacking NADH, was processed through heating, cooling and mixing steps similar to those described above. This mixture showed a 4% loss in H₂O₂ during processing and this loss was considered in calculating the amount of H₂O₂ produced in the NADH oxidase assay.

Assay of superoxide production. Superoxide formation was measured by the reduction of nitro blue tetrazolium (NBT) to formazan (Hillar & Loewen, 1995). The assay was conducted at 83 °C and various pHs (6–10) in a 0.8 ml reaction mixture with 1.4 mM O₂, 200 µM NBT, 150 µM NADH, and the constant ionic strength buffer described above in Methods, Nox assays. The reaction was initiated by the addition of enzyme. The reduction of NBT was monitored at 560 nm. An absorption coefficient of 15 mM⁻¹ cm⁻¹ for the formazan product at 560 nm (Hillar & Loewen, 1995) was used to calculate the initial velocity.

Analysis of kinetic data. All initial rate data were analysed according to Cleland (1979) using KinDist, a PC graphics program obtained from Professor Bryce V. Plapp, University of Iowa. The data were fitted to the Henri-Michaelis-Menten relationship: $v = V_{\max} \times S / (K_m + S)$.

Determination of the identity and content of flavin. The identity of the flavin bound to purified rMjNox was determined according to Mayhew & Massey (1969), with some modifications. During the following steps, exposure of the sample to light was avoided as much as possible. To extract flavin from the enzyme, 0.05 ml of a solution of rMjNox (1 mg ml⁻¹) was mixed with 5.5 µl 50% TCA. The mixture was held on ice for 5 min and then centrifuged at 10 000 g for 10 min to pellet the precipitated protein. The resulting supernatant of 50 µl was neutralized with 20 µl 1 M K₂HPO₄. This product was analysed via TLC on silica gel 60 F254 (Merck) using 1-butanol:acetic acid:water (10:5:5) as the resolving solvent. The resolved bands were viewed under a hand-held short-wavelength UV light. Solutions (0.1 mM) of FAD and FMN in 5% TCA and 5% K₂HPO₄ were used as standards.

The quantity of FAD bound per mole of rMjNox was determined as follows. For this work the following two homogeneous enzyme preparations were analysed: as isolated and FAD-incubated. The latter preparation was generated by incubating 300 µg purified protein with excess FAD (0.2 mM) at 4 °C for 5 h. Free flavin was removed by filtration and three 1 ml washes with 50 mM potassium phosphate buffer, pH 7.0, on the membrane of a YM-10 concentrator (Amicon). The product was recovered in 50 mM potassium phosphate buffer, pH 7.0, and assayed for protein content. From each of these two preparations, bound flavin was extracted as described in the preceding paragraph, except that the pellet obtained after the first centrifugation step was resuspended in 50 µl 5% aqueous TCA and recentrifuged to recover an additional supernatant that was combined with that from the first centrifugation. Flavin in this combined supernatant was quantified by measuring the A₄₅₀. The calculation was based on an absorption coefficient value of 10.6 mM⁻¹ cm⁻¹ for FAD in 5% aqueous TCA as determined from the reported value of 11.3 mM⁻¹ cm⁻¹ at pH 7 (Dawson *et al.*, 2002).

RESULTS

Expression and purification of rMjNox

MJ0649 was expressed in *E. coli* C41(DE3)(pRIL) as an NH₂-terminal His₆-tagged protein (rMjNox) (Fig. 2a, lane 2). When a cell lysate of *E. coli* C41(DE3)(pRIL)(pMJ0649-2) containing overexpressed rMjNox was centrifuged at 18 000 g for 1 h at 4 °C, the recombinant protein was almost exclusively found in the supernatant. This conclusion was based on an SDS-PAGE analysis (data not shown). Therefore, rMjNox was expressed in *E. coli* as a soluble protein. The 18 000 g supernatant exhibited NADH oxidase activity. However, a supernatant similarly obtained from *E. coli* C41(DE3)(pRIL) also had NADH oxidase activity. When the two cell extract preparations were heat-treated at 80 °C the resulting supernatant of *E. coli* C41(DE3)(pRIL)(pMJ0649-2), but not that of *E. coli* C41(DE3)(pRIL), contained the NADH oxidase activity. These results established that overexpressed MJ0649 carrying a His₆-tag had NADH oxidase activity. From a heat-treated cell extract supernatant, rMjNox was purified to homogeneity through Ni-NTA and Cibacron Blue agarose chromatography steps (Fig. 2b). Typically, from a wet cell paste corresponding to a dry cell weight of 0.3 g, up to 2 mg of rMjNox (Supplementary Table S1) with specific NADH oxidase activity of 36–60 units mg⁻¹ was obtained. The kinetic data reported in this paper were generated with a preparation exhibiting a specific activity of 60 units mg⁻¹. At a later stage of this work it was found that chilling of the heat-treated extract on ice for 2 h prior to its centrifugal clarification led to the generation of

homogeneous enzyme at the Ni-NTA chromatography step, and therefore removed the need for the Cibacron Blue agarose chromatography step.

Molecular properties of rMjNox

The data from SDS-PAGE (Fig. 2b) showed that rMjNox had a subunit molecular mass of ~50 kDa. This value is consistent with the nucleic acid sequence-derived mass of 50.5 kDa for the recombinant protein. In size-exclusion chromatography on a HiPrep 16/60 Sephacryl S-300 HR column (Amersham Pharmacia Biotech), the homogeneous enzyme exhibited a single peak. From the retention time in this column, the apparent molecular mass of the native protein was determined to be 86 ± 2 kDa. This value could indicate that the protein was a compact dimer or a monomer with a non-globular structure. With the latter possibility, the hydrodynamic radius of the protein would be larger than would be expected for a globular protein of equivalent mass. These possibilities will be investigated in the future through a more accurate method such as analytical ultracentrifugation (Cantor & Schimmel, 1980).

Flavin content of rMjNox

The UV-visible spectra of the enzyme as isolated from Ni-NTA chromatography as well as after incubation with FAD were characteristic of a flavoprotein (Fig. 3a, b). The absorbance at >500 nm exhibited by rMjNox has been seen with other group 3 flavin-dependent disulfide reductase (FDR) Nox enzymes, where it is indicative of a weak charge-transfer interaction between the active Cys thiolate and FAD (Ahmed & Claiborne, 1989a, b; Mallett & Claiborne, 1998). The absorbance peaks at 368 and 443 nm indicated that the enzyme contained bound flavin. TLC analysis of a TCA extract of the protein as isolated identified the bound flavin to be FAD. Further analysis showed that rMjNox as isolated contained 0.55 ± 0.02 mole FAD per mole of the dimeric protein. After incubation with excess FAD, the flavin content value of rMjNox increased to 1.9 ± 0.2 moles per dimer.

Kinetic characterization of rMjNox

Unless mentioned otherwise, the activity of rMjNox refers to the oxidation of NADH with oxygen, which was also termed simply NADH oxidase. Under standard assay conditions the enzyme, as isolated, showed a specific activity of 16 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. Upon incubation for 30 min with FAD, the enzyme exhibited increased activity. The maximum stimulation was observed at an FAD concentration of 0.1 mM in the incubation mixture, and the resulting specific activity was 55 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. Incubation with FMN did not change the specific activity of the enzyme from that as isolated. All kinetic data reported below were obtained with an enzyme preparation that was incubated with FAD at a concentration of 0.1 mM prior to assay. A carry-over from this preparation provided FAD to the assays at a concentration of 1.0 μM .

Fig. 2. SDS-PAGE analysis of expression and purification of rMjNox. (a) Expression of rMjNox in *E. coli* C41(DE3)(pRIL) (pMJ0649-2). Whole-cell lysates: lane 1, before induction; lane 2, after induction with 1 mM IPTG for 3 h; M, molecular mass standard. (b) Purification of rMjNox. Preparations from (amount of protein): lane 1, Ni-NTA chromatography (4 μg); lane 2, Cibacron Blue agarose chromatography (4 μg); M, molecular mass standard. The mass values for the standards (kDa) are shown to either the right (a) or the left (b) of the gel.

Fig. 3. UV-visible spectra of rMjNox. A solution of the enzyme in 25 mM potassium phosphate buffer, pH 7.0, was analysed. (a) Enzyme (2.5 mg ml^{-1}) as isolated by Ni-NTA chromatography. The full spectrum (inset) and an expanded version for the 300–800 range are shown. (b) FAD-incubated enzyme (1.0 mg ml^{-1}).

The response of the NADH oxidase activity of rMjNox to changes in pH is shown in Fig. 4(a). In the pH range 6.0–7.5, the enzyme showed an activity peak at pH 7.0 (Fig. 4a). However, a rise in pH above 8.0 increased activity steadily and to substantially higher values. The activity at pH 10 was $178 \mu\text{mol min}^{-1} \text{ mg}^{-1}$, which was nearly threefold higher than that at pH 7.0. Activities at pH higher than 10.0 were not determined. rMjNox showed significant activity over a broad temperature range (Fig. 4b). The optimum temperature was $>95^\circ\text{C}$ (Fig. 4b); activities at higher temperatures were not determined due to technical limitations. From the straight-line section of the Arrhenius plot corresponding to the range $25\text{--}65^\circ\text{C}$, the activation energy of the rMjNox-catalysed NADH oxidase reaction was determined to be 46 kJ mol^{-1} .

The apparent K_m value for O_2 , as determined at a saturating level ($150 \mu\text{M}$) of NADH, was 1.9 mM . The conditions employed did not allow for the attainment of a saturating level of dissolved O_2 for rMjNox in the assay. The calculated apparent V_{max} value with respect to saturating dissolved O_2 was $140 \mu\text{mol min}^{-1} \text{ mg}^{-1}$ (Fig. 5a). However, the enzyme was extremely sensitive to

NADH concentration (Fig. 5b). When dissolved O_2 was fixed at 1.4 mM (subsaturating), rMjNox showed an apparent K_m of $3 \mu\text{M}$ for NADH. The apparent V_{max} value for these assays was 66 units mg^{-1} . The enzyme also oxidized NADPH with oxygen. However, at dissolved O_2 and NADPH concentrations of 1.4 and 0.15 mM , respectively, the activity of rMjNox was only 3 units mg^{-1} , a value 95 % lower than that obtained with 0.15 mM NADH. Since the chemical breakdown of NADPH at 83°C was faster than the enzymic reaction, a detailed kinetic analysis for the NADPH oxidation activity was not pursued.

H_2O_2 and superoxide production by rMjNox in the Nox reaction

A measurement employing horseradish peroxidase and ABTS dye detected H_2O_2 in a standard O_2 -reducing NADH oxidase assay. For $110 \mu\text{M}$ NADH consumed, $66 \mu\text{M}$ H_2O_2 was produced. A control experiment showed that the heating and cooling steps of the assay caused a 4 % loss in H_2O_2 . Considering this loss, the $\text{H}_2\text{O}_2/\text{NADH}$ ratio in the NADH oxidase reaction was 0.62. An assay based on NBT reduction (Hillar & Loewen, 1995) showed that rMjNox produced a minor amount of superoxide while oxidizing NADH with oxygen. For the reason given in the Discussion, this activity was measured in the pH range 6–10. As shown in Fig. 4(a), superoxide formation activity reached a plateau at pH 7–8.5, and at higher pH values it increased about 2.5-fold above this plateau value. Although the nature of the pH profile for the superoxide formation activity was not exactly similar to that for the NADH oxidase activity, the fold increases between pH 7 and 10 were similar for both.

Alternate electron acceptors of rMjNox

The ability of rMjNox to use electron acceptors other than oxygen was tested. These assays were performed in the absence of oxygen and with NADH as the electron donor and the results are shown in Table 1. rMjNox catalysed the peroxidase reaction, although the value of this activity was much lower than that of the NADH oxidase activity. It exhibited high activity with ferricyanide and plumbagin, which have been used as artificial electron acceptors for enzyme assays. rMjNox also reduced DTNB, a disulfide compound and an artificial electron acceptor. However, no activity was seen with disulfide coenzyme A, disulfide coenzyme M or *P. furiosus* rubredoxin as electron acceptor.

DISCUSSION

The results presented above show that MJ0649 encodes an unusual NADH oxidase that belongs to the group 3 FDRs (Argyrou & Blanchard, 2004; Claiborne *et al.*, 1999). We discuss this finding below. In the literature, a Nox enzyme from *P. furiosus* has been called Nox1 (Ward *et al.*, 2001) and NoxA-3 (Maeder *et al.*, 1999; Robb *et al.*, 2001); we refer to this enzyme as NoxA-3.

Fig. 4. Effects of pH and temperature on rMjNox activity. (a) pH study: Nox (●) and superoxide formation (○) activities of rMjNox. (b) Temperature study: assays were performed at various temperatures under otherwise standard conditions; inset, a replot of temperature data according to the Arrhenius relationship. The activation energy value was calculated from the slope of the linear segment (at 25–65 $^{\circ}\text{C}$) of the replot; slope = $-E_a/R$, where E_a is the activation energy in kJ mol^{-1} and R is the universal gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$).

Group 3 of the FDR family includes NADH oxidases (Nox), NADH peroxidases (Npx) and CoADRs (Argyrou & Blanchard, 2004; Claiborne *et al.*, 1999) (Fig. 1). These enzymes use a reactive cysteine that lies within a conserved SFXXC element and form a Cys-sulfenic acid or Cys-SOH in the catalytic cycle (Claiborne *et al.*, 1999). They also possess FAD and NADH binding sites (Fig. 1) (Argyrou & Blanchard, 2004; Claiborne *et al.*, 1999). MJ0649 and its archaeal homologues possessed all these attributes and additionally certain distinct features (Fig. 1). In these proteins, the reactive Cys-bearing sequence element of MJ0649 ($^{39}\text{YSPC}^{42}\text{AIPY}^{46}$) was more strictly conserved than that seen in their bacterial homologues (Fig. 1). Also, MJ0649 had CoADR-type sequence features. In the CoADR of *Staphylococcus aureus* the Tyr³⁶¹ and Tyr⁴¹⁹ residues of the $^{361}\text{YYPG}^{364}$ and $^{419}\text{YAPP}^{422}$ elements help to stabilize Cys⁴³-thiolate, and during the CoA-disulfide reduction, Tyr³⁶¹ is the primary player in the protonation of the CoASH thiolate (Mallett *et al.*, 2006). These Tyr residues and the corresponding sequence elements were almost fully conserved in archaeal Nox homologues, but are absent in the *Enterococcus faecalis* Npx and Nox (Fig. 1). Also, the Arg³⁹⁵ and Asp⁴²⁸ of *S. aureus* CoADR, which further differentiate this enzyme from *Ent. faecalis* Npx and Nox (Mallett *et al.*, 2006), were almost fully conserved in the archaeal proteins and corresponded to Arg³⁹⁹ and Glu⁴³² of MJ0649 (Fig. 1). However, rMjNox did not reduce the

disulfide of coenzyme A or disulfide of coenzyme M, but oxidized NADH with molecular oxygen (Table 1). These mixed properties make MJ0649 an unusual member of the FDR family. Interestingly, when tested with DTNB, a disulfide compound of no physiological relevance, rMjNox showed significant disulfide reductase activity. MJ0649 exhibits 62 % identities and 16 % strong similarities to a putative NAD(P)H:rubredoxin oxidoreductase of *Methanococcus maripaludis* (MMP1259) (Fig. 1). Since a clone of *P. furiosus* rubredoxin (PfRd; ORF PAF1282) is available (Jenney & Adams, 2001), the ability of rMjNox to reduce PfRd was tested. PfRd shows high homologies to putative *M. jannaschii* rubredoxins (49 % identities and 19 % strong similarities to MJ0740, and 33 % identities and 11 % strong similarities to MJ0735). However, rMjNox did not reduce recombinant PfRd (Table 1).

In its subunit size, flavin content (~ 1 mole per subunit), and requirement for FAD for activity as established through an enhancement of activity via incubation with this coenzyme, rMjNox was similar to most group 3 FDR enzymes (Table 2). The described group 3 FDR enzymes contain one FAD per subunit (Argyrou & Blanchard, 2004; Claiborne *et al.*, 1999) and transfer electrons from nicotinamides via flavin for the purpose of oxygen, peroxide or disulfide coenzyme A reduction (Argyrou & Blanchard, 2004; Claiborne *et al.*, 1999). The observed

Fig. 5. Substrate kinetics of rMjNox. Assays were performed at 83 °C and under standard conditions, except that the concentration of a substrate was varied. (a) Dissolved O₂ kinetics; (b) NADH kinetics. Each dataset was fitted to the standard Henri-Michaelis-Menten equation, $v = V_{\max} \times S / (K_m + S)$.

Table 1. Activity of rMjNox with various electron acceptors

Assays were performed under standard conditions, except that the electron acceptors were supplied at the concentrations indicated

e^- acceptor, concentration (activity name)	Specific activity* (μmol min ⁻¹ mg ⁻¹)	Activity (%)†
O ₂ , 1.4 mM (Nox)	60	100
H ₂ O ₂ , 1.4 mM (Npx)	1.5	2.5
Ferricyanide, 1.4 mM	135	225
Plumbagin, 0.2 mM	40	67
DTNB, 0.7 mM	20	33
Disulfide coenzyme M, 1.4 mM (CoMDR)	0.2	0.3
Disulfide coenzyme A, 0.6 mM (CoADR)	0.6	1.0
<i>P. furiosus</i> rubredoxin	0	0

*Values were obtained from an average of two assays.

†Activity with 1.4 mM oxygen was considered to be 100%.

temperature optimum for the activity of rMjNox (95 °C) was consistent with the growth temperature range (48–94 °C) and optimal growth temperature (85 °C) for the host organism (Jones *et al.*, 1983). The pH versus activity data for rMjNox showed two activity peaks (at pH 7.0 and pH >10.0) and the NADH oxidase activity dramatically increased as pH was increased above 7.0 (Fig. 4a). It is possible that in the pH ranges of ≤7.0 and >7.0, two different sets of amino acid residues of the protein play critical roles in catalysis. Another explanation is that at pH >7.0, rMjNox produces superoxide; certain Fe-containing Nox enzymes, which do not belong to the FDR family, exhibit such a behaviour (Singh *et al.*, 2004). rMjNox did produce superoxide and the activity was elevated at pH >8.5. However, this action accounted for only 3.0 % of the total NADH oxidation activity.

rMjNox showed high sensitivity to NADH. The reported apparent K_m value for NADH was determined at a subsaturating concentration of O₂ and is therefore an overestimate; the actual value would be <3 μM. A comparison of this value with the apparent K_m of a homologous Nox enzyme (Table 2) clearly indicated that NADH is a physiologically relevant electron donor for MJ0649. rMjNox also oxidized NADPH with O₂, but the rate of this reaction was about 5 % of that found with NADH. This property is consistent with the presence of a characteristic NADH binding motif (GXGXXG) (Bellamacina, 1996) in MJ0649 (GAGAIG, Fig. 1). In an NADP-utilizing enzyme the C-terminal Gly of this motif is replaced with Ala, Pro or Ser, perhaps to accommodate the pyrophosphate group of NADP (Bellamacina, 1996). It should be noted that CoADRs from *Bacillus anthracis* and *P. horikoshii* carry the GXGXXG motif, yet oxidize both NADH and NADPH (Harris *et al.*, 2005; Wallen *et al.*, 2008).

The apparent K_m for O₂ of rMjNox (1.9 mM) was much higher than that for Nox enzymes for which a role in oxygen detoxification has been proposed (Table 2). It is possible that in rMjNox, bound FAD is not easily accessible to the oxygen. However, the $V_{\max}$ value of this enzyme (60 μmol min⁻¹ mg⁻¹, at 1.4 mM dissolved O₂) was much higher than that of many NADH oxidases (Table 2) and rMjNox produced H₂O₂. Also only 62 % of the reducing equivalents provided by NADH were recovered as H₂O₂ and the rest likely yielded H₂O; *P. furiosus* NoxA-3 behaves similarly (Ward *et al.*, 2001). As is true for the NADH oxidases of the FDR family (Mallett & Claiborne, 1998), the oxidation of NADH with O₂ by MjNox probably involved intermediary formation of enzyme-bound C(4a)-peroxyflavin, which was attacked by the thiolate of the reactive Cys residue (Cys⁴²), generating H₂O and a Cys-SOH species (Mallett & Claiborne, 1998); reduction of Cys-SOH with NADH generated H₂O and the overall reaction produced 2H₂O. Perhaps in MjNox and *P. furiosus* NoxA-3, the attack by the Cys thiolate is slow or very inefficient, which causes peroxyflavin to release H₂O₂. This hypothesis is supported by the observation that the C42S

Table 2. Biochemical properties of various group 3 FDR enzymes

–, Not applicable or not measurable.

Organism	Enzyme	NADH or NADPH oxidation with O ₂ (except Npx with H ₂ O ₂ and CoADR with disulfide coenzyme A)					Reactive electron acceptors other than oxygen (other than H ₂ O ₂ for Npx and CoA-S-S-CoA for CoADR)*	Quaternary structure	Subunit size (kDa)	Source or reference	
		<i>K_m</i> (apparent) (μM)			<i>V_{max}</i> (μmol min ⁻¹ mg ⁻¹)						Product H ₂ O/ H ₂ O ₂
		NADH	NADPH	O ₂	NADH	NADPH					
<i>M. jannaschii</i>	Nox	3	ND†	1900	140	ND†	H ₂ O and H ₂ O ₂	DTNB, H ₂ O ₂ , plumbagin, Fe(CN) ₆ ³⁻	Unknown‡	50.5	This work
<i>S. aureus</i>	CoADR	—	2	—	—	816	—	DPP, CoASSG	Dimer	49	delCardayre <i>et al.</i> (1998)
<i>Ent. faecalis</i>	Nox	41	ND†	ND†	690§	—	H ₂ O	DCIP, Fe(CN) ₆ ³⁻	Monomer	51	Schmidt <i>et al.</i> (1986)
	Npx	4	1000	—	44.3	<4.4	—	DCIP, Fe(CN) ₆ ³⁻	Tetramer	49	Parsonage <i>et al.</i> (1993); Poole & Claiborne (1986)
<i>A. fulgidus</i>	NoxA-1	130	—	60	8.7	—	H ₂ O ₂	DCPIP, Fe(CN) ₆ ³⁻ , DTNB, BV	Dimer	48	Kengen <i>et al.</i> (2003)
	NoxA-2	3.1	—	—	1.3	—	H ₂ O ₂	NBT, Fe(CN) ₆ ³⁻ , menadione, Cyt c	ND†	48	Reed <i>et al.</i> (2001)
<i>P. furiosus</i>	NoxA-3	<4	—	>110	12	—	H ₂ O and H ₂ O ₂	None	Dimer	50	Ward <i>et al.</i> (2001)
<i>Thermotoga hypogea</i>	Nox	7.5	—	85	37	—	H ₂ O ₂	Not tested	Dimer	50	Yang & Ma (2005)

*BV, benzyl viologen; CoASSG, coenzyme A : glutathione heterodisulfide; Cyt c, cytochrome c; DCIP, dichloroindophenol; DCPIP, 2,6-dichlorophenolindophenol; DPP, 4,4'-diphosphopantethine; Fe(CN)₆^{3–}, ferricyanide.

†ND, Not determined.

‡Gel filtration analysis-derived apparent native molecule mass, which could represent a compact dimer or a non-globular monomer.

§Specific activity of purified enzyme.

variant of *Ent. faecalis* Nox produces exclusively H₂O₂ from the NADH-dependent reduction of oxygen (Claiborne *et al.*, 1999; Mallett & Claiborne, 1998); Cys⁴²-SOH/SH (sulfenic acid/thiol) acts as the peroxidatic centre for the wild-type enzyme. Release of H₂O₂ from the peroxyflavin intermediate has been proposed previously for *P. furiosus* NoxA-3 (Ward *et al.*, 2001). However, it is not clear how the observed low-level peroxidase activity (Table 2) and superoxide formation activity (Fig. 4a) of MjNox fit the mechanism described above or whether the enzyme utilizes yet unknown ways of accomplishing these tasks.

It is tempting to speculate about an oxygen detoxification role for MjNox or MJ0649. Geological data (Corliss *et al.*,

1979; Jannasch & Mottl, 1985; Jones *et al.*, 1983) suggest that *M. jannaschii* could experience minor oxygen exposure within the submarine hydrothermal vents (Johnson & Mukhopadhyay, 2005) or during transport through cold oxygenic water (Huber *et al.*, 1990; Stetter *et al.*, 1993). Also, during low-temperature storage, hyperthermophilic anaerobes are relatively resistant to O₂ exposure (Huber *et al.*, 1990; Jannasch *et al.*, 1992). However, a high *K_m* value (1.9 mM) of the MjNox enzyme for O₂ makes an oxygen-detoxification role of MJ0649 less likely. At a typical seawater dissolved O₂ concentration of 8 μM and temperature of 2 °C (McCollom & Shock, 1997), the activity of the enzyme would be only 0.006 μmol min^{–1} mg^{–1}; this calculation is based on the values of *K_m* for oxygen

(1.9 mM) and activation energy (46 kJ mol^{-1}) for rMjNox. The abilities of rMjNox to catalyse the reduction of plumbagin, ferricyanide and DTNB with NADH suggest that MJ0649 might reduce an as yet unidentified substrate.

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