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SHORT COMMUNICATION

Andrei Brioukhanov · Alexander Netrusov
Melanie Sordel · Rudolf K. Thauer · Seigo Shima

Protection of *Methanosarcina barkeri* against oxidative stress: identification and characterization of an iron superoxide dismutase

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Abstract *Methanosarcina barkeri* is a methanogenic archaeon that can only grow under strictly anoxic conditions but which can survive oxidative stress. We have recently reported that the organism contains a monofunctional catalase. We describe here that it also possesses an active iron superoxide dismutase. The enzyme was purified in three steps over 130-fold in a 14% yield to a specific activity of 1500 U/mg. SDS-PAGE revealed the presence of only one band, at an apparent molecular mass of 25 kDa. The primary structure determined from the cloned and sequenced gene revealed similarity to iron- and manganese superoxide dismutases. The highest similarity was to the iron superoxide dismutase from *Methanobacterium thermoautotrophicum*. The enzyme from *M. barkeri* was found to contain, per mol, 1 mol iron, but no manganese – in agreement with the general observation that anaerobically growing organisms only contain iron superoxide dismutase. The enzyme was not inhibited by cyanide (10 mM), which is a property shared by all iron- and manganese superoxide dismutases. The presence of superoxide dismutase in *M. barkeri* is noteworthy since a gene encoding superoxide dismutase (*sod*) has not been found in *Archaeoglobus fulgidus*, a sulfate-reducing archaeon most closely related to the Methanosarcinaceae.

Key words Superoxide dismutase · Iron · Catalase · *Methanosarcina* · Methanogenic archaea

Abbreviation *sod* Gene encoding superoxide dismutase

A. Brioukhanov · M. Sordel · R. K. Thauer · S. Shima (✉)
Max-Planck-Institut für terrestrische Mikrobiologie
and Laboratorium für Mikrobiologie
des Fachbereichs Biologie der Philipps-Universität,
Karl-von-Frisch-Strasse, 35043 Marburg, Germany
e-mail: shima@mailer.uni-marburg.de,
Tel.: +49-6421-178200, Fax: +49-6421-178209,
URL: http://www.uni-marburg.de/mpi

A. Brioukhanov · A. Netrusov
Microbiology Department, Moscow Lomonosov State University,
Moscow, Russia

Introduction

Methanogenic archaea can grow only under strictly anoxic conditions, but some, however, can survive oxidative stress. Zhilina (1972) determined that the death rate of *Methanosarcina* in air was surprisingly low. Fetzer et al. (1993) found that *Methanosarcina* species in oxic paddy field soils rapidly began to produce methane and to grow again as soon as anoxic conditions were re-established. Kiener and Leisinger (1983) showed that other methanogenic archaea are also quite oxygen-tolerant. Leadbetter and Breznak (1996) described that in the hindgut of the termite *Reticulitermes flavipes*, species of *Methanobrevibacter* thrive in the microoxic zone directly adjacent to the intestinal mucosa, indicating that some methanogenic archaea can not only survive but also tolerate oxic conditions.

When strictly anaerobic microorganisms come in contact with O_2, generally O_2^- and H_2O_2 are generated by autoxidation of reduced iron-sulfur proteins and/or flavoproteins (Storz and Imlay 1999). The reduced oxygen species are strong oxidants ($E_0'(O_2^- / H_2O_2) = +890$mV; $E_0'(H_2O_2 / H_2O) = +1355$mV, which will damage the cells if not immediately removed (Elstner 1990) by the enzymes superoxide dismutase and catalase, which catalyze the disproportionation of 2 O_2^- to O_2 and H_2O_2 and of 2 H_2O_2 to O_2 and 2 H_2O, respectively. The presence or absence of one or both of these enzymes determines whether an anaerobe is more or less aerotolerant (Rocha et al. 1996). Instead of a superoxide dismutase, *Pyrococcus furiosus* contains a superoxide reductase that catalyzes the reduction of O_2^- to H_2O_2 with reduced rubredoxin (Jenney et al. 1999).

Initially, methanogenic archaea were thought to contain neither superoxide dismutase nor catalase. In 1981, Kirby's group reported the presence of an iron superoxide dismutase in *Methanobacterium bryantii* (Kirby et al. 1981). Later, Takao et al. (1990) cloned and sequenced the *sod* gene from *Methanobacterium thermoautotrophicum* strain ΔH and heterologously overexpressed it in *Escherichia coli* (Takao et al. 1991). *sod* and *kat* genes

were, however, not found in *Methanococcus jannaschii* (Bult et al. 1996). A catalase gene was not found in the genome of *M. thermoautotrophicum* strain ΔH (Smith et al. 1997), but a bifunctional catalase-peroxidase was found in the genome of *Archaeoglobus fulgidus*, a sulfate-reducing archaeon most closely related to the Methanosarcinacaea (Woese et al. 1991). A *sod* gene was not found in the genome of *A. fulgidus* (Klenk et al. 1997).

Recently we purified and characterized a monofunctional catalase from *Methanosarcina barkeri* and cloned and sequenced the encoding gene (Shima et al. 1999). In this communication, we describe that *M. barkeri*, besides a catalase, also contains an iron superoxide dismutase.

Materials and methods

Organism and culture conditions

Methanosarcina barkeri strain Fusaro (DSMZ 804), from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), was grown on 1% methanol at 37 °C as described previously (Karrasch et al. 1989). The cells were generally harvested anaerobically in the exponential phase at a cell concentration of 2 g (wet mass)/l.

Purification of superoxide dismutase

Superoxide dismutase was purified from 8 g (wet mass) methanol-grown cells of *M. barkeri* suspended in 30 ml of 50 mM Tris/HCl pH 8.0. The cell suspension was passed three times through a French pressure cell at 110 MPa. Cell debris was removed by centrifugation for 20 min at 20,000×*g* at 4 °C; membranes were removed by centrifugation at 240,000×*g* and 4 °C for 60 min. Ammonium sulfate powder was added to the supernatant (80% saturation). After incubation at 0 °C for 10 min, precipitated protein was removed by centrifugation at 25,000×*g* and 4 °C for 15 min. Ammonium sulfate powder was added again (100% saturation). After incubation at 0 °C for 10 min, the precipitated protein was collected by centrifugation at 25,000×*g* and 4 °C for 15 min.

The precipitated protein was dissolved in 50 mM Mops/KOH, pH 7, concentrated to 5 ml by ultrafiltration (30-kDa cut-off), and applied to a Resource Q column (6 ml volume) equilibrated with 50 mM Mops/KOH, pH 7.0. Superoxide dismutase eluted from the column with 150 ml of a linearly increasing gradient of NaCl (0–0.8 M; flow rate 3 ml/min; 4-ml fractions). Superoxide dismutase activity eluted at 0.34–0.4 M NaCl. The two fractions containing the activity were combined and diluted with 70 ml of 50 mM potassium phosphate, pH 7.0. The solution was applied to a Ceramic Hydroxyapatite column (1.3 cm×10 cm; Bio-Rad Laboratories) equilibrated with 50 mM potassium phosphate, pH 7.0. Superoxide dismutase was eluted from the column with 200 ml of a linearly increasing gradient of potassium phosphate, pH 7.0 (0.05–0.5 M; flow rate 3 ml/min; 5-ml fractions). Superoxide dismutase activity eluted at 0.2–0.29 M potassium phosphate. The eight fractions containing the activity were combined and concentrated by ultrafiltration (30 kDa cut-off).

The enzyme was stored at –20 °C; the superoxide dismutase activity remained constant for at least 3 months.

Determination of enzyme specific activity

Superoxide dismutase activity was determined spectrophotometrically at 25 °C by the xanthine oxidase-cytochrome *c* method (McCord and Fridovich 1969). The 0.7-ml assay mixture contained: 50 mM potassium phosphate, pH 7.8; 0.1 mM EDTA; 50 μM xanthine; 1.7 mU xanthine oxidase (from Serva); 10 μM cytochrome *c* (from Serva); and, where indicated, 10 mM potassium cyanide or 2–10 mM sodium azide. The reduction of cytochrome *c* by O_2^-, which was generated from O_2 by reduction with xanthine, was followed by absorption at 550 nm. One unit of superoxide dismutase is defined as the amount required to inhibit the reduction rate of cytochrome *c* by 50%.

Protein concentrations were determined by the method of (Bradford 1976) using the reagents from Bio-Rad Laboratories and bovine serum albumin as standard.

Cloning and sequencing of the *sod* gene

A probe for the *sod* gene was obtained by PCR using genomic DNA from *M. barkeri* as template, which was isolated as described by Jarrell et al. (1992). The oligonucleotides 5′AAGTTTGGTTATGGTGATTTTGCTCCTTATAT (sense) and 5′-ACGTATGCTTGGTGGTGTTTGTCGTGGTG (antisense) were derived from the N-terminal amino acid sequence of purified superoxide dismutase using G or T as third letter. Degenerated primers were not used successfully. A 83-bp PCR product was obtained, amplified, and cloned into the pCR-Blunt vector using the Invitrogen Zero Blunt PCR cloning kit. The identity of the cloned fragment was determined by DNA sequencing. The 23-base oligonucleotide probe 5′–TGTAACTTGAGCTGCTCTTCAGA designed from the PCR product was 3′-end-labeled with digoxigenin-dUTP following the protocol provided by Boehringer Mannheim and then used for Southern hybridization (Sambrook et al. 1989) of *M. barkeri* genomic DNA digested to completion. The 23-base probe for *sod* was used to screen the λZAP Express *Sau*3AI genomic library of *M. barkeri* (Vorholt et al. 1996). Of 10,000 plaques, 55 were positive. One of the positive plaques generated the plasmid pAB1 harboring the *sod* gene, which was sequenced. The nucleotide sequence is available under accession No. AJ272498 in the EMBL database.

Results and discussion

Cell extracts of methanol-grown *M. barkeri* exhibited approximately 10 U/mg protein superoxide dismutase activity as determined by the xanthine oxidase-cytochrome *c* method. The specific activity was very similar in cell extracts of acetate- or H_2/CO_2-grown cells (20–30 U/mg when the cells were harvested in the stationary phase). The specific activity of superoxide dismutase in cell extracts of *M. barkeri* was similar to that in cell extracts

Table 1 Purification of superoxide dismutase from *Methanosarcina barkeri*. Cell extract was prepared from 8 g (wet mass) of methanol-grown *M. barkeri* and superoxide dismutase was purified as described in Materials and methods

Fraction	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
240,000×*g* supernatant	330	3500	11	1	100
80–100% $(NH_4)_2SO_4$	34	3420	100	9	98
Resource Q	2.8	740	260	24	21
Hydroxyapatite	0.3	480	1500	136	14

of aerobically grown *E. coli* (13.5 U/mg) (Gregory and Fridovich 1973).

More than 95% of the activity in cell extracts of *M. barkeri* was recovered in the 240,000×*g* supernatant. The activity was stable under oxic conditions.

Superoxide dismutase from *M. barkeri* was purified to a specific activity of 1500 U/mg, as described in Table 1. In comparison, the specific activity of purified superoxide dismutase from *M. bryantii* was reported to be 2060 U/mg (Kirby et al. 1981).

Via gel filtration, superoxide dismutase from *M. barkeri* was found to have an apparent molecular mass of 70 kDa (Table 2). SDS-PAGE revealed the enzyme to be composed of only one type of subunit, with an apparent molecular mass of 25 kDa (Fig. 1). When the enzyme was boiled in SDS for only 5 min and then analyzed by SDS-PAGE, bands at 25 and 70 kDa were observed. When the enzyme was boiled longer than 5 min in SDS, the 70-kDa band slowly disappeared and the 25-kDa band increased in intensity. This property has also been reported for the iron superoxide dismutase from *M. bryantii* (Kirby et al. 1981).

Superoxide dismutases can contain iron, manganese, copper plus zinc, or nickel. The iron and manganese enzymes are phylogenetically related. The superoxide dismutase in anaerobic organisms is generally an iron enzyme. The purified *M. barkeri* enzyme contained iron but not manganese (Table 2).

Table 2 Molecular and catalytic properties of purified superoxide dismutase from *M. barkeri*. The N-terminal amino acid sequence was determined on a 477A protein/peptide sequencer from Applied Biosystems by Dr. D. Linder (University of Gießen, Germany). Iron and manganese were determined by atomic adsorption spectrometry using Zeeman correction. Inactivation of the enzyme by H_2O_2 was determined in 50 mM potassium phosphate pH 7.8 at 24 °C. In time intervals of 10 min, samples were withdrawn and assayed for superoxide dismutase activity under standard assay conditions. For comparison, the iron superoxide dismutase from *Escherichia coli* was inhibited to 50% at 3 mM azide and lost 50% of its activity in 10 min in the presence of 0.5 mM H_2O_2

Apparent molecular mass	70 kDa (gel filtration) 25 kDa (SDS/PAGE)
N-terminal sequence	AKELYKLP(?)LKFGYGD LAPYISE(E)QL(K)(L)HHD (K)(H)(H)QAYV(T)N
Metal content	Fe 0.8 mol/mol subunit Mn <0.1 mol/mol subunit
Isoelectric point	5.6 (calculated)
Specific activity	1500 U/mg
Inhibition by	
KCN	No
NaN_3 ($[I]_{0.5v}$)	10 mM
Inactivation by H_2O_2 (0.5 mM)	50% in 60 min
Active site sequence	$H^{29}X_3H^{33}H^{34}X_2Y^{37}X_{39}H^{77}X_3W^{81}$ $X_{56}W^{128}X_{34}D^{163}X_3H^{167}X_1Y^{169}$

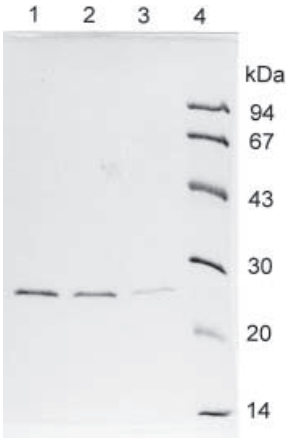

Fig. 1 SDS/PAGE of purified superoxide dismutase from *Methanosarcina barkeri*. Protein was separated on a 12% polyacrylamide gel and subsequently stained with Coomassie brilliant blue. *Lane 1* 1.5 μg purified enzyme, *lane 2* 1.0 μg, *lane 3* 0.3 μg, *lane 4* low molecular mass standards (Amersham Pharmacia Biotech). The protein was boiled for 20 min in SDS prior to electrophoresis (see text)

Iron superoxide dismutases are inhibited by azide and inactivated by H_2O_2. Copper/zinc and nickel superoxide dismutases are inhibited by cyanide and inactivated by H_2O_2 (Youn et al. 1996). Manganese superoxide dismutases are generally not affected by either of these compounds (Takao et al. 1991). Superoxide dismutase from *M. barkeri* was inhibited by azide and H_2O_2 but to a lessr extent than the iron superoxide dismutase from *E. coli* (Sigma), which we used as control (Table 2). A relative insensitivity to azide and H_2O_2 has also been reported for the iron superoxide dismutases from *Methanobacterium thermoautotrophicum* and *Sulfolobus solfataricus* (Takao et al. 1991; Yamano and Maruyama 1999). Superoxide dismutase from *M. barkeri* was not inhibited by 10 mM cyanide, which is a property shared among all iron and manganese superoxide dismutases.

The N-terminal amino acid sequence (Table 2) was determined by Edman degradation and was used to clone and sequence the encoding gene. The determined and deduced amino acid sequences only differed in the N-terminal methionine. The *sod* gene sequence predicts that the enzyme without the N-terminal methionine has a subunit molecular mass of 23,814 Da. The deduced amino acid sequence is most similar (57% identical) to that of the iron superoxide dismutase from *M. thermoautotrophicum* (Takao et al. 1990; Meile et al. 1995) and contains a sequence (Table 2) which is conserved in the active site region of all iron- and manganese superoxide dismutases analyzed to date (Parker and Blake 1988a, b). This sequence contains amino acids involved in metal ligation, as revealed by their crystal structures (Stallings et al. 1983; Parker and Blake 1988b; Lim et al. 1997).

The homologous probe used to screen for the *sod* gene was used for Southern hybridizations. In each restriction digest (*Acc*I, *Hin*dIII, *Bam*HI, *Kpn*I, *Eco*RI, *Eco*RV, or *Pst*I) of genomic DNA, only one band was observed, in-

dicating that the genome of *M. barkeri* harbors only one *sod* gene.

The comparison of the amino acid sequences of all known iron- and manganese superoxide dismutases revealed that the iron superoxide dismutase from *M. barkeri* is more closely related to the enzymes from other archaea than to those from bacteria and eukarya. The finding indicates that a *sod* gene was probably already present in archaea before differentiation into the different branches occurred. The situation is thus different from that of the *kat* gene, which, based on sequence comparisons and codon usage differences, was probably acquired by the methanogen only relatively recently by lateral gene transfer.

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