Molecular cloning and sequence analysis of the gene of the molybdenum-containing aldehyde oxido-reductase of *Desulfovibrio gigas*

The deduced amino acid sequence shows similarity to xanthine dehydrogenase

Ulrich THOENES¹,², Orfeu L. FLORES¹, Ana NEVES¹, Bart DEVREES³, Jozef J. VAN BEEUMEN⁴, Robert HUBER³, Maria J. RODRIGUES-POUSADA⁴, Jean LeGALL⁵, José J. G. MOURA⁴ and Claudia RODRIGUES-POUSADA¹

¹ Instituto Gulbehan de Ciencia, Laboratório de Genética Molecular, Oeiras, Portugal
² Max-Planck-Institut für Biochemie, Martinsried, Germany
³ Vakgroep Biochemie, Fysiologie en Microbiologie, Gent, Belgium
⁴ Instituto de Tecnologia Quimica e Biológica, Oeiras and Instituto Superior Tecnico, Dep. Quimica, Lisboa, Portugal
⁵ Departamento de Quimica, FCT, Universidade Nova de Lisboa, Monte da Caparica, Portugal
⁶ Department of Biochemistry, University of Georgia, Athens, Georgia, USA

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In this report, we describe the isolation of a 4020-bp genomic *PstI* fragment of *Desulfovibrio gigas* harboring the aldehyde oxido-reductase gene. The aldehyde oxido-reductase gene spans 2718 bp of genomic DNA and codes for a protein with 906 residues. The protein sequence shows an average 52% (∓ 1.5%) similarity to xanthine dehydrogenase from different organisms. The codon usage of the aldehyde oxidoreductase is almost identical to a calculated codon usage of the *Desulfovibrio* bacteria.

The molybdenum enzymes are ubiquitous proteins in a variety of species e.g. bacteria, plants, animals and man. They are classified according to their molybdenum cofactor. The nitrogenases contain the iron-molybdenum cofactor (FeMoco) as described recently [1, 2]. All other known enzymes contain the molybdopterin cofactor (Moco). They catalyze redox reactions like xanthine dehydrogenase, sulphite oxidase, nitrate reductase, dimethylsulfoxide reductase and formate dehydrogenase. Additional cofactors may be present in the molybdenum enzymes; i.e. flavin, b-type cytochrome and iron-sulphur centers. The molybdenum iron-sulphur protein (MOP) first described by Moura et al. [3] is an aldehyde oxidase [4] from *Desulfovibrio gigas*. *D. gigas* is a sulphate-reducing, strictly anaerobic Gram-negative bacterium [5]. MOP contains two [2Fe-2S] centers as additional cofactors and has a molecular mass of approximately 100 kDa [6]. The first 34 N-terminal amino acids of MOP have been determined by amino acid sequence analysis [6]. A related protein has been isolated from *D. desulfuricans* ATCC 27774 (our unpublished results), but no sequence information is available. So far 25 proteins of the *Desulfovibrio* family are known by their primary structure. The genes of 22 proteins have been isolated.

In this work, we describe the isolation and sequence analysis of a gene coding for MOP. The deduced amino acid sequence of MOP is compared with other proteins containing the Moco cofactor. The codon usage of the MOP gene is compared to other genes of *D. gigas* and other species of *Desulfovibrio*.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

*D. gigas* (NCIB 9332, DSM 1382) from Deutsche Sammlung von Mikroorganismen und Zellkulturen was grown anaerobically on Defined Multipurpose Medium as described by Widdel [7]. A 250-ml liquid culture was grown at 30°C for three days.

Isolated DNA fragments were cloned into the polylinker of *pUC19* [8] and used for transformation of *Escherichia coli* strain JM109 [9]. Competent cells were prepared according to standard protocols [10].

**Preparation and analysis of DNA**

*D. gigas* genomic DNA was isolated as described elsewhere [11]. Plasmid DNA was prepared using the plasmid purification kit Qiagen (Diagen). For isolation and characterization of the gene of MOP *D. gigas* DNA was digested with *PstI* and fragments of 3.5–4.5 kbp, positively hybridizing with an oligonucleotide derived from the N-terminus in a Southern-blot analysis were cut from the gel. DNA was extracted using the gel-extraction kit (Diagen). DNA was precipitated and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). About 60 ng DNA were ligated to 30 ng *pUC19* which had been linearized with *PstI* and dephosphorylated with alkaline phosphatase prior to ligation. Recombi-
nent plasmids were used to transform JM109 and plated on Luria-Bertani ampicillin plates containing 1 mM isopropyl β-D-galacto-pyranoside and 150 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galacto-pyranoside (X-gal). Positive clones were selected and grown in overnight cultures.

Plasmids were isolated as described above, digested with PsiI, and electrophoresed on 0.8% agarose gels. For further analysis, the resulting plasmid pMOP1, positively hybridizing in Southern-blot analysis, was digested with EcoRI and with EcoRI/SacI. The fragments obtained were subcloned into pUC19, resulting in pMOP2 to pMOP6. Fragments were also cloned in M13mp18/mp19.

The DNA sequences of the oligonucleotides (Table 1) were deduced from the peptide sequences of the N-terminus and also making use of primers which were derived from parts of the sequence after sequencing (Table 1). The 984-bp labeled 981-bp EcoRI fragment it was 65°C.

Membranes were either washed in 6× NaCl/Cit at 25, 30 and 40°C (DO1), or at 25, 28 and 35°C (DO2) each for 10 min. Membranes were washed for 10 min in 2× NaCl/Cit, 0.1% SDS at approx. 50°C when DNA fragments were used as probes. Plasmids were sequenced using the supplied primers for the M13/pUC-system (Pharmacia and Boehringer) and also making use of primers which were derived from the sequence after sequencing (Table 1). The 984-bp and 1400-bp EcoRI fragments were also cloned in the replicative form of M13mp19. Double-stranded as well as single-stranded DNA was sequenced with the dideoxy-chain-termination procedure [13] using the T7-sequencing kit (Pharmacia). To improve the gel reading of the C-terminal fragments which are rich in G/C, the terminal deoxyribonucleotidyl transferase technique without dGTP analogues was used. Sequencing experiments using analogues of dGTP (dITP and 7-deazaGTP were used in parallel) were performed in addition whenever a compression was suspected.

Computer methods

DNA and protein analysis were performed either on a DEC-VAX computer using the UWGCC-system (Genetics Computer Group) or on a MS-DOS PC using the DNASIS-program (Pharmacia LKB).

Peptide sequencing

Internal sequences of MOP were obtained using two different digests, 8 nmol native protein, purified as described [14], were used for the digestion with Lysobacter enzyme-
Table 2. List of Lys-C protease proteolytic and acid hydrolysis peptides of MOP. Molecular mass is only shown in those cases where it was determined by mass spectroscopy. Numbers 1, 2, 12, 13, 21, 22 are overlapping peptides.

<table>
<thead>
<tr>
<th>Number</th>
<th>Location</th>
<th>N-terminal sequences found by peptide sequencing: (These do not always match the corresponding peptide sequence deduced from the DNA sequence)</th>
<th>Molecular mass (experimental by MS) (calculated from DNA deduced peptides, range in brackets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-term</td>
<td>1−34</td>
<td>MIQKVTNVGIEQNLFDVDAEALLSDVL(R)(Q)−LT</td>
<td>1760.62 (54−69) 1760.00 (54−69)</td>
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<tr>
<td>1</td>
<td>54−68</td>
<td>GKVVRATVKKV</td>
<td>2671.46 (65−90) 2670.42 (65−90)</td>
</tr>
<tr>
<td>2</td>
<td>66−89</td>
<td>GVA(D)AQIITTEVGQOPENL?(P)?(L)?</td>
<td>2360.27 (133−132) 2361.14 (133−132)</td>
</tr>
<tr>
<td>3</td>
<td>113−130</td>
<td>GSLDITNAFPS?(E)(E)(V)(V)?(DF)(FDQ)(?)</td>
<td>1583.04 (144−159) 1583.87 (144−159)</td>
</tr>
<tr>
<td>5</td>
<td>160−167</td>
<td>KPEI(D)LEFK</td>
<td>1214.88 (169−179) 1216.61 (169−179)</td>
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<tr>
<td>6</td>
<td>169−178</td>
<td>MPADG?(E)(GS)(NLN)</td>
<td>1001.15 (180−188) 1001.57 (180−188)</td>
</tr>
<tr>
<td>7</td>
<td>180−188</td>
<td>YPRPTVAK</td>
<td>1422.60 (189−202) 1421.75 (189−202)</td>
</tr>
<tr>
<td>8</td>
<td>189−200</td>
<td>V?(G)(T)?Y?GADLG</td>
<td>1000.80 (100−101) 1001.57 (100−101)</td>
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<tr>
<td>9</td>
<td>203−208</td>
<td>MPAP−LI−FP</td>
<td>3216.32 (224−243) 3216.63 (224−243)</td>
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<tr>
<td>10</td>
<td>217−222</td>
<td>(V)SAANN</td>
<td>1001.15 (180−188) 1001.57 (180−188)</td>
</tr>
<tr>
<td>12</td>
<td>245−257</td>
<td>VKGKRNITGLITF</td>
<td>3795.54 (389−424) 3792.98 (389−424)</td>
</tr>
<tr>
<td>13</td>
<td>249−259</td>
<td>(NA)F(GG)LIT(T)(P)(Y)?</td>
<td>3909.97 (425−459) 3909.97 (425−459)</td>
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<tr>
<td>14</td>
<td>267−274</td>
<td>RPIIL6DEK</td>
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<td>275−284</td>
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<td>16</td>
<td>289−308</td>
<td>?EANARAAEAKV?KVPLELP</td>
<td>1105.38 (160−168) 1106.25 (160−168)</td>
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<td>310−329</td>
<td>YMSGPA(W)(E)(L)(T)?P?GT(P)</td>
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<td>389−403</td>
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<td>425−443</td>
<td>FSPITSEALV(A)AM?(TG)(R)??P</td>
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<td>3384.65 (366−395) 3386.73 (366−395)</td>
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<tr>
<td>23</td>
<td>596−602</td>
<td>YQAALK</td>
<td>1105.38 (160−168) 1106.25 (160−168)</td>
</tr>
<tr>
<td>24</td>
<td>721−732</td>
<td>PGGGY(C)TYDG(LT)K</td>
<td>1886.70 (1760−1760) 1886.90 (1760−1760)</td>
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<tr>
<td>25</td>
<td>736−748</td>
<td>KPTKIGN?TA?G</td>
<td>1208.64 (849−858) 1208.85 (849−858)</td>
</tr>
<tr>
<td>26</td>
<td>829−841</td>
<td>(R)ATLVGAG(PF)(PF)IPNI(Y)GL</td>
<td>2417.80 (859−882) 2416.90 (859−882)</td>
</tr>
<tr>
<td>27</td>
<td>842−845</td>
<td>QIPD</td>
<td>1208.64 (849−858) 1208.85 (849−858)</td>
</tr>
<tr>
<td>28</td>
<td>849−857</td>
<td>IVYVNHPRP</td>
<td>1208.85 (849−858) 1208.64 (849−858)</td>
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<tr>
<td>29</td>
<td>859−872</td>
<td>(G)PGFAGVGTLPLT</td>
<td>2416.90 (859−882) 2417.80 (859−882)</td>
</tr>
</tbody>
</table>

genes lysyl-C protease (Wako; E/S 1:100). The peptide mixture was separated on a RoSil TMS 3-μm column (Bio-Rad) using a DuPont Instruments separation system consisting of a 870 chromatographic pump, a 8800 gradient controller and an ultraviolet spectrophotometer. Precipitation was observed after this digestion. The precipitate was further digested by partial acid hydrolysis with 2% formic acid at 108°C for 2 h. We also performed partial acid hydrolysis on the total protein under the same conditions as for the precipitate. Peptide mixtures in all cases were chromatographed on an PTC C18 column using a 140A solvent delivery system and 1000S diode array detector (all Applied Biosystems).

Sequence analysis was performed on either a 475A or a 477A pulsed-liquid protein sequencer with on-line analysis of the phenylthiohydantoin–amino-acids on a 120A analyser (all Applied Biosystems):

Mass spectra

The electrospray mass spectra were obtained on 100 pmol sample dissolved in 10 μl 50% acetonitrile/1% formic acid in water. Samples were introduced into the mass spectrometer using a flow rate of 6 μl/min, pumped by a 140A solvent delivery system (Applied Biosystems). The mass spectrometer was a VG BIO-Q triple quadrupole instrument (Fisons Instruments). Spectra were obtained scanning m/z values over 600−1500 during 9 s using only the first quadrupole as the mass analyser. Scans were accumulated for 3 min. Calibration was performed using horse myoglobin (Sigma).

RESULTS AND DISCUSSION

Isolation and analysis of the gene encoding the MOP of D. gigas

The N-terminal sequences determined for all 29 peptides obtained from the MOP digest using Lys-C protease are summarized in Table 2, together with 34 amino acids of the N-terminus of MOP [6]. The sequence data were used to construct oligonucleotides. These degenerated oligonucleotides were designed to be complementary to the combinations of mRNA that could encode the protein. We have therefore taken into account the codon usage of D. gigas as deduced from the genes for desulforedoxin [15], flavodoxin [16] and [NiFe]hydrogenase [17].

Starting with the methionine of the N-terminus, a 45-bp-long degenerated oligonucleotide was synthesized (DO1). Three additional degenerated oligonucleotides (DO2−DO4) were synthesized based on the information obtained from the
sequence data of the ‘Lys-C peptides’. Additional oligonucleotides designated a–p (Table 2, Fig. 2) were synthesized for complete sequence analysis.

As an initial step to clone the MOP gene from *D. gigas*, we have examined the ability of the probe D01, (see Table 2) to hybridize the genomic DNA. DNA was thus digested with *SacI, PstI, BamHI, HindIII* and *BamHI*, and the fragments were separated by electrophoresis on an 0.8% agarose gel. The DNA fragments were transferred to nylon membranes and hybridized with labeled D01 as described in Materials and Methods. The hybridization pattern is shown in Fig. 1a, revealing one single band in each lane. The expected size of the gene of MOP is about 2500-2600 bp, corresponding to the band 1 in lane 5 (*EcoRI* digest).

As we did not expect that the entire gene was located on this fragment, we also isolated longer fragments corresponding to the band at approximately 4 kb in lane 2 (*PstI* digest). The DNA fragments from the region 3.5–4.5 kb were cut from the preparative gel and subcloned into pUC19. 40 white colonies were obtained and 20 randomly selected which were further analyzed by digestion with *PstI*. After Southern blotting and hybridization using labeled D01, two of them labeled, which were identical. The clone was designated pMOP1. Plasmid pMOP1 was further digested with *EcoRI, EcoRI–HindIII, EcoRI–PstI, EcoRI–SacI, HindIII–SacI, PstI–SacI* and *SacI*. The partial restriction map of pMOP1 deduced from the result is shown in Fig. 2. To localize the start codon of the gene on one of the restriction fragments, the gel was Southern blotted. The resulting membrane was hybridized with labeled oligonucleotide D01 as probe. The start codon was identified on the *EcoRI–PstI* fragment (approximately 1440 bp). The coding direction of the gene in pMOP1 was identified by hybridizing a similar Southern blot with labeled DO2 as probe. DO2 is localized on the 1166-bp *PstI–SacI* fragment. In this way, the coding direction of the MOP gene on the cloned *PstI* fragment was determined as shown in Fig. 2. Using the results from the described digests five different fragments of pMOP1 were subcloned generating pMOP2–pMOP6.

- pMOP2: approx. 1440 bp (*EcoRI–PstI*)
- pMOP3: 981 bp (*EcoRI–EcoRI*)
- pMOP4: 1597 bp (*EcoRI–PstI*)
- pMOP5: 431 bp (*EcoRI–SacI*)
- pMOP6: 1166 bp (*SacI–PstI*)

pMOP1–pMOP6 were used to determine the complete DNA sequence of the MOP gene including its flanking regions. The total length of the *PstI* fragment was determined to be approximately 4020 bp harboring the 2721-bp long coding region of MOP. The sequence is shown in Fig. 3, together with the resulting amino acid sequence of MOP and the location of the peptides. Sequencing was started with pMOP1 using the reverse primer and the universal primer (see Table 1). Within the resulting sequence information, none of the given peptides could be localized. The sequencing was continued with clone pMOP2 using the same primers as well as oligonucleotide D01. Here the positions of peptides 1 and 2 could be found. Using oligonucleotide D03 as sequencing primer, the N-terminus was confirmed and precisely located. The site of the start codon was found to be 502 bp upstream of the *EcoRI* site of pMOP2. During further improved sequencing reactions, the peptides 3–5 could also be discovered in the part of the gene cloned in pMOP2.
Sequence analysis was continued with pMOP3, again using the universal primer and the reverse primer. About two thirds of the sequence part cloned into pMOP3 could be determined using those two primers. The missing part between them could be sequenced on both strands using primer b and primer c.

In this 981-bp-long part of the sequence, the positions of the majority of the peptides, namely peptides 6-20, were found (see Table 2).

Using pMOP4 and pMOP5, the sequence of the smallest cloned part of the gene, the 431-bp EcoRI-Sacl fragment was determined using the universal primer, the reverse primer, primers i and k. In this fragment, three of the given peptides namely 21-23 were found.

Finally, the 1166-bp Sacl-PstI fragment in pMOP6 was sequenced using the reverse, the universal primer, the primers f, l, m, n and the degenerated oligonucleotide DO3. In this sequence part, the positions peptides 24-29 and the stop codon of the MOP protein were found.

In order to confirm the two EcoRI sites and the Sacl site, specified regions of pMOP1 were sequenced using the primers a, d and e.

As outlined in Table 2, some amino acid sequences obtained from the peptides did not exactly match the DNA sequence. In those cases the molecular masses of the peptides were determined by mass spectroscopy. These corresponded very closely to the values deduced from the gene sequence, providing good evidence for its accuracy. All the given peptides were found in the deduced amino acid sequence of MOP. They are unequally distributed over the whole sequence. Most of them are clustered in two regions extending from P144-P329, harboring peptides 4-17, and from H829-T872 containing peptides 26-29. This may indicate that Lys in these regions are more exposed and therefore more accessible for the Lys-C protease.

The molecular mass of MOP was also determined by electrospray ionisation mass spectroscopy to be 97210.7 Da. This is only 76.18 Da smaller than the value of 97286.88 Da deduced from the gene, a difference of less than 0.1% in accord with the expected accuracy of the mass spectrometric method [18]. These values are also in good agreement with electrophoresis and sedimentation data [6].

Codon usage

The DNA-sequence information was used to examine the codon usage of the MOP gene. It was compared to the codon usages of the three known genes of *D. gigas* and of all known genes of the *Desulfovibrio* bacteria (22 genes). Considering twofold degeneracy, MOP *D. gigas* and *Desulfovibrio* show identical codon usage, with the exception of Gln and Asp. At higher degeneracy the two most often used codons are identical with the exception of Arg, Ala and Gly where only the first most used codon is the same.

Overall, the frequency of the two most often used codons is in a range 50-70%. Major differences in the codon frequencies are only present in the remaining codons which are not used often. It should be noted that the relative frequency of the triplets AGA(R), ATA(I), ACA(T), AGT(S), TTG TTA CTA(L) is low (below 10%) and of GAA(E), GAT(D), GCC(A), AAG(K), AAC(N), TGC(C), TTC(F); CAA(Q) is high (above 50%) compared to the other triplets coding for the same amino acid.

Genomic organization

To confirm the Southern-blot pattern of DO1, additional Southern-blot analysis was performed. We now used the 981-bp EcoRI-fragment (Fig. 2) from pMOP3 which was labeled using Klenow fragment of DNA polymerase I. The results are shown in Fig. 1b.

As the EcoRI fragment is part of all restriction fragments containing the MOP gene, the hybridization pattern is similar as that of DO1 (Fig. 1a), with the exception of EcoRI itself. Here, we expect to have hybridization with the intra-gene fragment, while DO1 hybridizes with a larger 5'-fragment. Due to the longer and not degenerated DNA fragment used as probe, the bands are much more intense.
Our results clearly indicate that the aldehyde oxidoreductase is present as a single copy gene on the genome of *D. Gigas*.

Further sequence analysis of the region upstream of the coding region of MOP (not shown) did not result in any information about promoter-like regions. This data are strong indications for a polycistronic organization of the MOP gene.

**Sequence alignment with xanthine dehydrogenase**

The deduced amino acid sequence of MOP was used in a database search. Four different xanthine dehydrogenases from rat, mouse, *Drosophila melanogaster* and *Caliphora vicina* were found to give the highest similarity scores. The average similarity between MOP and the xanthine dehydrogenases was found to be 52% (25.6% identity). In xanthine dehydrogenases [19], the binding of molybdenum to the protein is mediated by the perin cofactor. A NAD/FAD-binding domain is suggested to be located N-terminal to the putative Mo-pterin-binding domain. In addition, two different [2Fe-2S] domains are located in the N-terminal segment. The four amino acid sequences of xanthine dehydrogenase were aligned with MOP.

The result can be seen in Fig. 4. In two regions, 16–179 and 627–1364 (general numbers) the sequences show particularly high similarity. The segment 180–626 encompasses the putative flavin-binding domain in xanthine dehydrogenase. In MOP for which no flavin cofactor has been demonstrated, this region is absent except for four short peptide regions of only 12–18 amino acids which are positioned rather arbitrarily in Fig. 4. Due to the lack of sequence information, it was not possible to compare MOP to the aldehyde oxidase from rabbit liver [20] containing molybdenum, non-heme iron and FAD. Furthermore, a comparison between
MOP and a putative FAD-free aldehyde oxidase containing tungsten an Fe/S-centers from *T. litoralis* [21] could not be carried out because of the missing sequence information.

In the N-terminal region of the alignment, eight well conserved cysteines were found. The first four cysteines (58, 63, 66 and 88; general numbers) carry the signature of plant ferredoxins (*Aphanothece* *sacrum* and *Spirulina platensis* [22, 23] where the four cysteines ligating the iron atoms are at the positions 41, 46, 49 and 79 [23]. They might, therefore, be considered to bind the first [2Fe-2S] center. The second
Fig. 4. Alignment of MOP and xanthine dehydrogenases from mouse (MOUSXDH), rat (RATXDH), C. vicina (CVXDH), D. melanogaster (DMXDH). Conserved amino acids are marked by black background. The cysteins binding the [2Fe-2S] centers are marked by "o".

four well conserved cysteines (129, 132, 166, 168; general numbers) might bind to the second [2Fe-2S] center, but do not show similarity to other structurally defined iron-sulfur proteins. In the C-terminal region of the alignment, the putative molybdo-pterin-binding domain [19] of the xanthine dehydrogenases (747–1256; general numbers) aligns well with the MOP sequence, suggesting similar structural cofactor-binding properties. Significant similarities to other prokaryotic molybdopterin-containing proteins [19] were not found. It's similarity to xanthine dehydrogenase suggests that MOP might have a similar enzymic activity to that already reported by some of us [4]. Xanthine dehydrogenase cata-
lyzes the oxidation of xanthine to hypoxanthine and subsequent ly to uric acid with simultaneous reduction of NAD to NADH. This so-called D-type activity (NAD reductase activity) in the xanthine dehydrogenase may be converted irreversibly or reversibly to O-type activity (O2 reductase activity) in xanthine oxidase [24]. Irreversible conversion can be achieved by treatment of xanthine dehydrogenase with tryptophan, resulting in decreased NAD-binding activity of xanthine dehydrogenase [25]. The lack of NAD binding in MOP would similarly suggest an O-type activity for this enzyme. However, D. gigas is a strictly anaerobic bacterium which excludes the O-type reaction. The physiological electron acceptor of MOP is not known and may be NAD or menaquinone which is present in D. gigas [26] and assumed to be the acceptor for the malate dehydrogenase of the Desulfovibrio bacterium [27]. It may be that a separate cofactor-binding protein takes the role of the flavoprotein domain of xanthine dehydrogenase in D. gigas.

However, a flavo-hemoprotein has been purified from D. gigas that reduces O2 to water and this activity is linked to NADH oxidation [28]. It should be noted that, although classified as a strict anaerobe, D. gigas has the capability of synthesizing nucleotide triphosphate from the degradation of polyglucose in the presence of oxygen [29]. Based on the observation that glyceraldehyde is a substrate for MOP, it has been recently proposed that its physiological role is linked to the degradation of polyglucose by D. gigas [30]. The protein is capable of reducing flavodoxin and cytochrome c; H2 can be produced from aldehydes in the presence of hydrogenase, an essential component in this complex electron-transfer chain. Thus, any eventual link between MOP and pyridine nucleotides remains to be demonstrated.

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