A prokaryotic alternative oxidase present in the bacterium *Novosphingobium aromaticivorans*[1]

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Received 1 August 2003; accepted 4 August 2003

First published online 27 August 2003

Edited by Stuart Ferguson

Abstract The alternative oxidase (AOX) is a terminal oxidase present in the respiratory chain of all plants as well as some yeasts and trypanosomes, but has not previously been found in a prokaryote. We have identified an AOX homologue in *Novosphingobium aromaticivorans*, the first AOX found in a prokaryote. We have cloned the gene for the *N. aromaticivorans* AOX and showed it to have a terminal oxidase activity when heterologously expressed in *Escherichia coli*. We have also shown that this novel AOX is expressed in *N. aromaticivorans* cells, and that its expression level is greatly influenced by the oxygen level and carbon source of the growth media.

Key words: Alternative oxidase; Prokaryote; Respiration; *Novosphingobium aromaticivorans*

1. Introduction

The alternative oxidase (AOX) is an alternative terminal electron acceptor in the respiratory chain of all higher plants as well as some yeasts, algae and trypanosomal species. AOX catalyzes the conversion of ubiquinol and molecular oxygen to ubiquinone and water and is normally found in the inner membrane of mitochondria. AOX does not pump protons and instead the energy available in the pool of reductants, normally utilized by complex III and IV of the respiratory chain, is lost as heat. AOX is able to maintain oxygen consumption when cytochrome *c* oxidase is inhibited by e.g. cyanide. In most organisms the exact in vivo function of AOX is not yet clear although several plausible theories exist. One possibility for a general function is that AOX keeps the respiratory chain components from being over-reduced when energy is abundant. This helps to balance carbon metabolism and electron transport at these conditions [1]. An over-reduced respiratory chain is also potentially harmful to the organism, when it can generate reactive oxygen species [1,2]. In some plants AOX is up-regulated to generate heat in specialized tissues [3].

The AOX has been shown to have an iron-dependent activity [4,5] and the protein has been suggested to be a diiron carboxylate protein [6]. The presence of the diiron site has recently been shown using EPR spectroscopy [7]. The plant AOXs are dimeric while the fungal AOXs are monomeric [8]. A plausible model of the active site and a suggested membrane-binding region of AOX have been proposed [9,10]. This model suggests that the protein is an interfacial integral membrane diiron protein. For a recent review on this family of enzymes see Berthold et al. [11]. We have regularly conducted BLAST searches of both finished and unfinished bacterial genome sequences at the http://www.ncbi.nlm.nih.gov/web page in an attempt to find a bacterial AOX. Some time ago we found in the *Novosphingobium aromaticivorans* genome under processing a gene homologous to the plant AOXs. (This gene has recently been deposited by the NCBI microbial genomes annotation project with accession number ZP_00095227). *N. aromaticivorans* was first isolated from a sample obtained from a depth of 410 m at a drill site in South Carolina. The bacterium was shown to be able to utilize a variety of aromatic hydrocarbons as sole carbon and energy sources [12] and as other bacteria of the genus *Novosphingobium* it is Gram-negative and a strict aerobe.

2. Materials and methods

2.1. Materials

N. aromaticivorans (DSM No. 12444) was obtained from DSMZ, Braunschweig, Germany. The *Escherichia coli* strains DH5α and TOP10 were used during cloning (Invitrogen). The vector pUNI-10 and the host strain BW23474 were gifts from Stephen Elledge (Baylor College of Medicine, Houston, TX, USA). The *Sauromatum guttatum* AOX antibody was obtained from Thomas Elthon (University of Nebraska, Lincoln, Nebraska, USA). The plasmids pHB-MALc2X, pHB-MALc2M, and pAtAOn3 are described previously [7,13]. The expression strain C43 (DE3) was obtained from J.E. Walker (Medical Research Council Laboratory).

2.2. Cloning and expression

N. aromaticivorans AOX (NaAOX) was cloned into pUNI-10 using the method of in vivo recombination [14]. *N. aromaticivorans* cells were used as template in the PCR reaction. The cloning was carried out in the same way as described by Stenmark et al. [15] using the forward primer (gttatctggaattcatatgatcccaccgttcatcgatc) and the reverse primer (gcggccgcggatccttatcaggcagccttcttcc). One hundred and forty bases extra, upstream of the start codon, were cloned using the alternative forward primer (gttatctggaattcatatgacgacgaatgccacagc) to verify the sequence upstream of the start codon. These two vectors were named pUNI-NaAOX and pUNI-NaAOX-big, and the inserts were sequenced. pUNI-NaAOX was recombined in vitro with the

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[1] Supplementary data associated with this article can be found at 10.1016/S0014-5793(03)00920-7

Abbreviations: AOX, alternative oxidase; MBP, maltose binding protein; NaAOX, *Novosphingobium aromaticivorans* alternative oxidase

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00920-7

high level expression vector pHB-MALc2X and the low level expression vector pHB-MALc2M as previously described for other pUNI vectors [15] forming the expression vectors pNaAOX-C2X and pNaAOX-C2M. Successful recombination was verified by restriction digest. The expression vectors were transformed into C43 (DE3). A colony was picked and grown in 25 ml LB with 60 μg/ml ampicillin in a 250 ml flask to an OD_{600} of 0.7–0.8. The cultures were then cooled down to 18°C and induced with 0.3 mM IPTG. The cells were harvested after 18 h of incubation and resuspended in 50 mM Tris–Cl, pH 7.5 containing 2× Complete™ protease inhibitor cocktail (Roche Diagnostics). Cells were broken at an OD_{600} of 20 using sonication. The samples were centrifuged at 18 000×*g* for 10 min to pellet inclusion bodies and unbroken cells. The supernatant was then centrifuged at 200 000×*g* for 90 min to obtain the membrane pellets. These were resuspended in sample buffer [13] to the original volume.

2.3. Complementation assay

The complementation assay was carried out on M63-SG plates as previously described [13,16]. Approximately 2% abnormally large colonies reproducibly appeared on the plates with the induced low expression construct; presumably due to mutations in *E. coli* or the plasmid as was previously seen when isolating the low expressing pHB-MALc2M [13]. These colonies were not used in calculating the average colony size. As a positive control the vector pAtAOn3 was used, which expresses *Arabidopsis thaliana* AOX. The plates were analyzed after 6 days of incubation at 37°C. A large container of water had to be present in the incubator during this time to prevent the plates from drying. The TotalLab software (Amersham Biosciences) was used to analyze colony sizes.

2.4. Growth of N. aromaticivorans and immunoblotting

A single colony of *N. aromaticivorans* was picked from a plate containing modified media 464 DSMZ (plus 1 g $MgSO_4 \times 7H_2O$, 70 mg $CaCl_2 \times 2H_2O$, 5 g NaCl/l H_2O, see http://www.dsmz.de/strains/no012444.htm for DSMZ medias). Two pre-cultures were grown in 15 ml tubes containing 10 ml of minimal media 457 DSMZ, one with 0.5% glucose and one with 30 mM lactate as carbon source. The tubes were incubated without agitation at 37°C for 3 days. These cultures were used to inoculate larger cultures containing the same kind of media. To obtain samples grown at different levels of oxygenation, the cultures were grown in 100 ml flasks with no agitation and in 250 ml flasks with agitation. In all cases the flasks contained 100 ml of media. The flasks were incubated for 3 days at 37°C and then the cells were harvested and broken and the membrane fractions isolated in the same way as for the *E. coli* samples except that the final sample was diluted in 1/4 of the original volume. Ten μl of these four samples and 5 μl of the pNaAOX-C2M *E. coli* membrane sample were run on a 2.5 M urea 10% polyacrylamide gel [17]. The gel was transferred to a nitrocellulose membrane and blocked in 5% fat-free milk, incubated with AOX antibody for 1 h and incubated with peroxidase labeled anti-mouse antibody for 1 h. The protein antibody complex was visualized using chemiluminescence (Super signal west Dura, Pierce). A FlourS ccd camera from Bio-Rad was used. Western blots of uninduced *E. coli* membrane preparations showed no reaction with the AOX antibody. Expression level of the pNaAOX-C2X was analyzed from a Coomassie stained gel. Quantification of the bands on the Western blot and the Coomassie gel was done using the TotalLab software (Amersham Biosciences).

3. Results and discussion

The sequence of the AOX which we found in the partial genome sequence of the Gram-negative bacterium *N. aromaticivorans* is very similar to the sequences of the plant AOX family. It has 58% identity with *A. thaliana* (AOX1a) in a 216 amino acid overlap. All proposed iron ligands and the second sphere ligands making hydrogen bonds to the histidines are conserved in the NaAOX. A sequence alignment is available as supplementary information. Sequencing of the cloned gene and a stretch of approximately 140 bases upstream of the start codon in the *N. aromaticivorans* genome confirmed that our clone is identical with the one present in the database and that our predicted start site is correct. The NaAOX is 229 amino acids long, lacking the N-terminal ~134 amino acids present in the *A. thaliana* protein. The bacterial AOX lacks the whole N-terminal domain that has been proposed to be the site of dimer formation [8] and contains the conserved cysteine located at position 127 in the *A. thaliana* sequence, which has been proposed to be involved in regulatory events [18–20]. The small size of the *N. aromaticivorans* AOX and the constraints of the diiron carboxylate four helix bundle fold makes this protein approach what appears to be the minimal size for a functional AOX. This puts the ubiquinone binding site close to the diiron center and it is possible that the location of the ubiquinone binding site is in a similar location as the substrate binding site of 5-demethoxyquinone hydroxylase (Coq7) [15], i.e. only a few angstroms from the diiron binding site.

We were able to show activity of the NaAOX using a complementation assay where the ability to restore aerobic respiration in a heme-deficient strain of *E. coli* (SASX41B) is analyzed. Since hemes are cofactors of the terminal oxidases of the *E. coli* respiratory chain these cannot be functionally expressed by SASX41B, while the expression of a vector coding functional AOX in SASX41B will permit aerobic growth [16,19,21]. We cloned the NaAOX gene and introduced it into a high level expression vector and a low level expression vector, both with a N-terminal maltose binding protein (MBP) fusion. We introduced our IPTG inducible high and low level expression plasmids into SASX41B and assayed growth on plates as described in Section 2. The induced low level expression construct restores aerobic respiration and the uninduced cells showed no complementation (Fig. 1). The high level expression construct shows complementation from the leaky expression of the uninduced cells, while induction appeared to put too much strain on the cells resulting in that no colonies are formed at all. This behavior is similar to what was seen when using the same vectors to express the *A. thali-*

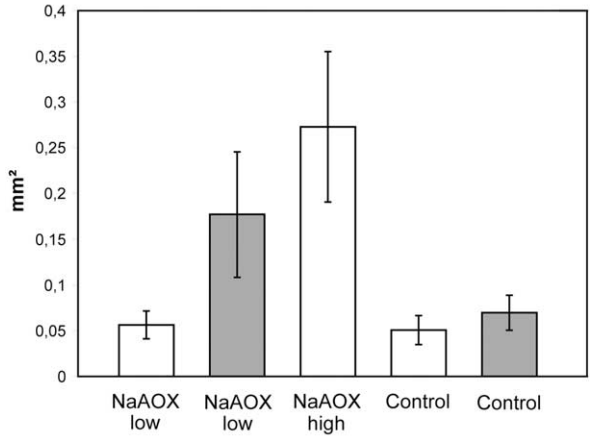

Fig. 1. Colony sizes of the *E. coli* strain SASX41B complemented with NaAOX expressed as a MBP fusion. Both the high level expression construct and the low level expression construct are shown, indicated by 'low' or 'high' below the bars. As a control SASX41B cells with the unrecombined pHBMAL-c2M vector were used. The plates were analyzed after 6 days instead of the normal 3 days; this was necessary because of the slow growth of the colonies. This is likely to be caused by a lower activity of the NaAOX compared to the *A. thaliana* AOX in the *E. coli* system. An area containing 15 colonies was chosen on the different plates. The average size and standard deviation is presented in the diagram. Gray bars indicate that the plates contained 0.1 mM IPTG for induction of expression.

ana AOX in SASX41B [13]. The high level expression variant of the NaAOX MBP fusion expressed to very high levels when it was induced and grown in liquid LB medium. The protein constituted approximately 15% of the protein of the *E. coli* membranes making this construct potentially useful for further structural and biochemical studies.

Why does *N. aromaticivorans* have an AOX gene? To find out under what conditions NaAOX is expressed we used the monoclonal antibody raised against the *S. guttatum* AOX [22]. This was possible since the sequence of the antibody binding site has been determined [23] and is completely conserved to the NaAOX sequence (amino acids 187–198). We grew *N. aromaticivorans* in minimal media using two different carbon sources, glucose and lactate, and at different oxygenation levels. A Western blot of membrane preparations from *N. aromaticivorans* grown under these conditions showed single bands when incubated with the AOX antibody and the different conditions gave very different levels of expression (Table 1 and Fig. 2). The oxygenation level of the cultures had strong effects on the expression level, agitating the culture drastically lowered the expression levels. Also, growth on lactate gave significantly lower expression levels than growth on glucose.

One reason for *N. aromaticivorans* to have acquired this gene is that this bacterium sometime during its lifecycle is exposed to conditions were the normal oxidases are inhibited. Complex I of the respiratory chain is present in the genome of *N. aromaticivorans* and this complex together with the AOX activity would make it possible to pump protons without the use of the normal terminal oxidases. *N. aromaticivorans* is a strictly aerobic bacterium but it prefers microaerobic conditions when grown on minimal media [12]. If AOX was solely functioning as an oxygen scavenger, one would expect it to be expressed at higher levels when the oxygen level is higher. In our study we get the opposite result where expression is higher when the level of oxygen is low. Glucose, which has a higher energy content per carbon atom than lactate, up-regulates AOX in *N. aromaticivorans*. Since *N. aromaticivorans* can utilize a variety of compounds as its sole energy and carbon sources, it is likely that AOX is used to adapt to the imbalances between carbon metabolism and electron transport. AOX could function by lowering the pools of reductants, thereby eliminating the potential inhibition of the carbon metabolism at high energy conditions.

Is the NaAOX a bacterial ancestor of all AOX genes or has it been acquired by bacteria through horizontal gene transfer from eukaryotes? We believe that the very high sequence identity between the NaAOX and the plant AOXs makes it very likely that the gene was, indeed, acquired through horizontal gene transfer directly from plants. Other members of the genus *Novosphingobium* and *Sphingomonas* have plants as their normal habitat [24,25] and horizontal gene transfer between plant and bacteria is not uncommon, especially if the bacterium is a plant pathogen [26].

Table 1
Expression levels of NaAOX in *N. aromaticivorans*

Relative level of expression (%)	Agitation	Carbon source	Lane in Fig. 2
100	No	Glucose	3
~76	No	Lactate	4
~48	Yes	Glucose	2
0	Yes	Lactate	1

The relative levels of expression are presented as percent of the highest level of expression. *N. aromaticivorans* was grown in minimal media with two different carbon sources and with or without agitation to modulate different oxygen levels.

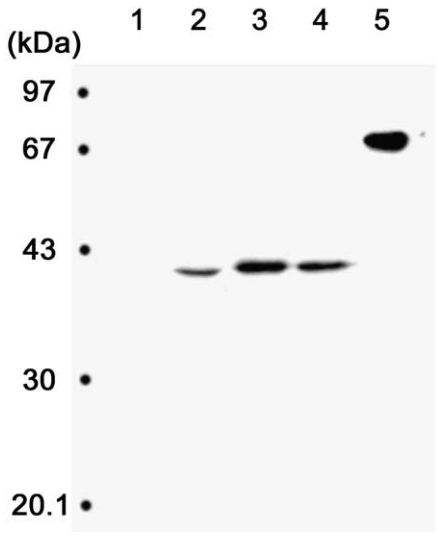

Fig. 2. Western blot of NaAOX expressed in *N. aromaticivorans* cells (lanes 1–4) and expressed as a MBP fusion in *E. coli* (lane 5). Molecular weight standards are shown to the left. Lane 1: cells grown with agitation and with lactate as a carbon source. Lane 2: cells grown with agitation and with glucose as a carbon source. Lane 3: cells grown without agitation and with glucose as a carbon source. Lane 4: cells grown without agitation and with lactate as a carbon source. Lane 5: membrane fraction of *E. coli* expressing the MBP NaAOX fusion; the low expressing construct was used. The NaAOX has an expected mass of 26 kDa but runs at 41 kDa. The MBP fusion expressed in *E. coli* has an expected mass of 64 kDa but runs at 77 kDa. Membrane proteins are known to often run abnormally on SDS–PAGE gels [27]. This is the most likely explanation for our observed mass difference, since the difference between the MBP fusion's expected and observed mass is almost the same as the difference between the observed and expected mass of the NaAOX as expressed in *N. aromaticivorans*.

4. Conclusions

In the present study we show that the NaAOX has a terminal oxidase activity and that its expression is regulated in *N. aromaticivorans* cells in response to growth conditions. We thereby demonstrate that there is a role for AOX also in the bacterial kingdom. *N. aromaticivorans* now provides an additional and potentially more simple system for studies of the in vivo function of AOX. The high level expression of the NaAOX MBP fusion obtained in the present study could also provide recombinant protein as a source for more detailed biochemical and structural studies of this novel AOX.

Acknowledgements: The authors would like to thank Tobias Cornvik, Martin Högbom and Susanne Fröjdendal for helpful discussions and technical assistance. We would also like to acknowledge the Swedish Research Council for financial support.

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