The Terminal Quinol Oxidase of the Hyperthermophilic Archaeon *Acidianus ambivalens* Exhibits a Novel Subunit Structure and Gene Organization

WERNER G. PURSCHKE, 1*, CHRISTIAN L. SCHMIDT, 1 ARND PETERSEN, 2 AND GÜNTER SCHÄFER 1

Institute of Biochemistry, Medical University of Lübeck, 23538 Lübeck, 1 and Research Center Borstel, 23845 Borstel, 2 Germany

Received 8 October 1996/Accepted 6 December 1996

A terminal quinol oxidase has been isolated from the plasma membrane of the crenarchaeon *Acidianus ambivalens* (DSM 3772) (formerly Desulfurolobus ambivalens), cloned, and sequenced. The detergent-solubilized complex oxidizes caldariella quinol at high rates and is completely inhibited by cyanide and by quinolone analogs, potent inhibitors of quinol oxidases. It is composed of at least five different subunits of 64.9, 38.0, 18.8, and 7.2 kDa; their genes are located in two different operons, *doxB*, the gene for subunit I, is located together with *doxC* and two additional small open reading frames (*doxE* and *doxF*) in an operon with a complex transcription pattern. Two other genes of the oxidase complex (*doxD* and *doxA*) are located in a different operon and are cotranscribed into a common 1.2-kb mRNA. Both operons exist in duplicate on the genome of *A. ambivalens*. Only subunit I exhibits clear homology to other members of the superfamily of respiratory heme-copper oxidases; however, it reveals 14 transmembrane helices. In contrast, the composition of the accessory proteins is highly unusual: none is homologous to any known accessory protein of cytochrome oxidases, nor do homologs exist in the databases. *DoxA* is classified as a subunit II equivalent only by analogy of molecular size and hydrophobicity pattern to corresponding polypeptides of other oxidases. Multiple alignments and phylogenetic analysis of the heme-bearing subunit I (DoxB) locate this oxidase at the bottom of the phylogenetic tree, in the branch of heme-copper oxidases recently suggested to be incapable of superstoichiometric proton pumping. This finding is corroborated by lack of the essential amino acid residues delineating the putative II*-pumping* channel. It is therefore concluded that *A. ambivalens* copes with its strongly acidic environment simply by an extreme turnover of its terminal oxidase, generating a proton gradient only by chemical charge separation.

The terminal oxidases of all oxygen-respiring organisms belong to a superfamily of membrane-residing heme-copper oxidases (9, 54). They function as primary energy converters commonly coupling the reduction of oxygen to water, with the electrogenic translocation of protons across the membrane. Thereby, depending on the organism, either cytochrome c or reduced quinones may serve as electron donors (44). The three-dimensional (3D) structures of the mitochondrial enzyme (61) as well as that from the bacterium *Paracoccus denitrificans* (28) have been resolved, revealing that despite substantially different complexity, their functional core structures are extremely similar with regard to subunits I, II, and III. While in subunit I the low-spin heme a and the binuclear heme-copper center are buried within the canonical 12-helix motif, involving six strictly conserved histidine residues, subunit II in cytochrome c oxidases contains a binuclear mixed-valence copper center which is missing in quinol oxidases (62). Recently, on the basis of protein-based phylogenetic analyses, it was proposed that in contrast to conventional thinking, the origin of terminal oxidases predates the occurrence of oxygenic photosynthesis in evolution (12, 13). This hypothesis was fundamentally supported by oxidase sequences determined from archaea populating the lowest branches of the phylogenetic tree.

The first archaean *aa*₃-type oxidase has been isolated from the extreme thermoacidophile *Sulfolobus acidocaldarius* and characterized (2, 3). It is a quinol oxidase of unusual composition, combining core structures of the two otherwise separated respiratory complexes III and IV (38), as concluded from the cloned genes. By genetic analysis, a second oxidase complex was detected in the same organism and partially characterized (36); it also displayed unusual complexity and gene organization (11, 37). No catalytically active oxidases from halobacteria have been prepared in isolated form; however, DNA-derived sequences of the subunit I homologs clearly revealed them to be members of the superfamily of heme-copper oxidases (17, 40). In general, the terminal oxidases from archaea apparently exhibit a larger diversity among each other with respect to structure and gene organization than other prokaryotic examples.

In this respect, the oxidase of the extreme thermoacidophilic sulfur-metabolizing archaeon *Acidianus ambivalens* deserves special interest. The enzyme has been purified in our laboratory (4) and characterized as a quinol oxidase. *A. ambivalens* is a chemolithoautotrophic archaeon that is alternatively capable of growing anaerobically or aerobically. It derives metabolic energy either from reduction of sulfur by elemental hydrogen or by aerobic oxidation of sulfur to sulfate (67, 68). In the latter case, the *aa*₃-type quinol oxidase, representing the only membrane-residing hemoprotein detectable in significant amounts, is expressed. Neither *b*-type nor *c*-type cytochromes or other respiratory complexes could be found (4). *A. ambivalens* may contain the simplest respiratory system discovered so far, consisting only of a hitherto unknown quinol reductase and the terminal quinol oxidase. Moreover, its phylogenetic position falls even below that of S. acidocaldarius (23); therefore, its...
terminal oxidase is expected to show the shortest evolutionary distance from a hypothetical ‘uroxidase.’

We here report on the isolation of the *A. ambivalens* terminal quinol oxidase as well as the cloning, sequencing, and transcription analysis of the corresponding genes, which resulted in a detailed insight into the molecular structure of this archaean oxidase. The enzyme can clearly be identified as a member of the superfamily of heme-copper terminal oxidases. However, it is extremely distant related even to other archaean oxidases, and the genes are organized in split operons; none of the auxiliary subunits has any known homologs in terms of sequence similarity. The results are discussed with respect to function and evolutionary implications.

**MATERIALS AND METHODS**

**Strain and growth conditions.** *A. ambivalens* (DSM 3772) was grown aerobically at 78°C and an initial pH value of 2.5 as described previously (4). After cell harvesting, membranes were prepared as described by Anemuller et al. (1).**

**Isolation, enzymatic activity, and amino acid sequencing of the terminal oxidase.** The terminal oxidase was isolated from membranes of *A. ambivalens* essentially by means of hydrophobic interaction chromatography as described earlier (4). **(i)** Ammonium sulfate at a concentration of 10% saturation was added to the solubilization buffer. **(ii)** The heylx agarose column was directly connected to the propyl agarose column in order to speed up the purification procedure. **Enzymatic activity with caldariella quinol or N,N,N',N'-tetramethyl-p-phenylenediamine mass of 45 kDa served for the construction of the two partially degenerate oligonucleotides 45k sense (5'-GAAGCTTCCCTTGGAGGATAAG-3') and 45ksense (reverse complementary: 5'-ARCCACTYCTCTTYTTIRCATC-3').** Both oligonucleotides hybridized to a corresponding 8.2-kb BamHI/HindIII fragment, which was first subcloned with pBluescript II SK- and found to contain the doxH locus of the terminal oxidase (Dox stands for oxidase of Desulfovibrio ambivalens, the formerly valid name for *A. ambivalens*).

**Analysis of mRNA.** RNA from aerobically grown *A. ambivalens* cells was isolated by the guanidinium thiocyanate procedure as described previously (4). The mRNAs of both oxidase loci were analyzed by Northern blotting with probes specific for different open reading frames. Additionally, the transcripts of the doxH locus were studied by reverse transcription (RT)-PCR, using primers spanning the borders of different genes from this locus. Two micrograms of RNA from *A. ambivalens* was reverse transcribed with Superscript II (Life Technologies) according to the manufacturer's protocol with the gene-specific primers antidoxC (5'-TTGCTCAAGCTATCTCATC-3') and antidoxF (5'-CTCTAGA CCTTGGAGGATAAG-3'). Ten percent of the PCR was performed as a positive control. The PCR was performed in a total volume of 50 μl with 0.2 mM deoxynucleoside triphosphates (Pharmacia), 100 nM Tris-HCl (pH 8.3), 50 mM KCl, 15 pmol of each primer, and 4 U of Taq DNA polymerase (Boehringer Mannheim). After an initial denaturation of 5 min at 95°C the reaction was hot started with the following cycling parameters: 30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 20 s to 50 s of extension at 72°C, followed by a final extension for 5 min at 72°C; 30 cycles were carried out. The reaction products were examined by Tris-acetate-EDTA agarose gel electrophoresis and subsequent Southern hybridization.

**Sequence analysis.** Multiple alignments for sequence analysis, determination of transmembrane helical segments, and the construction of phylogenetic trees were achieved by use of neuronal network algorithms (51, 52) and the CLUSTAL-W program package (59) as provided by EMBL via the Internet.

**RESULTS**

**Isolation and properties of the quinol oxidase.** Membranes of *A. ambivalens*, when grown aerobically, apparently contain one prominent hemoprotein which is a cytochrome of the aa type. As described previously (4), this protein can be purified by hydrophobic interaction chromatography or alternatively by hydroxylapatite chromatography from membranes solubilized by dodecyl maltoside or *N*-dodecyl-N,N-dimethylammonio-3-propanesulfonate (SB-12), respectively. As shown in Fig. 1, the final preparation contains four major polypeptides with apparent relative molecular masses of 45,000, 40,000, 28,000, and 20,000 Da. It was previously observed that the abundance of the 45-kDa polypeptide varied between preparations without influencing spectroscopic properties. Therefore, this polypeptide was considered a copurifying contaminant rather than a constituent subunit of the oxidase. However, further investigations demonstrated that it exhibited a suppressing effect on the TMPD-oxidizing activity of the enzyme published earlier (4). The modified purification scheme described here reproducibly resulted in an enzyme preparation containing all four subunits. This form of the isolated protein exhibits essentially the same substrate specificity as the oxidase in its
membrane-bound state (Table 1). In both cases, the highest activities were measured with caldariella quinol as the substrate, whereas no TMPD oxidase activity could be detected. The enzyme is very effectively inhibited by quinolone analogs as reported for the *E. coli* quinol oxidases (42). 2-Methyl-3-decyl-quinolone and 3-decyl-4-hydroxyl-quinolone were the most efficient inhibitors, resulting in an inhibition of 95% of the enzyme activity at concentrations of 1 mM with decyl-ubiquinol as the substrate, whereas stigmatellin was much less effective (78% inhibition at 62 mM) (data not shown). This sensitivity to inhibitors acting at quinone binding sites clearly proves that the enzyme is a quinol oxidase.

All four subunits are identified below as constituent members of the oxidase complex by genetic analysis. N-terminal sequences could be obtained from three of the above-mentioned polypeptides. The 20-kDa peptide was N-terminally blocked, but internal peptide sequence information was obtained after digestion with endoproteinase Lys-C.

**Cloning of two oxidase loci.** With two gene-specific oligonucleotides, deduced from the N-terminal amino acid sequence of the 28-kDa polypeptide (Fig. 1), a 600-bp *Pst*I fragment which contained the coding region of the protein chemically determined N terminus was detected. To isolate the additional oxidase genes, which were expected to be located in a single operon as in many other prokaryotes (12), the cloned *Pst*I fragment served as a total-fit probe to detect a 2.4-kb *Hind*III fragment of genomic DNA, which was subcloned and sequenced on both strands. Unexpectedly, the cloned fragment contained only two genes of the oxidase complex (*doxA* and *doxD*) and was named the *doxA* locus (Fig. 2B). A gene for subunit I which bears the strongly conserved signatures identifying the membrane protein as the catalytic subunit of heme-copper oxidases was not detected within this DNA fragment. However, two oligonucleotides deduced from the N-terminal amino acid sequence of the 45-kDa polypeptide (Fig. 1) hybridized to a 8.2-kb *Bam*HI/*Hind*III fragment. Subcloning and sequencing of both strands confirmed that the missing genes of the oxidase complex are located in an operon-like manner on this second locus, called the *doxB* locus, after the gene (*doxB*) for the subunit I of the oxidase (Fig. 2A).

**The doxB locus.** Figure 2A shows a physical map of the cloned genomic region with the *doxB* locus together with the locations of oxidase genes and neighboring open reading frames.

---

**TABLE 1. Substrate specificity of the A. ambivalens terminal oxidase in membranes and in the purified state**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity in membranes (U/nmol of heme a)</th>
<th>Purified enzyme (U/nmol of heme a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMPD</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>Duroquinol</td>
<td>0.90</td>
<td>0.10</td>
</tr>
<tr>
<td>Decyl-ubiquinol</td>
<td>0.48</td>
<td>0.19</td>
</tr>
<tr>
<td>Caldariella quinol</td>
<td>2.38</td>
<td>1.24</td>
</tr>
</tbody>
</table>

* ND, no activity detectable.
frames. doxB codes for the subunit with an apparent molecular mass of 40 kDa (Fig. 1). The N-terminal amino acid sequence, as well as the sequence of an internal peptide stretch obtained after proteolytic digestion, confirms the correct assignment of the gene to the DoxB protein as the subunit I homolog of this terminal oxidase. It contains 587 amino acid residues; the calculated molecular mass is 64.9 kDa. Alignment to subunits I of terminal oxidases from different organisms (not shown) reveals homology to these proteins with respect to the six invariant histidine residues at positions 70, 244, 293, 294, 379, and 381, located in appropriate positions of the putative transmembrane helices II, VI, VII, and X. doxB is preceded by typical archaeal promoter elements (boxA and boxB [49]). Directly following the stop codon at position 2885, a putative transcription termination element (48) is present. The coding region of doxC, the gene for the oxidase subunit with an apparent molecular mass of 45 kDa, starts 44 bp downstream of doxB. DoxC migrates abnormally in SDS-gels since the calculated molecular mass of the 344-residue membrane protein is 38 kDa. doxC is preceded by typical promoter elements (boxA at position 2881 within the coding region of doxB; boxB at position 2906). A putative ribosomal binding site at position 2921, complementary to 16S rRNA (45), is also found. The stop codon of doxC is directly followed by the start codon of doxE, a gene that codes for a small, extremely hydrophobic peptide with a relative molecular weight of 7,178. Downstream of the gene, putative terminator signals are located around position 4190; finally, an open reading frame named doxF, starts coding for a putative peptide of relative molecular weight of 7,265, 143 bp 3′ of doxE. Downstream of doxF, possible transcription termination signals are located around positions 4590 and 4635.

The doxA locus. The physical map and locations of open reading frames at the doxA locus are shown in Fig. 2B. Two genes of the oxidase complex, doxD and doxa, are present. Possible promoter elements 5′ upstream of doxa, putative ribosomal binding sites just upstream of the coding region of each gene, and a terminator element downstream of doxa at position 2050 reveal an operon structure. doxa codes for a protein with an apparent molecular mass of 28 kDa (Fig. 1), as verified by N-terminal protein sequencing. However, the calculated molecular mass is 18.8 kDa. As discussed below, the protein has been designated pseudo-subunit II. doxD, located 5 bp upstream of doxa, codes for a membrane protein with 184 amino acid residues and a molecular mass of 20.4 kDa. DoxD is identical to the 20-kDa (apparent) subunit (Fig. 1), as demonstrated by internal peptide sequencing after endoproteinase Lys-C treatment.

Open reading frames flanking the oxidase genes. At the doxB locus (Fig. 2), an open reading frame (orf1) ends 611 bp upstream of doxB, beyond the 5′ end of the sequenced region. A database search found no homolog. Since Northern blotting revealed no transcript of this open reading frame in RNA from aerobically grown cells (not shown), we assume that it has no relationship to the terminal oxidase. On the opposite strand downstream of the oxidase genes, overlapping doxF, is an open reading frame (antiorf) coding for a putative nonmembrane protein of 244 amino acid residues with a molecular mass of 28 kDa. Database searches suggest homology of the gene product to hypothetical proteins of unknown function from different organisms (8, 22). As for the open reading frame at the 5′ end of the doxB locus, we could not find a transcript of antiorf in RNA from aerobically grown cells.

At the doxa locus, an open reading frame (orf2) ends 308 bp 5′ from the start codon of doxD, orf2 extends 5′ beyond the cloned and sequenced region (Fig. 2). Again, no clue as to its function was obtained from database searching. The open reading frame is not part of a common operon with the dox genes because an mRNA of doxD and doxa (see Fig. 4A) does not include transcribed sequence information of orf2. Therefore, it is unlikely that orf2 is related to the terminal oxidase.

The oxidase loci are duplicated. A. ambivalens DNA was digested with different restriction enzymes and hybridized after Southern blotting with total-match probes, specific for both oxidase loci. The doxA locus was examined with a probe overlapping doxA and the 3′ region of doxD (Fig. 3A). The Southern blot (Fig. 3B) indicates that in each genomic restriction, the Probe hybridized to two restriction fragments of different sizes under high-stringency conditions (see Materials and Methods). The result clearly indicates that the probed gene locus is present in multiple copies in the genome. The 2.4-kb HindIII fragment was determined by cloning and sequencing to contain the doxA locus. To study the second copy of this gene locus, the 2.6-kb HindIII fragment (Fig. 3) was cloned and completely sequenced (not shown). The two sequenced copies are almost identical from positions 1 to 2158. Within this region, only five positions differ between the two loci. Three silent point mutations in the coding region of doxa and one silent point mutation in the coding region of doxD occurred. A fifth point mutation was found at position 841 in a noncoding region, inactivating the recognition sequence for the restriction enzyme AccI in the 2.4-kb HindIII fragment that is functionally present in the cloned 2.6-kb HindIII fragment. Downstream from position 2158 to the border of the cloned fragments, the sequences of the two loci are strikingly different; no similarity exists between positions 2158 and 2403 of the
2.4-kb *Hind*III fragment and positions 2158 and 2608 of the 2.6-kb *Hind*III fragment. An open reading frame, coding for a peptide of 12 kDa with no known homolog, is located in the latter fragment from positions 2135 to 2455.

The *doxB* locus was also examined for the presence of a second gene copy with four different probes completely spanning the cloned region. In all cases, a second fragment of different size in addition to the expected restriction fragment was detected (not shown). Thus, we assume that the *doxB* locus, like the *doxA* locus, is duplicated in the *A. ambivalens* genome.

**Transcription analysis of oxidase genes.** The transcription patterns of the *doxA* and *doxB* loci were analyzed by Northern blotting with RNA isolated from aerobically grown cells. Transcription of the *doxA* locus was studied with three different probes as schematically represented in Fig. 4A. Each probe detects only one unique transcript of 1.2 kb (Fig. 4B), in good agreement with the size of *doxD* plus *doxA*. Thus, the two genes are shown to be transcribed into a single mRNA. For the *doxB* locus, the corresponding experimental approach revealed a much more complex transcription pattern. As shown in Fig. 4C, mRNA from this locus was hybridized with probes covering *doxB* (I), *doxC* (II), and *doxE* and *F* (III); this analysis revealed that four different mRNAs are transcribed from the oxidase genes (Fig. 4D). Probes upstream of *doxB* or downstream of *doxF* did not detect any of these four mRNAs (not shown), indicating that the coding region for each of the four transcripts lies within the outer limits of the *dox* genes between *doxB* and *doxF*. The *doxB*-specific probe I hybridizes with high signal strength to an mRNA of 1.8 kb, in good agreement with the size of the *doxB* gene. In addition, a second mRNA of 3.1 kb, also detected by the *doxC*-specific probe II, is hybridized with the *doxB*-specific probe, but with very weak signal intensity. This result may indicate that *doxB* and *doxC* are transcribed into a common mRNA, which may be present in comparatively minor amounts. Aside from the 3.1-kb species, *doxC* transcripts are detected mainly at 1.6 kb and to a lesser extent at 1.3 kb. Since probe III, covering *doxE* and *doxF*, also detects the 1.6- and 1.3-kb mRNAs, it is assumed that *doxC* is transcribed mainly together with both *doxE* and *doxF* and to a lesser extent together with only *doxF*.

To verify the assumptions concerning the different transcripts of the oxidase genes from the *doxB* locus, we performed an RT-PCR analysis with different primers spanning the borders of *doxB/doxC*, *doxC/doxF*, and *doxE/doxF* (Fig. 5A). In Fig. 5B (left), the amplification products following PCR with two primer pairs, spanning *doxB* and *doxC*, are shown. With cDNA as the template (lanes RT), the expected DNA fragments of 910 and 391 bp, the same fragments as amplified when genomic DNA as a positive control was used as the template (lanes 1), were clearly produced. This is direct proof for the existence of a transcript containing both *doxB* and, downstream of it, *doxC*. RNA without RT served as a negative control; no amplification product was achieved with either primer pair, indicating the specificity of the PCR conditions. With the same high specificity, the expected DNA fragments (680 and 469 bp) were amplified by PCR with primer pairs spanning *doxC/doxF* and *doxE/doxF* (Fig. 5B, right), demon-
showing that these genes are also located on common transcripts. The identities of the amplified DNA fragments were confirmed by Southern blotting (not shown). The results of the RT-PCR, in accordance to those of Northern blotting, reveal the complex transcription pattern of the oxidase genes at the doxB locus. As schematically represented in Fig. 5C, doxB is mainly transcribed to an mRNA without an additional gene (1.8 kb). doxC is mainly transcribed to an mRNA together with doxE and doxF (1.6 kb) and also to an mRNA that contains only doxC and doxE (1.3 kb). A fourth transcript of 3.1 kb contains doxB, doxC, and doxE but is present in minor amounts.

The oxidase proteins. While from both gene loci, a total of six genes are transcribed into mRNA, the pattern of polypeptides found in oxidase preparations comprises only four prominent protein bands on SDS-gels. The bands of 45, 40, 28, and 20 kDa could be unequivocally assigned to proteins with calculated molecular masses of 38 (DoxC), 64.9 (DoxB), 18.8 (DoxA), and 20.4 (DoxD) kDa, consistent with the frequent observation that membrane proteins display abnormal migration behavior upon denaturating SDS-PAGE (25). Except for the DoxB polypeptide, we were unable to determine the functions of the translated gene products. DoxB represents the catalytic core of the A. ambivalens oxidase, exhibiting the typical consensus sequences for the heme- and copper-binding sites. These are located in putative transmembrane helices II and X for the hexacoordinated low-spin heme a (His70 and His381) and in helices VI, VII, and X for the high-spin heme a3 and the copper-ion (His379, His244, His293, and His294), as found for well-investigated terminal oxidases of known structure (14, 28, 60). Figure 6 depicts a partial alignment of these sequence stretches with a selection of prokaryotic cytochrome c or quinol oxidases; an alignment of the loop region between helices II and III is also shown and is discussed below. The strict conservation of the metal ligand positions is obvious, as well as their distances and locations within the overall secondary structure. The hydrophobicity profile, however, suggests a total of 14 possible membrane-spanning α-helices (not shown); 2 C-terminal helices are added, compared to the minimum of 12 helices in most other heme-copper oxidases (53). Despite an essentially conserved hydrophobicity profile and the invariant histidines, the overall similarity to other oxidases in terms of conserved amino acid positions is extremely low; the best pairwise alignment was obtained with the ba3-type oxidase from Thermus thermophilus (23.7% identity); for the related archaeon S. acidocaldarius and for P. denitrificans, values of only 20.4% (SoxB) and 17.7% (cytochrome aa3 oxidase) were calculated.

DoxA (18.8 kDa) is designated pseudo-subunit II. The protein is definitely present as a constituent of the oxidase preparations and is nearly identical in both hydrophobicity profile (not shown) and size to subunits II of both terminal oxidases oxidases from the aerobic archaeon S. acidocaldarius. Like these proteins and the counterpart polypeptide from T. thermophilus, it contains only one putative transmembrane α-helix located at its N terminus. It shares with other quinol oxidases also the absence of the essential cysteines and methionine, which in cytochrome c oxidases serve as ligands to the binuclear copper A center of subunit II (34, 63, 65). Otherwise, the primary sequence is dissimilar to a degree preventing any useful sequence alignment.

The failure to achieve useful alignments with commonly known constituents of terminal oxidase complexes holds for all other polypeptides translated from the respective A. ambiv-
algenes. Accordingly, none of these subunits did we find a homolog in the databases. The hydropathy profile of DoxD predicts two transmembrane \( \alpha \)-helices, one in the center and the other in the C-terminal part of the integral membrane protein.

In many prokaryotic oxidase operons, the gene for subunit I is followed by the gene for subunit III, an integral membrane protein which normally exhibits up to seven membrane-spanning \( \alpha \)-helices. However, DoxC is not a typical subunit III and therefore is designated pseudo-subunit III due to the adjacent location of the respective gene, \( \alpha_3 \) to doxB. As concluded from its hydropathy profile (not shown), DoxC presumably contains only three transmembrane \( \alpha \)-helices; no further similarities to other subunits III were found. In contrast to the Sulfolobus SoxC polypeptide, the translated sequence of doxC has no similarity to a \( \beta \)-type cytochrome, the product of the correspondingly localized gene \( \alpha_3 \) next to soxB in the soxA/BCD operon (38). In line with this, there is no indication for another chromophore in the alternative oxidase complex besides the two hemes \( \alpha \) of subunit I (4).

DoxE is an extremely hydrophobic peptide with only 65 amino acids. Although there is no homology to DoxE, the latter is reminiscent of SoxD, a hydrophobic peptide of 40 amino acid residues from the SoxABC terminal oxidase of Sulfolobus. SoxD migrates in SDS-PAGE (15% gel) with an apparent molecular mass of about 14 kDa (39). Upon SDS-PAGE, a weak band from the Acidilamnas terminal oxidase (Fig. 1) which may be DoxE is found in this location. Secondary structure predictions for DoxE reveal two transmembrane \( \alpha \)-helices connected by a very short polar loop. No equivalent for the gene product of doxF was found, and it remains undetermined whether its primary transcript is indeed translated into protein.

In summary, our results demonstrate that assuming a 1:1 stoichiometry of all encoded subunits, the \( A. \) ambivalens \( aa_5 \)-type terminal oxidase represents a complex with a calculated molecular mass of 149 kDa, with subunit I the only constituent displaying significant sequence similarity to other members of the superfamily.

**DISCUSSION**

In contrast to the related archaeon \( S. \) acidocaldarius, membranes of \( A. \) ambivalens apparently contain only one \( aa_5 \)-type terminal oxidase and no other respiratory cytochromes. It was therefore considered the archaeal organism with the most primitive respiratory chain (4, 56). In view of that belief, our results were surprising in that the terminal oxidase reveals unexpected and unusual features with regard to complexity of gene organization and structural properties, classifying it as one of the most distant members within the superfamily of heme-copper oxidases.

**Genes.** The six structural genes encoding the oxidase complex are divided into two gene loci, \( \alpha \)doxA and \( \alpha \)doxB. This differs from the genomic organization of the best-characterized archaecal terminal oxidase (SoxA/BCD from \( S. \) acidocaldarius), where all genes are grouped within a single operon. On the other hand, the genes of the alternate oxidase of \( S. \) acidocaldarius are also split in separate transcription units, clustered within the same genomic region but scattered over both DNA strands (11).

As demonstrated here, both oxidase gene loci exist in duplicate on the \( A. \) ambivalens genome. A detailed sequence analysis of the second \( \alpha \)doxA locus revealed that the \( \alpha \)dox genes as well as their regulatory DNA elements are identical in both copies. Only a few silent substitutions were found. For \( A. \) ambivalens, the presence of naturally occurring plasmids has been described (68). However, the idea that one copy might be located extrachromosomally had to be rejected, because the strain used in our studies does not bear plasmids (data not shown). This is in contrast to the situation in \( P. \) denitrificans, where a second gene copy of the cytochrome c oxidase subunit I was reported to be located on a megaplasmid (57, 58). Since the regulatory regions are identical and the transcription pattern is unambiguous (Fig. 4A and C), we assume no differential expression for the two \( \alpha \)doxA loci. The same may hold for both \( \alpha \)doxB loci. Thus, as a regulatory principle, amplification of gene expression seems to be achieved by duplication of the genes. In this context, it is important to note that \( A. \) ambivalens is a facultative aerobe with a preference for anaerobic growth; transition to aerobiosis and sulfur oxidation requires fast adaptation which may be facilitated by an enhanced gene dose. However, the signalling pathway switching the genes on or off is not known. Gene duplication is a well-known phenomenon in methanogenic archaea and is thought to be an adaptation to variations in substrate availability and other environmental factors (18).

Coordinated expression of the genes of an integrated functional complex would be optimally accomplished if they are cotranscribed from an operon. In \( A. \) ambivalens, however, a strict operon structure with one single mRNA, containing the...
relevant genes, is found only for the doxA locus. While doxD and doxA are fully transcribed into a common mRNA, the transcription pattern of the doxB locus is highly complex. Considering that there are no indications of differential transcription from the two doxA loci, there is no reason to assume that the situation at the doxB locus is different, and the complex transcription pattern (Fig. 4D) may result from differential transcription of the two doxB loci. Interestingly, complex and inhomogeneous transcription patterns are also known for other archaeal operons: for example, in Sulfolobus for the RNA polymerase genes together with three open reading frames with unknown homologs and the gene for the ribosomal RNA gene (5). A similar case was reported for the operon of the halophilic archaeon Halococcus morrhuae (35). The complex transcription pattern within the doxB locus may reflect an evolutionary relict such that the process of condensing larger operons for functionally related gene products from formerly independent expressed genes is incomplete.

Proteins. The terminal quinol oxidase of A. ambivalens is unique with regard to subunit composition and primary structure compared to other prokaryotic members of the superfamily. In fact, the justification for classifying this enzyme within this family is based essentially on its catalytic properties and the homology characteristics of subunit I (DoxB) only. None of the sequences of the auxiliary subunits (DoxA, -C, -D, -E, and -F) allows a meaningful alignment with corresponding subunits of any known terminal oxidase. Also, the number of poly-peptides exceeds those of other prokaryotic quinol or cytochrome c oxidases. Typically the core structures of these oxidases comprise three poly-peptides, with subunit I bearing the catalytic heme centers and subunit II hosting the binuclear mixed-valence copper A site which is missing in quinol oxidases; subunit III does not contribute functional residues involved in the redox process but is always present as an auxiliary protein with up to seven transmembrane α-helices. We have classified DoxA and DoxC as pseudo-subunits II and III, respectively, for reasons outlined in Results. For DoxA, these were essentially its size and the typical hydrophobicity pattern; no homologous proteins could be detected from sequence alignments. For DoxC, the reason was mainly the arrangement of the gene, 3’ next to the termination of the subunit I gene. However, no conserved amino acid positions, including the otherwise strictly conserved DCCD binding residue (46), were detected; moreover, the predicted folding pattern suggests only three putative transmembrane α-helices. Nevertheless, presence of the DoxC polypeptide was shown to have a significant effect on substrate specificity, which confirms its identification as an essential subunit.

Hence, the criteria for functional attribution of DoxB as subunit I are sound despite some specific peculiarities. As outlined in Results, the array of histidines complexing heme a and the binuclear heme a,a/copper B redox centers aligns perfectly with all known subunit I sequences (7, 9, 10, 53) and is located within the canonically found positions in helices 2 to 10 (Fig. 6), verified by the recently resolved 3D structures (28, 61). However, an inspection of the hydrophobicity profile based on algorithms for transfer energy (19) locates His70 in a rather polar environment predicted to fall outside a transmembrane segment of helix 2. This is in conflict with usual predictions for other terminal oxidases. His70 and His81 provide the axial ligands to the low-spin heme a. Using prediction methods based on neuronal network algorithms (51), however, one can determine a location within a long α-helical region which exceeds the length of the membrane-spanning sequence stretch predicted from hydrophobicity. This is of interest, because on one hand, available 3D structures of the oxidases from P. denitrificans (28) and from beef heart mitochondria (61) have shown that most transmembrane α-helices extend beyond the range deduced from simple hydrophobicity profiles; on the other hand, helix II is shielded between surrounding α-helices.

Therefore, we assume for His70 also a location inside the membrane, opposite the predictions from the plot of transfer energy. Actually, molecular modeling using a scaffold based on the coordinates of the P. denitrificans subunit I (28) yields an energy-minimized structure which is in line with the above-noted assumptions (29). However, the positions of the additional C-terminal helices 13 and 14 of the A. ambivalens oxidase cannot be deduced from present structural models. Also, the location of the quinol binding site within the overall structure of quinol oxidases is still unknown.

Functional implications. The physiological necessities of aerobically growing A. ambivalens define a series of bioenergetic requirements. (i) Since A. ambivalens can grow at a pH as low as 1, effective mechanisms for proton extrusion are necessary to maintain the cytoplasm near neutral. (ii) Strictly lithoautotrophic growth needs mechanisms to provide reducing equivalents for CO₂ fixation coupled to metabolic intermediate formation, e.g., by a reductive citric acid cycle. (iii) No reducing equivalents for cellular respiration can be obtained from reduced substrates. (iv) The obligate oxidation of elemental sulfur to H₂SO₄ is the only source of reducing equivalents which may provide electrons for the reduction of the ferredoxin/NADH pool as well as for the pool of caldariella quinol as the substrate of the terminal oxidase; none of these pathways is known in any detail, though a sulfoxygenase located in the cytoplasm has been characterized (30, 31). (v) Equivalents to the respiratory complexes I and III of organotrophic organisms apparently are absent (55, 56). Thus, respiratory energy conservation is restricted to the terminal quinol oxidase described here.

Figure 7 illustrates the location of this terminal oxidase within a proposed electron transport scheme, which raises the question of whether the aa₃-type oxidase of A. ambivalens can be a proton pump. Based on its primary structure, the likely answer is no. Site-directed mutagenesis studies have indicated that oxidases acting as H⁺ pumps exhibit distinct structural features forming two separate proton channels, one for protons consumed for water formation (substrate protons) and one for superstoichiometrically pumped protons (21, 24, 26). This view has received substantial support from the 3D structure of the P. denitrificans cytochrome c oxidase (28). Essential parts of the pumping channel are a motif in the helix 2 → 3 loop -N(X₁₀₆)D(X₁₀₅)N- sequence with the central aspartate located at the putative entrance to this channel; the other is a glutamate residue in helix 6 involved in proton conductance at the output side. These residues are underlined in Fig. 6, which also shows that none of them is conserved or replaced by an isomorphous amino acid in the quinol oxidase of A. ambivalens. Recent site-directed mutational studies of Rhodobacter spha-
organisms with alternate oxidases, each may have descended from either of the two evolutionary lines.

ACKNOWLEDGMENTS

We thank W. Oettmeyer (Ruhr-University, Bochum, Germany) for the kind gift of quinolone analogs. The excellent technical assistance of Tanja Henninger, Birgit Schenk, Ying Shan, and Lars Komorowski is gratefully acknowledged. We acknowledge the communication of sequence data (subunit I of *N. pharaonis*) prior to publication by S. Mattar and M. Engelhard (Max Planck Institute, Dortmund, Germany). We thank J. Miller (University of California, Los Angeles) for providing the sequence segment of subunit I from the *Pyrobaculum aerophilum* genome project.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Schäfer/17/2) and the European Commission (project BIO2-CT93-0274).

REFERENCES

40. Mattar, S.
35. Kletzin, A.
Kannt, A., and H. Michel.
34. Laemmli, U. K.