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Characterization of two genes encoding the mitochondrial alternative oxidase in *Chlamydomonas reinhardtii*

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Abstract Two cDNA clones (*AOX1* and *AOX2*) and the corresponding genes encoding the alternative oxidases (AOXs) from *Chlamydomonas reinhardtii* were isolated and sequenced. The cDNAs, *AOX1* and *AOX2*, contained open reading frames (ORFs) encoding putative proteins of 360 amino acids and 347 amino acids, respectively. For each of the ORFs, a potential mitochondrial-targeting sequence was found in the 5′-end regions. In comparison to AOX enzymes from plants and fungi, the predicted amino acid sequences of the ORFs showed their highest degree of identity with proteins from *Aspergillus niger* (38.1% and 37.2%) and *Ajellomyces capsulatus* (37% and 34.9%). Several residues supposed either to be Fe ligands or to be involved in the ubiquinol-binding site were fully conserved in both *C. reinhardtii* putative AOX proteins. In contrast, a cysteine residue conserved in the sequences of all higher plants and probably involved in the regulation of the enzyme activity was missing both from the AOX1 and AOX2 amino acid sequences and from protein sequences from various other microorganisms. The transcriptional expression of the *AOX1* and *AOX2* genes in wild-type cells and in mutant cells deficient in mitochondrial complex III activity was also investigated.

Key words Alternative oxidase · Mitochondrial protein · Gene expression · *Chlamydomonas reinhardtii*

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Introduction

In addition to the cytochrome respiratory pathway, the mitochondria of higher plants, many protists and fungi possess an alternative form of respiration, which is resistant to cyanide but sensitive to salicylhydroxamic acid and *n*-propylgallate. Cyanide-resistant respiration is mediated by the alternative oxidase (AOX), which transfers electrons from reduced ubiquinone to molecular oxygen. No proton motive force is generated during this reaction and the free energy is dissipated into heat. Thus, when electrons produced from the oxidation of NADH flow through the alternative pathway, two of the three sites of energy conservation are by-passed and the level of ATP is decreased (reviewed by Vanlerberghe and McIntosh 1997).

The biochemical nature of AOX and the physiological role of the alternative respiratory pathway are still poorly understood. In *Sauromatum guttatum* and other Araceae, the alternative pathway is involved in heat production during anthesis (Meeuse 1975). In non-thermogenic plants, most tissues examined to date contain some level of the alternative respiratory pathway. This cyanide-resistant respiration can be increased under various conditions, such as inhibition of the cytochrome pathway, low temperature, wounding, pathogen attack, elevated carbohydrate status, ripening, addition of ethylene, or elevation of salicylic acid levels (McIntosh 1994). It has been proposed that the activation of AOX may be a mechanism to prevent the over-reduction of respiratory chain components, which might otherwise result in the production of harmful reactive oxygen species (Wagner and Krab 1995). In some fungi, like *Neurospora crassa* (Lambowitz et al. 1972; Bertrand et al. 1983), *Pichia anomala* (Minagawa and Yoshimoto 1987) and *Magnaporthe grisea* (Yukioka et al. 1998), the alternative respiration is detected only when the cytochrome pathway is blocked by mutation or by inhibitors like antimycin A and cyanide.

Recently, the cDNA sequences for AOX from several higher plants and fungi have been reported (Vanlerberghe and McIntosh 1997; Yukioka et al. 1998; Huh and Kang 1999; Kirimura et al. 1999). In several cases, the accumulation of *AOX* transcripts could be correlated with the level of the alternative respiratory pathway. In some higher plants, like soybean (Finnegan et al. 1997) and *Arabidopsis thaliana* (Saisho et al. 1997), AOX enzymes are encoded by a multigene family which are differentially expressed in a tissue-dependent manner. The unicellular green alga *Chlamydomonas reinhardtii* also possesses a cyanide-resistant salicylhydroxamic acid-sensitive respiration. A protein of 36 kDa which is recognized by a monoclonal antibody raised against *S. guttatum* AOX has been identified (Derzaph and Weger 1996). The alternative respiration is present under normal growth conditions but considerably decreases when the cells are cultivated at high CO_2 concentration (Goyal and Tolbert 1989).

Mutants lacking functional mitochondrial genes encoding apocytochrome b or subunit I of cytochrome oxidase have been characterized at our laboratory (reviewed by Remacle and Matagne 1998). These mutants lack the cytochrome pathway of the mitochondrial electron transport chain, but their respiratory activity is partially maintained via the alternative pathway.

The genes encoding AOX from *C. reinhardtii* or other algae have never been characterized. We here demonstrate that two *AOX* genes are present in *C. reinhardtii*. The two AOX deduced protein sequences are discussed in comparison with those from higher plants and fungi. Moreover, we show that the abundance of *AOX1* and *AOX2* transcripts in each is very different and that *AOX1* transcripts are more abundant in mutant cells lacking mitochondrial complex III activity.

Materials and methods

Algal strains and growth conditions

Three *C. reinhardtii* strains were used in this work: the wild-type strain (612) derived from strain 137c, the cell wall-less mutant *cw15* (25) and the mitochondrial mutant *dum15* (201) lacking complex III activity. The cells were grown on agar plates or in liquid medium, under continuous illumination (70 $\mu E\ m^{-2}\ s^{-1}$) at 25 °C. Two culture media were used: Tris-minimal phosphate medium and Tris-acetate phosphate medium (Harris 1989).

Isolation of *C. reinhardtii* genomic DNA and total RNA

Genomic DNA (for PCR amplification using the degenerate primers) and total RNA (for Northern blot analysis) were extracted following a procedure modified from Newman et al. (1990; http://www.botany.duke.edu/chlamy/methods/dna.htm). Genomic DNA for PCR amplification of the *AOX1* gene was purified using the Nucleon Phytopure kit (Amersham Life Science).

Amplification by PCR

For PCR amplification of *C. reinhardtii* genomic DNA, two oligonucleotides were designed, according to two highly conserved amino acid sequences of AOX from other organisms. The structure of the degenerate forward primer was: 5′-GC(G/Y)GS-(G/Y)GT(G/Y)CC(G/Y)GG(G/Y)ATGGT-3′ (primer 1) and the structure for the reverse primer was: 5′-GCYTCYTCYTCNAR-RTA(C/R)CC-3′ (primer 2). PCR was carried out in a total volume of 50 µl containing 100 ng of genomic DNA, 2.5 mM $MgCl_2$, 6% dimethyl sulfoxide (DMSO), 200 µM of each dNTP, 1 µM of each primer and 2 units of *Taq* DNA polymerase (Gibco BRL Life Technologies) in standard reaction buffer. PCR was initiated by denaturation at 94 °C for 3 min. Then, 35 cycles were carried out at 95 °C for 1 min, 55°C for 1 min and 72 °C for 1 min. The last synthesis step was extended for 7 min at 72 °C.

For PCR amplification of the *C. reinhardtii* cDNA library, two specific primers were designed from exons of the amplified 930-bp genomic fragment: 5′-GAGGCTGAGAACGAGCGCATGCAC-3′ (primer 3) and 5′-TCCTCCTCCAAGAAGCCCACCGCG-3′ (primer 4). PCR amplification was performed in the same conditions as described above.

For PCR amplification of the *AOX1* gene sequence, the forward primer, 5′-AAGTAGTCGACGCCTGTAGACATTCTTGCG-3′ (primer 5), was designed from the 5′-end of *NRT2;3*, a gene (GenBank accession number: AJ223296) which has been shown to be in an inverse orientation upstream from the *AOX1* gene (E. Fernández, pers. comm.). The reverse primer, 5′-GTTCATTCTCAAAG-CATTACACTTTTCTCCAGAC-3′ (primer 6), was designed from the 3′-UTR of *AOX1* cDNA. PCR was carried out in a 50-µl reaction volume containing 2 µg of genomic DNA, 1.5 mM $MgCl_2$, 6% DMSO, 200 µM of each dNTP, 0.3 µM of each primer, 1 × Q solution (Qiagen) and 4 units of Super *Taq* Plus DNA polymerase in commercial buffer (HT Biotechnology). The conditions for PCR were as follows: 94 °C for 6 min (once), 92 °C for 20 s, 62 °C for 1 min, 68 °C for 4 min (10 times), 90 °C for 20 s, 57 °C for 1 min, 68 °C for 4 min (25 times). The elongation time was increased by an additional 10 s in each of the 25 final cycles; and the last extension step was extended for 7 min at 68 °C.

The PCR products were cloned into the pGEM-T Easy vector (Promega).

Reverse transcriptase PCR

First strand cDNA was synthesized from 3 µg total RNA isolated from *cw15* cells as described by Wegener et al. (1989) using the Superscript II RNase H-reverse transcriptase (200 units) and 250 ng oligo-dT primer (Gibco BRL Life Technologies) at 42 °C for 50 min. Reverse transcriptase (RT)-PCR was performed with the *Taq* polymerase (Westburg) using two primers designed from the *AOX2* gene: the forward primer 5′-TGCTCCTGCTGTCGT-TGCTCG-3′ (primer 7) and the reverse primer 5′-TGCCTATC-TCTTCTTCTGTTC-3′ (primer 8). PCR was carried out for 35 cycles in the same conditions as described above for PCR amplification of genomic DNA using the degenerate primers.

Screening of *C. reinhardtii* cDNA and genomic libraries

Screening of the *C. reinhardtii* λgt10 cDNA library (a gift from Dr. L.G. Franzén) and the λEMBL3 genomic library (provided by Dr. M. Goldschmidt-Clermont) was done by plaque hybridization, using either the 173-bp or the 930-bp fragment, respectively, ^{32}P-labeled as a probe. The cDNA clones isolated from the positive plaques were excised from the λ-phage DNA and subcloned into the *Eco*RI site of the pBluescript KS (+) plasmid vector (Stratagene). The genomic DNA isolated from the positive plaques was digested with *Kpn*I. The 4.4-kb fragment of one clone was inserted into the pBluescript KS (+) plasmid vector.

DNA sequencing and analysis

Sequencing of the cDNA and genomic clones was performed by the dideoxynucleotide chain-termination method using a T7 sequenc-

ing kit or an automated DNA sequencer. Sequence analyses and comparisons were performed using the GCG suite of software packages (Genetics Computer Group), available through the Belgian EMBnet Node.

mRNA isolation and primer extension

Messenger RNA was isolated from total RNA using the polyAT-tract mRNA isolation system (Promega).

The primer 5′-TATGGAGCCCAGAAGACGAGAAGC-3′ (primer 9) was radiolabelled at the 5′-terminus by a T4 polynucleotide kinase (Amersham Pharmacia Biotechnology) and [γ-^{32}P] ATP. Then, poly (A)$^+$ mRNA (2 μg) prepared from the *dum15* strain was hybridized with 1×10^7 cpm of this primer and first strand cDNA was synthesized, using Superscript II RNase H-reverse transcriptase (Gibco BRL Life Technologies). The extension products were run on a 5% polyacrylamide/urea sequencing gel using dideoxy sequencing reactions as markers. The sequencing reaction was done after annealing primer 9 with a 1.5-kb PCR product obtained by using primer 9 and primer 10 (5′-AAGTAGTCGACGCCTGTAGACATTC-3′) located in the *NRT2;3* gene.

Northern blot analysis

Approximately 50 μg of total RNA were separated on each lane of 0.8% agarose gels containing 1% (v/v) formaldehyde and were then vacuum transferred to Hybond-N membranes (Amersham Pharmacia Biotechnology). Northern blots were hybridized with DNA probes labeled with [α-^{32}P] dCTP, according to Brown and McKey (1997). The probe for the *AOX1* transcript was the 855-bp *Kpn*I-*Eco*RI fragment (located at the 3′-UTR of the *AOX1* cDNA); and the probe for the *AOX2* transcript was the 570-bp *Not*I-*Eco*RI fragment (located at the 3′-UTR of the *AOX2* cDNA). The membranes were washed to a final stringency of 0.1 × SSC, 0.1% SDS at 68 °C.

Results

Isolation and sequence analysis of the *AOX1* cDNA

The alignment of AOX protein sequences from several species revealed a number of highly conserved regions of amino acids. Two of these regions, A(G/A)VPGMV and GYLEEEA, respectively, were used to design two degenerate primers (primers 1 and 2 in Materials and methods) for PCR amplification of *C. reinhardtii* genomic DNA. Following the amplification, a single PCR product with a size of ~930 bp was obtained, whose sequence corresponded to a segment of the *AOX* gene containing four exons and three introns (data not shown). Using primers 3 and 4, designed from two different exons, a PCR amplification of a *C. reinhardtii* cDNA library yielded a fragment of the expected size (173 bp) and sequence. This fragment was used as a probe to screen a cDNA library and allowed the isolation of six clones (with inserts up to 2.2 kb), all sharing the same sequence. Surprisingly, comparison of the sequence in the 173-bp cDNA fragment with the corresponding one in the 2.2-kb insert indicated only 80% identity. Thus, at least two different loci for the AOX protein were present and transcribed in the nuclear genome of *C. reinhardtii*. Moreover, comparison of the 2.2-kb sequence with that of a genomic fragment containing the 5′-end of the *AOX1* gene (E. Fernández, pers. comm.) showed that the 2.2-kb cDNA clone was probably chimeric, with a 189-bp sequence at the 5′-end linking to an unknown DNA sequence (data not shown). The two *AOX* genes from *Chlamydomonas* were designated *AOX1* (for the gene corresponding to the 2.0-kb cDNA) and *AOX2* (for the gene containing the 930-bp genomic DNA and the cDNA containing the 173-bp sequence).

Sequence analysis of the 2,010-bp cDNA of *C. reinhardtii AOX1* (GenBank accession number AF047832) revealed an ORF of 1,080 nucleotides with a potential ATG start codon at position 63. The ORF encoded a protein of 360 amino acids with a calculated mass of 38.4 kDa.

A potential mitochondrial-targeting presequence of 50 amino acid residues could be identified in the protein sequence. Four amino acid residues (Arg at −3, Phe at −1, Ala at +1 and Thre at +2) around the putative cleavage site fitted the consensus motifs proposed by Chaumont and Boutry (1995) and Glaser et al. (1998) for mitochondrial presequences. Moreover, the 50-amino-acid-long N-terminal sequence was rich in Leu (eight residues), Ala (seven residues) and Ser (four residues) and had a region (LASASRLLGS) with high potential to form a positively charged amphiphilic α-helix (data not shown), two characteristics which are typical of mitochondrial-targeting sequences (Chaumont and Boutry 1995; Whelan and Glaser 1997). If the mature AOX1 from *C. reinhardtii* corresponded to the sequence of 310 amino acids, its predicted molecular mass should be 33.4 kDa.

The cDNA possessed a long 3′-untranslated region (868 bp), as found in many nuclear genes of *C. reinhardtii* (Franzén and Falk 1992). The motif TGTAA, considered to be a potential polyadenylation signal in *C. reinhardtii* (Silflow et al. 1985), was found 18 bases upstream from the 3′-end and also at position 1,225 in the cDNA sequence.

The G + C content of the cDNA sequence was 62.4%; and the pattern of codon usage in the ORF showed a very strong bias for C and G at the third position [%NN(C/G) = 87.5%] and matched that found for other nuclear genes of the alga (Rochaix 1987).

Isolation and sequence analysis of the *AOX2* cDNA

As the isolation of the *AOX2* cDNA by screening the cDNA library with the 173-bp specific probe ended in failure, a RT-PCR was performed on total RNA with primers 7 and 8 designed from the *AOX2* genomic sequence. It led to the isolation of a 1.7-kb fragment, which was found to correspond to the *AOX2* cDNA (GenBank accession number AF285187). Sequence analysis of the cDNA revealed an ORF of 1,041 nucleotides with a potential ATG start codon at position 50. The ORF encoded a protein of 347 amino acids with a calculated molecular mass of 37.6 kDa. As for AOX1 protein, the 50-

amino-acid-long N-terminal sequence of AOX2 was rich in Ala (eleven residues) and Leu (nine residues) and contained a segment (LASTGRLLGS) susceptible to forming a positively charged amphiphilic α-helix. Just upstream from this segment, a repeated sequence (LPALVPFAA) was found. However, the putative cleavage site of the targeting presequence is difficult to define.

We also performed a PCR analysis using primers designed from regions homologous to *AOX1* and *AOX2* cDNAs. Two PCR products, whose sequences corresponded to the sequences of *AOX1* and *AOX2* segments, respectively, were obtained (data not shown). This could suggest that only two copies of the *AOX* gene are present in the genome of *C. reinhardtii*.

Isolation and sequence analysis of the *AOX1* and *AOX2* genes

The PCR performed on genomic DNA using primers 5 and 6 allowed us to isolate a 5,253 bp fragment whose sequence corresponded to the *AOX1* gene.

The screening of a *C. reinhardtii* genomic library with the 930-bp fragment from *AOX2* as a probe led to the isolation of clones containing a 4.4-kb *Kpn*I fragment recognized by the probe on Southern blot. The 4.4-kb fragment was sequenced and found to correspond to the *AOX2* gene. As shown in Fig. 1, the coding regions of the *AOX1* and *AOX2* genes (GenBank accession numbers AF314254 and AF314255 for *AOX1* and *AOX2*, respectively) contained 7 introns and 11 introns, respectively. It is noteworthy that five introns had the same position in both genes. The sizes of the introns ranged from 103 bp to 398 bp. The splice sites for the exon/intron and intron/exon 18 junctions were $G_{14}\downarrow G_{18}T_{18}G_{12}A_{11}G_{18}$ and $G_9C_{17}A_{18}G_{18}\downarrow G_{11}$, respectively. These sequences perfectly fitted the two consensus motifs G↓**G T** G A **G** and (G/A)C **A G**↓G (invariant bases are shown in bold) identified in *C. reinhardtii* nuclear genes (Silflow 1998).

Protein sequence similarities

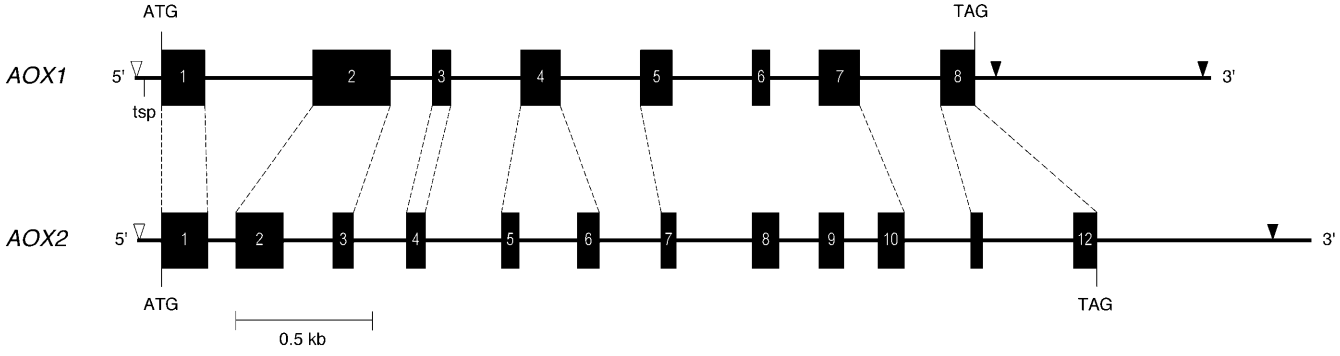
Pairwise alignments of the deduced protein sequences of *C. reinhardtii* AOX1 and AOX2 revealed a high similarity for certain segments, but only 57.6% identity was found for the whole sequence (Fig. 2). It is also interesting to note that despite the low sequence similarity in the N-terminal end of the *C. reinhardtii* AOX1 and AOX2, 9 out of 11 amino acid residues (residues 23–33 in Fig. 2) were conserved in the potential targeting presequence. These residues could be involved in the formation of the amphiphilic α-helix.

The two *C. reinhardtii* deduced protein sequences were compared to the AOXs from two higher plants and six yeasts and fungi (Fig. 2). The use of these sequences produced a somewhat different alignment in one-third of the protein at the N-terminal, as compared to previous alignments obtained by combining several plant sequences with two or three yeast and fungus sequences (Li et al. 1996; Yukioka et al. 1998; Kirimura et al. 1999). Several regions of close similarity were found, the most conserved regions residing in the central and C-terminal parts of the protein. The AOX1 and AOX2 from *C. reinhardtii* showed 28.5–29.4% and 30.4–30.6% similarity with the AOXs from *Arabidopsis thaliana* and *S. guttatum*, respectively. A greater degree of identity was found with the proteins from *Ajellomyces capsulatus* (37–34.9%), *Aspergillus niger* (38.1–37.2%), *Candida albicans* (31.1–31.4%), *M. grisea* (33.6–34.3%), *N. crassa* (33.9–34%) and *Pichia anomala* (33.2–33.2%).

Expression of *AOX1* and *AOX2* genes and determination of the transcription start site for *AOX1*

The transcription of *AOX1* and *AOX2* genes was also investigated. Northern blot analysis was performed with probes specific to *AOX1* and *AOX2* (i.e. fragments located at the 3′-UTR of each cDNA). Total RNAs were extracted from wild-type cells and from *dum15* mutant cells lacking mitochondrial complex III activity. In wild-type cells, a transcript of 2.3 kb (Fig. 3, A1), which probably corresponds to the mature *AOX1* mRNA, was

Fig. 1 Structure of the *AOX1* and *AOX2* genes of *Chlamydomonas reinhardtii*. *Black boxes* indicate coding regions, *lines* represent introns and 5′- and 3′-flanking regions. The putative start codon (*ATG*) and the in-frame stop codon (*TAG*) are shown at their respective positions. The putative TATA boxes and polyadenylation signals are marked by *open* and *black triangles*, respectively. The transcription start point (*tsp*) in *AOX1* gene is located 62 bases upstream from the initiation codon. Homologous exons are shown by *dashed lines*



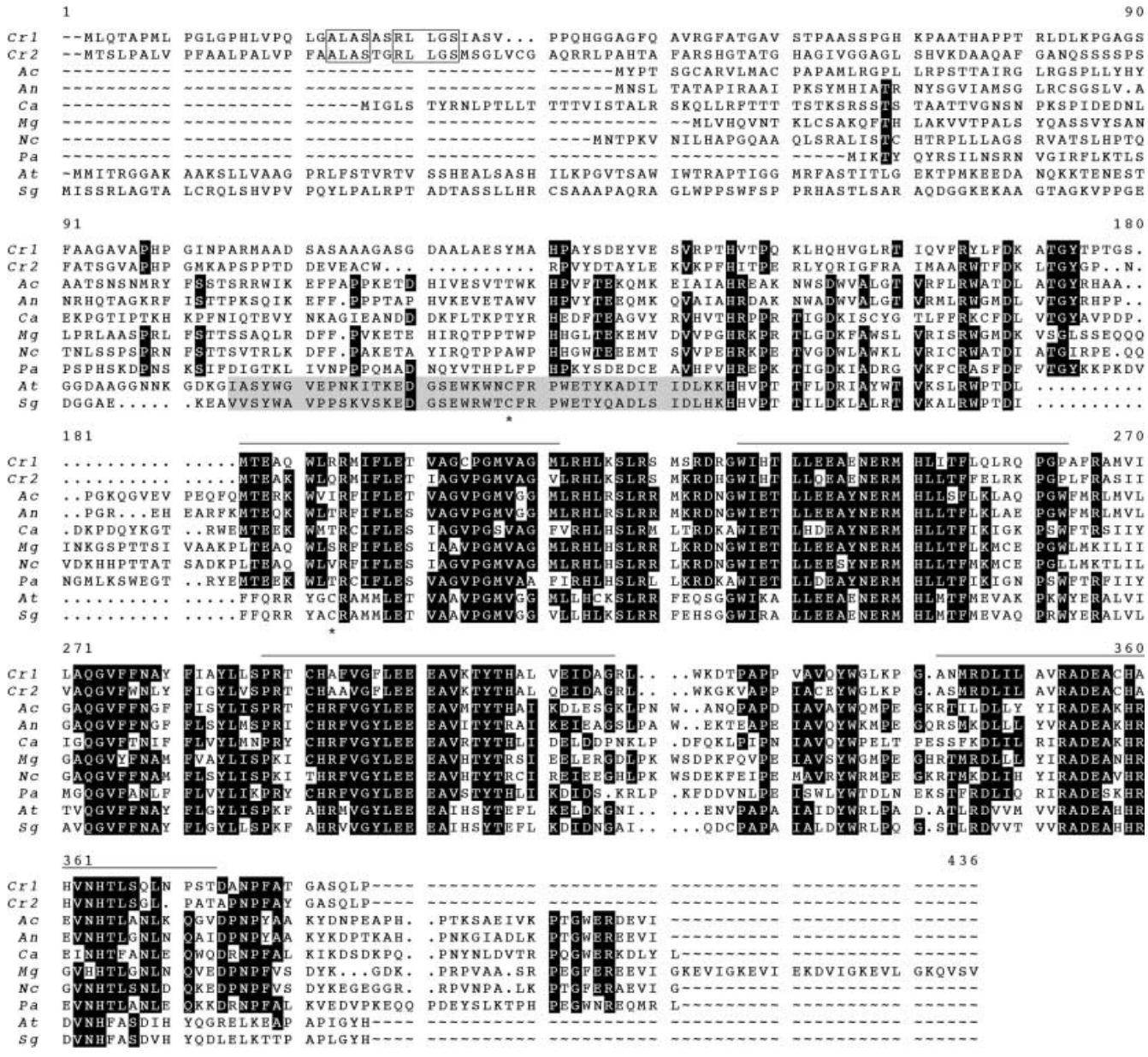


Fig. 2 Multiple protein sequence alignment of the *C. reinhardtii* AOX1 and AOX2 (*Cr1* and *Cr2*) with the alternative oxidases of the yeasts and fungi *Ajellomyces capsulatus* (*Ac*; McEwen, unpublished data), *Aspergillus niger* (*An*; Kirimura et al. 1999), *Candida albicans* (*Ca*; Huh and Kang 1999), *Magnaporthe grisea* (*Mg*; Yukioka et al. 1998), *Neurospora crassa* (*Nc*; Li et al. 1996) and *Pichia anomala* (*Pa*; formerly known as *Hansenula anomala*; Sakajo et al. 1991) and the higher plants *Arabidopsis thaliana* (*At*; Kumar and Söll 1992) and *Sauromatum guttatum* (*Sg*; Rhoads and McIntosh 1991). Amino acid residues identical in at least 50% of sequences are shown on a *black background*. Cysteine residues conserved in higher plants are marked by an *asterisk*. The protein domain including the regulatory cysteine in the plant sequences is shown on a *gray background*. The four helices presented in the model of Andersson and Nordlund (1999) are indicated by *bars*. The nine conserved residues in the potential targeting sequences of AOX1 and AOX2 are *boxed*

detected with the probe specific to *AOX1*. A weak signal of about 1.9 kb appeared after a much longer exposure time when the same membrane was hybridized with the probe specific to *AOX2* (Fig. 3, B1). The signal probably corresponded to the mature *AOX2* mRNA, since the size of *AOX2* cDNA can be estimated to be approximately 1.8–1.9 kb.

The amount of 2.3-kb transcript was about four times higher in mutant cells deprived of complex III activity, whereas the 1.9-kb signal was not significantly modified (Fig. 3, A2, B2).

The transcription start site for *AOX1* was determined by primer extension analysis. A 24-mer oligonucleotide (primer 9) complementary to nucleotides 135–158 of *AOX1* cDNA was annealed with *Chlamydomonas reinhardtii* mRNA and extended with RT. The extension products were compared with sequencing reactions as markers. The sequencing reaction was done after annealing the same oligonucleotide with a 1.5-kb genomic fragment amplified by PCR using primers 9 and 10. The results indicated that the 5′-end of the *AOX1* mRNA

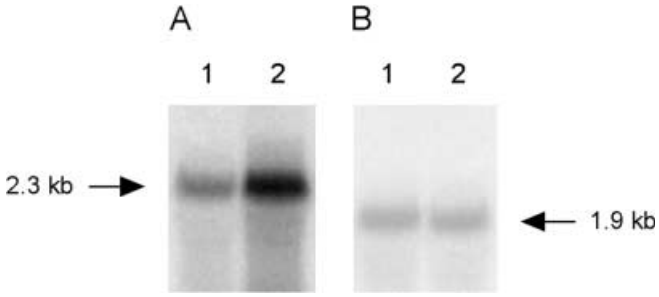


Fig. 3 Northern blots of total *C. reinhardtii* RNA (50 μg per lane) hybridized with probes specific either to the *AOX1* gene (**A**) or to the *AOX2* gene (**B**). Total RNA was isolated from wild-type cells (*lane 1*) and *dum15* mutant cells (*lane 2*) cultivated under phototrophic conditions. Exposure times of the membrane to an imaging plate were 8 h for **A** and 5 days for **B**

was located 62 bases upstream from the initiation codon (Fig. 4, see also Fig. 1).

Discussion

In this study, we isolated and sequenced two genomic fragments and two cDNAs (*AOX1* and *AOX2*) encoding the AOXs of *C. reinhardtii*. These are the first *AOX* sequences recorded from algae. It is not surprising to find two copies of the *AOX* gene in the genome of *Chlamydomonas*, since multiple *AOX* genes have also been identified in various higher plants (Whelan et al. 1996; Ito et al. 1997; Saisho et al. 1997). The deduced amino acid sequences of *AOX1* and *AOX2* cDNAs from *Chlamydomonas* allowed us to detect typical mitochondrial-targeting sequences and to identify protein segments displaying high similarity with those of AOX enzymes from higher plants, fungi and yeasts, especially in the central and C-terminal parts of the protein.

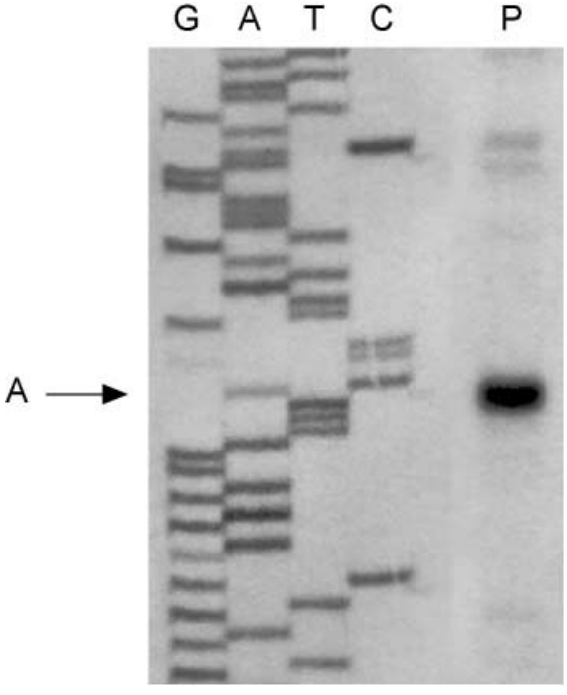


Fig. 4 Analysis of the transcription start site of *AOX1* by primer extension. The sequencing gel received primer extension products (*lane P*) or sequencing reaction products (*lanes G*, *A*, *T*, C). The primer extension product corresponds to the *A* at the *arrow*

The AOXs from higher plants and microorganisms are thought to be iron-containing proteins. Two ($E_{301}XXH_{304}$ and $E_{356}XXH_{359}$ in Fig. 2) of the three EXXH motifs conserved in *M. grisea*, *N. crassa* and all higher plant sequences have been suggested to be involved in forming the di-iron center of the enzyme (Siedow et al. 1995). These motifs appear in the C-terminal hydrophilic domain of the protein, just after the second proposed membrane-spanning helical region (Siedow et al. 1995). It is interesting to note that the $E_{301}XXH_{304}$ motif is absent not only in both AOX1 and AOX2 sequences of *C. reinhardtii* but also in four of the yeast and fungus protein sequences presented in Fig. 2. Based on a comparison of recent sequences and structural data on di-iron carboxylate proteins, Andersson and Nordlund (1999) have proposed a new model for the alternative oxidase, with a four-helix bundle in the most conserved region of the protein. These four helices are indicated by bars in Fig. 2. Six residues of the four helical regions (E_{209}, E_{248}, H_{251}, E_{300}, E_{356} and H_{359} in Fig. 2) are conserved in all of the proteins analyzed, including AOX1 and AOX2 from *C. reinhardtii*. According to Andersson and Nordlund (1999), these residues could be the ligands to the di-iron center of the AOX protein. The D_{355} and N_{247}, which are proposed to make hydrogen bonds with H_{251} and H_{359} (Andersson and Nordlund 1999), are also conserved in all sequences. The conserved residues A_{212}, P_{215}, A/S_{245}, H_{292}, G_{296} and E_{299} (all positioned one or two helical turns from the Fe ligands on helices 1, 2 and 3 in the Andersson and Nordlund model) are also present in the two algal sequences. These residues line a crevice which could serve as an ubiquinol-binding site (Andersson and Nordlund 1999).

The AOX from higher plants is present as a homodimer which exists under two distinct states: an oxidized state in which the dimer is covalently cross-linked by an intermolecular disulfide bridge, and a reduced state in which the disulfide bond is reduced to its component sulfhydryls and the dimeric structure is maintained through non-covalent interactions (Umbach and Siedow 1993). The reduced form of AOX is four-to five-fold more active than the oxidized form (Umbach and Siedow 1993). The more N-terminal cysteine residue highly conserved in higher plants (C_{128} of *S. guttatum* and *Arabidopsis thaliana* sequences in Fig. 2) could possibly be the site of this redox regulation (Umbach and Siedow 1996). The other cysteine present in all higher plant AOX amino acid sequences (C_{203} in Fig. 2) has been postulated to be involved in AOX activation by pyruvate and other α-keto acids (Umbach and Siedow 1996). More recently, site-directed mutagenesis showed that the highly conserved C_{128} residue is responsible for

both disulfide bond formation and organic acid activation of the oxidase (Vanlerberghe et al. 1998).

The situation is very different for the microorganism AOX amino acid sequences, since both the cysteine residue C_{128} and the conserved domain of about 40 amino acids surrounding the regulatory cysteine in the plant sequences are absent, both from the fungal sequences (Umbach and Siedow 2000) and also from both *C. reinhardtii* sequences (Fig. 2). An insertion of 18–25 amino acids (residues 171–195 in Fig. 2) typical of fungal sequences and not found in plants (Umbach and Siedow 2000) is also present in algal sequences, but with a reduced size (residues 171–179 in Fig. 2). Finally, the C-terminal extension of the fungal sequences beyond the C-terminus of the plant sequences is also absent in the *Chlamydomonas* sequences. Whether these structural differences are related to different regulatory patterns of AOX activities among plant and microorganisms remains to be determined.

The transcription of the two *AOX* genes from *Chlamydomonas* has also been investigated. Both genes are transcribed under normal growth conditions, but the amount of *AOX1* transcripts was much higher than that of *AOX2* transcripts. It is therefore not surprising that using the 173-bp fragment from *AOX2* as a probe, the screening of a cDNA library led to the isolation of six clones having the *AOX1* sequence.

Few data have been published on the transcriptional expression of *AOX* genes in higher plants and microorganisms. It has been reported that *AOX* gene transcription is induced in plant cells by various stimuli, like low temperature (Ito et al. 1997), or by natural chemicals such as hydrogen peroxide, salicylate, citrate and cysteine (Vanlerberghe and McIntosh 1996). In *P. anomala* (Sakajo et al. 1991), *M. grisea* (Yukioka et al. 1998), tobacco (Vanlerberghe and McIntosh 1992) and *Arabidopsis thaliana* (Saisho et al. 1997), accumulation of *AOX* mRNA was also observed when the cyanide-sensitive pathway was blocked by inhibitors interacting with complex III. We found that the amount of *AOX1* transcripts is increased in a complex III mutant of *C. reinhardtii*, which indicates that here also the absence of complex III activity determines an overexpression of the gene.

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