An alternative oxidase monoclonal antibody recognises a highly conserved sequence among alternative oxidase subunits

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Received 8 February 1999

Abstract The alternative oxidase is found in the inner mitochondrial membranes of plants and some fungi and protists. A monoclonal antibody raised against the alternative oxidase from the aroid lily *Sauromatum guttatum* has been used extensively to detect the enzyme in these organisms. Using an immunoblotting strategy, the antibody binding site has been localised to the sequence RADEAHHRDVNH within the soybean alternative oxidase 2 protein. Examination of sequence variants showed that A2 and residues C-terminal to H7 are required for recognition by the monoclonal antibody raised against the alternative oxidase. The recognition sequence is highly conserved among all alternative oxidase proteins and is absolutely conserved in 12 of 14 higher plant sequences, suggesting that this antibody will continue to be extremely useful in studying the expression and synthesis of the alternative oxidase.

Key words: Higher plant; Protozoan parasite; Mitochondrium; Respiration; Alternative oxidase; Epitope

1. Introduction

The alternative oxidase (AOX) is a quinol oxidase located in the inner mitochondrial membrane of plants, some fungi and a range of protists [1,2]. The enzyme has been studied most intensively in plants, where activity levels are quite variable among species [3] and also between the tissues within a species [4,5]. The enzyme activity is by far highest during thermogenesis in the flower spadices of aroid lilies, such as *Sauromatum guttatum* and *Arum italicum*, but is also found in non-aroid species, where it may have a role in preventing oxidative stress [6,7]. The AOX activity in various plant tissues can be induced by a number of stress conditions, including chilling [8,9], inhibition of the cytochrome pathway of electron transport [10,11], herbicide treatment [12], the inhibition of macromolecule synthesis [13–15] and the presence of reactive oxygen species [10,16]. Induction of AOX activity is directly associated with an increase in the AOX protein abundance, which in turn typically correlates with an increase in the steady state amount of the corresponding transcripts [10,12,17], suggesting the involvement of gene activation.

In plants, multigene families encoding AOX are common, although AOX may be encoded by a single gene in yeasts and protists [1,18]. For example, in soybean, three AOX genes have been identified [19], two have been identified in tobacco [19], rice [20] and tomato (R.C. Holtzapffel, P.M. Finnegan and D.A. Day, unpublished results) and four genes have been found in *Arabidopsis* [21]. Multiple immunoreactive AOX protein bands, suggesting the presence of an AOX multigene family, have been seen on immunoblots of mitochondrial proteins from numerous non-thermogenic plants, including wheat [15], potato [22], mango [23] and siratro [24], as well as thermogenic species, such as *S. guttatum*, *A. italicum* and *Symplocarpus foetidus* [3].

AOX has been studied in some detail in the protozoan parasite *Trypanosoma brucei* [25]. Recently, AOX has been detected in a range of other protists, many of them medically important parasites, including *Plasmodium falciparum* [26,27]. The insensitivity of AOX to nitric oxide (NO) has led to the hypothesis that the function of the oxidase in these parasites may be to avoid detection by the immune system [28].

The abundance of AOX protein has typically been estimated by immunoblotting mitochondrial proteins with the monoclonal antibody against AOX (AOA). This antibody was raised against the thermogenesis-induced AOX of *S. guttatum* flower spadices [3] and recognises AOX subunits from a wide range of species. It detects one or more mitochondrial proteins of 30–35 kDa on immunoblots from nearly all plant tissues that have been examined and also recognises a mitochondrial protein in all fungi and protists which have AOX activity. Often, however, the number of protein bands seen on immunoblots does not reflect the number of *AOX* genes present in that species. The question arises, therefore, whether the AOA antibody recognises all AOX proteins. This is particularly important to determine since AOA is a potentially useful diagnostic tool for parasites containing AOX. We have identified the binding site for the antibody on one of the soybean AOX proteins and show that it contains 12 highly conserved amino acid residues near the C-terminus of all characterised AOX proteins.

2. Materials and methods

2.1. Plant materials and isolation of mitochondria

Uninoculated soybean (*Glycine max* L. Merr. cv. Stevens) was germinated on vermiculite in a controlled environment cabinet at 26°C with a 16 h light (300 μmol quanta min^{-1} m^{-2})/8 h dark photoperiod. Soybeans inoculated with *Bradyrhizobium japonicum* strain USDA110 were grown on nutrient-supplemented river sand in a glasshouse under natural light [4]. Mitochondria were isolated from cotyledons, roots and nitrogen-fixing root nodules using published methods [29,30].

2.2. DNA manipulations

Standard methods were used throughout for the manipulation, purification and sequencing of DNA fragments [31]. All constructs were verified by DNA sequencing. The full length *AOX2* cDNA was digested with *Bst*EII which digests after the N23 codon (Fig. 1) and *Spe*I, which digests within the H333 codon. The resulting 934 bp

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Abbreviations: AOX, alternative oxidase; pBSII, pBluescript KS(+); MBN, mung bean nuclease; IPTG, isopropyl-β-D-thiogalactoside

PII: S0014-5793(99)00259-8

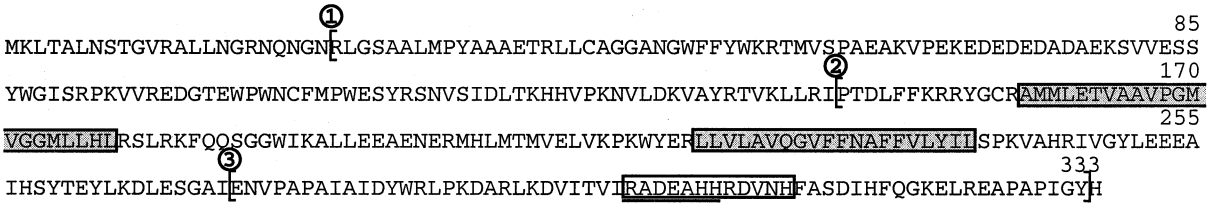

Fig. 1. The soybean AOX2 protein fragments used to define the binding site of the AOA antibody. The end points of the AOX2 fragments cloned in frame and internal to the α-fragment of β-galactosidase are shown on the sequence of the entire AOX2 primary translation product. The left hand brackets show the N-terminal AOX2 residue in constructs pAOX2ΔN23 (1), pAOX2ΔN144 (2) and pAOX2ΔN271 (3) while the right hand bracket marks the C-terminal AOX2 residue in all these clones. The AOX2 sequence in construct pAOX2-12 is within the open box, while that in pAOX2-8 is underlined. The shaded boxes show the putative transmembrane spanning domains of AOX2.

cDNA fragment was blunt-ended with mung bean nuclease (MBN, Promega) and cloned into *Sma*I-digested pBluescript II KS(+) (pBSII, Stratagene) to form pAOX2ΔC1. By this means, the 310 C-terminal amino acid residues of the *AOX2* ORF, excluding H333 (Fig. 1), were fused in frame at the C-terminus with the β-galactosidase α-peptide sequence of pBSII. Digestion of pAOX2ΔC1 at the single *Cla*I site in the pBSII polylinker, followed by digestion with MBN and re-ligation, resulted in the deletion of 11 bp from the pBSII polylinker to form pAOX2ΔN23, in which N23 of the *AOX2* ORF was brought into frame with the α-peptide sequence. Digestion of pAOX2ΔC1 with *Eco*RI, followed by digestion with MBN and re-ligation, deleted 17 bp of the pBSII polylinker and 358 bp of *AOX2* cDNA to form pAOX2ΔN144 in which the 188 C-terminal amino acid residues of AOX2 in pAOX2ΔC1 were fused in frame with the α-peptide. Digestion of pAOX2ΔC1 with *Eco*RI and *Mun*I, followed by digestion with MBN and re-ligation, deleted 17 bp from the pBSII polylinker and 745 bp from the *AOX2* cDNA to form pAOX2ΔN271 in which the C-terminal 61 amino acid residues of AOX2 in pAOX2ΔC1 were fused in frame with the α-peptide. The oligonucleotides 5′-GGGCCGATGAGGCCCACCATCGCGACGTTAACCATAGCT-3′ and 5′-ATGGTTAACGTCGCGATGGTGCGCCTCATCCGCCCGTAC-3′ were annealed, leaving *Kpn*I and *Sac*I compatible overhangs, and ligated into pBSII digested with *Kpn*I and *Sac*I to form pAOX2-12. Oligonucleotides 5′-TCGACGGTTCGCGCGGATGAAGCGCAGCAG-3′ and 5′-GATCCTGCTGCGCTTCATCCGCGCGAACCG-3′ were annealed, leaving *Sal*I and *Bam*HI compatible overhangs, and ligated into pBSII digested with *Sal*I and *Bam*HI to form pAOX2-8. Thus, in the latter two clones, residues 300–312 and residues 300–306 of the AOX2 ORF, respectively, became fused in frame within the α-peptide in pBSII.

2.3. *Escherichia coli* growth for immunoblotting

Overnight cultures of *E. coli* strain DH10B (Invitrogen) transformed with various pBSII derivatives were subcultured in 20 ml LB [31] medium containing 60 μg/ml ampicillin. The cultures were grown at 37°C with shaking to an OD_{600} of 0.2 before splitting into two equal portions. One portion was supplemented with 1 mM isopropyl-β-D-thiogalactoside (IPTG) and both portions were incubated further at 37°C with shaking until an OD_{600} of 0.7–0.9 was obtained. The cells were then collected by centrifugation at $4000 \times g$ for 10 min at 4°C and the supernatant was entirely removed. Cell pellets were stored at −20°C until analysed.

2.4. Immunoblotting

Purified mitochondria or *E. coli* cells were lysed by resuspending in SDS-PAGE sample buffer containing 2% (w/v) SDS and 20% (w/v) 2-mercaptoethanol. Proteins were separated by SDS-PAGE, blotted to nitrocellulose, probed with the AOA monoclonal antibody (a kind gift of Dr. T. Elthon, University of Nebraska, NE, USA) and the immunoreactive polypeptides identified by chemiluminescence as previously described [32].

3. Results and discussion

3.1. Mapping the AOA binding site in soybean AOX

Soybean contains three *AOX* genes and AOA detects three separate bands in immunoblots of mitochondrial proteins (Fig. 2). The ability of AOA to recognise AOX on immunoblots of SDS-PAGE gels indicates that the antibody binds to denatured proteins. This suggests that the AOA binding site involves a linear array of closely spaced amino acid residues within the AOX primary sequence. To broadly define the AOA binding site, progressively longer N-terminal deletions of the immunoreactive AOX2 protein (Fig. 1) were tested for their ability to react with AOA. Conveniently sized *AOX2* cDNA fragments were cloned in frame within the α-peptide of the β-galactosidase in the pBSII cloning vector and the fusion proteins (described in Fig. 1) were expressed in *E. coli*. Immunoblot analysis of strains expressing the fusion proteins showed that each of the constructs tested, pAOX2ΔN23, pAOX2ΔN144 and pAOX2ΔN271, produced an immunoreactive polypeptide of the predicted size: 56, 42 and 27 kDa, respectively (Fig. 3). The induction of the synthesis of these proteins by IPTG provides further evidence that they are the products of the gene fusions. Thus, the AOA antibody recognises and binds to the 61 C-terminal amino acid residues of AOX2.

In addition to the predicted immunoreactive polypeptides, a number of other protein bands were detected on the immunoblot in Fig. 3. Except those bands described below, all the additional bands were also evident when extracts of cells containing unmodified pBSII were probed with the secondary antibody alone (Fig. 3, lanes 7 and 8), demonstrating that they do not arise through specific interactions with AOA. The IPTG inducible immunoreactive polypeptides at 44 kDa in cells containing pAOX2ΔN23 and at 29 and 26 kDa in cells

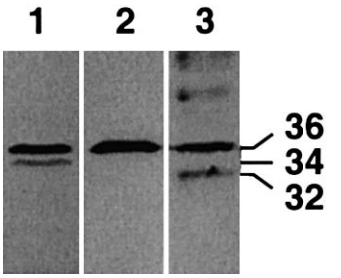

Fig. 2. Soybean AOX proteins recognised by the AOA antibody. Mitochondrial protein (50 μg) from cotyledons (lane 1) and roots (lane 2) of 14 days old seedlings and from N-fixing root nodules (lane 3) of 8 weeks old plants were separated by SDS-PAGE, immunoblotted with the AOA antibody and visualised by chemiluminescence. Each lane is from a different exposure of the same immunoblot. Images were digitised using a high performance CCD video camera (Cohu, San Diego, CA, USA) and manipulated using Photoshop 3.0 software (Adobe Systems, Mountain View, CA, USA).

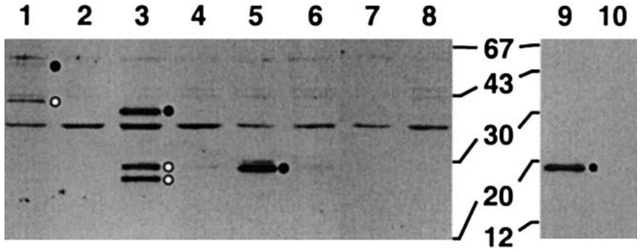

Fig. 3. Detection of AOX2-β-galactosidase fusion proteins by the AOA antibody. Protein extracts from *E. coli* strains containing pAOX2ΔN23 (lanes 1 and 2), pAOX2ΔN144 (lanes 3 and 4), pAOX2ΔN271 (lanes 5 and 6), pBSII (lanes 7 and 8) or pAOX2-12 (lanes 9 and 10) grown in the presence (odd numbered lanes) or absence (even numbered lanes) of 1 mM IPTG were separated by SDS-PAGE, immunoblotted with the AOA antibody and visualised using chemiluminescence. The positions of the molecular size markers are shown between the panels. Filled circles indicate the expected location of the constructed fusion proteins, while open circles indicate probable specific degradation products of the fusion proteins (see text for details). Images were digitised and manipulated as stated in the legend to Fig. 2.

containing pAOX2ΔN144, were specific to these strains, were not detected by the secondary antibody alone and therefore must contain the AOA binding domain. These proteins probably represent specific degradation products of the AOX2/α-peptide fusion proteins synthesised by these cells and may have arisen from intolerance of *E. coli* to high levels of the integral membrane AOX protein.

3.2. Identification of the AOA binding site in AOX from other species

The 61 residue C-terminal sequence of AOX2 was compared to the corresponding regions of the other AOX subunits which have been sequenced including those from *N. crassa*, *H. anomala*, *T. brucei*, *Chlamydomonas* sp. and *Aspergillus niger* (Fig. 4). Within this region, there is a single 12 amino acid sequence which is highly conserved among all AOX subunits. Since AOA recognises at least one protein on immunoblots of mitochondrial proteins from each of these species that have been tested, it seemed likely that the AOA recognition site would involve this highly conserved sequence. To test this, *E. coli* extracts from cells containing pAOX2-12, a pBSII derivative in which the 12 amino acid sequence from soybean AOX2 was fused internal to and in frame with the α-peptide of β-galactosidase, were immunoblotted (Fig. 3). These cells produced an IPTG inducible immunoreactive band at 19.5 kDa, very similar in size to the 18.7 kDa predicted from the sequence of the gene fusion. These results show that this 12 amino acid sequence is sufficient for AOA recognition.

3.3. Identification of key amino acid residues in the AOA binding site

During the construction of pAOX2-12, two mutants were inadvertently created (Table 1). The fusion protein produced by pAOX2-12A2V, in which A2 of the 12 residue sequence was converted to valine, was not recognised by AOA, indicating that A2 has an essential role in the antibody-AOX interaction. The abolition of AOA binding by the addition of two relatively small methyl groups to the side chain of A2 suggests that AOA binding to this residue is highly specific. All AOX subunits sequenced to date have Ala at this position (Fig. 4). The second mutant sequence, in pAOX2-12FS6, had a frame shift which caused the sequence of the fusion protein to diverge from the expected sequence after A5 of the 12 residue sequence. The fusion protein produced by pAOX2-12FS6 containing cells was also not recognised by AOA. Moreover, cells containing pAOX2-8, in which the sequence RADEAHH was fused into the α-fragment, also failed to produce a fusion protein recognised by AOA (Table 1). Together, these results indicate that residues C-terminal to H7 are required for AOA binding.

Dissection of the AOA binding site indicates that the minimum sequence that may be sufficient for binding is ADEAHHR, although the entire 12 residue sequence RADEAHHRDVNH may be required. The latter sequence is absolutely conserved in 12 of the 14 AOX subunit sequences that have been completely determined from higher plant sources (Fig. 4) and entirely encompasses helix 4 of the four helix bundle postulated to comprise the AOX active site [33]. In fact, the AOA antibody has previously been shown to inhibit the activity of detergent-solubilised AOX by 50% [3], consistent with the idea that helix 4 has an important role in the

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                     272                                                                        333
Soybean AOX1         E-NVP----APAIAIDYWQLPPG-STLRDVVMVVRADEAHHRDVNH-------------FASDIHYQGRELREAAA------PIGYH*
Soybean AOX2         E-NVP----APAIAIDYWRLPKD-ARLKDVITVIRADEAHHRDVNH-------------FASDIHFQGKELREAPA------PIGYH*
Soybean AOX3         E-NVP----APAIAIDYWRLPKD-ATLKDVVTVIRADEAHHRDVNH-------------FASDIHHQGKELKEAPA------PIGYH*
Tobacco              E-NVP----APAIAIDYCRLPKD-STLLDVVLVVRADEAHHRDVNH-------------FASDIHYQGQQLKDSPA------PIGYH*
Mango                K-NIP----APAIAIDYWRLPKD-ATLKDVITVVRADEAHHRDVNH-------------FASDVQVQGKELRDAPA------PVGYH*
Catharanthus roseus  E-NVP----APAIAIDYWQMPPD-STLRDVVMVVRADEALHRDVNH-------------YASDIHYKGLELKEAAA------PLDYH*
Arabidopsis AOX1a    E-NVP----APAIAIDYWRLPAD-ATLRDVVMVVRADEAHHRDVNH-------------FASDIHYQGRELKEAPA------PIGYH*
Arabidopsis AOX1b    E-NVP----APAIAIDYWRLEAD-ATLRDVVMVVRADEAHHRDVNH-------------YASDIHYQGRELKEAPA------PIGYH*
Arabidopsis AOX1c    E-NVP----APAIAVDYWRLEAD-ATLRDVVMVVRADEAHHRDVNH-------------YASDIHYQGHELKEAPA------PIGYH*
Arabidopsis AOX2     E-NVA----APAIAIDYWRLPKD-ATLKDVVTVIRADEAHHRDVNH-------------FASDIRNQGKELREAAA------PIGYH*
Potato               D-NVP----APAIALDYWRLPPG-STLRDVVMVVRADEAHHRDVNH-------------FASDVHYQGHQLKEAPA------PLGYH*
S. guttatum          Q-DCP----APAIALDYWRLPQG-STLRDVVTVVRADEAHHRDVNH-------------FASDVHYQDLELKTTPA------PLGYH*
Rice AOX1a           D-NVP----APAIAIDYWRLPAN-ATLKDVVTVVRADEAHHRDVNH-------------FASDIHYQGMELKQTPA------PIGYH*
Rice AOX1b           E-NTP----APAIAIDYWRLPAD-ATLKDVVTVIRADEAHHRDLNH-------------FASDIQQQGMKLKDTPA------PIGYH*
Chlamydomonas        WKDTP----APPVAVQYWGLKPG-ANMRDLILAVRADEACHAHVNHTLSQLNPSTDANPFATGASQLP*
Chlamydomonas        WKDKP----APKTGIAYWKLSPD-ATMRDLILAVRADEASHSLVNHTLSEI-PSDAPNPFIEPAKADAFSKAENKL*
Neurospora           PKWSDEKFEIPEMAVRYWRMPEGKRTMKDLIHYIRADEAVHRGVNHTLSNLDQKEDPNPFVSDYKEGEGGRRPVNPALK---PTGFER...
Hansenula            PKFDD--VNLPEISWLYWTDLNEKSTFRDLIQRIRADESKHREVNHTLANLEQKKDRNPFALKVEDVPKEQQPDEYSLKTPHPEGWNR...
Aspergillus          WEKTE----APEIAVQYWKMPEGQRSMKDLLLYVRADEAKHREVNHTLGNLNQAIDPNPYAAKYKDPTKAHPNKGIADLK--PTGWER...
Trypanosoma          PYQKCMFPKWLACTGTSAKMPHS----ATSSTCIRADEAEHRVVNHTFVTCTKN-----ACKTVSTPSLYLKKNPEEMYSNQPSGKTR...
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Fig. 4. An alignment of reported AOX subunit C-terminal amino acid sequences. Gaps were introduced to maximise the alignment and the most abundant residue at each position is shaded. Numbering is relative to the soybean AOX2 sequence. The highly conserved 12 amino acid sequence is boxed. The sequences presented have been compiled previously [2,20], except *Catharanthus roseus* (accession AB009395), *Chlamydomonas* sp. (accessions AB009087 and AF047832) and *Aspergillus niger* (accession AB016540).

Table 1
Recognition by the AOA monoclonal antibody of soybean AOX2 sequences within β-galactosidase fusions

AOX2 sequence in fusion protein	Recognition by AOA
RADEAHHRDVNH	Yes
RVDEAHHRDVNH	No
RADEA	No
RADEAHH	No

AOX activity. The high conservation of this sequence, and therefore the ability of AOA to recognise AOX subunits from diverse sources, may be due to structural constraints on this segment of the AOX active site.

3.4. Can AOA be used with confidence to identify new AOX proteins?

The finding that the single amino acid change in the A2V mutant eliminates the antibody-AOX interaction indicates that care must be taken when assessing immunoblots from species in which the 12 residue sequence diverges from that of *S. guttatum*, the original antigen source. For example, the rice AOX1b subunit has a divergent leucine at position 10 of the conserved sequence, while the rice AOX1a protein contains the V10 found in all other AOX subunits (Fig. 4). This variation may result in differential recognition of these subunits by AOA, leading to potentially inaccurate estimates of the relative abundance of these subunits from immunoblots. This is not a concern in soybean, where all three subunits have the highly conserved 12 residue sequence present in the *S. guttatum* protein. Therefore, the signal obtained on immunoblots from the soybean AOX subunits is a direct reflection of the overall protein amount. This verifies that, in soybean, the AOX protein amount is a true reflection of transcript abundance, as concluded in earlier studies [4,32,34].

Although A2V abolishes AOA binding, this is apparently not true of all variations to the AOA binding sequence. The antibody appears to recognise an AOX protein from *H. anomala* [35], *N. crassa* [36], *T. brucei* [25] and *Chlamydomonas* sp. [37] However, in all these species, the sequence corresponding to the AOA binding domain differs from the consensus plant sequence at positions 6 and 9 (Fig. 4). Moreover, the diversity of side chains present at these two positions suggests that the antibody may interact differentially with these residues. It must be pointed out, however, that no non-plant AOX protein has yet been matched with certainty to its cognate gene, so it is possible that the proteins recognised by AOA in these species do not correspond to the sequences that have been determined. In any event, the finding that the AOA antibody recognises a sequence that is highly conserved in all species that have been examined to date confirms that the antibody will continue to be an invaluable reagent in studies on AOX.

Acknowledgements: This research was supported by a grant from The Australian Research Council to D.A.D.

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