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A novel functional element in the N-terminal region of *Arum concinnatum* alternative oxidase is indispensable for catalytic activity of the enzyme in HeLa cells

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ABSTRACT

Alternative oxidase (AOX) is a quinol-oxygen oxidoreductase, which is known to possess a dicarboxylate diiron reaction center held in structurally postulated α-helical bundle. However, little is known about the structural or functional features of its N-terminal region in any organism, with the exception of a regulatory cysteine residue (CysI) in angiosperm plants. Here, we show that transcripts of two AOX1 isozymes (*AcoAOX1a* and *AcoAOX1b*) are coexpressed in thermogenic appendices of *Arum concinnatum*, while their enzymatic activities seem to be distinct. Namely, *AcoAOX1a*, an abundantly expressed transcript in vivo, shows an apparent cyanide-insensitive and *n*-propyl gallate-sensitive respiration during ectopic expression of the protein in HeLa cells, whereas *AcoAOX1b* exhibits a lower transcript expression, and appears to be totally inactive as AOX at the protein level. Our functional analyses further reveal that an E83K substitution in AcoAOX1b, which is located far upstream of CysI in the N-terminal region, is the cause of this loss of function. These results suggest the presence of a naturally occurring inactive AOX homologue in thermogenic plants. Accordingly, our results further imply that the N-terminal region of the AOX protein functionally contributes to the dynamic activities of respiratory control within the mitochondria.

1. Introduction

Alternative oxidase (AOX) of eukaryotic taxa is a terminal quinol oxidase of mitochondrial electron transport chains, which catalyzes the oxidation of ubiquinol and the four-electron reduction of molecular oxygen to water in a coupling manner [1]. Unlike cytochrome *c* oxidase (COX), AOX does not translocate protons across the inner mitochondrial membrane, thus, no redox energy is conserved into the electrochemical proton gradient by this enzyme. Despite such a dissipative nature in energy metabolism, there have been a number of reports suggesting that AOX plays a variety of physiological roles in higher plants, for example, contribution to floral thermogenesis [2], alleviation of reactive oxygen species (ROS) formation [3,4] and maintenance of TCA cycle turnover under restriction of COX-mediated respiration [5]. In fact, recent work on thermogenic receptacles of sacred lotus (*Nelumbo nucifera*) has shown that an increase in respiration through AOX is largely responsible for an increase in heat production [6].

Comparative analyses and site-directed mutagenesis studies have been successful in elucidating the structural and mechanistic nature of AOX, although no data from X-ray crystallography are available for analysis to date. Andersson and Nordlund presented a structural model of AOX in which they postulated four α-helices and two possible membrane binding domains [7]. In this model, they also proposed ligands to a catalytic diiron center in analogy with other diiron carboxylate proteins. A number of site-directed mutagenesis studies have confirmed that all of these iron binding residues are necessary for AOX activity, except for the glutamic acid in the first helix region [1,8,9]. Besides the active center, two highly conserved cysteines in plant AOX, termed CysI and CysII [10], have received attention. These cysteines are considered to play key roles in post-translational regulatory systems of AOX, namely redox regulation and activation by α-keto acids. Previous investigations have revealed that CysI is responsible not only for formation of disulfide bonds for redox regulation, but also for interaction with α-keto acids such as pyruvate, whereas CysII can act only as a target of activation by small α-keto acids, such as glyoxylate [11–13]. The AOX activity is, indeed, largely affected by these post-transcriptional regulation mechanisms, both in vivo and in vitro.

Mutagenesis studies have demonstrated that a single amino acid substitution in AOX drastically alters its enzymatic characteristics. For instance, substitutions of CysI by charged amino acids results in remarkably increased basal activity and insensitivity to pyruvate [13] while the substitution by serine yields an enzyme that is activated by succinate but not by pyruvate [14,15]. Another representative case of a single substitution having a large impact is reported in the G303E mutant of *Arabidopsis thaliana* AOX, which shows a 4.6-fold increase in resistance to salicylhydroxamic acid (SHAM) [16], a specific inhibitor of AOX. Site-directed mutagenesis studies with respect to

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potential catalytic mechanisms of AOX also revealed several residues that have impacts on the enzymatic activity both in plant AOXs [9,17] and in typanosomal AOXs (TAOs) [18].

With such a great deal of experimental effort, there has been notable progress in our understanding of the structure–function relationships of AOX, however, little is known about the biochemical characteristics of its N-terminal region other than the CysI residue. In this study, we demonstrate that a single amino acid substitution (E83K) in the N-terminal region causes a loss of activity in AOX from *Arum concinnatum*, whose intense thermogenic activity was recently discovered [19] but has not been investigated at the molecular level to date. This substitution seems to occur naturally in a pair of AOX isozymes in thermogenic appendices of this plant. Our findings suggest that an additional element in the N-terminal region is essential for the activity of AOX, and a translation product which shows homology to known AOXs may not always be functionally active within the mitochondria.

2. Materials and methods

2.1. Plant materials

A. concinnatum from a population near Panormos, a village on Crete, an island of Greece, were observed in the field for characterizing their thermogenic and morphological properties. Tissue samples for RNA extraction were collected from the plants grown in a population near the campus of University of Crete in Heraklion. All samplings and observations were conducted in May 2007.

2.2. Thermal imaging and temperature measurement

To verify thermogenicity of *A. concinnatum*, thermal images were obtained using Avio TVS-500 infrared camera (Nippon Avionics Co, Ltd., Tokyo, Japan) then saved as IRI files. The IRI images were subsequently analyzed with Goratec Thermography Studio Professional software (Goratec Technology GmbH and Co., KG, Erding, Germany). The temperatures were measured with copper-constantan thermocouples in the air and in the appendix. They were recorded with Grant Squirrel data logger (Grant Instruments, Cambridge, UK).

2.3. Isolation and sequencing of the full-length AcoAOX1a and AcoAOX1b

For the isolation of transcripts encoding AOX proteins by RT-PCR, total RNA was first extracted using an RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) from thermogenic appendix samples that had been flash-frozen in liquid nitrogen in the field, immediately after sampling. First strand cDNAs were generated with a PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa Bio Inc., Otsu, Japan) using an oligo (dT) primer. By aligning conserved cDNA sequences of *AOX* transcripts across several aroid species, namely *Dracunculus vulgaris AOX* [20], *Philodendron bipinnatifidum AOX* [20] and *Sauromatum guttatum AOX* [21], primers were designed to amplify approximately 550 bp fragments from the ORF region (Step 1 in Fig. S1): AcoPartF1 and AcoPartR1 (for all primer and probe sequences, see Table 1). The fragments were cloned into pCR 2.1 with a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), then sequenced.

Based on the partial sequence data, 5′- and 3′-RACE reactions were performed using a SMART RACE cDNA Amplification Kit (Clontech Laboratories Inc., Palo Alto, CA, USA) with these primers (Step 2 in Fig. S1): AcoRV2 and AcoRV1 for the *AcoAOX1a* 5′-fragment, AcoFW1 for the *AcoAOX1a* 3′-fragment, AcoF3re and AcoF2re for the *AcoAOX1b* 5′-fragment and AcoFW5 and AcoR4re for the *AcoAOX1b* 3′-fragment. Nested PCR was conducted when necessary. As both of AcoAOX1a- and AcoAOX1b-derived products had been amplified simultaneously with the primers, AcoAOX1b 5′- and 3′-fragments were screened by restriction enzymes *Ava*I and *Bst*XI (both from TaKaRa Bio Inc.), respectively. RACE products were also cloned into pCR2.1 and sequenced.

Table 1
Primer and probe sequences used for RT-PCR and real-time PCR.

Name	Sequence (5′ to 3′)
AcoPartF1	GCCCCCACCACCATCCTCGACA
AcoPartR1	TCGCGGTGGTGTGCCTCGTCGG
AcoRV2	ACCGCCGCCACCGTCTCCA
AcoRV1	TGTACAAGGCCAGCTTGTCGAGGACGGT
AcoFW1	CCGGGCGATGATGCTGGAGA
AcoF3re	AACTCGGTGTAGGAGTGGATGGCCTCCT
AcoF2re	ACCCGGTGGGCGAACTTGGGGGAGA
AcoFW5	TGCCGGGCGATGATGCTGGAGA
AcoR4re	AGAACGAGCGGATGCACCTGATGACCTT
Aco5′E	ACGATCGAAGGAGCCAGTGCAGT
Aco3′A	TAGAGTTCGCCAGTAGTAATGCTAATGCGT
Aco5′C	AGCCAGTGCAGTCCCCT
Aco3′C	TTCCCACTGTGGATTCACGTGAA
SalI-Aco5′	CTGCAGGTCGACAGCACGCTGTCAGATCCCGCCCA
SalI-Aco3′	CTCGAGGTCGACTGGATTCACGTGAAGCCTCCGCGCT
efFW1	ACATTGTGGTCATTGGCCA
efRV3	ACCAGTTGGGTCCTTCTT
Aco1aF	TTCCTCGGGTACCTCATC
Aco1aR	GGCACGTTCTCGATGGTG
Aco1bF	GGGGCCATCCAGGACAC
Aco1bR	GTCCGAGGCGAAATGGT
ef1aF	ACGGTTATGCTCCTGTCCT
ef1aR	TTCAAGAACTTGGGCTCCT
Aco1aP	AGATCGACAAGG
Aco1bP	CGAGGGCGAT
ef1aP	TGACGAAGATCG

For the probes, positions at which RNA nucleotides are allocated are indicated by underbars.

To obtain full-length cDNAs of *AcoAOX1a* and *AcoAOX1b*, another PCR amplification was performed using KOD -Plus- (TOYOBO Co., Ltd., Osaka, Japan) with Aco5′E and Aco3′A primers for the first reaction, followed by Aco5′C and Aco3′C primers for the nested PCR (Step 3 in Fig. S1). The final PCR products of almost 1.2 kb were purified by gel extraction, then cloned into the *Hinc*II site of pUC118 (TaKaRa Bio Inc.) following phosphorylation by calf intestinal alkaline phosphatase (New England Biolabs, Inc., Ipswich, MA, USA). The molecular species of the insert, i.e. either *AcoAOX1a* or *AcoAOX1b*, was identified by applying *Ava*I to PCR products amplified from each single clone with AcoF3re and AcoR4re primers before they were sequenced (Step 4 in Fig. S1). Nucleotide sequence data were analyzed with GENETYX software (Genetyx Corp., Tokyo, Japan). Phylogenetic analysis was conducted with MEGA version 4 [22].

2.4. Construction of vectors

DNA fragments that carry sequences corresponding to the mature forms of the AcoAOXs were amplified with SalI-Aco5′ and SalI-Aco3′ primers, following prediction of their mitochondria targeting sequences with Mitoprot [23]. The fragments were then digested with *Sal*I restriction enzyme to be cloned into *Sal*I site of pCMV/myc/mito (Invitrogen).

To construct vectors for expression of chimera proteins, the vectors carrying wild-type AcoAOX inserts were digested sequentially with *Ksp*I, *Eco*RI and *Nar*I (*Ksp*I and *Nar*I from Roche Diagnostics GmbH, Mannheim, Germany; *Eco*RI from TaKaRa Bio Inc.). The starting plasmid linearized with *Ksp*I was digested firstly with *Eco*RI to cleave at the middle of the inserts, and the fragments that contain sequences encoding N-terminal domain were subsequently digested with *Nar*I to generate further segmented inserts. The fragments were re-associated in appropriate combinations to generate the designed chimeric inserts. Ligation reactions were performed with a TaKaRa DNA Ligation Kit Ver.2.1 (TaKaRa Bio Inc.). During the construction of the expression plasmids, an additional IHSLLQVD sequence was added