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Alternative Oxidase (*At*Aox) c78s Mutant Expression at *Escherichia coli* (SASX41DB)

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Abstract

Alternative oxidase (AOX) is the terminal oxidase operating in the mitochondrial electron transport chain. The enzyme is activated by organic acid such as pyruvate and by reduction process. Based on sequences alignment of alternative oxidase gene (*Aox*) found in several organisms, there are 2 conserved cysteine residues. In order to investigate the importance of those cysteine residues on the activity of AOX, mutation at cysteine residue number 78 of *Aox* gene isolated from *Arabidopsis thaliana* (*AtAox*) was conducted. Cysteine at position number 78 was changed into serine and the c78s mutant was expressed in *Escherichia coli* strain SASX41DB. This particular *E. coli* strain is unable to grow aerobically unless transformed with Arabidopsis *Aox* gene (*AtAox*). Expression studies on c78s mutant showed that this mutant cannot be oxididized and can not be activated by pyruvic acid. This mutant is acivated by succinate instead of pyruvate. Mutation at cysteine closer to the N residue is affecting both organic acid and redox activation. Therefore, it is concluded that cysteine residue closer to the N residue is the site for both activation by pyruvate as well as activation by reduction process.

Keywords : Alternative oxidase, *site-directed mutation, SASx41DB, cysteine residues*

Introduction

Alternative oxidase (AOX) is the terminal oxidase operating in the mitochondrial electron transport chain. AOX catalyses the oxidation of ubiquinone with a subsequent reduction of oxygen to water that is not coupled with proton translocation (Figure 1).

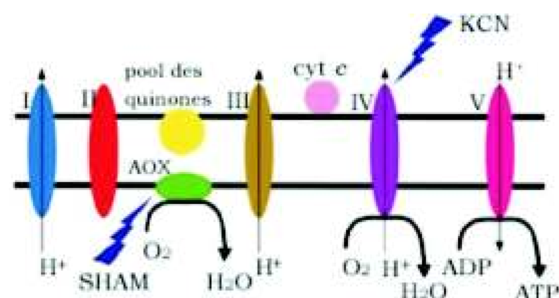

Figure 1. Alternative oxidase in plant mitochondrial electron transport chain

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At the protein level, AOX activity is controlled by the redox status of the Q pool (Dry *et al.*, 1989) and directly stimulated by certain a-keto acids e.g. pyruvate (Millar *et. al.*, 1993) which subtantially increases the apparent V_{max} of this enzyme (Hoefnagel *et al.*, 1997). The oxidase can be inactivated when disulphide bonds formed between neighbouring subunits under certain conditions (Umbah and Siedow, 1993). The covalently linked AOX dimer is largely insensitive to pyruvate (Umbach *et al.*, 1994) but can be reduced and activated during oxidation of malate and isocitrate (Vanlerberghe *et al.*, 1998), presumably via matrix NAD(P)H and thioredoxin (Moller and Palmer, 1981).

Based on the homologous alignment of amino acid sequences deduced from cDNA sequences, alternative oxidases of higher plants contain only two absolutely conserved cysteine residues (Vanlerberghe and McIntosh, 1997). Based on assumptions about the enzyme's structure, it was predicted that the cysteine which may be

exposed to the mtochondrial matrix and which is closer to the N-terminal (cys1), is the one involved in the sulfhydryl/disulfide system (Umbach and siedow, 1996). In contrast, it was suggested that the other cysteine (cys2), which may reside closer to the postulated catalytic site near the membrane, is the site of á-keto acid action (Umbach and siedow, 1996). Furthermore, Umbach and Siedow (1996) suggested that á-keto acids interact with cysteine through the formation of thiohemiacetal.

Researchers have used site-directed mutagenesis to confirm that cys1 is responsible for the disulfide linkage of subunits (Rhoads *et al.*, 1998) and (Vanlerberghe *et al.*, 1998). The same studies have shown that this residue also participates in activation by a-keto acids. Expression in *E. coli* of Arabdopsis AOX sequences in which the cysteine residue had been converted to alanine resulted in production of an enzyme which could not be covalently link but did not respond to pyruvate activation (Rhoads *et al.*, 1998). Conversion of the same cysteine to glutamate resulted in a permanently active enzyme even in the absence of pyruvate, although the activity was somewhat less than that of the wild-type enzyme (Rhoads *et al.*, 1998).

The *E.coli* strain SASX41DB has been used as an important tools to study AOX mutation. The *hem* A mutation in this strain makes this bacteria auxotrophic for 5-aminolevulenic acid (ALA). ALA is the precursor for both chlorophyll and heme biosynthesis (Jahn *et al.*, 1992). The ALA auxotrophy in this strain is caused by the absence of glutamyl-tRNA ehydrogenase activity (Avisar and Beale, 1989). The inability to synthesize heme in this strain renders the cytochrome-mediated respiration pathway inoperative. For this reason, *hem* A trains fail to grow aerobically (Sasarman *et al.*, 1968). The introduction of Arabidopsis AOX, a protein not found in *E. coli*, restores respiration through bypassing the inactive cytochrome apoenzyme in the transfer of electrons to oxygen (Kumar and Soll, 1992). Since *heme* is also required for the functional assembly of succinate dehydrogenase (Nakamura, 1996), the electron transport chain of this strain consists only of NADH dehydrogenase, ubiquinone (Q) and AOX. In this case, the expression in the *E.coli* system is much simpler than the plant system.

Site directed mutation of cysteine at residue 78 of *Arabidopsis Aox* to serine was undertaken to observe the possible effects on the activation of the *Arabidopsis* AOX. The data on this experiment is intended to gain a better perspective on the roles of the 2 conserved cysteine especially the one closest to the N terminal residue.

Materials and Methods

Site-directed mutation

Site directed mutation of the C78 codon of *AtAox1* to serine was done using the method described by Berthold (1998). The resulting mutation was then sequenced to make sure there were no additional mutation occured. The c78s *AtAox* mutant (KX-C78S), wild type *AtAox* (KX) and the negative control which is an empty plasmid pCDNAII (KX-) were used to rescue SASX41DB growth in an aerobic condition.

Colony growth and size mesurement

The *E. coli* strain SASX41DB was grown on LB agar with 60 ìl/ml ampicillin for membrane isolation or GA agar (1.2% agar, 1% NaCl, 1% bactotryptone, 0.1% yeast extract, 0.3% disodium succinate hexahydrate, 0.3% DL sodium lactate, 5 mg/l $FeSO4.7H2O$ and 60 ìg/ml ampicillin) for colony size measurement. Colonies of *E. coli* were incubated at 37°C for 63 – 64 hours before he size of individual colony was measured using Zeiss Axioskop

connected to an incident/transmitted light microscopy.

For the liquid culture, *E. coli* was grown in M63 + SG, M63 (Silhavy *et. al.*, 1984), with 0.1 mM $CaCl_2$, 0.3% disodium succinate hexahydrate, 0.2% (v/v) glycerol, 0.1% yeast extract, 0.1% casamino acids and 11.2 mg/l $FeSO_4.7H_2O$ (total concentration), pH 7.2. Solid and liquid media were supplemented with 50 mg/ml ALA (5-amino levulenic acid), 60 mg/ml ampicillin, 40 mg/ml methionine and 1mg/ml thiamine.

Membrane vesicles isolation, O_2 consumption and western blot

Membrane vesicles of *E. coli* strain SASX41DB were isolated according to the method described by Bertold (1998). O_2 consumption was measured essentially as described for plant mitochondria (Day *et al.*, 1988). The reaction medium used was *E. coli* reaction buffer composed of 100 mM NaCl, 50 mM Kpi (pH 7.0), 10 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA. The protein content estimation from *E. coli* membrane vesicle was performed similarly to the ne for mitochondria (Peterson, 1977) except that the 72% precipitation step was included before reacting with reaction solution A. Western blot was done using *Sauromatum guttatum* AOA antibody at a dilution of 1:1000 according to method by Towbin *et al.* (1979).

Results and Discussion

Mutant *AtAox* (KX-C78S) as well as the wild type (KX) and the negative control (KX-) were used to complement the growth of SASX41DB in an aerobic condition. The negative control (KX-) plasmid failed to complement the growth of SASX41DB whereas the mutant (KX-C78S) and the wild type (KX) were succesfully rescue the growth of this strain (Figure 2). This data indicates that the whole *AtAox* cDNA is important for the expression in *E. coli.*

Figure 2. SASX41DB transformed with KX-C78S (left), KX- (middle) dan KX (right)

Data of the relative size of the colonies growth are presented in table 1. Data in table 1 showed that the colony sizes of mutant and wild type did not differ significantly. This data indicates that the mutation did not affect the metabolism of the *E. coli.* However, the respiration profiles especially the activation by a-keto acids and the activation by reduction process will probably different.

Table 1. Colony areas from *E.coli* SASX41DB with the indicated plasmids, growing on GA media. Colonies were measured at 63 – 64 hours after dilution onto plates and incubation at 37°C

No	Plasmid	Area (mm2)	Replications
1	PAtAox(KX)	0.75 + 0.150	6
2	Pc78sAtAox(KX-C78S)	0.78 + 0.088	6
3	pCDNAII	not growing	6

Membrane vesicles were prepared from various *E. coli* strains and AOX activity was measured as O_2 consumption upon addition of NADH as respiratory subsrate. In vesicles containing wild type AOX, a low rate of O_2 uptake was seen upon addition of NADH but this was stimulated by the subsequent addition of 0.5 mM pyruvate (Figure 3). This oxygen uptake was abolished by n-propyl galate. The oxygen uptake of the wild type showed a typical activation by pyruvate at a concentration of 0.5 mM, 5.5 mM and 30.5 mM (Figure 3). Succinate had no significant effect on NADH-driven O_2 uptake by vesicles containing the wild type oxidase, confirming both the spesificity of organic activation in the enzyme and the lack of

succinate dehydrogenase in the heme mutant.

On the other hand, the c78s mutant of *AtAox* was not affected by pyruvate addition which is the potent activator. This result is in agreement with the results published by Rhoads *et al.* (1998) which showed an activation by succinate. Since these membranes lack succinate dehyrogenase, no O_2 uptake was observed with succinate alone (Figure 2). Half stimulation by succinate in c78s *AtAox* expressed in *E. coli* was achieved at 5 mM.

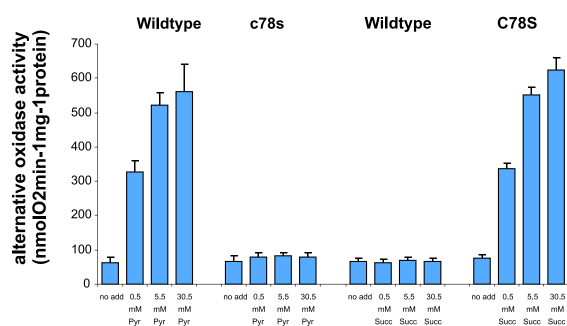

Figure 3. Oxygen consumption of the wildtype and mutant c78s*AtAox* membrane vesicles

Western blot analysis of the wild type *AtAox* and c78s *AtAox* expressed in the *E. coli* membrane did not indicate any substantial differences in expression of the different construct nor the relative amount of degradation products which were usually observed with both constructs. Repeated freezing and thawing of the membrane vesicles decreased alternative oxidase activity dramatically (data not shown). Therefore, membrane vesicles were stored as small aliquots and used only once after initial freezing in liquid Nitrogen either for western blot or O_2 consumption measurement. The presence of a degradation product is one of the dwarback of the *E. coli* heterologous system. The presence of a degradation product was probably not due to omission of a protease inhibitor during isolation, since the proteolytic product was detected in the expression of *AtAox* and trypanosome *Aox* in DH5á *E. coli* (Rhoads *et al.*, 1998); (Chauduri *et al.*, 1998). The degradation product was detected at each time interval of induction with IPTG and even when cells were grown in the presence of ALA (D. Berthold, personal comunication). Optimizing this system to abolish the proteolytic process as well as the use of a densitometer to measure the relative mount of the expressed protein in each preparation would be very useful in the future.

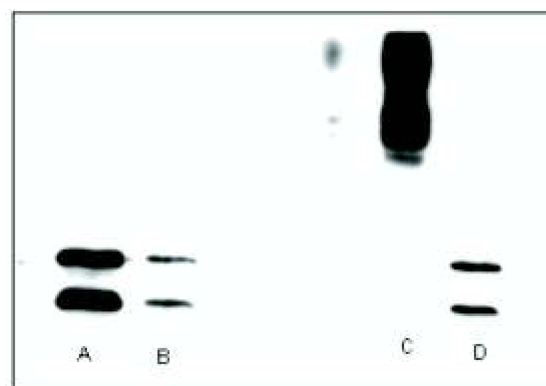

Figure 4. Western blot of reduced AOX from c78sAtAox mutant (A) and wildtype (B) and oxidized AOX from c78sAtAox mutant (C) and wildtype (D)

Western blot analysis also revealed that the mutant protein could not form an intersubunit disulfide bond when treated with diamide (a strong oxidant), as opposed to the wild type protein (Figure 4). Rhoads *et al.* (1998) also reported this finding when they used the c78a (cysteine 78 to alanine) *AtAox* and it was determined that the mutant could not be activated by pyruvate but instead was activated by succinate. Therefore, all the data confirm that succinate is an activator of AOX proteins containing a serine in place of the most N-terminal cysteine residue and show that this residue is essential for both covalent linkage (and thereby inactivation) of AOX and its activaton by pyruvate. Therefore, it is then hypothesized that the second cysteine residu

(closer to C terminal) influences the interaction of organic acids with the oxidase. Further experiments to test this hypothesis are needed

Expression of *AtAox* in *E. coli* allowed examination of a range of amino acid substitutions at the onserved cysteine sites, free from complications by endogenous AOX expression and succinate metabolism. The results confirm that succinate is an organic acid activator of an AOX proteins containing a serine in place of the most N-terminal cysteine residue and show that this residue is essential for both covalent linkage of AOX and its activation by pyruvate. The results suggests that the second cysteine may indirectly involved in the organic acid activation of AOX.

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