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Probing the ubiquinol-binding site of recombinant *Sauromatum guttatum* alternative oxidase expressed in *E. coli* membranes through site-directed mutagenesis☆

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ABSTRACT

In the present paper we have investigated the effect of mutagenesis of a number of highly conserved residues (R159, D163, L177 and L267) which we have recently shown to line the hydrophobic inhibitor/substrate cavity in the alternative oxidases (AOXs). Measurements of respiratory activity in rSgAOX expressed in *Escherichia coli* FN102 membranes indicate that all mutants result in a decrease in maximum activity of AOX and in some cases (D163 and L177) a decrease in the apparent K_m (O_2). Of particular importance was the finding that when the L177 and L267 residues, which appear to cause a bottleneck in the hydrophobic cavity, are mutated to alanine the sensitivity to AOX antagonists is reduced. When non-AOX anti-malarial inhibitors were also tested against these mutants widening the bottleneck through removal of isobutyl side chain allowed access of these bulkier inhibitors to the active-site and resulted in inhibition. Results are discussed in terms of how these mutations have altered the way in which the AOX's catalytic cycle is controlled and since maximum activity is decreased we predict that such mutations result in an increase in the steady state level of at least one O_2-derived AOX intermediate. Such mutations should therefore prove to be useful in future stopped-flow and electron paramagnetic resonance experiments in attempts to understand the catalytic cycle of the alternative oxidase which may prove to be important in future rational drug design to treat diseases such as trypanosomiasis. Furthermore since single amino acid mutations in inhibitor/substrate pockets have been found to be the cause of multi-drug resistant strains of malaria, the decrease in sensitivity to main AOX antagonists observed in the L-mutants studied in this report suggests that an emergence of drug resistance to trypanosomiasis may also be possible. Therefore we suggest that the design of future AOX inhibitors should have structures that are less reliant on the orientation by the two-leucine residues. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

The alternative oxidase is a ubiquinol oxidoreductase that catalyses the four electron reduction of oxygen to water. It is now generally recognized that the distribution of the alternative oxidase is substantially wider than previously thought [1]. It is ubiquitous amongst plants, and also found in some agrochemically important fungi (such as *Septoria tritici* — a wheat pathogen) and protists [1,2]. Importantly it is also widespread amongst human parasites such as *Trypanosoma brucei* (the causative agent of African Sleeping Sickness) [3,4], intestinal parasites such as *Cryptosporidium parvum* [5,6] and *Blastocystis hominis* [7] and opportunistic human pathogens such as *Candida albicans* [8]. It should be noted that immunocompromised individuals are particularly susceptible to these opportunistic human diseases, and new drugs that are well tolerated and have clearly defined biochemical targets are therefore urgently required [9]. Since the alternative oxidase is absent from the human host and is essential for the life-cycle of the trypanosomal parasite within the blood-stream [4] there is growing support for this protein to be considered as a viable target for the treatment of trypanosomiasis and indeed other diseases in which the alternative oxidase plays a key metabolic role [10–12]. Indeed treatment of mice infected with trypanosomes by the antibiotic ascofuranone at sub-nM concentrations rapidly clears the parasite from the blood-stream without any adverse effects upon the animal [13]. Furthermore the chemotherapeutic efficacy of ascofuranone *in vivo* has also been confirmed [14].

Abbreviations: $Q(H_2)$, (reduced) ubiquinone; OG, octyl gallate; SHAM, salicylic hydroxamic acid; CB, colletochlorin B; Asco, ascofuranone; AOX, alternative oxidase; rSgAOX, recombinant *Sauromatum guttatum* alternative oxidase

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Prior to the recent structural elucidation of the trypanosomal AOX [15], generally accepted structural models predicted that the AOX is an integral interfacial monotopic protein that interacts with a single leaflet of the lipid bilayer and contains a non-haem diiron carboxylate active site [1,16,17]. Such models are supported by extensive site-directed mutagenesis and spectroscopic studies [18–25]. Non-haem diiron-containing enzymes are a ubiquitous and diverse super-family of metalloenzymes [26]. They can be divided into different sub-families with a wide range of distinct catalytic functions such as oxidation, hydroxylation or desaturation and act on a wide variety of substrates. Despite their different activities, many of the enzymes in this family share very common structural elements. These include a common fold involving a four-helix bundle, a bridging carboxylate group in the diiron site and the presence of common ligands [26]. In addition they all possess a common catalytic function namely the activation of molecular oxygen [9,10]. The alternative oxidases are the newest and currently largest sub-class of the diiron protein family. The acquisition of a high-resolution structure of the alternative oxidase is a major breakthrough since it not only represents the first structure of any AOX or a membrane-bound diiron protein but also is the last of the mitochondrial respiratory quinol oxidases to be solved [15]. Crystal structures confirmed that the AOX is a homo-dimer with each monomer being comprised of 6 long α-helices, 4 of which form a 4 helix bundle which acts as a scaffold to bind the two iron atoms (connected by hydroxo bridge). The iron atoms within the active-site under oxidised conditions are co-ordinated by 4 glutamate residues but no histidine residues which is an unusual co-ordination for a diiron protein [15,26]. Of particular significance was the finding that the redox-active tyrosine (Y275 — *Sauromatum guttatum* numbering) is within 4 Å of the active-site consistent with its proposed role in the oxygen reduction cycle [1,27]. In addition to the wild-type enzyme, high-resolution structures of the active site of the protein in the presence of ascofuranone-derivatives, AF2779OH and colletochlorin B have also been described [15]. Such structures revealed the presence of a hydrophobic channel that connects the diiron centre with the interior of the lipid bilayer in a manner analogous to the channels observed in the yeast NADH dehydrogenase (Ndi1) [28], prostaglandin H_2 synthase [29] and Complex I [30]. All of the inhibitors appeared to enter the AOX active-site *via* this hydrophobic channel and docking studies suggest that it is also the channel through which ubiquinol enters and binds to the active-site [15,27].

In an attempt to further probe the nature of the substrate-binding site, we have generated a number of mutants within the diiron centre and at the entrance to the hydrophobic pocket in rSgAOX. Mutagenesis of highly conserved residues (R159, D163, L177 and L267) to alanine, all of which line the hydrophobic inhibitor/substrate cavity in AOX, resulted in a decrease in maximum activity of AOX and in the case of D163 and L177 a decrease in the apparent K_m (O_2). Of particular interest was the finding that the mutation of both L177 and L267 severely reduced the sensitivity of rSgAOX to AOX antagonists colletochlorin B and ascofuranone whilst increasing the sensitivity to bulky quinolone antimalarials. Such results are consistent with these residues acting as a gate to orientate substrates and inhibitors into the correct conformation to enter the active-site.

2. Materials and methods

2.1. Strains

The *Escherichia coli* strains DH5α and JM110 were used for amplification of plasmids and FN102 [31] for expression of rSgAOX.

2.2. Site-directed mutagenesis and plasmid construction

The *S. guttatum* AOX lacking the mitochondrial targeting signal sequence was used for expression in *E. coli*. Prediction of the cleavage site was performed using MitoProt (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html) [32]. In order to remove the leader sequence and facilitate cloning, a recognition site for *Nde*I was introduced at the cleavage site of the AOX. Firstly the orientation of the AOX cDNA in pAOSG81 [33] was reversed by digestion with *Eco*RI followed by ligation of the resulting fragments to give pAOSG/R. This plasmid, together with primers 5′-GTTCTCGCCCCCCCGCCATATGAGCACGCTGTCAGC-3′ and 5′-GCTGACAGCGTGCTCATATGGCGGGGGGGCGAGAAC-3′ was used to incorporate the *Nde*I site (alteration underlined) and was performed using the Quick-Change mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mature AOX sequence was removed on an *Nde*I–*Bam*HI fragment and ligated to *Nde*I–*Bam*HI digested pET15b (Novagen) to produce the expression construct pET.SgAOX. The recognition sequence of the *Nco*I site present in the pET vector was removed, by altering to GCATGG, in order to facilitate the construction of AOX mutants.

Construction of pET.T179A was carried out by removal of an *Nco*I–*Bam*HI fragment from pREP1-T179A [34] and ligation to *Nco*I–*Bam*HI digested pET.SgAOX.

Mutagenesis of AOX was performed using the Quick Change mutagenesis kit (Stratagene) according to the manufacturer's instructions. The list in Section 2.3 describes the oligonucleotides used for each mutation, with altered codons in bold and underlined. Mutagenesis was carried out with pSLM-AOR [21] yielding pQC.L177A, pQC.L267A, pQC.R159A and pQC.D163A respectively.

pQC.L177A and pQC.L267A were used initially to construct plasmids for expression in *Schizosaccharomyces pombe*. Each full-length mutant AOX was excised on a *Bsp*HI–*Bam*HI fragment and ligated to the yeast expression vector pREP1/N (a modified version of pREP1 [35] in which the *Nde*I site was replaced with *Nco*I), which had been digested with *Nco*I and *Bam*HI, yielding pREP1-L177A and pREP1-L267A. For *E. coli* expression, the mutant AOX fragment was removed from the yeast vector on an *Nco*I–*Bam*HI fragment and ligated to *Nco*I–*Bam*HI digested pET.SgAOX to produce pET.L177A and pET.L267A, respectively. Construction of pET.R159A and pET.D163A was carried out by removal of the mutant AOX on an *Nco*I–*Bam*HI fragment from pQC.R159A and pQC.D163A. The mutant AOX fragments were ligated to *Nco*I–*Bam*HI digested pET.SgAOX to produce pET.R159A and pET.D163A, respectively.

2.3. Primers

Oligonucleotides used for site-directed mutagenesis to produce mutated forms of *S. guttatum* AOX. Altered codons are in bold and underlined.

Mutation	Primer sequence	
L177A	L177A/F	CGG GCG ATG ATG **GCG** GAG ACG GTG GC
	L177A/R	GC CAC CGT CTC **CGC** CAT CAT CGC CCG
L267A	L267A/F	GTT GTG GGC TAC **GCG** GAG GAG GAG GCC
	L267A/F	GGC CTC CTC CTC **CGC** GTA GCC CAC AAC
R159A	R159A/F	C GTC AAG GCC CTC **GCG** TGG CCC ACC GAC
	R159A/R	GTC GGT GGG CCA **CGC** GAG GGC CTT GAC G
D163A	D163A/F	CGG TGG CCC ACC **GCG** ATC TTC TTC CAG C
	D163A/R	G CTG GAA GAA GAT **CGC** GGT GGG CCA CCG

2.4. Expression of rSgAOX in E. coli membranes

E. coli (FN102) cells were transformed with the pET.SgAOX construct, and grown overnight on selective Luria agar supplemented with 100 μg/ml amino-levulinic acid (ALA), 50 μg/ml kanamycin and 100 μg/ml ampicillin. A single colony was used to streak a fresh agar plate with the same supplements, and was incubated for 12 h at 37 °C. A scrape of cells from the streak plate was used to inoculate 50 ml starter culture (Luria broth, 100 μg/ml ALA, 50 μg/ml kanamycin, 50 μg/ml ampicillin). The starter culture was grown at 37 °C with shaking for ~4 h, followed by centrifugation at 8000 *g* for 5 min and resuspension

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