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Rapid report

Compelling EPR evidence that the alternative oxidase is a diiron carboxylate protein

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Abstract

The alternative oxidase is a respiratory chain protein found in plants, fungi and some parasites that still remains physically uncharacterised. In this report we present EPR evidence from parallel mode experiments which reveal signals at approximately $g=16$ in both purified alternative oxidase protein ($g=16.9$), isolated mitochondrial membranes ($g=16.1$), and in trypanosomal AOX expressed in *Escherichia coli* membranes ($g=16.4$). Such signals are indicative of a dicarboxylate diiron centre at the active site of the enzyme. To our knowledge these data represent the first EPR signals from AOX present in its native environment.

Keywords: Alternative oxidase; Diiron; Electron transfer; Respiration; Monotopic integral membrane protein

It is well established that mitochondria from all higher plants studied to date possess, in addition to the conventional cytochrome *c* oxidase, a second terminal oxidase [see 1–3]. In thermogenic plants the alternative oxidase (AOX) plays a key role in the release of heat for pollination purposes or for maintaining a warm environment within the flower at low ambient temperatures. In non-thermogenic plants, although its function is still the subject of much debate, proposed roles include maintaining TCA cycle turnover under high cytosolic phosphorylation potentials, defence against oxidative stress [see 1] and growth rate and energy charge homeostasis [4]. The occurrence of AOX is not restricted to plants since it is also found in fungi, green algae, bacteria and protozoa [see 5]. Of particular importance is the finding that within protozoa many are pathogenic organisms including the blood parasite *Trypanosoma brucei* [6] and the intestinal parasite *Cryptosporidium parvum* [7,8]. More recently AOX encoding genes have also been detected in molluscs, nematodes and chordates [9] but importantly are absent from their mammalian host making AOX a suitable chemotherapeutic target [6–8].

It is generally accepted that AOX is a non-haem diiron carboxylate protein in which the metal atoms are ligated by amino acid residues that all reside within a 4-helix bundle [*cf.* 1,2,10]. The requirement of such a tertiary structural motif, as well as the necessary spacing between the iron-ligating amino acids, imposes considerable constraints upon a possible overall fold of AOX and, consequently upon its membrane topology [10]. For this reason the current model of the AOX, supported by mutagenesis studies, predicts that the enzyme is a monotopic integral membrane protein [2,10,11] associating with one leaflet of the lipid bilayer. In comparison to other respiratory chain complexes, the alternative oxidase is poorly characterised due to the difficulties encountered in purification of the enzyme from any source to any degree of purity or stability. Although earlier characterisations of the yeast and trypanosomal enzyme

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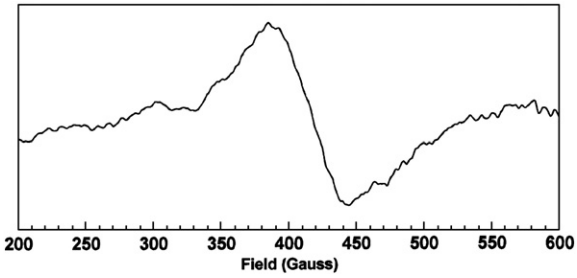

Fig. 1. EPR spectrum of AOX in purified *A. maculatum* protein. Approximately 15 mg of purified protein was made strictly anaerobic and then reduced with 1 mM DT and 1 mM PMS before freezing. EPR spectra were recorded on a Bruker Elexsys equipped with an Oxford Instruments liquid helium cryostat using a dual mode cavity and detecting in parallel mode. The instrument parameters were: temperature, 5 K; power, 50 mW; modulation, 1 mT.

established that iron was required for activity [12,13] the first direct demonstration that AOX was an iron-containing protein was provided by Berthold et al. [14] who reported EPR signals characteristic of a binuclear iron centre in *E. coli* membranes overexpressing a truncated but active *Arabidopsis thaliana* alternative oxidase (AOX1a) fused to a maltose-binding protein. More recently Affourtit and Moore [15] described a protocol for the preparation of pure AOX protein from *Arum maculatum* mitochondria which was highly active, exhibited an exceptional stability upon storage and was potently inhibited by iron chelators. Such observations support the suggestion that iron is essential for AOX catalysis and agree with the current belief that the enzyme's active site comprises a non-haem diiron centre. It should be emphasised that prior to the EPR observations of Berthold et al. [14] all previous spectroscopic attempts on either mitochondria or partially purified protein did not reveal any signal attributable to the AOX [16,17] and furthermore since this publication there have been no additional reports to substantiate these EPR observations in any AOX containing systems. Given the emerging importance of AOX not only in the metabolism of plants but for survival of pathogenic organisms within the mammalian host, further spectroscopic characterisation is required in order to establish the structural features of the alternative oxidase in clearly defined systems that are physiologically relevant. With this aim in mind we searched for EPR signals associated with diiron proteins in AOX protein purified from *A. maculatum* mitochondria, intact *Arum* mitochondria and the trypanosomal AOX expressed in *E. coli* membranes.

Although diiron proteins can exist in three oxidation states namely diferric resting, mixed valence and fully reduced we were specifically interested in detecting the low field signal detected in parallel EPR mode. This provides a sensitive structural probe for the presence of the fully reduced, but normally EPR silent, diferrous centre and previous studies have shown that the low field signal represents the majority of the iron in fully reduced diiron proteins [18]. While the EPR-active mixed valence species is often used as an indicator of diiron proteins, μ-hydroxo-bridged diiron clusters have relatively weak antiferromagnetic coupling with broad signals and corresponding low amplitude and are, in general, a minority species [19].

As discussed above it has been difficult to confirm the nature of the metal in AOX spectroscopically until recently due to the lack of suitably purified and stable protein. However as previously indicated we have developed a purification procedure [15] that results in a pure and stable protein displaying activities in excess of 3 μmol O_2 min^{-1} mg^{-1} protein with duroquinol as substrate. For EPR analysis AOX was solubilised from purified *Arum* mitochondria inner membranes using 0.5% (w/v) deoxy-BigCHAP and purified by FPLC using a DEAE-Sepharose column installed on a AKTA purifier system as described by Affourtit and Moore [15]. Fig. 1 shows that when 15 mg of purified AOX protein is made strictly anaerobic by repeated flushing with argon in the presence of 1 mM dithionite (DT) and 1 mM phenazine methosulphate (PMS), a low field EPR signal detected in the parallel mode similar to that reported by Berthold et al. [14] is observed at $g=16.9$. This EPR signal is typical of a site containing two exchange-coupled high spin ferrous ions and is only observed under fully reducing conditions and at low temperature (5 K), and is a characteristic signal for diiron carboxylate proteins [20]. Although the lineshape and temperature dependence (lost above 15 K) of the signal are similar to that observed by Berthold et al. [14], slight differences in the *g*-value are seen possibly reflecting the different environment of AOX between the two preparations. As observed previously [14] the $g=16.9$ signal is rapidly lost upon exposure to oxygen (not shown).

Mitochondria isolated from thermogenic spadices of *A. maculatum* possess a high content of alternative oxidase and are a major source for the generation of purified AOX protein. It was therefore of importance to determine if the signal illustrated in Fig. 1 could also be detected in isolated mitochondria. As in the experiments described in Fig. 1, samples containing 12 mg of purified *Arum* mitochondria that had been previously frozen were added directly to the EPR tube and made strictly anaerobic by a continuous flow of argon followed by the addition of 1 mM DT and 1 mM PMS. It is readily apparent from Fig. 2 that the characteristic signal observed with purified AOX protein is also

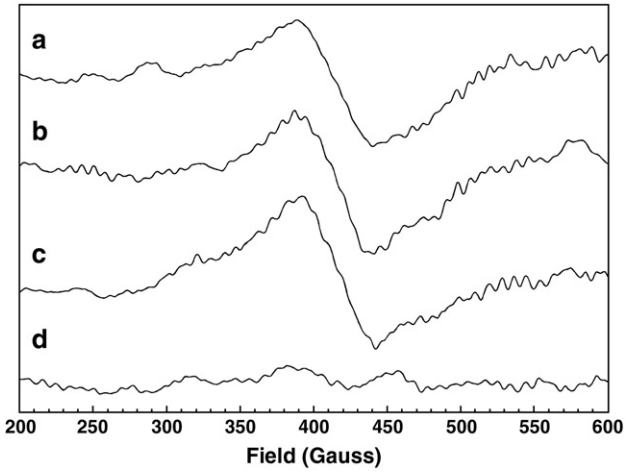

Fig. 2. EPR spectra of AOX in isolated *A. maculatum* mitochondria. Mitochondrial samples containing approximately 12 mg of purified protein were made strictly anaerobic by a continuous flow of argon with the following additions; a. 1 mM DT and 1 mM PMS. b. As in (a) but then exposed to air for 20 min. c. As in (a) but without DT or any added reductant. d. As in (a) but incubated with 4 μM octylgallate in air prior to freezing in presence of argon, DT and PMS. The instrument parameters were: temperature, 5 K; power, 50 mW; modulation, 1 mT.

seen in intact mitochondria ($g=16.1$) strongly suggesting that the diferrous signal is physiologically relevant. We noted that a similar signal could also be generated when mitochondria were incubated with 1 mM NADH as the reductant in the presence of PMS and furthermore that PMS was essential to observe the signal (results not shown). In contrast to Fig. 1, however, the EPR signal associated with the fully reduced diiron site in intact mitochondria did not disappear when the sample was exposed to air for 20 min (Fig. 2b). Furthermore we also found that in freshly harvested *A. maculatum* mitochondria no exogenous reductants were required to observe the signal (Fig. 2c). It should be noted that the mitochondria used in these experiments possessed respiratory rates in excess of 1 μmol O_2 min^{-1} mg^{-1} protein in the presence of NADH (1 mM) and 2 μM antimycin A and hence would rapidly exhaust all oxygen in the EPR tube even after exposure to air and rapid mixing. Even in the absence of added reductant the fact that the signal is still observed (Fig. 2c) suggests that freshly isolated *Arum* mitochondria contain sufficient endogenous substrates to maintain some respiratory activity and at the high protein concentrations necessary for EPR measurements rapidly achieve anaerobiosis when added to an EPR tube.

In order to ascertain if the diferrous signal was truly attributable to the alternative oxidase, a mitochondrial sample was treated with 4 μM octylgallate, a potent inhibitor of AOX activity, during exposure to air for 10 min at room temperature. The sample was then added directly to the EPR tube under a continuous flow of argon. Fig. 2d shows that in the presence of octylgallate, 1 mM PMS and 1 mM NADH the $g=16.1$ signal is no longer observed. To our knowledge the results presented in Fig. 2 are the first to report on the presence of an EPR signal attributable to the fully reduced diiron centre in AOX in intact plant mitochondria that is sensitive to AOX inhibitors.

In Fig. 3 we investigated the extent to which the diferrous signal attributable to the AOX could be detected in systems other than plants. In recent years the trypanosomal AOX (rTAO) [21,22] has also received considerable attention due to its potential role in African sleeping sickness and its similarity in sequence, and sensitivity to AOX inhibitors, to the plant protein [6]. It is readily apparent from Fig. 3 that in a sample of *E. coli* membranes overexpressing rTAO at high levels (66 mg/ml with a specific activity of approximately 11 μmol O_2 min^{-1} mg^{-1} protein with duroquinol) ran under the same EPR conditions as Figs. 1 and 2 generated a $g=16.4$ signal in the parallel mode typical of two exchange-coupled diferrous centres. As in Fig. 1 the signal disappeared following exposure to air for 10 min. Importantly the signal could also not be detected in *E. coli* membrane preparations in which rTAO expression was uninduced (results not shown).

Although it is well established that AOX catalyses the four electron reduction of oxygen to water thereby implicating the presence of a transition metal at the active site it is only relatively recently that the nature of this metal ion has been established [see 1]. To date Berthold et al. [14] have been the only laboratory to provide direct EPR spectroscopic evidence in favour of the idea that the active site of AOX comprises a non-haem diiron centre. In their study oxidation of a reduced sample of *E. coli* membranes expressing an *Arabidopsis* AOX-MBP hybrid protein generated an EPR signal detected in perpendicular mode with *g*-values 1.86, 1.67 and 1.53 characteristic of a mixed valent Fe(II)/Fe(III) binuclear centre. We have searched for such a signal following oxidation of our samples but, to date, have not been able to detect any mixed valent species. It should be noted that the mixed valent species reported by Berthold et al. [14] was only observed following the partial 1-electron reoxidation of the fully reduced AOX, whereas it is normally detected with other diiron carboxylate proteins when the mixed valent state is generated by the 1-electron reduction of the diferric centre [18–20]. Furthermore the formation of this signal specifically required a reaction with molecular oxygen that could not be fully replaced by ferricyanide. It is difficult to reconcile the detection of such a mixed valent species, attributable to a 1-electron oxidation, with what is known about the interaction of oxygen with other diiron proteins in which oxygen undergoes a 2-electron reduction upon binding to the diferrous centre to form a peroxy intermediate [20]. Nevertheless the result that the signal could be detected only in membranes containing the AOX-MBP fusion protein and not in samples lacking AOX or containing mutated AOX is evidence that the signal was indeed generated as a result of AOX activity. However, given the significant mechanistic difference between the AOX-MBP fusion protein and other diiron proteins (ie the mixed valent signal being observed only under oxidising conditions) it is also conceivable that the appearance of the signal observed by Berthold et al. [14] (and the lack of it in mitochondria, purified protein or membrane system presented here) maybe a reflection of the heterologous fusion system having a stabilising effect on the signal. Evidence that such an effect can indeed occur arises from the observation that similar signals have been detected when the oxygenase component of methane monooxygenase (protein A) is stabilised by catalytic additions of proteins B and C [23]. Nevertheless, whatever the reason for the appearance of the signal, the search for mixed valent species in clearly defined AOX systems will continue since the outcome of such studies does have significant implication with respect to our understanding of the mechanism of oxygen reduction by this enzyme [3].

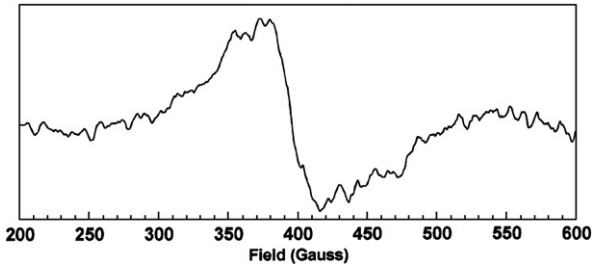

Fig. 3. EPR spectra of TAO expressed in *E. coli* membranes. Approximately 22 mg of *E. coli* membranes expressing rTAO [21,22] was made strictly anaerobic and then reduced with 1 mM DT and 1 mM PMS before freezing. EPR spectra were recorded on a Bruker Elexsys equipped with an Oxford Instruments liquid helium cryostat using a dual mode cavity and detecting in parallel mode. The instrument parameters were: temperature, 5 K; power, 50 mW; modulation, 1 mT.

What is certain however is that a low field EPR signal with *g*-values of approximately 16 can be detected in purified AOX protein, isolated *Arum* mitochondria and importantly in *E. coli* membranes overexpressing the trypanosomal AOX (rTAO), the lineshape and amplitude of these signals being comparable to that observed in other diiron proteins [20]. Although it has long been known that TAO shares some significant similarities with plant and fungal AOXs including sequence homology, sensitivity to AOX inhibitors and the ability to complement haem-deficient mutants of *E. coli* [see 6] the detection of an EPR signal similar to that observed with the purified plant AOX is the first direct physical evidence to support the notion that the trypanosomal enzyme possesses a binuclear iron centre. Of equal significance is the observation that the diferrous signal observed in reduced intact mitochondria disappears upon treatment with octylgallate under aerobic conditions confirming it is directly attributable to alternative oxidase protein. Such a result suggests that the inhibitor interacts with the catalytic centre of the enzyme thereby preventing it from becoming fully reduced even under the anaerobic conditions likely to exist in the EPR tube prior to freezing. Although this interpretation is consistent with what is known about the interaction of alternative oxidase inhibitors such as octylgallate with the active site of the enzyme the exact mechanism whereby this occurs will, however, have to await further detailed experimental examination of the purified protein.

In summary this is the first report, to our knowledge, to provide direct experimental evidence that the alternative oxidase, either as a purified protein or in its native membrane environment (ie intact mitochondria), contains an antiferromagnetically coupled binuclear iron centre at its active site. Furthermore the demonstration that a similar signal is found in trypanosomal AOX protein expressed in *E. coli* not only confirms that the enzyme is indeed present in these metabolically active parasites but also presents a potential target for future rational drug design. It is obvious, however, that before the design of structure-based specific AOX inhibitors can be achieved further work on the structure of this protein is urgently required and it is hoped that the availability of purified protein will enable this to occur.

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