

Origins, evolutionary history, and taxonomic distribution of alternative oxidase and plastoquinol terminal oxidase

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Abstract

Alternative oxidase (AOX) and plastoquinol terminal oxidase (PTOX) are related quinol oxidases associated with respiratory and photosynthetic electron transport chains, respectively. Contrary to previous belief, AOX is present in numerous animal phyla, as well as heterotrophic and marine phototrophic proteobacteria. PTOX appears limited to organisms capable of oxygenic photosynthesis, including cyanobacteria, algae and plants. We propose that both oxidases originated in prokaryotes from a common ancestral di-iron carboxylate protein that diversified to AOX within ancient proteobacteria and PTOX within ancient cyanobacteria. Each then entered the eukaryotic lineage separately; AOX by the endosymbiotic event that gave rise to mitochondria and later PTOX by the endosymbiotic event that gave rise to chloroplasts. Both oxidases then spread through the eukaryotic domain by vertical inheritance, as well as by secondary and potentially tertiary endosymbiotic events. © 2006 Elsevier Inc. All rights reserved.

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1. Introduction

As early as 1929, observations were made that respiration in plants was not fully inhibited by cytochrome oxidase inhibitors and these findings were gradually extended to a wide taxonomic range of organisms (Henry and Nyns, 1975). The “alternative oxidase” (AOX) responsible for this “CN-resistant” respiration has since been extensively characterized at the biochemical and molecular level (for recent review, see Finnegan et al., 2004). AOX is localized to the inner mitochondrial membrane and is a member of the di-iron carboxylate group of proteins, characterized by an active site that includes two iron atoms coordinated by several highly conserved glutamate and histidine residues (Berthold and Stenmark, 2003). AOX catalyzes the oxidation of ubiquinol and reduction of oxygen to water. Hence, the electron transport chain is branched, such that electrons in the ubiquinone pool are passed to O₂ via either the cytochrome pathway (using Complex III, cytochrome c and cytochrome

oxidase) or AOX. The AOX branch of respiration is non-energy-conserving and while its physiological role is still a matter of debate, a developing idea from plant studies is that it may act to dampen the rate of electron transport chain-generated reactive oxygen species (Finnegan et al., 2004).

In 1999, it was reported that the *Arabidopsis thaliana* chloroplast contains a di-iron carboxylate protein with sequence similarity to AOX (Carol et al., 1999; Wu et al., 1999). This protein has been variously termed IMMUTANS (Carol et al., 1999; Wu et al., 1999), plastid terminal oxidase (Carol and Kuntz, 2001) and plastoquinol terminal oxidase (PTOX) (McDonald et al., 2003). Analogous to the biochemical activity of AOX in the inner mitochondrial membrane, PTOX is a thylakoid membrane protein that couples the oxidation of plastoquinol with the reduction of oxygen to water (for recent review, see Aluru and Rodermel, 2004). Recently, site-directed mutagenesis has verified that the PTOX glutamate and histidine residues that are a conserved feature of di-iron carboxylate proteins are indeed necessary for PTOX activity (Fu et al., 2005). PTOX provides an important electron sink during early chloroplast biogenesis and likely also plays a supporting role in photosynthesis and plastid metabolism in the dark.

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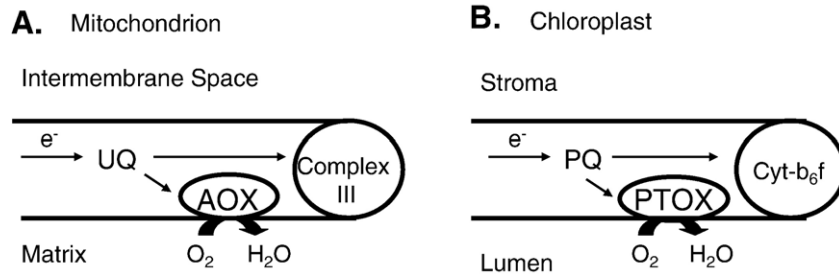


Fig. 1. AOX and PTOX each represent non-energy conserving branches in their respective respiratory (A) and photosynthetic (B) ETCs. Both act as quinol oxidases, able to pass electrons from a quinol pool (ubiquinol [UQ] or plastoquinol [PQ]) to O₂, producing H₂O. Note that this figure is based on the eukaryotic ETCs found in mitochondria and chloroplasts. Note also that only a portion of the ETC components is being shown to emphasize the branch point to AOX or PTOX.

The placement of AOX and PTOX in their respective respiratory and photosynthetic ETCs is illustrated in Fig. 1. The metabolic and physiological role of these two non-energy-conserving oxidases remains an area of intensive investigation. For a more thorough discussion of potential roles, the reader is directed to several recent reviews (Simons and Lambers, 1999; Peltier and Cournac, 2002; Aluru and Rodermel, 2004; Finnegan et al., 2004).

In 2003 the presence and functionality of AOX in a prokaryote, the soil proteobacterium *Novosphingobium aromaticivorans*, was demonstrated (Stenmark and Nordlund, 2003). Given no other example of a prokaryotic AOX (or PTOX), it was hypothesized that the bacterium may have acquired AOX by horizontal gene transfer, likely from plants. However, several groups later reported the presence of PTOX in cyanobacteria, sparking the suggestion that AOX and PTOX may have each entered the eukaryotic lineage via the endosymbiotic events that gave rise to mitochondria and chloroplasts (McDonald et al., 2003; Finnegan et al., 2003; Atteia et al., 2004). Despite the parsimonious nature of this hypothesis, the known taxonomic distribution of AOX and PTOX was still quite restricted, leaving considerable uncertainty about the evolutionary events that may have contributed to their distribution in nature (Suzuki et al., 2005). Here, we report the most comprehensive analysis of AOX and PTOX distribution to date and suggest that this expanded range can be used to infer more confidently the origins and evolutionary history of these related organelle-localized oxidases.

2. Materials and methods

2.1. Data retrieval

AOX and PTOX sequences were identified using tBLASTn and discontinuous megablast searches of the non-redundant, trace archive, and environmental samples databases at the National Center for Biotechnology Information, at the *Cyanidioschyzon merolae* Genome Project, at CyanoBase, at the Taxonomically Broad EST Database, at the Institute for Genomic Research, at the U.S. Department of Energy Joint Genome Institute, at the *Phaeodactylum tricornutum* EST database and at the *Ciona savignyi* genome project. A summary of all AOX and PTOX sequences uncovered as of August 2006, along with supporting information (including website addresses

of the above databases) is available as Supplementary Data (Table 1SD–3SD). Table 1SD includes >410 different AOX and PTOX genes from ~270 species across five kingdoms. Table 2SD includes >100 different AOX and PTOX genes from three different marine environmental sample datasets. Table 3SD summarizes the >45 pathogenic species found to contain AOX.

3. Results and discussion

3.1. Prokaryotic origins of AOX and PTOX

We suggest that extant AOX and PTOX proteins arose from a common ancestral protein present in ancient prokaryotes and that diversification toward AOX and PTOX occurred prior to the primary endosymbiotic events that gave rise to eukaryotes. This is based on the following evidence:

- i. Di-iron carboxylate proteins (of which AOX and PTOX are clearly members) are likely ancient in origin since they are present in all kingdoms of life (including Archaeobacteria) and in both aerobes and anaerobes (Gomes et al., 2001). Other members of this protein family include ribonucleotide reductases, ferritins, bacterioferritins, Δ^9 -desaturases, methane mono-oxygenases, and rubrerythrins. A combination of sequence and structure-based analyses show that this family is characterized by the presence of a non-haem di-iron centre (in which residues involved in metal binding are highly conserved) contained within a structurally conserved four-helix-bundle conformation (Gomes et al., 2001). Significantly, diverse members of this protein family retain a capacity to reduce oxygen to water, even though these same proteins are thought to primarily have other physiological functions (Broadwater et al., 1998; Gassner and Lippard, 1999; Gomes et al., 2001). Based on these observations, it was hypothesized that oxygen reductase activity is an ancient feature of this protein family and that the ancestral proteins may have acted to scavenge oxygen during the early transition from an anaerobic to aerobic world (Gomes et al., 2001). This could have been the driving force for the evolution of a more efficient oxidase, the ancestor of AOX and PTOX. An additional benefit is that this early oxidase activity may have been resistant to sulfide in a sulfide-rich world.

Unlike cytochrome oxidase, extant AOX is sulfide-resistant (Azcón-Bieto et al., 1989).

- ii. While the amino acid identity between di-iron carboxylate proteins is generally too low to be used for conventional phylogenetic analyses, this is clearly not the case in comparing AOX and PTOX. These proteins share ~25% amino acid sequence identity within the core protein (delimited by the first and fourth iron-binding motifs). Clearly, AOX and PTOX are more similar to one another than to any other members of this protein family. Nonetheless, they can also be clearly defined from one another by conventional phylogenetic analyses or analysis of amino acid sequence flanking conserved iron-binding residues (McDonald et al., 2003).
- iii. Based upon previous work (Stenmark and Nordlund, 2003; McDonald and Vanlerberghe, 2005; and Table 2SD) and now newly-found AOX genes in *Vibrio angustum*, *Vibrio fischeri*, *Vibrio splendidus*, *Sphingomonas* sp., *Roseovarius* sp., *Erythrobacter* sp., *Photobacterium* sp., *Photobacterium profundum*, *Psychromonas ingrahamii*, *Roseobacter denitrificans*, and *Thiobacillus denitrificans* (Table 1SD), it is becoming evident that AOX is widespread amongst proteobacteria. Similarly, PTOX genes have now been described in many cyanobacterial genomes (McDonald and Vanlerberghe, 2005; Tables 1SD and 2SD).
- iv. Phylogenetic analyses that include a broad taxonomic range of both prokaryotic and eukaryotic proteins show that AOX and PTOX clearly fall into two separate clades (Fig. 2). This is consistent with the hypothesis that an ancestral protein diversified to AOX and PTOX prior to the primary endosymbiotic events that gave rise to

eukaryotes. If diversification to AOX and PTOX had occurred following the primary endosymbiotic events, we might expect clades not as strictly defined along the AOX/PTOX divide but rather along the prokaryotic/eukaryotic divide.

3.2. Endosymbiotic events and vertical inheritance of AOX and PTOX in Eukaryotes

We suggest that AOX and PTOX arose in eukaryotes via the primary endosymbiotic events that gave rise to mitochondria and chloroplasts, respectively. Subsequently, these proteins spread through the eukaryotic domain by vertical inheritance and, in some cases, via secondary and tertiary endosymbiotic events. This model is summarized in Fig. 3 and is supported by the following evidence:

- i. Extant proteobacteria and extant cyanobacteria are proposed to be the descendants of the ancient prokaryotes that, via two separate endosymbiotic events, gave rise to mitochondria and chloroplasts, respectively (Precht and Maier, 2001). It is indeed intriguing then that extant prokaryotic AOXs exist but thus far appear limited to proteobacteria, not being found in the sequenced genomes of any other eubacterial groups. Similarly, extant prokaryotic PTOXs exist but are limited to cyanobacteria and are, for example, not present in several sequenced phototrophic bacterial genomes (Tables 1SD and 2SD).
- ii. It is generally accepted that the primary endosymbiotic event that gave rise to the mitochondrion preceded that giving rise to the chloroplast (Precht and Maier, 2001).

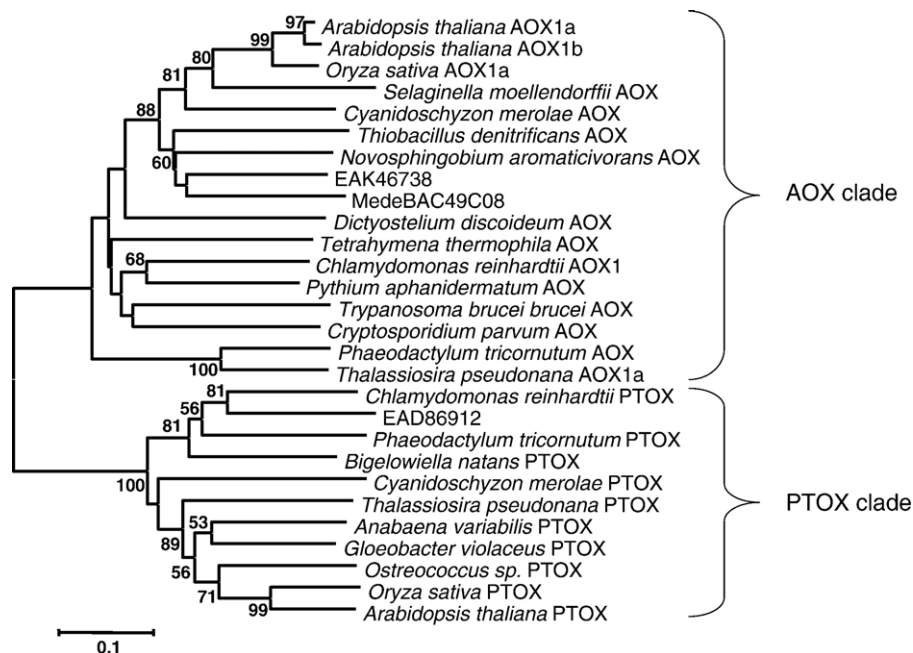
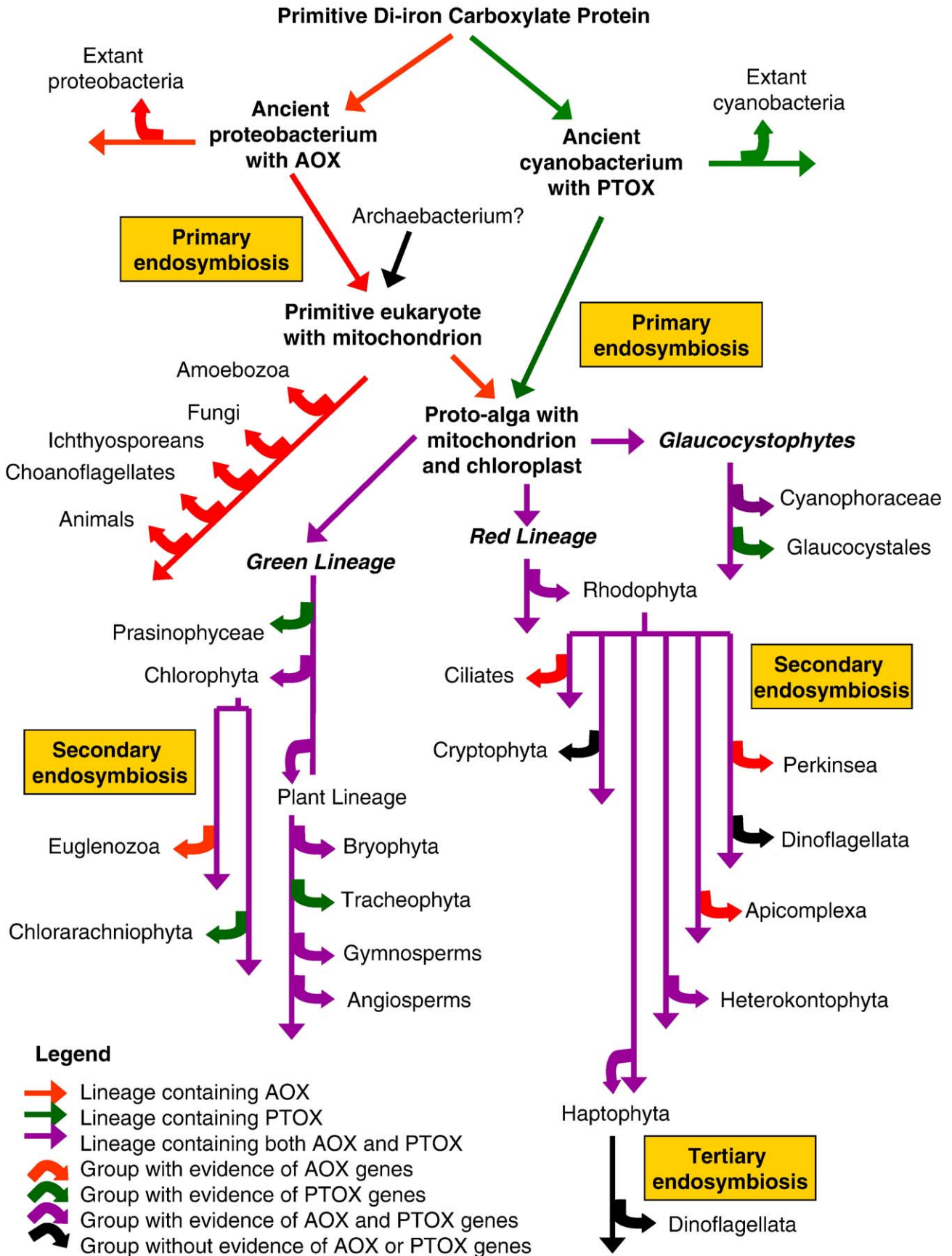


Fig. 2. A protein phylogeny generated with a wide taxonomic range of prokaryotic and eukaryotic organisms, demonstrating the separation of AOX and PTOX clades. Phylogenies were generated as previously described (McDonald et al., 2003; McDonald and Vanlerberghe, 2004) using 99 amino acid sites comprising the 1st to 3rd iron binding motifs of AOX and PTOX (e.g. amino acids 182–291 of *A. thaliana* AOX1a, 135–244 of *A. thaliana* PTOX).



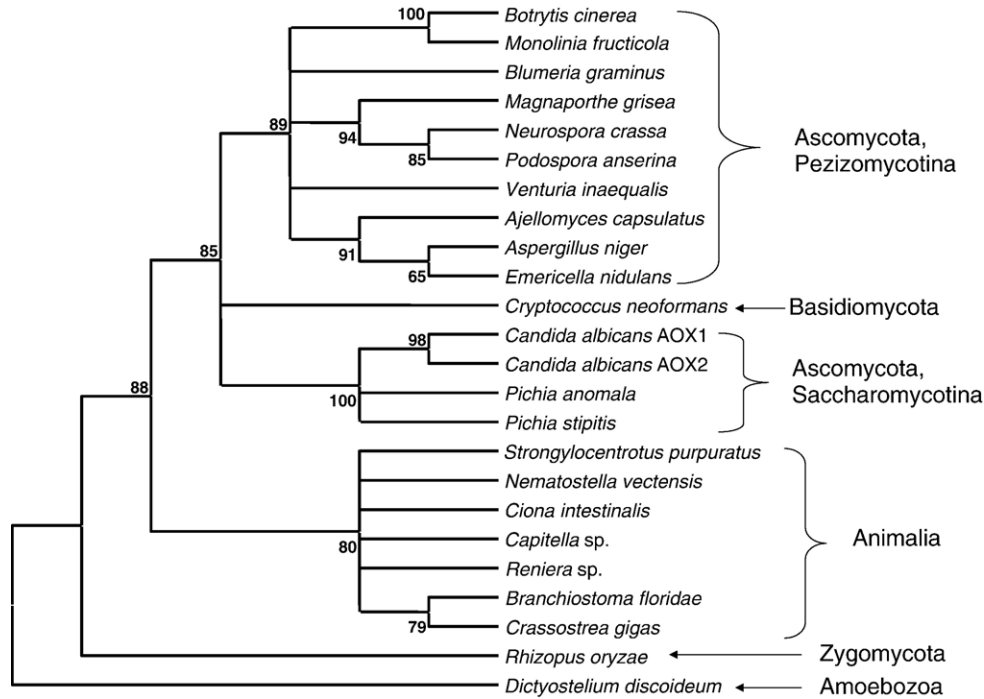


Fig. 4. A protein phylogeny demonstrating that animal AOXs and the AOXs from the fungi (Ascomycota and Basidiomycota) form monophyletic clades. The single known AOX from the Zygomycota is basal to the rest of the fungal AOXs. The slime mold *Dictyostelium discoideum* has an AOX that is divergent from all fungal and animal AOXs. Phylogenies were generated as previously described (McDonald et al., 2003; McDonald and Vanlerberghe, 2004; McDonald and Vanlerberghe, 2005) using 104 amino acid sites comprising the 1st to 3rd iron binding motifs of AOX (e.g. amino acids 182–296 of *A. thaliana* AOX1a).

As a result, some eukaryotic lineages (including amoebozoa, some protists, fungi, ichthyosporeans, choanoflagellates, and animals) are believed to have never been exposed to the cyanobacterial endosymbiont that gave rise to chloroplasts. We would expect, then, that the extant members of these “aplastidic” lineages could have AOX but not PTOX. The available evidence supports this prediction. While the known distribution of AOX amongst the aplastidic lineages including animals continues to mount, we find no evidence of PTOX within any of these groups (Fig. 3; Table 1SD).

- iii. We define “plastidic” eukaryotes as those groups that have contained a “chloroplast” at some time during their evolutionary history and hence this includes all of the lineages resulting from the primary endosymbiotic event involving a cyanobacterium (Rodríguez-Ezpeleta et al., 2005). This includes the Glaucocystophyta, the “green” lineage and the “red” lineage (Fig. 3). Unlike the “aplastidic” lineages, we would expect that these organisms could contain both AOX and PTOX. Again, this prediction is supported by our analysis. In the primary plastidic lineages we found evidence of AOX and PTOX in the glaucocystophytes, AOX and PTOX in red algae, and PTOX in the prasinophytes *Mesostigma viride* and

Micromonas sp., the most basal ancestors to green algae and plants (Table 1SD). These findings are consistent with the idea that the common ancestor of all of the primary plastid lineages (the proto-alga) contained both AOX and PTOX.

- iv. The plastidic eukaryotes also include those organisms believed to have acquired a plastid via secondary and tertiary endosymbiotic events, in which a non-photosynthetic host eukaryotic cell engulfed a green or red alga (Archibald, 2005). Here again, we would expect that these organisms could not only contain AOX, but PTOX as well (Fig. 3). Extant green algae (Chlorophyta) contain both AOX and PTOX and two separate secondary endosymbiotic events involving ancient green algae are proposed to have resulted in the Chlorarachniophyta and the Euglenozoa. PTOX has been identified in the chlorarachniophyte *Bigeloviella natans* while *Euglena gracilis* and *Euglena longa* have AOX (Castro-Guerrero et al., 2004; Table 1SD). The extant red alga *Cyanidioschyzon merolae* contains both AOX and PTOX. Groups hypothesized to contain secondary red plastids are the Cryptophyta, ciliates, Apicomplexa, Heterokonts, Haptophyta, and Dinoflagellata. Clear evidence of AOX and/or PTOX genes is found in all these groups, with the

Fig. 3. A hypothesis for the origins and evolutionary history of AOX and PTOX, based upon current evidence regarding the taxonomic distribution of these oxidases in nature. Straight arrows refer to the lineages proposed to contain only AOX (red), only PTOX (green) or both AOX and PTOX (purple). Curved arrows summarize the current evidence (as gene sequence data) for different taxonomic groups. A curved red arrow indicates evidence of AOX, a curved green arrow indicates evidence of PTOX and a curved purple arrow indicates evidence of both AOX and PTOX. Black arrows indicate no current evidence for AOX or PTOX. See text for full details.

exception of the Cryptophyta and Dinoflagellata (Table 1SD). We do find AOX, however, in *Perkinsus marinus*, considered a sister group to the dinoflagellates (Fig. 3).

4. Taxonomic distribution and function

Given the ancient nature of the primary endosymbiotic events, we predict that the taxonomic distribution of AOX and PTOX could be widespread. Indeed, the current deluge of newly arriving DNA sequence data is supporting this expectation. The broadening distribution also strengthens our assumption that AOX and PTOX have spread through the prokaryotic and eukaryotic domains by vertical inheritance. Below, we provide a few further comments about the distribution of these proteins and what this might indicate regarding their functional significance.

Previous work and our analyses indicate the widespread presence of AOX in the aplastidic lineage. AOX is present in the jakobids *Reclinomonas americana* and *Seculamonas ecuadoriensis* and the jakobid-like *Malawimonas jakobiformis* (Table 1SD). Jakobids are free-living mitochondriate protists and the *R. americana* mitochondrial genome contains more genes than any other eukaryote (Archibald et al., 2002). This suggests that jakobids represent one of the earliest diverging eukaryotic lineages, although this view has been contested (Simpson et al., 2006). AOX is also found in the Amoebozoa (e.g. *Dictyostelium discoideum*, *Hyperamoeba dachnaya*, and *Hartmanella vermiformis*) and is widespread in the fungal groups Ascomycota and Basidiomycota. This fungal distribution can now be further extended to the more basal Zygomycota and Chytrids (Fig. 4; Table 1SD).

AOX is present in two Ichthyosporeans, *Capsaspora owczarzaki* and *Sphaeroforma arctica*, as well as the choanoflagellate *Monosiga ovata* (Table 1SD). Molecular phylogenies have demonstrated that ichthyosporeans diverged after fungi, but before choanoflagellates and animals (Lang et al., 2002; Fig. 3). The choanoflagellates are considered to be the sister group of animals (Lang et al., 2002; Fig. 3).

Until recently (McDonald and Vanlerberghe, 2004), it was assumed that AOX was not present in the Animalia. We have now identified AOX genes in 18 animal species, representative of seven different phyla: Chordata, Echinodermata, Nematoda, Mollusca, Annelida, Cnidaria and Porifera (Fig. 4; Table 1SD). Recently, the AOX gene of *Ciona intestinalis* (sea squirt) was expressed in human cultured cells and confirmed to target to mitochondria and possess biochemical activity (Hakkaart et al., 2006). The identification of putative AOXs in several sponges (e.g. *Ephydatia muelleri*, *Oscarella carmela* and *Reniera* sp.), which are members of the most basal animal phylum, suggests that the presence of AOX is the ancestral state, rather than the lack of AOX exemplified by several vertebrate and arthropod genomes (Table 1SD).

The broad distribution of AOX emerging in animals is somewhat surprising but consistent with past biochemical studies showing a CN-resistant component to respiration in some animals (see references in McDonald and Vanlerberghe, 2004). A large number of these studies have taken place in

animals that readily tolerate sulfide-rich habitats. This includes animals in the Mollusca and Annelida, in which we find several species containing AOX genes. We predict that, in many cases, the CN- and sulfide-resistant respiration being noted in previous animal studies results from AOX. Other potential roles of AOX in the animal kingdom have been previously discussed (McDonald and Vanlerberghe, 2004).

A striking feature of the organisms being found to contain AOX is that many are pathogenic organisms (Table 3SD). These include *Vibrio splendidus* (eubacteria), *Prototheca wickerhamii* (Chlorophyta), *Trypanosoma brucei brucei* (kinetoplastida), *Cryptosporidium parvum* (Apicomplexa), *Phytophthora infestans* (Oomycota), *Candida albicans* (Ascomycota), *Cryptococcus neoformans* (Basidiomycota), *Rhizopus oryzae* (Zygomycota) and *Meloidogyne hapla* (Nematoda). In part, this may represent a bias in the dataset because of intensive investigation of such organisms. However, in several cases, AOX has been shown or suggested to play an active role in pathogenesis (Akhter et al., 2003; Fang and Beattie, 2003; Missall et al., 2004; Felipe et al., 2005; Henriquez et al., 2005).

An interesting finding of our analysis is that PTOX is thus far limited strictly to organisms carrying out oxygenic photosynthesis, including cyanobacteria, algae and plants (Tables 1SD and 2SD). Whole genome data is available for several bacteria capable of non-oxygenic photosynthesis (e.g. *Chlorobium tepidum* TLS, *Chloroflexus aurantiacus* J-10-fl) and these all lack PTOX. In several cases in which plastidic eukaryotes (the ancestors of which we predict to have contained PTOX) have lost their capacity for photosynthesis (while in some cases still retaining a relic plastid such as the apicoplast), whole genome analyses confirm that PTOX is absent. This is the case, for example, in *Theileria parva* (a red lineage apicomplexan) and *Trypanosoma brucei* (a green lineage euglenozoan). There are no confirmed cases of a photosynthetic eukaryote lacking PTOX since, in all five cases in which complete genomes are available, PTOX is present. In the case of cyanobacteria, however, there are examples in which PTOX is clearly absent (e.g. *Synechocystis* PCC6803, *Thermosynechococcus elongatus* BP-1). However, these same cyanobacterial genomes do encode other oxidases (e.g. cytochrome bd oxidase) that may be functionally analogous to PTOX (Howitt and Vermaas, 1998; Hart et al., 2005). There is no evidence of a cytochrome bd oxidase in any eukaryote.

We did find clear evidence of one horizontal gene transfer event, albeit an exceptional case. The genome of cyanophage P-SSM4 encodes a PTOX gene that shares a high degree of sequence similarity with the PTOX gene of its host *Prochlorococcus marinus* and also contains a specific indel seen only in *Prochlorococcus marinus* PTOX genes (McDonald et al., 2003). Interestingly, this viral genome contains several *Prochlorococcus marinus* genes encoding proteins that play fundamental roles in photosynthesis (e.g. Psb, Psa, hli, PcyA) and it is believed that expression of these genes during infection acts to maintain host photosynthesis and thus improve phage fitness (Lindell et al., 2005). That PTOX is amongst the genes being recruited in this manner indeed suggests that it may play

an important role in the photosynthetic process of this cyanobacterium. Rapid changes in PTOX expression in response to changes in light intensity have been noted in both higher plants (Rizhsky et al., 2002) and cyanobacteria (McDonald and Vanlerberghe, unpublished), perhaps indicating a role for PTOX in adjustment of the photosynthetic apparatus to changes in electron flux.

As exemplified by Fig. 3, there are several major lineages in which AOX and/or PTOX genes might be predicted to exist, but for which no molecular evidence has been forthcoming. In some cases this may simply be a consequence of the paucity of genome sequence data available for any members of that group (e.g. Cryptophyta, Dinoflagellates). For other groups in which sequence data is abundant (e.g. vertebrates), the lack of AOX and/or PTOX sequence presumably reflects a specific gene loss from the lineage.

Numerous gene phylogenies show that AOX and PTOX proteins form two separate clades, with monophyletic groups within each clade (McDonald et al., 2003; McDonald and Vanlerberghe, 2004; McDonald and Vanlerberghe, 2005 and Fig. 2). Nonetheless, it has been difficult to resolve the positions of the bacterial and protist proteins. Limited data from many lineages, the occurrence of paralogs within multi-gene families, gene loss from some lineages, and gene replacements (during secondary and tertiary endosymbiotic events) may all contribute to this difficulty. Presumably, better data sampling will serve to clarify the topology of gene trees and to address critiques about, for example, the placement of bacterial AOXs (Suzuki et al., 2005; Henriquez et al., 2005).

In summary, it appears that the distribution of AOX and PTOX in nature is much broader than previously thought and we expect that complete loss of these genes has only occurred in a minority of lineages. This exposes a large gap in our understanding of the physiological role of these terminal oxidases in a wide range of organisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbd.2006.08.001.

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