The Plastid Terminal Oxidase: Its Elusive Function Points to Multiple Contributions to Plastid Physiology

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Abstract

Plastids have retained from their cyanobacterial ancestor a fragment of the respiratory electron chain comprising an NADPH dehydrogenase and a di-iron oxidase, which sustain the so-called chlororespiration pathway. Despite its very low turnover rates compared with photosynthetic electron flow, knocking out the plastid terminal oxidase (PTOX) in plants or microalgae leads to severe phenotypes that encompass developmental and growth defects together with increased photosensitivity. On the basis of a phylogenetic and structural analysis of the enzyme, we discuss its physiological contribution to chloroplast metabolism, with an emphasis on its critical function in setting the redox poise of the chloroplast stroma in darkness. The emerging picture of PTOX is that of an enzyme at the crossroads of a variety of metabolic processes, such as, among others, the regulation of cyclic electron transfer and carotenoid biosynthesis, which have in common their dependence on the redox state of the plastoquinone pool, set largely by the activity of PTOX in darkness.

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INTRODUCTION

Oxidases in Energy-Transducing Membranes

Oxidases: enzymes that reduce oxygen; they are either bioenergetic enzymes engaged in oxidative phosphorylation or scavenger enzymes decreasing the local oxygen concentration

Alternative oxidases: quinol oxidases contributing to an electron transfer pathway alternative to cytochrome oxidase; mitochondria harbor a unique oxidase of this kind named AOX

n-side: the negatively charged side of the inner membrane of a mitochondrion after this membrane has been electrically polarized by the turnover of the respiratory enzymes

Oxygen, found in its reduced form as water (−2) and in its oxidized form as gaseous molecular oxygen in the atmosphere (0), is the most abundant element on earth. It is involved in many redox processes throughout the life realm, prominent among which are respiration and oxygenic photosynthesis, which respectively use oxygen as an electron acceptor and water as an electron donor. These metabolic processes involve a membrane-borne electronic flow that is coupled to the synthesis of ATP, thus supplying cells with the energy currency required to sustain their metabolism, development, and division (86, 101). As a consequence of its two unpaired electrons occupying two antibonding orbitals, the dioxygen molecule is highly reactive and can be found in many different chemical states and, in particular, in reactive oxygen species (ROS)—primarily singlet oxygen ($^1O_2$), superoxide ($O_2^{\cdot -}$), and peroxide derivatives (8, 135), which may trigger oxidative reactions in an uncontrolled manner and are potentially harmful to their biological environment. This may account for the cellular enzymatic arsenal involved in the active or passive detoxification of ROS or in the scavenging of their oxygen precursor (85, 114, 116, 130).

Oxidases use dioxygen as an electron acceptor. Whereas bacteria encode a variety of oxidases, some of which are primarily oxygen-scavenging enzymes (49), mitochondria harbor two oxidases (for a review, see 134): the cytochrome *c* oxidase (complex IV) and the alternative oxidase (AOX), first described in plant mitochondria (12; for a recent review, see 99). Complex IV is a diheme dicopper oxidase. It is a transmembrane, electrogenic protein complex that couples dioxygen reduction to proton pumping across the membrane (160, 164). A water-soluble cytochrome *c* found in the intermembrane space acts as its electron donor. AOX is a simple diiron protein, bound monotopically to the n-side of the inner mitochondrial membrane (92, 154, 166). It sustains a non-electrogenic, cyanide-insensitive (in contrast to complex IV) respiration that uses quinols, which

are soluble in the lipid bilayer, as electron donors. At first sight, the quinol-oxidizing activity of AOX may be seen as futile from a thermodynamic standpoint or as a partially decoupling electron flow from proton pumping, because it lowers the ATP yield of the respiratory pathway by diverting reducing equivalents from the sequential electrogenic activities of complexes III and IV. However, its affinity for oxygen being lower than that of complex IV (45), it is a poor competitor to the downstream cytochrome bc_1 oxidase complexes unless the quinone pool is overreduced (72). Thus, it may act as a safety valve that would function only when the mitochondrial electron transfer chain is congested. There are, however, many other possible roles for AOX that stem from its nonelectrogenic behavior, including thermogenesis (156), oxygen scavenging (45), stress signaling (29), reactive oxygen and nitrogen species homeostasis (89, 154), gametophyte development (26), fruit ripening (112), or, when working as an overflow device, a damper of metabolite fluctuations (119).

A counterpart of this mitochondrial AOX is specifically found in plastids: the plastid terminal oxidase (PTOX). PTOX, which is also a diiron-containing protein, takes up electrons from plastoquinol (PQH_2) to reduce oxygen via a nonelectrogenic process. Originally called IMMUTANS, it is a nucleus-encoded, 6α-helical-bundle, peripheral membrane protein. Its mass varies from 40 to 50 kDa depending on the species. PTOX binds strongly to the stromal side of thylakoid membranes in their nonappressed regions (82). Its expression level in unicellular organisms and in mature tissues of vascular plants is substoichiometric with respect to the major protein complexes of the photosynthetic electron transfer chain, with a PTOX/photosystem II (PSII) protein ratio in spinach of approximately 1/100 (82). Yet, as discussed below, a variety of stresses impact its abundance. PTOX participates in a light-independent electron transfer pathway called chlororespiration, a term coined by Bennoun (13) more than 30 years ago by analogy with mitochondrial respiration. Chlororespiration involves the reduction of a pool of plastoquinones (PQs), which are soluble in the lipid phase of thylakoid membranes, by a PQ reductase and their reoxidation by PTOX, which acts as a PQH_2:oxygen oxidoreductase.

Two types of reductases have been identified. The NADH dehydrogenase-like (NDH) enzyme, present in land plants and some unicellular algae (Streptophyta and *Nephroselmis*), uses NADPH or reduced ferredoxin (163) as an electron donor and pumps protons similarly to its mitochondrial homolog. The type II NAD(P)H dehydrogenase-like (NDA2) enzyme, present in most unicellular algae (88), oxidizes NADPH (56) and is a monotopically bound, nonelectrogenic enzyme. Thus, the overall chlororespiratory process by which NADPH (or ferredoxin) is oxidized and oxygen is reduced is expected to be electrogenic in plant chloroplasts but not in algae. Strangely enough, there has been no study so far of the potential implication of these differences in electrogenicity in the physiological control of chlororespiration between plants and NDA2-bearing algae.

Several reviews have been devoted to PTOX (16, 37, 90, 109, 115, 129, 146), but its role(s) and interplay with the photosynthetic function remain enigmatic. Here, we describe some structural features of the enzyme in light of the recent structural data obtained for its mitochondrial counterpart. We discuss its enzymatic function and its possible physiological integration into various metabolic pathways, taking into account, in particular, a quantitative comparison of its activity with the redox fluxes sustained by the pathways to which it may directly or indirectly contribute.

THE EVOLUTION, STRUCTURE, AND FUNCTION OF THE PTOX ENZYME

Terminal Oxidases and Their Common Ancestor

PTOX and AOX are both diiron quinol oxidases (137, 167). With the exception of the diiron-binding motif made of four glutamic acid and two histidine residues, protein sequences within the

nonheme diiron protein family are poorly conserved (18, 77). Other members in this family include methane monooxygenase, the R2 subunit of ribonucleotide reductase, ferritin, rubrerythrin, the 5-demethoxyquinone hydrolase, and the aerobic Mg-protoporphyrin IX monomethylester hydroxylase involved in chlorophyll biosynthesis (18, 77, 78). As in AOX and ribonucleotide reductase, a conserved tyrosine residue is necessary for PTOX function (40), suggesting that quinol oxidation proceeds similarly in PTOX and AOX. The two enzymes are found in both prokaryotes and eukaryotes. Their common ancestor—a primitive diiron-carboxylate oxidase, perhaps an oxygen scavenger during the transition from the anoxic to the oxic world—is of prokaryotic origin. As pointed out by McDonald & Vanlerberghe (91), the divergence of these two oxidases occurred before the two primary endosymbiotic events that respectively led to the emergence of the plastid and the mitochondrion. Indeed, PTOX and AOX are phylogenetically more distant than their respective prokaryotic and eukaryotic versions, contrary to what would be the case if the divergence had occurred after primary endosymbiosis. PTOX is present in cyanobacteria; green, red, and brown algae; flowering plants; and cyanophages. Although chloroplasts evolved from the engulfment by a eukaryotic cell of a symbiotic photosynthetic cyanobacterium, which likely had both a cytochrome oxidase and an alternative oxidase (48), their thylakoids display a single type of oxidase—PTOX. Assuming that the loss of cytochrome oxidase activity in chloroplasts is an evolutionary consequence of the integration of both the photosynthetic and respiratory metabolisms within a single eukaryotic cell, the same logic raises the question, addressed below, of why there has been evolutionary pressure to conserve alternative oxidases such as PTOX in the entire photosynthetic eukaryotic lineage.

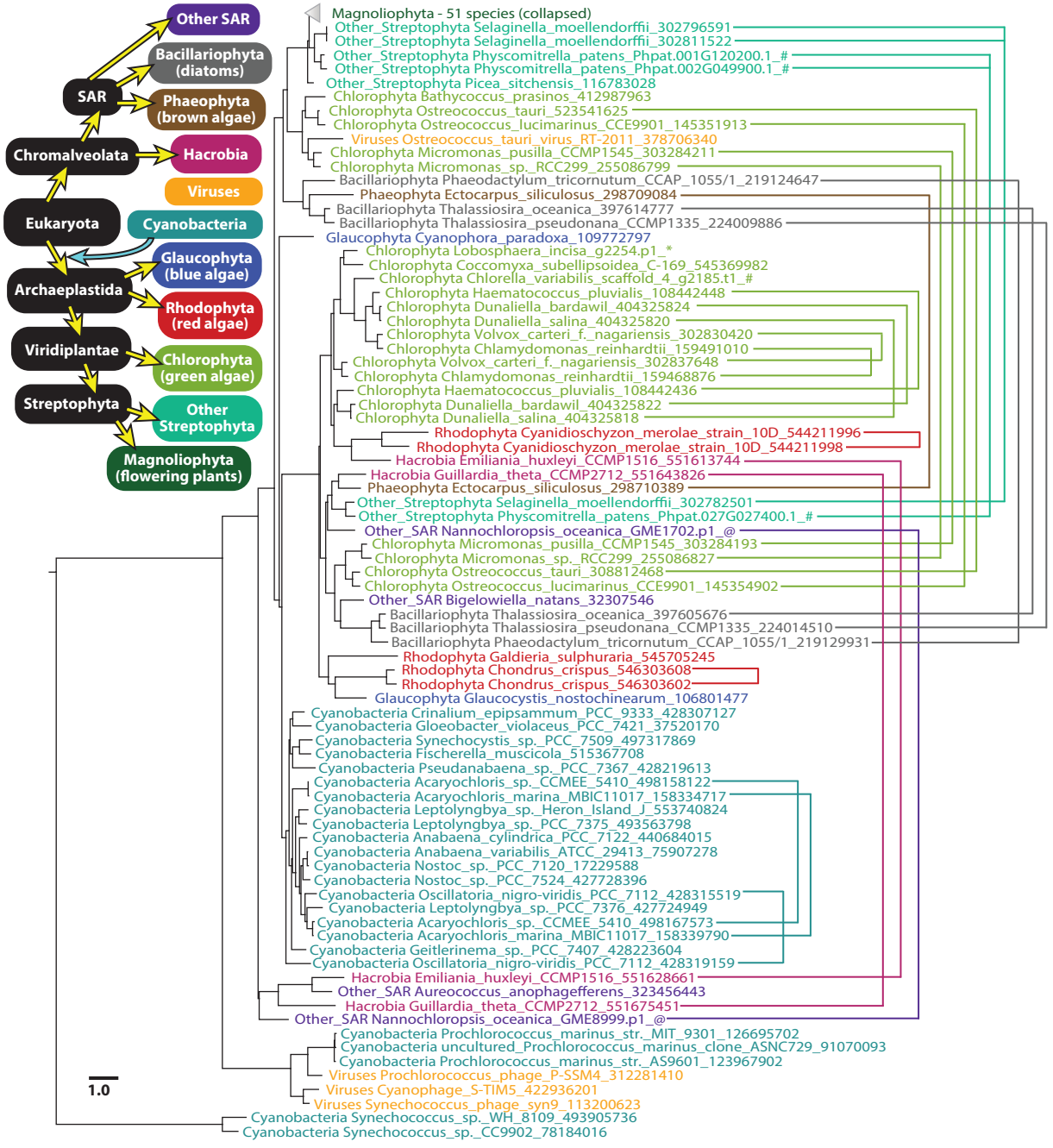
The Number of PTOX Genes

Similarly to multiple AOX copies in plants (27), some organisms have two copies of PTOX (157). Because PTOX shares homology with AOX, they must be discriminated to correctly assign sequences to PTOX. We designed position-specific scoring matrices for PTOX and AOX that allowed the specific retrieval of each of these oxidases from protein sequence databases (see the online **Supplemental Material**; follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). In agreement with previous studies (21, 90, 157), we identified several organisms from various taxonomic groups that encode two (or sometimes three) versions of the PTOX gene (**Figure 1**). Although the majority of cyanobacteria and plants contain a single PTOX gene, species carrying two PTOX copies were found in green, red, and brown algae; diatoms; and hacrobians (cryptophytes and haptophytes). The moss *Physcomitrella* also contains three copies.

Supplemental Material

Figure 1

Phylogenetic tree of PTOX protein sequences. The unrooted tree was reconstructed using the maximum likelihood method based on a matrix of amino acid substitutions computed from the multiple alignment of PTOX sequences. Nonhomologous C and N termini were excluded, and 325 sites were used for analysis. **Supplemental Figure 1** additionally shows the statistical confidence in branching computed by approximate likelihood ratio tests. Organisms are color coded by taxonomic group. The inset shows an evolutionary scheme of photosynthetic organisms based on the present classification of eukaryotes (1, 70), using the same color coding. Most sequences were retrieved from GenBank, and the GI number is given at the end of each organism description. For the remainder, specific symbols have been appended to identify the source: #, data from Phytozome; @, data kindly provided by the authors of the genome publication (107); *, data from unpublished genomic sequence (N.J. Tourasse & O. Vallon, personal communication). The scale bar is the average number of amino acid substitutions per site. For additional details about sequence identification and analysis, see the online **Supplemental Material**.



Inparalogs: paralogs in a given lineage that all evolved by gene duplications that occurred after the speciation event that separated this lineage from another lineage (141)

Strikingly, phylogenetic analysis based on more than 100 PTOX protein sequences indicates that, in most cases, the multiple PTOX sequences in a single organism do not appear to be inparalogs (as also shown in 21, 141). Indeed, the individual copies of PTOX in a given species can be distantly related and scattered in the phylogenetic tree, whereas PTOX sequences from organisms belonging to different taxonomic groups can be closely related (**Figure 1**). Phylogenetic intermixing is also seen in cyanobacteria (*Acaryochloris* and *Oscillatoria*). Thus, species encoding several PTOX proteins may have acquired them independently from different sources, possibly by horizontal gene transfer.

Because PTOX genes are present in cyanophages and algal viruses, one may hypothesize that viruses could have acted as vectors in such gene transfers. However, this transduction hypothesis is not in line with the facts that (*a*) a majority of cyanobacteria possess a single copy of PTOX and (*b*) the viruses sequenced to date do not bear a copy of PTOX homologous to any of the duplicates. PTOX was likely present in the ancestor of the Archaeplastida (91), which comprise three monophyletic groups (1, 70) where PTOX is present (21, 90). Therefore, the scattered distribution of sequences from three red and two blue algae (Rhodophyta and Glaucophyta, respectively) in the PTOX tree (**Figure 1**) is unexpected and, unless this is an artifact caused by sampling bias, suggests an early loss of the gene and subsequent independent reacquisitions. According to Keeling & Palmer (71), the endosymbiotic and nonendosymbiotic horizontal transfer of plastid-targeted genes can be relatively common in algae.

Nevertheless, in a few cases, the two PTOX copies from the same species are more closely related to each other than to PTOX proteins from other organisms, and this reflects organism-specific PTOX duplications (21) (**Figure 1**). This is the case, e.g., for maize, *Eucalyptus*, *Physcomitrella*, red algae, and green algae belonging to the Chlorophyceae class (including *Chlamydomonas*), where the duplication seems to have occurred in the ancestor of this group. In the case of *Selaginella moellendorffii* and *Glycine max*, the copies are nearly identical, so although they are found in different genomic contexts, they may be false positives resulting from artifacts in genome assembly. We note that the cyanobacterial oxidase sequences of two *Synechococcus* strains (and to a lesser extent those of *Prochlorococcus* strains) branch remotely and may thus represent early-diverging or fast-evolving PTOX (**Figure 1**).

In conclusion, PTOX has followed a complex evolutionary history, with several independent duplication events. The phylogenomic survey of numerous plastid-targeted proteins shows that, in fact, such genes often display a complex evolutionary pattern involving duplications and lateral transfers (see the supplementary material for Reference 21). The duplication, represented in every group of photosynthetic organisms for which the protein sequences are retrievable, begs the question of the functional importance of these enzymes, inasmuch as they may display differential expression patterns or responses to the environmental conditions of the two genes (84). We discuss this further below (see The Function of PTOX in Thylakoid Membranes).

PTOX and AOX: Similarities and Differences

The *AtPTOX* gene (originally called *IMMUTANS*) is distantly related to the gene encoding the mitochondrial AOX enzyme (25, 162). The ExxEH. . .ExxEH–specific motif, responsible for iron chelation, is conserved in both oxidases and required for function (40). As discussed above, the two enzymes have a compact four-helix scaffold that harbors the chelation site of the active transition metal center (18). In addition, two amphipathic helices, found in both PTOX and AOX, dock them to the membrane (137) (**Figure 2*a***) to a depth of approximately 8 Å.

The structure of AOX from the parasite *Trypanosoma brucei* was recently solved at atomic resolution (137). Bearing 16% identity (26% similar residues) to the *Chlamydomonas* PTOX2

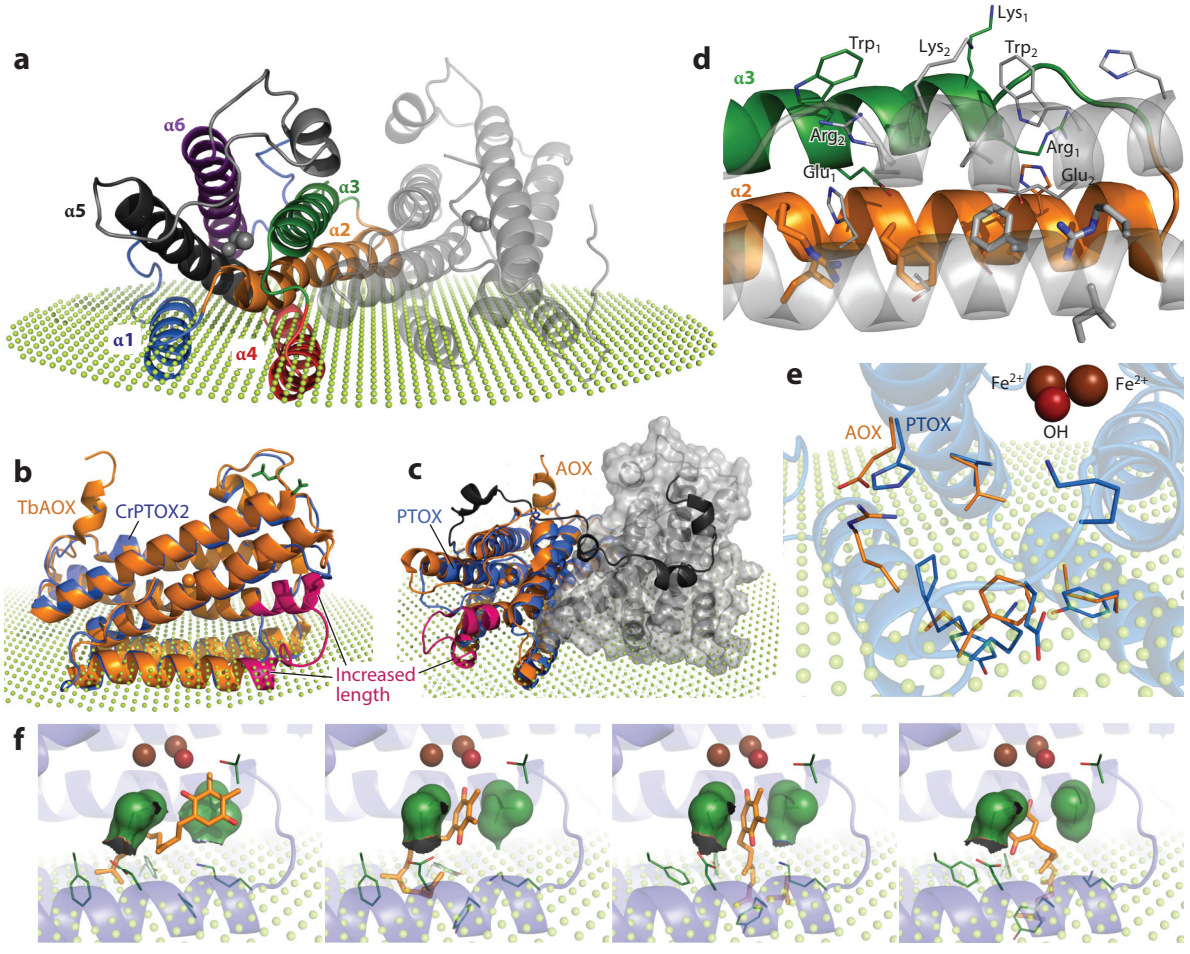


Figure 2

A model structure of CrPTOX2. The stromal surface of the membrane is schematically depicted as light green spheres. (*a*) Overview of the PTOX dimer in the membrane. Helix numbering is from N to C terminus. (*b*) Major structural differences between CrPTOX2 (*blue*) and TbAOX (*orange*; Protein Data Bank ID 3VV9). Green sticks show the last residue before and first residue after the loop in PTOX. The increased length of the helices next to the binding pocket in AOX is shown in pink. (*c*) PTOX (*blue*) superimposed on AOX (*orange*). The dimerization domain of AOX (*black*) spans the second AOX monomer (*gray surface*). Because of its low sequence homology, the corresponding domain of PTOX was not included in the model. (*d*) The interface of two monomers. The colors of the helices in one monomer correspond to the colors in panel *a*; helices from the other monomer are shown in transparent gray. Conserved residues (in 93% of PTOX sequences) in the interface are shown as thick sticks; other residues that could make contact are shown as thin sticks. The distance between two pairs of phenylalanine residues between the monomers does not exceed 4 Å. Note the two pairs of glutamic acid and arginine opposite one another and the two pairs of lysine opposite the tryptophan. (*e*) Differences in the binding pocket between PTOX (*blue*) and AOX (*orange*). (*f*) Docking software model of plastoquinol (PQH_2)–binding modes in PTOX. Residues flexible during the docking are shown as sticks. Residues orienting PQH_2 with regard to the diiron active site by hydrophobic stacking are shown as surfaces.

sequence in a 227-amino-acid conserved region, the crystal structure can be used to build a structural model of PTOX, which could not be derived before owing to the relatively weak conservation between PTOX and the other members of the diiron-carboxylate family that have a known three-dimensional structure (**Figure 2**). We used the predicted structure and

docking software to gain insight into the structural determinants of the binding site of PQH_2 in *Chlamydomonas reinhardtii* PTOX2, the major terminal oxidase of this green alga (51). Because AOX crystallizes as a dimer (137), we show the enzyme in its dimeric form (**Figure 2*a***). Owing to a lack of sequence conservation with AOX, the N-terminal part of PTOX as well as a loop corresponding to a PTOX-specific insertion could not be modeled.

The modeled PTOX structure differs in some instances from early structural predictions for AOXs (7, 17, 18). As expected, the predicted scaffold binding the diiron active center is very similar to that of TbAOX. Yet two major macroscopic differences arise. In PTOX, helices 1 and 2 next to the entrance of the binding pocket are shorter than they are in TbAOX (**Figure 2*b***, pink). This narrows down the space available for PQ. In addition, the 16-amino-acid sequence corresponding to exon 8 of AtPTOX is located on the side of the protein opposite the membrane (**Figure 2*b***, loop flanking the amino acids at the top right corner, shown in green). Although the N-terminal part of the PTOX protein could not be included in the model, we suggest that it contributes to the interaction between monomers within a dimer. Indeed, it should interact with the loop corresponding to exon 8 of AtPTOX in the other monomer, as shown in **Figure 2*c***, which highlights the structural proximity between this loop in one monomer and the superposed N-terminal part of AOX in the other. Importantly, this hypothetical interaction between two different domains in each monomer would account for the finding that the deletion of the loop corresponding to exon 8 of AtPTOX—possibly in proximity to the N terminus of the second PTOX monomer—results in the destabilization of the protein (40).

Figure 2*d* shows the residues conserved in 93% of PTOX and AOX sequences in GenBank. As proposed earlier (17, 18, 38), they likely play a role in the chelation of the two iron atoms comprising the active center. However, as shown by Shiba et al. (137), the conserved histidines are not directly implicated in iron binding, but rather stabilize the second shell of the active site. The TbAOX structure shows that, as first hypothesized by Young et al. (165), two leucine residues (TbAOX L122 and L212, the former of which is conserved in PTOX and AOX) are located next to the diiron center and orient ubiquinol (UQH_2) so that it correctly interacts with the metal center, presumably by hydrophobic stacking. The corresponding two amino acids in CrPTOX2 are leucine (L220; AthL135) and valine (V312; AthV226), respectively. Besides valine, leucine or isoleucine is found in other PTOXs in place of CrPTOX V312. In AOX, the corresponding position is almost invariably occupied by leucine or phenylalanine. In *Arabidopsis*, the L135A mutation drastically decreases PTOX activity in vitro. The increased distance between the aromatic group of the quinol and the shorter and less bulky side chain in the mutant may possibly destabilize quinol binding (compare with **Figure 2*f***).

Another macroscopic difference highlighted by the comparison of the structures is the position of the large polar residue at the entrance of the quinone-binding pocket. In CrPTOX2, lysine 212 narrows the entry but is quite flexible (**Figure 2*f***). In other PTOX proteins, this position is in most cases occupied by another bulky and basic residue, arginine. This is, however, at variance with AOX, where the corresponding position (TbAOX F99) is occupied by hydrophobic residues, mostly phenylalanine, alanine, or leucine. Moreover, the AOX entry site is wider, as according to the model, helices 1 and 2 are longer than they are in PTOX. The presence of a conserved, polar, and possibly charged residue facing the lipid phase is unusual and suggests some critical function. It may contribute to the recruitment of PQs (see below for discussion) or anchor the enzyme at the membrane surface. Indeed, in the case of AOX, positively charged residues—arginines—on the interface between the membrane and proteins have been proposed to interact with the polar head groups of phospholipids (137).

Despite this list of differences, in general, PTOX and AOX are structurally similar, as expected from their homologous function. Yet, as we discuss below, they are not identical from either a

biochemical or enzymatic standpoint, and the limited but significant structural differences shown by the present comparison may constitute the structural determinants of these functional differences.

Functional Properties of PTOX as Deduced from In Vitro and Heterologous Expression Studies

The first report of an in vitro study of PTOX came from Josse et al. (67), who expressed the *Arabidopsis* protein in bacterial membranes. The reconstitution of PTOX in liposomes, i.e., without any interference with other oxidase activity, has been achieved only recently (167). In addition to the enzymatic studies of heterologously expressed enzymes, the complementation of knockout *Arabidopsis thaliana* lines with a site-directed PTOX mutant protein provided insights into the residues necessary for the enzyme function (38–40).

Interestingly, these reports supported a pronounced specificity of PTOX toward PQH_2 as opposed to other quinols (67, 167). This may be a notable difference between PTOX and AOX because AOXa1, when targeted to plastids, can complement a Δ*PTOX Arabidopsis* line, suggesting that it remains active when PQH_2 substitutes for UQH_2 (39; for a recent review, see 115). The same applies when using an overexpressed AOX2 that shows dual targeting (to chloroplasts and mitochondria) despite its endogenous mitochondria-targeting sequence. Thus, AOX can substitute for PTOX and act as a PQH_2 oxidase even though its affinity for UQH_2 remains higher than for PQH_2, as shown by the decrease in the oxygen consumption rate in bacterial membranes upon addition of PQH_2 in excess (39). PTOX would therefore be more stringent than AOX in its specificity toward its electron donor, and it is tempting to propose that this specificity stems, at least partly, from the location at the entrance of the quinol-binding site of a positively charged residue in PTOX in place of a hydrophobic one in AOX, as discussed above. A closer look at the two binding pocket environments shows that the residues surrounding the bound PQH_2 are less hydrophobic in PTOX than in AOX (**Figure 2*e***). This is surprising because the methoxybenzoquinone substituents in ubiquinone (UQ) make it more polar than PQ, suggesting an opposite trend for the substrate specificity of the two enzymes (122). Hypothetically, though, the hydrophobic interaction between the lipid phase and the isoprenoid chain may not be sufficient to pull the polar head of the UQ out of the binding pocket, thus preventing PTOX from using UQ as a substrate (67, 167).

The oligomerization state of the enzyme is another structural issue that deserves attention. PTOX dimers have been found in transgenic tobacco plants overexpressing *Chlamydomonas* PTOX1 (2). Yu et al. (167) recently reported that purification of a heterologously expressed PTOX–maltose-binding protein fusion yields mainly a tetramer and to a lesser extent a dimer and a monomer. This stands in stark contrast to data from Fu et al. (39), who reported that PTOX is uniquely found as a monomer in nondenaturing biochemical analysis. Notably, the cysteine residues involved in dimerization and, consequently, in the regulation of the activity of AOX (40, 153) are absent in PTOX. Yet the dimerization may, as mentioned above, involve the N-terminus part of one monomer and the loop corresponding to exon 8 of the other one (**Figure 2*b,c***). Even though it is not required from a mechanistic standpoint (see below), dimerization may take part in the regulation and/or the (de)stabilization of the protein. Finally, one could envisage the formation of heterodimers in species that express several isoforms of PTOX, but this issue has not been addressed.

According to Moore et al. (92), quinol oxidation into semiquinone by these diiron oxidases is initiated by a superoxo-diiron center upon oxidation of the diferrous state by O_2. After the release of a water molecule, the peroxo-bridged diiron oxidizes the tyrosine residue (Y220 of CrPTOX2) in proximity to the dimetal center; this radical then oxidizes the semiquinone to eventually produce

a quinone and the resting state, oxo-diiron (3+). The latter subsequently oxidizes another quinol into a quinone, and this closes the catalytic cycle by regenerating the diferrous center. Notably, the proposed mechanism involves no high-valence iron intermediates, but does involve radical transients such as tyrosyl [as in ribonucleotide reductase (75)] and semiquinone. Importantly, according to the present views, these two radicals are formed during the reaction sequence initiated by the binding of oxygen to the diferrous center and terminated by the formation of the oxo-diiron state. They are thus transient and thought to be short lived, thereby minimizing the production of ROS—quite a chemical feat, given the need to couple two reactions that sequentially donate two electrons to a reaction that requires four electrons (92).

There are, however, conditions where PTOX seems unable to take up this mechanistic challenge. When overexpressed in tobacco lines or when having to cohabit in the thylakoid membrane with a heterologously expressed bacterial phytoene desaturase, PTOX is a strong ROS producer (41, 50). Interestingly, in vitro studies recently provided further insights into the balance between the antioxidant (oxygen-reducing) and prooxidant (ROS-producing) activity of PTOX. At basic pH, increasing the concentration of the electron donor increased the prooxidant activity, whereas the opposite applied at acidic pH (167).

$\Delta\tilde{\mu}_{H^+}$: the transmembrane electrochemical potential difference for H^+

P_{700}: a dimer of chlorophyll in photosystem I, which is oxidized (P_{700}^+) following charge separation and reduced by plastocyanin

Water–water cycle: a redox cycle with water acting as the electron donor and oxygen as the electron acceptor, thereby producing water

THE FUNCTION OF PTOX IN THYLAKOID MEMBRANES

Chlororespiration

Because PTOX, by oxidizing PQH_2, potentially diverts reducing power from the photosynthetic electron transfer chain, it may interfere in vivo with photosynthetic electron transport and CO_2 fixation in the Calvin-Benson-Bassham cycle. Yet, as discussed above, PTOX is present in the thylakoid membranes of the vast majority of photosynthetic organisms, and the mere fact that it has been retained throughout evolution suggests that, although possibly detrimental in some instances, it fulfills beneficial functions. Contrary to the original proposal (14), chlororespiration as such is not electrogenic in green algae in darkness (15, 117), as neither NDA2 nor PTOX is a proton-pumping enzyme (**Figure 3*d***). However, the chlororespiratory pathway in organisms possessing NDH can probably generate $\Delta\tilde{\mu}_{H^+}$ in the dark. In the light, irrespective of their intrinsic electrogenicity, chlororespiratory enzymes can contribute to $\Delta\tilde{\mu}_{H^+}$ when coupled to the activity of either photosystem: NDA2 can do so by sustaining the turnover of the cytochrome b_6f complex and the reduction of P_{700}^+ when PSII is poorly active or inactivated (an extreme experimental case being under PSII-inhibited conditions) (60), and PTOX can do so when involved in an electrogenic water–water cycle together with PSII (23). In a marine alga (*Ostreococcus*) and a cyanobacterium (*Synechococcus*) that have low PSI/PSII ratios, likely stemming from their adaptation to iron-limited conditions in the ocean, electrons from PSII are redirected toward oxygen via PTOX. Notably, however, such a cycle provides less ATP per oxidized H_2O molecule than does linear electron flow (LEF) from H_2O to $NADP^+$. In *Ostreococcus*, this water–water cycle accounts for up to 30% of the total electron flow in these conditions. This is commensurate with the estimates obtained in *Thellungiella*, a halotolerant relative of *Arabidopsis*, which, when salt stressed, can also use PTOX as a safety valve to redirect PSII-generated electrons to oxygen (142) by upregulating PTOX fourfold. We note, incidentally, that a number of species with duplicated PTOX are halotolerant or can cope with high salt stress (**Figure 1**). Moreover, lodgepole pine has attracted attention for its enhanced oxygen-dependent electron flow, particularly in winter (132), but contributions from the Mehler reaction (O_2 reduction at the level of PSI) were not ruled out.

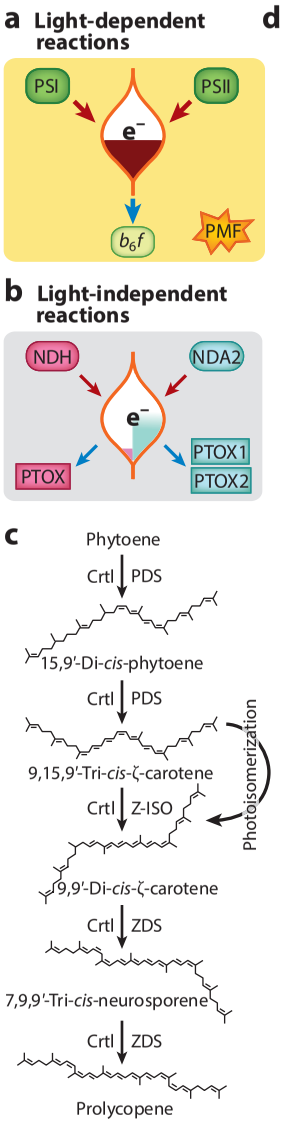
Because electron flow, ATP synthesis, and the buildup of a proton-motive force are so intricately coupled, PTOX must serve multiple functions as soon as it is engaged in a water–water cycle

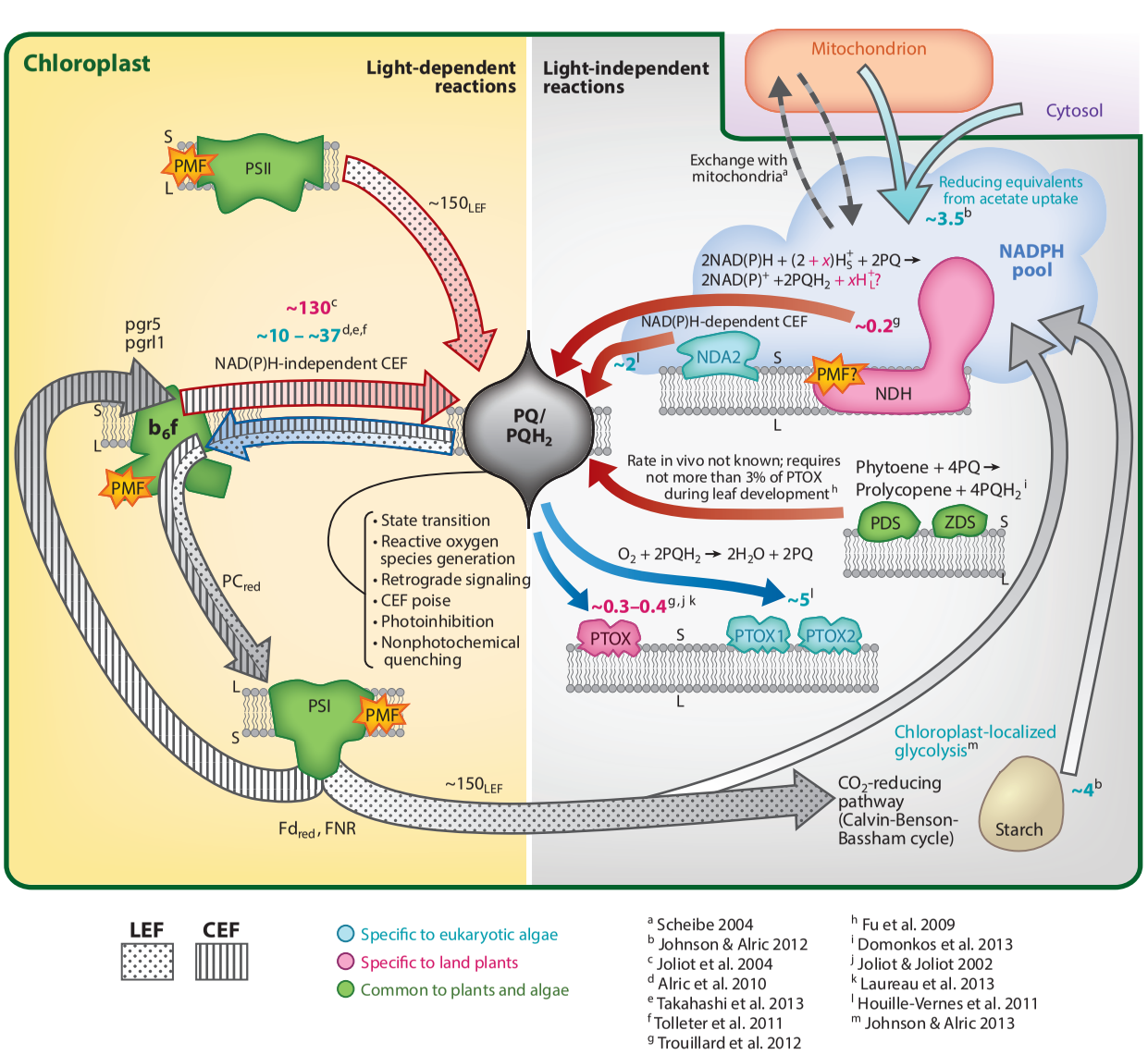
with PSII, and considering it merely as a safety valve seems excessively restrictive. In line with this, the role of cyclic electron flow (CEF) has been recently proposed to go beyond that of a supplier of $\Delta\tilde{\mu}_{H^+}$ and to extend to photoprotection owing to the photosynthetic control exerted at the level of the cytochrome b_6f complex upon acidification of the lumen (for reviews, see 63, 138). When involved in a water–water cycle, PTOX could be seen as a part of any photoprotective processes triggered by the built-up $\Delta\tilde{\mu}_{H^+}$ across the membrane. These include the activation of the violaxanthin de-epoxidase and the consecutive onset of nonphotochemical quenching, photosynthetic control, etc. (42). In plants, where chlororespiration is electrogenic, it may also play a significant role in sustaining the polarization state of the thylakoid membrane in the dark (**Figure 3*b*,*d***). The upkeep of a $\Delta\tilde{\mu}_{H^+}$ across the thylakoid membrane is well documented (for *Chlamydomonas*, see 34; for vascular plants, see 66; for a review, see 76) and may serve all processes requiring a protonmotive force, such as, to mention a few, protein import into the lumen (149), activation of the CF_oF_1 ATP synthase (43), and pH-dependent nonphotochemical quenching (94, 128). Thus, in NDH-containing organisms, chlororespiration may contribute to polarizing the thylakoid membranes and thereby indirectly take part in the various $\Delta\tilde{\mu}_{H^+}$-dependent processes. In the case of pH-dependent nonphotochemical quenching or activation of the CF_oF_1 ATP synthase, the latter of which requires the $\Delta\tilde{\mu}_{H^+}$ to reach values exceeding specific thresholds, chlororespiration would act as a primer. It would set a polarization state at values remaining below, but close to, these thresholds, thereby sensitizing the activation processes. Importantly, the upkeep of this permanent $\Delta\tilde{\mu}_{H^+}$ in the dark would develop at the expense of reducing power, whereas in organisms with nonelectrogenic NDA in place of NDH, it would rely on ATP consumption (117).

The Electron Sink Hypothesis

Because the enzymology of PTOX in vitro remained poorly characterized until recently (167), most of our knowledge of the activity of PTOX comes from in vivo studies. In vivo assays based on fluorescence methods (51, 79, 152, 158) showed that the maximum rate of light-independent PQH_2 oxidation varies among species, reaching up to 5 electrons transferred to oxygen per second per PSII center ($e^-\ s^{-1}\ PSII^{-1}$) in *Chlamydomonas* (two PTOX enzymes exist in this algae, PTOX2 being the major oxidase, with a rate of 4.5 $e^-\ s^{-1}\ PSII^{-1}$) (51) (**Figure 3*d***). These values were obtained in mutants lacking cytochrome b_6f complexes to ensure the full reduction of the PQ pool upon illumination and thereby allow the study of its light-independent reoxidation. In the case of vascular plants, the assessment of PTOX activity does not require the absence of cytochrome b_6f because the inactivation of CO_2 fixation in the dark creates the bottleneck required for the full reduction of the PQ pool. The PTOX-mediated oxidation rate in plants is much lower than in *Chlamydomonas*, reaching only 0.3 $e^-\ s^{-1}\ PSII^{-1}$ in tomato (152) and 0.4 $e^-\ s^{-1}\ PSII^{-1}$ in *Arabidopsis* (64). However, the alpine plant *Ranunculus glacialis* exhibits a threefold-higher PTOX abundance when grown in high light than when grown in low light (79). This is mirrored at the functional level by a V_{max} of light-independent PQH_2 oxidation in high-light-grown plants of 1.3 $e^-\ s^{-1}\ PSII^{-1}$, threefold larger than that in shade-grown plants. In line with the above discussion, this light-dependent regulation of PTOX activity led Streb et al. (143) to propose that, by acting as an additional electron exit pathway, PTOX could provide photoprotection to the reaction centers.

Several authors have proposed the hypothesis that PTOX acts as a safety valve in plants (25, 102, 106). However, the PTOX-driven PQ oxidation rates do not exceed a few electrons per second per PSII, and this seems far from sufficient to provide an efficient safety valve against high excitation pressure, a situation encountered when the excitonic influx at the level of the photosystems overcomes the chemical outflux of the CO_2 assimilation pathway: Under saturating light conditions, the maximal rate of photosynthesis—and thus the reduction of PQ or the oxidation of





[a] Scheibe 2004
[b] Johnson & Alric 2012
[c] Joliot et al. 2004
[d] Alric et al. 2010
[e] Takahashi et al. 2013
[f] Tolleter et al. 2011
[g] Trouillard et al. 2012
[h] Fu et al. 2009
[i] Domonkos et al. 2013
[j] Joliot & Joliot 2002
[k] Laureau et al. 2013
[l] Houille-Vernes et al. 2011
[m] Johnson & Alric 2013

PQH_2, the rates of each step in the overall process being equal by definition—is approximately two orders of magnitude larger (~150 e^- s^{-1} $PSII^{-1}$). This is illustrated by an early study showing that the electron flow through PTOX decreases at the onset of illumination (110). This decrease stems from the drain of reducing equivalents from quinol by the oxidized cytochrome *f*, highlighting the competition between the speedy light-driven electron transfer and the sluggish PTOX pathway. Because it is a poor competitor of the photosynthetic mainstream, PTOX is unlikely to be a major player in photoprotection, at least under constant and saturating light conditions.

Another feature of PTOX that argues against the safety valve function is its contribution to ROS production (33, 41, 50, 167), which may damage the photosynthetic apparatus. However, according to Li et al. (84), PTOX may consume up to 10% of photochemically produced oxygen via the astaxanthin biosynthesis pathway, which would lower the oxygen partial pressure in the cell and consequently decrease ROS production (84). One could also argue that PTOX contributes to PSII photoprotection by shifting the site of ROS production from the appressed membranes, where the majority of PSII resides, to the nonappressed membranes, where PTOX would be located (57, 82). This hypothesis must be qualified, though, by the limited diffusion of PQH_2 along thylakoid membranes caused by molecular crowding, demonstrated by Lavergne & Joliot (81; for a recent review, see 74). Thus, under sustained photosynthetic electron flow at saturating light intensity, those PQs that are reduced by PSII in the appressed membranes would only rarely be reoxidized by a remote PTOX enzyme located in the nonappressed membranes.

On the one hand, the abundance of PTOX seems to be positively correlated to abiotic stresses such as cold, high light, and high salt (54); on the other hand, however, the artifactual overexpression of PTOX did not provide additional protection in cold-stressed, cold-adapted, or control *Arabidopsis* plants (127). These seemingly conflicting observations may be reconciled if, in natural conditions, the increased abundance of PTOX is coupled to the upregulation of enzymes such as superoxide dismutase. PTOX, as a link in this detoxifying pathway, would contribute to lowering the yield of ROS production by providing a safer way out for the reducing power produced in

Figure 3

Plastoquinone (PQ) pool central to photosynthesis. In photosynthetic organisms, two major modes of electron transfer can be distinguished: linear electron flow (LEF) and cyclic electron flow (CEF). LEF involves the photoinduced extraction of electrons from water and reduction of PQ by photosystem II (PSII), plastoquinol (PQH_2) oxidation and plastocyanin (PC) reduction by cytochrome b_6f, PC shuttling in the lumen and reduction of photooxidized photosystem I (PSI), and light-induced electron transfer from PSI to $NADP^+$ via ferredoxin (Fd) and ferredoxin-$NADP^+$ oxidoreductase (FNR). CEF, which is light dependent, consists of recycling electrons from the stromal side back to the PQ pool (28, 144). The PQ pool is depicted as an amphora (80). Red arrows indicate PQ-reducing pathways, and blue arrows indicate PQH_2-oxidizing pathways. Cyan indicates enzymes, rates, or reactions specific to eukaryotic algae; pink indicates enzymes and rates specific to land plants; and green indicates enzymes common to plants and algae. "PMF" highlights the fact that the turnover of the enzyme(s) contributes to the buildup of the proton-motive force. (*a*) Major PQ-related redox pathways in the light. (*b*) PQ-related redox pathways in the dark. The redox steady state of the PQ pool is shown for dark-adapted organisms. (*c*) The carotenoid biosynthesis pathway in plants. (*d*) A consensus view on the major reducing-equivalents-exchange pathways in the chloroplast. The maximal rates of electron transfer (in units of electrons per second per photosystem) are shown as measured in vivo under oxic, nonstress conditions. The positions of the enzymes in thylakoid membranes and locations of NDH-pumped protons are shown with respect to the stromal (S) and lumenal (L) side. Gray arrows indicate electron transport not directly involving quinones, and gray dashed arrows indicate transport via the malate/oxaloacetate shuttle and other pathways of reducing power exchange with the mitochondria and cytosol. For clarity, the quinone-reducing activity of cytochrome b_6f fueling the Q cycle is not shown. For the PDS and ZDS proteins, arbitrary shapes and positions in the membrane were chosen based on References 131, 145, and 151. Note that in algae, the more negative overall redox balance is due to mixotrophy and the idiosyncratic localization of the upper part of glycolysis in the chloroplast (extensively reviewed in References 60 and 61). Additional abbreviations: Fd_{red}, reduced ferredoxin; PC_{red}, reduced plastocyanin.

excess by PSII. However, such a concerted and complementary pas de deux will not take place when a strong constitutive promoter drives PTOX overexpression, unless ROS or PTOX directly functions as a *trans*-acting regulatory element on the genes coding for detoxifying enzymes, which does not seem to be the case (50, 57).

At this stage, an excess of PTOX under low-light conditions may be as detrimental as (or even more detrimental than) a low amount of PTOX under high light. PTOX activity may even generate more ROS per enzyme turnover in low light if, as substantiated by Feilke et al. (33), the stepwise delivery of the two quinols lengthens the lifetime of reactive intermediates so that ROS production increases with the time delay between flashes. Thus, ROS production by PTOX, which is not an asset for a safety valve, should probably be considered from another perspective: It could be a starting point for redox signaling within the cell before the PQ pool becomes overreduced, as shown for singlet oxygen production in other physiological circumstances (8, 73).

The Carotenoid Biosynthesis Hypothesis

In contrast to the elusive role of PTOX in fully grown plants, there is little doubt that it plays a role in chloroplast development. *Immutans* mutants of *Arabidopsis*, eventually characterized as lacking PTOX (25, 162), were obtained years ago by random mutagenesis (121). The recessive mutation resulted in variegated leaves, the macroscopic and visual signature of undeveloped chloroplasts, in which neither carotenoids nor chlorophyll was synthesized. Interestingly, only some of the chloroplasts within a given leaf were colorless, with the rest displaying wild-type characteristics, and there was no intermediate organelle state between these two extremes. This phenotype was correlated to the light intensity, as shown later by Wetzel et al. (159) and Ross et al. (126), because plants grown in low light developed leaves similar to those of wild-type plants.

The stage at which PTOX activity is critical for chloroplast biogenesis has been studied by examining the expression pattern of the *ptox* gene. Notably, only a few percent of the wild-type content of PTOX is sufficient to warrant the proper development of leaves tissues (38). Although in *Arabidopsis* its expression hardly changes in response to most environmental stresses (127), it is regulated during plant development, and its pattern strongly supports its involvement in early plastid biogenesis. Moreover, as shown by Wetzel et al. (159), in PTOX-less plants, high light is harmful to developing chloroplasts only during the 24 h following seed coat breakage, i.e., during the differentiation of proplastids into chloroplasts (see 37 and references therein).

Cell imaging recently showed that young leaf primordia of PTOX-less *Arabidopsis* in high light present a severely reduced number of chloroplasts, further supporting the involvement of PTOX in the development of the leaf tissue (37). Thus, PTOX activity is critical at an early stage of chloroplast development, but its molecular action in this respect remains a matter of debate. The mainstream view is that, in the absence of light-driven oxidation of PQH_2, the oxidase activity of PTOX provides the electron-accepting substrate of phytoene desaturase (**Figure 3*d***), which, in the carotenoid biosynthesis pathway (**Figure 3*c***), catalyzes the conversion of phytoene to zeta-carotene using PQ as the electron acceptor (31, 44, 131, 139). In the absence of PTOX, the PQ pool is fully reduced owing to the activity of PSII in the light or that of the dehydrogenase in the dark, and this blocks the carotenoid biosynthesis pathway at its very early steps (159). Consistent with this assumption, the rate of carotenoid synthesis is markedly lower in an etiolated PTOX-less *Arabidopsis* (24). This negatively impacts photoprotection, thereby inducing photooxidative stress and preventing the proper maturation of developing chloroplasts. One should keep in mind, however, that any process that depends on the redox state of the PQ pool and is critical for chloroplast development, such as chloroplast-to-nucleus retrograde signaling (36, 103), would be similarly affected in the lack of PTOX (**Figure 3*d***).

Even though the carotenoid biosynthesis hypothesis straightforwardly relates the lack of PTOX to a defect in chloroplast biogenesis, it fails to account for the fact that some plastids apparently cope with the lack of PQ oxidase, as evidenced by the variegated phenotype. This latter observation is likely the origin of the hypothesis that a threshold exists above which both carotenoid biosynthesis and plastid development take place (159; for a recent review, see 37). This idea is consistent with the observations that (*a*) healthy leaves develop in PTOX-lacking plants when grown in low light (6, 37, 121, 126, 127, 159); (*b*) variegation becomes more pronounced at lower temperatures, when the light-induced PQH_2 oxidation is hampered by the lower turnover rate of the Calvin-Benson-Bassham cycle (126); and (*c*) de-etiolation is similarly impaired in high light in a PTOX-lacking strain, in which cotyledons develop variegation and the kinetics of the rise in PSII photochemical efficiency develop more slowly and to a lesser extent than in the wild type (126). Each of these findings is readily accounted for if, at the early stage of chloroplast development, the stammering photosynthetic electron transfer chain is sufficient to ensure a level of oxidized PQ compatible with carotenoid biosynthesis, at least under low-light conditions. When in excess, illumination would increase the reducing pressure, and PTOX would then become a major player determining the redox state of the PQ pool. As development would go on, the light-driven electron flux would take over, and PTOX would be relegated to other physiological roles.

Notably, by providing an electron sink downstream of the PQ pool, PTOX could contribute to the photoprotection of the nascent organelle in other ways than merely supporting the carotenoid biosynthesis pathway. For instance, an analysis of greening in etiolated pea (*Pisum sativum*) shoots showed that both PTOX and the NdhI subunit of the NDH complex were present in dark-grown plants (82). During de-etiolation, PSII subunits were more abundant than PSI subunits after 6 h of greening (69). Therefore, as in the *Ostreococcus* and *Synechococcus* cases described above (10, 23), basal PTOX levels in etioplasts, increasing rapidly upon transition to light (82), may partly compensate for this unbalanced stoichiometric ratio and thereby contribute to photoprotection. Importantly, such a hypothesis requires that the activity of the NDH complex does not counteract the oxidation of PQ by PTOX, which seems likely given that its substrates (reduced ferredoxin and/or NADPH) are provided mainly by PSI activity. Conversely, in etiolated barley, where PSI activity would precede PSII activation upon transition to the light (104), PTOX could act as a regulator of CEF before a shift to the linear regime in the developed organ (see **Figure 3*a*,*d***).

Testing the above hypothesis will require decoupling the indirect involvement of PTOX in carotenoid biosynthesis from its role as an electron sink. Galzerano et al. (41) took a first step in this direction by expressing in wild-type *Arabidopsis* a bacterial phytoene desaturase (CRTI; see **Figure 3*d***) that does not use PQ for either phytoene oxidation or zeta-carotene oxidation (131). Unexpectedly, this led to increased PTOX activity, an increased sensitivity of the ROS production to propyl-gallate (an inhibitor of PTOX), and reduced growth rates. The next step will be to overexpress CRTI in a PTOX-defective background, but this remains to be undertaken.

The biosynthesis of carotenoids has also been investigated from a PTOX perspective in algae. Obviously, issues related to chloroplast development do not hold in algae, in which the single chloroplast divides in a mitotic manner with a division furrow halving the chloroplast (47). After each cell division, a significant light-dependent PQH_2-oxidizing activity segregates in the mitotic progeny, making PTOX less essential for providing the required electron acceptors for the PDS protein. In fact, carotenoids accumulate in *Chlamydomonas* mostly during the first part of the photoperiod. The synthesis rates during the dark period are approximately two orders of magnitude lower than in the light, and the absolute quantities of carotenoids hardly change during the dark part of the cycle (55). Accordingly, strains lacking PTOX would produce only ~2.5% less carotenoids when grown in a 12-h–12-h dark-light cycle, and their pigment content would barely differ from that of the wild type, as reported for a *Chlamydomonas* mutant lacking PTOX2 (51).

Perhaps less expectedly, the mutant displayed no change in carotenoid accumulation when grown in heterotrophic conditions (acetate and darkness), i.e., in the absence of light-driven oxidation of the PQ pool (51). The presence of PTOX1, another PTOX paralog in this organism (see Terminal Oxidases and Their Common Ancestor, above), operating at a rate approximately tenfold slower than that of PTOX2, may account for this observation. In this respect, it is worth pointing out that PTOX1 and PTOX2 differ from an enzymological standpoint, the latter being more sensitive to propyl-gallate than the former (51), raising the possibility that PTOX1 is specifically devoted to the carotenoid biosynthesis pathway. Along these lines, in the Chlorophycea *Haematococcus pluvialis*, the PTOX1-specific transcription pattern follows the carotenoid biosynthesis time course, supporting its assignment to the synthesis of astaxanthin (157).

Upper part of glycolysis: the conversion of glucose, glucose-1-phosphate, or glucose-6-phosphate to glyceraldehyde-3-phosphate, which in algae is specifically located in the chloroplast

State transitions: changes in the distribution of light energy between the two photosystems in response to changes in the redox state of the PQ pool

PTOX Activity in Darkness

The redox steady state of the PQ pool, which is poised in the dark by the chlororespiratory pathway, differs between algae and vascular plants (**Figure 3*b*,*d***). In the latter, after a long period of darkness, the pool is almost completely oxidized [but see reports indicating that 20% of the PQ pool remains reduced in darkness in tomato (152)]. In *Chlamydomonas reinhardtii*, 30% of the PQ pool remains in its reduced state in darkness when the algae are kept under vigorous stirring (51). This contrast may simply reflect differences in the light-independent electron influx, which themselves may stem from different metabolite exchange rates between the different cellular compartments. In algae, starch degradation and the upper part of glycolysis (glucose to glyceraldehyde-3-phosphate) take place exclusively in the chloroplast, whereas in vascular plants they are cytosolic (113). The reductive flux in darkness in the chloroplast is larger in algae (**Figure 3**), which, according to Johnson & Alric (60), may reflect the fact that starch is the major source of reduced carbon in algal cells, in contrast to vascular plants, which can metabolize sucrose in the cytosol. Alternatively, the differences in chlororespiratory fluxes between green algae and vascular plants may be a consequence of chlororespiration being electrogenic in the latter and not in the former. The fact that NDH has to work against the permanent $\Delta\tilde{\mu}_{H^+}$ it contributes to generating may be a limitation to its turnover rate under steady-state conditions.

In any event, by enabling electron transfer in the dark from NADPH to oxygen, chlororespiration links the redox steady state of the PQ pool to that of the stroma, which is linked to the overall cellular redox poise by metabolite exchanges. As an example of such a metabolic entanglement, PTOX has been proposed in algae to act as a carbon sink that adjusts the intracellular N/C ratio in conditions of unbalanced availability (111, 158). Indeed, the chlororespiration rate is enhanced in *Chlamydomonas* during nitrogen starvation. In these conditions, there is a profound remodeling of the photosynthetic apparatus together with a fourfold increase in PTOX and NDA2 accumulation levels (20, 111, 158). Accordingly, chlororespiration becomes more active during nitrogen deprivation (an increase from 1.4 to 3.4 $e^- s^{-1}$ $PSII^{-1}$; calculations are from data in Reference 158).

Other Functional Hypotheses Regarding PTOX

Other physiological responses that may involve PTOX are the process known as state transition and the switch between CEF and LEF. In plants and algae, state transitions are changes in the relative absorption cross section of each photosystem caused by the lateral migration of light-harvesting complex II (LHCII) between stacked and unstacked regions of the thylakoid membranes (reviewed in 124, 161). They are controlled by an LHCII kinase, the reversible activation of which depends on the occupancy of the Q_o site of cytochrome b_6f complexes by a PQH_2 (155, 168). The lateral migration of LHCII toward PSI is triggered by a reduction of the PQ pool, thereby decreasing the reducing pressure exerted by PSII and increasing the oxidizing pressure resulting from PSI

turnover (30). Chlororespiration's control over the redox state of the PQ pool in darkness makes PTOX a key enzyme in the bioenergetics of the photosynthetic cell because its activity is expected to set the appropriate light-harvesting balance. Moreover, PTOX, by acting as a sensor of the redox and ATP status in the chloroplast stroma, helps to trigger the ultrastructural changes undergone by the thylakoid in response to changes in the energetic status of the entire cell (19, 161).

When deprived of ATP, algal cells undergo an activation of glycolysis, known as the Pasteur effect, that releases NADPH in the chloroplast stroma (120), thus eliciting a reduction of the PQ pool, activation of the LHCII kinase, and eventually an increase in PSI antenna size relative to that of PSII. These conditions are well suited, at least in nonsaturating light conditions, to favor CEF around PSI, a mode enabling the production of ATP that is not committed to carbon fixation, and thus contributes to restoring suitable intracellular ATP levels in these ATP-depleted cells (19). Subsequent studies on the relationship between state II conditions and CEF in *Chlamydomonas* added support to the view that a state I–state II transition may correspond to a switch from LEF to CEF (32, 35, 87). However, further studies showed that the metabolic conditions required for transition to state II (i.e., reducing conditions in the chloroplast stroma), rather than the lateral redistribution of LHCII per se, were responsible for the enhancement of CEF in *Chlamydomonas* (147), making the possible role of PTOX in CEF even more direct. Indeed, by oxidizing NADPH in the dark and thereby allowing the redox equilibration between the stroma and the thylakoid membrane, chlororespiration in general and PTOX in particular regulate the partition between LEF and CEF at the onset of illumination (152). PTOX could also regulate CEF directly by preventing overreduction of the pool, but the degree of its direct involvement in CEF remains to be specified.

As an illustration of the complex interplay between chlororespiration and CEF, the inactivation of a protein thought to contribute to CEF, PGR5 (9, 95, 97), rescues the variegated phenotype of Δ*PTOX Arabidopsis* (105). The deletion of *NDH* in the Δ*PTOX* context also rescues this phenotype, albeit to a lesser extent, likely because of its less prominent contribution to CEF (105). Importantly, PTOX, similarly to the major CEF actors (PSI and cytochrome b_6f), is localized in nonappressed membranes (3, 59, 82, 100); this favors its involvement in CEF if, as proposed by various authors (32, 65), the segregation of PSII and PSI between the appressed and nonappressed membranes is an ultrastructural requirement for efficient CEF.

PTOX AS AN ENVIRONMENT-RESPONSIVE ENZYME

If, as discussed above, PTOX is indeed involved either directly or indirectly in a variety of physiological functions (carotenoid biosynthesis, photoprotection of the nascent electron transfer chain, redox poise, etc.), it would be unsurprising that its abundance varies with environmental conditions. Both the protein and transcript level of PTOX respond to environmental conditions (reviewed in 146), but in *Arabidopsis*, its transcript level is rather insensitive to a wide range of stresses (127). This initially led to the conclusion that its activity has minimal influence in mature plants. However, PTOX has since been shown to be upregulated in response to high-light treatment—sometimes coupled to other stresses—in a variety of other organisms (46, 53, 79, 96, 108, 152, 157). In addition, it is retained in the genomes of high-light- but not low-light-ecotype cyanobacteria (90). Changes in the oxidase abundance in response to chilling have also been documented (22, 54, 132). As mentioned above, PTOX is differentially expressed during nitrogen or phosphate starvation in *Chlamydomonas* (93, 111, 158), during drought in *Coffea* (140), and during salt stress in halophilic *Thellungiella* (142). It is notable that in each of these conditions the PQ pool potentially undergoes overreduction, either because of the excessive influx of light-generated reducing equivalents or because of the insufficient outflux hampered by the stress-induced downregulation of the Calvin-Benson-Bassham cycle (52).

Importantly, in many of these cases, PTOX is not the only enzyme whose abundance changes. Its chlororespiration partner (either NDA2 or NDH) is often similarly regulated, which suggests that what matters is the acclimation of the chlororespiratory flux as a whole rather than the oxidase activity per se. In stress conditions, chlororespiratory enzymes would be upregulated to cope with the increasing reducing pressure in the chloroplasts in the dark, and this would provide a redox buffer, thereby smoothing the sudden changes induced by the onset of the light-driven electron flow following illumination. Moreover, this could indirectly play a photoprotective role by setting the redox poise so as to promote CEF and the ensuing photoprotective mechanisms (52, 58, 63, 123, 129).

PTOX IN NONGREEN PLASTIDS

The role of PTOX in poising the redox state of the stroma, combined with the fact that the chloroplast is the site of several biosynthetic pathways involving electron redox reactions, makes it an obligate actor in several plant cell processes beyond those discussed above. Recent studies have implicated it in strigolactone metabolism in rice (148). Strigolactone, a recently discovered plant hormone, derives from carotenoids and plays an important role in root biogenesis and shoot branching (118, 136). Both rice and *Arabidopsis* Δ*PTOX* plants are more branched, similarly to other mutants defective in strigolactone response (148). Notably, the interplay between chlororespiration and strigolactone biosynthesis may go beyond the fact that they are both linked to carotenoid biosynthesis, because *Arabidopsis* mutants defective in NDH are less strongly affected in carotenoid biosynthesis but still show defects in strigolactone biosynthesis. Indeed, Roose et al. (125) showed that a lack of one of the NDH-related proteins results in different branching of the roots together with a slightly altered PQ redox state. Interestingly, *PTOX* mRNA is also present in nongreen tissues such as roots in abundances commensurate with those in photosynthetic tissues (5). The *PTOX* promoter operates in several tissues and at various developmental stages, with no obvious correlation to the tissue's carotenoid content (5). Finally, apart from PTOX, at least some of the enzymes of the carotenoid biosynthesis pathway are present in the nongreen tissues of plants (83), supporting their role in strigolactone biosynthesis and not simply in carotenoid production for photoprotective purposes.

Not only chloroplasts (variegation) and amyloplasts are affected in mutants of either chlororespiratory enzymes in higher plants. Chromoplasts, another functional type of plastids, accumulate high amounts of carotenoids. Consistent with their implication in the carotenoid biosynthesis pathway, the genes coding for PTOX, PDS, and ZDS are upregulated in tomato and pepper fruits, which display increased carotenoid levels (68). As expected, the carotenoid content of chromoplasts is strongly affected in plants lacking PTOX activity (11). The accumulation of pigments is also impaired when NDH is not functional (98). Because carotenoid biosynthesis is an energy-consuming process, this may reflect the need for a permanent $\Delta\tilde{\mu}_{H^+}$ to sustain this biosynthetic pathway and the involvement of chlororespiration in its generation (108a, 121a).

CONCLUDING REMARKS AND RESEARCH FRONTIERS

The unquestionable piece of data regarding PTOX is its involvement in the oxidation of the PQ pool. Thus, together with NDH/NDA2, it sets the redox poise in the dark not only of the PQ pool but also of the entire stroma. This is expected to impact, to different degrees, all the processes that either involve redox reactions or are redox controlled. There are many such processes, of which CO_2 fixation and CEF are the most obvious because they rely on the function of protein complexes found in the same membrane as PTOX. Because these two processes are driven by light and have turnover rates that are much larger than the chlororespiration flux, their interplay

with chlororespiration is more likely to be transient and to become particularly important during transitions between different light intensity regimes, the most extreme case being dark to light. The other, light-independent processes are biosynthetic pathways, prominent among which is carotenoid biosynthesis, but the recent results on strigolactone suggest that the implication of chlororespiration may extend beyond that. Between these two extremes stands retrograde signaling, which is commonly thought to be triggered by the redox status of the chloroplast. The picture that emerges for PTOX is that of a housekeeping function. It sets the appropriate redox poise and electron transfer flow in the dark to directly or indirectly allow several other vital functions to take place smoothly.

SUMMARY POINTS

1. The plastid terminal oxidase (PTOX) has a complex evolutionary history, with several independent duplication events.
2. PTOX and the alternative oxidase (AOX) are structurally similar, as expected from their homologous function, but they display profound differences from both a biochemical and enzymatic standpoint.
3. The carotenoid biosynthesis hypothesis straightforwardly relates the lack of PTOX to a defect in chloroplast biogenesis but fails to account for the fact that some plastids apparently cope with the lack of plastoquinone (PQ) oxidase, as evidenced by a variegated phenotype.
4. Because chlororespiration controls the redox state of the PQ pool in darkness, PTOX is a key enzyme in the bioenergetics of the photosynthetic cell.
5. Together with NADH dehydrogenase-like (NDH)/NAD(P)H dehydrogenase-like (NDA), PTOX sets the redox poise in the dark not only of the PQ pool but also of the entire stroma and thereby potentially impacts, to different degrees, all the processes that either involve redox reactions or are redox controlled.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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