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REVIEW

# Ascorbic acid oxidase: an enzyme in search of a role

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## Abstract

Ascorbic acid oxidase (AAO) has been fully characterized at molecular level, yet its functional role is unclear. The properties of the enzyme and the main hypotheses on its function are discussed. Recent data and reappraisal of previous observations suggest that AAO could be part of a dynamic mechanism operating whenever plant cells have to control oxygen availability.

*Additional key words*: ascorbic acid, dioxygen.

## From hexoxidase to ascorbic acid oxidase

The long and controversial history of ascorbic acid oxidase (AAO) begins in 1931, when Albert von Szent-Györgyi identified in cabbage leaf extract an enzyme called hexoxidase. This enzyme could oxidize hexuronic acid, a mysterious substance isolated from adrenal glands of beef and from plant tissues (Szent-Györgyi 1931). At that time, Szent-Györgyi was convinced that both hexuronic acid and hexoxidase were involved in plant respiration. Not much later, he discovered that hexuronic acid was actually the antiscorbutic factor vitamin C, so this substance was eventually named ascorbic acid (AA).

Hexoxidase followed the same fate, and changed its name into L-ascorbate:$O_2$ oxidoreductase (EC 1.10.3.3), generally referred to as ascorbic acid oxidase (AAO). Soon it appeared clear that this enzyme was typically present only in plant tissues. For about three more decades, AAO was considered the terminal oxidase of a plant-specific respiratory pathway of electron transport (James and Boulter 1955). However, in parallel with new studies on AA distribution in plant tissues and the increasing persuasion that AA was not an actual respiratory substrate, also the role of AAO was questioned.

Mertz (1961) reported that AAO activity was mainly associated to the cell wall fraction, and excluded that AAO could be significantly involved in cellular respiration. In addition, he observed an apparent correlation between AAO activity and cell enlargement in maize root cells.

Since then, AAO has been extensively studied at molecular level. Enzyme purification has been obtained from different sources (Kroneck *et al.* 1982); protein sequence has been deduced from cDNA (Ohkawa *et al.* 1989) and crystal structure investigated by X-ray analysis (Messerschmidt *et al.* 1989). We now know that AAO, together with laccase (present in plants and fungi) and ceruloplasmin (detected in the sera of mammals and birds), belongs to the blue oxidases, a class of multi-copper enzymes catalyzing the four-electron reduction of molecular oxygen to water with concomitant one-electron oxidation of the substrate (Messerschmidt and Huber 1990). The reaction catalyzed by AAO (Fig. 1) proceeds by a sequential mechanism in which single electrons are taken up from the reducing substrate by the oxidized [Cu(II)] type-1 copper centre, and transferred via a highly optimized intramolecular electron transfer pathway to the type-3 copper centers, where dioxygen reduction takes place (Farver and Pecht 1991). Remarkably, such reaction mechanism occurs without the release of intermediate reaction products of dioxygen reduction, *i.e.* activated oxygen species such as superoxide ions or hydrogen peroxide. Furthermore, in the presence of a slight excess of dioxygen over AAO, the rate of intramolecular electron transfer from type 1 Cu(I) to type 3 Cu(II)

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*Abbreviations*: AA - ascorbic acid; AAO - ascorbic acid oxidase; AFR - ascorbate free radical; DHA - dehydroascorbic acid; GR - glutathione reductase; GSH - glutathione; IAA - indole-3-acetic acid; QC - quiescent centre.

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increases 5-fold, as compared to that of the same process under anaerobic conditions (Farver *et al.* 1994). Notably, dioxygen is the obligate electron acceptor of AAO, whereas, beside AA, several other electron donors containing a functional *o*-diphenolic group can be oxidized in the reaction. For instance, AAO catalyzes (+)-catechin oxidation with a $K_m$ of $3.076 \times 10^{-3}$ M (Marchesini *et al.* 1977). When AA is the substrate of the reaction, 4AA are oxidized to ascorbate free radical (AFR), which in turn rapidly disproportionates to AA and dehydroascorbic acid (DHA).

## Proposed functions of AAO

Paradoxically, molecular characterization of AAO and detailed analysis of its catalytic mechanism resulted of little use for the identification of its function. The present state of research on the physiological role of AAO resembles the situation occurring with its animal counterpart ceruloplasmin: so many hypotheses, but no conclusive evidence (Floris *et al.* 2000).

The hypothesis that AAO activity could be in some way related to cell expansion, proposed by Mertz four decades ago, is still the most accredited. Lin and Varner (1991) reported that AAO is more abundant in expanding cells of ripening zucchini fruits. It has also been observed that AAO activity is higher during elongation of suspension cultured tobacco cells (Kato and Esaka 1999), and that transformation of these cells with the cucumber AAO gene induced cell expansion (Kato and Esaka 2000). It has been suggested that AFR formed in the reaction catalyzed by AAO could act in a trans-membrane electron transport mechanism leading to vacuolization and eventually cell enlargement (Hidalgo *et al.* 1991). However, it should be considered that contrasting data have been reported in two recent studies on the effects of AAO overexpression in tobacco plants: Sanmartin *et al.* (2003) could not observe any effect of AAO overexpression on plant growth; Pignocchi *et al.* (2003) observed in AAO-overexpressing plants an increase in stem growth as compared to wild-type plants only under conditions of relatively low irradiance (250 $\mu$mol $m^{-2}$ $s^{-1}$).

A few alternative hypotheses on AAO function have been proposed. Reddy *et al.* (1986) observed a rise in AAO activity and a concomitant decrease in AA content in rice plants during panicle initiation, suggesting that AAO activity could be necessary to decrease AA content in that specific developmental stage. More recently, Kerk and Feldman (1995) proposed an intriguing model for the maintenance of the quiescent centre (QC) in maize root tip, based on the observations that cell cycle progression is delayed in QC cells because of low AA content (Liso *et al.* 1984, 1988) and that AAO activity, protein and mRNA are apparently abundant in the QC of maize roots. According to Kerk and Feldman, therefore, AAO activity in the QC should have the function of lowering AA content, thus slowing down the rate of cell division. Later on, the same authors (Kerk *et al.* 2000) proposed that AAO could also work in the inactivation of the plant hormone indole-3-acetic acid (IAA) by catalyzing its oxidative decarboxylation. Indeed, auxin is known to stimulate the transcription of the AAO gene (Esaka *et al.* 1992, Esaka 1998).

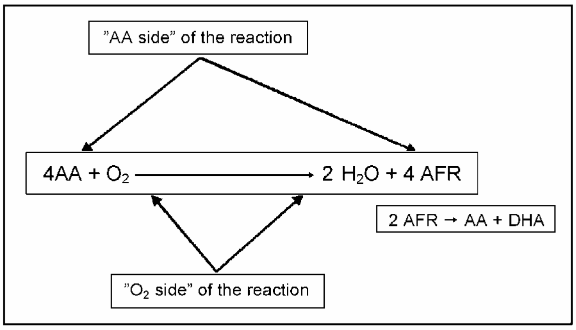

Fig. 1. The reaction catalyzed by AAO. Four AA molecules are oxidized to ascorbic acid free radical (AFR), yielding four electrons that are used to reduce 4 copper ions covalently bound to the protein. The four electrons are then transferred to molecular oxygen, four protons are taken up from the surrounding medium and two water molecules are released. AFR spontaneously disproportionates to AA and dehydro-ascorbic acid (DHA). See text for details.

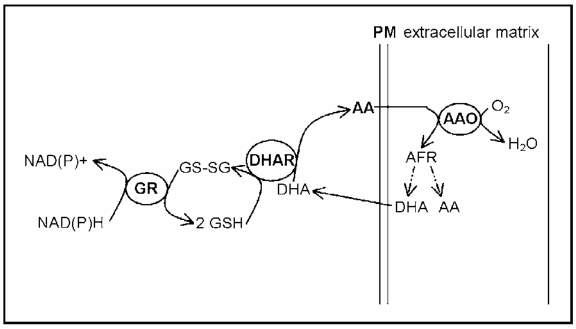

Fig. 2. The possible role of AAO in the control of intracellular redox status. GR: glutathione reductase; DHAR: dehydro-ascorbic acid reductase; PM: plasma membrane.

Lately, some investigators reconsidered the role of AAO as a possible factor controlling intracellular redox state (Potters *et al.* 2002). According to this hypothesis, AAO would be the terminal oxidase in an electron transport chain in which reducing equivalents are transferred from pyridine nucleotides to oxygen *via* the glutathione (GSH) and the AA systems (Fig. 2). Interestingly, glutathione reductase, dehydroascorbate reductase and AFR reductase are all present in the cytosol (and other compartments as well), whereas AAO is located in the extracellular matrix (Chichiriccò *et al.* 1989). The presence of an AA (out)-DHA (in) exchange system at the plasma membrane (Horemans *et al.* 1998) suggests that this system could actually operate. Clearly, the up- or down-regulation of AAO activity should affect the redox state of AA, GSH and pyridine nucleotides in opposite ways. This is apparently not consistent with recent data reported by Kanellis and co-workers (Sanmartin *et al.* 2003), showing that overexpression of AAO (up to 380-fold increased activity) in tobacco plants completely oxidized AA to DHA in the apoplast, but in the symplast induced a limited (5 %) decrease in the AA redox state and, surprisingly, raised GSH and lowered GSSG content, thus increasing the GSH/GSSG ratio. Similar results have been reported by Pignocchi *et al.* (2003). The decrease in apoplastic AA content (and corresponding increase in DHA) is probably the basis of enhanced sensitivity to ozone observed in AAO-over-expressing plants (Sanmartin *et al.* 2003).

## More roles for AAO?

Almost all the above-mentioned hypotheses on AAO function are based on the assumption that the enzyme is required either to decrease AA content, or to generate AFR and/or DHA. Generation of both AFR and DHA has been correlated to growth (Hidalgo *et al.* 1991, Lin and Varner 1991). Surprisingly, huge AAO overexpression resulted in higher DHA (and presumably AFR) content, but did not induce any change in growth, development, flower and seed production in tobacco plants (Sanmartin *et al.* 2003). Moreover, no clear correlation could be observed between AA content, AAO activity and growth of pumpkin roots subjected to different treatments affecting these three parameters (Fig. 3).

| | control | DHA | IAA | GalL |
|---|---|---|---|---|
| |  | | | |
| AAO activity [a.u.] | 92 ± 2.1 | 124 ± 2.7 | 147 ± 6.5 | 98 ± 3.1 |
| AA [nmol $g^{-1}$(f.m.)] | 550 ± 38 | 1500 ± 88 | 750 ± 43 | 980 ± 66 |
| Length increase [cm] | 1.1 ± 0.2 | 0.4 ± 0.1 | 0.63 ± 0.1 | 2.6 ± 0.5 |

Fig. 3. Relationship between AAO activity, root growth and AA content in pumpkin. Seedlings (72 h after germination) were grown for 24 h with roots immersed in distilled water (control), 1 mM dehydroascorbic acid (DHA), 1 mM L-galactono-γ-lactone (GalL), or 100 μM indoleacetic acid (IAA). Root length increase was measured and root tips (3 mm) were excised, homogenized and analyzed for AAO activity and AA content as previously reported (Arrigoni *et al.* 2003). AAO activity (measured by native-PAGE/activity staining and analysis of the relative intensity of protein bands, in arbitrary units, a.u.) was slightly increased by DHA and significantly enhanced by IAA. Both DHA and GalL increased AA content. Overall root growth was inhibited by treatment with DHA and IAA, and stimulated by GalL.

All these data say that AAO function in plants is still unknown. Indeed, AAO seems to be there just to catalyze a "waste" of AA, thus thwarting its celebrated antioxidant activity. Yet, how can such complex and perfect molecular machinery be just a "blind alley" in plant evolution?

In many years of research on AAO, little attention has been given to the most striking feature of the enzyme: its capability to specifically reduce dioxygen to water without releasing toxic reactive oxygen intermediates. The $O_2$ side of the reaction (see Fig. 1), rather than the AA side, is probably the key to understand AAO function in plants. Data obtained in our laboratory (Arrigoni *et al.* 2003) show that AAO activity is not influenced by increased AA content, whereas it is elicited either by light or by oxygen (in dark) in leaf disks of *Cucurbita pepo*. Interestingly, light stimulation of AAO activity was prevented by treatment with DCMU, which, by inhibiting photosynthetic electron transport, decreases dioxygen generation. Such data point at a possible, unexpected relationship between AAO activity and photosynthesis.

How can an enzyme typically located in the extracellular matrix interact with photosynthetic activity? Clearly, AAO is not directly involved in the photosynthetic process. Nevertheless, the enzyme could be beneficial to avoid the risks of hyperoxia, potentially occurring in photosynthetic cells (Raven *et al.* 1994). Dioxygen continuously produced in chloroplasts exceeds the amount required for respiration. Dioxygen diffuses outside the chloroplasts as a consequence of the increase in its concentration, crosses the cytosol, joins the extracellular matrix and escapes through the stomata from the intercellular spaces. $O_2$ transfer from chloroplasts to intercellular spaces occurs by simple diffusion in aqueous medium, which is a relatively slow process. AAO, by acting in the apoplast as an $O_2$-consuming enzyme, would ensure the establishment of a steep $O_2$ concentration gradient between the production site (chloroplasts) and the delivery site (apoplast/intercellular spaces). Apoplastic AAO, by accelerating $O_2$ diffusion, could prevent the risks of $O_2$ stagnation in photosynthesizing leaves (Raven *et al.* 1994). Our recent data (Arrigoni *et al.* 2003) also show that $O_2$-mediated increase in AAO activity seems to be due to activation of the extant protein, rather than new protein synthesis (or gene expression). As the rates of $O_2$ production and utilization change upon environmental conditions, AAO activity should be able to change accordingly. Regulation of AAO activity could represent a dynamic strategy for biochemical prevention of damage potentially caused by oxygen.

Plants can counteract the possible dangerous consequences of hyperoxia induced by photosynthesis in different ways, ranging from anatomical adaptations (compactness of leaf mesophyll; number and density of the stomata) to biochemical repair mechanisms (activities of enzymes scavenging reactive oxygen species). It is conceivable that different plants have evolved a concoction of different strategies. This could possibly explain why AAO activity is expressed in some plants more than in others.

The existence of a correlation between AAO and photosynthesis is further supported by data reported some years ago by Arrigoni and co-workers, concerning the AA system in the heterotrophic angiosperm *Cuscuta reflexa* Roxb. (Tommasi *et al.* 1990). This plant shares with other (green) angiosperms the basic features of the AA system: *de novo* AA biosynthesis from sugars; the presence of AA recycling enzymes (AFR reductase and DHA reductase) and a significant AA peroxidase activity. Notably, AAO activity is totally absent in *C. reflexa* (Tommasi *et al.* 1990). Yet, this obligate parasitic plant grows, *i.e.* forms new cells undergoing differentiation, joins sexual maturity and forms many viable seeds. AAO is clearly not essential for the growth and the completion of *C. reflexa* life cycle. We hypothesize that *Cuscuta* can do without AAO because it is a non-photosynthetic plant. It is possible to hypothesize that the loss of AAO could have occurred in parallel with the loss of photosynthetic activity during evolution to holoparasitism.

In conclusion, although not essential, AAO could be very useful to plants performing dioxygenic photosynthesis and in all conditions in which $O_2$ must be kept under control. This is well in accordance with the observation that hyperoxia induces a 2-fold increase in ceruloplasmin gene expression in rat lung (Fleming *et al.* 1991).

## AAO down-regulation and its possible physiological significance

If up-regulation of AAO activity can be very useful when too much oxygen is around and namely in photosynthesizing leaves, data showing that AAO expression is decreased during fruit ripening and wound healing open a new interesting scenario.

Diallinas *et al.* (1997) observed high transcription of the AAO gene in preclimacteric melon fruit and subsequent repression in the climacteric stage, in which intense respiration and high ethylene synthesis occur. The repression of AAO expression and activity could also have an important physiological significance in connection with the activity of 1-aminocyclopropane-1-carboxylate oxidase (ACCO), the terminal enzyme of ethylene biosynthetic pathway, which is known to specifically require AA. AAO repression in climacteric fruits could be an elegant mechanism to increase at once $O_2$ availability for respiration and AA availability for ethylene synthesis, thus switching on the ripening process.

Wound healing is another phenomenon potentially controlled by AAO down-regulation. Diallinas *et al.* (1997) reported that AAO expression was dramatically repressed in wounded melon fruit within few hours. Once more, such AAO repression could increase both $O_2$ and AA availability. Indeed, this could be beneficial to increase the rate of respiration (wound respiration) and the synthesis of hydroxyproline containing proteins, an event that specifically requires AA for the activity of peptidyl-prolyl-4-hydroxylase (P4H), the enzyme catalyzing the post-translational modification of proline residues incorporated in polypeptide chains (Arrigoni and De Tullio 2002). The synthesis of hydroxyproline-containing proteins is one of the best characterized responses to wounding.

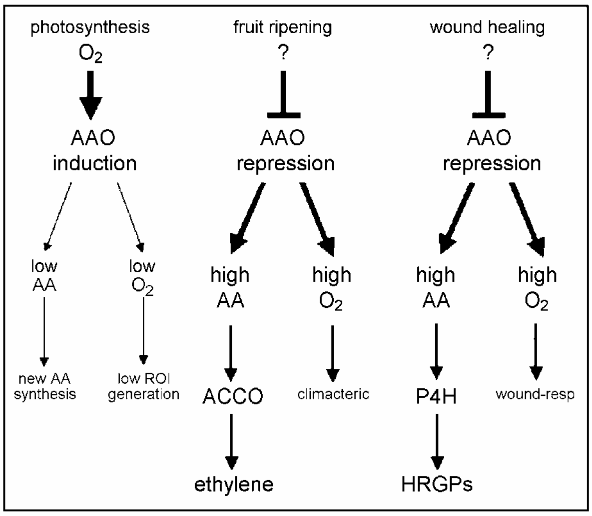

Fig. 4. A scheme of some possible functions of AAO up- or down-regulation. Dioxygen is likely to be the trigger inducing AAO activity in photosynthetic leaves, whereas the mechanisms leading to AAO down-regulation in fruit ripening and wound healing are still unclear. Higher AAO activity in photosynthetic leaves results in lowering both AA content, which is replenished by new AA biosynthesis, and $O_2$ concentration, which in turn can limit the formation of reactive oxygen species. On the other hand, lower AAO activity increases the availability of both AA and $O_2$, which are used for the activity of AA-dependent dioxygenases 1-aminocyclopropane-carboxylate oxidase (ACCO, catalysing ethylene production) and peptidyl-prolyl-4-hydroxylase (P4H, necessary for the synthesis of hydroxyproline-rich glycoproteins, HRGPs) and for increased respiratory activity, respectively.

## New directions for a classical enzyme

In these years of genomic and proteomic analysis, molecular identification of a gene/protein is often considered a goal *per se*. Nevertheless, in many cases molecular knowledge, disjoined from functional analysis, cannot help us in the difficult task of understanding how organisms really work. In this context, the story of AAO is a paradigm of the distance between molecular analysis and biological understanding.

Available data strongly suggest that the regulation of AAO activity is likely to be involved in dioxygenic photosynthesis, fruit ripening (climacteric rise) and wound healing (wound respiration) (Fig. 4). It seems that plants use AAO in different tissues and under different developmental conditions as a dynamic tool for oxygen management. This opens new fascinating directions to further investigations on this old-fashioned and probably misunderstood enzyme.

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