Indian Journal of Biochemistry & Biophysics
Vol. 47, October 2010, pp. 306-310

# Reinstate hydrogen peroxide as the product of alternative oxidase of plant mitochondria

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*Received 02 December 2009; revised 27 September 2010*

Chill treatment of potato tubers for 8 days induced mitochondrial $O_2$ consumption by cyanide-insensitive alternative oxidase (AOX). About half of the total $O_2$ consumption in such mitochondria was found to be sensitive to salicylhydroxamate (SHAM), a known inhibitor of AOX activity. Addition of catalase to the reaction mixture of AOX during the reaction decreased the rate of SHAM-sensitive $O_2$ consumption by nearly half, and addition at the end of the reaction released half of the $O_2$ consumed by AOX, both typical of catalase action on $H_2O_2$. This reaffirmed that the product of reduction of $O_2$ by plant AOX was $H_2O_2$ as found earlier and not $H_2O$ as reported in some recent reviews.

**Keywords**: Mitochondrial alternative oxidase, Chilling treatment, Potato, SHAM-sensitive activity, Catalase-dependent $O_2$ release, $H_2O_2$

A significant portion of respiration in plant mitochondria, active under biotic and abiotic stress conditions has been known to be insensitive to cyanide, was initially called "alternate" oxidase, but later changed to the more appropriate name, alternative oxidase (AOX). Some ambiguity existed on the product of reduction of $O_2$ consumed in this reaction between hydrogen peroxide ($H_2O_2$) and water ($H_2O$). Using mitochondria from chilled potato with enhanced AOX activity[1], we have reaffirmed that $O_2$ is indeed reduced to $H_2O_2$, a reactive oxygen species (ROS) now recognized as important signal molecule regulating diverse metabolic processes.

Cyanide-insensitive respiration in mitochondria, as alternative to cytochrome oxidase, is distinguished by potent inhibition by hydroxamates[2] in plants and by phenolates[3] in animals. AOX pathway uses the redox of flavoprotein-dehydrogenases[4] and of coenzyme Q[5] for transporting electrons to $O_2$, similar to mammalian mitochondria[6], but apparently dissipates energy as heat[4]. Indeed, there are many reports correlating increase in cyanide-insensitive respiration and in AOX activity with keeping higher temperature localized to reproductive tissues in thermogenic plants[7-10] in members of Araceae (*Sauromatum guttatum, Symplocarpus foetidus, Arum, Dracunculus vulgaris*, *Xanthosoma robustum, Philodendron),* and recently in *Nelumbo nucifera*. Under cold stress, this activity in mitochondria is found to increase in animals[11] and to a large extent in plants[12], accounting in some cases half of the total oxygen consumed[1,13].

From the time AOX was found to be ubiquinol oxidase that taps electrons from ubiquinol[5], a 2-electron donor $H_2O_2$ as the reduction product of $O_2$ was self-evident. Rich and coworkers[14] attempted to measure $H_2O_2$ in the reaction mixture of cyanide-insensitive respiration with mungbean mitochondria and found that it accounted for only a small fraction of the total oxygen consumption. This was ascribed to the presence of $H_2O_2$-consuming enzymes such as catalase in these mitochondria. Similar experiments performed with submitochondrial particles devoid of matrix proteins and possibly also catalase, demonstrated "a rate of $H_2O_2$ production which could easily account for the net flux of electrons through the alternate pathway"[14]. The AOX purified from *Arum maculatum* was indeed found to be $H_2O_2$-producing quinol oxidase[15,16].

Reports started appearing in 1990's that the product of oxygen reduction by AOX is water, more due to insufficient experimental evidence for

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stoichiometric match of either superoxide or $H_2O_2$ with $O_2$ consumption. To quote some of these: (i) the metabolic consequences of electron flow through the alternative pathway would be to keep the Krebs cycle running with a minimal ATP production. Whilst the end product of oxygen reduction is known to be water, the nature of the redox components involved in this reduction remains unknown[17], (ii) this 'alternative' is believed to bring about a four-electron reduction of $O_2$ to water, which, unlike Cyt *c* oxidase is sensitive to inhibition by SHAM and completely insensitive to cyanide[18], (iii) when cyanide resistant pathway is engaged, electrons diverge from the main, cyanide-sensitive electron transport pathway at the UQ pool and instead of continuing to the terminal Cyt *c* oxidase, flow through an 'alternative oxidase" that catalyzes the reduction of molecular $O_2$ to water[19], and (iv) "the sole enzyme activity of the alternative pathway ubiquinol oxidase, transfers electrons from reduced ubiquinone (ubiquinol) to molecular $O_2$, producing water as the reduced product"[20]. In addition, the illustrations showing AOX reducing $O_2$ to water have further made a strong impact on this notion[20,21]. This misleading assertion with hardly any experimental evidence in support appears to be based on the inability to find the product of the consumed $O_2$ from the medium.

Recently, strong views were expressed by Moller *et al*[22] on our finding that AOX reduces $O_2$ to $H_2O_2$, citing in support the work of Huq and Palmer[23] on the stoichiometry of 2:1 for NADH:$O_2$ during oxidation of NADH by AOX. However, on reexamination of the data of Huq and Palmer, it was obvious that their experiments supported the presence of NADH peroxidase activity in the preparation[24] which would consume $H_2O_2$ produced by AOX.

To demonstrate formation of $H_2O_2$ during AOX activity, in this study, we have studied the effect of adding the $H_2O_2$-specific enzyme catalase using chilled potato tuber mitochondria with increased AOX activity[1,13]. When added during the reaction, catalase should halve the cyanide-insensitive rate of $O_2$ consumption, and at the end of the reaction it should release half-equivalent of $O_2$ from $H_2O_2$ present[25]. The results of our experiments[1] have reaffirmed the original observation of Rich *et al*[14] and have justified reinstating $H_2O_2$ as the product of plant mitochondrial AOX activity. The objective of this work is to draw attention to this conceptual paradigm shift, expected to provide basis for understanding the physiological role of AOX.

## Materials and Methods

Potato tubers (*Solanum tuberosum* L.) were collected from the standing crop in the field. The tubers were stored at 4°C for the specified days to induce AOX activity and mitochondria were isolated according to the procedure described previously[26]. All the chemicals used were purchased from Sigma-Aldrich Fluka. Potassium phosphate, $MgCl_2$, KCl and sucrose were of analytical grade. Solutions of SHAM in dimethyl sulfoxide and of KCN in water were prepared fresh and the pH was adjusted to 7.8.

Mitochondrial protein was estimated by modified biuret method using BSA as the standard. Consumption and release of $O_2$ were measured at 25ºC, polarographically in Hansatech oxygen electrode, model Oxygraph. The samples of mitochondria (~5 mg of protein/ml) were initially incubated to consume the endogenous substrate in the aerated buffer system consisting of potassium phosphate (10 mM, pH 7.8), sucrose (0.3 M), potassium chloride (10 mM), $MgCl_2$ (5 mM), and BSA (0.1%). The rate of total $O_2$ uptake was measured in the same buffer system after adding a suitable amount of mitochondrial sample and the respiratory substrate succinate (40 mM) without any inhibitor was added. The rate of $O_2$ uptake sensitive to KCN (1 mM) was taken as the capacity of cytochrome pathway, whereas the rate of $O_2$ uptake sensitive to SHAM (3 mM) in the presence of KCN (1 mM) was taken as the capacity of AOX. The non-specific residual $O_2$ uptake was less than 10% of the total $O_2$ consumption. The concentration of dissolved $O_2$ under these conditions of temperature and atmospheric pressure at Pune was calculated to be 250 nmole/ml.

## Results

### Mitochondrial oxidation rates increase during chill treatment of potato tubers

The rates of total $O_2$ consumption by mitochondria remained unchanged for 3-days exposure of potato tubers to chill treatment and then significantly increased at 5-and 10-days exposure. The AOX activity showed similar increase after 5 days, and at 10 days about three-fold increase in the rate was observed. The AOX activity increased from 17% in

the fresh to 54% at 8≤ days of total $O_2$ consumption. In other words, AOX activity accounted for half of the $O_2$ consumption in the mitochondria from potato under cold-stress. These results explained why earlier workers could find little AOX activity in fresh potato mitochondria[5] and also confirmed the findings of increased AOX activity on prolonged chill treatment[1,13].

**Effect of inhibitors, KCN and SHAM, and catalase on mitochondrial oxidation**

The effects of KCN, SHAM and catalase on $O_2$ consumption by mitochondria obtained from 10-days chilled potato tubers, typical of several experiments, are illustrated in Fig. 1. The initial rate of $O_2$ consumption with succinate as substrate is given as nmole/min indicated by numbers on the trace. The numbers along the traces indicate the rate of $O_2$ consumption in nmol/min. The decrease by 40 nmol/min in the rate on addition of KCN corresponds to cytochrome oxidase activity. On addition of catalase, the cyanide-insensitive rate decreased to 20 nmol/min from 41 nmol/min, nearly half, as expected of formation of $H_2O_2$. And this rate was further inhibited by the subsequent addition of SHAM confirming that it was obtained by AOX activity.

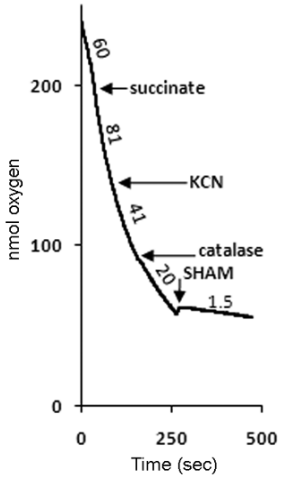


Fig. 1—Effect of inhibitors on the $O_2$ consumption by mitochondria isolated from potato tubers pre-chilled for 10 days at 4°C [Oxygen consumption was measured in an oxygraph in the reaction mixture (1 ml) consisting of phosphate buffer system (pH 7.8), succinate (40 mM) as substrate and mitochondria (0.8 mg protein). Addition of the inhibitor KCN (1 mM), enzyme catalase (5000 u/ml) and inhibitor SHAM (3 mM) are shown by arrows. The numbers along the traces indicate the initial rates of $O_2$ consumption in nmol/min. Note the rate of $O_2$ consumption is nearly halved upon addition of catalase, indicating the formation of $H_2O_2$]

**Release of $O_2$ on addition of catalase at the end of the reaction**

Oxygen in the reaction mixture was allowed to be completely consumed by the respiring mitochondria, obtained from tubers chilled for different periods, and catalase was then added. Presence of $H_2O_2$ was indicated by the immediate release of half-equivalent

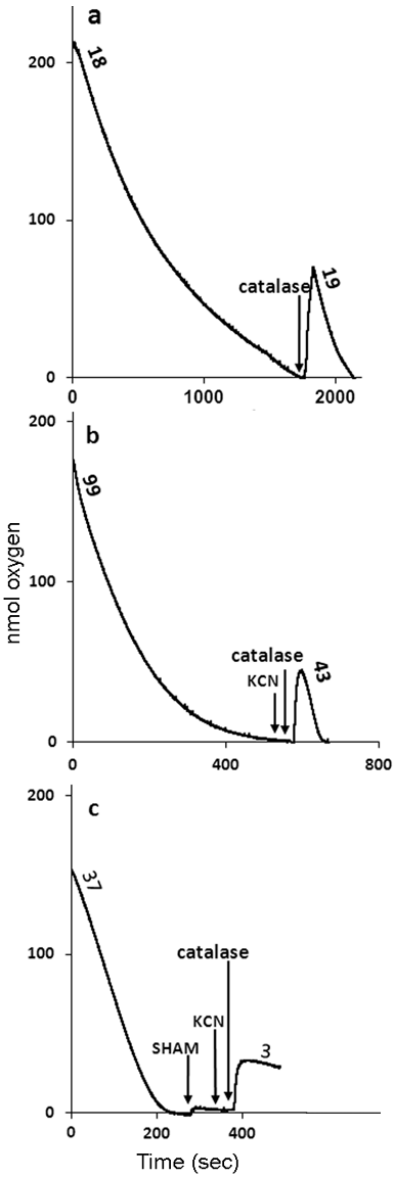


Fig. 2—Release of part of $O_2$ consumed by potato tuber mitochondria on addition of catalase indicative of presence of $H_2O_2$ [The reaction mixture (1 ml) consisted of phosphate buffer system (pH 7.8) and the specified amount of mitochondria. [(a) 0.2 mg protein, (b) 2.5 mg protein and (c) 1 mg protein obtained from pre-chilled of potato tubers for stated periods (a) 8-days, (b) 3-days and (c) 1-day. The reaction was started by adding succinate (40 mM) and consumption of oxygen was allowed to go to completion. The numbers along the traces indicate the initial rates of $O_2$ consumption in nmol/min. Oxygen released back into the medium was recorded after adding catalase (5000 units/ml), and where mentioned, KCN (1 mM) and SHAM (3 mM)]

of $O_2$ back into the medium in all the cases tested. With no inhibitor added (Fig. 2a), significant amount of $O_2$ was released and this was again consumed by the respiring mitochondria at the same initial rate. KCN was added after complete consumption of $O_2$ in the reaction mixture and subsequently catalase was added. (Fig. 2b). The released $O_2$ was again consumed, but at a reduced rate, since only AOX and residual activity continued (Fig. 2b). When both SHAM and KCN were added before catalase (Fig. 2c), the released $O_2$ was retained in the medium as both the oxidases were inhibited.

## Discussion

No single area of research relating to plant mitochondria has seen as much progress in recent years as the study of the alternative pathway[20]. Consumption of $O_2$ in large quantities in respiration is essential for aerobic life and partially reduced oxygen species are paradoxically toxic. Molecular $O_2$, a bi-radical with an unpaired electron on each oxygen atom is mercifully inert. Its chemical reactivity requires placing an electron in its anti-bonding orbital that needs a metal centre or another radical. Nature has designed the unique cytochrome oxidase system whose heme and copper centres in cytochrome *a* and $a_3$ afford the concerted 4-electron transfer to $O_2$ acting as electron sink. Indeed, most other $O_2$-reducing systems produce $H_2O_2$. To our knowledge, the only chemical model known to reduce $O_2$ to $H_2O$ is hemin-catalyzed oxidation of dithiothreitol[27]. As a component of the mammalian mitochondrial electron transport chain, cytochrome oxidase accounts for the bulk of consumed $O_2$ and produces non-toxic water as the product.

A small fraction of about 1% of total $O_2$ is consumed by a shunt pathway in mammalian mitochondria that is insensitive to cyanide and, therefore, not dependent on cytochrome oxidase. This is considered a minor leak in electron transport chain and reduces $O_2$ to superoxide and its dismutation product, $H_2O_2$. The credit goes to Chance[28] for bringing out the conceptual change that the small quantity of $H_2O_2$ invariably produced in all aerobic tissues, is necessary with meaningful physiological role and certainly not a waste product of metabolism as surmised before. The multiple roles of $H_2O_2$ in cell metabolism, such as phagocytosis, hormonal action and thermogenesis has already been recognized and it has gained the status of a metabolic regulating molecule (see Ramasarma for a review)[29]. Interest in understanding the cellular functions of $H_2O_2$ has increased enormously ever since.

Differential expression of AOX gene is induced upon biotic and abiotic stresses throughout the higher plants[30,31]. Rapid progress has been made in recent years in defining ROS as a major signal in diverse biological processes in plants[32]. The large amounts of $H_2O_2$ thus available must be useful in some functions of the plant, in addition to providing local heat that it does. Most suggestions consider this AOX activity for secondary functions such as control of electron overflow[33]. It is time we consider direct implication of regulated production by AOX of large amounts of $H_2O_2$ as an active metabolite capable of selective oxidation. The findings of Sitaramam and coworkers[34] have substantiated the need for AOX activity in salt tolerance and growth of plants. An unusual example of respiration is provided by mitochondria of some parasites, wherein consumption of $O_2$ is found to be sensitive to SHAM, but not to cyanide, and to produce $H_2O_2$ like plant AOX[35,36]. This is interpreted as part of $O_2$ toxicity in *Ascardia galli*[36]. A cattle parasite *Setaria digitata* survives in 30 mM cyanide and its respiration produces $H_2O_2$ that seems to provide energy for its vigorous muscular action[35]. Possibly, many other new functions of AOX and its product $H_2O_2$ will be uncovered.

In the light of the present findings that $H_2O_2$ is the product of AOX activity, some dogmas need reevaluation such as the presumed role of AOX in overcoming oxidative stress and its implication in prevention of generation of ROS of which $H_2O_2$ is a likely component[37,38]. This hypothesis stands challenged by the recent finding that oxidative stress conditions do not induce AOX in the fungus *Podospora anserina*, in contrast to plant oxidase. On the other hand, overexpression of AOX is found to increase ROS production[39] as expected.

It appears that degradation of $H_2O_2$ in the potato tuber mitochondria is relatively small and this allowed the retention of $H_2O_2$ in the medium and oxygen release from it by catalase. The success of our experiments is also due to use of a high concentration of catalase (5000 units/ml) to release $O_2$ from $H_2O_2$ almost instantly, even in the presence of KCN, a non-competitive inhibitor of catalase that does not compete with $H_2O_2$ for binding heme in contrast to other hemoprotein-enzymes, a not so well-known fact[40]. The results of the

above experiments of catalase effect on the rate, consumption and release of oxygen commend reinstating the neglected observation of Rich and coworkers[14] that $H_2O_2$ is indeed the product of reduction of oxygen by AOX of plant mitochondria, already appropriately referred as ubiquinol oxidase[20] and thus should be renamed.

**Acknowledgements**

This work was supported by the Department of Science and Technology (DST), Govt. of India, New Delhi under the WOS-A scheme and the award of the fellowship to Radha Bhate. T Ramasarma is Honorary Scientist of the Indian National Science Academy, New Delhi. This article is based on the presentation at the 8th Annual Meeting of Society for Free Radical Research in India held in Lucknow, 19th -21st March 2009.

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