Inhibition of the alternative oxidase stimulates H_2O_2 production in plant mitochondria

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Abstract The hypothesis that a non-coupled alternative oxidase of plant mitochondria operates as an antioxygen defence mechanism [Purvis, A.C. and Shewfelt, R.L., Physiol. Plant. 88 (1993) 712–718; Skulachev, V.P., Biochemistry (Moscow) 59 (1994) 1433–1434] has been confirmed in experiments on isolated soybean and pea cotyledon mitochondria. It is shown that inhibitors of the alternative oxidase, salicyl hydroxamate and propyl gallate strongly stimulate H_2O_2 production by these mitochondria oxidizing succinate. Effective concentrations of the inhibitors proved to be the same as those decreasing the cyanide-resistant respiration. The inhibitors proved to be ineffective in stimulating H_2O_2 formation in rat liver mitochondria lacking the alternative oxidase.

Key words: Alternative oxidase; Antioxygen defence; Reactive oxygen species; H_2O_2; Plant mitochondrion

1. Introduction

A cyanide-resistant oxidase (the so-called alternative oxidase) is localized in the inner membrane of plant mitochondria. It catalyzes four-electron reduction of O_2 by two ubiquinols with no energy conserved in spite of the fact that in the same membrane there are energy-coupled $CoQH_2$-cytochrome *c* reductase and cytochrome *c* oxidase which catalyze this reaction in an energy-coupled fashion. Although the alternative oxidase is probably the most studied system of non-coupled respiration in plant mitochondria, the functional significance of this pathway still remains unclear. This is not likely to be thermoregulation which in plants is inherent only in flowers of a few species (for a review, see [1]).

In 1993 Purvis and Shewfelt hypothesized that the alternative oxidase is used in the antioxygen defence by transporting to oxygen the excess of reducing equivalents [2]. In line with this assumption, the alternative oxidase was shown to be operative at much higher $CoQH_2/CoQ$ ratios than the energy-coupled $CoQH_2$-cytochrome *c* reductase [3,4].

Independently, one of us suggested [5] that the alternative oxidase can be involved in the antioxygen defence of plant mitochondria by decreasing concentrations of O_2 and its one-electron reductants in these organelles. It was proposed that the alternative oxidase, in co-operation with the non-coupled NAD(P)H-CoQ reductases, also found in plant mitochondria, organize a respiratory chain which bypasses all three energy-coupling sites of the main respiratory chain and, hence, can perform its defensive function being no more restricted by ADP availability. It is also important that the non-coupled chain does not include the $O_2^{-\bullet}$-producing components, which become long-lived in state 4, such as CoQH [5–8].

In 1995–1996 Wagner [9], Vanlerberghe and McIntosh [10] showed the induction of the alternative oxidase by H_2O_2. This explains earlier findings that such an induction in plant [11–13] and yeast [14] cells can be achieved by adding antimycin A, which is known to strongly increase the H_2O_2 production by mitochondria [15]. In the same way, i.e. by accumulation of H_2O_2, Wagner [9] accounted for induction of the alternative oxidase by salicylate [16–19], an inhibitor of catalase [19]. These data are consistent with the above suggestion that the alternative oxidase participates in the antioxygen defence system, the role of which becomes especially important when the ROS level increases [7,8,20,21]. In this paper, we directly show that operation of the alternative oxidase in parallel with the energy-coupled respiratory chain indeed strongly lowers the H_2O_2 production of plant mitochondria.

2. Materials and methods

Soybean mitochondria and pea cotyledon mitochondria were isolated according to Bonner [22]. Rat liver mitochondria were isolated as described [23]. Mitochondrial oxygen consumption was measured with a Clark-type electrode and a polarograph (Rank Brothers, Oxford, UK). Mitochondrial hydrogen peroxide production was monitored using the H_2DCF-DA [24] or scopoletin [25] methods. Both methods gave qualitatively similar results. In the figures, the data obtained with H_2DCF-DA are shown as more illustrative due to the higher sensitivity of this probe.

All reagents used in our experiments were first tested for possible interference with the H_2O_2 measuring system (H_2DCF-DA/peroxidase or scopoletin/peroxidase) to avoid possible artifacts. Mitochondrial protein concentration was measured by the biuret method. Horseradish peroxidase was from Reanal, scopoletin from Serva, H_2DCF-DA from Molecular Probes.

3. Results

As is shown in Fig. 1A, the succinate-supported respiration of soybean mitochondria can be partially inhibited by SHAM, an inhibitor of the alternative oxidase. Subsequent addition of KCN results in complete inhibition of the oxygen consumption. The respiration of pea mitochondria is practically insensitive to KCN, whereas the subsequent addition of 0.6 mM SHAM strongly inhibits respiration (Fig. 1B). It should be noted that both pea and soybean mitochondria were uncoupled, possibly due to the presence of high concentrations of free fatty acids. Only when the incubation medium was supplemented with BSA (0.2 mg/ml) some energy coupling

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Abbreviations: H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species; SHAM, salicyl hydroxamate

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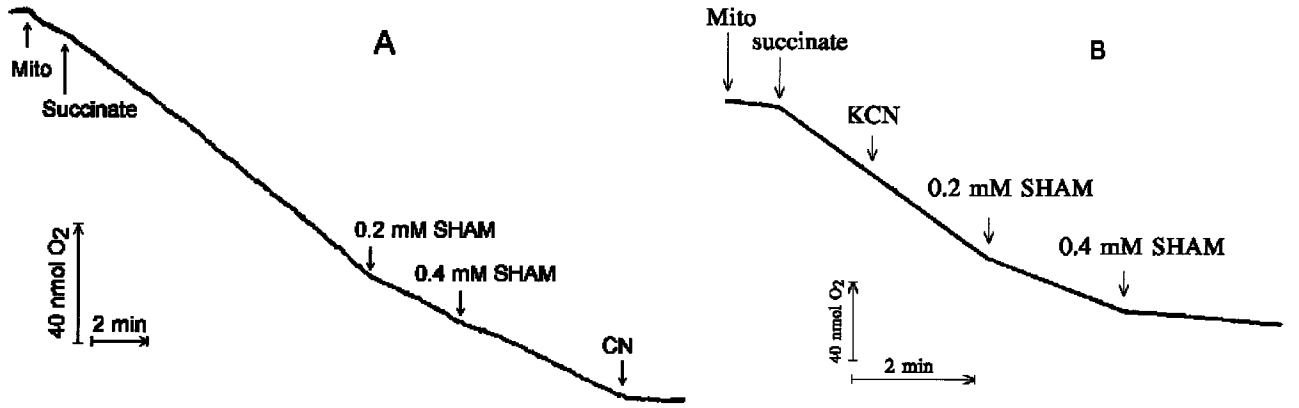

Fig. 1. The effect of SHAM and KCN on respiration of soybean (A) and pea (B) mitochondria. The incubation medium contained 400 mM sucrose, BSA (0.2 mg/ml), 1 mM EGTA, 20 mM HEPES/Tris (pH 7.4). Additions: mitochondria (2 mg protein/ml); 1 mM KCN; 5 mM succinate.

was observed (1.5-fold stimulation of respiration upon addition of the uncoupler, 60 nM SF6847, data not shown).

Fig. 2A shows that addition of succinate to pea mitochondria initiates H_2O_2 production. This H_2O_2 generation is strongly enhanced by the addition of SHAM. The concentrations of SHAM which gave maximal rates of H_2O_2 production were the same as those which maximally inhibited mitochondrial respiration (cf. Fig. 1). This was true also for soybean mitochondria (data not shown).

Fig. 2B shows that H_2O_2 production by soybean mitochondria was significantly enhanced by adding antimycin A. Subsequent addition of SHAM further stimulated formation of H_2O_2.

Similar results were obtained using another inhibitor of the alternative oxidase, propyl gallate (not shown).

Rat liver mitochondria were used as a control. These mitochondria do not contain a cyanide-insensitive oxidase. As expected, the succinate oxidation by these mitochondria was not sensitive to SHAM up to a concentration of 2 mM. When hydrogen peroxide production in the presence of antimycin A was measured, it appeared that SHAM was inhibitory rather than stimulatory (Fig. 2C). Propyl gallate was without effect on animal mitochondria (not shown).

4. Discussion

The data presented here clearly shown that inhibition of the alternative oxidase strongly stimulates production of H_2O_2 by plant mitochondria. This does not occur in animal mitochondria. Just these relationships could be expected within the framework of the concept considering the alternative oxidase as a part of an antioxygen defence system of plant mitochondria.

Being independent of the ADP phosphorylation process, the alternative oxidase can be involved in such a defence by (i) removal of excess reducing equivalents that could be used to reduce O_2 to $O_2^{-\bullet}$ [2], (ii) bypass of energy coupling in complex I and complex III, i.e. in those steps of the respiratory chain that produce $O_2^{-\bullet}$ at high protonic potential [5–8] and (iii) decrease of the intramitochondrial [O_2] level [5–8]. All these functions may be carried out in cooperation with non-coupled NAD(P)H-CoQ reductases also present in plant mitochondria [5–8].

Interestingly, saturation by O_2 of the alternative oxidase occurs at much higher [O_2] than that of cytochrome oxidase [26,27]. Hence, there is no risk that non-coupled respiration will compete for O_2 with the coupled oxidase under conditions of O_2 limitation. Thsi situation resembles that in *Azotobacter* which also possesses an alternative respiratory chain specialized in lowering the intracellular [O_2]. Such a lowering is required, first of all, to protect the *Azotobacter* nitrogenase system which is extremely sensitive to O_2-induced damage. This O_2-lowering function, called respiratory protection [28,29], is catalyzed, according to our data, by the non-coupled NADH-quinone reductase and the partially coupled *bd* oxidase. The energy-conserving efficiency of this respiratory chain was found to be 5 times lower than that of the 'classical' chain composed in *Azotobacter* of the coupled NADH-quinone reductase, the Q cycle and the *o* oxidase. H^+/e^- ratios of the former and the latter chains seem to be 1 and 5, respectively [30]. Just as in the case of plant mitochondria, the low-efficiency *bd* oxidase has a lower affinity to oxygen and requires a much higher QH_2/Q ratio than the *o* oxidase (see [7,8] for reviews).

The problem of oxygen danger is more dramatic for the plant cell and *Azotobacter* than for the animal cell which does not produce O_2 and possesses no nitrogenase. This is apparently why animal mitochondria, as a rule, are not equipped with an alternative respiratory chain. Instead, they are assumed to have a mechanism of 'mild' uncoupling. This mechanism can prevent maintenance of a high level of protonic potential at state 4 which strongly stimulates $O_2^{-\bullet}$ production [5–8,31].

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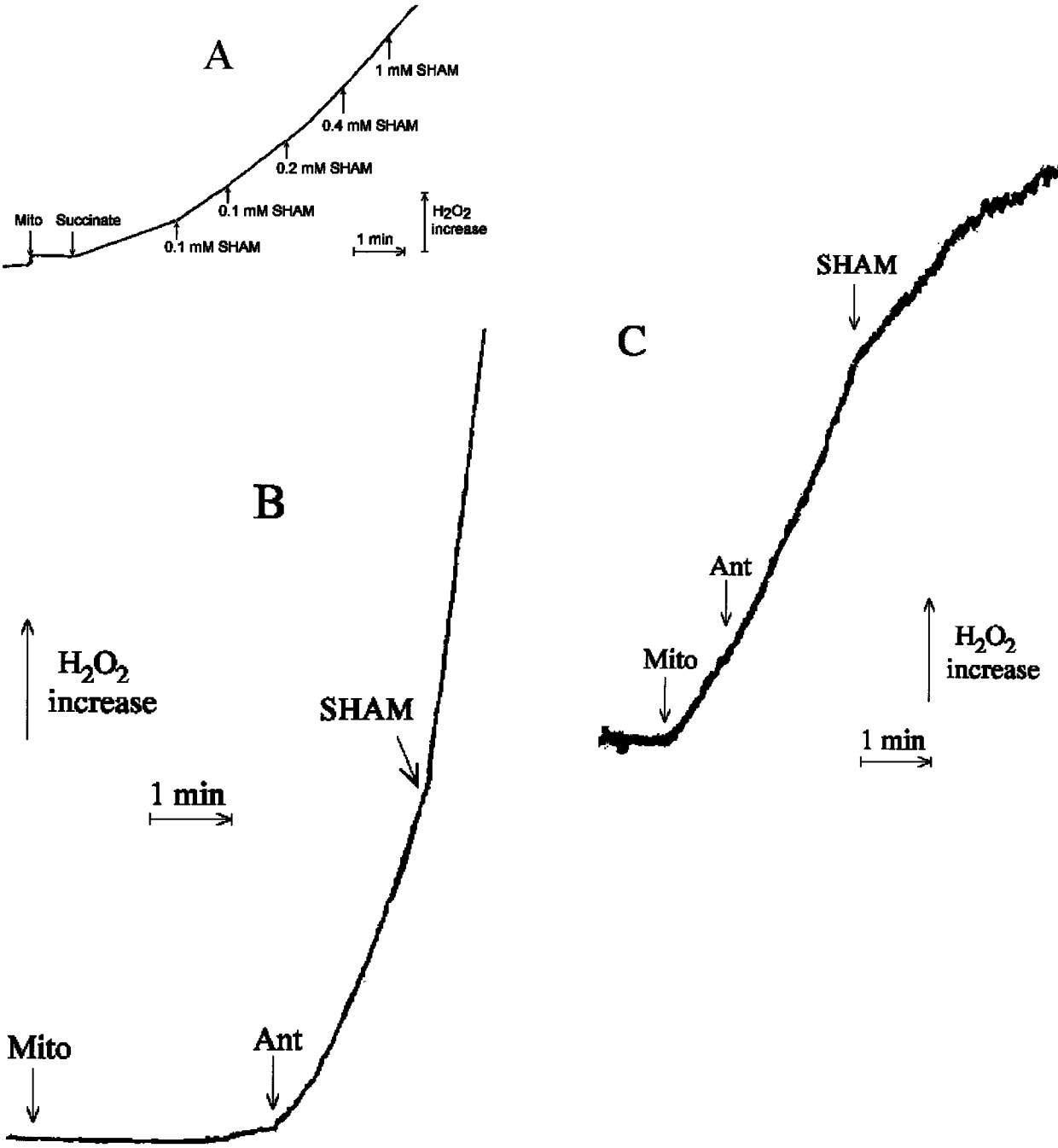

Fig. 2. The effect of SHAM and antimycin A on H_2O_2 generation by rat liver and plant mitochondria. The incubation medium (see Fig. 1) was supplemented with 10 U horseradish peroxidase and 2 μM H_2DCF-DA. A: Soybean mitochondria. B: Pea mitochondria. C: Rat liver mitochondria. In B and C, the incubation mixture contained 5 mM succinate. Additions: Mito, mitochondria (2 and 1 mg/ml protein in plant and animal mitochondria, respectively); 5 mM succinate; Ant, antimycin A; 0.8 mM SHAM (B,C).

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