Hydrogen peroxide generation by higher plant mitochondria oxidizing complex I or complex II substrates

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Abstract The generation of H_2O_2 by isolated pea stem mitochondria, oxidizing either malate plus glutamate or succinate, was examined. The level of H_2O_2 was almost one order of magnitude higher when mitochondria were energized by succinate. The succinate-dependent H_2O_2 formation was abolished by malonate, but unaffected by rotenone. The lack of effect of the latter suggests that pea mitochondria were working with a proton motive force below the threshold value required for reverse electron transfer. The activation by pyruvate of the alternative oxidase was reflected in an inhibition of H_2O_2 formation. This effect was stronger when pea mitochondria oxidized malate plus glutamate. Succinate-dependent H_2O_2 formation was ca. four times lower in *Arum* sp. mitochondria (known to have a high alternative oxidase) than in pea mitochondria. An uncoupler (FCCP) completely prevented succinate-dependent H_2O_2 generation, while it only partially (40–50%) inhibited that linked to malate plus glutamate. ADP plus inorganic phosphate (transition from state 4 to state 3) also inhibited the succinate-dependent H_2O_2 formation. Conversely, that dependent on malate plus glutamate oxidation was unaffected by low and stimulated by high concentrations of ADP. These results show that the main bulk of H_2O_2 is formed during substrate oxidation at the level of complex II and that this generation may be prevented by either dissipation of the electrochemical proton gradient (uncoupling and transition state 4-state 3), or preventing its formation (alternative oxidase). Conversely, H_2O_2 production, dependent on oxidation of complex I substrate, is mainly lowered by the activation of the alternative oxidase.

Key words: Alternative oxidase; Antioxygen defence; Hydrogen peroxide; Plant mitochondrion; Reactive oxygen species; Uncoupling

1. Introduction

Higher plants are aerobic organisms that consume (mitochondria) or evolve (chloroplasts) oxygen. Up to 2% of total oxygen consumed by mitochondria is converted to reactive oxygen species (ROS) [1,2]. This value greatly increases when plants are under abiotic or biotic stress [3–5]. Similarly to what happens in mammalian mitochondria [1,6,7], the main sites of ROS generation are complexes I and III of the respiratory chain [8]. By interacting with one of these sites, molecular oxygen may undergo a univalent reaction to form superoxide anion (O_2^-), which then spontaneously or enzymatically dismutates to hydrogen peroxide (H_2O_2). The effects of ROS are usually minimized by both enzymatic and non-enzymatic defences [9]. However, these mechanisms act only after ROS have been generated. This production is particularly high when mitochondria, oxidizing succinate, are in a resting (respiratory control or state 4) condition, when the electrochemical proton gradient ($\Delta\bar{\mu}H^+$) is high and respiration rate is limited by a lack of ADP [1,10,11]. A threshold value for the electrical component ($\Delta\Psi$) of $\Delta\bar{\mu}H^+$ has been found [11]. These conditions favor the generation of long-lived ubisemiquinone ($UQH^\bullet$) which, in turn, is capable of directly reducing O_2 [12,13]. Thus complex III appears to be the major site of ROS generation.

It has been proposed by Skulachev that the maintenance of a safely low level of oxygen and its one-electron reductants may be obtained by special mechanisms of 'mild' uncoupling or by non-coupled oxidations that prevent $\Delta\bar{\mu}H^+$ to become high [12,13]. In higher plant mitochondria these mechanisms of prevention of ROS generation may be achieved by free fatty acid-induced uncoupling (mild uncoupling), which can be mediated by the plant uncoupling mitochondrial protein (PUMP) [14], or possibly by ANT [15], as recently shown for mammalian mitochondria [16]. In addition, plant mitochondria, possessing a cyanide-resistant oxidase (alternative oxidase) [17], are also able to perform a non-coupled respiration which, as suggested [18,19], may contribute to the prevention of ROS generation. Recent results appear indeed to corroborate this hypothetical role [20,21].

In this paper, the contribution of the oxidation of complex I (malate plus glutamate) and complex II (succinate) substrates to the H_2O_2 formation by higher plant mitochondria and the role of uncoupled and non-coupled respiration in preventing such a generation were examined.

2. Materials and methods

Pea (*Pisum sativum* L., cv. Frimousse) mitochondria were isolated from 7-day-old etiolated seedlings, as previously described [22]. Briefly, 70 g of stems were cut and homogenized by a mortar with a pestle in 120 ml of a medium composed of 20 mM HEPES-Tris (pH 7.6), 0.4 M sucrose, 5 mM EDTA, 25 mM potassium metabisulfite and 0.3% bovine serum albumin (BSA). The homogenate was then filtered through eight gauze layers. Debris was again homogenized in 100 ml of the medium and filtered once more. The filtrate was centrifuged at $28\,000\times g$ for 5 min in a Sorvall RC-5B centrifuge, at 4°C. The pellet was resuspended in 120 ml homogenization medium in a Potter homogenizer. This fraction was centrifuged at $2500\times g$ for 3 min and the supernatant centrifuged at $28\,000\times g$ for 5 min. The pellet (mitochondrial fraction) was suspended in a final volume of 2 ml

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Abbreviations: ANT, adenine nucleotide translocator; CAtr, carboxyatractylate; $\Delta\bar{\mu}H^+$, electrochemical proton gradient; $\Delta\Psi$, electrical potential difference; OLIGO, oligomycin; PHPA, *p*-hydroxyphenylacetate; P_i, inorganic phosphate; PUMP, plant uncoupling mitochondrial protein; PVPP, polyvinylpolypyrrolidone; ROS, reactive oxygen species; TPP^+, thiamine pyrophosphate; $UQH^\bullet$, ubisemiquinone

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Table 1
H_2O_2 formation induced by succinate or malate plus glutamate oxidation in pea stem mitochondria

Addition	H_2O_2 formation [pmol (mg protein min)$^{-1}$]	
	5 mM SUCC	10 mM MAL+ 10 mM GLU
None	1360	205
5 mM malonate	0	–
40 μM rotenone	1339	136
1 μM antimycin A	544	46

of 20 mM HEPES-Tris (pH 7.5), 0.4 M sucrose and 0.1% BSA (fatty acid-free). The suspension was stored on ice at about 3 mg protein/ml.

Mitochondria from *Arum maculatum* L. and *A. italicum* Mill. were obtained from spadices with opened spathae, collected from spontaneously growing plants. The isolation method was that above described for pea mitochondria, except that 30 g of spadices were homogenized in 300 ml homogenization medium supplemented of 0.6% PVPP (polyvinylpolypyrrolidone). The final mitochondrial suspension contained about 5 mg protein/ml.

Oxygen consumption was monitored, at room temperature, by a Clark-type oxygen electrode (YSI, model 4004). The incubation mixture was composed of 20 mM HEPES-Tris (pH 8.0), 0.4 M sucrose, 5 mM $MgCl_2$, 5 mM K-phosphate, 0.1% BSA and 0.1 mg/ml mitochondrial protein in a final volume of 2 ml. Succinate (5 mM) or 10 mM malate plus 10 mM glutamate were used as respiratory substrates. When the oxidation of malate plus glutamate was followed, 300 μM thiamine pyrophosphate (TPP^+) and 200 μM NAD^+ were supplied to the incubation medium.

Hydrogen peroxide was measured by the method described in [23]. Briefly, experiments were run at 25°C for ca. 10 min in 2 ml (final volume) of the incubation medium described above, supplemented with 100 μM $MnCl_2$, 200 IU horseradish peroxidase and 50 μg/ml *p*-hydroxyphenylacetate (PHPA). Hydrogen peroxide formation was followed as fluorescence increase by a Perkin-Elmer spectrofluorimeter (model LS 50 B). The excitation and emission wavelengths were 320 and 400 nm, respectively, with a 10 nm slit width. Each experimental run was calibrated by the addition, at the end of the experiment, of 50 nM H_2O_2.

The mitochondrial protein was determined by Bradford method, using the Sigma protein assay (Sigma, St. Louis, MO, USA).

3. Results

The generation of H_2O_2 was followed in isolated pea stem mitochondria oxidizing either malate plus glutamate or succinate (Table 1). The level of H_2O_2 recovered was almost one order of magnitude higher when mitochondria were energized by succinate. Malonate, an inhibitor of complex II [24], completely inhibited this formation. Rotenone, a partial inhibitor of complex I [24], caused only a 40% inhibition, due to non-coupled electron transport pathway by-passing complex I. In addition rotenone did not affect the succinate-dependent H_2O_2 formation, showing that no reverse electron transport took place in these conditions. Antimycin A induced an inhibition of ca. 60 and 80% of H_2O_2 generation linked, respectively, to succinate or malate plus glutamate oxidations. These results show, therefore, that pea stem mitochondria formed H_2O_2 by oxidizing complex I or complex II substrates, albeit the generation mediated by complex II substrate was much higher than that dependent on complex I substrate oxidation.

The role of the alternative oxidase in preventing H_2O_2 formation was then checked in pea stem and *Arum* sp. spadix mitochondria (Table 2). As is known [25], *Arum* sp. mitochondria possess, when oxidizing succinate, a high level of the alternative oxidase (78%), while in pea stem mitochondria this activity was undetectable. The level of H_2O_2 evaluated in mitochondria from *Arum* sp. was four times lower than that recovered in pea mitochondria. Pyruvate, known for activating the alternative oxidase [19], induced a KCN-insensitive respiration (15%) also in pea mitochondria oxidizing succinate, which was reflected in an inhibition of H_2O_2 formation. In agreement, the stimulation by pyruvate of the KCN-insensitive malate plus glutamate oxidation (from 59% to 72%) was linked to a concomitant decrease of H_2O_2 formation. From these results, it appears that the alternative oxidase contributes to the prevention of H_2O_2 formation, the latter being inversely related to the presence and activation of the alternative oxidase.

The effect of an uncoupler of oxidative phosphorylation (FCCP) or ADP plus inorganic phosphate (P_i) on H_2O_2 formation by pea stem mitochondria, oxidizing complex I and II substrates, was evaluated (Table 3). ADP caused an inhibition of the succinate-dependent H_2O_2 formation in a concentration-dependent manner, while that linked to malate plus glutamate oxidation was unaffected by 0.05–0.15 mM ADP and stimulated at higher concentrations. The inhibition caused by ADP on the succinate-dependent H_2O_2 formation was partially prevented by oligomycin (OLIGO) and completely prevented by OLIGO plus carboxyatractylate (CAtr). Conversely, the malate plus glutamate-dependent H_2O_2 formation was unaffected. Fig. 1 shows the effects of increasing concentrations of FCCP on H_2O_2 formation (panel A) or oxygen uptake (panel B) by pea stem mitochondria, when oxidizing succinate or malate plus glutamate. The succinate-dependent H_2O_2 formation was abolished by 2 μM FCCP (a concentration which caused maximal stimulation of oxygen consumption for both substrates), while malate plus glutamate-dependent H_2O_2 formation was only 40–50% inhibited even at higher concentrations.

4. Discussion

Recent results show that mitochondria generate superoxide radical in chilling-sensitive plant tissues exposed to low temperatures [26]. The results presented in this paper confirm that

Table 2
Effect of the induction of the alternative oxidase on H_2O_2 formation during succinate or malate plus glutamate oxidation by pea (*P. sativum*) stem and *Arum* sp. spadix mitochondria

Source	Substrate	O_2 uptake [nmol (mg protein min)$^{-1}$]	H_2O_2 formation [pmol (mg protein min)$^{-1}$]	KCN-insensitive respiration (%)
Arum sp.	5 mM SUCC	92	360	78
P. sativum	5 mM SUCC	81	1470	0
	SUCC+10 mM PYR	86	890	15
	10 mM MAL+10 mM GLU	29	188	59
	MAL+GLU+10 mM PYR	29	86	72

Table 3
Effect of ADP on H_2O_2 formation induced by succinate or malate plus glutamate oxidation in pea stem mitochondria

ADP (mM)	H_2O_2 formation [pmol (mg protein min)$^{-1}$]	
	5 mM SUCC	10 mM MAL+ 10 mM GLU
None	1372	173
0.05	931	180
0.15	515	199
0.25	442	263
1.00	317	354
0.25+2.5 μg/ml OLIGO	895	257
0.25+2.5 μg/ml OLIGO+ 5 μM CAtr	1310	251

plant mitochondria are a major site of H_2O_2 formation, when oxidizing succinate or malate plus glutamate. The succinate-dependent H_2O_2 formation is, however, ca. one order of magnitude higher than that recovered in pea mitochondria oxidizing malate plus glutamate. The H_2O_2 formation by succinate-dependent oxidation appears to be performed at the level of complex III (ubisemiquinone) and does not seem to be associated to a reverse electron transport, because it is rotenone-insensitive. This result differs from that obtained by others on mammalian mitochondria [11,13], where rotenone strongly inhibited the succinate-dependent H_2O_2 formation. This discrepancy can, however, be explained by considering that pea stem mitochondria were working with a proton motive force below the threshold value required for reverse electron transfer and fast H_2O_2 formation. Indeed, FCCP causes only an increase less than 2-fold of state 4 respiration. Conversely, the oxidation linked to malate plus glutamate appears to involve complex I, as recently shown for rat heart and pigeon brain mitochondria [23,27]. Since complex I of plant mitochondria resembles that of mammalian ones [28], the single-electron reaction, generating $O_2^{\cdot-}$ which then dismutates to H_2O_2, could occur at the level of the flavoprotein or of a Fe-S cluster [1,6,7]. In any case, this reaction seems to take place before charge separation (H^+ ejection), because H_2O_2 generation is not inhibited by ADP and only partially prevented by uncoupling.

The results presented in this paper are in line with the view that uncoupled and non-coupled respiration may have a physiological role in preventing ROS formation by mitochondria [12,13]. The activation of the alternative oxidase (non-coupled respiration) appears to be the main mechanism for ROS prevention during oxidation of complex I substrates. Conversely, the succinate-dependent H_2O_2 formation may be prevented by both uncoupled and non-coupled respiration. The prevention accomplished by the alternative oxidase is increased by pyruvate, which stimulates this oxidase [19], particularly when the cytochrome pathway is inhibited [29]. In these conditions, electrons coming from substrate oxidation are preferentially channeled towards the alternative oxidase.

The observations that oxidation of complex I substrates produces per se low levels of ROS and that this generation is further minimized by the activation of the alternative oxidase open the possibility that complex I may perform a major role under oxidative stress of plants. Oxidation of substrates by complex I and activation of the alternative oxidase (cytochrome pathway inhibited by stress) may, hence, favor the survival of plants. This role appears to be particularly relevant, because substrate oxidation at the level of complex I, via the alternative oxidase, is not completely non-phosphorylating [30] and activation by pyruvate of this pathway stimulates the synthesis of ATP [31].

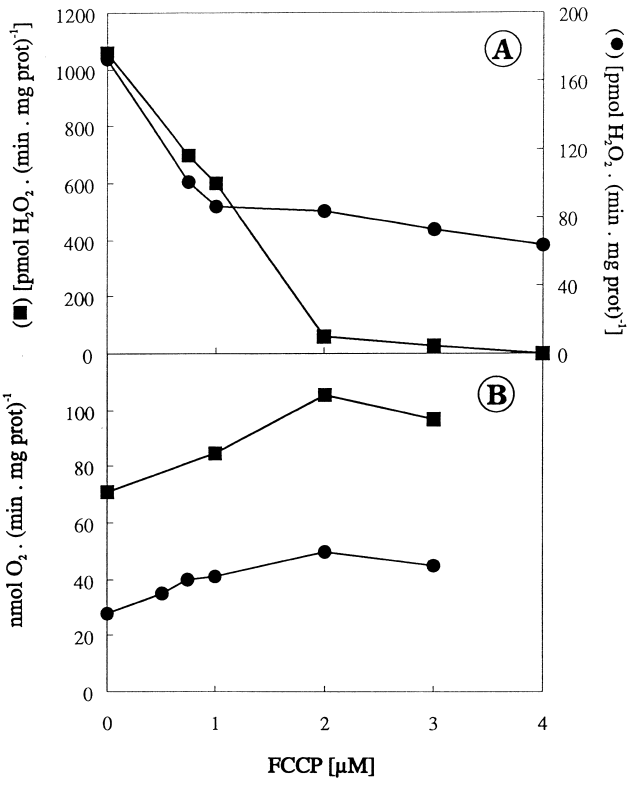

Fig. 1. Effect of FCCP-induced uncoupling on H_2O_2 formation (A) or oxygen consumption (B) by pea stem mitochondria oxidizing 5 mM succinate (■) or 10 mM malate plus 10 mM glutamate (●).

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