The role of mild uncoupling and non-coupled respiration in the regulation of hydrogen peroxide generation by plant mitochondria

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Abstract The roles of mild uncoupling caused by free fatty acids (mediated by plant uncoupling mitochondrial protein (PUMP) and ATP/ADP carrier (AAC)) and non-coupled respiration (alternative oxidase (AO)) on H_2O_2 formation by plant mitochondria were examined. Both laurate and oleate prevent H_2O_2 formation dependent on the oxidation of succinate. Conversely, these free fatty acids (FFA) only slightly affect that dependent on malate plus glutamate oxidation. Carboxyatractylate (CAtr), an inhibitor of AAC, completely inhibits oleate- or laurate-stimulated oxygen consumption linked to succinate oxidation, while GDP, an inhibitor of PUMP, caused only a 30% inhibition. In agreement, CAtr completely restores the oleate-inhibited H_2O_2 formation, while GDP induces only a 30% restoration. Both oleate and laurate cause a mild uncoupling of the electrical potential (generated by succinate), which is then followed by a complete collapse with a sigmoidal kinetic. FFA also inhibit the succinate-dependent reverse electron transfer. Diamide, an inhibitor of AO, favors the malate plus glutamate-dependent H_2O_2 formation, while pyruvate (a stimulator of AO) inhibits it. These results show that the succinate-dependent H_2O_2 formation occurs at the level of Complex I by a reverse electron transport. This generation appears to be prevented by mild uncoupling mediated by FFA. The anionic form of FFA appears to be shuttled by AAC rather than PUMP. The malate plus glutamate-dependent H_2O_2 formation is, conversely, mainly prevented by non-coupled respiration (AO).

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Key words: Alternative oxidase; Antioxygen defence; Hydrogen peroxide; Plant mitochondria; Uncoupling

1. Introduction

Mitochondria, together with chloroplasts, are a major site of ATP synthesis in plant cells. This process is associated with the generation of reactive oxygen species (ROS) [1]. During substrate-dependent electron transport, superoxide anion ($O_2^{\bullet-}$) appears to be generated, by a univalent reduction of oxygen, at the level of Complex I or Complex III of the mitochondrial respiratory chain [2]. Superoxide anion is then converted to hydrogen peroxide (H_2O_2), either spontaneously or by a reaction catalyzed by superoxide dismutase.

Both $O_2^{\bullet-}$ or H_2O_2 are potentially involved in several physiological or pathological processes, such as signal transduction or oxidative damage (oxidative stress) [3–6]. Therefore, their intracellular level must be continuously controlled. Under physiological conditions, ROS are usually removed by the mitochondrial antioxidant systems [1,7]. However, under pathological conditions, ROS generation can be highly increased causing the impairment of cell functions [3].

The rate of ROS generation by isolated mitochondria is dependent on their metabolic state and occurs strongly when the transmembrane electrical potential ($\Delta\Psi$) is above a threshold value [6]. However, according to a hypothesis formulated by Skulachev [8,9], mitochondria possess special mechanisms of 'mild' uncoupled respiration or non-coupled respiration which keep $\Delta\Psi$ below this threshold value, thus preventing ROS generation.

In this context plant mitochondria appear to be able to perform both non-coupled respiration and mild uncoupled respiration. Indeed, the presence of a cyanide-insensitive oxygen consumption, named alternative oxidase (AO), has been well recognized [10]. AO diverges from the cytochrome chain at the level of ubiquinone and electron flux through this pathway bypasses two of the three sites coupled to ATP synthesis. The role of AO is not completely understood. The only confirmed function concerns its role in the thermogenic inflorescence of *Araceae* [11]. A few years ago, an additional function for AO was suggested [12,13]. It could be involved in a dissipating mechanism to prevent ROS formation. Indeed, subsequent experimental works from several laboratories have confirmed this hypothesis [8,14–17].

In addition to this mechanism, plant mitochondria can perform mild uncoupling mediated by free fatty acids (FFA). According to the FFA circuit hypothesis [18], FFA cross the inner membrane, from the cytosol leaflet to the matrix leaflet, in their protonated form and then can recross the membrane in their anionic form only if transported by specific carrier proteins [19–21]. The latter function is accomplished in plant mitochondria by a 32 kDa membrane protein, named PUMP (plant uncoupling mitochondrial protein), which is stimulated by FFA and inhibited by GDP and ATP [22]. PUMP, reconstituted into liposomes, allows for proton translocation via the FFA cycling mechanism [23]. The activity of this dissipating system has been correlated to an oxygen burst and thermogenesis that occurs during fruit ripening [24], and to the prevention of ROS formation [25]. These dissipating systems seem to work sequentially during the life of the plant cell, since a high level of FFA-induced uncoupling activity excludes the AO activity [26].

Mammalian mitochondria appear to perform only mild uncoupling through proteins that are able to carry the anionic

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Abbreviations: AAC, ATP/ADP carrier; AO, alternative oxidase; CAtr, carboxyatractylate; $\Delta\Psi$, transmembrane electrical potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FFA, free fatty acids; ROS, reactive oxygen species; UCP, uncoupling protein; PUMP, plant uncoupling mitochondrial protein

form of FFA. The best known is the uncoupling protein (UCP1) identified in the inner membrane of mitochondria from brown adipose tissues which is involved in heat production [27]. This function may also be accomplished by the ATP/ADP carrier (AAC) which appears to be involved in thermoregulatory uncoupling in tissues different from brown adipose tissue [28] and also in the prevention of ROS formation [29]. In addition FFA-induced uncoupling may be due to the opening of the mitochondrial permeability transition pore by a direct interaction of FFA with the pore assembly [30]. Several isoforms of UCP (2–4) have been identified in mitochondria from several tissues [31]. In particular, one of these (UCP2) is less involved in thermogenesis than the AAC, being related to the regulation of mitochondrial H_2O_2 generation [32].

In previous work [16], it has been shown that the main bulk of H_2O_2 by pea mitochondria is formed during substrate oxidation at the level of Complex III and that this generation may be prevented by the dissipation of the electrochemical proton gradient by FCCP. Conversely, H_2O_2 production at the level of Complex I is mainly lowered by the activity of AO. In addition, it has been demonstrated that these mitochondria may be uncoupled by low concentrations of palmitate with a mechanism which is mediated by AAC [33]. Therefore, in this work the roles of mild uncoupling caused by FFA (mediated by PUMP and/or AAC) and of non-coupled respiration (AO) in preventing H_2O_2 formation by plant mitochondria were examined.

2. Materials and methods

Pea (*Pisum sativum* L., cv. Alaska) stem mitochondria were isolated from 5-day-old etiolated seedlings as previously described [16], with minor changes concerning the media. The extraction medium was composed of 20 mM HEPES–Tris (pH 7.6), 0.3 M mannitol, 5 mM EDTA, 1 mM dithioerythritol and 0.3% bovine serum albumin (BSA), while the resuspending medium (1 ml) was composed of 20 mM HEPES–Tris (pH 7.5), 0.3 M mannitol and 0.1% BSA (fatty acid-free). The suspension containing about 5 mg protein/ml was stored on ice.

Oxygen consumption was monitored, at 25°C, by a Clark-type oxygen electrode (YSI, model 4004). The incubation mixture was composed of 20 mM HEPES–Tris (pH 7.5), 0.3 M mannitol, 5 mM MgCl₂, 0.5 mM K-phosphate and 0.1 mg/ml mitochondrial protein in a final volume of 2 ml. Succinate (5 mM) or malate plus glutamate (10 mM) were used as respiratory substrates. When the oxidation of malate plus glutamate was followed, 300 μM thiamine pyrophosphate and 200 μM NAD⁺ were supplied to the incubation medium.

H_2O_2 was measured by the method described in [34]. Briefly, experiments were run at 25°C for ca. 5 min in 2 ml (final volume) of the incubation medium above described, supplemented with 100 μM MnCl₂, 200 IU horseradish peroxidase, 50 μg/ml *p*-hydroxyphenylacetate and 0.4 mg/ml mitochondrial protein. H_2O_2 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 slitwidth for both emission and excitation. Each experimental run was calibrated by the addition of 50 nM H_2O_2, at the end of the experiment.

Membrane potential measurements were performed using safranin O, as previously described in [33]. The fluorescence intensity change of safranin O was recorded at 25°C using excitation and emission wavelengths of 495 and 586 nm, respectively. A slitwidth of 10 nm for both emission and excitation was used. The data are presented as arbitrary units of fluorescence. The incubation medium was the same described in oxygen consumption experiments except that the amount of mitochondrial protein was 0.4 mg/ml.

Succinate-dependent reverse electron transfer was determined evaluating fluorimetrically the redox state of matrix nicotinamide nucleotides, according to the method described in [35]. The excitation and emission wavelengths were 340 and 460 nm, respectively. Experimental conditions were the same used for membrane potential determinations.

The mitochondrial protein was determined by the Bradford method, using the Bio-Rad protein assay (Bio-Rad, Germany).

3. Results

Fig. 1A, shows that the addition of succinate to isolated pea stem mitochondria induced a time-dependent production of H_2O_2 (trace a) which was completely inhibited by oleate (trace b) and laurate (trace c) and only partially inhibited by rotenone (trace d). The inset shows the complete relationship between FFA concentration and inhibition of H_2O_2 formation. Oleate was more effective than laurate, complete inhibition being obtained, respectively, at 40 and 80 μM FFA. Fig. 1B shows that also malate plus glutamate induced, although to a lesser extent, a rapid generation of H_2O_2 which was followed by an apparent slight but progressive decrease (trace a). Both oleate (trace b) and laurate (trace c) caused a very slight inhibition of H_2O_2 formation. The inset shows that, even at 100 μM FFA, the inhibitory effect did not exceed 40%. The inhibitory effects of FFA on H_2O_2 formation dependent on the oxidation of both substrates was abolished by the presence of BSA (results not shown).

In order to verify if the FFA-induced uncoupling was mediated by AAC or/and PUMP, the effects of carboxyatractylate (CAtr, inhibitor of AAC) and GDP (inhibitor of PUMP) were assayed on FFA-induced stimulation of oxygen consumption in mitochondria energized by succinate (Fig. 2A). CAtr completely inhibited both oleate- (trace a) and laurate-stimulated (trace b) O₂ consumption, while GDP (traces c and a) caused only a partial inhibition (ca. 30%). These results were confirmed by assaying the effects of CAtr and GDP on oleate-inhibited H_2O_2 formation by pea mitochondria (Fig. 2B). In-
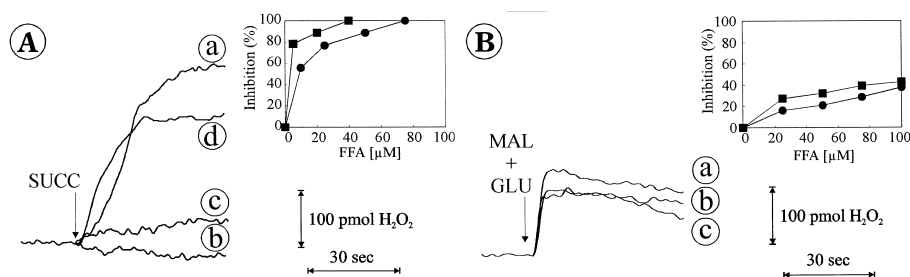

Fig. 1. Effect of oleate and laurate on the time-course of H_2O_2 formation by pea stem mitochondria energized by succinate (A) or malate plus glutamate (B). Additions: 5 mM succinate (SUCC); 5 mM malate plus glutamate (MAL+GLU). Traces: a, control; b, 40 μM oleate; c, 40 μM laurate; d, 10 μM rotenone. Insets show the concentration-dependent inhibition caused by oleate (■) or laurate (●).

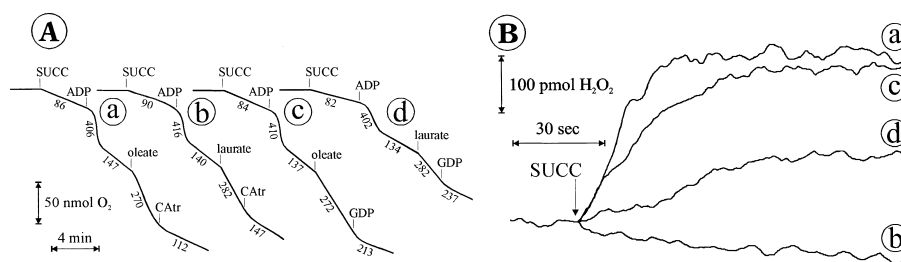Fig. 2. Effect of CAtr and GDP on oleate- or laurate-stimulated O_2 consumption (A) and on the oleate-inhibited H_2O_2 generation (B) by pea stem mitochondria energized by succinate. Additions of A: 5 mM succinate (SUCC); 100 μM ADP; 5 μM oleate; 20 μM laurate; 0.5 μM CAtr; 100 μM GDP. Figures next to each trace are expressed as nmol O_2 (mg protein min)$^{-1}$. B: 5 mM succinate (SUCC). Traces: a, control; b, 40 μM oleate; c, 40 μM oleate plus 0.5 μM CAtr; d, 40 μM oleate plus 100 μM GDP.

deed, CAtr almost completely restored the H_2O_2 formation in oleate-uncoupled mitochondria (trace b), while GDP caused only a 30% of restoration (trace c).

From these results it appears that both saturated (laurate) and unsaturated (oleate) FFA can act as natural uncouplers, causing mild uncoupling that reflects in a lower generation of ROS. This contention is confirmed by results presented in Fig. 3A. Oleate induced a slight decrease of $\Delta\Psi$, when compared to control (trace a), which was then followed, after 4 min, by a complete dissipation (trace b). The typical sigmoidal kinetic obtained resembles that linked to the aperture of a pore/channel. Laurate also caused a very slight decrease of $\Delta\Psi$ which was followed by a complete dissipation which occurred by a slower kinetic (trace c).

Since the succinate-dependent H_2O_2 formation was partially inhibited by rotenone (an inhibitor of Complex I), the effects of FFA on reverse electron transport were assayed (Fig. 3B). The addition of succinate to mitochondria induced a reverse electron transfer (trace a) which was completely inhibited by both oleate (trace c) and laurate (trace d), but only partially inhibited by rotenone (trace b). The complete relationship between FFA concentration and inhibition of the reverse electron transport (inset), shows that this transport was abolished at 40 μM FFA.

As seen in Fig. 1B, the malate plus glutamate-dependent H_2O_2 formation was lower than that induced by succinate and its generation was then followed by slight but progressive decrease. This result could depend on the concomitant scavenging activity of AO. Indeed, these mitochondria exhibited a cyanide-insensitive oxygen consumption which was increased by pyruvate (inducer of AO) and inhibited by diamide (inhib-

itor of AO) (Fig. 4A). In agreement, malate plus glutamate H_2O_2 formation (Fig. 4B, trace a) was lowered by pyruvate (trace b) and increased by diamide (trace c). In particular, the latter effect induced a progressive increase of H_2O_2 formation after the initial rapid burst.

4. Discussion

The data presented in this paper corroborate and extend previously obtained results [16]. In particular, it is confirmed that the main bulk of the H_2O_2 appears to be generated by succinate oxidation rather than by malate plus glutamate oxidation. However, it is now shown that the succinate-dependent H_2O_2 formation does not occur, as previously suggested [16], at the level of ubiquinone (Complex III), but proceeds by a reverse electron transport towards Complex I. This transfer is, differently from mammalian mitochondria [29,34], only slightly inhibited by rotenone, albeit, reverse electron transport is a well recognized phenomenon in plant mitochondria [35]. This partial inhibition could depend on the fact that rotenone per se is only a partial inhibitor of Complex I of plant mitochondria [36], but also on the fact that its effect on NAD^+ reduction by reverse electron flow can be increased (ADP) or decreased (ATP) as a function of the levels of adenylates [37].

In the light of these results, Complex I becomes, as previously shown for mammalian mitochondria [29,34], the major site of H_2O_2 generation by plant mitochondria. This implies, however, that forward and reverse electron flow in this Complex proceeds through different pathways [38]. The forward electron transfer, energized by the oxidation of NAD^+-linked
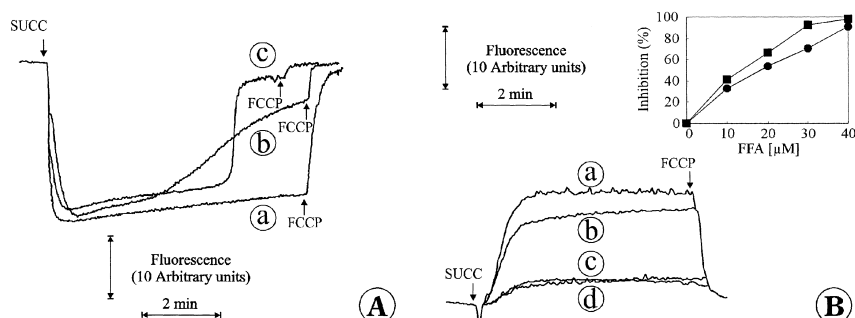

Fig. 3. A: Effect of oleate and laurate on the electrical potential of pea stem mitochondria energized by succinate. Additions: 5 mM succinate (SUCC); 1 μM FCCP. Traces: a, control; b, 40 μM laurate; c, 20 μM oleate. B: Effect of oleate or laurate on succinate-driven reverse electron transport. Additions: 5 mM succinate (SUCC); 1 μM FCCP. Traces: a, control; b, 10 μM rotenone; c, 40 μM laurate; d, 40 μM oleate. Inset shows the concentration-dependent inhibition caused by oleate (■) or laurate (●).
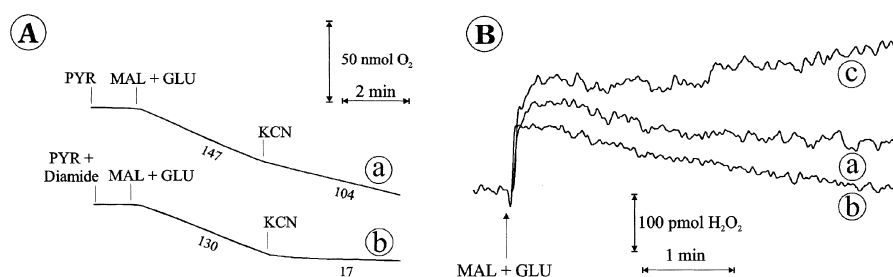
Fig. 4. A: AO activity (cyanide-resistant respiration) by pea stem mitochondria energized by malate plus glutamate. Additions: 10 mM malate plus glutamate (MAL+GLU); 1 mM KCN; 0.1 mM pyruvate (PYR); 0.1 mM diamide. Figures next to each trace are expressed as nmol O_2 (mg protein min)$^{-1}$. B: Effect of AO activity on malate plus glutamate-dependent H_2O_2 generation. Traces: a, control; b, 1 mM pyruvate; c, 0.1 mM diamide.

substrates, generates low levels of H_2O_2. This could depend on the concomitant activity of AO, which prevents H_2O_2 formation more than uncoupling, when plant mitochondria are oxidizing substrates of Complex I [16]. The overlapping of AO activity can also help to understand the biphasic kinetic of malate plus glutamate-dependent H_2O_2 generation. The low but progressive decrease of H_2O_2 generation, that follows the initial rapid burst, appears to be linked to the activity of AO and does not seem to depend on a direct interaction of H_2O_2 with constituents of the respiratory chain [39]. Indeed, this decrease is potentiated by pyruvate (inducer of AO) and converted to an increase of H_2O_2 production by diamide (an inhibitor of AO).

The succinate-dependent reverse electron transfer is mainly prevented by mild uncoupling induced by FFA. This uncoupling seems to require only a very slight dissipation of $\Delta\Psi$, although this effect is then followed by a complete collapse, which seems to be linked to the FFA-induced opening of a pore [40] overlapping to the protonophoric effect of FFA [33]. The protective effect of FFA on ROS formation can be mediated by PUMP, because activation of this protein leads to an inhibition of H_2O_2 generation [25]. Here, it is also shown that this uncoupling may be mediated by AAC. In this context, the ability of CAtr to almost completely restore the FFA-inhibited H_2O_2 formation and to inhibit FFA-stimulated O_2 uptake shows that AAC could accomplish a major role. Indeed, the inhibition of PUMP by GDP caused only ca. 30% restoration of FFA-inhibited H_2O_2 formation and FFA-stimulated O_2 consumption.

From these and other results, it appears that plant mitochondria can prevent ROS formation by both non-coupled respiration (AO) [8,14–17] and FFA-uncoupled respiration (mediated by AAC or PUMP [22,23]). In the latter case, the uncoupling effects of FFA can be interpreted in the framework of the FFA-cycling mechanism, in which the anionic form of the FFA is translocated by PUMP or AAC. The activity of PUMP has been demonstrated in storage tissues (potato) [22] or in ripening fruits [24], where its activity appears to be coordinated with that of AO [26]. Pea stem mitochondria, on the contrary, were isolated from actively growing tissues (young tissues). Therefore, it is suggested that these two uncoupling mechanisms could accomplish their protective effects as a function of the type of tissue and the developmental state of the plant.

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