Reactive oxygen species inhibit the succinate oxidation-supported generation of membrane potential in wheat mitochondria

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Abstract In order to gain a first insight into the effects of reactive oxygen species (ROS) on plant mitochondria, we studied the effect of the ROS producing system consisting of xanthine plus xanthine oxidase on the rate of membrane potential ($\Delta\Psi$) generation due to either succinate or NADH addition to durum wheat mitochondria as monitored by safranin fluorescence. We show that the early ROS production inhibits the succinate-dependent, but not the NADH-dependent, $\Delta\Psi$ generation and oxygen uptake. This inhibition appears to depend on the impairment of mitochondrial permeability to succinate. It does not involve mitochondrial thiol groups sensitive to either mersalyl or *N*-ethylmaleimide and might involve both protein residues and/or membrane lipids, as suggested by the mixed nature. We propose that, during oxidative stress, early generation of ROS can affect plant mitochondria by impairing metabolite transport, thus preventing further substrate oxidation, $\Delta\Psi$ generation and consequent large-scale ROS production. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Durum wheat mitochondria; Reactive oxygen species; Succinate carrier; Electrical membrane potential

1. Introduction

Reactive oxygen species (ROS) are generated during aerobic metabolism, mitochondria being one of the most important ROS sources as well as ROS targets (for references see [1–4]).

Although in plant mitochondria ROS production is high due to the high oxygen concentration in plant cells due to activity of chloroplasts, as well as to biotic and abiotic stress (for references see [5–7]), the knowledge of the ROS-induced effects on plant mitochondria is rather poor. Mitochondrial damage was reported due to H_2O_2 in castor bean endosperm [8]; moreover, high mitochondrial electrical membrane potential ($\Delta\Psi$) was shown to cause ROS production via succinate oxidation in state 4, due to reverse electron transfer in plant [9] as in animal mitochondria [10,11], thus necessitating the occurrence of a mitochondrial defence mechanism against ROS. In this regard, we have recently shown that in durum wheat mitochondria (DWM) ROS can activate both the plant mitochondrial potassium channel (PmitoK$_{ATP}$) [12] and the plant uncoupling mitochondrial protein (PUMP) [13], both of them working by collapsing the mitochondrial $\Delta\Psi$ in an uncoupling protein-like manner; this poses the question as to whether and how ROS production can elicit other defence mechanisms under conditions of high $\Delta\Psi$-related ROS generation. Thus, we investigated the effect of ROS produced by xanthine plus xanthine oxidase on $\Delta\Psi$ generation caused by either succinate of NADH addition to DWM.

We show that the early ROS production can decrease the rate of $\Delta\Psi$ generation by succinate, but not by NADH, possibly by impairing the carrier-mediated succinate uptake by mitochondria.

2. Materials and methods

2.1. Chemicals and plant material

All reagents were purchased from Sigma. Substrates were used as tris(hydroxymethyl)-aminomethane (Tris) salts at pH 7.20. Antimycin A and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were dissolved in ethanol. Certified seeds of durum wheat (*Triticum durum* Desf., cv. Ofanto) were from the Italian Cereal Crop Institute (Foggia, Italy).

2.2. Isolation of DWM

Mitochondria were isolated from 72 h old etiolated seedlings of durum wheat, as in [14]. The grinding buffer contained 0.5 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 4 mM cysteine, 0.1% (w/v) defatted bovine serum albumin (BSA), 0.6% (w/v) polyvinylpyrrolidone (PVP), 30 mM Tris–HCl pH 7.50 and washing buffer contained 0.5 M sucrose, 1 mM EDTA, 0.1% (w/v) defatted BSA, 10 mM Tris–HCl pH 7.40. Purification of washed mitochondria was obtained by isopycnic centrifugation in a self-generating 28% (v/v) Percoll (PVP-coated silica sol, Pharmacia) gradient as in [15]. The final mitochondrial suspension was diluted to obtain a 0.3 M sucrose concentration. This protocol gives intact and coupled mitochondria [14]. Mitochondrial protein content was determined by Lowry's method, modified as in [16], using BSA as a standard.

2.3. Superoxide anion production

Superoxide anion (and some hydrogen peroxide [17]) was generated by a system, already used in [12,13], consisting of 0.1 mM xanthine plus the amount of xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.1.3.22) from buttermilk (Sigma X-4376) required to generate superoxide anion at an initial rate of 20 nmol/min (usually about 100–200 μg). The rate of superoxide anion generation by the xanthine plus xanthine oxidase system was determined by measuring the rate of oxygen consumption due to xanthine oxidase reaction in a medium consisting of 0.3 M mannitol, 5 mM MgCl$_2$, 20 mM Tris–HCl pH 7.20. A check was made that antimycin A, phenylsuccinate and cysteine are no inhibitors of the xanthine oxidase. Mersalyl (0.3 mM) and

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Abbreviations: BSA, bovine serum albumin; DWM, durum wheat mitochondria; $\Delta\Psi$, electrical membrane potential; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; NEM, *N*-ethylmaleimide; PmitoK$_{ATP}$, plant mitochondrial potassium channel; PUMP, plant uncoupling mitochondrial protein; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; Tris, tris(hydroxymethyl)-aminomethane

N-ethylmaleimide (NEM) (0.1 mM) inhibited xanthine oxidase to different extents in different enzyme preparations (15–50%); in each experiment correction of enzyme amount was made for mersalyl and NEM inhibition.

2.4. Oxygen uptake

Oxygen uptake by DWM due to the addition of either 5 mM succinate or 1 mM NADH was measured at 25°C by means of a Gilson Oxygraph model 5/6-servo Channel pH 5, equipped with a Clark-type electrode (5331 YSI, Yellow Spring, OH, USA). Measurements were carried out in 2 ml of a medium consisting of 0.3 M mannitol, 5 mM $MgCl_2$, 20 mM Tris–HCl pH 7.20 and 0.2 mg of mitochondrial proteins.

2.5. Fluorimetric measurements of $\Delta\Psi$ changes

Mitochondrial $\Delta\Psi$ was monitored at 25°C essentially as in [18], by measuring safranin 'O' fluorescence changes (λ_{ex} 520 nm, λ_{em} 570 nm) by means of a Perkin-Elmer LS50B spectrofluorimeter. Measurements were carried out in a reaction medium (2 ml) containing 0.3 M mannitol, 5 mM $MgCl_2$, 20 mM Tris–HCl pH 7.20, 2.5 µM safranin 'O',

and 0.2 mg of mitochondrial proteins. The rate of $\Delta\Psi$ generation was expressed as rate of fluorescence change, ΔF/s, i.e. change in arbitrary units of fluorescence per second, and calculated as the higher slope of the experimental curve obtained after succinate or NADH addition to DWM. The rate obtained by using control DWM with 5 mM succinate was taken equal to 100. Succinate-respiring DWM showed a $\Delta\Psi$ of about 180–190 mV [12].

3. Results

The mitochondrial membrane potential ($\Delta\Psi$) of purified DWM was found to remain rather constant in the time as monitored by safranin fluorescence (Fig. 1). Either succinate (5 mM) or NADH (1 mM) was then added in the absence or presence of the ROS producing system consisting of xanthine plus xanthine oxidase, which per se have no effect on the safranin fluorescence. Both succinate and NADH were found to cause a fast increase in the mitochondrial $\Delta\Psi$ as shown by
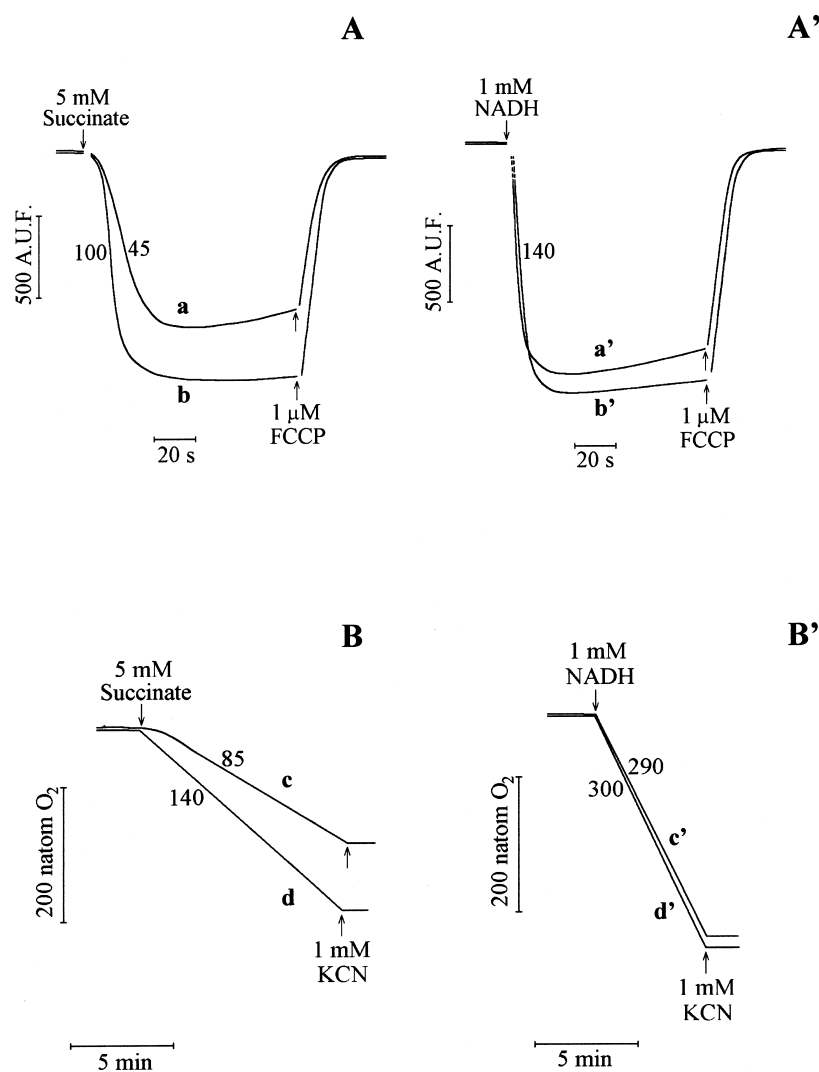

Fig. 1. Effect of xanthine plus xanthine oxidase on the rate of both $\Delta\Psi$ generation and oxygen uptake in succinate and NADH respiring mitochondria. A,A′: Measurements of mitochondrial $\Delta\Psi$ changes were carried out as reported in Section 2 by monitoring safranin 'O' fluorescence. Traces b and b′: DWM were suspended in the reaction medium, incubated 5 min, then 5 mM succinate or 1 mM NADH was added, with safranin 'O' fluorescence continuously monitored; finally, 1 µM FCCP was added. In traces a and a′ the reaction medium also contained the superoxide anion producing system xanthine plus xanthine oxidase, incubation time 5 min. The numbers alongside the curves indicate the rates of $\Delta\Psi$ generation (see Section 2). B,B′: Oxygen uptake measurements were carried out as reported in Section 2. DMW were either incubated 5 min in the medium, then succinate (trace d) or NADH (trace d′) was added, or incubated 5 min with xanthine plus xanthine oxidase, then succinate (trace c) or NADH (trace c′) was added. Then, at the arrows 1 mM cyanide was added. The numbers alongside the traces indicate the rates of oxygen uptake expressed as natom O/min/mg/ of protein.
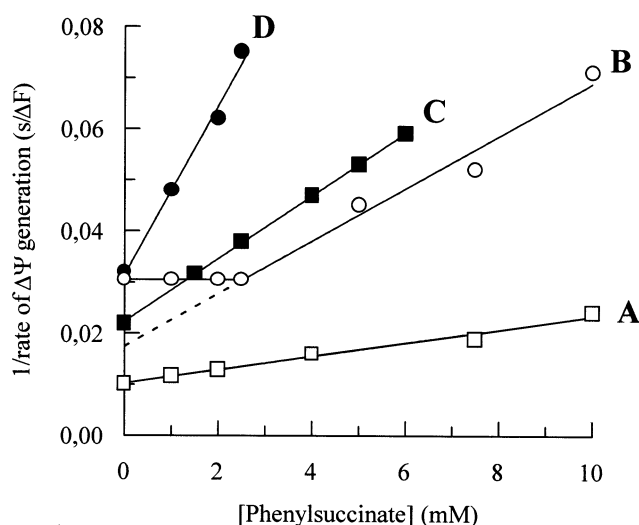
Fig. 2. Determination of the limiting step of the rate of $\Delta\Psi$ generation. The experiments were carried out as reported in Fig. 1A; phenylsuccinate was added, at the reported concentrations, 1 min before succinate (A). In B–D the reaction medium also contained: 4 ng antimycin A, added 1 min before succinate (B), or the superoxide anion producing system xanthine plus xanthine oxidase incubated 5 min (C), or xanthine plus xanthine oxidase plus antimycin A (D). The rates of $\Delta\Psi$ generation due to succinate addition to mitochondria are reported as Dixon plots.

the decrease of safranin fluorescence (traces b and b′, respectively). $\Delta\Psi$ was completely and rapidly collapsed by adding the uncoupler FCCP. When DWM were incubated for 5 min with xanthine plus xanthine oxidase, about 55% inhibition (trace a) and no inhibition (trace a′) of the rate of $\Delta\Psi$ generation caused by succinate and NADH, respectively, were found. Consistently, succinate-dependent oxygen uptake was found to be inhibited by 5 min incubation with xanthine plus xanthine oxidase (compare traces c and d), while no significant inhibition was observed in the case of NADH (traces c′ and d′); externally added cyanide was found to block oxygen uptake, showing the lack of the alternative oxidase pathway under our experimental conditions (see also [14,19]).

Incubation of DWM with either xanthine or xanthine oxidase alone gave no significant inhibition of the succinate-dependent and NADH-dependent $\Delta\Psi$ generation. The xanthine plus xanthine oxidase-dependent inhibition observed in succinate oxidising DWM was largely prevented by superoxide dismutase plus catalase (not shown), thus indicating that the inhibition involves superoxide anion/hydrogen peroxide production.

Since succinate and external NADH differ from each other with respect to the localisation of their dehydrogenases, located in the outer and in the inner face of the mitochondrial inner membrane, respectively [7], we investigated whether ROS production can affect the mitochondrial permeability to succinate.

In order to determine whether the rate of the succinate oxidation-supported $\Delta\Psi$ generation reflects the rate of succinate uptake and whether the inhibition found in Fig. 1A was dependent on the succinate uptake by mitochondria, the control strength criterion was applied as in [20] using phenylsuccinate, which inhibits the succinate transport in plant mitochondria, but cannot enter mitochondria [21] (Fig. 2). Thus, the rate of $\Delta\Psi$ generation was investigated as a function of increasing

phenylsuccinate concentrations in control DWM (line A). The intercept to the Y-axis of the line fitting the experimental points determined in the presence of phenylsuccinate proved to coincide with the experimental point measured at zero phenylsuccinate concentration: this shows that the rate of $\Delta\Psi$ increase mirrors the rate of succinate transport across the mitochondrial membrane (see [22]). As a control, in the same experiment, we partially inhibited electron flow via the respiratory chain by using 4 ng antimycin A (line B); as expected, no inhibition by phenylsuccinate up to about 2 mM was found, thus showing that in this case the electron flow limits the rate of $\Delta\Psi$ increase. Interestingly, under this condition, the maximal rate of succinate transport, as obtained by extrapolation of the line to the y-axis (dashed line), was found to be lower than in A.

When DWM were treated with xanthine plus xanthine oxidase (5 min incubation), either in the absence (line C) or in the presence of 4 ng antimycin A (line D), a significant inhibition was found at any investigated phenylsuccinate concentration, moreover, in both cases, the y-axis intercepts show that the transport of succinate is the rate limiting step of the rate of $\Delta\Psi$ generation. This result shows that succinate transport is the process inhibited by ROS.

The dependence of the rate of succinate uptake on increasing substrate concentrations was investigated either in the absence or in the presence of xanthine plus xanthine oxidase (5 and 10 min incubation) and plotted as double reciprocal plots (Fig. 3A). Saturation characteristics were found in control DWM, with K_m and V_{max} values equal to 0.23 mM and 105 ΔF/s, respectively; the rate of succinate-dependent $\Delta\Psi$ generation was inhibited by xanthine plus xanthine oxidase treatment in agreement with a mixed inhibition, with increased K_m and decreased V_{max} values with respect to control DWM: K_m and V_{max} values were 0.52 mM and 67 ΔF/s, 0.71 mM and 48 ΔF/s, in DWM exposed for 5 and 10 min to xanthine plus xanthine oxidase, respectively. As a control, in the same experiment, phenylsuccinate was found to inhibit succinate uptake in a competitive manner (K_i about 0.63 mM) (Fig. 3B). The mixed inhibition exerted by xanthine plus xanthine oxidase was confirmed by plotting the data according to Dixon, Eadie–Hofstee and Hanes plots and as a single-curve plot $[I](1-i)/i$ vs. $[S]$, where i is equal to $(v-v_i)/v$ and v and v_i are the rates of the uninhibited and inhibited reactions. In agreement with the nature of inhibition, showing a notable competitive component, no inhibition of mitochondrial swelling due to xanthine plus xanthine oxidase was observed in high concentration succinate medium (not shown).

The possible involvement of mitochondrial –SH groups in the xanthine plus xanthine oxidase-dependent inhibition was investigated (Fig. 4). The rate of $\Delta\Psi$ generation in the presence of mersalyl (0.3 mM), a thiol reagent that cannot enter mitochondria (trace e), was equal to 30% compared to the control (trace a). The mersalyl inhibition was completely prevented (not shown) and removed by cysteine (trace b), which per se caused no significant change in the rate of $\Delta\Psi$ generation (trace c). In the same experiment, incubation of DWM for 5 min with xanthine plus xanthine oxidase induced an about 50% decrease in the rate of $\Delta\Psi$ generation (trace d). When mersalyl plus xanthine plus xanthine oxidase were added, an about complete inhibition was found, with a partial removal due to cysteine (trace f), showing that mersalyl cannot prevent ROS inhibition. NEM (0.1 mM) was also used as
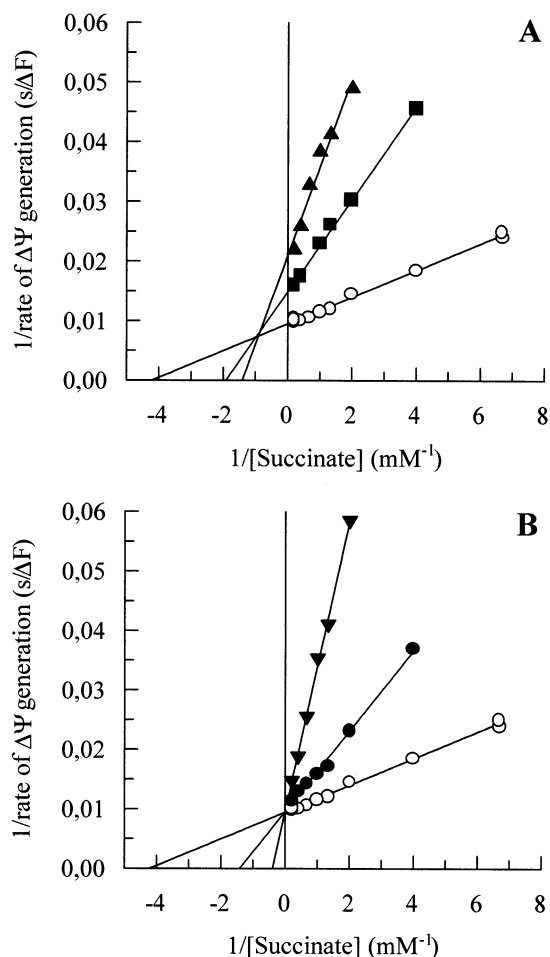
Fig. 3. Nature of inhibition by phenylsuccinate and xanthine plus xanthine oxidase of the rate of succinate transport. The experiments were carried out as reported in Fig. 1A. In control experiments (○) the reactions were started by adding DWM with succinate at the reported concentrations, with continuous measurement of safranin 'O' fluorescence made as a function of time. In other experiments the reaction medium also contained (A) xanthine plus xanthine oxidase incubated either 5 min (■) or 10 min (▲) or (B) phenylsuccinate either 1 mM (●) or 4 mM (▼). The rates of $\Delta\Psi$ generation are reported according to double reciprocal plots.

a lipophilic thiol reagent, which can penetrate mitochondrial membranes, with no prevention of ROS effect (not shown).

4. Discussion

In a model system properly developed, we show that ROS can decrease the rate of $\Delta\Psi$ generation supported by succinate oxidation. The inhibition found derives from ROS generated by xanthine plus xanthine oxidase, it is prevented by externally added superoxide dismutase and catalase; moreover, $\Delta\Psi$ generation impairment depends on the substrate uptake inhibition, as demonstrated by the failure of the early ROS production to inhibit NADH oxidation, thus showing that no effect occurs on the common electron carriers of the respiratory chain, and by the control strength experiment, in which xanthine plus xanthine oxidase can inhibit the same process inhibited by phenylsuccinate, i.e. succinate uptake by mitochondria. We do not show how succinate uptake occurs, nonetheless investigation as in [21] by monitoring the succi-

nate/malate exchange showed in several experiments a 35–70% xanthine plus xanthine oxidase-dependent inhibition (not shown), thus suggesting the involvement of the dicarboxylate carrier [23] in the ROS–mitochondria interaction.

We suggest that the inhibition by ROS of metabolite carriers devoted to fill up the mitochondrial pool of the respiratory substrates could be a feedback-like mechanism by which mitochondria preserve themselves under oxidative stress. Indeed, under stress conditions mitochondrial ROS generation increases, with some direct superoxide anion generation expected towards the intermembrane space [24], thus suggesting possible defence mechanisms involving mitochondrial components facing the outer face of the inner membrane. At present how ROS impair $\Delta\Psi$ generation remains to be established. ROS inhibition was found to be not dependent by ROS–mitochondrial thiol interaction, differently from the ADP/ATP carrier [25], as suggested by the lack of thiol reagent protection. Since ROS were found to inhibit succinate uptake according to a mixed inhibition, we suggest that the carrier impairment could depend on the ROS interaction with a variety of mitochondrial compounds including protein carrier domain(s) and membrane lipids close to the carrier. Consistently, an impairment of inner mitochondrial membrane by hydrogen peroxide due to activation of phospholipid degradative enzymes was found in plant mitochondria [8] and ROS proved to cause changes in cardiolipin content in the mitochondrial inner membrane [26,27].

The conclusion that mitochondrial inner membrane proteins are favourite targets of ROS is not unique. In rat liver mitochondria singlet oxygen was suggested to impair several carriers [25] and inhibition of the phosphate carrier was found
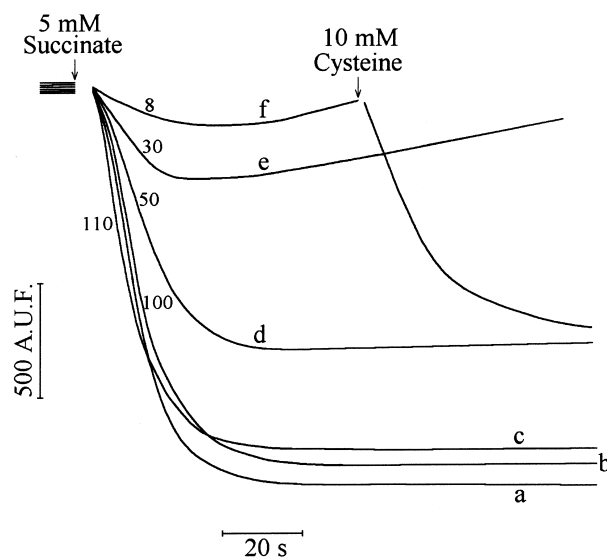

Fig. 4. Non-prevention by mersalyl of xanthine plus xanthine oxidase inhibition. The experiments were carried out as reported in Fig. 1A. DWM were suspended in the reaction medium without any addition (a) or with: 0.3 mM mersalyl plus 10 mM cysteine (b), 10 mM cysteine (c), xanthine plus xanthine oxidase incubated for 5 min (d), 0.3 mM mersalyl (trace e), 0.3 mM mersalyl plus xanthine plus xanthine oxidase (f). At the arrow 10 mM cysteine was added. The time of additions to the reaction medium was: mitochondria, after 1 min mersalyl, after 1 min xanthine plus xanthine oxidase, after 5 min cysteine and after 10 s succinate. The incubation times were also observed when mersalyl, xanthine plus xanthine oxidase or cysteine were not added. The numbers alongside the curves represent the rates of $\Delta\Psi$ generation.

to be due to the AZT (3′-azido-3′-deoxythymidine)-induced extra ROS production (Valenti et al., submitted); in myocardial mitochondria superoxide anion and hydroxyl radical damage Ca^{2+} uptake, with the transport more sensitive than are oxidative phosphorylation and respiratory chain [28]; in mitochondria from the flight muscles of the housefly, hydroxyl radical causes a severe loss of functional activity of the ADP/ATP translocator [29]. Moreover, ROS–membrane protein interaction can regulate mitochondrial functions. ROS induce opening of the permeability transition pores causing mitoptosis of ROS overproducing organelles ([30] and references therein). In DWM, the activation of both $PmitoK_{ATP}$ and PUMP, which can lower mitochondrial ROS generation [12,13,31], was found in the early phase of ROS production [12,13] suggesting the existence of a feedback mechanism to defend mitochondria against oxidative stress according to a mild uncoupling mechanism [32].

In conclusion, in the light of the carrier protein localisation and reactivity, we propose that the impairment of the mitochondrial translocators by ROS, together with the ROS-dependent $PmitoK_{ATP}$ and PUMP activation, may contribute to the plant defence mechanism by decreasing energy metabolism and consequent large-scale ROS production by mitochondria during oxidative stress in plants.

References

[1] Papa, S. and Skulachev, V.P. (1997) Mol. Cell. Biochem. 174, 305–319.

[2] Kowaltowski, A.J. and Vercesi, A.E. (1999) Free Radic. Biol. Med. 26, 463–471.

[3] Cardoso, S.M., Pereira, C. and Oliveira, C.R. (1999) Free Radic. Biol. Med. 26, 3–13.

[4] Atlante, A., Calissano, P., Bobba, A., Giannattasio, S., Marra, E. and Passarella, S. (2001) FEBS Lett. 497, 1–5.

[5] Scandalios, J.G. (1993) Plant Physiol. 101, 7–12.

[6] Alscher, R.G., Donahue, J.L. and Cramer, C.L. (1997) Physiol. Plant. 100, 224–233.

[7] Möller, I.M. (2001) Annu. Rev. Plant Physiol. Plant Mol. Biol. 52, 561–591.

[8] Kwon, H.J., Lee, W.S. and Shin, S. (1998) Plant Cell Physiol. 39, 655–659.

[9] Casolo, V., Braidot, E., Chiandussi, E., Macrì, F. and Vianello, A. (2000) FEBS Lett. 474, 53–57.

[10] Korshunov, S.S., Skulachev, V.P. and Starkov, A.A. (1997) FEBS Lett. 416, 15–18.

[11] Korshunov, S.S., Korkina, O.V., Ruuge, E.K., Skulachev, V.P. and Starkov, A.A. (1998) FEBS Lett. 435, 215–218.

[12] Pastore, D., Stoppelli, M.C., Di Fonzo, N. and Passarella, S. (1999) J. Biol. Chem. 274, 26683–26690.

[13] Pastore, D., Fratianni, A., Di Pede, S. and Passarella, S. (2000) FEBS Lett. 470, 88–92.

[14] Pastore, D., Trono, D. and Passarella, S. (1999) Plant Biosyst. 133, 219–228.

[15] Moore, A.L. and Proudlove, M.O. (1987) in: Purification of Plant Mitochondria on Silica Sol Gradients (Packer, L. and Douce, R., Eds.), Methods Enzymol. 148, 415–420.

[16] Harris, D.A. (1987) in: Spectrophotometry and Spectrofluorimetry: A Practical Approach (Bashford, C.L. and Harris, D.A., Eds.), pp. 59–61, IRL Press, Oxford.

[17] Wippich, N., Peschke, D., Peschke, E., Holtz, J. and Bromme, H.J. (2001) Int. J. Mol. Med. 7, 211–216.

[18] Moore, A.L. and Bonner Jr., W.D. (1982) Plant Physiol. 70, 1271–1276.

[19] Pastore, D., Trono, D., Laus, M.N., Di Fonzo, N. and Passarella, S. (2001) Plant Cell Physiol. 42, 1373–1382.

[20] Valenti, D., Barile, M., Quagliariello, E. and Passarella, S. (1999) FEBS Lett. 444, 291–295.

[21] Fratianni, A., Pastore, D., Pallotta, M.L., Chiatante, D. and Passarella, S. (2001) Biosci. Rep. 21, 81–91.

[22] La Noue, K.F. and Schoolwerth, A.C. (1984) in: Metabolite Transport in Mammalian Mitochondria (Ernster, L., Ed.), Bioenergetics, pp. 221–226, Elsevier, Amsterdam.

[23] Laloi, M. (1999) Cell. Mol. Life Sci. 56, 918–944.

[24] Han, D., Williams, E. and Cadenas, E. (2001) Biochem. J. 353, 411–416.

[25] Atlante, A., Passarella, S., Moreno, G. and Salet, C. (1992) Photochem. Photobiol. 56, 471–478.

[26] Paradies, G., Petrosillo, G., Pistolese, M., Di Venosa, N., Serena, D. and Ruggiero, F.M. (1999) Free Radic. Biol. Med. 27, 42–50.

[27] Paradies, G., Ruggiero, F.M., Petrosillo, G. and Quagliariello, E. (1997) Biochim. Biophys. Acta 1362, 193–200.

[28] Yang, Z. and Yang, F. (1997) Biosci. Rep. 17, 557–567.

[29] Yan, L.J. and Sohal, R.S. (1998) Proc. Natl. Acad. Sci. USA 95, 12896–12901.

[30] Skulachev, V.P. (2000) IUBMB Life 49, 365–373.

[31] Kowaltowski, A.J., Costa, A.D.T. and Vercesi, A.E. (1998) FEBS Lett. 425, 213–216.

[32] Skulachev, V.P. (1997) Biosci. Rep. 17, 347–366.