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Lipid Peroxidation Induced by NAD(P)H and NAD$^+$-Dependent Substrates in Soybean Mitochondria

Angelo Vianello[1], Francesco Macrì[1] and Alberto Bindoli[2]

[1] *Section of Plant Physiology and Biochemistry, Institute of Plant Protection, University of Udine, I-33100 Udine, Italy*
[2] *Centre for the Study of Mitochondrial Physiology, C.N.R., I-35100 Padova and Institute of Biological Chemistry, University of Padova, I-35100 Padova, Italy*

Lipid peroxidation induced by Fe^{2+}/ADP in soybean mitochondria is stimulated by pyruvate, malate (in the presence of NAD^+) and by NAD(P)H. This lipid peroxidation is almost completely inhibited by EDTA indicating that iron is essential. Also salicylhydroxamic acid, a specific inhibitor of the alternate oxidase, is a strong inhibitor of lipid peroxidation but its effect should be related to chelation or to a general antioxidant action. Rotenone does not show any effect on the malate/NAD^+/Fe^{2+}/ADP-induced lipid peroxidation. From the reported data, it is possible to conclude that, in soybean mitochondria, the peroxidation of unsaturated lipids can be modulated through a balance between the systems sparking lipid peroxidation, like NAD(P)H/Fe^{2+}/ADP, and the systems which protect against it, i.e. quinones, maintained at the reduced state by the substrates.

Key words: Flavin dehydrogenase — *Glycine max* (L.) Merril — Lipid peroxidation — Malondialdehyde — Mitochondria — Soybean.

Free radical reactions are responsible of many physiological and pathological conditions of plant tissues, e.g. senescence, ethylene synthesis, wounding and herbicide effects (Elstner 1982, Harman and Mattick 1976, Konze and Elstner 1978, Thompson et al. 1987). The oxidative degradation of unsaturated fatty acids leading to the damage of biological membranes is one of the major effects of free radical reactions.

It has long been known that NADPH, in the presence of Fe^{2+}/ADP, can induce lipid peroxidation in rat liver microsomes (Hochstein and Ernster 1963). More recently, it has been shown that rat heart submitochondrial particles (Takayanagi et al. 1980), lutoidic tonoplast from latex of *Hevea brasiliensis* (Chrestin et al. 1984) and soybean mitochondria and microsomes (Vianello et al. 1986) can undergo lipid peroxidation in the presence of NADH or NADPH and Fe^{3+}/ADP.

In all cases, lipid peroxidation is referred to the continuous reduction of Fe^{3+}/ADP to Fe^{2+}/ADP; iron is then reoxidized in the peroxidative process.

In the present paper we have studied the conditions in which two NAD^+-dependent substrates (malate and pyruvate) and NAD(P)H can induce lipid peroxidation in soybean mitochondria. Iron ions seem essential for the peroxidative process which is mediated by NAD(P)H oxidized either through the internal or the external flavin dehydrogenases.

Abbreviations: BSA, bovine serum albumin; EGTA, ethylene-bis(oxyethylenenitrile)tetraacetic acid; DTT, dithiothreitol; MDA, malondialdehyde; SHAM, salicylhydroxamic acid.

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Materials and Methods

Soybean seeds (*Glycine max* (L.) Merril, cv Asgrow A3127) were germinated on moisted sheet of paper at 30°C for 3 days.

Fifty grams of seedlings were rinsed in distilled water, ground in an ice-cold mortar with 180 ml of 20 mM HEPES-Tris (pH 7.6), 5 mM $MgCl_2$, 0.4 M mannitol, 1 mM DTT, 1 mM NaEDTA, 0.1% BSA and then filtered through eight layers of gauze. The filtrate was centrifuged at 28,000 × *g* for 5 min. The pellet was resuspended in half of the initial volume of the grinding medium and centrifuged at 2,500 × *g* for 3 min. The supernatant was finally centrifuged at 28,000 × *g* for 5 min. The mitochondrial pellet was resuspended in approx. 3 ml of 20 mM HEPES-Tris (pH 7.5), 0.125 M KCl.

Proteins were determined by the biuret method (Gornall et al. 1949) using BSA as a standard.

Oxygen uptake was measured with a Clark-type oxygen electrode (Estabrook 1967).

Malondialdehyde was assayed by the thiobarbituric acid method as described by Buege and Aust (1978).

Results

As reported in Table 1 the addition of L-malate to soybean mitochondria, in the presence of NAD^+, slightly increases lipid peroxidation measured as MDA formation; malate or NAD^+ alone are without effect. Fe^{2+}/ADP, instead, greatly increase lipid peroxidation and, when added to the malate/NAD^+ system act synergistically since MDA production is stimulated three times. The chelating agent EDTA inhibits almost completely both the malate/NAD^+- and malate/NAD^+/Fe^{2+}/ADP-stimulated lipid peroxidation. This indicates that ions such as iron are essential for the induction of lipid peroxidation. On the other hand, when malate, without NAD^+, is added to the Fe^{2+}/ADP-inducing lipid peroxidation system, it does prevent lipid peroxidation probably by reducing quinones (Vianello et al. 1986), known as powerful antioxidants

Table 1 Effect of malate on malondialdehyde formation in soybean mitochondria

Additions	Malondialdehyde formation (nmol/mg protein)	%
None	1.22±0.10 (6)	100
Malate	1.13±0.20 (6)	92
NAD^+	1.12±0.16 (6)	92
Malate+NAD^+	1.43±0.15 (6)	117
Malate+NAD^++EDTA	1.26±0.23 (3)	103
Fe^{2+}+ADP	2.40±0.22 (9)	196
Malate+Fe^{2+}/ADP	1.96±0.18 (3)	161
NAD^++Fe^{2+}/ADP	2.32±0.26 (3)	190
Malate+NAD^++Fe^{2+}/ADP	3.65±0.37 (4)	299
Malate+NAD^++Fe^{2+}/ADP+EDTA	1.34±0.30 (4)	110

Soybean mitochondria (1 mg protein/ml) were incubated for 30 min at 30°C in 0.125 M KCl, 20 mM HEPES-Tris (pH 7.6) and 1 mM KCN. Other additions were: 10 mM malate, 0.5 mM NAD^+, 1 mM EDTA, 10 μM $FeSO_4$, 0.1 mM ADP. Values are mean±S.D. and figures in brackets show the number of estimations. The per cent values with respect to the control are also reported.

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(Mellors and Tappel 1966). The protective effect of malate in the absence of NAD^+ on the Fe^{2+}/ADP system was studied in the presence of increasing concentrations of Fe^{2+}/ADP. As reported in Fig. 1, malate is able to protect at all the tested concentrations of Fe^{2+}/ADP and the protective effect is proportionally greater as lipid peroxidation increases, because of the higher concentrations of iron.

Like malate, also pyruvate stimulates lipid peroxidation, but some differences are apparent (Table 2). First of all, pyruvate gives rise to a lipid peroxidation in which the addition of NAD^+ does not appear essential, even if inducing a slight increase of peroxidation. Moreover, pyruvate stimulates, rather than inhibit, the lipid peroxidation induced by Fe^{2+}/ADP both in the absence and, to a greater extent, in the presence of NAD^+. Succinate, used as a reducing agent, induces no stimulation while it inhibits the Fe^{2+}/ADP-stimulated lipid peroxidation (Table 2).

Since the lipid peroxidation stimulated by malate and pyruvate appears to be dependent on the reduction of NAD^+, we tried the effect of NAD(P)H on the Fe^{2+}/ADP-induced lipid peroxidation in soybean mitochondria (Table 3). Both NADPH and NADH stimulate lipid peroxidation, the latter being much more effective; EGTA, a specific Ca^{2+} ion-chelating agent,

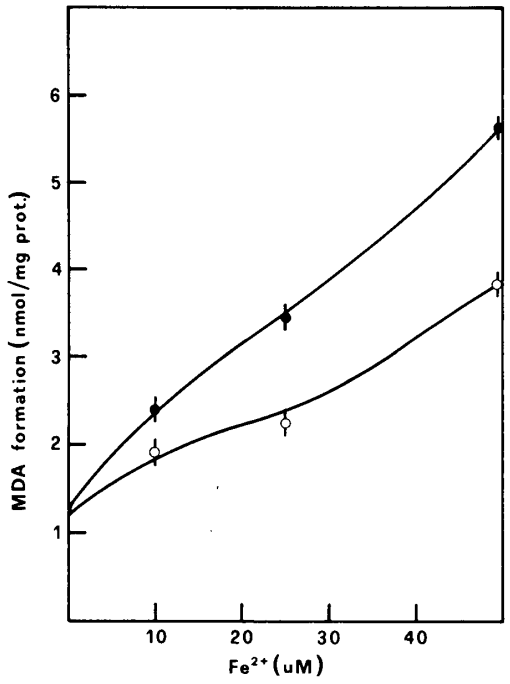

Fig. 1

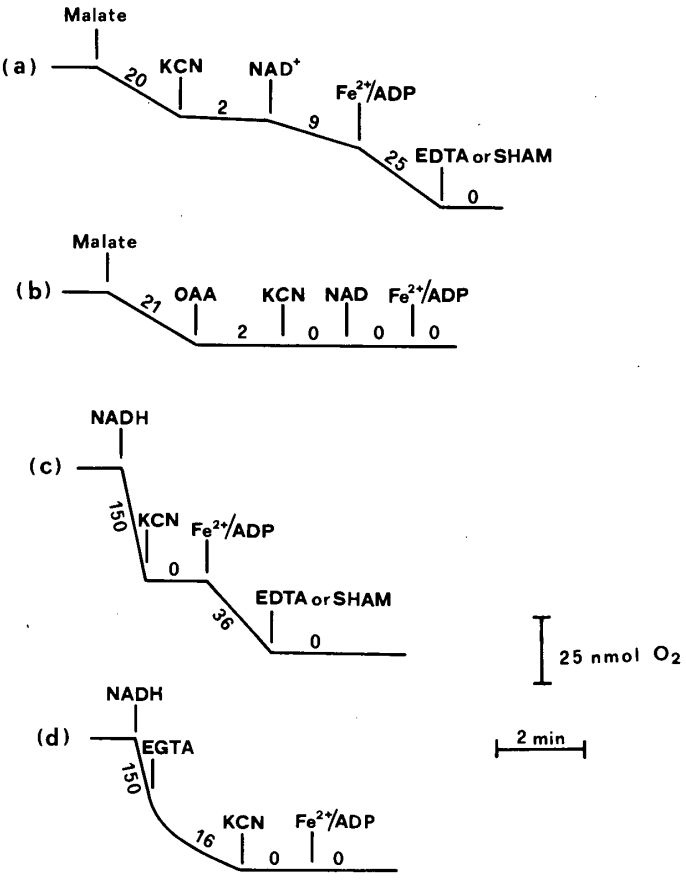

Fig. 2

Fig. 1 Protective effect of malate and malondialdehyde formation induced by increasing concentrations of Fe^{2+}/ADP. Conditions were as in Table 1. The concentration of ADP was ten times that of $FeSO_4$. Each value represents the mean±S.D. of four experiments. (●) Control; (○) 10 mM malate.

Fig. 2 Oxygen uptake by soybean mitochondria. Soybean mitochondria (1 mg/ml) were incubated at room temperature in 20 mM HEPES-Tris (pH 7.6), 0.125 M KCl. Concentrations of the substrates added were: 10 mM malate, 1 mM KCN, 0.5 mM NAD^+, 10 μM $FeSO_4$, 0.1 mM ADP, 1 mM EDTA, 2 mM oxaloacetate, 2 mM SHAM and 2 mM EGTA. Figures by each trace are expressed as nmol O_2/min × mg protein.

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Table 2 Effect of pyruvate and succinate on malondialdehyde formation in soybean mitochondria

Additions	Malondialdehyde formation (nmol/mg protein)	%
None	1.13±0.06 (7)	100
Pyruvate	2.51±0.13 (3)	222
Pyruvate+NAD^+	2.66±0.18 (3)	235
Fe^{2+}/ADP	2.26±0.22 (9)	200
Pyruvate+Fe^{2+}/ADP	3.23±0.24 (3)	286
Pyruvate+NAD^++Fe^{2+}/ADP	3.95±0.30 (3)	350
Succinate	1.20±0.05 (4)	106
Succinate+Fe^{2+}/ADP	1.81±0.23 (4)	160

Conditions were as in Table 1; other additions were: 10 mM pyruvate, 10 μM $FeSO_4$, 0.1 mM ADP, 0.5 mM NAD^+, 10 mM succinate. Values are mean±S.D. and figures in brackets show the number of estimations. The per cent value with respect to the control are also reported.

strongly inhibits lipid peroxidation elicited by NADH, but it is ineffective in the Fe^{2+}/ascorbate system (Table 4), known for inducing a lipid peroxidation independent of the activity of any enzyme.

In several plant mitochondria, at variance with mitochondria prepared from animal sources, the reducing equivalents delivered by the various substrates can follow either the respiratory chain pathway or the alternate oxidase pathway; the latter is well known to be cyanide insensitive. In Table 4 the effect of some specific inhibitors of both pathways and of EGTA on MDA formation is reported. MDA production was stimulated both with the malate+NAD^++Fe^{2+}/ADP system and with the Fe^{2+}/ascorbate system. Oxaloacetate, which competitively inhibits the malate dehydrogenase activity, also inhibits lipid peroxidation, while rotenone, which simply blocks the electron flux to ubiquinone, has no effect indicating that the complete electron flux through the respiratory chain is not needed for this type of lipid peroxidation. Salicylhydroxamic acid, the classical inhibitor of the alternate oxidase pathway, strongly inhibits the MDA formation; its effect should be referred both to a chelating action towards iron ions, since it inhibits the Fe^{2+}/ascorbate-

Table 3 Effect of NADH and NADPH on malondialdehyde formation in soybean mitochondria

Additions	Malondialdehyde formation (nmol/mg protein)	%
None	1.18±0.10 (6)	100
NADH	1.66±0.11 (6)	140
NADPH	1.20±0.30 (3)	102
Fe^{2+}/ADP	2.40±0.22 (9)	203
NADH+Fe^{2+}/ADP	3.71±0.40 (6)	314
NADPH+Fe^{2+}/ADP	2.84±0.32 (3)	240
NADH+Fe^{2+}/ADP+EGTA	1.80±0.25 (3)	152

The conditions were as in Table 1. Other additions were: 1 mM NAD(P)H, 10 μM $FeSO_4$, 0.1 mM ADP, 1 mM EGTA, Values are mean±S.D. and figures in brackets show the number of estimations. The per cent values with respect to the control are also reported.

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Table 4 Effect of different inhibitors on malate+NAD^++Fe^{2+}/ADP-induced and Fe^{2+}/ascorbate-induced malondialdehyde formation

Additions	Malondialdehyde formation (nmol/mg protein)			
	Malate+NAD^++Fe^{2+}/ADP	%	Ascorbate/Fe^{2+}	%
None	3.69±0.32 (6)	100	10.64±0.80 (6)	100
Oxaloacetate	1.82±0.11 (3)	49	—	—
Rotenone	3.55±0.13 (3)	96	—	—
SHAM	0.97±0.07 (3)	26	1.02±0.09 (3)	10
EGTA	2.09±0.19 (3)	56	10.78±0.35 (3)	101

Conditions were as in Table 1; other additions were: 10 mM malate, 0.5 mM NAD^+, 10 μM $FeSO_4$ (in the malate system), 30 μM $FeSO_4$ (in the ascorbate system), 0.1 mM ADP, 2 mM oxaloacetate, 8 μg/mg protein rotenone, 2 mM SHAM, 2 mM EGTA and 0.1 mM ascorbate. Values are mean±S.D. and figures in brackets show the number of estimations. The per cent values with respect to the control are also reported.

induced lipid peroxidation, and to a direct antioxidant activity.

The effect of malate on oxygen uptake was followed with an oxygen electrode. As reported in Fig. 2a, KCN almost completely inhibits the respiratory oxidation of malate; if NAD^+ is added, a substantial stimulation of oxygen uptake is apparent and this is greatly increased by Fe^{2+}/ADP addition; the addition of EDTA, on the contrary, abruptly stops the consumption of oxygen. It is interesting to note that the oxidation of malate alone is not accompanied by MDA production (Table 1), so it simply reflects the respiratory consumption, while the addition of NAD^+ and Fe^{2+}/ADP increases MDA formation (Table 1) indicating that this oxygen uptake is of the peroxidative type. In Fig. 2b the addition of oxaloacetate almost completely inhibits the oxidation of malate, since it competitively blocks the activity of malate dehydrogenase. The subsequent addition of NAD^+ or of Fe^{2+}/ADP gives rise to no further oxygen uptake.

NADH is oxidized at an high rate by plant mitochondria (Fig. 2c) and KCN almost completely inhibits this, indicating that the alternate oxidase has a very low activity in this type of mitochondria. The addition of Fe^{2+}/ADP to this system strongly stimulates the oxygen uptake and MDA production (Table 3) which is, once again, inhibited by EDTA. EGTA is able to inhibit the activity of the external flavin dehydrogenase (Møller and Lin 1986); as reported in Fig. 2d, EGTA slows down the oxidation of NADH which is further inhibited by KCN. The subsequent additon of Fe^{2+}/ADP does not elicit any stimulation of lipid peroxidation.

Discussion

In soybean mitochondria the Fe^{2+}/ADP-dependent lipid peroxidation elicited by the NAD^+-dependent substrates and NAD(P)H appears to be strictly dependent on the presence of iron ions, since EDTA completely prevents MDA formation. The action of malate and pyruvate appears to be mediated by the reduction of NAD^+; NADH by itself is in fact sufficient to stimulate the Fe^{2+}/ADP-induced lipid peroxidation. Similarly to NADH, NADPH is able to spark lipid peroxidation, but to a lower extent.

The oxidation of malate and pyruvate to produce NADH may be carried out by some enzymes. Three are located in the mitochondrial matrix (malate dehydrogenase, malic enzyme and pyruvate dehydrogenase), while another (malate dehydrogenase) is outside the inner membrane (Palmer 1980). The different response of malate with respect to pyruvate in stimulating lipid peroxidation, may be related to the fact that the equilibrium of malate dehydrogenase strongly disfavors product formation (Møller and Lin 1986). The addition of

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NAD^+, which enters the mitochondrial matrix (Neuburger and Douce 1978), might shift the equilibrium of the internal and/or external malate dehydrogenase toward NADH formation. The oxidation of succinate does not produce NADH and, therefore, is unable to trigger lipoperoxidation.

Plant mitochondria contain, in the inner membrane, three different NAD(P)H-dependent dehydrogenases (Møller and Lin 1986); two located on the inner face and the other associated with the outer face. The exogenous dehydrogenase is not specific for NADH but also can oxidize NADPH. Besides their main function of transporting electrons to the terminal oxidase, these dehydrogenases, after interaction with added NAD(P)H or with NADH produced by the oxidation of malate or pyruvate, can transfer electrons either to oxygen, which is transformed to the superoxide anion (Rustin et al. 1983), or to the iron/ADP complex, which, in this way, is maintained at a reduced state. It is well known that reduced ion can both initiate and propagate the chain reaction of lipid peroxidation (Svingen et al. 1979).

Recently, Rustin et al. (1983, 1984) proposed a new hypothesis to explain the mechanism of cyanide resistance in plant mitochondria. They claimed that the alternate oxidase does not require a terminal oxidase, does not utilize unsaturated fatty acids and depends on the couple lipid radical/lipid hydroperoxyl radical which cyclically oxidizes ubiquinol to ubiquinone; the latter is then continuously reduced by the respiratory substrates. In soybean mitochondria the alternate oxidase activity, measured as an oxygen uptake in the presence of KCN, is very low. In this condition the NAD^+-dependent substrates or NAD(P)H can induce lipid peroxidation, measured as a time-increasing production of MDA, which indicates that unsaturated fatty acids are continuously utilized for the peroxidative process. It is thus possible to conclude that in soybean mitochondria and, probably, in all types of mitochondria, the peroxidation of unsaturated fatty acids can be modulated through a balance between a peroxidation-inducing and a peroxidation-preventing system. The former reduces iron-ADP by the reduced flavoprotein (Table 1-3) and the latter depends on the substrate-bound reduction of quinones (Vianello et al. 1986) known to be extremely powerful antioxidants (Mellors and Tappel 1966). The availability of iron ions or of other metals is crucial in shifting the balance towards lipid peroxidation (Table 1).

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