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NAD(P)H oxidation elicits anion superoxide formation in radish plasmalemma vesicles

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Radish plasmalemma-enriched fractions show an NAD(P)H-ferricyanide or NAD(P)H-cytochrome *c* oxidoreductase activity which is not influenced by pH in the 4.5–7.5 range. In addition, at pH 4.5–5.0, NAD(P)H elicits an oxygen consumption (NAD(P)H oxidation) inhibited by catalase or superoxide dismutase (SOD), added either before or after NAD(P)H addition. Ferrous ions stimulate NAD(P)H oxidation, which is again inhibited by SOD and catalase. Hydrogen peroxide does not stimulate NADH oxidation, while it does stimulate Fe^{2+}-induced NADH oxidation. NADH oxidation is unaffected by salicylhydroxamic acid and Mn^{2+}, is stimulated by ferulic acid, and inhibited by KCN, EDTA and ascorbic acid. Moreover, NADH induces the conversion of epinephrine to adrenochrome, indicating that anion superoxide is formed during its oxidation. These results provide evidence that radish plasma membranes contain an NAD(P)H-ferricyanide or cytochrome *c* oxidoreductase and an NAD(P)H oxidase, active only at pH 4.5–5.0, able to induce the formation of anion superoxide, that is then converted to hydrogen peroxide. Ferrous ions, sparking a Fenton reaction, would stimulate NAD(P)H oxidation.

Introduction

Increasing evidence suggests that redox activities bound to plasma membranes may be related to some important physiological processes [1–5]. NAD(P)H, used as a source of reducing equivalents, can react with oxidoreductases localized on both sides of the plasmalemma. Such a reduction is involved in the uptake of Fe^{3+} by iron-deficient dicotyledonous plants (Turbo reductase): electrons originating from the cytoplasmic NAD(P)H reduce extracellular ferric salts, which may then be taken up by plant cells [6], it has also been proposed that NAD(P)H oxidoreduction generates a transplasmalemma proton gradient [7–12] which would drive uptake of solutes. There is no conclusive evidence supporting the latter possibility [13–15] while, according to an alternative interpretation, the redox chain, which exports electrons from cellular NAD(P)H to ferricyanide, causes a drop in the intracellular pH which would be regulated by the activation of the ATP-dependent proton pump of plasmalemma [16–18]. Recently, it has been shown that NADH oxidation by soybean plasma membranes is stimulated by auxins and, therefore, it has been suggested that it may play a role in growth processes [19].

In plants, the components of the redox chains responsible for NAD(P)H oxidoreductase activities of the plasmalemma are partially known. Cytochromes of the *b*-type, flavoproteins, quinones and cytochromes *P*-450/*P*-420 have been described, but it is not known which function in oxidoreductions [20–26]. In particular, cytochromes of the *b*-type appear to be related to blue-light-dependent processes [5].

The involvement of NAD(P)H oxidation in the generation of activated oxygen species and H_2O_2 has been established in different systems. An NAD(P)H oxidase that generates species of oxygen radicals is localized in lutoidic tonoplast [27]. In mitochondria, NAD(P)H may sustain $O_2^{\cdot-}$ formation at the level of NAD(P)H dehydrogenase(s) or ubisemiquinone [28–30]; alternatively, by the activity of a mitochondrial or microsomal cytochrome *P*-450 and mitochondrial NAD(P)H dehydro-

Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CCR, cytochrome *c* reductase; DTT, dithiothreitol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid; IDPase, inosine diphosphatase; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid; SHAM, salicylhydroxamic acid; SOD, superoxide dismutase (EC 1.15.1.1); Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

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genase(s), it can reduce Fe^{3+}/ADP to Fe^{2+}/ADP, which sparks lipid peroxidation in both animals [31] and plants [32,33]. The latter process has an absolute requirement for ionic iron, which acts both in the initiation and propagation phase of lipid oxidation [30,31]. Human neutrophils possess a plasma membrane NAD(P)H oxidase that performs an electrogenic single-electron reduction of oxygen to superoxide radical [34,35], which is an important asset to the bactericidal capacity of these cells [36]. Similarly, a plasma-membrane-bound NAD(P)H oxidase generates $O_2^{\cdot-}$ upon infection of potato tubers with incompatible races of *Phytophtora infestans* [37]. Cell-wall [38–40] and, as recently shown, plasmalemma [41–43] peroxidases are able to oxidize NADH, catalyzing the formation of anion superoxide, which then dismutases to H_2O_2. The direct involvement of superoxide radicals in extracellular NADPH-dependent ferric reduction by iron-deficient been roots has been also shown [44].

Recently, it has been shown that radish plasmalemma vesicles have an oxidoreductase able to transfer electrons across the membrane from ascorbate to ferricyanide, generating an electrical potential [45].

The objective of this work was to verify whether NAD(P)H oxidation in radish plasma membrane vesicles is related to the generation of oxygen free radicals and hydrogen peroxide. A preliminary report of this work has been published [46].

Materials and Methods

Plant material. Radish seeds (*Raphanus sativus* L., cv. Tondo Rosso Quarantino) were germinated for 24 h as described in Ref. 47.

Isolation of plasmalemma-enriched fractions. 50 g of 24-h-old radish seedlings were ground with mortar and pestle in 250 ml of 20 mM Hepes-Tris (pH 7.6)/5 mM $MgCl_2$/0.4 M mannitol/1 mM DTT/1 mM EDTA/0.1% BSA and then filtered through eight layers of gauze. The filtrate was centrifuged at $12\,000 \times g$ for 10 min. The supernatant was re-centrifuged at $80\,000 \times g$ for 30 min and the pellet (microsomal fraction) resuspended in 2 ml of 10 mM Hepes-Tris (pH 7.5)/0.4 M mannitol/1 mM DTT/0.1% BSA. The microsomes were further purified by separation on a sucrose step-gradient consisting of 9 ml each of 20/35/45% sucrose (w/v) in the latter medium minus mannitol. After centrifugation at $80\,000 \times g$ for 150 min (Beckman SW 25.1 rotor), the 35–45% sucrose interface was collected by a Pasteur pipette, diluted in 20 mM Hepes-Tris (pH 7.5)/0.4 M mannitol and re-centrifuged at $100\,000 \times g$ for 1 h. The pellet (plasmalemma-enriched fraction) was resuspended in the latter buffer and stored at −40 °C.

Oxygen consumption. NAD(P)H oxidase and NADH-duroquinone reductase were monitored as oxygen uptake by a platinum electrode of the Clark-type. The conditions to assay NADH-duroquinone reductase were as described in Ref. 25.

Absorbance measurements. NAD(P)H-ferricyanide and NAD(P)H-cytochrome *c* reductase were followed by absorbance changes at 420 and 550 nm, respectively, using a Perkin-Elmer model 554 spectrophotometer. Non-covalently bound iron was measured as changes in absorbance at 540 nm caused by Fe^{2+}-bathophenanthroline sulfonate chelate formation, following the method described by Tangerås et al. [48]. Absorbance values were converted by standard calibration curves. The formation of anion superoxide was measured as change in absorbance at 480 nm following the conversion of epinephrine to adrenochrome.

Enzyme assays. Vanadate-sensitive ATPase (marker enzyme of plasmalemma) was estimated from the release of inorganic phosphate (P_i) evaluated by the method of Cross et al. [49]. The activity of antimycin-insensitive cytochrome *c* reductase (marker enzyme of endoplasmic reticulum) was measured following the method of Lord et al. [50]. Latent IDPase (marker enzyme of Golgi vesicles) was measured as described in Ref. 51 and P_i determined as above [49]. Cytochrome *c* oxidase (marker enzyme of mitochondria) was assayed as in Ref. 52.

Protein determination. Protein was estimated by the biuret method described by Gornall et al. [53].

Chemicals. NADH, SHAM, ferulic acid, *p*-coumaric acid, NEM, duroquinone, PCMB, PCMBS, mersalyl, oligomycin, BHT, bathophenanthroline sulfonate, FCCP and catalase were purchased from Sigma, St. Louis, MO, USA. Cytochrome *c* and epinephrine were obtained from Fluka Chemie, Buchs, Switzerland. SOD and NADH were supplied by Boehringer, Mannheim, F.R.G. and BDH Italia, Milan, Italy, respectively.

Data presentation. The experiments were repeated at least three times. Standard deviation did not exceed 10%.

Results

Microsomal vesicles from 24-h-old seedlings were enriched with the plasmalemma marker, vanadate-sensitive ATPase, and devoid of nitrate-sensitive ATPase (enzyme marker of tonoplast) [47]. In an attempt to further purify this fraction from the endoplasmic reticulum, mitochondrial fragments and Golgi vesicles, the microsomes were fractionated by a sucrose step-gradient. The high-density fraction collected (at the 35–45% sucrose interface) was enriched with vanadate-sensitive, nitrate-insensitive ATPase and contained negligible levels of antimycin-insensitive cytochrome *c* reductase (enzyme marker of the endoplasmic reticulum), latent IDPase (enzyme marker of Golgi), oligomycin-sensitive ATPase and cytochrome *c* oxidase (marker enzymes of mitochondria), when compared with

TABLE I

ATPase, cytochrome c reductase (CCR), latent IDPase and cytochrome c oxidase (CCO) activity in low- and high-density microsomes from 24-h-old seedlings

The medium for ATPase was: 10 mM Hepes-Tris (pH 7.0)/5 mM $MgSO_4$/1 mM EGTA/100 μM molybdate/50 mM KBr/2 μM nigericin and 50 μl of vesicle preparation (approx. 0.17 mg protein) in a final volume of 1 ml. The reaction was started by the addition of 3 mM MgATP and proceeded for 20 min at 37°C. CCR, latent IDPase and CCO were assayed as described in Refs. 50–52, respectively. n.d.: not detectable.

Activity	Fractions		
	microsome	low density (20–35%)	high density (35–45%)
ATPase (μmol P_i/mg protein per h)			
control	3.41	2.64	7.36
100 μM Na_3VO_4	0.71	1.92	1.60
50 mM KNO_3	3.31	2.32	7.12
4 μg/ml oligomycin	2.75	2.57	7.27
CCR (μmol/mg protein per h)	19.00	9.00	4.20
IDPase (μmol P_i/mg protein per h)	0.82	0.54	0.48
CCO (μmol/mg protein per h)	0.47	n.d.	n.d.

the activities found in microsomes and in the low-density fraction (20–35% sucrose interface) (Table I).

Fig. 1 shows that this high-density fraction exhibited an NADH-ferricyanide oxidoreduction which was insensitive to the pH of the medium in the 4.5–7.5 range. In addition, this membrane showed an oxygen consumption (NADH oxidation), but only at pH 4.5–5.0. NADH oxidase assayed at pH 5.0 was stimulated by ferrous ions and stopped by the subsequent addition of catalase or SOD. In particular, catalase caused a slight

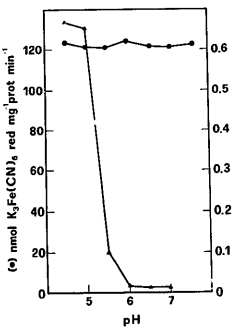

Fig. 1. NADH-ferricyanide oxidoreduction (●) or NADH oxidation (▲) as a function of the pH of the incubation mixture. The media for NADH oxidation were: 20 mM sodium acetate (pH 4.5, 5.0 and 5.5)/20 mM Mes-Tris (pH 6.0, 6.5)/20 mM Hepes-Tris (pH 7.0, 7.5), respectively, plus 0.25 M sucrose and 200 μl of membrane preparation (approx. 0.8 mg protein) in a final volume of 1.5 ml. For NADH-ferricyanide oxidoreduction, the buffers were as above plus 0.5 mM $K_3Fe(CN)_6$ and 50 μl membrane preparation (approx. 0.2 mg protein) in a final volume of 2 ml. The reactions were started by the addition of 1 mM NADH (NADH-ferricyanide oxidoreduction) or 4 mM NADH (NADH oxidation), respectively.

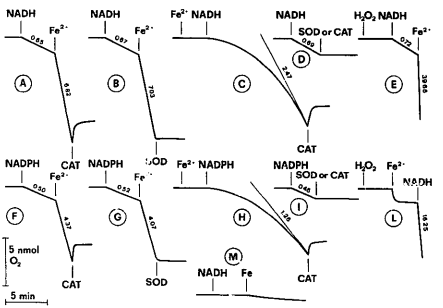

Fig. 2. NAD(P)H oxidation in radish plasma-membrane-enriched fractions. The medium was: 40 mM sodium acetate (pH 5.0)/0.25 M sucrose and 200 μl of membrane preparation (approx. 0.8 mg protein) in a final volume of 1.5 ml. Additions were 4 mM NAD(P)H, 40 μM $FeSO_4 \cdot 7\ H_2O$ or $FeCl_3 \cdot 6H_2O$, 300 IU catalase, 200 IU SOD, 250 μM H_2O_2. Figures next to each trace are expressed in nmol O_2/mg protein per min.

initial release of oxygen (Fig. 2, traces A and B). This indicates that both hydrogen peroxide and anion superoxide were involved in this oxidation. At pH 7.0, no NADH oxidation was recovered but an NADH- and duroquinone-dependent oxygen uptake was observed (data not shown). When ferrous ions were added before NADH, the oxygen consumption proceeded with a slower kinetic (trace C). SOD and catalase, added either before (not shown) or after NADH supply, were also able to block NADH-elicited oxygen uptake (trace D), further supporting the contention that H_2O_2 and $O_2^{\cdot}$ were involved in NADH oxidation. Hydrogen peroxide did not modify NADH-dependent oxygen consumption, which was, conversely, strongly stimulated by the subsequent addition of iron ions (trace E). Ferrous ions added before NADH, in the presence of H_2O_2, caused a rapid but rather modest oxygen consumption, which was again greatly stimulated by the subsequent addition of NADH (trace L). NADPH exhibited the same pattern of responses (traces F, G, H and I). In the absence of membranes, no NADH oxidation was observed and the subsequent addition of ferrous or ferric ions caused only a negligible oxygen consumption, probably caused by a very slow autooxidation of NADH in the presence of iron (trace M).

Ferulic acid slightly stimulated NADH-dependent O_2 consumption (Fig. 3, trace A), while SHAM and Mn^{2+} had no effect (traces B and C). KCN, added before or after the substrate, inhibited NADH-dependent oxygen consumption (traces D and E). Similarly, the presence of EDTA in the incubation medium abolished NADH oxidation, suggesting that contaminant ferrous ions are essential for this activity (traces G and H). The subsequent addition of Fe^{2+} caused a rapid increase of oxygen uptake which later came to a stop. The known antioxidant BHT did not inhibit Fe^{2+}-stimulated NADH-dependent oxygen consumption (trace F). Ascorbate, a scavenger of free superoxide radicals, inhibited NADH oxidation, further confirming the involvement of $O_2^{\cdot}$ (trace I). The NADH oxidase was also found in membranes washed with 0.5 M NaCl, indicat-

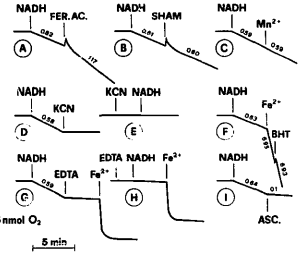

Fig. 3. Effect of ferulic acid, SHAM, $MnCl_2$, KCN, BHT, EDTA and ascorbic acid on NADH oxidation in plasma-membrane-enriched fractions. The conditions were as in Fig. 2. Other additions: 1 mM ferulic acid, 2 mM SHAM, 0.25 mM $MnCl_2$, 1 mM KCN, 1 mM BHT, 1 mM EDTA and 1 mM ascorbic acid. Figures next to each trace are expressed in mmol O_2/mg protein per min.

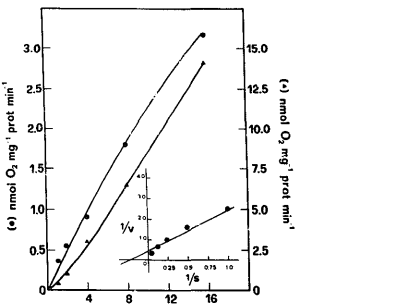

Fig. 4. NADH oxidation (●) and Fe^{2+}-dependent NADH oxidation (▲) as a function of NADH concentration. The conditions were as in Fig. 2. The inset shows a double-reciprocal plot of the NADH dependent oxygen uptake.

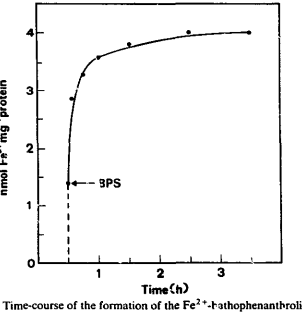

Fig. 5. Time-course of the formation of the Fe^{2+}-bathophenanthroline sulfonate chelate in radish plasma-membrane-enriched fractions. The membranes (approx. 1.4 mg protein) were incubated in 40 mM sodium acetate (pH 5.0)/0.25 M sucrose/5 μM FCCP/5 μl of saturated solution dithionite in a final volume of 1.5 ml. After 20 min of preincubation at 37°C, 50 μM bathophenanthroline sulfonate (BPS) was added.

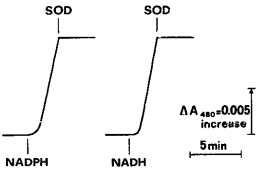

Fig. 6. Conversion of epinephrine to adrenochrome induced by NAD(P)H in radish plasma-membrane-enriched fractions. The medium was: 40 mM sodium acetate (pH 5.0)/0.25 M sucrose/80 μM $FeSO_4 \cdot 7\ H_2O$/2 mM epinephrine and 200 μl of membrane preparation (approx. 0.8 mg protein) in a final volume of 1.5 ml. The reaction was started by the addition of 4 mM NAD(P)H. The addition was 100 IU SOD.

ing that the enzyme is bound to the membranes (results not shown). Fig. 4 shows that both NADH oxidation and Fe^{2+}-stimulated NADH oxidation increased in parallel with the increasing NADH concentration. The double-reciprocal plot of the data of the NADH-dependent oxygen uptake gave a K_m of approx. 4 mM (inset).

The presence of non-covalently bound iron in radish plasma membrane fractions is shown in Fig. 5. As described for rat liver mitochondria [49], the formation of Fe^{2+}-bathophenanthroline sulfonate chelate was biphasic, suggesting that there was an amount of iron which reacted readily because it was loosely bound, while the pool which chelated in the low phase appeared to be better bound.

The direct involvement of anion superoxide in NAD(P)H-dependent oxygen consumption by radish plasmalemma vesicles was evaluated by assaying the conversion of epinephrine to adrenochrome. In the presence of ferrous ions, NADH or NADPH elicited an increase of absorbance at 480 nm which was stopped by the addition of SOD (Fig. 6).

NADH-ferricyanide oxidoreduction, at pH 5.0, was stimulated by ferulic acid, PCMB, PCMBS, mersalyl, and, at pH 7.0, also by SHAM and *p*-coumaric acid, while it was unaffected by duroquinone (Table II). NADH oxidoreduction could also be appreciated by using cytochrome *c* as an electron acceptor (data not shown). Both NADH-ferricyanide and NADH-cytochrome *c* oxidoreductases were insensitive to monovalent and divalent cations and were not inhibited by KCN, NEM, oligomycin and vanadate (data not shown), assayed in the same conditions of experiments of Table II.

TABLE II

Effect of several compounds on NADH-ferricyanide oxidoreduction in radish plasma-membrane-enriched preparations

The medium was 20 mM sodium acetate (pH 5.0)/0.25 M sucrose or 20 mM Hepes-Tris (pH 7.0)/0.25 M sucrose and 50 μl of membrane preparation (0.2–0.25 mg protein) in a final volume of 2 ml. The reactions were started by 1 mM NADH.

Additions (0.5 mM)	nmol $K_3Fe(CN)_6$ reduced per mg protein per min	
	pH 5.0	pH 7.0
Control	142	128
SHAM	140	290
Ferulic acid	214	326
p-Coumaric acid	143	258
Duroquinone	138	130
PCMB	550	1047
PCMBS	662	2094
Mersalyl	266	408

Discussion

Recently, Hassidim et al. [45] suggested that electrons from ascorbate are transported electrogenically to ferricyanide across the membrane of radish microsomal vesicles. In this paper we show that radish plasma membranes have NAD(P)H-ferricyanide or NAD(P)H-cytochrome *c* oxidoreductases which exhibit characteristics similar to those described by others [54]. In addition, NADH elicit an oxygen consumption which may be interpreted in different ways: NADH oxidation has been used as evidence for the presence of redox systems in the plasmalemma, which by a flavoprotein, may reduce, directly or through a short chain of reactions, molecular oxygen [55]; alternatively, it has been suggested that NADH oxidation by plasmalemma vesicles involves the cytochrome *P*-450/420 system in a monooxygenase reaction [56]; in a third explanation, put forward by Yamazaki and Yokota [57] and then adopted by others [41–43], NAD(P)H oxidation is attributed to peroxidase activity catalyzing the oxidation of NAD(P)H by molecular oxygen in a complex series of reactions. The main lines of evidence supporting the latter hypothesis are: the stimulation by SHAM and other phenolic compounds [38,42], stimulation by Mn^{2+} [38] or SHAM plus Mn^{2+} [43], inhibition by SOD but only if added before NADH [38,42]. Although some of our findings are compatible with this third possibility, e.g., inhibition by KCN and catalase, or by SOD only if added before NADH, the whole of our results may be rationalized in a different way.

According to Cakmak et al. [44], NADH would cause the reduction of a flavoprotein of radish plasmalemma:

$$Fp + NADH + H^+ \rightarrow FpH_2 + NAD^+ \quad (1)$$

which, by reacting with contaminant iron ions, generates ferrous ions:

$$FpH_2 + Fe^{3+} \rightarrow Fe^{2+} + FpH^{\cdot} + H^+ \quad (2)$$

The radical flavoprotein may then directly react with oxygen to produce $O_2^{\cdot}$.

$$FpH^{\cdot} + O_2 \rightarrow O_2^{\cdot} + Fp + H^+ \quad (3)$$

Following the scheme of reactions outlined by Halliwell and Gutteridge [58], ferrous ions, in turn, autooxidize to produce $O_2^{\cdot}$:

$$Fe^{2+} + O_2 \Leftrightarrow Fe^{2+}\text{-}O_2 \Leftrightarrow Fe^{3+} + O_2^{\cdot} \quad (4)$$

Evidence for this is provided by the fact that NADH oxidation generates anion superoxide, is inhibited by SOD, added either before or after NADH supply, and by ascorbate, and is prevented by EDTA. In addition, the presence of non-covalently bound iron in radish plasma membranes further supports the involvement of this ion in these reactions. Alternatively, NAD(P)H oxidation, always through a flavoprotein, might bring about the univalent reduction of oxygen, as shown in human neutrophils [34,35]. However, this possibility has been ruled out because it cannot explain the inhibitory effect of EDTA. Anion superoxide then undergoes a non-enzymic dismutation leading to hydrogen peroxide:

$$2O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

Ferrous ions, which greatly stimulate NADH oxidation, can allow, through a Fenton reaction, the formation of hydroxyl radicals:

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+} \quad (6)$$

Ferric ions can be reduced as follow:

$$Fe^{3+} + O_2^{\cdot} \rightarrow Fe^{2+} + O_2 \quad (7)$$

Catalase and SOD, by eliminating from the medium H_2O_2 and $O_2^{\cdot}$, inhibit reactions (6) and (7), respectively. The lack of effect of BHT on this series of reactions has to be expected, since this antioxidant reacts with peroxy radicals by converting them to less active products [59]. The rapid but limited increase in NADH oxidation-induced Fe^{2+}, after the addition of EDTA, may be related to the fact that Fe^{2+}, in equimolar concentrations with EDTA, is rapidly oxidized [31]; however, EDTA concentration in our experiment being higher than that of Fe^{2+}, a subsequent complete inhibition takes place.

The biosynthesis of lignin in plant cell wall is a phenol-radical-mediated process requiring H_2O_2 [39,40]. The peroxidase of cell wall has been suggested to produce H_2O_2 [38–40], utilizing NADH as a substrate, although Gross et al. [39], in their model of lignin biosynthesis, hypothesize that H_2O_2 may be also formed from NADH via a non-enzymic reaction or through a flavoprotein catalyzing this reaction. Recent evidence suggests that peroxidases may be also associated with plasmalemma [41–43]. The physiological role of these activities has not been established and the authors speculate that they may be implicated in the process of lignification or resistance to pathogens. Similarly, in the light of our results, we can infer that the NADH oxidation of radish plasma membrane vesicles can represent another possibility to produce hydrogen peroxide, whose metabolism, inside the cell, appears important at the onset of seed germination [60,61] and for lignin biosynthesis outside the cell [39,40]. In addition, it may represent, as happens for human neutrophils [34,35], another source of anion superoxide, which appears also related to the mechanism of defence to plant pathogens [37]. The catalytic site of the NADH oxidase seems to be localized on the external face of the plasmalemma in connection with the acidic environment of the cell wall (approx. pH 5). NADH for this activity can be provided by a cell wall-bound malate dehydrogenase utilizing malate exported from the cytoplasm in exchange with oxaloacetate [62,63].

Acknowledgments

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