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Hydrogen peroxide formation and iron ion oxidoreduction linked to NADH oxidation in radish plasmalemma vesicles

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Previously, we showed the presence in radish (*Raphanus sativus* L.) plasmalemma vesicles of an NAD(P)H oxidase, active at pH 4.5–5.0, which elicits the formation of anion superoxide (Vianello and Macrí (1989) Biochim. Biophys. Acta 980, 202–208). In this work, we studied the role of hydrogen peroxide and iron ions upon this oxidase activity. NADH oxidation was stimulated by ferrous ions and, to a lesser extent, by ferric ions. Salicylate and benzoate, two known hydroxyl radical scavengers, inhibited both basal and iron-stimulated NADH oxidase activity. The iron chelators EDTA (ethylenediaminetetraacetic acid) and DFA (deferoxamine melysate) at high concentrations (2 mM) inhibited the NADH oxidation, whereas they were ineffective at lower concentrations (80 μM); the subsequent addition of ferrous ions caused a rapid and limited increase of oxygen consumption which later ceased. Hydrogen peroxide was not detected during NADH oxidation but, in the presence of salicylate, its formation was found in significant amounts. NADH oxidase activity was also associated to a Fe^{2+} oxidation which was only partialy inhibited by salicylate. Ferrous ion oxidation was partially inhibited by catalase and prevented by superoxide dismutase, while ferric ion reduction was abolished by catalase and unaffected by superoxide dismutase. These results show that during NADH oxidation iron ions undergo oxidoreduction and that hydrogen peroxide is produced and rapidly consumed. As previously suggested, this oxidation appears linked to the univalent oxidoreduction of iron ions by a reduced flavoprotein of radish plasmalemma which is then converted to a radical form. The latter, reacting with oxygen generates the superoxide anion which dismutates to H_2O_2. Hydrogen peroxide, through a Fenton's reaction, may react with Fe^{2+} to produce hydroxyl radicals, or with Fe^{3+} to generate the superoxide anion.

Introduction

Plant plasma membranes contain some oxidoreductase activities which are related to important physiological functions [1–6]. They appear to be involved in the uptake of Fe^{3+} by iron-deficient dicotyledonous and monocotyledonous (not grasses) plants [1,2]; proton and electron extrusion [7–12], although on this there are conflicting results [13–17]; modulation of the H^+-ATPase acting as a proton pump [18–20]; elongation growth [21] and generation of an electrical potential [22].

In isolated plasma membranes, electron transport systems are capable of transferring reducing equivalents from NAD(P)H to several electron acceptors, i.e., ferricyanide, cytochrome *c*, duroquinone, tetrazolium derivative, dichlorophenol and oxygen [23–27]. In addition, some similar oxidoreductase activities have been found in glyoxysomal and endoplasmic reticulum membranes [28].

The ability of NAD(P)H oxidases of different plant cell organelles to generate the superoxide anion and hydrogen peroxide has been reviewed [29,30]. Recently, this ability has been described in plasma membranes from wheat [25], cauliflower [31], radish [32], guard cell protoplasts of *Commelina communis* [34] and in watermelon glyoxysomes [35]. The NAD(P)H oxidase activity of plasmalemma has been interpreted as due to a free radical chain reaction initiated by a peroxidase [31] or an NAD(P)H dehydrogenase activity [32]. The latter requires iron ions which are essential for inducing the process.

Abbreviations: BSA, bovine serum albumin; DETAPAC, diethylenetriaminepentaacetic acid; DFA, deferoxamine melysate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PMFS, phenylmethylsulfonyl fluoride; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)aminomethane

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In higher plants, the generation of the anion superoxide and/or hydrogen peroxide appears involved in several processes at the plasmalemma level or in the wall surrounding the cell. These are: (i) ferric reduction for uptake of iron [36], (ii) dessication injury [37], (iii) zinc deficiency in roots [38], (iv) lignin biosynthesis [39–41] and (v) defence mechanisms against pathogens [42].

Hydrogen peroxide and the superoxide anion can interact in an iron-catalyzed reaction (Haber-Weiss reaction) to form hydroxyl radicals:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$2\,O_2^- + 2\,H^+ \rightarrow O_2 + H_2O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

The final step of these reactions is also referred to as Fenton's reaction. Hydroxyl radical is highly reactive toward polyunsaturated fatty acids. However, its ability to spark lipid peroxidation has been questioned [43,45] because of the high reactivity that does not allow it to migrate [46]. On the other hand, some iron chelators (EDTA and DETAPAC) stimulate hydroxyl radical formation, but they inhibit lipid peroxidation which appears closely associated with light emission [47,48]. In agreement, many in vitro lipid peroxidation systems are not inhibited by superoxide dismutase, catalase or $OH^{\cdot}$ scavengers [43–45]. In the light of recent results, the initiation of lipid peroxidation by Fe^{2+} and H_2O_2 is mediated by an oxidant which requires both ferric and ferrous ions [49].

In this work we studied the role of hydrogen peroxide and iron ions during NADH oxidase activity in radish plasmalemma vesicles. A preliminary report of these findings has been presented at the 8th International Workshop on Plant Membrane Transport, Venice, 1989.

Materials and Methods

Plant material. Radish (*Raphanus sativus* L., cv. Tondo Rosso Quarantino) seeds were germinated as described in Ref. 50.

Preparation of plasma membrane vesicles. 50 g of seedlings were ground in 250 ml of 50 mM Tris-HCl (pH 8.0)/0.3 M sucrose/1 mM $MgCl_2 \cdot 6\,H_2O$/1 mM Na-EDTA/1 mM DTT/1 mM PMFS/0.1% BSA and then filtered through eight layers of gauze. The filtrated was centrifuged at 15 000 × *g* for 10 min and the resulting supernatant was recentrifuged at 80 000 × *g* for 30 min. The pellet (microsomes) was resuspended in 2 ml of 10 mM Tris-HCl (pH 7.5)/0.25 M sucrose/1 mM $MgCl_2 \cdot 6\,H_2O$/1 mM DTT/0.1% BSA and layered onto a discontinuous sucrose gradient consisting of three aliquots of 9 ml each of 20, 35 and 45% (w/v) sucrose in 10 mM Tris-HCl (pH 7.5)/1 mM $MgCl_2 \cdot 6\,H_2O$/1 mM DTT/0.1% BSA. After centrifugation at 80 000 × *g* for 120 min (Beckman SW 25.1 rotor), the 35–45% sucrose interface fraction was collected by a Pasteur pipette, diluted in 10 mM Hepes-Tris (pH 7.0)/0.25 M sucrose/5 mM $MgCl_2 \cdot 6\,H_2O$ and recentrifuged at 100 000 × *g* for 1 h. The pellet was resuspended by a Potter-type homogenizer in 2.5 ml of the above buffer and stored at −40 °C.

NADH-dependent oxygen consumption. Oxygen uptake was monitored by a platinum electrode of the Clark-type, at room temperature (22 °C). The medium consisted of 40 mM sodium acetate (pH 5.0)/0.25 M sucrose, and 200 μl of membrane vesicles (approx. 0.8 mg protein) in a final volume of 1.5 ml. The reaction was started by 4 mM NADH.

Hydrogen peroxide determination. Assay for H_2O_2 was performed as described by Minotti and Aust [49]. In H_2O_2 decomposition experiments the medium was 40 mM sodium acetate (pH 5.0)/0.25 M sucrose and 45 nmol H_2O_2 in a final volume of 1.5 ml. The reaction was initiated by 40 μM $FeSO_4 \cdot 7\,H_2O$ and proceeded at 37 °C. The assay mixture for H_2O_2 formation experiments was 40 mM sodium acetate (pH 5.0)/0.25 M sucrose/40 μM $FeSO_4 \cdot 7\,H_2O$ and 200 μl of membrane vesicles (approx. 0.8 mg protein) in a final volume of 1.5 ml. The reaction was started by the addition of 4 mM NADH and proceeded at 37 °C. In both experiments, 0.5-ml aliquots were withdrawn and added to 1 ml of reactive (24.8 mM phenol, 4.3 mM 4-aminoantipyrine and 19 IU/ml horseradish peroxidase in 1 mM potassium phosphate buffer (pH 6.9)) and the absorbance was read at 505 nm. A standard curve with known amounts of H_2O_2 was used for calculation.

Ferrous ion assay. The incubation mixture was as described for NADH-dependent oxygen uptake assays plus 60 nmol $FeSO_4 \cdot 7\,H_2O$ or $FeCl_3 \cdot 6\,H_2O$. Aliquots (0.5 ml) were removed and mixed with 1 ml of 15 mM 1,10-*o*-phenanthroline. The absorbance of the ferrous-phenanthroline complex was measured at 510 nm. Absorbance values were converted by a standard calibration curve.

Superoxide anion formation. The formation of the superoxide anion was monitored by assaying the conversion of epinephrine to adrenochrome [51]. The incubation mixture contained 40 mM sodium acetate (pH 5.0)/0.25 M sucrose/40 μM H_2O_2/2 mM epinephrine in a final volume of 2 ml. The reaction was started by the addition of 40 μM $FeCl_3 \cdot 6\,H_2O$ and followed continuously at 480 nm, at room temperature.

Protein determination. Protein was evaluated by the biuret method described by Gornall et al. [52].

Chemicals. Phenol, rat liver catalase (EC 1.11.1.6), horseradish peroxidase (EC 1.11.1.7), deferoxamine melysate, EDTA, 4-aminoantipyrine, 1,10-*o*-phenan-

throline were purchased from Sigma Chemical, St. Louis, MO, U.S.A. Sodium benzoate and sodium salicylate were from Merck, Darmstadt, F.R.G. Epinephrine was obtained from Fluka Chemie, Buchs, Switzerland. Other chemicals were reagent grade.

Results

In a preceding work we showed that radish plasma membrane vesicles sustain an NAD(P)H-dependent oxygen uptake which is stimulated by ferrous ions and inhibited by catalase or superoxide dismutase, thus indicating the involvement of the superoxide anion and hydrogen peroxide [32].

Fig. 1, trace A, also shows that NADH-dependent oxygen consumption (NADH oxidation) was strongly stimulated by Fe^{2+} and, to a lesser extent, by Fe^{3+}. An intermediate value was obtained by using a mixture (1 : 1) of Fe^{2+} and Fe^{3+}. Salicylate and benzoate, two hydroxyl radical scavengers, partially inhibited the basal and the Fe^{2+}-stimulated NADH oxidation (traces B and C). This inhibition was also evident if the scavengers

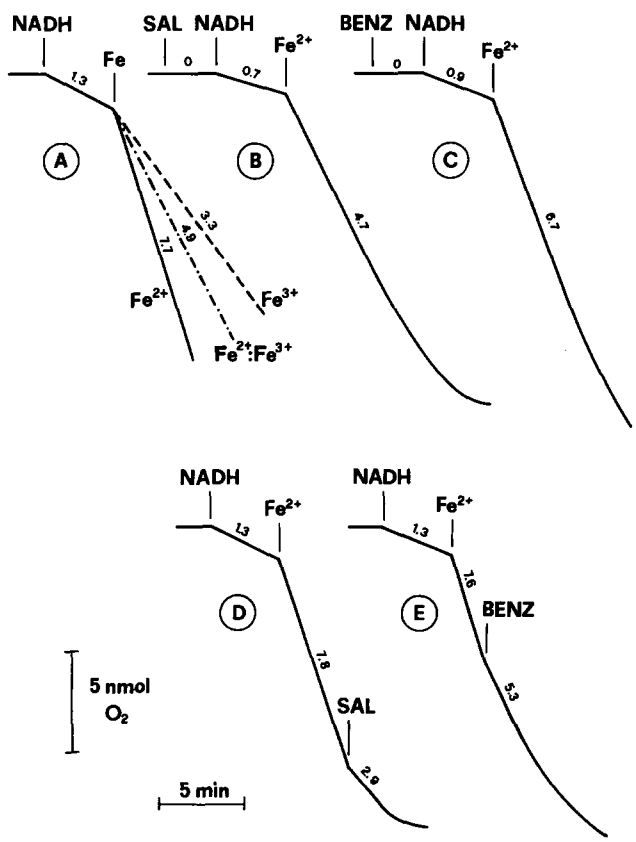

Fig. 1. Effect of iron ions, salicylate and benzoate on NADH oxidation in radish plasmalemma-enriched vesicles. Additions were: 4 mM NADH, 40 μM $FeSO_4 \cdot 7\ H_2O$, 40 μM $FeCl_3 \cdot 6\ H_2O$ or a mixture (1 : 1) of both (trace A); 15 mM salicylate (trace B); 15 mM benzoate (trace C); 30 mM salicylate (trace D); 30 mM benzoate (trace E). Figures next to each trace are expressed in nmol O_2/mg protein per min and are representative of one typical experiment.

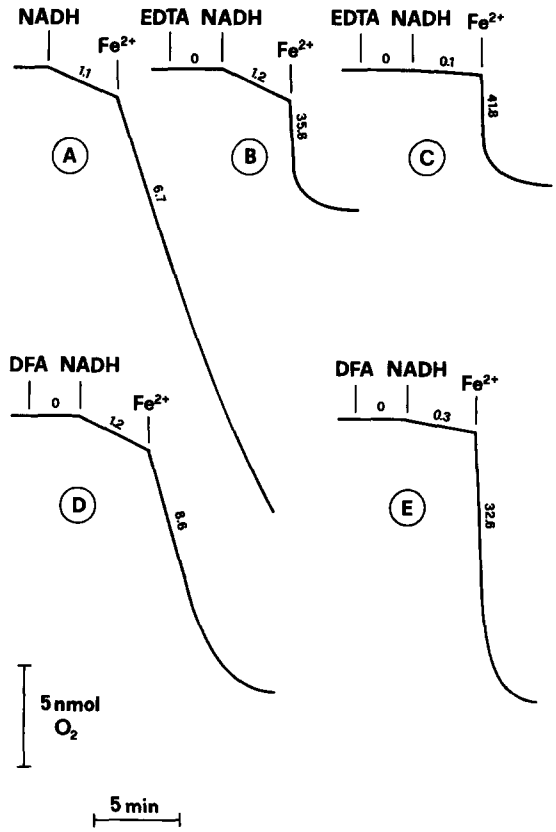

Fig. 2. Effect of EDTA and DFA on NADH oxidation and Fe^{2+}-dependent NADH oxidation in radish plasmalemma-enriched vesicles. Additions: control (trace A), 80 μM EDTA (trace B), 80 μM DFA (trace D); 2 mM EDTA (trace C); 2 mM DFA (trace E); 40 μM $FeSO_4 \cdot 7\ H_2O$ and 4 mM NADH (all traces). Figures next to each trace are expressed in nmol O_2/mg protein per min and are representative of one typical experiment.

were added during Fe^{2+}-stimulated NADH oxidation (traces D and E).

The need of trace iron ions for NADH oxidase activity in our membrane preparation has been already demonstrated [32]. The iron chelator EDTA, at low concentration (80 μM), did not inhibit NADH oxidation (Fig. 2, trace B) when compared with the control (trace A); the subsequent addition of Fe^{2+} (40 μM), caused only a rapid but limited increase of oxygen consumption when then came to a complete inhibition. Another chelating agent, DFA (80 μM), showed a similar pattern, but the inhibition of Fe^{2+}-stimulated oxygen uptake occurred after a longer period (trace D). In the presence of high concentrations of EDTA or DFA (2 mM), NADH oxidation was inhibited (traces C and E). The addition of Fe^{2+} (40 μM) also caused a rapid and limited increase of oxygen consumption.

No H_2O_2 was found during Fe^{2+}-dependent NADH oxidation (Table I). However, in the presence of salicylate, which prevents the conversion of H_2O_2 to hydroxyl radicals in a reaction catalyzed by ferrous ions (Fenton's reaction), a significant amount of H_2O_2 was detected, and its formation was completely abolished by catalase.

TABLE I

Effect of salicylate on ferrous ion oxidation and hydrogen peroxide formation dependent upon NADH oxidase activity of radish plasmalemma-enriched vesicles, and on hydrogen peroxide decomposition

n.d., not determined. Data are means of three replicates ± S.D.

Additions	H_2O_2 formation (nmol/mg protein)	Fe^{2+} oxidation (nmol/mg protein)	H_2O_2 decomposition (nmol/ml)
Control	0	52 ± 4.4	40 ± 4.1
Salicylate (20 mM)	116 ± 5.9	40 ± 3.7	0
Salicylate (20 mM) + catalase (300 IU)	0	n.d.	n.d.

Therefore, it appears that H_2O_2 was rapidly formed and consumed. In addition, NADH oxidation, in the presence of Fe^{2+}, implied the concomitant oxidation of Fe^{2+} to Fe^{3+} and salicylate partially inhibited such a Fe^{2+} oxidation. Table I also shows that H_2O_2, supplied to the incubation system without membranes, was rapidly utilized through a Fenton's reaction and that salicylate blocked this decomposition. In agreement, the addition of Fe^{2+} was crucial for initiating H_2O_2 decomposition, which proceeded with a rapid kinetic. The same pattern was observed in the presence or absence of membranes, indicating that the interaction between H_2O_2 and Fe^{2+} did not require the enzymic asset of the membranes (Fig. 3).

The direct involvement of Fe^{2+} oxidation and its dependence from NADH oxidase activity is shown in

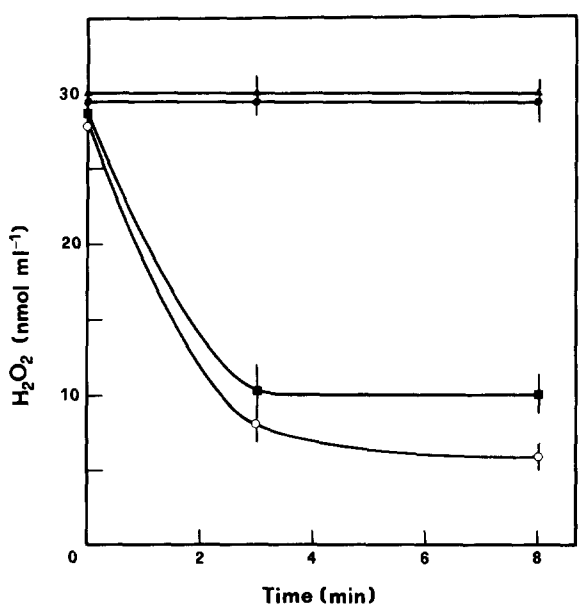

Fig. 3. Time-course of hydrogen peroxide decomposition induced by ferrous ions. ●, 45 nmol H_2O_2; ○, 45 nmol H_2O_2 plus 40 μM $FeSO_4 \cdot 7\ H_2O$; ▲, 45 nmol H_2O_2 plus 200 μl of membranes (approx. 0.8 mg protein); ■, H_2O_2 plus $FeSO_4 \cdot 7\ H_2O$ plus 200 μl of membranes. Data are means of three replicates ± S.D.

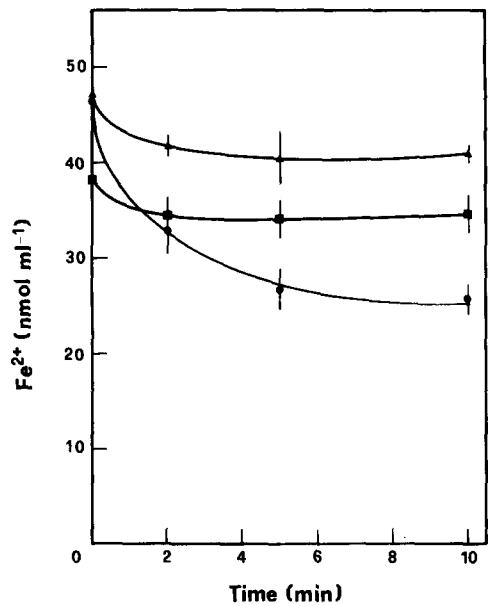

Fig. 4. Time-course of Fe^{2+} oxidation in radish plasmalemma-enriched vesicles. ■, 60 nmol $FeSO_4 \cdot 7\ H_2O$ plus 4 mM NADH; ▲, 60 nmol $FeSO_4 \cdot 7\ H_2O$ plus 200 μl of membranes; ●, 60 nmol $FeSO_4 \cdot 7\ H_2O$ plus 4 mM NADH in the presence of membranes. Data are means of three replicates ± S.D.

Fig. 4. In the absence of membranes or NADH, negligible levels of Fe^{2+} oxidation were found, while in the presence of NADH and membranes Fe^{2+} was rapidly oxidized to Fe^{3+}. The different level of Fe^{2+} detected in the samples without membranes with respect to those with membranes, may be related to the fact that radish plasmalemma vesicles, as previously shown [32], contain endogenous iron ions. Table II shows that Fe^{2+} oxidation was partially inhibited by catalase and almost completely inhibited by SOD. Ferric iron reduction was, instead, inhibited by catalase and unaffected by SOD. Therefore, superoxide dismutase did not alter Fe^{3+} reduction, while apparently it inhibited the Fe^{2+} oxidation. The low level of Fe^{2+} oxidation recovered in the presence of SOD may be due to a rapid conversion of Fe^{3+}, formed during NADH oxidation, to Fe^{2+} in a reaction requiring H_2O_2 and producing the superoxide anion. This possibility was confirmed in the experiment

TABLE II

Effect of catalase and SOD on iron ion oxidoreduction dependent upon NADH oxidase activity of radish plasmalemma-enriched vesicles

Data are means of three replicates ± S.D.

Addition	Fe^{2+} oxidation (nmol/mg protein)	Fe^{3+} reduction (nmol/mg protein)
Control	46 ± 3.9	23 ± 2.0
Catalase (100 IU)	22 ± 5.2	3 ± 1.5
SOD (100 IU)	5 ± 2.6	26 ± 2.7

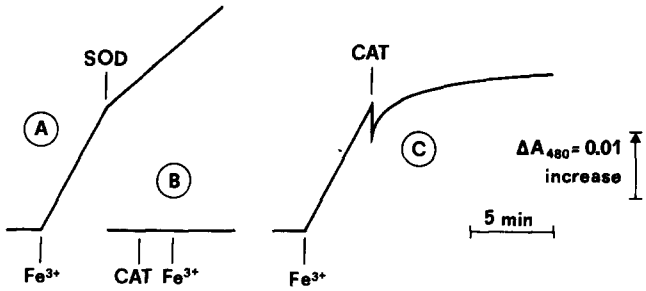

Fig. 5. Conversion of epinephrine to adrenochrome induced by ferric ions and hydrogen peroxide. Additions: 40 μM $FeCl_3 \cdot 6\ H_2O$; 100 IU SOD; 100 IU catalase.

of Fig. 5. The addition of Fe^{3+} to the medium at pH 5.0 in the presence of H_2O_2, caused a rapid conversion of epinephrine to adrenochrome, indicating the formation of the superoxide anion (trace A). The subsequent addition of SOD caused a 50% inhibition. Catalase added before or after Fe^{3+} addition, completely inhibited the superoxide anion formation (traces B and C). The partial inhibition caused by SOD may be explained by considering that the superoxide anion dismutases, regenerating H_2O_2, that hence appears cyclically consumed and formed.

Discussion

In a previous paper we demonstrated that NADH oxidase activity of radish plasmalemma vesicles implies the formation of the superoxide anion [32]. This activity appears strictly dependent upon the presence of iron ions, which seem essential for the generation of such an oxygen radical form and, as inferred, for the subsequent formation of hydroxyl radicals via a Haber-Weiss reaction.

In this study we show that the NADH oxidase activity of radish plasmalemma led to the generation of hydrogen peroxide which was rapidly consumed. The formation of hydrogen peroxide required the presence of the membranes, while its decomposition was independent, thus indicating that the latter process was of a chemical type.

The essentiality of iron ions in initiating lipid peroxidation and hydroxyl radical formation has been established, although these two processes appear unrelated [43–45]. Our preceding results [32] and the data presented here, demonstrate that oxidoreduction of iron ions occurred and was associated to NADH oxidase activity, being almost completely inhibited by high concentrations of EDTA or DFA. In addition, ferric or ferrous ions strongly stimulated NADH oxidation. However, a mixture of Fe^{2+}/Fe^{3+} (1 : 1), which appears essential to promote lipid peroxidation without the involvement of hydroxyl radicals [49], gave only an intermediate value of NADH oxidation. Therefore, NADH oxidase activity does not seem related to lipid peroxidation, but rather to the generation of hydroxyl radicals. In agreement, NADH oxidase and Fe^{2+}-stimulated NADH oxidase activity are inhibited by superoxide dismutase and catalase which, as known, do not inhibit lipid peroxidation [43–45]. The inhibitory effect of salicylate and benzoate on the latter activities further supports the second possibility. However, the partial inhibition caused by the two hydroxyl radical scavengers suggests that H_2O_2, besides being utilized in a Fenton's-type reaction, may be consumed in another manner. In our experimental condition, hydrogen peroxide can react with Fe^{3+} to produce the superoxide anion and Fe^{2+}.

These results may be rationalized by the scheme of reactions outlined in Fig. 6. As previously suggested [32], NAD(P)H would reduce a flavoprotein of plasmalemma which, through two univalent reactions, reduces the contaminating iron to ferrous ions, converting itself to a radical form; subsequently, the radical flavoprotein reacts with molecular oxygen to produce the superoxide anion. Ferrous ions, thus formed, may be taken up by the plant cell or, alternatively, utilized to generate again the superoxide anion by reacting with oxygen. Then, the superoxide anion disproportionates to give hydrogen peroxide which is converted to hydroxyl radicals. Alternatively, hydrogen peroxide can react with ferric ions to generate the superoxide anion and ferrous ions.

The involvement of the superoxide anion and/or hydrogen peroxide in several physiological and patho-

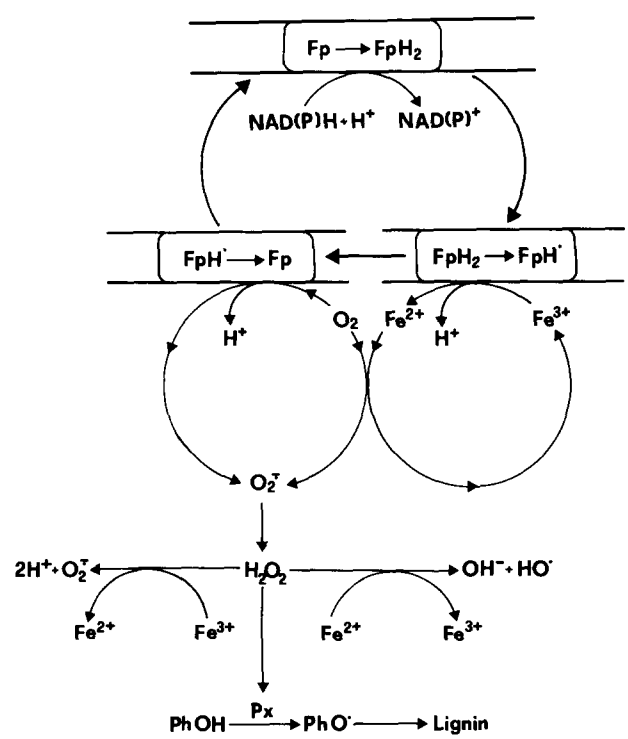

Fig. 6. Hypothetical scheme of reactions induced by NADH oxidation in radish plasmalemma vesicles. Fp, flavoprotein; PhOH, phenol; Px, peroxidase.

logical processes at the plasmalemma level or in the wall surrounding the cell has been established [36–42]. The reactions described above may constitute, hence, another mechanism to produce such oxygen forms, in addition to the activity of the cell wall [39–41] or plasmalemma peroxidases [31,34] outside the plasmalemma in the acidic environment of the cell wall.

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