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Redox Activities in Plasma Membrane Vesicles Isolated from Papaya (*Carica papaya*) Leaves

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Abstract. Plasma membrane vesicles were isolated from papaya (*Carica papaya*) leaf cells by a two-phase partition system. Redox activities of these vesicles were determined by ferricyanide reduction and NADH oxidation. Ferricyanide reductase activity was accompanied by continuous acidification of the medium and was stimulated by fusicoccin. NADH oxidase activity was inhibited by catalase.

Plasma membrane redox systems (PMRS) have been found in plant cells (Barr et al. 1984, Craig et al. 1981, Thiel and Kirst 1988), as well as in animal cells (Crane et al. 1985). The physiological role of these systems is still under discussion (Crane et al. 1985, Komor et al. 1987, Møller and Lin 1986). Evidence exists pointing out to the involvement of PMRS in iron reduction and uptake, and superoxide generation (Bienfait 1985, Cakmak and Marschner 1988, Crane et al. 1991).

Most of the studies with plant cells have been carried out by using whole cells or tissue fragments (Barr et al. 1984, Craig et al. 1981, Lüthen and Böttger 1988a,b, Morré et al. 1987a, Thiel and Kirst 1988); in addition, most of these works have focused on roots (Lüthen and Böttger 1988a,b; Rubinstein and Stern 1986), although PMRS have been also investigated in some photosynthetic cells (Marré et al. 1988, Neufeld and Bown 1987, Trockner and Marré 1988). Only a few works have been done with isolated vesicles (Askerlund et al. 1987, Morré et al. 1987, Sandelius et al. 1986, Vianello and Macri 1989).

In this work, plasma membrane vesicles were isolated from papaya leaf cells by fractionation in a two-phase polymer system, which is a powerful tool for the rapid and efficient separation and purification of membrane fractions (Briskin et al. 1987, Larsson 1985, Larsson et al. 1987). Redox activities of this plasma membrane fraction were studied by using spectrophotometric and polarographic techniques.

Abbreviations: FC, fusicoccin; PCMBS, parachloromercuribenzenesulfonic acid.

Materials and Methods

Obtention of the Microsomal Fraction

Papaya (*Carica papaya*) leaves were cut and homogenized in a weight:volume ratio 1:2 of the homogenization medium, consisting in 0.25 M sucrose, 70 mM Tris (pH 8), 3 mM EDTA, 4 mM DTT, and bovine serum albumine (0.1% wt/vol). The homogenization was carried out with an Omni-Mixer (Sorvall) homogenizer (8 × 20 s pulses). The homogenate was filtered through muslin and centrifuged at 10,000 *g* for 10 min. The pellet was discarded and the supernatant fraction was centrifuged at 50,000 *g* for 30 min. All procedures were carried out at 4°C. The microsomal pellet obtained was resuspended in a 3 mM KCl, 0.33 M sucrose, 5 mM phosphate buffer (pH 7.8). This was the starting material for the two-phase partition of membranes.

Two-phase Partition of Membranes

The method described by Larsson (1985) was followed with slight modifications. Two grams of the microsomal suspension were added to a 30 g phase mixture to give the 32 g phase system with a final composition of 6.2% (wt/wt) Dextran T500, 6.2% (wt/wt) polyethylene glycol 3350, 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8. The phase system was thoroughly mixed by 40 inversion of the tube, and the two phases were separated by centrifugation at 4°C in a swinging bucket centrifuge at 2000 *g* for 3 min. The upper phase was removed and added to a centrifuge tube loaded with new bottom phase. At the same time, new upper phase was added over the bottom phase in the original tube. The phases were mixed and the procedure was repeated twice. Finally, the upper phases with pure plasma membranes were combined and diluted at least twofold with 3 mM KCl, 0.33 M sucrose, 5 mM phosphate buffer (pH 7.8). Plasma

membranes were collected by centrifugation at 100,000 *g* for 30 min. The membrane pellet was resuspended in the same medium, divided in 300–400 μl aliquots, immediately frozen in liquid nitrogen, and stored at −70°C until utilization.

Ferricyanide Reductase Assay

The reduction of 0.5 mM ferricyanide was assayed spectrophotometrically at room temperature in 20 mM Tris-Hepes buffer (pH 7) with a Shimadzu UV-160 double beam spectrophotometer. The sample cuvette containing 4–6 μg of membrane protein per assay was measured against a blank with no vesicle added. The baseline was allowed to stabilize for a few minutes. Thereafter, the assay was started by adding NADH to a final concentration of 0.5 mM.

NADH Oxidase Assay

NADH oxidase activity was followed by measuring oxygen consumption at 25°C by means of an oxygen electrode (Oxygraph, Gilson); 50–60 μg of membrane protein per assay was added to acetate buffer (pH 5). NADH, $FeSO_4$, and catalase were used at final concentrations 4 mM, 40 μM, and 3 mg/ml, respectively.

Continuous Monitoring of Extravesicular pH

Membrane vesicles (50–60 μg of protein) were added to 6 ml of 0.5 mM ferricyanide in 0.5 mM Hepes buffer (pH 7) and maintained at 25°C with continuous stirring. The pH was monitored continuously with a pH electrode connected to a recorder. A baseline was established and the experiment was initiated by adding NADH to a final concentration of 1 mM.

Protein Assay

Protein was determined according to the method described by Bradford (1976).

Results and Discussion

The advantages of two-phase polymer partition have been previously stated (Briskin et al. 1987, Larsson 1985, Larsson et al. 1987, Sandelius et al. 1986). Plasma membrane vesicles obtained from papaya leaf cells by this procedure showed ferricyanide reductase activity when assayed in the presence of NADH. This NADH:ferricyanide oxidoreductase activity showed hyperbolic kinetics with a very high affinity K_m for ferricyanide (59 μM), and a V_{max} (800 nmol · min^{-1} · mg^{-1} protein) consistent with the values obtained in other plant systems (Sandelius et al. 1986).

As shown in Table 1, Triton X-100, a non-ionic detergent which dissociates membranes, increased the rate of ferricyanide reduction by 50%. It is well known that a great percentage of the vesicles obtained by the two-phase method are right side-out sealed (Larsson 1985, Larsson et al. 1987). This stimulatory effect of Triton X-100 on ferricyanide reduction may be explained because the vesicles obtained by the two-phase partition system seem to be sealed too tightly to allow maximum rates, as discussed previously (Sandelius et al. 1986). The low concentrations of non-ionic detergent could cause a rearrangement of the proteins into the membrane. In Table 1, it is also shown that PCMBS, an agent that blocks sulfhydryl groups, inhibited more than 85% the ferricyanide reduction rate at 50 μM. In agreement with the findings in plasma membranes from other cells (Sun et al. 1984, Sandelius et al. 1986), this inhibitory effect of PCMBS seems to show that one or more sulfhydryl groups are essential in this plasma membrane redox activity.

Table 1. NADH:ferricyanide reductase activity in plasma membranes—Effects of Triton X-100 and PCMBS.

Compound added	Ferricyanide reduced (nmol · min^{-1} · mg^{-1} protein)
None (control vesicles)	632 ± 4
Triton X-100 (0.01% (vol/vol))	969 ± 52
PCMBS (50 μM)	85 ± 19

Data are given as means ± SD of three different measurements.

Fusicoccin (FC) is a fungal metabolite that affects several physiological processes normally controlled by plant hormones (Ballio et al. 1964, Marré 1979, Aducci et al. 1982, 1988). It seems that the first step in the mechanism of action of FC is its recognition by high-affinity and specific-binding sites of membranes (Aducci et al. 1982). As it has been recently shown in a reconstituted system (Aducci et al. 1988), there is a functional relationship in the plasma membrane between the FC-binding protein and the H^+-ATPase. This relationship explains why there is an increased H^+ extrusion by cells (Lüthen and Böttger 1988, Marré et al. 1988) or vesicles (Aducci et al. 1988) in the presence of FC. Trockner and Marré (1988) have shown that FC also induces an increase in oxygen uptake by *Elodea densa* leaves. It has been reported that FC also induces an increase in the rate of ferricyanide reduction in several plant cells or tissues tested (Craig and Crane 1982, Marré et al. 1988). As Böttger and Hilgendorf (1988) point out, only at ferricyanide levels far higher than the K_m of the reductase, FC should increase the reduction rate. We tested the influence of 50 μM FC on ferricyanide reductase activity in plasma membrane vesicles from papaya leaf incu-

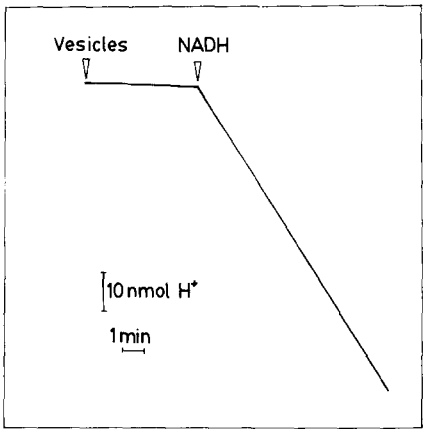

Fig. 1. Proton extrusion to the medium by papaya plasma membrane vesicles in the presence of 0.5 mM ferricyanide and 1 mM NADH. The experiment was carried out as described in Materials and Methods. The acidification rate remained constant for at least 20 min.

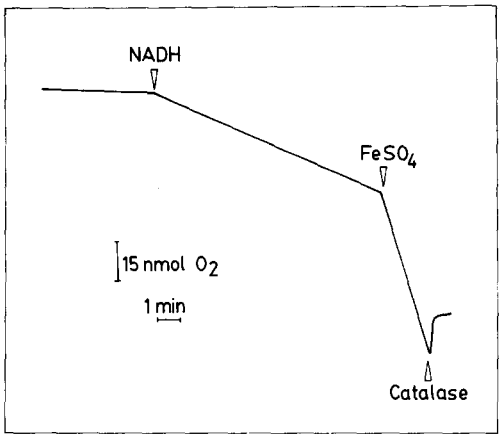

Fig. 2. Oxygen consumption by plasma membrane vesicles as determined by an oxygen electrode at 25°C and pH 5. The initial concentration of O_2 in the buffer medium was assumed to be 0.25 μmol/ml. Arrows indicate the addition of 4 mM NADH, 40 μM $FeSO_4$, and catalase (3 mg/ml).

bated in the presence of 0.5 mM ferricyanide, a concentration far higher than the K_m obtained for this system. There was a very clear and significant increase by 20% in the rate of ferricyanide reduction. Since experiments were carried out with plasma membrane vesicles in the absence of ATP, an indirect effect of FC on PMRS through its effect on plasma membrane ATPase should be ruled out as the only explanation. Thus, this stimulation seems to be a direct effect on one of the electron carriers of the PMRS, according to the hypothesis suggested by Böttger and Hilgendorf (1988). To our knowledge, at least one other different and direct effect of FC has been recently described—namely, a rapid stimulation of ethylene production in detached rice leaves (Chen and Kao 1993).

The redox reaction

$$2\ Fe(CN)_6^{3-} + NADH + H^+ \rightarrow 2\ Fe(CN)_6^{4-} + H^+$$

produces a net increase in proton concentration. This fact makes it possible to follow ferricyanide reductase activity by continuous measurement of the extravesicular pH in a weakly buffered medium. Figure 1 shows that there was a continuous acidification of the medium when ferricyanide and NADH were added to the vesicular suspension. The average rate of this proton increase was 130 nmol $H^+ \times min^{-1} \times mg^{-1}$ protein. In plant cells, ferricyanide reduction is accompanied by acidification of both cytoplasm and extracellular medium (Marré et al. 1988).

An NADH oxidase has been described in radish plasmalemma vesicles which is greatly stimulated by ferrous ions (Vianello and Macri 1989). In the present work, NADH oxidase activity was tested at pH 5 by using an Oxygraph oxygen electrode. As shown in Fig. 2, this activity was greatly increased upon the addition of $FeSO_4$, and was inhibited by the addition of catalase, as previously described in radish plasmalemma vesicles (Vianello and Macri 1989). The effect of catalase pointed out the presence of H_2O_2; the striking stimulation effect of NADH oxidation by ferrous ions could be explained by accounting for the formation of peroxide and superoxide ion radicals (Byczkowski and Gessner 1988; Cakmak and Marschner 1988; Halliwell and Gutteridge 1988; Vianello and Macri 1989). Ferrous ions would react with H_2O_2 to give very reactive hydroxyl radicals ($^{\cdot}OH$) in a Fenton reaction (Vianello and Macri 1989). The ferric ions produced in the Fenton reaction would increase both the NADH oxidation and the oxygen consumption, giving rise to more H_2O_2 through the intermediate formation of superoxide anion radicals ($O_2^{\cdot -}$). Figure 2 shows that catalase produced a slight initial release of oxygen due to the consumption of H_2O_2 which, on the other hand, would give rise to an inhibition of the Fenton reaction and hence of oxygen consumption.

In conclusion, plasma membrane vesicles isolated from papaya leaf show a NADH:ferricyanide oxidoreductase activity which responds to activation by FC and to inhibition by sulfhydryl group blockers, and a NADH oxidase activity that very probably gives rise to ion-radicals.

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