PLASMA MEMBRANE REDOX ACTIVITY: Components and Role in Plant Processes

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b-Type Cytochromes

Early investigations were focused on a search for a photoreceptor in higher plants that could mediate blue light-regulated processes such as phototropism (97). Studies with microsomal membrane fractions from maize coleoptiles identified a blue light-reducible membrane-bound cytochrome with a Soret band in the 423–428 region (29), which was subsequently termed the Light-Induced Absorbance Change or LIAC (145). The LIAC required reductants such as EDTA, methylene blue, or exogenous flavins for optimal response, and anoxic conditions were also necessary for maintenance of a stable signal (68). The action spectrum of the LIAC response coincided with the absorption spectrum for flavin, suggesting a flavin or flavoprotein blue light receptor (29). Furthermore, flavin was present in measurable quantities in PM-enriched, Renografin gradient membrane fractions that exhibited the LIAC response (82).

LIAC activity and the ascorbate-reducible b-type cytochrome co-sedimented on sucrose density gradients with vanadate-sensitive ATPase activity, considered a reliable PM marker (6). Subsequent detailed spectrophotometric investigations used highly enriched plant PM fractions isolated by two-phase aqueous partitioning. As a result, ascorbate-reducible cytochromes have been identified with distinct α-bands of between 557 and 561 nm in PM from various sources (6–8, 39). These values are similar to those for b-type cytochromes found in the outer mitochondrial membrane and ER. The evidence taken together suggests that ascorbate-reducible b-type cytochromes and the LIAC represent the same cytochrome species in the PM.

Direct and indirect evidence exists for the presence of cytochrome P-450 and its degradation product, cytochrome P-420, in PM from higher plants (8, 80; but see 7). Cytochromes P-450/P-420 belong to a family of b-type cytochromes that catalyze a variety of oxidative reactions in plant tissues, including the synthesis of phenolics, membrane sterols, hormones, phytoalexins, and terpenoids (59). The cytochrome P-450 plus P-420 content was estimated by difference spectroscopy to be in the range of 100 pmoles (mg protein)−1 in PM from cauliflower inflorescences, although not all preparations contained cytochrome P-450/P-420 (8). Furthermore, both cytochrome P-450/P-420 and its reductase (measured as NADPH:cytochrome c reductase) are considered marker enzymes for ER in other tissues and were present in cauliflower PM (8), raising the question of potential contamination of the PM fraction with ER.

Tetcyclacis, a putative specific inhibitor of cytochrome P-450-mediated reactions, inhibited ferricyanide reduction by mesophyll cells of Lemna gibba fronds preincubated in the inhibitor for 1–6 hr (22). The resulting inhibition by
Quinones

While mammalian liver PM has been shown to contain ubiquinone \([0.9 \text{ nmol (mg protein)}^{-1}]\) (53), no direct evidence exists for its presence in the PM of plant cells. There are indications, however, that components of the PM may transfer e\(^{-}\) to quinones. For example, the quinone vitamin K\(_3\) was shown to stimulate redox activity in PM isolated from soybean hypocotyls (18). Also, duroquinone (tetramethyl quinone) and menadione, which are artificial quinone e\(^{-}\) acceptors of small molecular weight, have been used by many groups to measure redox activity in PM isolated from a variety of plant tissues, particularly in PM-enriched microsomal fractions from Cucurbita (36).

There is evidence for a NADH-duroquinone reductase activity in PM-enriched preparations that may be distinct from other reductase activities. Thus, the specific activity of duroquinone reduction by NADH was not higher in PM-enriched fractions from Fe-starved, compared to Fe-sufficient, cotton roots, but NADH:ferricyanide and NADH:cytochrome c reductases were stimulated about two-fold (42). Furthermore, a duroquinone-stimulated NADH dehydrogenase was identified in maize root PM that was distinct from NADH:ferricyanide and NADH:ascorbate free radical reductase activities (86). This 27-kDa purified maize PM NADH-duroquinone reductase did not show absolute substrate specificity for quinones (85). These observations collectively suggest that PM redox proteins could reduce naturally occurring quinones that could then shuttle e\(^{-}\) between components of a PM redox chain or to dioxygen.

ORGANIZATION AND RELATIONSHIP OF REDOX COMPONENTS AT THE PM

PM redox chains can be expected to have domains or components capable of transferring e\(^{-}\) from reducing equivalents generated by cytoplasmic bioenergetic reactions across or within the plane of the membrane to apoplastic or cytosolic acceptors. It is also possible that e\(^{-}\) flow to PM proteins may be related to cytoplasmic redox changes.

Latency of Redox Activity

Studies of the pathway of e\(^{-}\) flow at the PM have benefited from the apparent right-side-out (RSO) orientation of vesicles isolated by aqueous two-phase partitioning. The orientation has been ascertained by electron microscopy with PM-specific stains such as phosphotungstic acid (38) and by the acute structural latency of the PM vanadate-sensitive ATPase, determined by comparison of measurements of enzyme activity in the absence and presence of detergents such as Brij 58 (100). It is possible to invert aqueous two-phase-isolated PM
ciency (23). The enzymatic Fe$^{3+}$ reduction step, and not the transport of Fe$^{2+}$ into the root, appears to be the rate-limiting factor in Fe uptake by roots of Fe-stressed (i.e., Fe-deficiency-stressed) plants (70). PM from various plant tissues reduce ferric chelates at appreciable rates; this even includes monocot roots which, in theory, do not require a reduction step for assimilation of Fe$^{3+}$ (34, 54). An enrichment or activation of redox activity has been observed in PM isolated from Fe-starved roots in all dicotyledonous species examined (35, 37, 42, 137) except Plantago (122).

The reduction rate for Fe chelates is always lower than that measured for ferricyanide reduction, and Fe chelate and ferricyanide reductase activities in PM from Fe-stressed roots can be separated by isoelectric focusing (77a). Contrary to reports measuring altered NADPH levels in roots of Fe-stressed beans (130), the tomato root Fe$^{3+}$ chelate reductase exhibits a distinct preference for NADH (35, 37, 137). A kinetic comparison of the PM enzymes from Fe-deficient vs Fe-sufficient dicot roots led to the conclusion that a constitutive reductase was amplified or activated under Fe stress (35, 42, 77a).

In addition to extensive characterization of the Fe$^{3+}$ chelate reductase, partial purification of the enzyme has been reported by several groups. An early report identified 25 and 34 kDa stress-associated polypeptides present in sugar beet PM (4). Brüggemann et al (35) used native gel electrophoresis of detergent-solubilized tomato root PM followed by SDS-PAGE to identify polypeptides of 100, 64, and 55 kDa associated with reductase enzyme-stained gel bands. Valenti et al (137) have used size exclusion and strong ion exchange chromatography to identify a lyso-PC solubilized protein of 70 kDa native molecular mass that exhibited ferricyanide reductase activity; this activity was amplified in PM fractions of Fe-stressed tomato PM. Using isoelectric focusing and Fe$^{3+}$ chelate reductase enzyme staining, Holden et al (77a) identified several isoforms of the Fe$^{3+}$ chelate reductase that were amplified in PM from Fe-stressed tomato plant roots. Polypeptides of 66, 34, and 27 kDa were reported to be associated with isoform activity in these PM fractions (77).

Recently, progress has been made toward identifying the genetic elements of the Fe stress response. As in dicots and nongraminaceous monocot plants, yeast cells exhibit amplified PM-localized Fe$^{3+}$ chelate reductase activity upon imposition of Fe-deficiency stress (50, 83). By complementing a yeast mutant lacking the reductase amplification response with DNA from wild-type cells, Dancis et al (55) were able to identify a nucleotide sequence (Fre-1) encoding either the reductase or an element controlling reductase activity in response to cellular Fe status. Nucleotide sequence analysis indicated that Fre-1 had transmembrane domains and sequence similarity to the b cytochrome heavy-chain component of the neutrophil respiratory burst oxidase (56).
after stimulation by elicitor molecules released following infection of potato tubers by the potato blight fungus Phytophthora infestans (58). However, no evidence has been provided for localization of the response at the PM. If an elicitor-activated $O_2^\cdot$ generating system does exist in the plant PM, it may require reconstitution of both cytosolic and membrane-bound components for activity, as observed for the neutrophil enzyme system (14).

**HYDROGEN PEROXIDE FORMATION** $H_2O_2$ has been the focus of hypotheses involving peroxidase-linked free-radical mechanisms leading to the polymerization of cell wall carbohydrates and proteins (e.g. 62). Evidence in support of $H_2O_2$ formation at the cell surface has led to the proposal that $H_2O_2$ generated at the plant PM in response to stresses such as pathogen attack may have an important function in lignification and/or structural protein polymerization that produces a barrier around the invaded area (27 and references therein).

$H_2O_2$ is a toxic species of activated $O_2$, and for this reason specific peroxidases with $H_2O_2$-scavenging capabilities (e.g. glutathione and ascorbate peroxidases, catalase) exist inside all living cells (e.g. 5). $H_2O_2$ is also an effective second messenger in animal cells, particularly with regard to insulin action (90). Thus, the production of $H_2O_2$ at the cell surface can lead to a cascade of reactions. The production of $H_2O_2$ by animal cell PM may provide a useful model for plant cells undergoing stress-related, defensive, or wound responses; this is particularly true with regard to peroxidase-mediated crosslinking of cell wall phenolics or structural proteins (28). Protein crosslinking mechanisms, catalyzed by peroxidases and using a PM-generated source of $H_2O_2$ occur in the zona pellucida proteins of sea urchin eggs (126, 134). In thyroid cells tyrosine residues on thyroglobulins are crosslinked into active hormones (61).

In what may be an analogous plant cell system, elicitation of plant suspension cells with fungal elicitors results in a rapid oxidative burst of $H_2O_2$ production (2, 140). Apostol et al (2) speculated that $H_2O_2$ production may be part of a signal transduction mechanism for coordination of cellular defenses. In support of this hypothesis, compounds such as mastoparan, which activate signal transduction pathways dependent on GTP-binding proteins, effectively mimicked the oxidative burst in soybean cells (81).

NAD(P)H-dependent "oxidase" activities that generate $H_2O_2$ have been well documented for PM isolated from a variety of tissues (6, 10, 92, 141). Furthermore, the activity of an NAD(P)H-dependent duroquinone-stimulated oxidase in PM vesicles from tobacco leaves was increased following infiltration of tobacco leaves by protein-lipopolysaccharide complexes extracted from pathogenic bacteria (136). Catalase stimulation of the activity was interpreted to indicate that $H_2O_2$ was produced by the enzyme during $e^-$ transfer to $O_2$ via
whether the potentiostat was on (i.e. no buildup of Fe(CN)$_6^{3-}$) or off (i.e. Fe(CN)$_6^{3-}$ was allowed to accumulate) (118). These results indicated that a biochemical process was involved in ferricyanide-induced acidification by mesophyll cells. It is still possible, however, that a requirement for charge balance in the bulk medium is an important component of acidification when Fe(CN)$_6^{3-}$ is not reoxidized by the coulometric device.

If the stimulation of H$^+$ excretion in the presence of ferricyanide is more closely related to biochemical events at the PM, redox activity may either lead to H$^+$ excretion by indirectly affecting the H$^+$/ATPase, or, in contrast, by processes similar to those of mitochondria and chloroplasts. H$^+$ excretion at these organelles is thought to be closely associated with redox activities of quinone and protein components in the membrane-bound e$^-$ transport chains (115).

Evidence that e$^-$ transport at the PM induces H$^+$ excretion by a mechanism similar to the mechanism of organelles may be inferred from data of Böttger & Hilgendorf (26). They showed that acidification of the medium around root segments was decreased by lowering the O$_2$ tension to a level that was still two orders of magnitude above the amount reported to inhibit cytochrome oxidase. If one assumes that O$_2$ is the natural acceptor for e$^-$ transport at the PM and that an oxidase exists at the PM with a higher $K_m$ than cytochrome oxidase, then the data are consistent with the hypothesis that this PM redox activity is important for H$^+$ excretion. However, ATP levels were not determined, and no evidence was presented to show that the lowered O$_2$ tension did not affect other cytosolic processes leading to H$^+$ excretion, exclusive of PM redox activity.

Indirect data using certain drugs provide further evidence that H$^+$ excretion at the PM is not solely dependent on H$^+$/ATPase activity. ATPase (15, 87) and mitochondrial e$^-$ transport (17) inhibitors as well as calmodulin antagonists (16) eliminate acidification by certain untreated cells, but not by cells exposed to oxidants such as ferricyanide or iridate (e.g. 15, 87). However, Fe(CN)$_6^{3-}$ accumulates in the medium after addition of the inhibitors, so it would be important to determine if the H$^+$ were traversing the PM merely in response to the need for charge balance in the bulk medium. This possibility could be tested with the coulometric device described earlier.

In an attempt to understand the role of redox agents in H$^+$ excretion processes closely associated with the PM, cultured carrot cells (18) and root segments (60, 87) were pretreated in various lipophilic e$^-$ donors (e.g. duroquinol, diphenylcarbazide, vitamin K$_3$). After washing, ferricyanide reduction was increased and, in carrot cells, ferricyanide-induced H$^+$ excretion occurred. However, it is difficult to draw any conclusions about quinone effects on H$^+$ excretion by the root cells, since some treatments led to alkalinations, some to acidifications, and some to both occurring sequentially (60,
But soluble phosphatase activity also increased in preparations from the same segments. It is not clear what causes the increase in ATPase and phosphatase activities and what role, if any, these activities play in the AFR effects.

In vitro work with isolated maize root PM has indicated that NADH-AFR oxido-reductase activity may have different properties from oxido-reductases that prefer other e\textsuperscript{−} acceptors (36), but the in vivo hyperpolarization of the onion cell membrane and the stimulation of H\textsuperscript{+} excretion were also induced by 1 mM AA and by 1 mM DHA added separately, although the kinetics were very different (69). Furthermore, the concentration of AFR, a very labile compound, was estimated from equimolar solutions of AA and DHA. It is likely that apoplastic enzymes (e.g. 41) as well as the pH of the wall space greatly affect the chemical equilibrium controlling the amount of AFR at the PM, especially over longer incubation periods. Finally, because some of the experiments were carried out under N\textsubscript{2} (69), the physiological condition of the cells in these experiments was altered compared to their usual aerated state.

Another type of interaction between redox agents and the H\textsuperscript{+}-ATPase was suggested by Elzenga et al (63). Using PM-enriched preparations, they showed that ATPase activity of Elodea is inhibited by NAD\textsuperscript{+}, NEM, and glutathione, and is stimulated by NADH. Because the H\textsuperscript{+}-ATPase has regulatory regions that contain SH groups (125), it is possible that the reduced pyridine nucleotides may activate this enzyme by affecting its redox state (24). Giannini & Briskin (66), however, observed no effect of NADH on Mg-ATP-induced H\textsuperscript{+} pumping into beet root PM vesicles.

Hormone Action

Attempts have been made to link the action of plant hormones such as cytokinins, gibberellins, and ethylene with e\textsuperscript{−} transport at the PM (reviewed in 19), but the data are largely fragmentary and inconclusive. However, more information has appeared regarding the role of auxins in relation to PM redox activity and growth mechanisms. Concentrations of cis-platin and p-nitrophenylacetate that retarded NADH:ferricyanide oxido-reductase activity in soybean hypocotyl segments inhibited control growth by only 10–20%, but these agents reduced the increment of 2,4-D-induced growth by 55% (94). Because the mode of action of cis-platin and p-nitrophenylacetate is unknown, however, one cannot be sure they are affecting only redox activity in intact tissues. What is more, there appears to be no effect of 2,4-D on NADH:ferricyanide oxido-reductase activity in preparations of PM (31).

A relationship between redox activity at the PM and auxin action is suggested by growth stimulations of onion root cells by AFR, a result of cell elongation rather than the production of new cells (74). AFR, however, has no significant effect on growth of soybean hypocotyl segments (94). It has already been pointed out that the AFR-induced acidification by onion cells can
PHOTOSYNTHETICALLY ACTIVE RADIATION (PAR). Much evidence exists from action spectra, specific inhibitors of photosynthesis, and photosynthetically incompetent leaves that activation of photosynthesis leads to a stimulation of trans-PM e⁻ transport to ferricyanide (116). While the close physical association between chloroplast and PM does not preclude some sort of direct communication between the two, the timing of the light-induced response indicates that a chemical signal is more likely to be responsible (114, 118). Suggestions have been made that the chemical component is NAD(P)H produced by cytoplasmic reactions using intermediates transported from the chloroplast (116).

Because reactions at the chloroplast can control PM redox activity assayed with ferricyanide, it is valid to ask whether this activity is involved with some of the effects of photosynthesis on cell biochemistry and development when an exogenous oxidant such as ferricyanide is not present. Using the patch-clamp apparatus in the whole-cell configuration with guard cell protoplasts, Serrano et al (124) proposed that a reduced intermediate, supplied by photosynthetic reactions, was an important component of light-induced H⁺ excretion. It was possible that the reduced compound initiated e⁻ transport at the PM, which then activated an H⁺-ATPase. Redox activity has been detected at the guard cell PM (101, 113, 139).

The patch-clamp technique also demonstrated that white light increased K⁺ channel activity in PM of Arabidopsis mesophyll cells, and this effect was mimicked by 1 mM DTT or 10 mM mercaptoethanol. Similar results with reducing agents were obtained using yeast PM (21), thus leading to the possibility that channel activity is regulated by endogenous reductants; sulphydryl groups on the channel proteins have been suggested as possible regulatory sites (131). Still to be determined, however, is whether DTT and mercaptoethanol at the concentrations used are satisfactory substitutes for the physiological conditions normally resulting in the light. Also unknown is the role, if any, played by e⁻ transport at the PM in the putative reduction of proteins associated with ion channels.

BLUE LIGHT The LIAC mentioned above (in the section on b-type cytochromes) has aided in characterizing cytochrome components of the PM, but the possibility exists that it is also part of a signal-transduction chain for blue light. Arguments for and against a role for the LIAC in fungal and higher-plant signal transduction have been summarized recently (116). It has also been reported that blue light stimulates tetrazolium reduction at the PM of guard cells (108, 138) and ferricyanide reduction at the PM of mesophyll cells (57, 114). The latter effect of blue light on trans-PM redox activity only occurred in segments pretreated with sphingosine or certain of its analogs—compounds that
SUMMARY AND CONCLUSIONS

Compounds capable of redox activity are present in PM-enriched preparations. There is direct evidence for the presence of $b$-type cytochromes, and indirect evidence for flavins, flavoproteins, and quinones. Measurements of redox activity in vivo and in PM-enriched preparations have pointed to the presence of at least two pyridine nucleotide oxidases and several NAD(P)H:ferricyanide and quinone reductases. There may also be a separate NADH:AFR reductase. The relationship between components of the various redox systems, i.e., whether they are shared or whether each system operates independently, is not known. The natural ultimate acceptor(s) may be Fe-chelates in some cases, and $O_2$ may be an acceptor in other systems.

There seems to be a clear role for trans-PM redox activity in the uptake of Fe by dicots and nongrainaceous monocots. In this case, PM-localized redox activity is always present, but the activity is increased by Fe starvation. No convincing evidence exists for expression of a novel redox system during Fe deficiency.

If $O_2$ were a natural, physiological acceptor for PM redox activity, then certain forms of active $O_2$ might be an end product. Evidence exists that $O_2$, $H_2O_2$, or both are produced at the cell surface after exposure to certain elicitors in a manner analogous to that seen during trans-PM redox activity in human neutrophils. $H_2O_2$ in the apoplast of the plant cell could then play a role in defense against disease and possibly in growth, although it must still be rigorously demonstrated that $H_2O_2$ is involved in these responses.

Exposure of various plant cells to certain impermeant oxidants accelerates acidification of the medium, thus implicating redox activity in $H^+$ excretion. There may be a separate, redox-associated pathway for $H^+$ or a redox activation of the $H^+$-ATPase. The ongoing debate concerning the nature of the $H^+$ excretion pathway during trans-PM $e^-$ transport should not obscure an important point: It has not been determined whether redox activity is involved with $H^+$ excretion when artificial redox agents are absent.

Growth-active auxins stimulate the NADH oxidase activity of purified PM vesicles. Furthermore, parallels have been noted between effects on this oxidase activity and effects on growth. Even with these observations, a causal relationship between an oxidase at the PM and auxin-induced elongation must be considered very tentative.

PAR can increase trans-PM $e^-$ transport, presumably by increasing the supply of a cytosolic donor. It is not known whether this redox activity participates in PAR effects on the PM. Blue light causes reduction of a $b$-type cytochrome in PM-enriched vesicles as well as a stimulation of $e^-$ transport across the PM of intact cells to ferricyanide. As yet, these observations have
ize redox-associated phosphorylation/dephosphorylation reactions in plant cells and PM vesicles.

Finally, evidence has been presented that GTP binding proteins exist in plant cells (e.g. 103) and that blue light can affect GTP binding (143). Furthermore, G-proteins may activate trans-PM redox activity in human neutrophils (14, 107) and participate in the elicitation of plant defense responses (81). Thus, it will be important to investigate interactions between GTP-binding proteins and redox reactions at the plant PM.

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