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CHLOROPLAST AND MITOCHONDRIAL MECHANISMS FOR PROTECTION AGAINST OXYGEN TOXICITY

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As a consequence of their oxygen rich environment, organelles of photosynthetic tissues are exposed to large fluxes of oxyradicals and reactive oxygen species. Superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen are all potential by-products of respiratory and photosynthetic systems. Strong reductants found in mitochondria and chloroplasts along with a steady flux of photosynthetically generated oxygen enhance the potential for oxyradical production. Unless neturalized by scavenger substrates or enzymes, these reactive intermediates pose a lethal threat.

The presence of superoxide dismutases, catalases, various peroxidases and scavenger substrates are all means of defences available to protect organelles. A balance between oxyradical production and neutralization should exist. Perturbations in generation or in sequestration caused by environmental or nutritional factors might profoundly alter the steady state level of oxyintermediates.

KEY WORDS: Oxyradical, superoxide dismutase, peroxidase, oxygen.

INTRODUCTION

Although molecular oxygen is not toxic, by-products of its metabolism such as oxyradical intermediates are reactive and pose a potential for cellular damage. Among the oxyintermediates known to cause deleterious effects are superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen. Microenvironments enriched in oxygen, such as those found in photosynthesizing organisms, are regions in which the potential for oxygen mediated damage is greatest. Survival in an environment containing potentially lethal molecules would necessitate the elaboration of defensive proteins, enzymes, or substrates. Superoxide dismutases, catalases, peroxidases and various low molecular weight reductants play a role in neutralizing harmful oxygen by-products.

OXYINTERMEDIATE PRODUCTION BY PLANT ORGANELLES

Chloroplast

The choloroplast with its strong photoxidative potential is the most likely organelle to examine for significant oxyintermediate production. A consumption of oxygen by illuminated chloroplasts was first demonstrated by Mehler.[1] Oxygen consumption is associated with photosystem I, and probably is related to the autoxidation of reduced ferredoxin. Ferredoxin undergoes oxidation, presumably in a 1 electron step, when

the availablitiy of $NADP^+$ is low. The reaction could be visualized as: $Ferredoxin_{reduced} + O_2 \rightarrow Ferredoxin_{oxidized} + O_2^-$.

The resulting superoxide could either spontaneously dismute or be enzymatically converted to H_2O_2. Both O_2^- as well as H_2O_2 have been shown to be produced by illuminated intact chloroplasts.[1,2] Perturbations to the photosynthetic electron transport system, i.e. adding ascorbate as the photooxidant or flavins, viologen dyes and phenols as acceptors resulted in oxygen consumption and the generation of superoxide and hydrogen peroxide.[3,4] Methyl viologen, because of its widespread herbicidal use, assumes a physiological importance in oxyradical and oxyintermediate production. Under intense illumination and high oxygen tensions, fluxes of oxygen radicals and active intermediates could result in toxicity to organelles or cells.[4,5-7]

Oxyintermediate production from the photosystem II side of the electron transport chain is possible at the quinone site. In mitochondria, the autoxidation of ubisemiquinone resulted in O_2^- production.[8] The addition of dibromothymoquinone to illuminated chloroplasts resulted in hydrogen peroxide formation emanating from the region of the plastoquinone-Rieske iron-sulfur center.[4] However, a direct oxidation of plastoquinone to generate superoxide and hydrogen peroxide has not been demonstrated.

Superoxide produced by chloroplasts could reduce components of the photoelectron transport chain such as cytochrome *b* or plastocyanin. Alternately, dismutation to produce H_2O_2 with further metal catalyzed reduction of H_2O_2 to the strong oxidant $OH\cdot$ is feasible. The later scenario would carry with it a potential toxicity.

Photodynamic generation of singlet molecular oxygen has been shown to occur under conditions of intense illumination, low CO_2 tensions, the presence of uncouplers and blockage of the photosynthetic electron transport system.[4,5,7,9] A simplified depiction of the process can be seen in the following equations whereby the photoexcited chlorophyll imparts its energy to dioxygen:

$$S_0 + h\nu \dashrightarrow S_1$$

$$S_1 \dashrightarrow T_1$$

$$T_1 + {}^3\Sigma O_2 \dashrightarrow S_0 + {}^1\Delta gO_2$$

where S_0 and S_1 are the ground and first excited singlet state of the chlorophyll molecule and T_1 represents the triplet state. Alternatively, singlet oxygen might be formed from the reaction of superoxide and hydrogen peroxide.[10]

Mitochondria

The termination of the mitochondrial electron transport chain is characterized by the tetravalent reduction of oxygen to water by the cytochrome a complex. There is no evidence of oxyintermediates being formed at this locus. However, superoxide, hydrogen peroxide and hydroxyl radical have been shown to be associated with the oxidation of various ubiquinone complexes, and perhaps cytochrome b_{566}.[4,8,11,12] Formation of oxygen by-products would be enhanced when electron flow is blocked or restricted. This would favor oxidation of the electron transport chain components by oxygen, resulting in the formation of O_2^-, H_2O_2 and $OH\cdot$. Flohe *et al.*[12] have shown that univalent reduction of oxygen predominates in mitochondria when electron flow is blocked. Therefore, hydrogen peroxide and hydroxyl radicals are presumably formed as a result of superoxide dismutation and further reaction of superoxide with hydrogen peroxide.

Plant mitochondria are unique because they possess a cyanide insensitive alternate respiratory pathway. The exact physiological role of this metabolism is still a mystery but several hypotheses have been put forth to rationalize its existence. Among proposed roles for the alternate pathway are the following: thermogenesis, fruit ripening, ethylene production by wounded tissue, and adaptation to an environment enriched in CN^-.[4,11,13] Two additional roles for the alternate pathway might involve a means of meeting energy requirements with minimal ATP production when electron transport is not fully operational or insuring that a sufficient supply of oxidized pyridine nucleotides are available during times of "energy overload".[13,14] The alternate pathway provides an additional means for the generation of active oxygen intermediates, although at present the exact loci are not known. Possible sites include ubiquinone and *b*-type cytochromes.[4,11] Moreover, the CN^- resistant lipoxygenase system of developing seeds is capable of generating oxygen intermediates in conjunction with the alternate pathway of respiration.[11] Model systems have shown that peroxy radicals, such as those generated by unsaturated fatty acid peroxidations, readily react with reduced quinones. In the presence of O_2 the semiquinones could yield superoxide and hydrogen peroxide.

Superoxide generation by mitochondria portends complex repercussions. Superoxide could reduce various components of the electron transport chain such as cytochromes *b* or *c*. The loss of a potential ATP through a bypass of the phosphorylation site might be a consequence of this mechanism. An alternate and potentially more damaging path would be through the reaction of superoxide and hydrogen peroxide to generate the strong oxidizing species, $OH^{\cdot}$. The resulting membrane destruction through direct and indirect actions of the radical species would be obvious. Mechanisms to neutralize oxygen by-products must be in place in order to insure that organelles survive the hazards of life in an oxygen atmosphere.

DEFENSIVE SYSTEMS

Chloroplast

Chloroplastic enzymatic systems which minimize the toxicity of oxyintermediates consist of superoxide dismutases and peroxidases. The presence of low molecular weight endogenous reductants and scavengers such as glutathione and carotenoids further enlarge the sphere of protection.

All three isozymes of superoxide dismutase (Cu-Zn,Mn, and Fe) are present in plants.[4,7,15] Every organelle contains at least one of the superoxide dismutases and are therefore provided a means of neutralizing superoxide radicals. The Cu-Zn form of the enzyme is the predominant plant cytosolic superoxide dismutase.[4,7,15,16] The Cu-Zn enzyme in the plant cytosol has recently been shown to be immunologically distinct from the chloroplastic form of the Cu-Zn protein.[17] In addition, there is recent evidence showing the cytosolic enzyme in rice might function in a monomeric form.[17]

Chloroplasts contain superoxide dismutases but depending upon the species, one, two or possibly all three of the isozymes might be present. The Cu-Zn enzyme is found in the soluble, stromal, fraction as well as in association with the thylakoid membrane.[4,7,15,18] The ubiquitous Cu-Zn enzyme should serve to effectively scavenge superoxide anions except under conditions of potential inactivation by excessive peroxide or elevated cyanide levels. The presence of alternate forms of the enzyme would therefore be advantageous.

Although a thylakoid associated, Mn— containing superoxide dismutase has been found in cyanobacteria and green algae, the presence of this enzyme in higher plant chloroplasts has been questioned as a presumed artifact of the assay system.[4,15]

The iron-containing superoxide dismutases, once thought to be restricted to prokaryotic organisms, are now known to be in at least five families of higher land plants in addition to *Euglena*.[4,7,15,19] The plant and euglenoid enzymes are similar in many physical aspects to the well studied procaryotic proteins.[15] In algae, *Euglena* and higher plants, the iron proteins are found in the soluble fraction.[4,15,20] Whereas a chloroplastic association of the Fe-containing superoxide dismutase has not been demonstrated in *Euglena*, the protein is found in the chloroplast stroma of higher plants.[15,20] The enzyme is not present in mitochondria and only small amounts have been detected in the cytoplasm by immunomicroscopic means.[15,21]

It would therefore appear that in regard to higher plant chloroplasts, the Cu–Zn form of superoxide dismutase predominates as the stromal enzyme. In certain plant families, a Cn^- resistant, Fe-containing protein is found. The presence of a Mn-containing superoxide dismutase which is specifically associated with chloroplasts remains questionable.

Removal of H_2O_2 in biological systems is accomplished through the actions of peroxidases and catalases. Although abundant in plant tissue, catalase is apparently absent in chloroplasts and therefore, disposition of peroxide is relegated to peroxidases.[22,23] Of major importance to the chloroplast in peroxide removal is the ascorbate cycle in which ascorbate is peroxidized and then reduced.[4,15,23] The following reactions could account for ascorbate mediated peroxide removal:

$$\text{Ascorbate} + H_2O_2 \dashrightarrow \text{Dehydroascorbate} + 2H_2O$$

$$\text{Dehydroascorbate} + 2GSH \dashrightarrow \text{Ascorbate} + GSSG$$

$$GSSG + NADPH + H^+ \dashrightarrow 2GSH + NADP^+$$

Reduced pyridine nucleotide phosphate is generated by the photosynthetic electron transport chain. Therefore, H_2O_2 is removed by photosynthetically generated NADPH.

Low molecular-weight cellular compounds might play a significant role in removal of toxic oxygen species in the chloroplast. Glutathione and ascorbate have been mentioned in relation to peroxidations. In addition, hydroquinones and carotenes are known to react with O_2^-.[24] Due to the high reactivity of the hydroxyl radical,[4,7,9,15,23,24] almost any available substrate could serve as a target. Protective substrates might become radicalized thereby propagating chain reactions. This might prove toxic to an organism if reacting substrates are integral membrane components or key metabolic intermediates. If scavenging substrates are not replenished, the consequences could be lethal. Therefore, it might be difficult to distinguish those effects that protect the cell from those which appear to protect but are deleterious in the long run.

Mitochondria

Plant mitochondria like their animal counterparts possess a Mn-containing superoxide dismutase.[7,25,26] The enzyme is localized in the mitochondrial matrix and would thus serve as an internal oxyradical scavenger. The intermembraneous space possesses a Cu–Zn containing superoxide dismutase[25,26] thereby acting as a trap for O_2^- that would be directed toward the outside of the organelle. There is no evidence

of an association of the iron-containing superoxide dismutase with mitochondria.[26]

Like chloroplasts, mitochondria do not contain appreciable amounts of catalase. Sequestration of H_2O_2 is therefore a peroxidative function. Glutathione would be peroxidized either through a specific glutathione peroxidase or a mitochondrial peroxidase with broad substrate specificity:[12,23,27]

$$2\,GSH + H_2O_2 \longrightarrow 2H_2O + GSSG.$$

The cycle would be complete with the regeneration of reduced glutathione by a glutathione reductase:

$$GSSG + NAD(P)H \longrightarrow 2GSH + NAD(P)^+.$$

Alternately it might be possible to envisage a cycle whereby a mitochondrial cytochrome *c* peroxidase[28] might utilize H_2O_2 to oxidize reduced cytochrome *c*:

$$2\ cyt\ c^{+2} + H_2O_2 \longrightarrow 2\ cyt\ c^{+3} + 2H_2O.$$

Cytochrome c^{+3} might then be reduced by O_2^-:

$$2\ cyt\ c^{+3} + 2O_2^- \longrightarrow 2\ cyt\ c^{+2} + 2O_2.$$

This cycle of peroxidation and reduction of cytochrome c would serve to remove both H_2O_2 and O_2^- that might be generated in mitochondria.

Finally, as in chloroplasts, the presence of endogenous non-enzymatic molecules might play a role in preventing significant accumulation of oxyintermediates. Hydroquinones, carotenoids or thiol groups could serve as substrates for the highly reactive

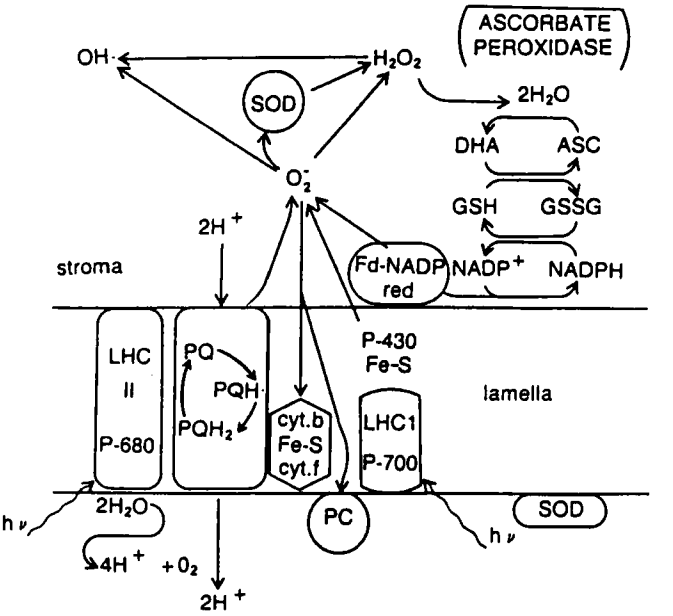

FIGURE 1 Scheme for the production and sequestration of active oxygen intermediates in chloroplasts. ASC, ascorbate; DHA, dehydroascorbate; Fd, ferredoxin; GSH, reduced glutathione; GSSG oxidized glutathione; PC, plastocyanin; PQ, plastoquinone; LHC1, light harvesting complex I; LHCII, light haversting complex II. The scheme was modified from Rabinowitch and Fridovich.[7]

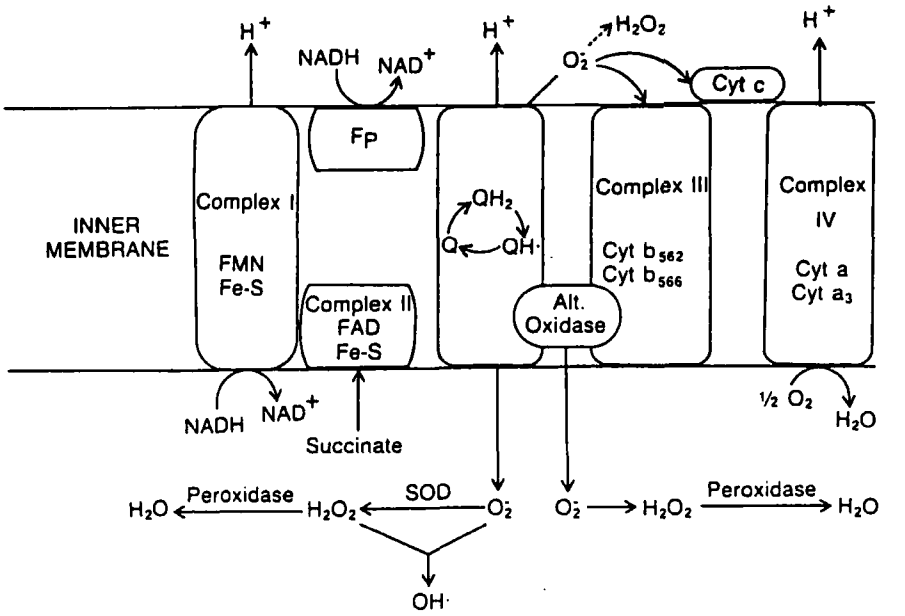

FIGURE 2 Scheme for the production and sequestration of active oxygen intermediates in mitochondria. Alt. oxidase, alternate oxidase; F_p, flavoprotein; Q, ubiquinone.

$OH^{\cdot}$. Singlet oxygen traps consist of fatty acids, metal complexes, sulfides, carotenoids or α-tocopherol. It should be noted however, that by scavenging oxygen radicals, these mitochondrial and chloroplastic substrates might also become "radicalized" thereby propagating a chain of peroxidations. Moreover, should the substrates be consumed without adequate replenishment, the consequences could be deleterious to the organelle.

CONCLUSIONS

In estimating oxyradical production in chloroplasts and mitochondria, interactive systems of generation and sequestration should be recognized. Perturbations brought about by environmental or nutritional factors might alter these relationships. Figure 1 shows a simplified scheme of presumed oxyradical production and scavenging in the chloroplast. Superoxide is produced by plastoquinone and by the strong reductant of photosystem 1. Possible reactions of O_2^- include reduction of the electron transport carriers or the disproportionation to generate H_2O_2. Reaction of superoxide and hydrogen peroxide would generate hydroxyl radical. Omitted, for simplicity, is the singlet oxygen generating capacity of chlorophyll molecules as well as singlet oxygen generation through oxyintermediate interaction. Also omitted is active oxygen production by exogenous autoxidizable substrates such as methyl viologen. Protective systems include superoxide dismutase and ascorbate peroxidase.

Figure 2 is a scheme depicting oxyradical production in mitochondria and the endogenous protection available. Superoxide generation is shown emanating predo-

minantly from ubiquinone. Alternate oxidase involving O_2^- production is also shown. Possible reactions involving superoxide include: dismutation to form H_2O_2 and the reduction of certain cytochromes. The production of $OH^\cdot$ from H_2O_2 and O_2^- is also depicted. Scavenging reactions include the superoxide dismutase sequestering of O_2^-, the peroxidation of H_2O_2 by peroxidases, as well as a superoxide and hydrogen peroxide sequestration via a cycle of cytochrome peroxidase and O_2^- mediated cytochrome *c* reduction.

The schemes depicted in *F*igures 1 and 2 were simplified for convenience. However, their simplicity also demonstrates that we are only at the early stages of understanding mechanisms of protective enzyme and metabolite control in relation to toxic by-products of oxygen.

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