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Role of Autooxidation of Protoporphyrinogen IX in the Action Mechanism of Diphenyl Ether Herbicides

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Protoporphyrinogen oxidase (EC 1.3.3.4), the last common enzyme in the biosynthesis of heme and chlorophyll[1,2] catalyzes the conversion of protoporphyrinogen IX into protoporphyrin IX by six-electron oxidation. It has generally been accepted that protoporphyrinogen oxidase is the primary target of diphenyl ether herbicides such as oxyfluorfen and acifluorfen. Protoporphyrinogen oxidase is known to be localized in the plastid envelope.[3] Paradoxically, *in vivo*, the inhibition of protoporphyrinogen oxidase by diphenyl ether herbicides leads to massive accumulation of protoporphyrin IX, the product of enzymatic reaction rather than the substrate.[4,5] It has been demonstrated that phytotoxic herbicidal effect of diphenyl ether herbicides is due to the abnomal accumulation of protoporphyrin IX which is known as a strong photosensitizer in the presence of light and and molecular oxygen, generating singlet oxygen. Singlet oxygen triggers photodynamic membrane lipid peroxidation and ultimate cellular death.[5] However, the mechanism by which protoporphyrinogen IX is converted to protoporphyrin IX *in vivo* is not clear. Two feasible explanations for the massive accumulation of protoporphyrin IX upon the herbicide inhibition of protoporphyrinogen oxidase have been be suggested: First, a non-enzymatic autooxidation of the protoporphyrinogen IX to protoporphyrin IX has been assumed since the protoporphyrinogen IX can readily be oxidized under certain conditions.[6] Second, an enzymatic oxidation of protoporphyrinogen IX by a herbicide-resistant plasma membrane associated peroxidase-like activity has recently been proposed.[2] In both cases, the substrate of protoporphyrinogen oxidase should be exported from the plastid to the plasma membrane and rapidly oxidized to protoporphyrin IX in the membrane.

In an effort to examine the role of non-enzymatic autooxidation for the accumulation of protoporphyrin IX in diphenyl ether-treated plants, we investigated the oxyfluorfen inhibition of protoporphyrinogen oxidase in barley etiochloroplasts, and effects of ionic strength and ethyl alcohol on the non-enzymatic oxidation rate of protoporphyrinogen IX. Barley etiochloroplasts were obtained according to the methods of Lee *et al.*[2] and briefly passed through Biogel P-30 gel filtration column to remove salts and used for the assay without further purification. The substrate, protoporphyrinogen IX, was prepared by the reduction of protoporphyrin IX with sodium amalgam[7] and stored in liquid nitrogen under dark condition. Protoporphyrinogen oxidase was assayed following the procedure of Sherman *et al.*.[8]

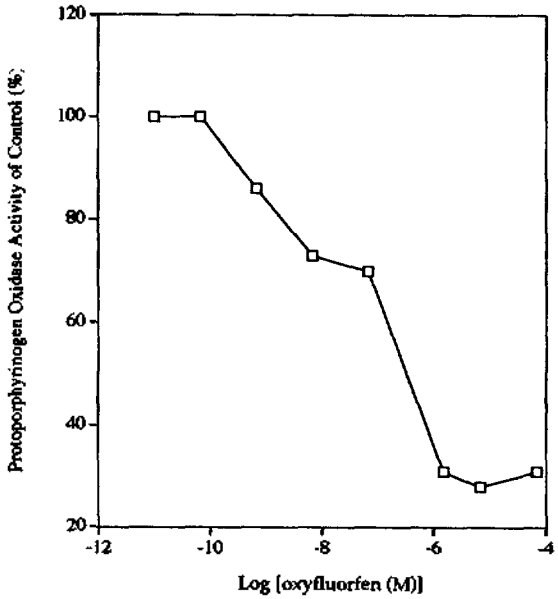

Figure 1. Effect of oxyfluorfen on the protoporphyrinogen oxidase activity of barley etiochloroplasts measured under initial velocity conditions. Reaction mixture consisted of 100 mM HEPES (pH 7.5), 5 mM EDTA, 1% Tween-20, and 2 mM DTT in 3 mL. The reaction was started by adding 300 uL of 200 uM substrate and 120 uL of etiochloroplast (0.62 mg/mL of protein). Fluorescence intensity was monitored using spectrofluorometer at 626 nm with excitation at 395 nm.

Figure 1 shows the oxyfluorfen inhibition of protoporphyrinogen oxidase of barley etiochloroplasts. As consistent with previous results of others,[3,5,6] the inhibition by oxyfluorfen was remarkable and I_{50} was about 0.5 uM. This clearly supports the idea that the inhibition of protoporphyrinogen oxidase by oxyfluorfen would cause the accumulation of protoporphyrinogen IX molecules, which diffuse out of their site of biosynthesis until they reach to plasma membrane. Once protoporphyrinogen IX molecules arrive at the plasma membrane, they would be oxidized by enzymatic and/or non-enzymatic reaction.[9] In order to address the importance of non-enzymatic oxidation of protoporphyrinogen IX, we examined whether hydrophobic environment, simulated by addition of ethyl alcohol and lowering ionic strength, has any effects on the non-enzymatic oxidation rate of protoporphyrinogen IX. As seen in Figure 2 and 3, non-enzymatic oxidation rates of protoporphyrinogen IX were highly dependent on concentrations of ethyl alcohol and ionic strength of reaction mix-

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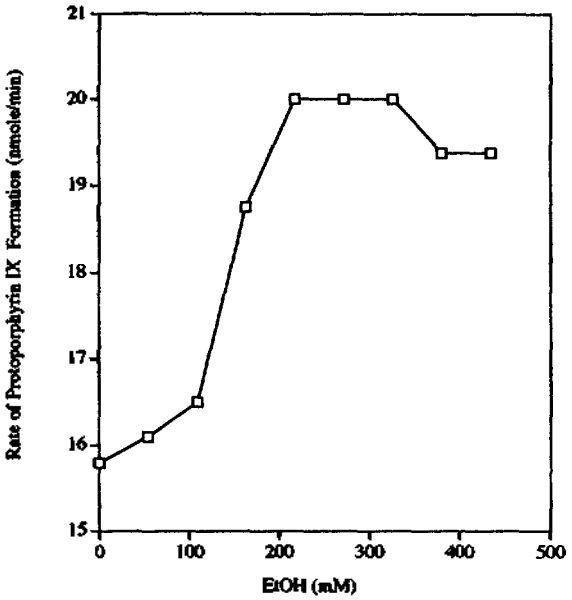

Figure 2. Effect of ethyl alcohol on the non-enzymatic oxidation of protoporphyrinogen IX. Experimental condition was the same as in Figure 1 except that the reaction mixture did not contain enzyme and Tween-20.

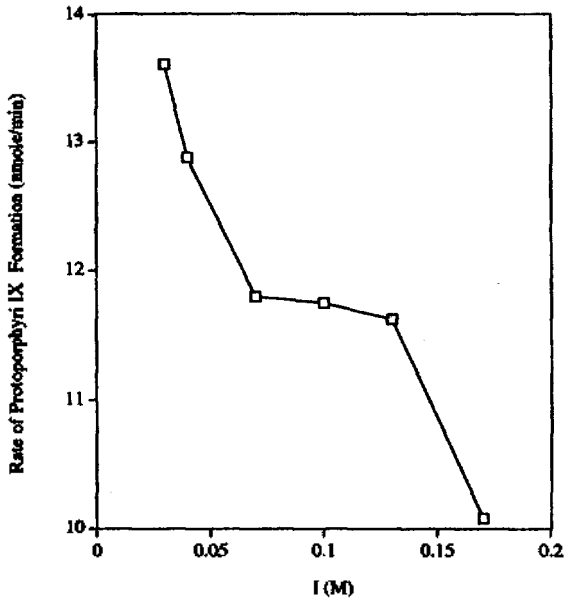

Figure 3. Effect of ionic strength on the non-enzymatic oxidation of protoporphyrinogen IX. Ionic strength was adjusted by adding appropriate amount of sodium chloride. Experimental condition was the same as in Figure 2.

ture. Ethyl alcohol increased the oxidation rate and the oxidation was more favoured in lower ionic strength. These results indicate that the non-enzymatic oxidation of protoporphyrinogen IX can be accelerated under hydrophobic conditions such as low ionic strength and high concentration of ethyl alcohol. Particularly, this rate enhancement was more apparent when fatty acid such as palmitate was added to the reaction mixture.[10] Since protoporphyrin IX is known to be more hydrophobic than protoporphyrinogen IX,[11] the transition state for the oxidation of protoporphyrinogen IX could be product-like and the oxidation would be accelerated by stabilization of the transition state under hydrophobic environments such as interior of the plasma membrane. In addition, the hydrophobic nature of the transition state would hinder the oxidation of accumulated protoporphyrinogen IX in hydrophillic environments such as cytosol and help to transport the accumulated protoporphyrinogen IX to the plasma membrane without being oxidized and then the facilitated oxidation occurs in the plasma membrane. In conclusion, protoporphyrinogen IX produced as a consequence of the inhibition of protoporphyrinogen oxidase in diphenyl ether-treated plants moves to the plasma membrane presumably as an unprocessed form and undergoes oxidation in the plasma membrane by non-enzymatic reaction as well as herbicide resistant potox-like isozyme.

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