IDENTIFICATION OF INTERMEDIATE SUBSTRATE FREE-RADICALS FORMED DURING PEROXIDATIC OXIDATIONS, BY ELECTRON PARAMAGENTIC RESONANCE SPECTROSCOPY

I. Yamazaki and H. S. Mason

Department of Biochemistry, University of Oregon Medical School, Portland

L. Piette

Instrument Division, Varian Associates, Palo Alto, Cal.

Received December 4, 1959

We have observed formation of free-radicals during peroxidatic oxidations of ascorbic acid, dihydroxyfumaric acid, and hydroquinone. The observations were made with a Varian V-4500 x-band spectrometer, using 100 k.c. field modulation and a sample cell 0.9" x 0.5" x 0.01" attached to a flow system for kinetic measurements and positioned to avoid dielectric losses due to water at 25° C. Our enzyme was recrystallized Japanese turnip peroxidase (R.Z. = 2.7). Acetate buffer and dihydroxyfumaric acid were specifically purified to avoid autoxidation*.

Peroxidatic oxidation of ascorbate at pH 4.8 produced a free-radical characterized by a sharp doublet with a splitting of 1.7 gauss, g = 2.0043. Peroxidatic oxidation of dihydroxyfumarate gave a free-radical with a single asymmetric line, while the peroxidatic oxidation of hydroquinone produced the semiquinone with a five-line hyperfine pattern identical with that obtained during the autoxidation of hydroquinone (Blois, 1955).

The rates of enzyme-catalyzed free-radical formation were too high to be measured by the present technique, but the steady state free-radical

* For which, we are grateful to Dr. F. Anan.

concentration from peroxidatic oxidation of ascorbic acid was measured as a function of substrate, enzyme, and hydrogen peroxide concentrations at constant flow rate (Table 1). These steady state free-radical concentrations were consistent with the expression:

$$\left[AH\cdot\right]_s = \left(\frac{k_3\left[AH_2\right]\left[\text{Complex II}\right]}{k_d}\right)^{1/2}$$

where $\left[AH\cdot\right]_s$ is the steady state concentration of free-radical, $\left[AH_2\right]$ is the concentration of ascorbic acid, k_3 is the rate constant for oxidation of AH_2 to AH· by Complex II, and k_d is the rate constant for free-radical decay. This relationship appears to fit the mechanism of peroxidatic oxidations proposed by Saunders (Saunders and Mann, 1940), George (George, 1953), and Yamazaki (Yamazaki, 1957).

Table 1

Free-radical concentrations produced during the peroxidatic oxidation of ascorbic acid in acetate buffer, pH 4.8, 0.1 M, at 25° C.

Peroxidase, M	H_2O_2, M	Ascorbic Acid, M	Free-Radical, M
1×10^{-8}	2×10^{-2}	2×10^{-2}	1.7×10^{-6}
4×10^{-8}	2×10^{-2}	2×10^{-2}	3.6×10^{-6}
1.6×10^{-7}	2×10^{-2}	2×10^{-2}	7.2×10^{-6}
4×10^{-8}	2×10^{-2}	5×10^{-3}	1.1×10^{-6}
4×10^{-8}	5×10^{-3}	2×10^{-2}	3.0×10^{-6}

Free-radicals have thus been detected and identified, for the first time, in enzyme-catalyzed oxidations of substrates. A complete account of these experiments has been submitted for publication. We propose to carry out more detailed examination of the systems here described, and to extend our studies to other peroxidases and oxidases.

REFERENCES

Blois, M.S., J. Chem. Phys., 23, 1351 (1955).

George, P., Biochem. J., 54, 267 (1953).

Saunders, B.C. and Mann, P.J.G., J. Chem. Soc., 769 (1940).

Yamazaki, I., Proc. Internat. Symp. Enzyme Chem., 224 (1958).