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THE HORSERADISH PEROXIDASE CATALYSED OXIDATION OF DEOXYRIBOSE SUGARS

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The ability of horseradish peroxidase (E.C. 1.11.1.7, Donor: H_2O_2 oxidoreductase) to catalytically oxidize 2-deoxyribose sugars to a free radical species was investigated. The ESR spin-trapping technique was used to demonstrate that free radical species were formed. Results with the spin trap 3,5-dibromo-4-nitrosobenzene sulphonic acid showed that horseradish peroxidase can catalyse the oxidation of 2-deoxyribose to produce an ESR spectrum characteristic of a nitroxide radical spectrum. This spectrum was shown to be a composite of spin adducts resulting from two carbon-centered species, one spin adduct being characterized by the hyperfine coupling constants a^N = 13.6 G and a_β^H = 11.0 G, and the other by a^N = 13.4 G and a_β^H = 5.8 G. When 2-deoxyribose-5-phosphate was used as the substrate, the spectrum produced was found to be primarily one species characterized by the hyperfine coupling constants a^N = 13.4 G and a_β^H = 5.2. All the radical species produced were carbon-centered spin adducts with a β hydrogen, suggesting that oxidation occurred at the C(2) or C(5) moiety of the sugar. Interestingly, it was found that under the same experimental conditions, horseradish peroxidase apparently did not catalyze the oxidation of either 3-deoxyribose or D-ribose to a free radical since no spin adducts were found in these cases.

KEY WORDS: ESR, spin trapping, horseradish peroxidase, deoxyribose sugars.

ABBREVIATIONS: HRP, horseradish peroxidase; DBNBS, 3,5-dibromo-4-nitrosobenzene-sulphonic acid.

INTRODUCTION

Horseradish peroxidase (HRP) has been widely used to investigate the peroxidase-catalysed oxidation of xenobiotics. Typically, peroxidases have a high turnover number, are generally non-specific and thus have a wide range of substrates. HRP-catalysed reactions almost serve as a definition of all peroxidase-catalysed reactions.[1] HRP catalyses the oxidation of a wide variety of substrates by hydrogen peroxide. The generally accepted mechanism for the enzymatic cycle is as follows, where AH_2 is the substrate:

$$\text{HRP} + H_2O_2 \rightarrow \text{HRP-compound I} + H_2O$$

$$\text{HRP-compound I} + AH_2 \rightarrow \text{HRP-compound II} + \cdot AH$$

$$\text{HRP-compound II} + AH_2 \rightarrow \text{HRP} + \cdot AH$$

The ability of a variety of peroxidases to oxidize chemical carcinogens and drugs to free radical species has recently received considerable attention.[2,3] Yamazaki[4] can be credited with providing the criteria necessary to prove that a free radical has been

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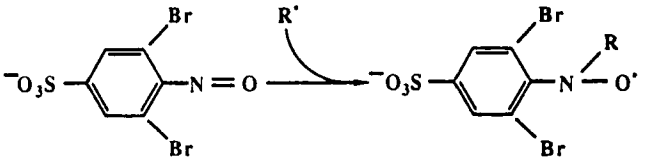
formed. By definition, the free radical species must be formed free of the enzyme. This makes it possible to detect the species by a variety of techniques, including ESR.

Surprisingly, so far no one has reported on the ability of HRP to catalyse the oxidation of nucleic acids or their constituent nucleotides. Free radical-induced strand breaks in DNA are thought to be initiated by free radical reactions at the sugar moiety.[5] In this study the ability of HRP to catalyse the oxidation of deoxyribose sugars was investigated using the ESR technique of spin trapping. This technique is used when it is desirable to detect free radials that have short lifetimes. The radical ($R\cdot$) is generated in the presence of the spin trap. The resultant spin adduct is a relatively stable nitroxide free radical which can be detected by conventional ESR spectroscopy. In this paper we present data obtained with the spin trap 3,5-dibromo-4-nitrosobenzene-sulphonic acid (DBNS). The general reaction scheme can be summarized below:



In this work we report on the ability of HRP to catalyze the oxidation of 2-deoxy-ribose and 2-deoxy-ribose-5-phosphate by hydrogen peroxide using the spin-trapping technique. We report here the ESR spectra detected and make tentative assignments for the site of radical formation.

MATERIALS AND METHODS

DBNBS, horseradish peroxidase (type I), hydrogen peroxide (30% solution), 2-deoxy-D-ribose (crystalline), 2-deoxy-D-ribose-5-phosphate (sodium salt), D-ribose and 3-deoxy-D-ribose were all obtained from Sigma Chemical Company (St. Louis, MO) and used without further purification. Solutions of DBNBS were made to a stock concentration of 26 mM in 0.075 M sodium-phosphate buffer (pH 7.4); 2-deoxy-D-ribose-5-phosphate was made to a stock concentration of 0.2 M in phosphate buffer; and HRP was made to a stock concentration of 20 mg/ml (1,900 units/ml). The hydrogen peroxide stock solution was 10 mM. All solutions were prepared immediately prior to the start of the experiment, and the enzyme solution was kept on ice.

ESR spectra were obtained using an IBM-200 spectrometer operating at 9.7 GHz with 100 kHz modulation frequency and equipped with an ER-4103 TM cavity. Samples were aspirated into the quartz flat cell using a Gilford rapid sampler system.[6]

TABLE I
Hyperfine Splitting Constants of Radical Adducts with DBNBS

	Hyperfine Splittings (Gauss)		
Radical Trapped	a^N	a_β^H	Source
ĊH	13.4	5.2	2-deoxyribose-5-phospate
ĊH	13.6	11.0	2-deoxy-ribose
ĊH	13.4	5.8	2-deoxy-ribose

All the experiments were performed at room temperature. The spectra were analyzed by a computer correlation technique, and hyperfine splitting constants were obtained from spectra simulated on a HP 9000 computer system.[7]

RESULTS AND DISCUSSION

When a radical is spin trapped, the site of the unpaired electron shifts from the parent radical to the nitroxide group of the radical adduct. This greatly increases the concentration of the free radicals formed due to the increased stability of the resulting nitroxide. Some loss of structural information about the parent radical also results as most of the free electron spin density is localized on the nitrogen and oxygen of the nitroxide group. The free electron interacts with the nitrogen of the nitroxide group and generates a primary triplet splitting. In this study the nitrogen splitting is between 13 and 14 G and is typical of a carbon-centered radical.[8] Identification of the trapped radical depends on the hyperfine structure within each triplet, which is due to the interaction of the electron localized on the nitroxide with the nuclei of the trapped parent radical.

The ESR spectrum detected when 2-deoxy-ribose-5-phosphate was incubated with HRP/H_2O_2 in the presence of the spin trap DBNBS is shown in Figure 1. The

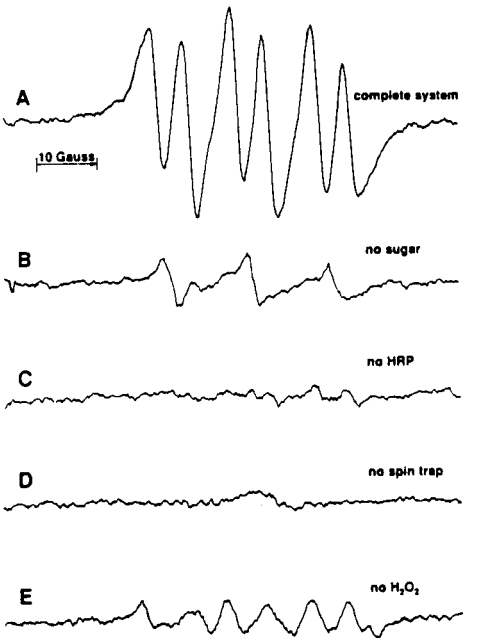
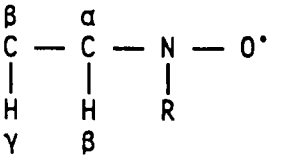


FIGURE 1 A. ESR spectrum of carbon-centered radical adducts formed from 120 mM 2-deoxy-D-ribose-5-phosphate, in the presence of 1.48 mg/ml HRP, 1.2 mM H_2O_2 and 4.8 mM DBNBS in 75 mM sodium phosphate, pH 7.4. ESR parameters were: modulation amplitude, 0.7 G, receiver gain 8×10^5; time constant 2.5 s; microwave power 20.9 mW; and scan time, 500 s; B. As A, but no 2-deoxy-ribose-5-phosphate; C. As A, but no HRP; D. As A, but no DBNBS; E. As A, but no H_2O_2.

spectrum is a triplet of doublets, indicating a carbon-centered radical with a β hydrogen. The α,β and γ positions are defined with relation to the unpaired electron on the nitrogen of the nitroxide group of the spin trap,[9] as shown in the figure below:



As can be seen from Figure 1, the spectrum is entirely dependent on the presence of the sugar, HRP and DBNBS. The spectrum produced in the absence of the sugar is most probably due to an oxidation product of the spin trap (Figure 1B). Furthermore, there is a weaker signal in the absence of hydrogen peroxide (Figure 1E). Computer simulations indicate that the spectrum produced by the HRP-catalyzed oxidation of 2-deoxy-ribose-5-phosphate is a composite of more than one species with each species consisting of a carbon-centered radical with a β hydrogen. The simulation of this spectrum is shown in Figure 2. The radical adduct has a primary nitrogen splitting of 13.4 G and a beta hydrogen of 5.2 G (Figure 2B). Possible sites for the primary radical center would be at C(2) or C(5) but, since every carbon is bonded to at least one hydrogen, no carbon position can be excluded.

When 2-deoxyribose was incubated with the HRP/H_2O_2/DBNBS system, the spectrum shown in Figure 3 was produced. This spectrum was dependent upon the presence of all reactants (Figure 3B-3E). The spectrum is essentially a triplet of doublets, with a weaker triplet of doublets intruding as shoulders on the major peaks. The composite simulation of this spectrum (Figure 4B) is a composite of two species shown in Figures 4C and 4D. Species 1 (Figure 4C) has a primary nitrogen splitting of 13.6 G and a β hydrogen splitting of 11.0 G. Species 2 (Figure 4D) has a primary nitrogen splitting of 13.4 G and a β hydrogen splitting of 5.8 G with a relative g-shift of 0.13 G. Both these species are radical adducts of carbon-centered radicals with a hydrogen β to the radical centre, i.e., $\dot{C}H$ radicals. However, to have two carbon-centered radicals with such vastly different β hydrogen splittings indicates that the

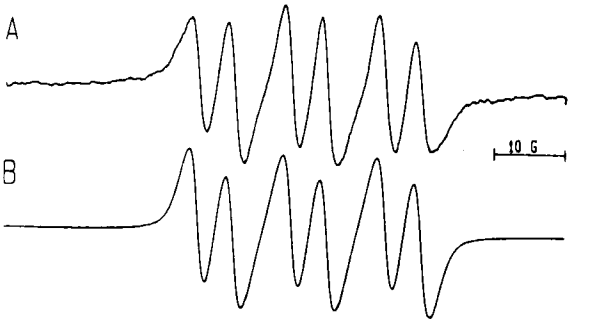


FIGURE 2. A. Experimental spectrum of 2-deoxy-D-ribose-5-phosphate radical adduct (same as in Figure 1A). B. Computer simulation of the 2-deoxy-ribose-5-phosphate radical adduct using the hyperfine coupling constants a^N = 13.4 G and a_β^H = 5.2 G with a linewidth of 2.1 G (78% Lorentzian-22% Gaussian).

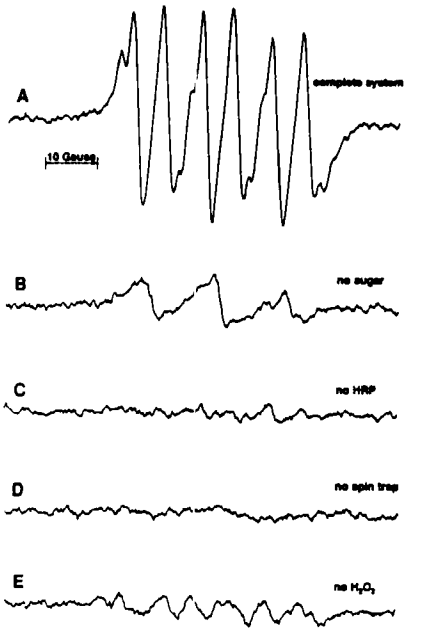


FIGURE 3. A. ESR spectrum of carbon-centered radical adducts formed from 120 mM 2-deoxy-D-ribose in the presence of 1.48 mg/ml of HRP, 4.8 mM DBNBS and 1.2 mM H_2O_2. ESR parameters: modulation amplitude 0.7 G. receiver gain 8×10^5, time constant, 2.5 s, microwave power 20.9 mW; and scan time. 500 s; B. As A, but no 2-deoxy-ribose; C. As A, but no HRP; D. As A, but no DBNBS; E. As A, but no H_2O_2.

species present are located at two different carbon atoms on the sugar molecule and not diastereoisomeric pairs. Possible sites for the formation of a ĊH radical are again at C(2) and C(5) after hydrogen atom abstraction by horseradish peroxidase.

The experiments were repeated with 3-deoxyribose, but no spectra could be produced with this sugar, implying the importance of the C(2) hydrogen. Experimentation with ribose and ribose-5-phosphate also produced no detectable radicals.

SUMMARY

It can be readily seen that 2-deoxyribose and 2-deoxyribose-5-phosphate can be oxidized by HRP/H_2O_2 to form a free radical species that can be detected with the ESR spin-trapping technique. There are two probable sites for the formation of a ĊH type radical on the 2-deoxyribose sugar, these being the C(2) and the C(5) carbons. The fact that there is a species produced from 2-deoxy-ribose, but not 2-deoxy-ribose-5-phosphate, suggests that there is an involvement of the C(5) carbon in the species with the 11.0 G β hydrogen. In the spectra formed from 2-deoxy-ribose, there is a big difference in the hyperfine splitting of the β hydrogens, suggesting that the radicals are formed at different carbon centers, while the addition of a phosphate group to the C(5) carbon seems to inhibit radical formation at one site. In related work, the chemiluminescence of monosaccharides in the presence of horseradish peroxidase was proposed to be the consequence of carbon-centered free radical formation (10).

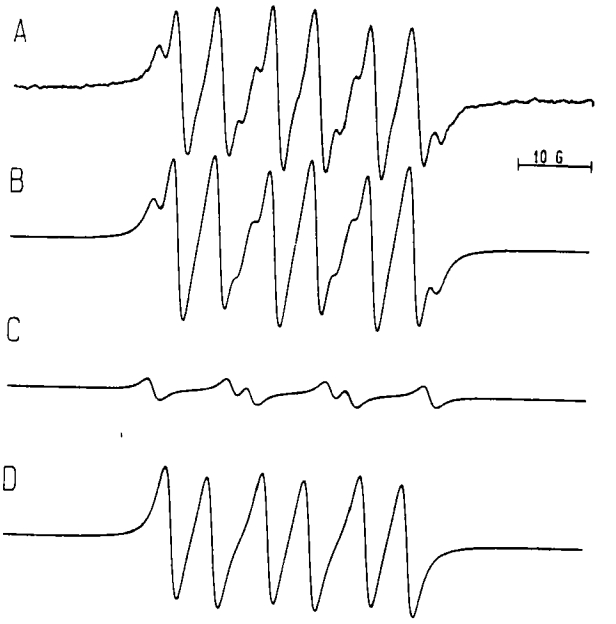


FIGURE 4. A. Experimental spectrum of the 2-deoxy-ribose radical adducts (same as in Figure 3A); B. Composite computer simulation of the 2-deoxy-ribose radical adduct using the hyperfine coupling constants given in Table 1; C. Computer simulation of species 1 using the hyperfine coupling constants a^N = 13.6 G and a^H_β = 11.0 G with a linewidth of 1.56 G (100% Lorentzian) and a relative concentration of 18; D. Computer simulation of species 2 using hyperfine coupling constants a^H_β = 11.0 G with a linewidth of 1.74 G (100% Lorentzian) and a relative concentration of 100.

References

1. Mason, R.P. and Chignell, C.F. *Pharmacol. Rev.* **33**,189–211, (1981).
2. Eling, T.E., Boyd, J. Reed, G. Mason, R.P. and Sivarajah, K. *Drug Metab. Rev.* **14**, 1023–1053, (1983).
3. Rao, D.N.R., Flitter, W.D., and Mason, R.P. in Cellular Antioxidant Defense Mechanisms (C.K. Chow, ed) CRC Press, Boca Raton, Florida, Vol. 1, pp. 59–71, (1988).
4. Yamazaki, I. in *Free Radicals in Biology* (W.A. Pryor, ed) Acedemic Press, New York, vol. 3, pp. 183–218, (1977).
5. Von Sonntag, C. *Int. J. Rad. Biol.***46,** 507–519, (1984).
6. Mason, R.P. *Methods Enzymol.*, **105,** 416–422, (1984).
7. Duling, D.R., Motten, A.G., and Mason, R.P. *J. Magn. Res.*, **77**,504–511, (1988)
8. Li, A.S.W., Cummings, K.B., Roethling, H.P., Buettner, G.R. and Chignell, C.F. *J. Magn. Res.*,**79,** 140–142, (1988).
9. Riesz, P. and Rustgi, S. *Radiat. Phys. Chem.*, **13,** 21–40, (1979).
10. Medeiros, M.H.G. and Sies, H. *Free Radical Biol. Med.*, **6,** 565–571, (1989).

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