HORSERADISH PEROXIDASE. XXV.

AN ELECTRON SPIN RESONANCE STUDY OF THE LOW TEMPERATURE PHOTOCHEMICAL REACTION OF COMPOUND I

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Received September 17,1976

SUMMARY: When Compound I of horseradish peroxidase in a glycerol/water glass at liquid nitrogen temperature is irradiated with white light an ESR doublet signal grows which is considerably more intense than the signal from the original glass. It is proposed that the doublet signal may be attributed to a species, called Intermediate Y, which is frozen in a conformation which prevents it being converted to the conventional form of Compound II.

INTRODUCTION

Recently, photochemical reactions of horseradish peroxidase Compounds I and II have been reported (1,2). It was found that Compound I was photoreduced to Compound II and that Compound II was photoreduced to the native HRP at room temperature. In low temperature glycerol/water glasses it was found that, at the low light intensities used, there was no photochemical reaction of Compound II but a reaction of Compound I was observed. This reaction was interpreted initially as a photoreduction to Compound II, distorted in the frozen lattice. However, a closer examination of the low temperature optical spectral results indicated perhaps a better explanation, namely that a low spin species is photochemically generated from Compound I (3). In particular, the development of a new Soret maximum at 416 nm, the tendency of two pairs of peaks to merge into single peaks centered near 540 nm and in the 640-650 nm region and the maintenance of a shoulder near 575 nm all agree with the spectral data for low spin species such as Compound III and alkaline peroxidase (4).

Compound I of horseradish peroxidase (5-7) can be represented as R·Fe(IV)O, where R· represents an organic free radical and O an iron-bound oxygen atom. Since equilibrium (8) and kinetic (9,10) studies show that Compound II of horseradish peroxidase is formed from Compound I via the addition of both a proton and an electron, Compound II might be represented as a structure of the type Fe(IV)OH. By analogy, the two species in equilibrium which represent Compound II of cytochrome *c* peroxidase would then be Fe(IV)OH and R·Fe(III)OH (11). The latter species would normally be expected to have a low spin optical spectrum and it has been postulated that the type of product obtained from the low temperature photolysis of horseradish peroxidase Compound I would have a similar structure (3)

$$H^+ + e + R\cdot Fe(IV)O \xrightarrow{h\nu} R\cdot Fe(III)OH \qquad (1)$$

(Compound I) (Intermediate Y)

This equation implies that Intermediate Y[a] should have a free radical ESR signal, as do Compounds I of horseradish peroxidase and cytochrome *c* peroxidase (5,13). The main purpose of this communication is to report the results of a test of the latter prediction. A few results involving the low temperature photolysis of Compound II and an attempt at the low temperature photolysis of native horseradish peroxidase are also reported.

MATERIALS AND METHODS

Horseradish peroxidase obtained as a lyophilized powder was purchased from Fluka Co. and purified by Mme. Claudette Job so that isoenzyme C was isolated with a final RZ value of 3.0. Glycerol, which enables one to obtain a glass at low temperatures was first vacuum distilled, then added to all samples. Solutions of horseradish peroxidase and H_2O_2 were made up in 50% v/v glycerol/water containing 0.01 ionic strength citric acid buffer (pH 5.0). Compound I was prepared by mixing equal volumes of horseradish peroxidase (0.35 mM) and H_2O_2 (0.5 mM) in dim light and at a temperature of 233°K. The resultant intensely green solution was placed in standard quartz ESR tubes (3 mm internal diameter) and rapidly frozen in liquid nitrogen.

(a) This terminology is chosen since Hager *et al.* have named a halogenating intermediate for horseradish peroxidase Compound X (12).

Samples of horseradish peroxidase Compound II were prepared from Compound I solutions in carbonate buffer of pH 10.2 by the addition of a half-molar equivalent of *p*-cresol (10). All operations were conducted in dim light.

ESR spectra were recorded at 77°K except when noted otherwise with a Bruker model ER 420 X-band spectrometer. A 150 W light bulb was used as a source of irradiation. Photolyses were attempted both with the sample inside the ESR instrument cavity and outside the cavity. When the sample was inside the cavity the light bulb was unshielded and placed about 12" from the sample. When irradiation took place outside the cavity, the sample in its liquid nitrogen Dewar flask and the light bulb were about 2" apart with both wrapped in aluminum foil.

Dark and light ESR spectra of the native HRP were also recorded under the same conditions and compared with those of Compound I.

RESULTS AND DISCUSSION

In the dark, Compound I of horseradish peroxidase gives a weak ESR signal at $g \simeq 2.005$ confirming the published results (5). This signal, which is not the one which correlates with the amount of Compound I present (5), stays the same in intensity even after being warmed up to 233°K where the sample is thawed. Upon irradiation of Compound I at 77°K inside the ESR cavity with white light for about 5 minutes, a new, light-induced, more intense, signal starts to grow. The intensity of this signal depends on the length of time of irradiation and reaches a maximum value after over 7 hours of irradiation. This signal has a g-value of 2.0025 ± 0.0005 (Fig. 1). (The same results are obtained, but in much less time, from irradiation outside the cavity.) It was observed that the irradiated sample had changed from a green to a brown color. The light-induced signal was found to decay slowly even at liquid nitrogen temperature but it regained intensity upon re-irradiation at this temperature. After being warmed to 233°K (a thawing process requiring about 5 - 10 minutes) the light-induced signal was lost almost completely leaving a signal which resembles the Compound I signal obtained before irradiation.

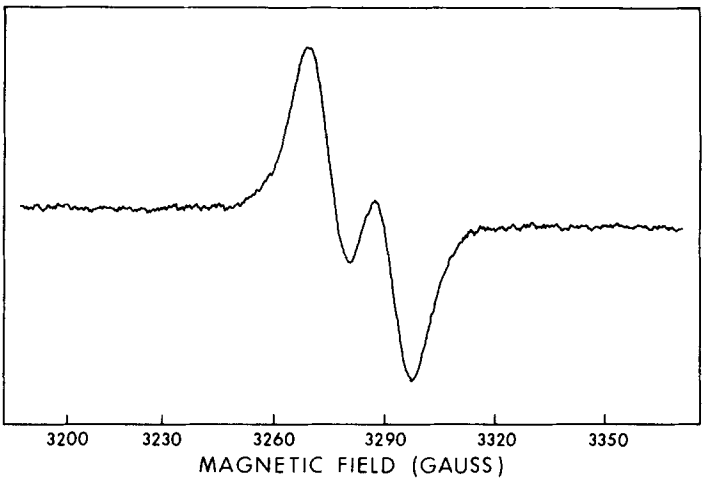

Figure 1: ESR spectrum of HRP-I after ~4 hours irradiation of 0.18 mM HRP in 0.01 citrate buffer (pH 5.0) and in 50% glycerol. Modulation amplitude 2G; microwave power 13 dB (12.5 mW) receiver gain 1.25×10^5 at microwave frequency 9206 MHz.

When Compound II was subjected to the intense irradiation outside the cavity for approximately one hour at 77°K, the same intense signal with the same g-value as displayed in Fig. 1 was obtained which would indicate, perhaps, that the process is

$$\underset{\text{(Compound II)}}{\text{Fe(IV)OH}} \xrightarrow{h\nu} \underset{\text{(Intermediate Y)}}{\text{R·Fe(III)OH}} \qquad (2)$$

Therefore, provided the same end product is obtained in Eqns. 1 and 2, the Intermediate Y of horseradish peroxidase can be formed either from Compound I or Compound II, although much more readily from the former species. Since the reverse of the process depicted in Eqn. 2 is not observed at 77°K either in the presence or absence of light, it would appear that a much larger energy barrier exists for this reverse process. One explanation is that R·Fe(III)OH is frozen in a conformation which prevents it being converted to Fe(IV)OH. Compound II of horseradish peroxidase, unlike Compound II of cytochrome

c peroxidase, cannot be represented as consisting of the two species R·Fe(III)OH and Fe(IV)OH in facile equilibrium.

In the dark, the native enzyme also shows an ESR signal at g ≃ 2.005, again in agreement with the literature (14) but the signal intensity is only about one quarter that of HRP-I before any photochemical reaction and it stays the same upon irradiation. This evidence serves to show that the brown photochemical product of Compound I and of Compound II is not the native enzyme.

The width of the doublet signal is in accord with a free electron on a single carbon atom which, with its adjacent carbon atoms, has a total of only one hydrogen atom attached (18).

These preliminary results show that the predicted ESR signal for the photochemical product of HRP-I does exist. They do not prove that the proposed mechanism is correct. The ultimate source of the electrons for the reduction process remains a mystery, but one must consider carefully the mechanism of photo-oxidation of proteins which involves the heme group (15-17) for it may be that the two processes are complementary.

We thank Mme. C. Job and Prof. J. Ricard of the University of Marseilles for the preparation of HRP and Prof. R.E.D. McClung for helpful discussions. One of us (H.B.D.) is indebted to the National Research Council of Canada for a research grant.

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