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EPR STUDIES ON COMPOUND I OF HORSERADISH PEROXIDASE

ROLAND AASA, TORE VÄNNGÅRD and H. BRIAN DUNFORD*

Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Fack, S-402 20 Göteborg 5, (Sweden)

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Summary

Compound I of horseradish peroxidase (donor: hydrogen-peroxide oxidoreductase EC 1.11.1.7) was studied by EPR at low temperatures. An asymmetric signal was found, about 15 Gauss wide and with a *g*-value of 1.995, which could be detected only at temperatures below 20 K and which had an intensity corresponding to about 1% of the heme content. In a titration with H_2O_2, the signal intensity was proportional to the concentration of Compound I, reaching a maximum when equivalent amounts of H_2O_2 were added. This indicates that the signal is not due to an impurity, and it is suggested that a free radical is formed, relaxed by a near-by fast-relaxing iron.

Introduction

Compound I, formed in the reaction between hydrogen peroxide and peroxidase, has been the subject of many investigations with different techniques such as optical, magnetic susceptibility, Mössbauer and EPR (see ref. 1). From Mössbauer studies [2,3] it was suggested that the iron is in the Fe(IV) state. EPR of Compound I of cytochrome *c* peroxidase [4,5] demonstrated the presence of a free radical in a concentration equal to that of the iron, and this was taken as evidence for the localization of the additional oxidizing equivalent in the protein part of the molecule [4]. However, the EPR spectrum of Compound I of horseradish peroxidase has no corresponding feature. The reason for this is not understood, although some models have been proposed [6—8].

With presently available preparation Compound I of horseradish peroxidase is relatively stable, and we have made a careful search for EPR signals in a titration with hydrogen peroxide. A small signal was found having free radical properties but with a very short relaxation time. The signal had an intensity proportional to the amount of Compound I. These findings are consistent with the presence of a free radical relaxed by a near-by iron.

* Permanent address: Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada

Materials and Methods

Chemicals. Horseradish peroxidase was obtained from Boehringer Mannheim as a highly purified suspension in aqueous ammonium sulphate and prepared for experiments as described elsewhere [9]. No effort to separate the isozymes [10] was made. All experiments were performed in 0.1 M phosphate buffer, pH 7, unless stated otherwise. Hydrogen peroxide, 30% by weight, was obtained from Riedel-de Haën AG (Seelze-Hannover) and diluted before use. Its concentration was determined by the horseradish peroxidase-catalyzed oxidation of iodide [11].

Optical and EPR spectra. Optical spectra were obtained with a Cary 15 spectrophotometer in 1-mm cells.

EPR spectra were recorded at temperatures between 4.2 and 20 K with a Varian X-band E-3 spectrometer equipped with a helium gas-flow system [12]. Some EPR spectra were obtained at temperatures between 5 and 30 K with a Varian V-4503 35 GHz spectrometer, also furnished with a similar helium gas-flow system [13].

For rapid-freeze experiments equal volumes of horseradish peroxidase (0.35 mM) and H_2O_2 (0.5 mM) were mixed and rapidly frozen within 50 ms in an apparatus described earlier [14]. The medium was 0.01 M acetate buffer (pH 4.9), chosen to simplify a comparison with earlier published kinetic data [15].

H_2O_2 titration experiments. Small amounts, varying between 10 and 100 μl, of 1.7 mM H_2O_2 were mixed with 600 μl of 0.14 mM horseradish peroxidase. The reaction mixture was rapidly transferred to an optical cuvette and an EPR tube. The Soret region was scanned in the spectrophotometer, and simultaneously the EPR sample tube was frozen in liquid nitrogen. The time elapsed between mixing and freezing was about 1 min. In a separate experiment it was verified that no significant changes in the optical spectrum occurred in this period of time.

Results

EPR spectrum. Fig. 1 shows typical EPR spectra recorded at about 4.2 K of horseradish peroxidase after addition of about one equivalent of H_2O_2. The peak at 3260 Gauss was found to be very variable in different experiments. It was saturated even at the lowest available power (2 μW) at 4.2 K and could be detected at 77 K with a g-value of 2.004. On the other hand, the asymmetric signal, which remains at high power in Fig. 1 could only be detected at temperatures below 20 K and was saturated only at powers in excess of 2 mW at 4.2 K. Its g-value at maximum absorption is 1.995.

At 35 GHz the EPR signal was observed for temperatures below 30 K. The shape and g-values were identical to those observed at 9 GHz, although the signal at $g = 1.995$ was saturated at lower microwave power.

H_2O_2 titration. The results of the H_2O_2 titration experiments are summarized in Fig. 2. The decrease in absorption at 403 nm after correction for the dilution has been taken as a measure of Compound I [9]. If the concentration of Compound I is measured from the absorbance at the isosbestic point at 411

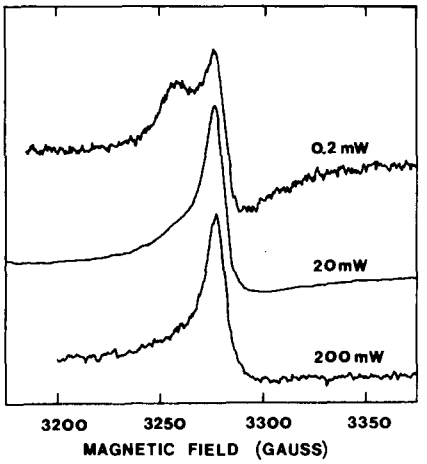

Fig. 1. EPR spectra at 4.2 K of 0.6 mM horseradish peroxidase in 0.1 M phosphate buffer, pH 7, frozen immediately after the addition of equivalent amounts of H_2O_2. The microwave frequency was 9165 MHz, and the microwave power is given in the figure.

nm of native horseradish peroxidase and Compound II, essentially the same result is obtained. This indicates that the concentration of Compound II is negligible in all samples. The shape of the g = 1.995 EPR signal remaining at high power was found to be unchanged throughout the titration. Therefore, the amplitude of this signal was taken as a measure of its intensity.

In a separate experiment the time courses of the optical and EPR spectra were followed after adding about two equivalents of H_2O_2. It was found that Compound I measured from the optical spectrum and the EPR signal at g = 1.995 decayed simultaneously within about one hour on standing in room temperature.

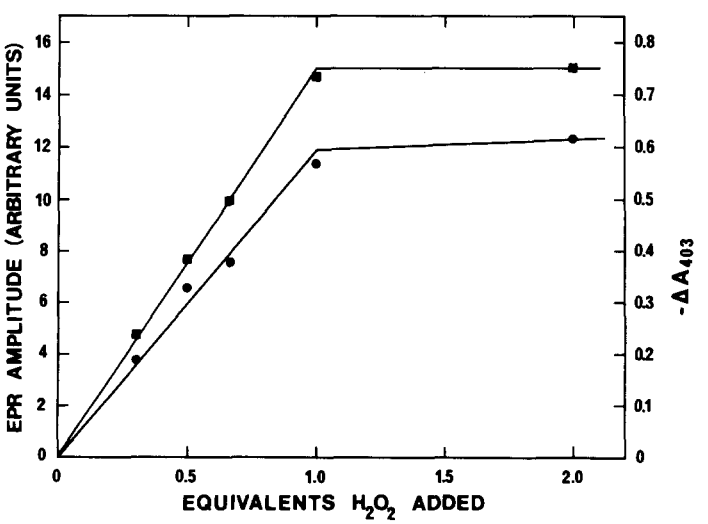

Fig. 2. Titration of 0.14 mM horseradish peroxidase with increasing amounts of H_2O_2 following the absorbance change at 403 nm (●) and the amplitude of the g = 1.995 EPR signal (■). The values have been corrected for dilution.

Intensity and temperature dependence of the g = 1.995 EPR signal. A determination of the number of unpaired spins corresponding to the $g = 1.995$ signal shown in Fig. 1 was made using a Cu^{2+} solution and the low-spin hemoglobin-azide complex as references. Precautions must be taken not to saturate the reference signals. Assuming that the signal arises from an isolated Kramers' doublet with $S = ½$ its maximum integrated intensity was found to correspond to about 1% of the horseradish peroxidase concentration used in the titration. The signal has an asymmetric shape with very long tails, which certainly gives a large uncertainty in this value.

The $g = 1.995$ EPR signal was broadened as the temperature was raised above 4.2 K and could not be observed for temperatures above 20 K. The integrated intensity of the signal was estimated between these temperature limits. Within experimental errors, the variation of the intensity could be described by a normal Boltzmann law dependence.

Rapid-freeze experiment. The EPR signal obtained from the sample which was rapidly frozen had essentially the same shape and intensity as those found in the titration experiments. The easily saturable signal at 3260 Gauss had, however, a somewhat higher intensity.

Discussion

The optical titration curve in Fig. 2 gives a direct measure of the amount of Compound I formed in the reaction with hydrogen peroxide. The shape of the curve and the total absorbance change are consistent with earlier published data [9].

The small and variable signal at $g = 2.004$ is most likely of no particular interest. Morita and Mason [16] observed a similar signal, which also could be detected at relatively high temperatures, and which was independent of the concentration of Compound I. Thus, in the following the discussion will be restricted to the properties of the $g = 1.995$ absorption. This signal might be the same as the one observed by Blumberg et al. at low temperatures [17]. These authors considered the signal to be of no importance because of its low intensity. However, the fact that the signal intensity is proportional to the amount of Compound I formed (see Fig. 2) clearly indicates that the signal is not derived from a protein impurity. Such an origin would not give a break in the titration curve at equivalent amounts of H_2O_2 as shown in Fig. 2. Furthermore, the decay of the EPR signal occurred simultaneously with the disappearance of Compound I. The fact that the signal observed in this work appears very rapidly as predicted from known kinetics of Compound I formation [15] is further evidence of its relevance. Thus, we believe that this EPR signal appears in a reaction between native horseradish peroxidase and H_2O_2.

Low EPR intensity as observed here is obtained if the signal is due to an excited state with low thermal population. Such a situation is related to the model proposed by Peisach et al. [8]. In this model two oxidizing equivalents are stored in the heme protein molecule and there is a ferric iron having an $S = 3/2$ ground state with $M_S = \pm 1/2$ higher in energy than $M_S = \pm 3/2$. A signal is expected only from the $M_S = \pm 1/2$ state, but it would be too small to be seen at low temperatures and too broad at higher temperatures. The signal observed

in this work has a temperature dependence predicted by the Boltzmann law and is therefore most likely due to low-lying energy levels. In fact, the similarity between the 9 and 35 GHz spectra suggests that we are dealing with an isolated Kramers' doublet more than about 10 cm^{-1} below other states. Thus, although the model in ref. 8 could give a small EPR signal it is not consistent with our findings. Similarly, a model with Fe(V) having $S = 3/2$ need not be considered.

Although it cannot be excluded that the EPR signal arises through a side reaction or from a small fraction of the molecules in chemical equilibrium with Compound I, the properties of the EPR signal itself suggest another possibility. The g-value and width are those of a free radical but the short relaxation time strongly indicates that iron is involved. A coupling of a radical to a fast-relaxing metal ion has been shown to reduce the observed radical intensity under certain conditions [18]. The signal observed here could then be representative of all the Compound I molecules.

The localization of the free radical is not apparent from the present study. From our data is it not possible to choose between a radical localized on the porphyrin ring as proposed by Dolphin et al. [19] or a localization on the protein part. Likewise, the electronic state of the iron does not follow from our experiments. However, Fe(IV) is a good candidate as it would certainly be fast relaxing and also is consistent with Mössbauer studies [2,3]. In order to get a fuller understanding of the electronic structure of Compound I of horseradish peroxidase, the EPR data must be correlated with low-temperature magnetic susceptibility measurements and Mössbauer studies on improved samples.

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