SHORT COMMUNICATIONS

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Electron spin resonance of intermediates in the catalytic reaction of peroxidase at low temperature

Recently, we reported the temporal resolution of individual steps in the reaction of peroxidase with hydrogen peroxide and luminol achieved by a cooling $\rightleftharpoons$ warming procedure in hydroorganic solutions[1]. We were able to quench the reaction and to isolate, consecutively, compounds I and II, and to record their respective absorption and optical rotatory dispersion spectra. We checked that our intermediates were identical to those obtained by B. CHANCE[2] using fast techniques. In the same experimental conditions, we have recorded the electron spin resonance spectra of compounds I and II and compared our results with those previously reported by MORITA AND MASON[3] in aqueous buffer solutions frozen 10 sec after mixing of the reactants. The new data may provide further information concerning the electronic structure of compounds I and II which have been already studied by absorption and optical rotatory dispersion, and show how the macromolecule is involved in the catalytic process.

Experimental

Two types of horse radish peroxidase were used: type VI (Sigma Chemicals) with a R.Z. value of 3.0 and an activity of 295 purpurogallin units per mg and type II (RZ value of 1,5), already used by MORITA AND MASON[3] in their ESR work. Hydrogen peroxide was a Merck product and luminol was obtained from Aldrich Chem. Co. The solvent was a mixture of dimethylformamide and water, in the volume ratio 70:30 (freezing point, $-67°$). The other mixtures such as ethyleneglycol–water (50:50 v/v, freezing point, $-48°$), dimethylsulphoxide–water (65:35 v/v, freezing point, $-97°$) are not suitable for peroxidase reactions since they act as hydrogen donors and thus do not permit the isolation of compound I. The methanol–water mixtures previously used to investigate haemoglobin derivatives[4] cause the precipitation of horse radish peroxidase and are thus also unsuitable.

The dimethylformamide–water solutions were prepared in $2 \cdot 10^{-3}$ M phosphate buffer (pH 8,0 in aqueous solution). The protonic activity varies by about $\pm$ 0.2 unit between $+20$ and $-60°$. The procedure used for dissolution of the enzyme has been previously described[1].

ESR spectroscopy was performed with a VARIAN E_3 spectrometer and with a temperature control device previously described and used for absorption and optical rotatory dispersion measurements[1]. Quartz sample tubes of 3 mm internal diameter were used and the spectra were recorded at $-60°$ to $-65°$ in the fluid hydroorganic solutions and at $-196°$ in frozen aqueous samples. We followed the same procedure with respect to temperature control previously to isolate and stabilize compounds I and II in order to obtain their spectra[1].

Results

Fig. 1 shows the ESR spectra of horse radish peroxidase in a frozen aqueous

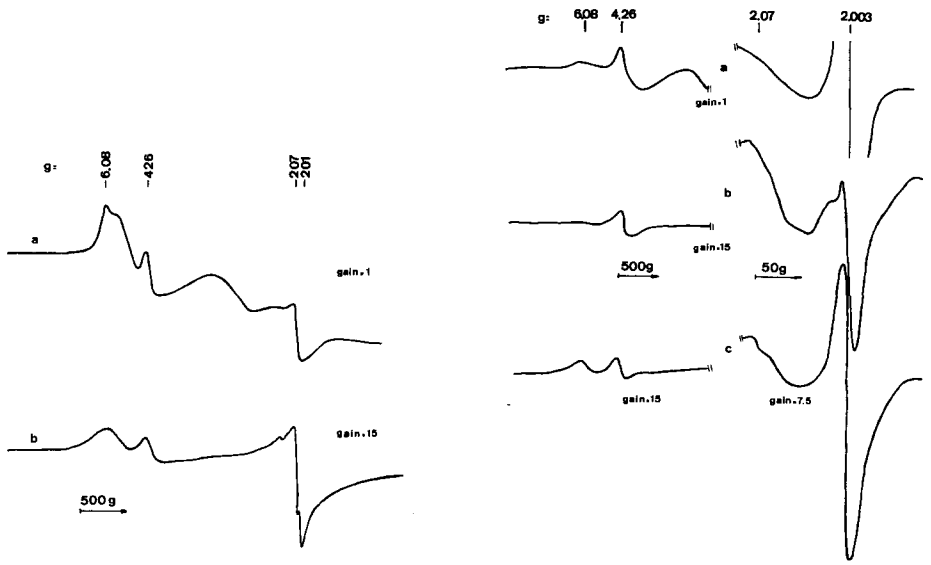

Fig. 1. EPR spectra of horse radish peroxidase type II (10^{-3} M): a) in phosphate buffer (pH 8) at $-196°$. b) in the mixture dimethylformamide–phosphate buffer (pH 8), volume ratio 70:30).

Fig. 2. EPR spectra of horse radish peroxidase type II (10^{-3} M): a) in phosphate buffer (pH 8) + H_2O_2 (10^{-3} M): rapid freezing 10 sec after mixing. b) in mixture dimethylformamide–phosphate buffer (pH 8, volume ratio 70:30) + H_2O_2 (10^{-3} M) and luminol (10^{-3} M) recorded at $-60°$. c) same preparation as b) after warming up to $-10°$ (5 min) and recooling down to $-60°$.

buffer solution at $-196°$ and in a still fluid mixture of dimethylformamide and buffer (volume ratio 70:30) at $-65°$. The 2 spectra are very similar. Since the amplitude is temperature-dependent (in fact, empirically exponential), the signal from the sample at $-65°$ is approximately 25 times weaker than that from the frozen solution (where $g = 6.08$).

Fig. 2 shows the ESR spectra obtained in the same way during the reaction of horse radish peroxidase with hydrogen peroxide and luminol. Aqueous buffer solutions are frozen 10 sec after the mixing of reactants. Hydroorganic mixtures are submitted to cooling $\rightleftharpoons$ warming cycles between $-65°$ and $-10°$ and the spectra recorded at $-65°$.

There are no fundamental differences between the shape of signals recorded in the 2 cases. Nevertheless, due to the temporal resolution obtained in the fluid mixture, it is shown that the formation of the free radical ($g = 2.003$) appears to occur only during the conversion of compound I to II. The amount of free radical obtained represents in both cases a very low percentage of the original concentration of horse radish peroxidase and also (as seen in the fluid mixture) of the concentration of compound II. Thus, it is confirmed that this long-lived free radical (lasting more than 10 h at $-30°$ in the absence of free hydrogen peroxide) is a minor component of compound II. Its signal is devoid of any fine structure. At a temperature of $-65°$ in a fluid solvent, this absence of structure might be due to a localisation of the free radical in the protein. Finally, the most useful information gained in fluid solution concerns the evolution of the $g = 6.08$ signal (high spin) during the reaction: it can be seen

that the ESR absorption of free horse radish peroxidase disappears when compound I is formed with a stoichiometric concentration of hydrogen peroxide at $-65°$. The signal reappears after the conversion compound I $\rightarrow$ compound II. These observations agree with the recordings by optical rotatory dispersion previously performed in the same conditions, indicating a reversible modification of the interaction between the haem and the apoprotein during the course of the enzymic process.

From the present work, it is difficult to draw general conclusions on the study of haemoproteins by ESR in fluid hydroorganic solvents without spectrophotometric determinations giving the ratio of high spin to low spin which may also be influenced by the solvents. In the present case, we have compared the spectroscopic data obtained to that recorded in aqueous buffer solutions by fast techniques or after rapid freezing, to insure that responses from intermediates are not influenced by the hydroorganic solvent.

Further experiments are planned using cooled fluid solvents to investigate the free radical associated with compound II and the behavior of luminol radicals arising from steps:

$$\text{Compound I} + \text{luminol}\cdot H_2 \rightarrow \text{Compound II} + \text{luminol}\cdot H\cdot$$

and

$$\text{Compound II} + \text{luminol}\cdot H_2 \rightarrow \text{horse radish peroxidase} + \text{luminol}\cdot H\cdot$$

since we are able to eliminate the presence or reactivity of hydrogen peroxide normally involved during these reactions at room temperature.

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