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Formation of compound I of horseradish peroxidase in AOT reverse micelles as studied by pulse radiolysis and stopped-flow methods

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Abstract

The kinetics of the formation of compound I of horseradish peroxidase entrapped in AOT reverse micelles has been studied by pulse radiolysis and stopped-flow methods. It has been found that the rate of the formation of HRP compound I in reverse micelles depends on w_o, regardless of the source of hydrogen peroxide and is up to ten times higher than in homogeneous aqueous solution.

Keywords: Horseradish peroxidase; Pulse radiolysis; Reverse micelles; Stopped-flow

1. Introduction

Most enzymes are known to be active only in aqueous solution and, as a rule, do not display any activity in organic solvents. Entrapment of enzymes in reverse micelles enables the use of these highly active and selective catalysts in organic media.

Peroxidases are enzymes whose primary function is catalyzing the oxidation of variety of substrates, AH, by peroxides.

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + AH \rightarrow \text{Compound II} + A^{\cdot} \quad (2)$$

$$\text{Compound II} + AH \rightarrow \text{Peroxidase} + A^{\cdot} \quad (3)$$

Most peroxidases contain ferric heme in the active site. The first intermediate in the catalytic cycle of peroxidase (reaction 1), compound I is a two electron oxidation product of the ferriheme enzyme and is described as a ferryl porphyrin Π-cation radical. One electron reduction of compound I gives a ferryl derivative known as compound II (reaction 2).

Reaction (1) was studied by us in aqueous solution of horseradish peroxidase (HRP) [1] with the use of pulse radiolysis method for H_2O_2 generation and the rate constant of compound I formation has been estimated to be 1.8×10^7 $M^{-1} \cdot s^{-1}$.

Determination of kinetic parameters of enzymatic reactions in reverse micelles revealed some interesting facts. In some cases the k_{cat} was found to exceed the aqueous value (so called 'superactivity'). A bell-shaped profiles of activity versus

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w_o (the molar water to surfactant ratio) were often observed. An outstanding 'superactivity' was found for HRP, where the rate of substrate oxidation was about 100 time higher in reverse micelles at optimum w_o ratio than in water [2].

For most substrates of peroxidases the rate-determining step is reaction (3). The aim of this work is to check whether the microenvironment of reverse micelles also influences the fastest step in the peroxidase cycle i.e. the formation of compound I (reaction 1). As it is known that the exchange process between water pools of reverse micelles proceeds on the same time scale as the formation of compound I in homogeneous aqueous solution [3], we have applied the experimental techniques which avoid this disturbance:

- pulse radiolysis – where H_2O_2 is produced in the hydrocarbon continuous phase;
- stopped-flow spectrophotometry – where the rapid mixing (within the 'dead time' of the apparatus) of the solution of H_2O_2 entrapped in water pools with the same volume of equimolar solution of the enzyme entrapped in water pools initiates the investigated process.

2. Experimental

Horseradish peroxidase (HRP) (Biolab, Poland) was a freeze-dried, salt free preparation with RZ (A_{403}/A_{275}) equal to 3.0. AOT [sodium bis(2-ethylhexyl)sulfosuccinate] was a product from Sigma and used as received [4]. Deionized and triply glass-distilled water was used throughout. AOT reverse micelles were formed by injection of appropriate amounts of aqueous stock solutions either of buffer alone (1 mM phosphate buffer, pH 7.0) or hydrogen peroxide in buffer or enzyme in buffer into 0.1 M AOT in n-heptane (Sigma) to obtain desired w_o. The mixture was shaken until a completely clear solution was obtained. One should remember, however, that the introduction of large hydrophilic enzyme molecule into reverse micellar system most likely causes the rearrangement of micellar aggregates. Thus, after a newly established equilibrium, w_o may differ from that of the system without enzyme. In this work we always give w_o values resulting from the amounts of water and AOT molecules added to the system.

All experiments were carried out at 23°C.

Pulse radiolysis experiments were performed with the linear accelerator at the Institute of Applied Radiation Chemistry of Łódź [5]. Pulses of 17 ns delivering a dose of ca. 40 J/kg were applied. Samples were Ar-saturated.

The stopped-flow experiments were performed on the DX-17MV (Applied Photophysics) spectrofluorimeter. Samples were air equilibrated.

3. Results and discussion

We have observed, with the use of pulse radiolysis, that in Ar-saturated reverse micellar system, compound I of HRP is formed. The concentration of HRP compound I has been estimated to be 2 μM taking its absorption coefficient at 660 nm from [6]. To obtain such an amount of HRP compound I, at least the same concentration of H_2O_2 is needed. H_2O_2 is a molecular product of water radiolysis ($G[H_2O_2] = 0.07$ μmol J^{-1}). In homogeneous aqueous solution irradiated with the dose of 40 J/kg ca. 2.8 μM H_2O_2 should be formed. In reverse micellar solution, however, most of energy is absorbed in the oil phase. The energy portion absorbed in water pools should be more or less proportional to the water content, which under our experimental conditions varied from 0.9% ($w_o = 5$) to 5.4% ($w_o = 30$). Thus, the organic phase is the main source of H_2O_2 in our pulse radiolysis experiments with reverse micelles. Taking into account that bubbling with Ar cannot remove all the oxygen dissolved in the system [7] the following mechanism of H_2O_2 formation in reverse micelles can be proposed:

$$H + O_2 \rightarrow HO_2^{\cdot} \quad (4)$$

$$H + HO_2^{\cdot} \rightarrow H_2O_2 \quad (5)$$

$$2HO_2^{\cdot} \rightarrow H_2O_2 \quad (6)$$

where H (hydrogen atom) is an intermediate in hydrocarbon radiolysis.

In pulse radiolysis experiments the rate of compound I formation was followed at 660 nm under pseudo-first order conditions ([HRP] varied from 1 to 4×10^{-5} M) (Fig. 1).

In stopped flow experiments the rate of compound I formation was measured at 405 nm (Söret band) under conditions where the reaction was second-order (Fig. 2).

On the Fig. 3 rate constants, $k_{1,obs}$, obtained by the pulse radiolysis and stopped-flow methods are plotted against w_o. In both cases the maximum value of the rate constant observed at $w_o = 20$ exceeds that in bulk water by the factor of ten.

The same $k_{1,obs}$ vs. w_o profiles have been obtained under conditions where the overall concentration of the enzyme and H_2O_2 have been kept constant and under conditions where the local concentrations of both reactants in water pools of reverse micelles have been kept constant. Hence, we conclude that higher $k_{1,obs}$ for reverse micellar system cannot be exclusively responsible for the high local concentrations of the enzyme or H_2O_2 in water pools.

Compound I shows lower stability in reverse micelles than in homogeneous aqueous solution. Half-life time of compound I in reverse micelles at $w_o = 20$ is about 200 ms and is followed by the formation of compound II (reaction 2). Compound I formed in bulk water remains stable during our longest observation time i.e. up to 100 s. The stability of compound I in reverse micelles does not depend on its concentration. It could mean that the entrapment of HRP into AOT reverse micelles causes such conformational changes which allow intramolecular electron transfer to porphyrin ring.

In conclusion, our results obtained using the two complementary techniques show, that the rate of the formation of HRP compound I in reverse micelles depends on w_o, regardless of the source of hydrogen peroxide and is up to ten times higher in reverse micellar system than that in homogeneous aqueous solution. The half-life of HRP compound I is shorter in reverse micelles than in bulk water. The observed changes in the reactivity of HRP and its compound I upon the entrapment into AOT reverse micelles are most likely due to conformational changes of the enzyme induced by

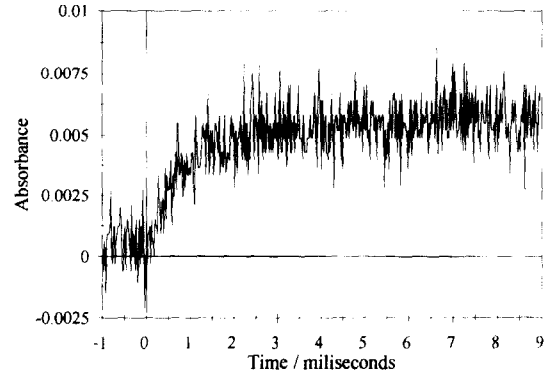

Fig. 1. Formation of compound I observed at 660 nm in pulse radiolysis experiments. ([HRP] = 4×10^{-5} M, $w_o = 20$).

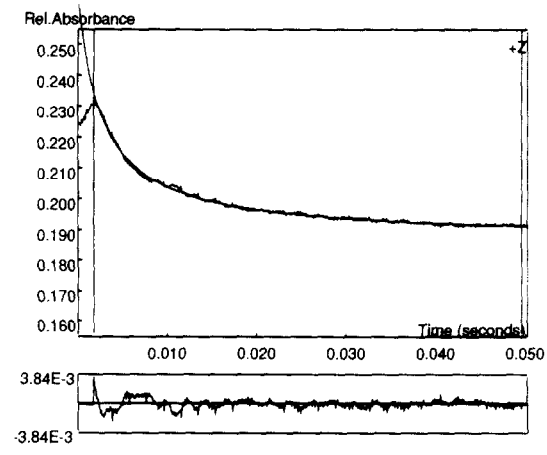

Fig. 2. Formation of compound I observed at 405 nm in stopped-flow experiments. ([HRP] = 2×10^{-6} M, $w_o = 20$).

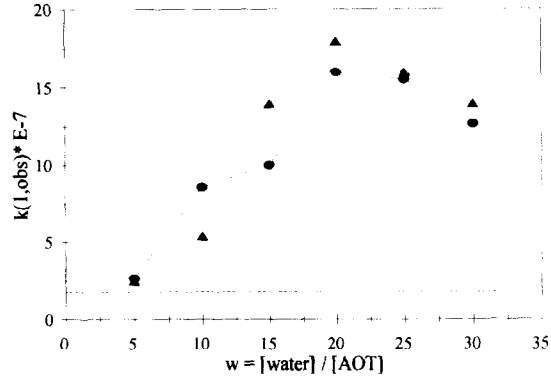

Fig. 3. Rate constants for HRP compound I formation ($k_{1,obs}$) vs. w_o. (▲ – pulse radiolysis and ● – stopped-flow). Dashed line denotes $k_{1,obs}$ in bulk water.

the specific properties of the water pool microenvironment.

Acknowledgements

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