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A stopped-flow study of the reaction of cytochrome *c* peroxidase with hydroperoxides

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Transient kinetic measurements show that cytochrome *c* peroxidase reacts with excess of hydroperoxides to produce compound ES in two phases. The activation energies for the fast and slow phases are calculated to be 6.3 and 20.5 kcal·mol^{-1}, respectively. The fast phase is assigned to the reaction of native active (pulsed) cytochrome *c* peroxidase with peroxides, whereas the slow phase is due to the presence of an inactive (aged, resting) enzyme. As the active species is exhausted, the equilibrium between the active and inactive enzymes is shifted by a slow conformational change to replenish the active enzyme. Since the rate-limiting step of the reaction of the inactive enzyme with peroxides is the conformation change, the overall reaction rate is independent of the nature and concentration of peroxides.

Cytochrome *c* peroxidase; Hydroperoxide; Compound ES; Activation energy; Stopped-flow; Heme coordination

1. INTRODUCTION

Yeast cytochrome *c* peroxidase (CCP) reacts stoichiometrically with hydroperoxides to form a stable peroxide intermediate, compound ES [1,2]. Initial steady-state and transient kinetic studies show that the rate of this reaction is very fast, with a second-order rate constant of 10^7–10^8 $M^{-1} \cdot s^{-1}$ [3–6]. The three-dimensional structure of CCP [7] suggests that the enzyme has a pentacoordinated high-spin heme, but electronic absorption and EPR spectra of CCP at 77 K are indicative of a mixture of hexa-coordinated high-spin and low-spin ferric heme compounds [8–10]. Although spectrophotometric studies of the reaction of CCP with hydroperoxides gave proportional titration curves [2], an EPR titration of CCP at low temperature showed that the low-spin component of CCP preferentially reacts with hydroperoxides over the high-spin one [11]. The present work has been carried out in order to unravel this inconsistent behavior of CCP. Our data show that native CCP with a pentacoordinated high-spin ferric heme group is reactive with hydroperoxides, whereas aged CCP preparations in a hexacoordinated high-spin state are quite inactive. The conversion of the aged CCP to the native form is accompanied by a protein conformation change.

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2. MATERIALS AND METHODS

Cytochrome *c* peroxidase was purified from bakers' yeast according to a modification of the method of Yonetani and Ray [1], recrystallized 3 times by dialysis against distilled water [12] and stored in a polycrystalline state at −20°C. The concentration of CCP was spectrophotometrically determined using $\epsilon_{408\,nm}$ = 98 $mM^{-1} \cdot cm^{-1}$ at pH 7.0 [13]. Hydrogen peroxide (Fisher, USA) and ethyl hydroperoxide (EtOOH, Ferrosan, Malmö, Sweden) were used without further purification.

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The concentrations of hydrogen peroxide and ethyl hydroperoxide were spectrophotometrically determined using CCP as described [2]. Potassium phosphate buffers, 0.1 M, were used throughout the experiments and their pH values were determined at 25°C. For experiments at lower temperatures the pH values were estimated according to published data [14].

Stopped-flow transient kinetic and rapid wavelength scanning measurements were performed with a Union Giken model 415-RA or 401-RA rapid scan spectrophotometer (Osaka, Japan) and a home-built stopped-flow apparatus for studies at subzero temperatures [15]. The dead time of the stopped-flow apparatus, with a 1.0 cm light path length, was less than 5 ms. The temperature of the syringes, mixing and observation chambers of the stopped-flow apparatus were maintained between 2 and 25 ± 0.1°C with a Haake model F3-Q circulating water bath (Berlin). Rapid scan absorption spectra were measured with a multichannel photodiode array, with a maximal speed of 95 $nm \cdot ms^{-1}$, stored and analyzed with a Sord model M223 microcomputer, mark III (Tokyo, Japan). The reaction kinetics were analyzed using $\Delta\epsilon_{424nm}$ (compound ES minus CCP) = $-42\ mM^{-1} \cdot cm^{-1}$ [2]. The observed rate constants were determined from a non-linear least-square analysis. Single values of rate constants reported here are averages of 5 experimental determinations with an estimated error of ± 10%.

3. RESULTS

3.1. *Stopped-flow experiments at 424 nm*

The reaction of CCP with hydroperoxides to form compound ES was examined spectrophotometrically at 424 nm by stopped-flow techniques as a function of (i) hydroperoxide concentration, (ii) pH and (iii) temperature.

In 0.1 M phosphate buffer, pH 6.1, compound ES was formed biphasically when the concentration of hydrogen peroxide or ethyl hydroperoxide exceeded that of CCP. The kinetics could be described as the sum of two exponential functions, each kinetic curve being considered to be first-order. The relative amplitude of the slow phase was 21 ± 1% of the total absorbance change at 424 nm, at 2°C and pH 6.1. Fig.1 illustrates the apparent rate constants of the fast and slow phases

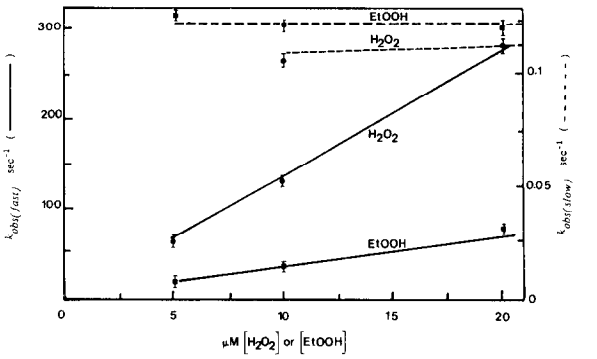

Fig.1. Plots of observed pseudo-first-order rate constants, k_{obs}, for the fast and slow phases of the reactions of cytochrome *c* peroxidase with hydrogen peroxide and ethyl hydroperoxide (EtOOH) as a function of hydroperoxide concentrations. Conditions: 5 μM cytochrome *c* peroxidase in 0.1 M potassium phosphate buffer, pH 6.1 at 2°C. The slow phase was unchanged up to 100 μM hydroperoxide (not shown).

of compound ES formation as a function of peroxide concentration. The observed pseudo-first-order rate constant for the fast process, $k_{obs(fast)}$, was directly proportional to the peroxide concentration, if the peroxide concentration was larger than the enzyme concentration. The second-order rate constant for the fast phase, $k_{1(fast)}$, was determined to be 1.38×10^7 and $3.38 \times 10^6\ M^{-1} \cdot s^{-1}$ at 2°C for hydrogen peroxide and ethyl hydroperoxide, respectively. On the other hand, the pseudo-first-order rate constant for the slow phase, $k_{obs(slow)}$, was found to be independent of the peroxide concentration over a wide range (10–100 μM for hydrogen peroxide and 5–100 μM for ethyl hydroperoxide) as well as the nature of hydroperoxides. The $k_{obs(slow)}$ value was determined to be 1.25×10^{-1} and $1.40 \times 10^{-1}\ s^{-1}$ at 2°C for hydrogen peroxide and ethyl hydroperoxide, respectively. At hydroperoxide concentrations lower than the enzyme concentration, the slow phase was not observed and $k_{1(fast)}$ remained independent of the peroxide concentration. The $k_{1(fast)}$ and $k_{obs(slow)}$ were found to be independent of enzyme concentration from 0.5 to 10 μM at 2°C.

Fig.2 shows the values of $k_{1(fast)}$ and $k_{obs(slow)}$ determined for hydrogen peroxide and ethyl hydroperoxide as a function of pH at 2°C. The $k_{1(fast)}$ values were relatively pH-independent,

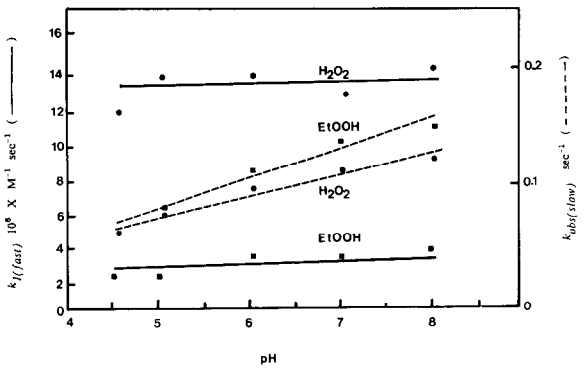

Fig.2. Plots of the second-order rate constant, $k_{1(fast)}$, and observed pseudo-first-order rate constant, $k_{obs(slow)}$, for the fast and slow processes of the reaction of cytochrome *c* peroxidase with hydroperoxides as a function of pH. Conditions: 10 μM cytochrome *c* peroxidase and 20 μM hydroperoxides in 0.1 M potassium phosphate buffers at 2°C.

whereas the $k_{obs(slow)}$ values increased with increasing pH, in the range from pH 4.5 to 8. The amplitude of the slow phase for the hydrogen peroxide reaction was pH-dependent [5], as presented in fig.3. An apparent pK_a value of ~6.7 was estimated for the pH-dependent range. The reaction with ethyl hydroperoxide gave similar results.

Fig.4 shows the Arrhenius plots of $k_{1(fast)}$ and $k_{obs(slow)}$ for ethyl hydroperoxide. The fast process had an activation energy of 6.3 ± 0.2 kcal·mol^{-1} (26.3 ± 0.8 kJ·mol^{-1}), whereas the slow phase had a much higher activation energy of 20.5 ± 0.2 kcal·mol^{-1} (85.9 ± 0.8 kJ·mol^{-1}).

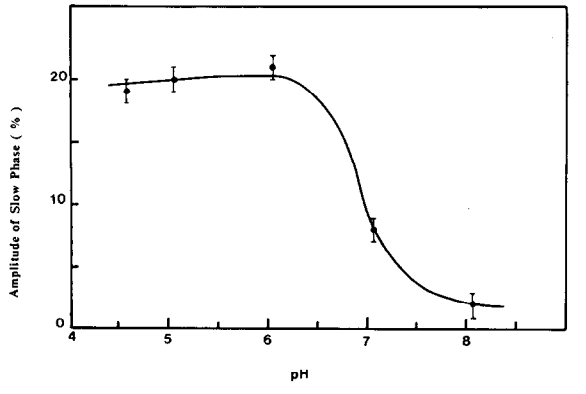

Fig.3. Plot of the relative amplitude (ΔA_{424nm}) of the slow phase as a function of pH. Conditions: 10 μM cytochrome *c* peroxidase and 20 μM hydrogen peroxide in 0.1 M potassium phosphate buffers at 2°C.

3.2. *Wavelength scanning stopped-flow experiments*

Fig.5 illustrates a series of successive difference absorption spectra of CCP reaction with excess ethyl hydroperoxide (CCP minus compound ES), recorded at different times after mixing. The difference spectra of the fast (fig.5A) and slow (fig.5B) phases were calculated using as a baseline the spectra at 194 ms and 32 s after mixing, respectively, when the two phases were virtually completed. The difference spectra of the fast phase were characterized by a broad absorbance change around 350–410 nm, with a maximum at 385 ± 10 nm, whereas those of the slow phase show a sharp maximal absorbance change at 405 nm in the Soret band of CCP. Three isosbestic points were observed at 345 ± 5, 414 ± 1 and 456 ± 3 nm (the last not shown). These isosbestic points, estimated kinetically, agree with the reported values from the statically determined difference spectrum between CCP and compound ES [8].

4. DISCUSSION

In order to obtain detailed kinetic and spectral information on the reaction between CCP and

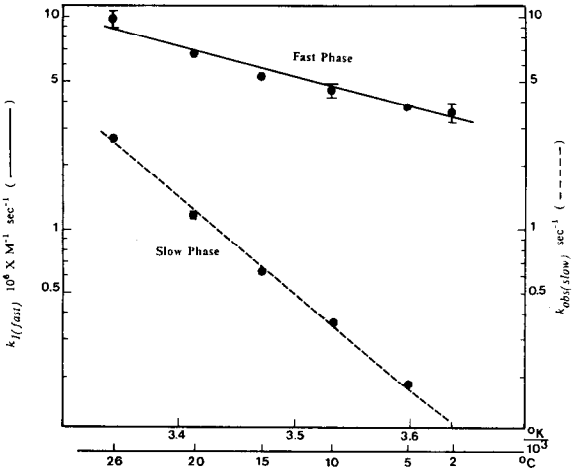

Fig.4. Arrhenius plots of $k_{1(fast)}$ and $k_{obs(slow)}$ for the fast and slow phases. Conditions: 5 μM cytochrome *c* peroxidase and 10 μM ethyl hydroperoxide in 0.1 M potassium phosphate buffer, pH 6.1.

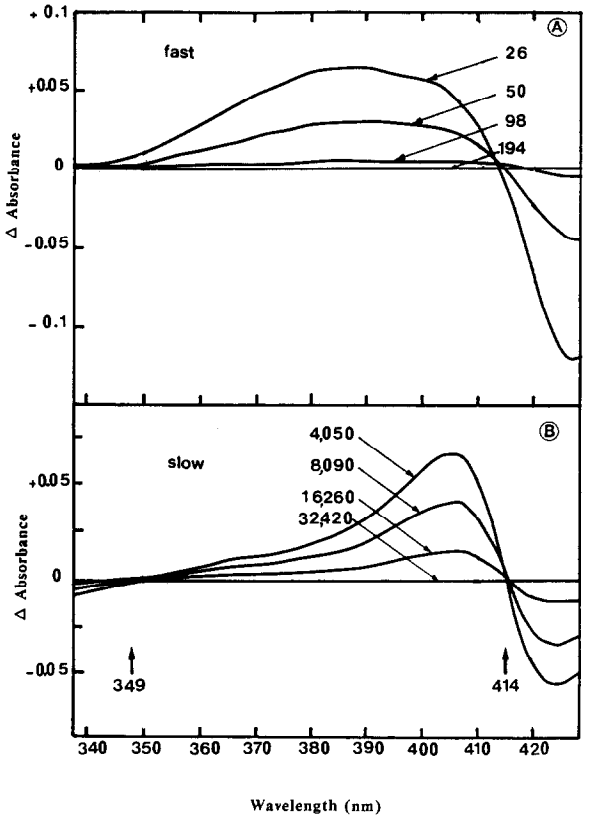

Fig.5. Difference absorption spectra between cytochrome *c* peroxidase and compound ES as a function of reaction times after mixing 10 μM cytochrome *c* peroxidase with 20 μM ethyl hydroperoxide in 0.1 M potassium phosphate buffer, pH 6.1 at 2°C. Spectra were recorded at the times (indicated in ms) after mixing. (A) The fast phase using the 194-ms spectrum as a baseline and (B) the slow phase using the 32-s spectrum as a baseline.

hydroperoxides, we have carried out the stopped-flow experiments with CCP concentrations substantially higher than those previously used [5,6]. The expected increase in apparent reaction rates was controlled by the extensive use of a less reactive substrate, ethyl hydroperoxide, and lower temperature, 2°C.

The observed second-order rate constant values for the fast reaction of CCP with hydroperoxides, $k_{1(\text{fast})}$, are of the same order of magnitude as reported in previous stopped-flow studies [4–6,16,17] (table 1). However, we found that the $k_{1(\text{fast})}$ value is pH-independent over a wide range from pH 4.5 to 8 (cf. fig.2). Although the $k_{1(\text{fast})}$ value at pH 4.5 appears to be slightly lower (by ~10%), a decrease in the acidic region with pK_a = 5.5 [5] has not been observed. The kinetic difference spectra for the fast phase (cf. fig.5A) are practically a mirror image of the statically determined difference spectra between compound ES and CCP [8]. It is reasonable to conclude that the fast phase of the reaction represents the bimolecular reaction between the enzyme and substrates, which is pH-independent in the range examined from pH 4.5 to 8. The calculated activation energy of the reaction (cf. fig.4) of 6.3 kcal·mol^{-1} is consistent with this assignment and other measurements in 0.1 M Bis-Tris buffer, pH 5.8, reporting an activation energy of 6.5 kcal·mol^{-1} [17].

Table 1

Second-order rate constants (k_1) for the reaction of cytochrome *c* peroxidase with hydroperoxides

Substrate	k_1 ($M^{-1} \cdot s^{-1}$)	Temperature (°C)	Reference
Hydrogen peroxide	1.4×10^8	20–25	[4][a]
	4.5×10^7	25	[5]
	3.4×10^7	25	[6]
	2.7×10^7	25	[16][a]
	3.0×10^7	20	[16]
	1.4×10^7	2	(this paper)
Ethyl hydroperoxide	2.5×10^7	20–25	[4][a]
	2.8×10^6	25	[6]
	8.5×10^6	25	(this paper)
	5.2×10^6	15	[17]
	3.0×10^6	2	[17]
	3.4×10^6	2	(this paper)

[a] Initial steady-state measurements

The slow phase of the reaction was independent of the concentration and nature of hydroperoxides (cf. fig.1), indicating that the bimolecular reaction between the enzyme and substrates is not the rate-limiting step in the slow phase. The kinetic difference spectra for the slow phase (cf. fig.5B) with a sharp Soret band at 405 nm are indicative of the enzyme in a hexacoordinated high-spin state. This state of CCP is produced upon prolonged storage, particularly in acidic pH and is characterized by a sharp, intensified Soret band with no distinct shoulder in the 360–390 nm region [13]. A larger

activation energy of 20.5 kcal·mol^{-1} calculated for the slow process (cf. fig.4) (or E_a = 19.1 kcal·mol^{-1} in 0.1 M Bis-Tris buffer, pH 5.8 [17]) suggests substantial changes in protein conformation in the process.

On the basis of these observations, we propose the reaction scheme for the fast and slow processes shown in fig.6.

The pentacoordinated native CCP reacts rapidly with hydroperoxides in a straightforward bimolecular reaction to form compound ES with a second-order rate constant, $k_{1(fast)}$, of 10^6–10^7 M^{-1}·s^{-1}. In stored enzyme preparations, CCP is partially converted to a hexacoordinated state, the relative fraction of which depends on the history of storage and pH [13]. The preparation used here was converted up to 20% to hexacoordinated CCP below pH 6, as indicated from the amplitude of the slow phase (cf. fig.3). The apparent insensitivity of the slow process to the concentration and nature of hydroperoxides indicates that the hexacoordinated aging-induced CCP is unreactive with hydroperoxides. As the pentacoordinated native CCP is exhausted in the fast bimolecular reaction with hydroperoxides, the hexacoordinated CCP is slowly converted to a pentacoordinated state via a slow conformational change of the enzyme, this event being the rate-limiting step in the slow phase. The newly generated pentacoordinated CCP is immediately converted to compound ES by hydroperoxides. We have recently demonstrated that the pentacoordinated native CCP is converted to a low-spin state with EPR extrema at g_x = 2.7, g_y = 2.2 and g_z = 1.78 at 77 K by a freezing-induced distortion of the coordination state of the heme group, whereas the aging-induced, hexacoordinated high-spin CCP exhibits a typical EPR spectrum of axial symmetry with extrema at $g_\perp$ = 6 and $g_\parallel$ = 2 [13].

Therefore, the proposed reaction scheme (cf. fig.6) is consistent with the previous EPR study showing that at 77 K the low-spin component of CCP, the native CCP, is preferentially converted to compound ES over the high-spin component [11], the latter deriving from the aged CCP. It should be pointed out that freshly crystallized CCP, which contains no age-induced hexacoordinated artifact, reacts with hydroperoxides in a fast single-exponential process [13]. In the case of horseradish peroxidase [18] the two-step formation of compound I in neutral conditions has been interpreted as follows: the first step corresponds to the formation of the collision complex ES followed by an isomerization (of the heme or protein) to compound I.

The distinct difference in reaction rates with hydroperoxides between hydroperoxidases and metmyoglobin/methemoglobin, all of which contain identical prosthetic groups, protohemes, has been recognized since the early 1950s [3,19,20]. The present work has demonstrated that the coordination state of the ferric heme group plays a fundamental role in the reactivity of CCP: CCP is highly reactive and unreactive with hydroperoxides in the penta- and hexacoordinated ferric states, respectively. In this respect, it is more than coincidental to note that reactive hydroperoxidases are generally considered to have pentacoordinated fer-

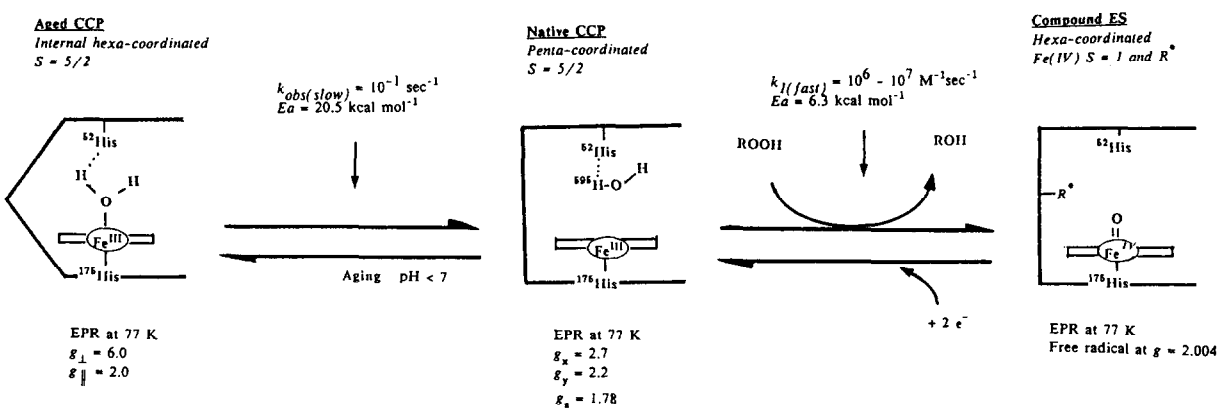

Fig.6. Schematic presentation of the reaction of cytochrome *c* peroxidase with hydroperoxides.

ric hemes, whereas less reactive metmyoglobin and methemoglobin are known to have a water molecule coordinated at the axial site in a manner similar to the aged CCP. It is interesting also that the free energy for the reduction of hydrogen peroxide by metmyoglobin is 17.9 $kcal \cdot mol^{-1}$ [20], comparable to 20.5 $kcal \cdot mol^{-1}$ calculated in this work for the aged hexacoordinated CCP slow-phase catalysis.

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