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Pseudomonas Cytochrome *c* Peroxidase

VII. Kinetics of the Peroxidatic Reaction Mechanism

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Initial rate data are reported for the *Pseudomonas* cytochrome *c* peroxidase reaction with substrates *Pseudomonas* ferrocytochrome *c* and hydrogen peroxide at pH 5.0, 6.0, and 7.0. The results indicate a sequential reaction mechanism involving a kinetically significant ternary complex for this enzyme.

In 1931 Mann [1] postulated the existence of a ternary complex in the reaction mechanism of horse radish peroxidase (HRP) on the basis of hyperbolic relationships between the over-all rate of reaction and the donor concentration. Chance [2] has later pointed out, however, that such a "saturation effect" could be expected even with binary complexes alone. Only with the arrival of the kinetic method of Alberty [3] and Dalziel [4] was it possible to detect ternary complexes of even very short life-times. Using the kinetic method of Dalziel,[4] Yonetani and Ray [5] have recently confirmed the presence of a ternary complex in the reaction mechanism of yeast cytochrome *c* peroxidase (YCCP).

The properties of *Pseudomonas* cytochrome *c* peroxidase (PsCCP) differ clearly from those of YCCP, *e.g.* the enzyme molecule of PsCCP contains two heme moieties present in the low spin form.[6] In addition, PsCCP does not form those spectrally distinguishable complexes with hydrogen peroxide which are characteristic of other peroxidases,[7] *e.g.* YCCP. Consequently, a somewhat different kinetic behaviour may be expected for PsCCP as compared with that of YCCP.

In order to simplify the interpretation of the kinetic data, these studies concern only that part of the peroxidatic reaction in which the enzyme was initially incubated with reduced cytochrome *c* before addition of H_2O_2, *i.e.* the phase of initial delay was excluded.[8]

MATERIALS AND METHODS

Pseudomonas cytochrome c peroxidase (PsCCP) was prepared from acetone-dried cells of *P. aeruginosa* as previously described.[6] The ratio A_{407}/A_{280} of the preparation used

was 4.4. The concentration of the enzyme was determined spectrophotometrically using $A(1\ \%,\ 1\ cm)$ equal to 12.1 at 280 nm.[9] The molar concentrations of the enzyme were calculated on the basis of a molecular weight of 43 200.[9]

Yeast cytochrome c peroxidase (YCCP) was prepared as described previously.[10] The ratio A_{407}/A_{280} of the preparation was 1.30. The concentration of the enzyme was determined on the basis of the total hematin content, measured as pyridine ferrohemochrome according to Paul *et al.*[11]

Pseudomonas cytochrome c-551 (Ps-cyt-551) was prepared from acetone-dried cells of *P. aeruginosa* by the method of Ambler.[12] The purity of the cytochrome preparations $[A_{551}(\text{red.}) - A_{570}(\text{red.})/A_{280}]$ were 1.12 – 1.19, while those of Ambler were 1.13 – 1.17. The cytochrome preparations were found to be homogeneous on disc electrophoresis. The concentration of Ps-cyt-551 was determined spectrophotometrically applying the extinction coefficients $\varepsilon_{551}(\text{red.}) = 26.9\ \text{mM}^{-1}\ \text{cm}^{-1}$ and $\Delta\varepsilon_{551}(\text{red.-ox.}) = 19.0\ \text{mM}^{-1}\ \text{cm}^{-1}$.[8]

Horse heart cytochrome c was a commercial preparation from Sigma (Type III, 98 %), and was used without further purification. The extinction coefficients $\varepsilon_{550}(\text{red.}) = 27.6\ \text{mM}^{-1}\ \text{cm}^{-1}$ and $\Delta\varepsilon_{550}(\text{red.-ox.}) = 19.6\ \text{mM}^{-1}\ \text{cm}^{-1}$ were used for the spectrophotometric determination of the cytochrome concentration.[13]

Reduced Ps-cyt-551 and horse heart cytochrome c were prepared according to Yonetani and Ray [14] using anaerobic gel filtration on Sephadex G-25.

Hydrogen peroxide solutions were prepared from Merck Perhydrol (30 % H_2O_2). Peroxide concentration was determined enzymatically with YCCP using horse heart cytochrome *c* as substrate according to the method of Yonetani.[13]

Measurements of reaction rates. The activity of *Pseudomonas* cytochrome *c* peroxidase was assayed spectrophotometrically by measuring the rate of peroxidatic oxidation of fully reduced *Pseudomonas* cytochrome *c*-551 by the enzyme in acetate and phosphate buffers, pH 5.0, 6.0, and 7.0, all at $\mu = 0.01$. The reaction was initiated by mixing 10 μl of hydrogen peroxide solution with the reaction mixture (2.0 ml) containing 5 μl of suitably diluted enzyme and varying amounts of Ps-cyt-551. No appreciable oxidation of ferrocytochrome *c* was observed in the absence of the enzyme under the experimental conditions used. The reaction was followed by measuring the decrease in absorbance of Ps-cyt-551 at 551 nm with time. Initial rates (v_0/e) were calculated from the slope of the reaction curve at zero time and were expressed in terms of mol ferrocytochrome *c* oxidized per mol of enzyme per second.

Disc electrophoresis of Ps-cyt-551 was carried out in polyacrylamide gel according to the procedure of Ornstein and Davis [15] and Maurer.[16] The basic gel system No. 1a of Maurer (pH 8.9; 7 % gel) was used.

Instruments. Spectrophotometric measurements were performed with a Beckman DU spectrophotometer. Enzymatic activities were measured with a Beckman DK-1 A or a Cary 15 recording spectrophotometer equipped with cell compartment thermostated at 25°C. pH was measured with a Radiometer TTT 1 C meter fitted with a combination glass-calomel electrode; Beckman pH 7 buffer No. 3581 was used for standardization.

Reagents used were of analytical grade if not otherwise stated.

THEORETICAL BASIS OF KINETIC ANALYSIS

Alberty,[3] as well as Dalziel,[4] has suggested a number of mechanisms for two-substrate enzyme reactions. They have also given criteria for evaluating different mechanisms on the basis of kinetic data. For two-substrate reaction systems in which both substrates interact with the enzyme before dissociation of either product, the following rate equation, in Dalziel's notation, should be obeyed:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \qquad (1)$$

where e is the enzyme concentration, S_1 and S_2 are the two substrates and ϕ_0, ϕ_1, ϕ_2, and ϕ_{12} represent the kinetic coefficients. The kinetic coefficients,

which are functions of rate constants in the mechanism, can be evaluated from primary and secondary plots of initial-rate data according to Dalziel.[4]

The Mann [1] mechanism of the peroxidatic reaction of HRP is a general form of compulsory order mechanism for two-substrate reactions involving a rate-limiting ternary complex. When the ternary complex becomes non-rate-limiting, the Chance mechanism [2] is obtained, which is a special case of the general compulsory order mechanism (eqn. 2).

$$\frac{e}{v_0} = \frac{\phi_1'}{[S_1]} + \frac{\phi_2'}{[S_2]} + \frac{\phi_{12}'}{[S_1][S_2]} \qquad (2)$$

In the Mann mechanism, the secondary plot of intercepts has an intercept value of ϕ_0 and a slope of ϕ_2; while in that of Chance, the replot of intercepts gives an intercept value of zero and a slope value of ϕ_2'. The two mechanisms are, therefore, clearly distinguishable from each other by determining an intercept value for the secondary plot of primary intercepts.

RESULTS

Initial rate data measurements were made in phosphate buffer, pH 6.0 ($\mu = 0.01$), with concentrations of Ps-cyt-551 in the range 5 – 30 μM and of hydrogen peroxide in the range 10 – 200 μM. Lineweaver-Burk plots with each reactant as variable substrate (Fig. 1) are linear within the experimental error, and secondary plots of the slopes and intercepts (Figs. 2 and 3) are also linear. Since the ϕ_0 value was found to be positive it was concluded that the

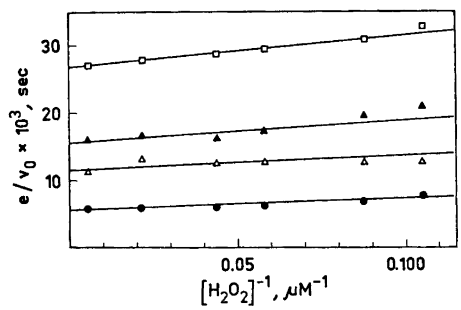

Fig. 1. Plot of reciprocal of specific initial rate at 25°C in sodium phosphate buffer, $\mu = 0.01$, pH 6.0 *versus* the reciprocal of H_2O_2 concentration at various concentrations of reduced Ps-cyt-551. [Ps-ferrocyt-551]: □, 5.2 μM; ▲, 10.2 μM; △, 15.0 μM; ●, 30.0 μM. The concentrations of other reagents used were: H_2O_2 10 – 200 μM and PsCCP 1.56 nM. Kinetic coefficients calculated from the plot are given in Table 1.

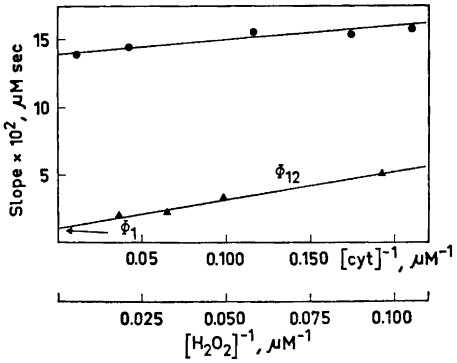

Fig. 2. Secondary plots of primary plot slopes *versus* the reciprocal of fixed substrate concentration in phosphate buffer, pH 6.0. Triangles and circles, respectively, represent data taken with Ps-cyt-551 and H_2O_2 as the fixed substrate.

Table 1. Kinetic coefficients for the peroxidatic oxidation reaction catalyzed by *Pseudomonas* cytochrome *c* peroxidase at 25°C in sodium acetate and sodium phosphate buffers. The kinetic coefficients are those in the initial-rate equation

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}.$$

Substrate	pH	Buffer	ϕ_0 sec	ϕ_1 M sec	ϕ_2 M sec	ϕ_{12} M^2 sec	ϕ_2/ϕ_0 M	ϕ_{12}/ϕ_2 M	$\phi_{12}/\phi_1\phi_2$ sec^{-1}
Ps-cyt-551	5.0	Acetate, $\mu = 0.01$	0.8×10^{-3}	6.0×10^{-8}	1.5×10^{-7}	0.4×10^{-12}	1.9×10^{-4}	2.7×10^{-6}	41
Ps-cyt-551	6.0	Phosphate, $\mu = 0.01$	1.6×10^{-3}	1.0×10^{-8}	1.4×10^{-7}	0.2×10^{-12}	8.8×10^{-5}	1.4×10^{-6}	365
Ps-cyt-551	7.0	Phosphate, $\mu = 0.01$	0.4×10^{-3}	3.8×10^{-8}	2.1×10^{-7}	0.3×10^{-12}	5.3×10^{-4}	1.4×10^{-6}	41

Pseudomonas cytochrome *c* peroxidase reaction follows the reaction scheme given in eqn. (1). The kinetic coefficients in eqn. (1), estimated from the slopes and intercepts of the secondary plots, are shown in Table 1.

Similar experiments were made in phosphate buffer, pH 7.0 ($\mu = 0.01$) and acetate buffer, pH 5.0 ($\mu = 0.01$). The Lineweaver-Burk plots for the first of these experiments, Fig. 4, are linear in the range of substrate concentrations

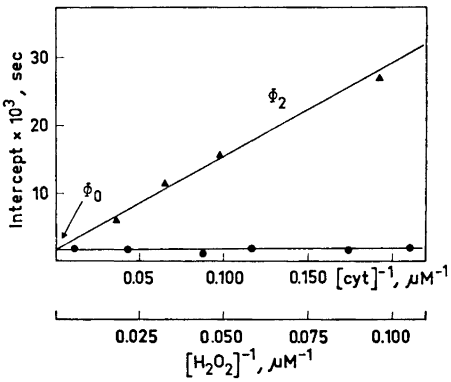

Fig. 3. Secondary plots of primary plot intercepts *versus* the reciprocal of fixed substrate concentration in phosphate buffer, pH 6.0. Triangles and circles, respectively, represent data taken with Ps-cyt-551 and H_2O_2 as the fixed substrate.

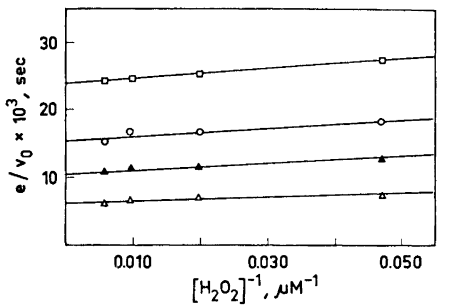

Fig. 4. Plot of the reciprocal of specific initial rate at 25°C in sodium phosphate buffer, $\mu = 0.01$, pH 7.0 *versus* the reciprocal of H_2O_2 concentration at various concentrations of reduced Ps-cyt-551. [Ps-ferrocyt-551]: □, 9.1 μM; ○, 13.8 μM; ▲, 18.4 μM; △, 34.4 μM. The concentrations of other reagents used were: H_2O_2 20 – 200 μM and PsCCP 1.56 nM. Kinetic coefficients calculated from the plot are given in Table 1.

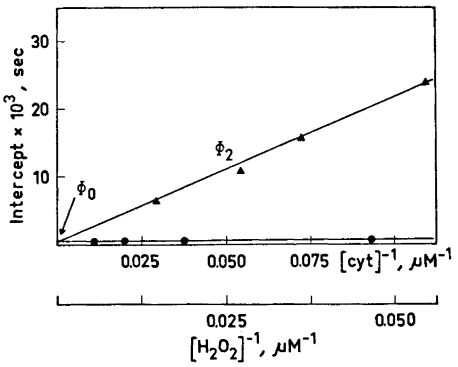

Fig. 5. Secondary plots of primary plot intercepts *versus* the reciprocal of fixed substrate concentration in phosphate buffer, pH 7.0. Triangles and circles, respectively, represent data taken with Ps-cyt-551 and H_2O_2 as the fixed substrate.

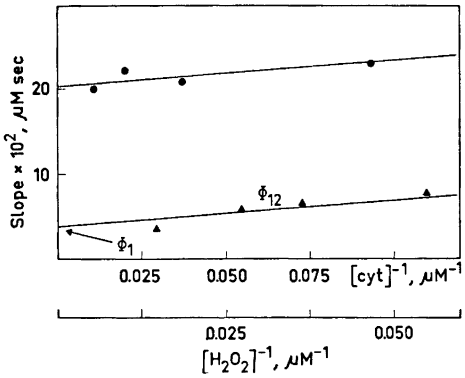

Fig. 6. Secondary plots of primary plot slopes *versus* the reciprocal of fixed substrate concentration in phosphate buffer, pH 7.0. Triangles and circles, respectively, represent data taken with Ps-cyt-551 and H_2O_2 as the fixed substrate.

used, and the slopes and intercepts (Figs. 5 and 6) allowed reliable estimates of the kinetic coefficients to be made from the secondary plots. This was also true of the experiments performed at pH 5 (Figs. 7, 8, and 9). The values obtained for the kinetic coefficients are given in Table 1.

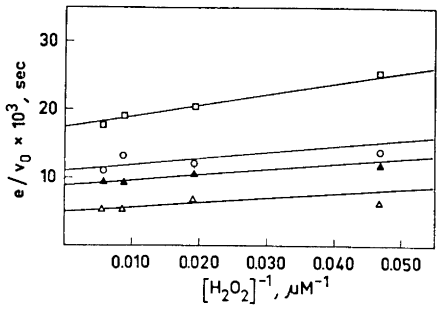

Fig. 7. Plot of the reciprocal of specific initial rate in sodium acetate buffer, $\mu = 0.01$, pH 5.0 *versus* the reciprocal of H_2O_2 concentration at various concentrations of reduced Ps-cyt-551. [Ps-ferrocyt-551]: □, 9.1 μM; ○, 14.4 μM; ▲, 16.9 μM; △, 34.1 μM. Other experimental conditions as given in the text of Fig. 4.

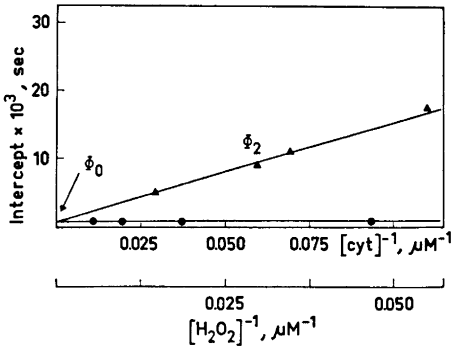

Fig. 8. Secondary plots of primary plot intercepts *versus* the reciprocal of fixed substrate concentration in acetate buffer, pH 5.0. Triangles and circles, respectively, represent data taken with Ps-cyt-551 and H_2O_2 as the fixed substrate.

The inhibition of the reaction by *Pseudomonas* ferricytochrome *c*, *i.e.* the product of one of the substrates, was studied by measuring initial rates of reaction in the presence of oxidized Ps-cyt-551 as well as in the absence of the inhibitor (Fig. 10). The primary Lineweaver-Burk plot indicates a competitive product inhibition.

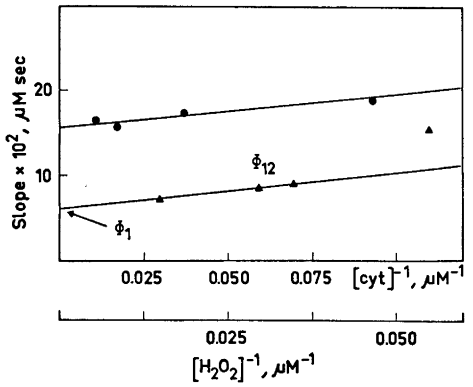

Fig. 9. Secondary plots of primary plot slopes *versus* the reciprocal of fixed substrate concentration in acetate buffer, pH 5.0. Triangles and circles, respectively, represent data taken with Ps-cyt-551 and H_2O_2 as the fixed substrate.

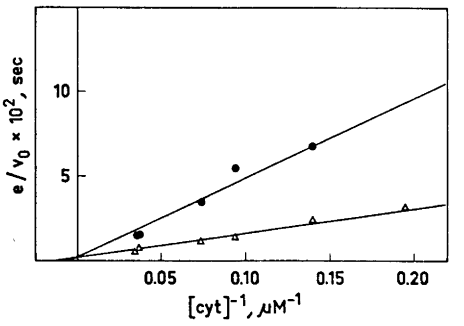

Fig. 10. Product inhibition of *Pseudomonas* cytochrome *c* peroxidase by oxidized Ps-cyt-551 with reduced Ps-cyt-551 as variable substrate. The concentration of H_2O_2 was kept constant, 143 μM. [Ps-ferricyt-551]: △, none; ●, 25.3 μM. The K_i value calculated is equal to 11.1 μM. Other experimental conditions as given in the text of Fig. 1.

DISCUSSION

The results of the kinetic studies indicate the reaction scheme given in eqn. (1) for the *Pseudomonas* cytochrome *c* peroxidase reaction, in agreement with the Mann [1] mechanism and excluding that of Chance.[2] The Mann mechanism involves the formation of a rate-limiting ternary complex according to the following equations:

$$E + S_1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES_1 \tag{3}$$

$$ES_1 + S_2 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES_1S_2 \tag{4}$$

$$ES_1S_2 \xrightarrow{k_3} E + P \tag{5}$$

where E = PsCCP, S_1 and S_2 are the two substrates, P = product, k_1, k_{-1}, k_2, k_{-2}, and k_3 = rate constants of the indicated reactions.

On the basis of our data, it cannot be decided whether S_1 represents H_2O_2 or Ps-cyt-551. In the Mann mechanism the following relationships exist between the kinetic coefficients and the rate constants: $\phi_0 = 1/k_3$; $\phi_1 = 1/k_1$; $\phi_2 = 1/k_2$; $\phi_2/\phi_0 = K_m$; $\phi_{12}/\phi_2 = k_{-1}/k_1 = K_s$; $\phi_{12}/\phi_1\phi_2 = k_{-1}$. The association rate of cytochrome *c* peroxidase and H_2O_2 at pH 6.0 ($k_1 = 1.0 \times 10^8$ $M^{-1}sec^{-1}$) was determined to be about the same as that of YCCP ($k_1 = 1.4 \times 10^8$ $M^{-1}sec^{-1}$).[5]

Most hematin-containing peroxidases are known to form two types of enzymatically active ES-complexes with peroxides,[2] Complex I and Complex II. Yeast cytochrome *c* peroxidase appears to form only one type of ES-complex with peroxide (Complex II).[13,17] With PsCCP no such hydrogen peroxide complexes have been identified.[7] It has earlier been shown [8] that with the experimental conditions used, 2 mol of ferrocytochrome *c* appear to be necessary to convert 1 mol of PsCCP-H_2O_2-complex into 1 mol of free cytochrome *c* peroxidase. However, a simultaneous interaction between a PsCCP-H_2O_2 complex and 2 molecules of reduced cytochrome *c* to form a quaternary complex PsCCP-H_2O_2-(cytochrome)$_2$ is excluded since the primary plots of e/v_0 *versus* reciprocal of cytochrome *c* at fixed H_2O_2 were found to be linear. It therefore seems that the PsCCP-H_2O_2 complex formed under the present conditions interacts with 2 molecules of ferrocytochrome *c* in a consecutive two step sequence. The ternary complex E-H_2O_2-cytochrome *c* is assumed to be the rate-determining Michaelis-complex when the concentration of $H_2O_2 \geq K_m$.

Alberty [18] has introduced the product inhibition method as an additional tool in deciding between different mechanisms, and it has shown its value in kinetic studies of, *e.g.*, ribitol dehydrogenase,[19] malic dehydrogenase,[20] as well as yeast and liver alcohol dehydrogenases.[21] With yeast cytochrome *c* peroxidase a mixed competitive (noncompetitive-competitive) inhibition has been observed for ferricytochrome *c*, and the inhibition pattern obtained confirms the compulsory order mechanism of that enzyme.[5] The inhibition studies of PsCCP showed Ps-ferricyt-551, one of the products, as a competitive inhibitor for

Ps-ferrocyt-551 in the reaction and this agrees with a random ternary complex sequence. However, the case of PsCCP is complicated. The end product might influence the reaction velocity in two different ways; by affecting the retardation phase or then competing with the ferrocytochrome *c* for the enzyme in the peroxidatic reaction. This ambiguity makes the diagnostic method less valuable for this particular case.

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