

Pseudomonas Cytochrome *c* Peroxidase. XI. Kinetics of the Peroxidatic Oxidation of *Pseudomonas* Respiratory Chain Components

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The steady state kinetics of cytochrome *c* peroxidase from *Pseudomonas aeruginosa* (PaCCP) has been studied by initial velocity techniques using several cytochromes *c* (550 and 555 *P. aeruginosa*; 551 *P. fluorescens*) and *Pseudomonas* azurin as electron donors and hydrogen peroxide as electron acceptor. From the initial velocity patterns a sequential mechanism with compulsory substrate-binding order is proposed for PaCCP. A comparative kinetic study of the peroxidatic oxidation of cytochrome *c*-551 (*P. aeruginosa*) by yeast cytochrome *c* peroxidase was made to evaluate the significance of electrostatic interactions in complex formation between the enzyme and substrates.

Pseudomonas cytochrome *c* peroxidase (PaCCP, cytochrome *c*:H₂O₂ oxidoreductase, EC 1.11.1.5) has been found to catalyze the peroxidatic oxidation of such bacterial respiratory chain components as cytochrome *c*-551 and azurin from *Pseudomonas*.¹ The kinetics of the peroxidatic oxidation of cytochrome *c* (551 *P. aeruginosa*) by PaCCP in buffers of different pH-values has recently been reported.² A sequential reaction mechanism in which both substrates add to the enzyme before either product is released was proposed for PaCCP. The present work was undertaken to differentiate between ordered and random substrate binding mechanisms.

The cytochromes used as substrates were characterized by their isoelectric points and extinction coefficients.

As in the earlier study,² all initial velocity measurements reported were performed in such a way that the phase of initial delay¹

was avoided, *i.e.* PaCCP was incubated with reduced substrates before the reaction was started by addition of H₂O₂.

MATERIALS AND METHODS

Pseudomonas cytochrome c peroxidase (PaCCP) was prepared from acetone-dried cells of *P. aeruginosa*, as previously described.³ The ratio A_{407}/A_{280} of the preparation was 4.4 and the specific activity was 115–130 U/mg, measured as reported earlier.⁴ The concentration of the enzyme was determined spectrophotometrically using A (1%, 1 cm) equal to 12.1 at 280 nm.⁵ The molar concentration of the enzyme was calculated using a molecular weight of 43 200.⁵

Yeast cytochrome c peroxidase (YCCP) was prepared as described previously.⁶ 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.*⁷

Horse heart cytochrome c (*H-cyt*) was a commercial preparation from Sigma Chemical Co., Type III, 98%, used without further purification. The extinction coefficients $\epsilon_{550}(\text{red.}) = 27.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\Delta\epsilon_{550}(\text{red.-ox.}) = 19.6 \text{ mM}^{-1} \text{ cm}^{-1}$ were used for the spectrophotometric determination of the cytochrome concentration.⁸

Pseudomonas cytochrome c-551 (*Pa-cyt-551*) and *Pseudomonas azurin* were prepared from acetone-dried cells of *P. aeruginosa* by the method of Ambler⁹ and Ambler and Brown.¹⁰ The purity of the cytochrome preparations [$A_{551}(\text{red.}) - A_{570}(\text{red.})/A_{280}$] was 1.12–1.19, and that of azurin [$A_{625}(\text{ox.})/A_{280}$] 0.4. The best azurin preparation of Ambler and Brown had a purity of 0.6. Both azurin and Pa-cyt-551, however, were found to be homogeneous on disc electrophoresis. The concentration of

Pa-cyt-551 was determined spectrophotometrically applying the extinction coefficients $\epsilon_{551}(\text{red.}) = 26.9 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\Delta\epsilon_{551}(\text{red.-ox.}) = 19.0 \text{ mM}^{-1} \text{ cm}^{-1}$, and the concentration of azurin by using $\epsilon_{625}(\text{ox.}) = 5.1 \text{ mM}^{-1} \text{ cm}^{-1}$.

Pseudomonas cytochromes c-550 (Pa-cyt-550) and c-555 (Pa-cyt-555) were prepared from *P. aeruginosa*. The methods of purification were essentially similar to those described for Pa-cyt-551 by Ambler.⁹ Cytochrome c-554,¹¹ which is a mixture of cytochromes c-550 and c-555, was eluted from CM-cellulose at pH 5.1 and the components were separated using either isoelectric focusing or, with slight modifications, the chromatographic purification method of Ambler and Taylor.¹² The purity of Pa-cyt-550 [$A_{550}(\text{red.}) - A_{570}(\text{red.})/A_{280}$] was 1.3 and that of Pa-cyt-555 [$A_{555}(\text{red.}) - A_{570}(\text{red.})/A_{280}$] 1.14. The extinction coefficients $\epsilon_{550}(\text{red.}) = 20.9 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\Delta\epsilon_{550}(\text{red.-ox.}) = 14.9 \text{ mM}^{-1} \text{ cm}^{-1}$, and $\epsilon_{555}(\text{red.}) = 24.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\Delta\epsilon_{555}(\text{red.-ox.}) = 17.4 \text{ mM}^{-1} \text{ cm}^{-1}$ were used for the spectrophotometric determination of Pa-cyt-550 and Pa-cyt-555, respectively.

Cultivation of Pseudomonas fluorescens (ATCC 17400). The bacteria were grown with stirring but without aeration, as described for *P. aeruginosa*.³ The cells were harvested 16 h after an 8 l inoculum had been transferred to 300 l of medium in a 400 l pilot plant fermentor (Getinge-Verken, Getinge, Sweden).

Cytochrome c-551 of P. fluorescens (Pf-cyt-551) was prepared from acetone-dried cells according to the method of Ambler and Wynn.¹³ The purity of Pf-cyt-551

[$A_{551}(\text{red.}) - A_{570}(\text{red.})/A_{280}$] was 1.2 and the preparation was homogeneous on disc electrophoresis. The concentration of the cytochrome was determined spectrophotometrically applying the extinction coefficients $\epsilon_{551}(\text{red.}) = 29.3 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\Delta\epsilon_{551}(\text{red.-ox.}) = 21.9 \text{ mM}^{-1} \text{ cm}^{-1}$.

Reduced cytochromes and azurin were prepared according to Yonetani and Ray¹⁴ 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 Yonetani.⁸

Measurements of reaction rates. The activities of *Pseudomonas* and yeast cytochrome c peroxidases were assayed spectrophotometrically by measuring the rate of peroxidatic oxidation of fully reduced substrates by the enzyme in phosphate buffer, pH 6.0, $\mu = 0.01$ (PaCCP) or in acetate buffer, pH 6.0, $\mu = 0.05$ (YCCP). The PaCCP-reaction was initiated by mixing 10 μl of hydrogen peroxide solution with the reaction mixture (2.0 ml), as previously described.³ The YCCP-reaction was initiated by mixing 5 μl of suitably diluted enzyme with the reaction mixture (2.0 ml) containing varying amounts of reduced cytochrome and peroxide. The reaction was

followed by recording the disappearance of electron donors or the appearance of products. All velocities were measured on a Cary 15 recording spectrophotometer with cell compartment thermostated at 25 °C. Reaction mixtures were incubated at 25 °C before the reaction was initiated. Initial velocities were determined from the slopes of the recorded lines and expressed in terms of mol reduced substrate oxidized per mol of enzyme per second.

Isoelectric focusing was carried out according to Vesterberg and Svensson¹⁵ in a LKB 8101 (110 ml) electrofocusing column, following the LKB manual. The density gradient was produced with sucrose (0–50 % w/v) and the pH gradient in the range 4–8 (for cytochromes) and 4–6 (for azurin) with carrier ampholytes (LKB-Produkter AB, Stockholm) at a concentration of 1 % (w/v). When necessary, the samples were dialyzed against 1 % glycine. Electrophoresis was usually performed for 22–24 h at 500 V and 9–15 °C. The pH of the fractions was measured with a Radiometer PHM 4 c pH meter at the same temperature as the electrophoresis was run.

Disc electrophoresis was carried out in polyacrylamide gel according to the procedures of Ornstein and Davis¹⁶ and Maurer.¹⁷ The basic gel system No. 1a of Maurer (pH 8.9, 7 % gel) was used. The protein bands were stained with Coomassie brilliant blue G-250 by the method of Diezel *et al.*¹⁸

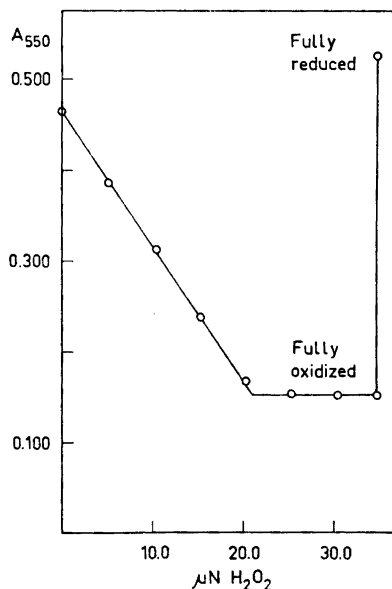


Fig. 1. Enzymatic titration of Pa-cyt-550 with H_2O_2 in the presence of 33 nM PaCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$ at 23 °C. Absorbances were measured with a Beckman DU-2 spectrophotometer.

Instruments. Spectrophotometric measurements were performed with a Beckman DU-2 spectrophotometer. Enzymatic activities were measured with a Cary 15 recording spectrophotometer equipped with cell compartment thermostated at 25 °C. pH was measured with a Radiometer pH meter 27 fitted with a combination glass-calomel electrode. Beckman pH 7 buffer No. 3501 was used for standardization.

Reagents were of analytical grade except those used in the cultivation medium for bacteria, which were of technical grade.

RESULTS

Properties of substrates. PaCCP was used for the determination of the extinction coefficients of Pa-cyt-550, Pa-cyt-555, and Pf-cyt-551 by enzymatic titration with standardized H_2O_2 . The PaCCP preparation used showed no oxidase or catalase activity, nor did the substrates undergo significant autoperoxidation under these conditions.

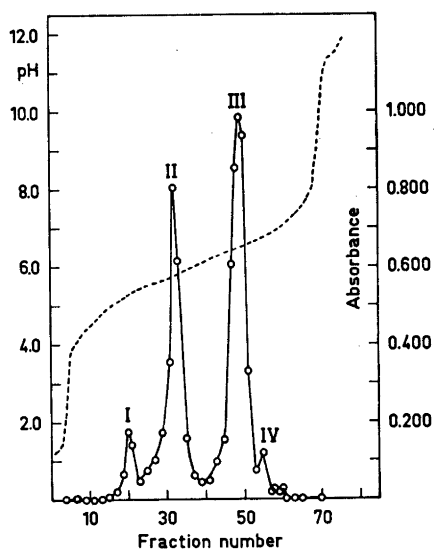


Fig. 2. Isoelectric focusing of the cytochrome *c*-554 "crude" preparation from *P. aeruginosa* in a pH 4 to 8 gradient at 14 °C. The experiment was carried out as described under Materials and Methods. pH is represented by the dotted line; O, absorbance at 416 nm. Fraction I represents reduced cytochrome *c*-555, fraction II the same cytochrome in oxidized form and fraction III oxidized cytochrome *c*-550. Fraction IV is due to a cytochrome (probably partly denatured) giving reduced α -peak at 553 nm.

Pa-cyt-550. Duplicate titrations were performed with Pa-cyt-550 in the presence of 0.033 μM PaCCP. The difference extinction coefficient, $\Delta\epsilon_{550}(\text{red-ox.})$, obtained from the slope of the titration curve was 14.9 $\text{mM}^{-1} \text{cm}^{-1}$ in both determinations. The values of the extinction coefficient of reduced Pa-cyt-550 were 21.0 and 20.8, average 20.9 $\text{mM}^{-1} \text{cm}^{-1}$, while those of oxidized Pa-cyt-550 were 6.1 and 5.9, average 6.0 $\text{mM}^{-1} \text{cm}^{-1}$. The titration curve is shown in Fig. 1.

The isoelectric focusing of cytochrome *c*-554 isolated from *P. aeruginosa*¹¹ gave three major components (Fig. 2), one of which corresponds to Pa-cyt-550 in the oxidized form and gives the isoelectric point of 6.5.

Pa-cyt-555. The extinction coefficients of Pa-cyt-555 were determined enzymatically in duplicate. Difference extinction coefficients, $\Delta\epsilon_{555}(\text{red-ox.})$, of 17.3 and 17.4 $\text{mM}^{-1} \text{cm}^{-1}$, average 17.4 $\text{mM}^{-1} \text{cm}^{-1}$, were obtained from the slopes of the titration curves (Fig. 3). The values for reduced Pa-cyt-555, $\epsilon_{555}(\text{red.})$, were 24.5 and 24.6 $\text{mM}^{-1} \text{cm}^{-1}$, average 24.6 $\text{mM}^{-1} \text{cm}^{-1}$, while 7.2 $\text{mM}^{-1} \text{cm}^{-1}$ was found for oxidized Pa-cyt-555, $\epsilon_{555}(\text{ox.})$, in both titrations.

The isoelectric points (Fig. 2) of reduced

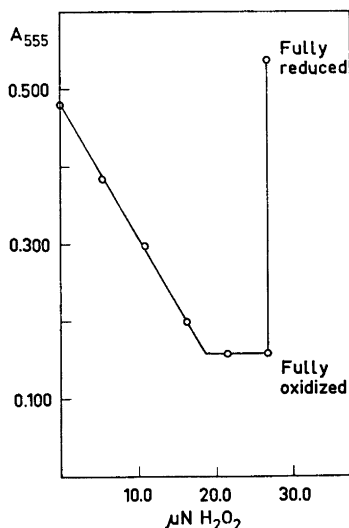


Fig. 3. Enzymatic titration of Pa-cyt-555 with H_2O_2 in the presence of 33 nM PaCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, at 23 °C. Absorbances were measured with a Beckman DU-2 spectrophotometer.

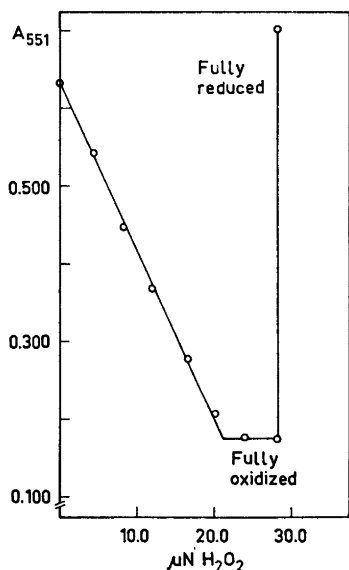


Fig. 4. Enzymatic titration of Pf-cyt-551 with H_2O_2 in the presence of 24 nM PaCCP in sodium phosphate buffer, pH 6.0, $\mu=0.01$, at 23 °C. Absorbances were measured with a Beckman DU-2 spectrophotometer.

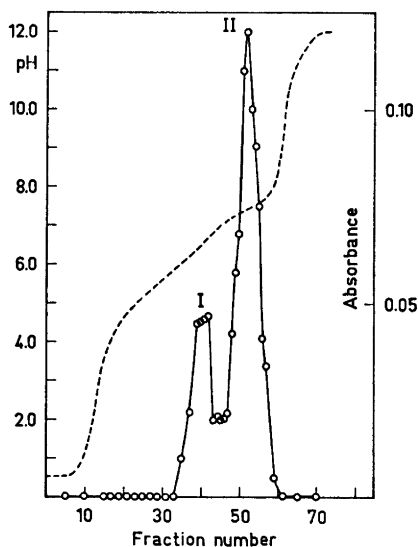


Fig. 5. Isoelectric focusing of cytochrome c (551 *P. fluorescens*) in a pH 4 to 8 gradient at 12 °C. Experimental details are described under Materials and Methods. \circ absorbance at 408 nm, pH is represented by the dotted line. Component I corresponds to the reduced and Component II to the oxidized form of the cytochrome.

and oxidized Pa-cyt-555 were 5.2 and 5.7, respectively.

Pf-cyt-551. Duplicate titrations (Fig. 4) of Pf-cyt-551 gave a difference extinction coefficient, $\Delta\epsilon_{551}(\text{red.-ox.})$, of 21.8 and 22.0 $\text{mM}^{-1} \text{cm}^{-1}$, average 21.9 $\text{mM}^{-1} \text{cm}^{-1}$. The values for the reduced Pf-cyt-551, $\epsilon_{551}(\text{red.})$, were 29.0 and 29.5, average 29.3 $\text{mM}^{-1} \text{cm}^{-1}$; those of the oxidized cytochrome, $\epsilon_{551}(\text{ox.})$, 7.2 and 7.5 $\text{mM}^{-1} \text{cm}^{-1}$, average 7.4 $\text{mM}^{-1} \text{cm}^{-1}$.

Isoelectric focusing of Pf-cyt-551 (Fig. 5) gave the pI-values 6.7 and 7.3 for the reduced and oxidized form of the cytochrome, respectively.

Azurin. A pI-value of 5.6 (measured at 18 °C) was obtained for *Pseudomonas azurin* in the oxidized form. This is in good agreement with the literature value of 5.4.¹⁹

Initial velocity patterns of PaCCP. All initial rate measurements were made in sodium phosphate buffer, pH 6.0, $\mu=0.01$. The concentrations of Pa-cyt-550 varied in the range 7–16 μM and hydrogen peroxide in the range 50–180 μM . Initial velocity measurements with Pa-cyt-555 as substrate were carried out in the concentration range 8–28 μM , while $[\text{H}_2\text{O}_2]$ was varied from 20–205 μM . The concentration range of Pf-cyt-551 was 7–23 μM and of H_2O_2 , 20–210 μM . Initial velocity measurements with *Pseudomonas azurin* as substrate were carried out in the concentration range 12–40 μM with H_2O_2 varied from 30 to 300 μM . The enzyme concentration employed (0.32 μM) was 200 times larger with horse heart cytochrome c than with bacterial cytochromes. The H-cyt concentration varied in the range 7–20 μM and H_2O_2 from 20 to 206 μM .

Within experimental error, and over the concentration ranges shown, all the plots are linear (Figs. 6, 7, 10, 11, 14, 15) and the results are therefore consistent with the initial-rate equation of Dalziel²⁰

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

The secondary plots of intercepts and slopes were linear as well, and the kinetic coefficients ϕ_0 , ϕ_1 , ϕ_2 , ϕ_{12} in eqn. 1 calculated from them are shown in Table 1. Even though the lines of the Lineweaver-Burk plots (Figs. 6 and 10) are only weakly intersecting when peroxide

Table 1. Kinetic coefficients for the peroxidatic oxidation reaction catalyzed by *Pseudomonas* cytochrome *c* peroxidase at 25 °C in sodium phosphate buffer, pH 6.0. The kinetic coefficients are those in the initial-rate equation

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

where S_1 is taken as H_2O_2 and S_2 as the electron donor. Assuming a compulsory-order mechanism, the following relationships exist between the kinetic coefficients (Dalziel coefficients) and the rate constants: $k_3 = 1/\phi_0$; $k_2 = 1/\phi_2$; K_m of $S_2 = \phi_2/\phi_0$; K_m of $S_1 = \phi_1/\phi_0$; $K_s = k_{-1}/k_1 = \phi_{12}/\phi_2$; $k_{-1} = \phi_{12}/\phi_1\phi_2$. Rate constants refer to the reactions in Scheme 1.

Substrate	ϕ_0 s	ϕ_1 M s	ϕ_2 M s	ϕ_{12} M ² s	ϕ_2/ϕ_0 M	ϕ_1/ϕ_0 M	ϕ_{12}/ϕ_2 M	$\phi_{12}/\phi_1\phi_2$ s ⁻¹
Pa-cyt-551 ^a	1.6×10^{-3}	1.0×10^{-8}	1.4×10^{-7}	0.2×10^{-12}	8.8×10^{-5}	6.3×10^{-5}	1.4×10^{-6}	143^b
Pa-cyt-550	1.6×10^{-3}	1.6×10^{-8}	1.1×10^{-7}	0.8×10^{-12}	6.9×10^{-5}	1.0×10^{-5}	7.3×10^{-6}	455
Pa-cyt-555	2.0×10^{-3}	1.8×10^{-8}	1.8×10^{-7}	0.6×10^{-12}	9.0×10^{-5}	0.9×10^{-5}	3.3×10^{-6}	185
Azurin	1.4×10^{-3}	1.5×10^{-8}	1.7×10^{-7}	0.5×10^{-12}	1.2×10^{-4}	1.1×10^{-5}	2.9×10^{-6}	196
Pf-cyt-551	1.6×10^{-3}	1.3×10^{-8}	1.1×10^{-7}	0.1×10^{-12}	6.9×10^{-5}	0.8×10^{-5}	0.9×10^{-6}	70
Horse heart cytochrome	0.1	4.0×10^{-6}	3.0×10^{-5}	3.2×10^{-10}	3.0×10^{-4}	4.0×10^{-5}	1.1×10^{-5}	2.7

^a The data for Pa-cyt-551 are from Ellfolk *et al.*² ^b The value of this relationship was misprinted in the earlier paper.²

Table 2. Kinetic coefficients for the peroxidatic oxidation reaction catalyzed by yeast cytochrome *c* peroxidase at 25 °C in sodium acetate buffer, pH 6.0. The kinetic coefficients are those in the initial-rate equation

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

where S_1 is the peroxide and S_2 the electron donor. The following relationships exist between the kinetic coefficients and the rate constants of Scheme 1: $k_3 = 1/\phi_0$; $k_1 = 1/\phi_2$; K_m of $S_2 = \phi_2/\phi_0$; K_m of $S_1 = \phi_1/\phi_0$; $K_s = k_{-1}/k_1 = \phi_{12}/\phi_2$; $k_{-1} = \phi_{12}/\phi_1\phi_2$.

Substrate	ϕ_0 s	ϕ_1 M s	ϕ_2 M s	ϕ_{12} M ² s	ϕ_2/ϕ_0 M	ϕ_1/ϕ_0 M	ϕ_{12}/ϕ_2 M	$\phi_{12}/\phi_1\phi_2$ s ⁻¹
Horse heart ^a cytochrome <i>c</i>	3.8×10^{-4}	7.1×10^{-9}	1.7×10^{-9}	—	4.5×10^{-6}	1.9×10^{-5}	$\sim 10^{-8}$	$\sim 10^0$
Horse heart cytochrome <i>c</i>	5.5×10^{-4}	9.8×10^{-9}	2.8×10^{-9}	1.3×10^{-14}	5.1×10^{-6}	1.8×10^{-5}	4.6×10^{-6}	474
Pa-cyt-551	0.5	5.0×10^{-6}	2.1×10^{-5}	7.0×10^{-11}	4.2×10^{-5}	1.0×10^{-5}	3.3×10^{-6}	0.7

^a Calculated from the data of Yonetani and Ray (1966).

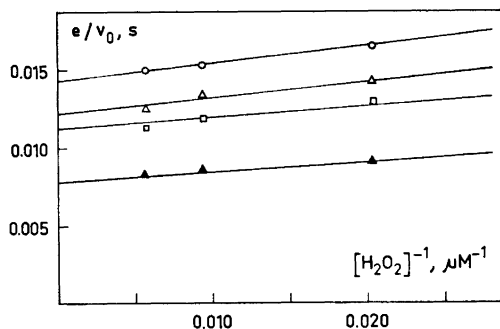


Fig. 6. Primary plots: variation of the reciprocal of the specific initial rate in sodium phosphate buffer at pH 6.0 and 25 °C with the reciprocal of the H_2O_2 concentration for several constant concentrations of reduced Pa-cyt-550 (μM): O, 8.0; Δ , 10.9; \square , 12.0; \blacktriangle , 16.4. The concentration of PaCCP was 1.51 nM.

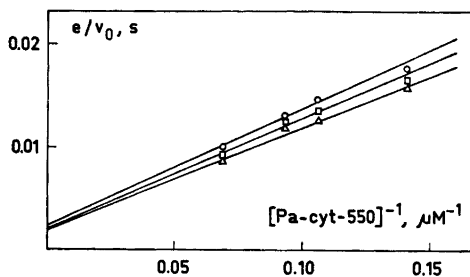


Fig. 7. Primary plots: variation of the reciprocal of the specific initial rate with the reciprocal of the Pa-cyt-550 concentration for several constant concentrations of H_2O_2 (μM): O, 49.3; \square , 108.0; Δ , 178.0.

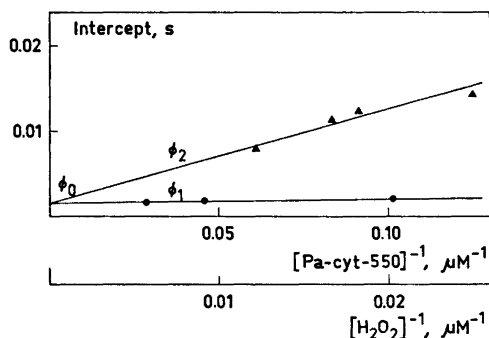


Fig. 8. Secondary plots of primary plot intercepts versus the reciprocal of fixed substrate concentration. Triangles and circles, respectively, represent data taken with Pa-cyt-550 and H_2O_2 as the fixed substrate. Kinetic coefficients calculated from the plot are given in Table 1.

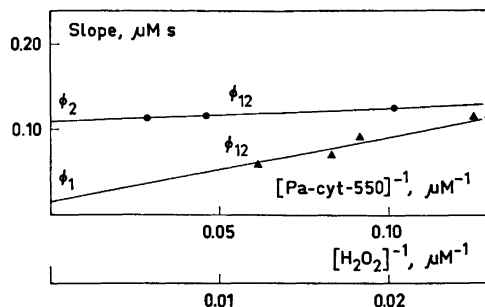


Fig. 9. Secondary plots of primary plot slopes versus the reciprocal of fixed substrate concentration. Triangles and circles, respectively, represent data taken with Pa-cyt-550 and H_2O_2 as the fixed substrate. Kinetic coefficients calculated from the plot are given in Table 1.

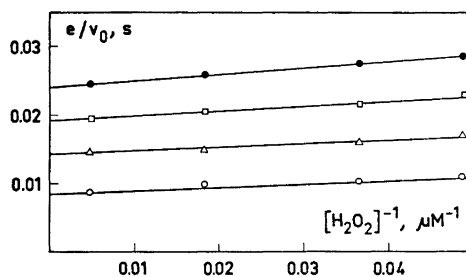
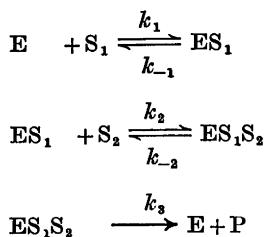


Fig. 10. Primary plots: variation of the reciprocal of the specific initial rate in sodium phosphate buffer at pH 6.0 and 25 °C with the reciprocal of the H_2O_2 concentration for several constant concentrations of reduced Pa-cyt-555 (μM): \bullet , 8.2; \square , 11.0; Δ , 15.8; \circ , 28.2. The concentration of PaCCP was 1.62 nM.

is the variable substrate, evidence for the presence of a ϕ_{12} -term in eqn. 1 is supplied from Slater-plots.²¹ These plots of apparent K_m values against apparent maximum velocities pass through the origin in the absence of the ϕ_{12} -term, whereas in the reactions of PaCCP and YCCP Slater-plots intersect the ordinate at a positive value. Thus a sequential reaction mechanism can be proposed for these peroxidases (Scheme 1).

Initial velocity patterns of YCCP. All initial velocity measurements were made in sodium acetate buffer, pH 6.0, $\mu = 0.05$. The concentration of horse heart cytochrome *c* varied in the range 2–12 μM and hydrogen peroxide in the range 2–180 μM . The YCCP-concentration (90.5 nM) used with Pa-cyt-551 was more



Scheme 1.

than 300 times that employed in the H-cyt-reaction; the concentration of Pa-cyt-551 varied in the range 5–17 μM and H_2O_2 from 16 to 170 μM . The primary Lineweaver-Burk plots drawn from the data (Figs. 14, 15) are linear, and the kinetic coefficients in eqn. 1 could be estimated from the secondary plots of intercepts (Fig. 16) and slopes (Fig. 17). The kinetic coefficients of the YCCP-reaction are shown in Table 2.

DISCUSSION

Steady state experiments alone can distinguish between a random substrate addition mechanism and an ordered one by using Dalziel coefficients,²⁰ Haldane relationships,²² and product inhibition studies.^{23,24} Although product inhibition studies may resolve some mechanistic differences when used in one reaction direction only, a full kinetic diagnosis for a particular enzyme system using any of these approaches requires data from both forward and reverse reactions.

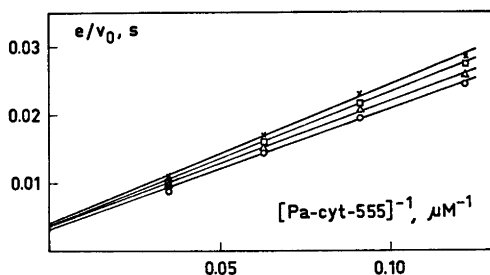


Fig. 11. Primary plots: variation of the reciprocal of the specific initial rate with the reciprocal of the Pa-cyt-555 concentration for several constant concentrations of H_2O_2 (μM): \times , 20.6; \square , 27.4; \triangle , 54.8; \circ , 205.5.

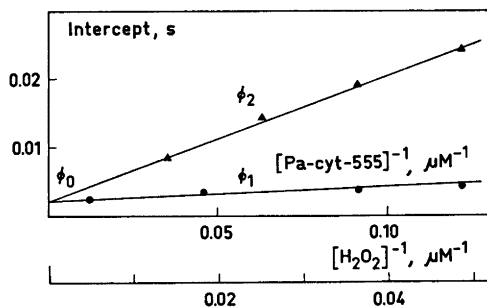


Fig. 12. Secondary plots of primary plot intercepts versus the reciprocal of fixed substrate concentration. Triangles and circles, respectively, represent data taken with Pa-cyt-555 and H_2O_2 as the fixed substrate. Kinetic coefficients calculated from the plot are given in Table 1.

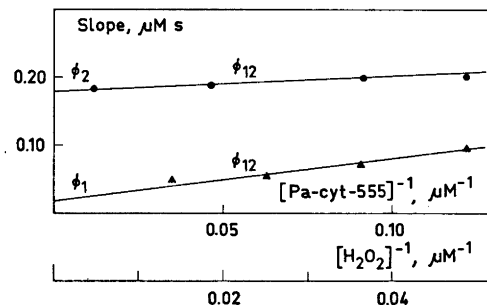


Fig. 13. Secondary plots of primary plot slopes versus the reciprocal of fixed substrate concentration. Triangles and circles, respectively, represent data taken with Pa-cyt-555 and H_2O_2 as the fixed substrate. Kinetic coefficients calculated from the plot are given in Table 1.

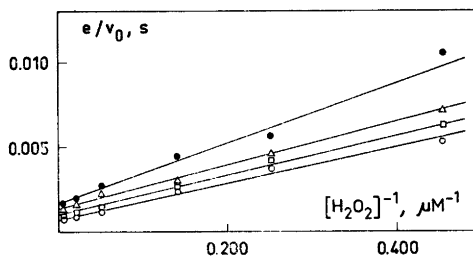


Fig. 14. Primary plots; variation of the reciprocal of the specific initial rate in sodium phosphate buffer pH 6.0 and 25 $^\circ\text{C}$ with the reciprocal of the H_2O_2 concentration for several constant concentrations of reduced horse heart cytochrome *c* (μM): \bullet , 2.1; \triangle , 3.8; \square , 8.2; \circ , 12.5. The concentration of yeast cytochrome *c* peroxidase was 0.35 nM.

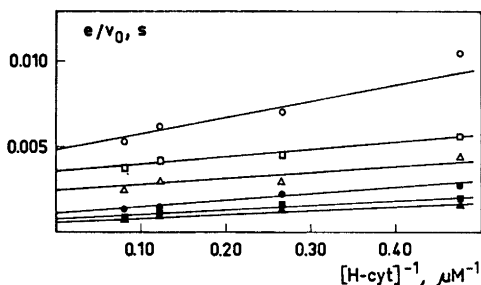


Fig. 15. Primary plots: variation of the reciprocal of the specific initial rate with the reciprocal of the horse cytochrome *c* concentration for several constant concentrations of H_2O_2 (μM): \circ , 2.2; \square , 4.0; \triangle , 7.1; \bullet , 19.6; \blacksquare , 49.5; \blacktriangle , 178.0.

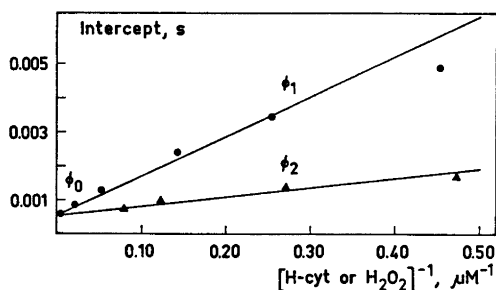


Fig. 16. Secondary plots of primary plot intercepts versus the reciprocal of fixed substrate concentration. Triangles and circles, respectively, represent data taken with horse heart cytochrome *c* and H_2O_2 as the fixed substrate. Kinetic coefficients calculated from the plot are given in Table 2.

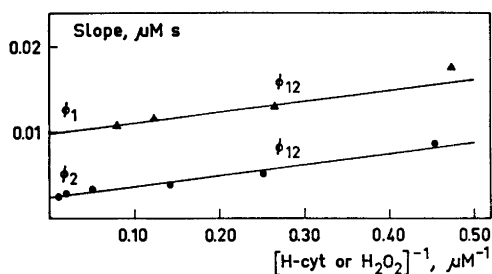


Fig. 17. Secondary plots of primary plot slopes versus the reciprocal of fixed substrate concentration. Triangles and circles, respectively, represent data taken with horse heart cytochrome *c* and H_2O_2 as the fixed substrate. Kinetic coefficients calculated from the plot are given in Table 2.

Intersecting initial velocity patterns led to the proposal of a sequential reaction mechanism for the peroxidatic oxidation of cytochrome *c* (551 *P. aeruginosa*) by *Pseudomonas* cytochrome *c* peroxidase.³ Because of the "irreversible" nature of the peroxidase reaction the conventional kinetic approaches mentioned above are not conclusive. Another way of tackling the mechanistic problem of the PaCCP-reaction is to determine the kinetic coefficients with several substrates, which we have done with cytochromes *c* 550 and 555 (*P. aeruginosa*) and cyt *c* (551 *P. fluorescens*) as well as with a copper-containing protein, the blue protein or azurin from *P. aeruginosa*.

The values of ϕ_0 and ϕ_1 for the PaCCP-reaction (Table 1) with each of these substrates are almost constant, and the identity of these values is expected for the general compulsory-order mechanism.²⁵ Nevertheless, for a compulsory-order mechanism the ratio ϕ_{12}/ϕ_2 should also be independent of the nature of the substrate. In fact, however, the ratio varies from substrate to substrate. This variation may, at least partly, be due to the fact that the ϕ_{12} -values are very small so that the ratio cannot be calculated with certainty.

PaCCP seems to have similar reactivity with all the bacterial protein substrates used. The K_m -values of the cytochromes vary from 69 μM (Pf-cyt-551) to 90 μM (Pa-cyt-555) and the Michaelis constant for *Pseudomonas* azurin is 120 μM . These values are remarkably similar considering that the protein substrates differ widely in some molecular properties, e.g. isoelectric point and molecular weight. Furthermore, azurin differs from cytochromes even with respect to the prosthetic group. In contrast, horse heart cytochrome *c* is a poor substrate for PaCCP since all the kinetic coefficients are greatly increased compared to those of the bacterial electron donors. The higher value of ϕ_0 also suggests that the intramolecular reaction of the ternary complex is slower than with bacterial cytochromes.²⁵

All the kinetic coefficients of the YCCP-reaction (Table 2) are substantially greater with the bacterial cytochrome Pa-cyt-551 than with horse heart cytochrome, indicating that Pa-cyt-551 is a poor substrate for YCCP. Nevertheless, both the Michaelis constant for H_2O_2 (ϕ_1/ϕ_0) and the ratio ϕ_{12}/ϕ_2 are roughly

independent of the nature of the electron donor. The constancy of the ratio ϕ_{12}/ϕ_2 agrees with a compulsory order mechanism. Because an ordered mechanism also requires the identity of the ϕ_0 and ϕ_1 values for different substrates,²⁵ and the bacterial cytochrome does not satisfy these criteria in the YCCP-reaction, the mechanism of the enzyme cannot be deduced from these results. The reaction mechanism of YCCP is currently a subject of some controversy. YCCP has been assumed to follow a general compulsory-order mechanism when mammalian-type cytochromes are used as substrates,²⁶ whereas Nicholls and Mochan,²⁷ working from the formation of reversible complexes between YCCP and cytochrome *c*, have recently proposed a random-addition mechanism for YCCP.

Electrostatic forces are known to be predominant in cytochrome *c* complex formation with yeast cytochrome *c* peroxidase.^{27,28} Accordingly, full activity is obtained with YCCP (pI 5.2)²⁹ and oppositely charged basic substrates like horse heart cytochrome *c* (pI 10.05),³⁰ but not with the acidic Pa-cyt-551 (pI 4.7).³¹ With the bacterial cytochrome, however, the selective specificity of YCCP may also affect the reaction rate. In the case of PaCCP (pI 6.7),³ forces of an electrostatic nature seem not to be of importance in the formation of enzyme-substrate complexes with acidic or neutral cytochromes, *i.e.* Pa-cyt-551 (pI 4.7), Pa-cyt-550 (pI 6.5), and Pf-cyt-551 (pI 6.7), since reaction velocities with these different substrates are closely similar. On the other hand, the reaction velocity is markedly lowered with horse heart cytochrome *c* (Table 1). As with YCCP, the specificity of the enzyme should also be considered when explaining reduced activity. Moreover, the isoelectric points of the substrates do not necessarily reflect the charge of the contact area with the enzymes.

It seems that YCCP and PaCCP are kinetically different and cannot be directly compared with each other even if both should follow an ordered mechanism. It has to be borne in mind, too, that PaCCP, unlike YCCP, exhibits an initial delay before reacting if the enzyme is not incubated with the electron donor before addition of H₂O₂. Because of this initial delay the true nature of the PaCCP-reaction remains to be established.

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