Pseudomonas Cytochrome c Peroxidase

IV. Some Kinetic Properties of the Peroxidation Reaction, and Enzymatic Determination of the Extinction Coefficients of Pseudomonas Cytochrome c-551 and Azurin

RITVA SOININEN and NILS ELLFOLK

Department of Biochemistry, University of Helsinki, Helsinki 17, Finland

Kinetic data are reported for the peroxidation reaction catalyzed by Pseudomonas cytochrome c peroxidase. The optimum pH of the peroxidation of Pseudomonas ferrocytochrome c-551 was found to be 6.0 and the optimum ionic strength 0.01 in sodium acetate and sodium phosphate buffers. Pseudomonas cytochrome c peroxidase reacts specifically with two Pseudomonas respiratory chain pigments, ferrocytochrome c-551 and reduced azurin. Horse heart ferrocytochrome c, yeast ferrocytochrome c, and conventional low molecular weight peroxidase substrates such as pyrogallol, guajacol, sodium ascorbate, ferrocyanide, and reduced 2,6-dichlorobenzenone-indo-3'-chlorophenol were found to be poor donors in the peroxidation. Ethyl hydroperoxide could not replace hydrogen peroxide as an electron acceptor in the peroxidation reaction. Maximal turnover rates for Pseudomonas ferrocytochrome c-551 and reduced azurin were found to be 710 sec^{-1} and 670 sec^{-1}, and apparent K_m-values were 91 μM and 125 μM, respectively, at a fixed hydrogen peroxide concentration (84 μM) in sodium phosphate buffer, pH 6.0, ionic strength of 0.01, 23°C. The maximal turnover rate for hydrogen peroxide was 118 sec^{-1} and the apparent K_m was 6 μM at a fixed Pseudomonas ferrocytochrome c-551 concentration (13.0 μM), under the same conditions. An initial delay in the peroxidation was observed when the enzyme was added to the reaction mixture containing electron donor and hydrogen peroxide, but there was no delay when the enzyme was incubated with donor before the addition of hydrogen peroxide. The extinction coefficients of Pseudomonas cytochrome c-551 and azurin were determined enzymatically by the reaction catalyzed by Pseudomonas cytochrome c peroxidase. The following values were obtained for cytochrome c-551; ε_{551} (reduced) = 26.9 mM^{-1} cm^{-1} and $\Delta\varepsilon_{551}$ (reduced — oxidized) = 19.0 mM^{-1} cm^{-1}; and for azurin: ε_{625} (oxidized) = 5.1 mM^{-1} cm^{-1}. The results are discussed.

Lenhoff and Kaplan [1] first showed the existence of cytochrome c peroxidase in Pseudomonas cells. They studied the characteristics of the reaction catalyzed by the enzyme, using a partially purified preparation containing

cytochrome c, catalase, and presumably other respiratory chain pigments. The activity was measured by means of a coupling reaction with an indophenol dye.

Cytochrome c peroxidase has recently been isolated in pure form from *Pseudomonas*.[2] We have studied its location in the cell and some of its physicochemical properties,[3,4] and now report on the kinetics of the peroxidation reaction. The extinction coefficients of the peroxidase substrates *Pseudomonas* cytochrome c-551 and azurin, determined enzymatically, are also reported.

MATERIALS AND METHODS

Pseudomonas cytochrome c peroxidase (PsCCP) was prepared from acetone-dried cells of *P. aeruginosa* [4] as previously described.[2] The ratio A_{407}/A_{280} of the preparation used was 4.28. The concentration of the enzyme was determined spectrophotometrically, using $A(1\%, 1\ cm) = 12.1$ at 280 nm.[4] The molar concentrations were calculated on the basis of a molecular weight of 53 500.[4]

Yeast cytochrome c peroxidase (YCCP) was prepared in crystalline form from baker's yeast by the method of Ellfolk;[5] 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 haematin content, measured as pyridine ferrohemochrome (Paul *et al.*[6]).

Horseradish peroxidase (HRP) was a commercial preparation from Sigma (Type II, RZ 1.40).

Pseudomonas cytochrome c-551 (Ps-cyt-551) and Pseudomonas azurin were prepared from acetone-dried cells of *P. aeruginosa* by the method of Ambler[7] and Ambler and Brown.[8] The purity of the Ps-cyt-551 preparation $(A_{551}(red.) - A_{570}(red.)/A_{280})$ was 1.09 − 1.13 and that of the azurin $(A_{625}(ox.)/A_{280})$ 0.38. Ambler and Brown obtained an azurin preparation of higher purity, namely 0.60.[8] Both Ps-cyt-551 and azurin showed only two protein bands (reduced and oxidized) on disc electrophoresis. The concentration of Ps-cyt-551 was determined spectrophotometrically, applying the following extinction coefficients: $\varepsilon_{551}(red.) = 26.9\ mM^{-1}\ cm^{-1}$ and $\Delta\varepsilon_{551}(red. - ox.) = 19.0\ mM^{-1}\ cm^{-1}$ and the concentration of azurin by using $\varepsilon_{625}(ox.) = 5.1\ mM^{-1}\ cm^{-1}$.

Horse heart cytochrome c (H-cyt) was a commercial preparation from Sigma (Type III, 98 %) and was used without further purification. The extinction coefficients $\varepsilon_{550}(red.) = 27.6\ mM^{-1}\ cm^{-1}$ and $\Delta\varepsilon_{550}(red. - ox.) = 19.6\ mM^{-1}\ cm^{-1}$ were used for the spectrophotometric determination of cytochrome concentration.[9]

Yeast cytochrome c (Y-cyt) was prepared in crystalline form from baker's yeast by the method of Hagihara *et al.*[10] The purity of the preparation $(A_{550}(red.)/A_{280})$ was 1.01, and only minor impurities were observed on disc electrophoresis. The concentration of Y-cyt was determined according to the following extinction coefficients: $\varepsilon_{550}(red.) = 29.0\ mM^{-1}\ cm^{-1}$ and $\Delta\varepsilon_{550}(red. - ox.) = 21.2\ mM^{-1}\ cm^{-1}$.[9]

Reduced cytochromes and azurin were prepared by anaerobic gel filtration of dithionite-reduced proteins on Sephadex G-25.[11] Colourless reduced azurin was localized in the fractions collected from the column by oxidizing small samples from the fractions with ferricyanide.

Low molecular weight electron donors. Sodium ascorbate (*purum*, Fluka), potassium ferrocyanide (*p.a.*, Merck), pyrogallol (crystalline, Merck), guajacol (crystalline, Rhone-Poulenc) and 2,6-dichlorobenzenone-indo-3'-chlorophenol (K & K Laboratories) were tested as peroxidase substrates without further purification. Solutions of sodium ascorbate, pyrogallol, and the indophenol dye (*i.e.* those substrates in which the presence of endogenous H_2O_2 or the generation of H_2O_2 during the reduction could be expected) in oxygen free buffer were prepared just before use. Reaction rates were measured by spectrophotometric determination at the appropriate wavelengths [12-14] of changes of substrate concentrations. The indophenol dye was reduced with hydrogen in the presence of palladium-asbestos as described by Smith and Stotz.[15] The concentration of the indophenol dye was determined at 575 nm by spectrophotometric titration with sodium ascorbate in sodium phosphate buffer, pH 6.0, $\mu = 0.01$.

Peroxides. Hydrogen peroxide solutions were prepared from Merck Perhydrol (30 % H_2O_2); ethyl hydroperoxide was synthesized from diethyl sulphate and hydrogen peroxide by the method of Rieche and Hitz;[16] solutions were made up with buffer. Peroxide concentration was determined enzymatically with YCCP as described by Yonetani.[9]

Measurements of reaction rates. Enzymatic activities were determined spectrophotometrically by following the disappearance of electron (or hydrogen) donors, or the appearance of products. The reaction was initiated, after the addition of $2-10$ μl of enzyme, by rapidly mixing $5-10$ μl of H_2O_2 or C_2H_5OOH with the reaction mixture of final volume 2.5 ml, containing varying amounts of electron donor if not otherwise stated. Any autoperoxidation of donors was recorded in the absence of enzyme under the same conditions. Initial rates were calculated from the slope of the reaction curve at zero time if not otherwise stated. Initial rates (v_0/e) were expressed in terms of equivalents of electron or hydrogen transferred per second per molecule of enzyme.

Buffers, ionic strength $0.01-0.3$: (i) sodium acetate, pH $4-6$; and (ii) sodium phosphate, pH $6-7.5$.

Disc electrophoresis in polyacrylamide gel was carried out according to the procedures of Ornstein and Davis [17] and Maurer.[18] The basic gel system No. 1a of Maurer (pH 8.9, 7 % gel) was used for the acidic proteins and the acid gel system No. 7 of Maurer (pH 4.3, 15 % gel) for the basic proteins. The runs were performed at $+4°C$ using a current of 1.5 mA per gel. The protein bands were stained with Coomassie brilliant blue by the method of Weber and Osborne,[19] the gel being destained electrophoretically at room temperature.

Instruments. Spectrophotometric measurements were performed with a Beckman DU-2 spectrophotometer. Enzymatic activities were followed with a Beckman DK-1 A or a Cary 15 recording spectrophotometer equipped with cell compartments thermostated at 23°C or 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 were of analytical grade if not otherwise stated.
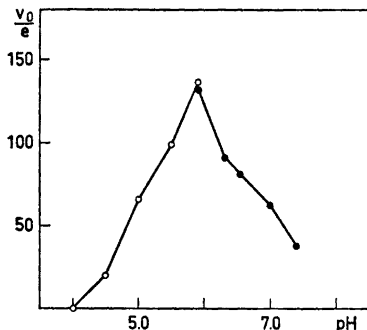
Fig. 1. Effect of pH on the peroxidation of Ps-cyt-551 catalyzed by PsCCP in a reaction mixture of ionic strength 0.01 at 25°C. The reactant concentrations used were: reduced Ps-cyt-551 21.5 μM, H_2O_2 101 μM, and PsCCP 1.44 nM. The reaction was initiated by adding the enzyme to the reaction mixture. Reaction curves were recorded with a Beckman DK-1 A spectrophotometer and the initial rates were estimated graphically from the reaction curves after the initial delay (see Fig. 3 *A*). The slight autoperoxidation of Ps-cyt-551 observed in acetate buffers was accounted for in calculating the results. O, sodium acetate buffers. ●, sodium phosphate buffers.
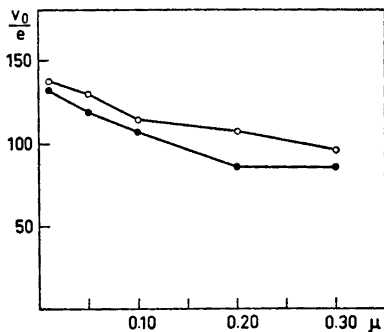
Fig. 2. Effect of ionic strength on the peroxidation of Ps-cyt-551 catalyzed by PsCCP; pH 6.0, 25°C. The reactant concentrations used were: reduced Ps-cyt-551 21.5 μM, H_2O_2 101 μM, and PsCCP 1.44 nM. Other details were the same as those described in Fig. 1. O, sodium acetate buffers. ●, sodium phosphate buffers.

RESULTS

Ionic strength and pH optimum. Effects of ionic strength and pH on the peroxidation of Ps-cyt-551 catalyzed by PsCCP were studied in sodium acetate and sodium phosphate buffers in the pH range 4.0 − 7.5 and ionic strengths 0.01 − 0.3. The PsCCP activity has a distinct pH optimum at pH 6.0 in both acetate and phosphate buffer, as shown in Fig. 1. The dependence of the reaction rate on ionic strength at the optimum pH is illustrated in Fig. 2. The maximum reaction rates were obtained at the lowest ionic strengths studied, the optimum being 0.01 in both buffers throughout the entire pH range. The reaction rate was considerably lower in distilled water than in buffer of the lowest ionic strength examined. The optimum conditions of pH 6.0 and $\mu = 0.01$ for the peroxidation reaction were used in further studies described in this paper.

Reaction curves of the peroxidation of Ps-cyt-551 catalyzed by PsCCP in sodium phosphate and sodium acetate buffers, pH 6.0, $\mu = 0.01$ were obtained; a reaction curve and a semilogarithmic plot of the curve are shown in Fig. 3 (phosphate buffer). The initial reaction rate observed when enzyme was added first differed from that found when hydrogen peroxide was added first to the reaction mixture. There was a considerable delay, of about 5 to 10 sec, when enzyme was added after hydrogen peroxide (Fig. 3). In the semilogarithmic plot two pseudo first-order reaction phases can be observed. In contrast, when the enzyme was added and incubated with Ps-cyt-551 for 10 − 30 sec before addition of hydrogen peroxide the reaction obeyed first-order kinetics
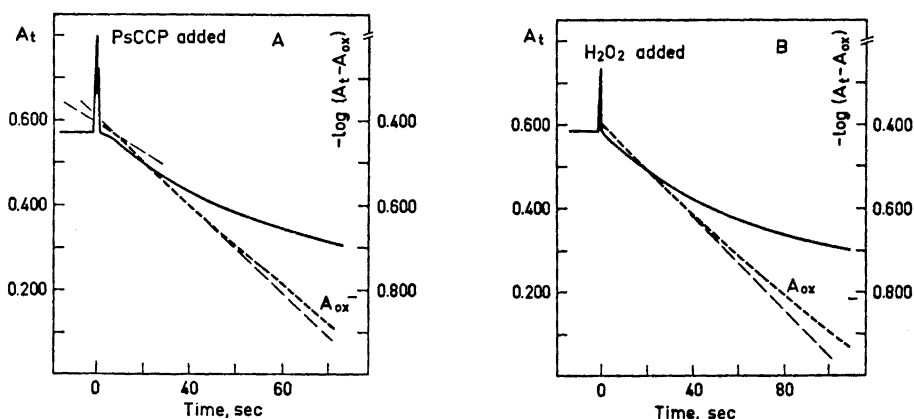

Fig. 3. Reaction curves and semilogarithmic plots of reaction curves of the peroxidation of Ps-cyt-551 catalyzed by PsCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, 23°C. The reactant concentrations used were: reduced Ps-cyt-551 21.0 μM, H_2O_2 84 μM, and PsCCP 1.52 nM. Curves were recorded with a Beckman DK-1 A spectrophotometer. Log $(A_t - A_{ox.})$ corresponds to the logarithm of the change in absorbance at 551 nm of Ps-cyt-551 at a given time (A_t) minus the absorbance of fully oxidized Ps-cyt-551 $(A_{ox.})$. *A.* Reaction initiated by adding enzyme to the reaction mixture. *B.* Reaction initiated by adding H_2O_2 to the reaction mixture. ———, reaction curve. − − −, semilogarithmic plot of reaction curve.

initially. Therefore reaction curves obtained after the latter sequence, that is enzyme first then peroxide, were used for calculation of initial reaction rates, which data were used in turn for the determination of maximal turnover rates and K_m-values reported below. In both cases the reaction curve showed a slight upward concavity later. Similar reaction curves were observed with sodium acetate buffer, pH 6.0, $\mu = 0.01$. Activity was slightly but not significantly greater in acetate buffer, as noted above. In the concentration range 5 − 25 μM Ps-cyt-551 the course of the peroxidation reaction, as indicated by the reaction curves, was the same, the initial delay increasing with increasing concentrations of Ps-cyt-551. Delay was also observed when the PsCCP activities were measured in the crude extracts from the acetone-dried cells and in the samples taken at various stages of the purification. At the Ps-cyt-551 concentrations used, however, the delay was so short that it could be neglected and the initial rates estimated graphically.

Similar reaction curves were observed with the other peroxidase substrate, azurin (Fig. 4).

Enzymatic determination of the extinction coefficients of Ps-cyt-551 and azurin. PsCCP can be used for the determination of the extinction coefficients of its substrates by enzymatic titration with H_2O_2. More than 90 % reduced Ps-cyt-551 and 100 % reduced azurin were titrated in the presence of 0.076 μM PsCCP with H_2O_2 solution which had been standardized by titrating enzymatically with YCCP, as described by Yonetani.[9] The PsCCP preparation used showed no oxidase or catalase activity, nor did the substrates undergo
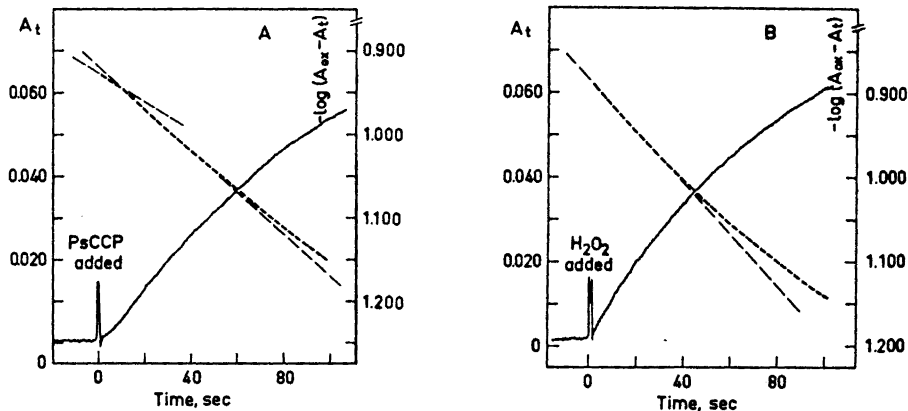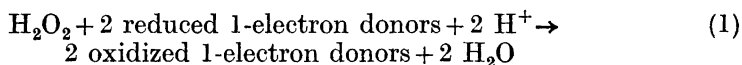

Fig. 4. Reaction curves and semilogarithmic plots of reaction curves of the peroxidation of azurin catalyzed by PsCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, 23°C. The reactant concentrations used were: reduced azurin 27.0 μM, H_2O_2 79 μM, and PsCCP 1.44 nM. Curves were recorded with a Cary 15 spectrophotometer. Log $(A_{ox} - A_t)$ corresponds to the logarithm of the change in absorbance at 625 nm of fully oxidized azurin $(A_{ox} = 0.138$ in both A and $B)$ minus the absorbance of azurin at a given time (A_t). A. Reaction initiated by adding enzyme to the reaction mixture. B. Reaction initiated by adding H_2O_2 to the reaction mixture. ——— , reaction curve. − − − , semilogarithmic plot of reaction curve.

autoperoxidation under these conditions. Triplicate determinations were performed with Ps-cyt-551. The results were calculated according to the stoichiometry of eqn. 1.

$$H_2O_2 + 2 \text{ reduced 1-electron donors} + 2 \text{ H}^+ \rightarrow \tag{1}$$
$$2 \text{ oxidized 1-electron donors} + 2 \text{ H}_2O$$

The difference extinction coefficients obtained from the slope of the titration curves were 18.8, 19.0, and 19.3 mM^{-1} cm^{-1} and averaged 19.0 mM^{-1} cm^{-1} for $\Delta\varepsilon_{551}$ (red. − ox.) of Ps-cyt-551. The values for the extinction coefficients at 551 nm of reduced Ps-cyt-551 were 26.8, 26.9 and 27.1 mM^{-1} cm^{-1}, the mean value of ε_{551} (red.) being 26.9 mM^{-1} cm^{-1}, and those of oxidized Ps-cyt-551, 7.8, 7.8, and 8.1 mM^{-1} cm^{-1}, averaged 7.9 mM^{-1} cm^{-1} for ε_{551}(ox.).

Similarly, the extinction coefficients for azurin from two determinations were found to be 4.9 and 5.3 mM^{-1} cm^{-1}, the mean value of which, 5.1 mM^{-1} cm^{-1}, was used as ε_{625} (ox.). Titration curves are shown in Figs. 5 and 6.
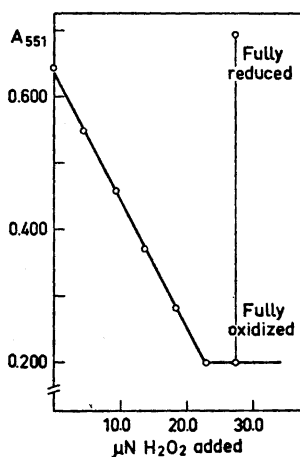

Fig. 5. Enzymatic titration of Ps-cyt-551 with H$_2$O$_2$ in the presence of PsCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, 23°C. Absorbance was measured with a Beckman DU-2 spectrophotometer. Other details are given in the text.

Fig. 6. Enzymatic titration of azurin with H$_2$O$_2$ in the presence of PsCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, 23°C. Absorbance was measured with a Beckman DU-2 spectrophotometer. Other details are given in the text.
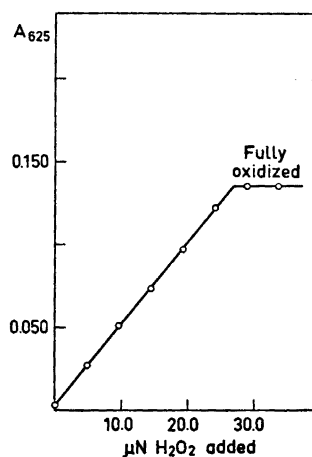
Donor specificity of PsCCP. Different pigments and commonly used low molecular weight peroxidase substrates were studied as electron and hydrogen donors in the PsCCP assay; the results are shown in Table 1. PsCCP was found to be highly specific for the *Pseudomonas* electron donors Ps-cyt-551 and azurin. Insignificant activities were observed with yeast and horse heart cytochromes. Classical peroxidase substrates such as pyrogallol, guajacol, and sodium ascorbate turned out to be poor donors in the peroxidation reac-

Table 1. Donor specificity of PsCCP. Apparent values of V_m/e and K_m are given at the indicated concentrations of H_2O_2 and an infinite donor concentration in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, 23°C. The reaction was initiated by adding H_2O_2 to the reaction mixture containing the enzyme and donor. Autoperoxidation and auto-oxidation of the donors were found to be neglible under these conditions.

Donor	V_m/e sec^{-1}	K_m μM	[H_2O_2] μM	[Donor] μM
1. Ps-cyt-551 [a]	710	91	84	5 − 22
2. Azurin [a]	670	125	84	5 − 26
3. Y-cyt [b,d,h]	17	200	85	7 − 19
4. H-cyt [b,d]	5	250	81	5 − 20
5. Ferrocyanide [b,f]	50	200	84	6 − 26
6. Guajacol [b,e]	0	—	84	24, 100
7. Na-ascorbate [b,e]	0	—	85	24, 100
8. Pyrogallol [b,e,h]	1	8	84	12 − 50
9. 2,6-Dichlorobenzenone-indo-3′-chlorophenol [c,f]	18	21	85	3 − 16
10. 9 + oxidized Ps-cyt-551 [c,g]	28	19	85	7 − 20

[a] With 1.52 nM enzyme.

[b] With 0.152 − 1.52 μM enzyme.

[c] With 6.82 − 30.4 nM enzyme

[d] Donor activity of substrate tested with YCCP.

[e] Donor activity of substrate tested with HRP.

[f] Extinction coefficient at 420 nm of ferricyanide was found to be 1.04 mM^{-1} cm^{-1} under these conditions and that of the oxidized indophenol dye 11.5 mM^{-1} cm^{-1} at 575 nm.

[g] Different amounts of oxidized Ps-cyt-551 were added to the reaction mixture containing a constant concentration of the reduced indophenol dye (7.8 μM). The peroxidation of the dye in the presence of oxidized Ps-cyt-551 catalyzed by PsCCP was followed at 575 nm.

[h] Non-linear Lineweaver-Burk plot. The constants were calculated from the linear part of the curve.

tion catalyzed by PsCCP, but some donor activity was observed with ferrocyanide and 2,6-dichlorobenzenone-indo-3′-chlorophenol. Addition of oxidized Ps-cyt-551 increased to some extent the peroxidation of the indophenol dye. A delay in the reaction similar to that observed with Ps-cyt-551 and azurin was noticed in studies of the peroxidation reaction with horse heart and yeast cytochromes and the indophenol dye as donors, the delay with horse heart cytochrome being independent of the sequence of the addition of the reactants. Non-linear Lineweaver-Burk plots were obtained with some substrates tested, as indicated in Table 1. Maximal turnover rates and apparent K_m-values were calculated from the linear part of the curve.

The acceptor specificity of PsCCP. Ethyl hydroperoxide could not replace hydrogen peroxide as an electron acceptor in the peroxidation of Ps-cyt-551, as shown in Table 2. Tests showed that ethyl hydroperoxide had acceptor activity in the peroxidation of horse heart cytochrome *c* catalyzed by YCCP.

Michaelis-Menten kinetics of the peroxidation reaction. The initial reaction rates of the peroxidation of Ps-cyt-551 and azurin were measured at various donor concentrations with a fixed H_2O_2 concentration (84 μM), and at various H_2O_2 concentrations with a fixed Ps-cyt-551 concentration (13.0 μM), and

Table 2. Acceptor specificity of PsCCP. Apparent values of V_m/e and K_m are given at the indicated concentrations of Ps-cyt-551 and an infinite acceptor concentration in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, 23°C. The reaction was initiated by adding acceptor to the reaction mixture containing the enzyme and Ps-cyt-551.

Acceptor	V_m/e sec^{-1}	K_m μM	[Ps-cyt-551] μM	[Acceptor] μM
1. H_2O_2 [a]	118	6	13.0	8 − 170
2. C_2H_3OOH [b,c]	1	450	12.8	2 − 200

[a] With 1.52 nM enzyme.
[b] With 0.152 μM enzyme.
[c] Acceptor activity tested with H-cyt and YCCP.

the data were analyzed by means of Lineweaver-Burk plots (Figs. 7 and 8). The relationship between the reciprocal of the initial velocity and the reciprocal of the donor concentration was linear in the concentration range $5 - 26$ μM. The initial rates were much less dependent on H_2O_2 concentration than on donor concentration, resulting in much smaller apparent K_m-values for
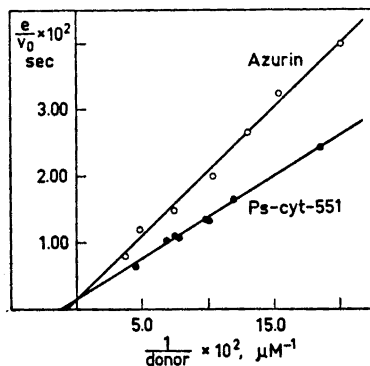

Fig. 7. Lineweaver-Burk plot of the initial rates of the peroxidation of Ps-cyt-551 and azurin catalyzed by PsCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$, 23°C. The concentrations of the reactants used were: reduced Ps-cyt-551 $5 - 22$ μM, reduced azurin $5 - 26$ μM, H_2O_2 84 μM, and PsCCP $1.44 - 1.52$ nM. The reactions were initiated by adding H_2O_2 to the reaction mixture. The peroxidation of Ps-cyt-551 was recorded with a Beckman DK-1 A spectrophotometer and that of azurin with a Cary 15 spectrophotometer. Kinetic constants calculated from the plots are given in Table 1.
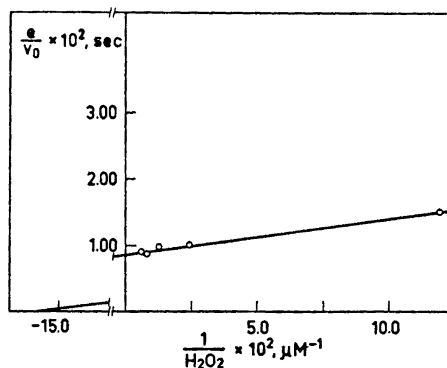
Fig. 8. Lineweaver-Burk plot of the initial rates of the peroxidation of Ps-cyt-551 catalyzed by PsCCP in sodium phosphate buffer, pH 6.0, $\mu = 0.01$. The concentrations of the reactants used were: reduced Ps-cyt-551 13.0 μM, H_2O_2 $8 - 180$ μM and PsCCP 1.52 nM. Other details as described in Fig. 7. Kinetic constants calculated from the plot are given in Table 2.

H_2O_2 than for Ps-cyt-551 and azurin (Tables 1 and 2). Substrate inhibition was observed when the concentration of H_2O_2 was greater than 200 μM (not shown in Fig. 8).

DISCUSSION

The kinetics of peroxidation reactions catalyzed by *Pseudomonas* cyto-chrome *c* peroxidase have been studied in this investigation. The optimum pH for the peroxidation of *Pseudomonas* cytochrome *c*-551 (6.0) lies between the isoelectric point of the substrate (4.7)[20] and that of the enzyme (6.7).[2] This indicates that electrostatic forces may be important in the interaction of the protein substrate and the enzyme in the enzymatic reaction. Yeast cytochrome *c* peroxidase is known to form a specific complex with horse heart cytochrome *c*, the predominant forces between the proteins being of electro-static nature.[21,22] Lenhoff and Kaplan [1] reported a pH optimum "in the region of neutrality" for PsCCP, when the activity was measured indirectly with 2,6-dichlorobenzenone-indo-3'-chlorophenol. PsCCP activity was only slightly dependent on the ionic strength of the reaction medium. Maximal activities were obtained at low ionic strength, the low activity in distilled water indicat-ing that ions are required to maintain the right milieu for the reaction. No difference between the effects of acetate and phosphate ions was observed in the peroxidation reaction catalyzed by PsCCP, in contrast to the peroxida-tion of yeast cytochrome *c* catalyzed by YCCP, in which the ionic strength for maximal reaction rate was found to depend on the pH in acetate but not in phosphate buffers.[23]

The peroxidation reaction of *Pseudomonas* pigments Ps-cyt-551 and azurin, horse heart and yeast cytochromes, and certain donors of low molecular weight showed an initial delay when the enzyme was added after hydrogen peroxide to the reaction mixture containing the electron donor. After in-cubation of the enzyme with the donor there was no delay. This feature of the peroxidation reaction fits the concept of the hysteretic enzyme, introduced by Frieden.[24] The mechanism of PsCCP peroxidation in this respect remains to be studied further. The reaction obeyed first-order kinetics only during its earlier part. The substrate concentrations used were smaller than the corre-sponding values of K_m, but not, however, as small ($[S] \ll K_m$) as those needed for a two-substrate reaction exhibiting first-order kinetics. The upward concavity during the later part of the reaction has been interpreted by Yonetani and Ray as being due to denaturation of YCCP.[23] This effect was observed with PsCCP, independent of the sequence of the addition of the enzyme and hydrogen peroxide to the reaction mixture, in both acetate and phosphate buffers. The initial reaction rates (v_0/e) obtained from the reaction curves when enzyme was added first (*i.e.* the enzyme was incubated with the donor before addition of hydrogen peroxide) were used in the kinetic analyses in this study. With YCCP there was no such initial delay in the reaction.[21,23]

PsCCP provided a simple enzymatic method of determining the extinction coefficients of its substrates. The enzyme preparation did not contain any oxidase or catalase activity. The extinction coefficients for Ps-cyt-551 and azurin obtained in this study are compared in Table 3 with those reported in

Table 3. Extinction coefficients of Ps-cyt-551 and *Pseudomonas* azurin (mM^{-1} cm^{-1}).

	ε_{551}(red.)	$\Delta\varepsilon_{551}$(red. − ox.)	Ref.
Ps-cyt-551	26.9[a]	19.0	This study
	30.0[b]	20.0	7
	28.3[c]	—	20
	ε_{625}(ox.)		
Azurin	5.1[a]		This study
	5.0[d]		8
	5.5[e]		25
	2.8 − 3.5[f]		26, 27
	6.95[g]		28

[a] Enzymatic determination.
[b] Based on tyrosine, histidine and arginine content.
[c] Based on iron content.
[d] Calculated from the data of Ambler and Brown,[8] based on dry weight, copper and amino acid content.
[e] Calculated from the data of Horio *et al.*[25] using A_{280}(1 %)= 6.0 of Ambler and Brown,[8] based on dry weight and copper content.
[f] Based on dry weight and copper content.
[g] No details of the calculation are given.

iterature. The extinction coefficients for Ps-cyt-551 based on the amino acid and iron content of the preparation differ slightly from the values obtained in this study. The values for azurin calculated from the data of Ambler and Brown [8] and Horio *et al.*[25] agree well with the values found in this study, but the other literature values, based on dry weight or copper content of the preparations, differ. The agreement between the enzymatic values and those based on the metal, amino acid or dry weight content of the pigments proves the validity of the stoichiometry of eqn. (1) in the peroxidation catalyzed by PsCCP.

Purified PsCCP was found to be highly specific for two *Pseudomonas* respiratory chain proteins with acidic isoelectric points, Ps-cyt-551 and azurin. It did not react with basic animal and yeast cytochromes or conventional peroxidase substrates, a finding which agrees with that of Lenhoff and Kaplan [1] with their partially purified preparation and an indirect method of measuring the activities with a reduced indophenol dye. We observed little direct reaction between the reduced dye and PsCCP. Ps-cyt-551 increases the peroxidation of the dye, as already suggested by Lenhoff and Kaplan.[1] YCCP is specific for basic horse heart and yeast cytochromes and does not oxidize the classical peroxidase substrates.[29] In contrast to YCCP, PsCCP was found to be highly specific for its electron acceptor; ethyl hydroperoxide cannot be substituted for hydrogen peroxide as is the case in the peroxidation of animal and yeast cytochromes catalyzed by YCCP.[23]

The maximal turnover rates 710 sec^{-1} for Ps-cyt-551 and 670 sec^{-1} for azurin at a fixed concentration of H_2O_2 (84 μM) were one order of magnitude lower than maximal turnover rates of the peroxidation reaction catalyzed by YCCP,[23] the highest rate obtained with yeast cytochrome *c* as substrate at infinite donor and acceptor concentrations being 1.4×10^4 sec^{-1}.[23] The apparent K_m for H_2O_2, 6 μM, at a fixed Ps-cyt-551 concentration (13.0 μM) was found to be considerably smaller than that for Ps-cyt-551 and azurin at a fixed concentration of H_2O_2 (84 μM), 91 μM and 125 μM, respectively, indicating greater affinity of the enzyme towards hydrogen peroxide than towards electron donors under these conditions. Lenhoff and Kaplan[1] obtained an apparent K_m-value of 5 μM for H_2O_2 by indirect activity measurements with partially purified peroxidase in the presence of catalase inhibitor. Their value is in good agreement with the value obtained in this study. K_m-values for the donors were one order of magnitude lower in the reaction catalyzed by YCCP[23] than in the reaction catalyzed by PsCCP, this also indicating the lower activity of PsCCP.

Non-linear Lineweaver-Burk plots of reciprocal initial rates (e/v_0) *versus* reciprocal initial donor concentrations were obtained; accordingly, additional kinetic studies on PsCCP in this respect are required.

REFERENCES

1. Lenhoff, H. M. and Kaplan, N. O. *J. Biol. Chem.* **220** (1956) 967.
2. Ellfolk, N. and Soininen, R. *Acta Chem. Scand.* **24** (1970) 2126.
3. Soininen, R., Sojonen, H. and Ellfolk, N. *Acta Chem. Scand.* **24** (1970) 2314.
4. Ellfolk, N. and Soininen, R. *Acta Chem. Scand.* **25** (1971) 1535.
5. Ellfolk, N. *Acta Chem. Scand.* **20** (1966) 1427; **21** (1967) 175.
6. Paul, K. G., Theorell, H. and Åkeson, Å. *Acta Chem. Scand.* **7** (1953) 1284.
7. Ambler, R. P. *Biochem. J.* **89** (1963) 341.
8. Ambler, R. P. and Brown, L. H. *Biochem. J.* **104** (1967) 784.
9. Yonetani, T. *J. Biol. Chem.* **240** (1965) 4509.
10. Hagihara, B., Horio, T., Yamashita, J., Nozaki, M. and Okunuki, K. *Nature* **178** (1956) 629.
11. Yonetani, T. and Ray, G. S. *J. Biol. Chem.* **240** (1965) 3392.
12. Yonetani, T. *J. Biol. Chem.* **241** (1966) 2562.
13. Maehley, A. C. *Methods Biochem. Anal.* **1** (1954) 357.
14. Chance, B. *Methods Biochem. Anal.* **1** (1954) 412.
15. Smith, F. G. and Stotz, E. *J. Biol. Chem.* **179** (1949) 865.
16. Rieche, A. and Hitz, F. *Ber.* **62** (1929) 2458.
17. Ornstein, L. and Davis, B. J. *Disc electrophoresis*, Distillation Product Industries, Eastman Kodak Co. 1962.
18. Maurer, H. R. *Disk-Elektrophorese*, Walter de Gruyter & Co., Berlin 1968.
19. Weber, K. and Osborne, M. *J. Biol. Chem.* **244** (1969) 4406.
20. Horio, T., Higashi, T., Sasagawa, M., Kusai, K., Nakai, M. and Okunuki, K. *Biochem. J.* **77** (1960) 194.
21. Nicholls, P. and Mochan, E. *Biochem. J.* **121** (1971) 55.
22. Mochan, E. and Nicholls, P. *Biochem. J.* **121** (1971) 69.
23. Yonetani, T. and Ray, G. S. *J. Biol. Chem.* **241** (1966) 700.
24. Frieden, C. *J. Biol. Chem.* **245** (1970) 5788.

25. Horio, T., Sekuzu, I., Higashi, T. and Okunuki, K. *Haematin Enzymes*, Pergamon, London 1961, p. 302.
26. Brill, A. S., Bryce, G. F. and Maria, H. *Biochim. Biophys. Acta* **154** (1968) 342.
27. Tang, S.-P. W., Coleman, J. E. and Myer, Y. P. *J. Biol. Chem.* **243** (1968) 4286.
28. Yamanaka, T., Kijimoto, S. and Okunuki, K. *J. Biochem. (Tokyo)* **53** (1963) 256.
29. Yonetani, T. and Ray, G. S. *J. Biol. Chem.* **240** (1965) 4503.