Pseudomonas Cytochrome c Peroxidase

V. Absorption Spectra of the Enzyme and of its Compounds with Ligands. Inhibition of the Enzyme by Cyanide and Azide

RITVA SOININEN and NILS ELLFOLK

Department of Biochemistry, University of Helsinki, SF-00170 Helsinki 17, Finland

A spectrophotometric study of *Pseudomonas* cytochrome c per-
oxidase has been made. Spectra and extinction coefficients of the
oxidized and reduced enzyme in sodium phosphate buffer pH = 6.0,
$\mu = 0.1$, are given. The effect of pH on the absorption spectra of ferri-
and ferroperoxidase has been studied. A pK value of 10.0 was ob-
tained for the transition of ferroperoxidase from the neutral to the
alkaline form. Ferroperoxidase reacts with carbon monoxide at pH
6.0; ferri- and ferroperoxidase react with cyanide and ferriperoxidase
with azide, the latter reaction being slow at pH 6.0, even at high
ligand concentrations. Peroxidase does not react with fluoride.
Cyanide inhibits the peroxidatic oxidation of reduced *Pseudomonas*
cytochrome c-551; the mechanism of the inhibition was found to be
of a mixed type (intermediate between competitive and noncompeti-
tive) in respect to hydrogen peroxide; the K_i value is 7.1 μM in
sodium phosphate buffer pH = 6.0, $\mu = 0.2$, at a fixed concentration
of reduced *Pseudomonas* cytochrome c-551 (18.6 μM). Azide inhibition
is noncompetitive in respect to hydrogen peroxide, the K_i value
being 3.2 mM. No stable hydrogen peroxide-peroxidase compound,
similar to hydrogen peroxide-horseradish peroxidase and hydrogen
peroxide-yeast cytochrome c peroxidase compounds, was detected.
The addition of hydrogen peroxide to the enzyme results only in a
decrease in the absorbance in the Soret region. At equimolar con-
centrations of hydrogen peroxide and peroxidase the decrease has
two stages: a rapid first-order decrease and an increase to a higher
absorbance level is followed by a slow, steady decrease. It appears
that during the first stage some kind of hydrogen peroxide-peroxidase
compound may be formed, and the second stage is the denaturation
of the enzyme by hydrogen peroxide.

Peroxidases like horseradish peroxidase, Japanese radish peroxidase, lacto-
peroxidase, and yeast cytochrome c peroxidase are hemoproteins having
non-covalently bound protoheme as the prosthetic group showing typical
high-spin spectra.[1-3] *Pseudomonas* cytochrome c peroxidase (PsCCP) is a low-
spin peroxidase with hemochrome-type spectra and a covalently bound heme
c as the prosthetic group.[4]

Some plant peroxidases have been classified as low-spin, b-type or para-peroxidases.[1],[5—7] Recently, the presence of cyanide as a ligand in purified preparations has been demonstrated.[6],[7] These low-spin peroxidases can be reversibly converted to high-spin peroxidases by the addition of mercaptide-forming agents.

Thiobacillus cytochrome peroxidase is another low-spin peroxidase which, in addition to PsCCP, has been isolated from bacteria.[8] The spectra of this peroxidase deviate considerably from those of PsCCP, but it is known to contain heme c as the prosthetic group.

In this publication the spectra of PsCCP and its oxidized, reduced, and alkaline forms, its reactions with hydrogen peroxide and other ligands, and its inhibition by ligands, are described.

MATERIALS AND METHODS

Pseudomonas cytochrome c *peroxidase (PsCCP)* was prepared by the method previously described,[4] using a slightly modified large scale procedure.[9] The preparation obtained was homogeneous when analysed by disc electrophoresis in 7 % gel pH 8.9 [10] with staining according to Weber and Osborne.[11] The spectral purity of the preparation (A_{407}/A_{280}) was 4.53 and the specific activity 110 — 125 U/mg (method described below). PsCCP concentration was determined spectrophotometrically, using $A(1 \%, 1$ cm$) = 12.1$ at 280 nm;[12] a molecular weight of 43 200, based on the iron content of the enzyme,[12] was assumed.

Peroxidase activity was measured spectrophotometrically at 25°C in sodium phosphate buffer pH 6.0, $\mu = 0.01$, H_2O_2 85 μM, *Pseudomonas* ferrocytochrome c-551 (ferro-Ps-cyt-551) 11 μM, unless otherwise stated. The reaction was initiated, after the addition of 2 — 5 μl of enzyme, by rapidly mixing 10 μl H_2O_2 into the reaction mixture (final volume of 2.0 or 2.5 ml), except in the studies of the effect of H_2O_2 on PsCCP when the reaction was initiated by the addition of enzyme which had been incubated with H_2O_2. In the inhibition studies the enzyme was incubated for 20 — 30 sec with the reaction mixture, containing the inhibitor and ferro-Ps-cyt-551, before the addition of H_2O_2. The reaction was followed by recording the oxidation of ferro-Ps-cyt-551 at 551 nm on a Beckman DK-1 A recording spectrophotometer equipped with a thermostated cell compartment. The reaction rates were calculated from (i) the slope of the reaction curve at zero time when the reaction was initiated by adding H_2O_2, or (ii) the final slope after the delay in the reaction [13] when it was initiated by adding enzyme. The peroxidase activity was expressed in terms of arbitrary units (decrease of absorbance at 551 nm per 10 sec) or as initial rates v_0/e (equivalents of electrons transferred per sec per molecule of enzyme).

Pseudomonas cytochrome c-551 (Ps-cyt-551) was prepared by the method of Ambler.[14] The spectral purity of the preparations used was 1.06 — 1.16 and their homogeneity was checked by disc electrophoresis as previously described.[13] The concentration of Ps-cyt-551 was determined as previously described.[13] Ferrocytochrome c-551 was prepared by anaerobic gel filtration of dithionite-reduced Ps-cyt-551 on Sephadex G-25.[15]

Hydrogen peroxide solutions were prepared from Merck "Perhydrol" (30 % H_2O_2). Peroxide concentration was determined enzymatically with yeast cytochrome c peroxidase (prepared according to Ellfolk [16]), using horse heart cytochrome c (Type III, Sigma) as substrate.[17]

Potassium cyanide (p.a., Baker) solution was prepared in distilled water and the pH of the solution was adjusted to pH 6.0 with *ortho*-phosphoric acid; its concentration was determined just before use by adding excess ammonia and titrating with standard silver nitrate. Working solution was prepared by diluting stock with buffer. At pH 6.0, KCN is completely (100.0 %) undissociated (pK_a of HCN $= 9.31$).

Sodium azide (Merck) and *sodium fluoride (p.a.,* Merck) solutions were prepared in buffer. At pH 6.0 95.0 % of sodium azide and 100.0 % of sodium fluoride is in the dissociated form (pK_a of $HN_3 = 4.72$ and of HF $= 3.17$).

Carbon monoxide. The buffer was saturated with carbon monoxide (*purum*, Fluka) before the addition of PsCCP.

Spectrophotometer. Cary 15 instrument, thermostated cell compartments set to 25°C. Absorption spectra were recorded by means of "0 − 1, 1 − 2" slidewires and the absorption maxima were determined from spectra recorded at a scanning speed of 3 − 6 nm/min, the chart scale being 1 nm/division. The wavelength indicator was checked with a mercury lamp. 0.4 ml micro quartz cells with beam masks, or rectangular quartz cells, both with 1.0 cm light path, were used. Most of the spectra were recorded with $4.3 − 6.8$ μM PsCCP in a final volume of 210 μl.

Buffers. The following buffers, of ionic strength 0.1, were used for the spectrophotometry: sodium phosphate pH 6.0 − 7.8, tris(hydroxymethyl)aminomethane (TRIS)-HCl pH 7.6 − 8.6, and glycine-NaOH pH 8.9 − 10.9. Enzyme activity measurements were made in pH 6.0 sodium phosphate buffers of ionic strength 0.01 and 0.2. The pH values were measured with a Beckman Zeromatic II pH meter standardized at pH 7.0 with Beckman Standard Buffer No. 3581 and at pH 9.2 with 0.01 M sodium borate.

Chemicals were of analytical grade unless otherwise stated.

RESULTS

Absorption spectra of ferri- and ferro-PsCCP are shown in Fig. 1, and corrected peak wavelengths, extinction coefficients, and relative absorbances in Table 1. The addition of potassium ferricyanide did not alter the spectrum of PsCCP, which shows it to be in the fully oxidized form. The spectrum of ferro-PsCCP was stable for at least 30 min after reduction with sodium dithionite.

Table 1. Spectral characteristics of *Pseudomonas* cytochrome *c* peroxidase.

	Medium	λ of maxima, nm	ε mM^{-1}cm^{-1}	Relative absorbances
Ferri-PsCCP	Sodium phosphate buffer pH 6.0, $\mu = 0.1$, 25°C	407	237	$A_{407}(\text{ox.})/A_{280} = 4.53$,[a]
		525	19.6	$A_{407}(\text{ox.})/A_{525}(\text{ox.}) = 12.1$
Ferro-PsCCP		420	273	$A_{420}(\text{red.})/A_{407}(\text{ox.}) = 1.16$,
		524	27.5	$A_{420}(\text{red.})/A_{557}(\text{red.}) =$
		551 (shoulder)	34.2	6.98, $A_{557}(\text{red.})/A_{551}$ (red.) = 1.15
		557	39.2	
Alkaline ferri-PsCCP	0.1 N NaOH, 25°C	410	−[b]	
		535	−[b]	
Alkaline ferro-PsCCP		417	−[b]	$A_{550}/A_{521} \sim 1.7$
		521	−[b]	
		550	−[b]	

[a] $\varepsilon_{280} = 52.3$ mM^{-1} cm^{-1} on a dry weight basis, calculated from $A(1 \%, 1 \text{ cm}) = 12.1$ at 280 nm.[12]
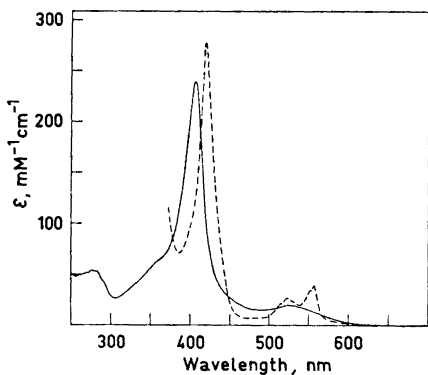[b] Spectrum unstable.

Fig. 1. Absorption spectra of ferri- and ferro-PsCCP in sodium phosphate buffer pH 6.0, $\mu = 0.1$, 25°C. — ferri-PsCCP. - - - - ferro-PsCCP; PsCCP was reduced by the addition of a minimum amount of solid sodium dithionite.
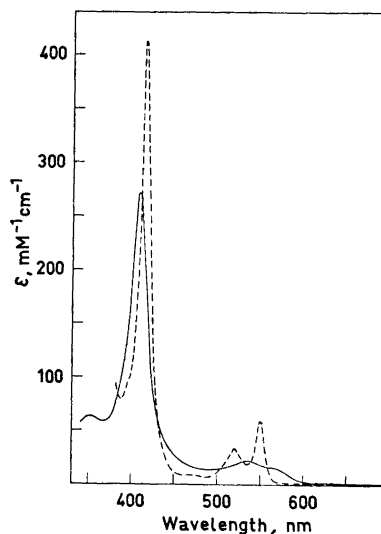
Fig. 2. Absorption spectra of ferri- and ferro-PsCCP in 0.1 N sodium hydroxide, 25°C. — ferri-PsCCP; recorded within 10 min of the mixing PsCCP with sodium hydroxide. - - - - ferro-PsCCP; recorded within 10 min of the reduction of PsCCP in sodium hydroxide with a minimum amount of solid sodium dithionite.

Effect of pH on the absorption spectra of ferri- and ferro-PsCCP. Spectrophotometric titration of ferro-PsCCP in the alkaline pH range. The effect of pH in the range 6.0 – 13.0 on the spectra of PsCCP was studied. With increasing pH the absorbances of the maxima of ferri- and ferro-PsCCP rose (Fig. 2); at the same time the maxima of the ferri-PsCCP spectrum shifted to longer wavelengths, and those of ferro-PsCCP to shorter wavelengths (Table 1). The double α-band disappeared above pH 10, becoming a single maximum at 550 nm (Fig. 3). Ferri-PsCCP was unstable above pH 10.8, the absorbances decreasing slowly ($\Delta\varepsilon_{524-525\ nm}$ less than 5 mM^{-1} cm^{-1} per 10 min) without a concomitant shift in the wavelengths of the maxima; similarly, ferro-PsCCP was unstable above pH 8.9 ($\Delta\varepsilon_{\alpha-band}$ less than 10 mM^{-1} cm^{-1} per 10 min). The pK value for the transition of ferro-PsCCP from the neutral to the alkaline form was determined from the change in absorbance at 550 nm (Fig. 4). A plot of log $[\alpha/(1-\alpha)]$ against pH in the range 7.5 – 10.3 gave straight line of slope 0.32, from which a pK value of 10.0 for the half-dissociated PsCCP was obtained.

Reaction of PsCCP with carbon monoxide, cyanide, azide, and fluoride. That ferro-PsCCP reacts with carbon monoxide at pH 6.0 is indicated by the difference spectrum shown in Fig. 5 A. The Soret band shifted to a shorter wavelength, the difference minimum being at 433 nm and the maximum at 415 nm
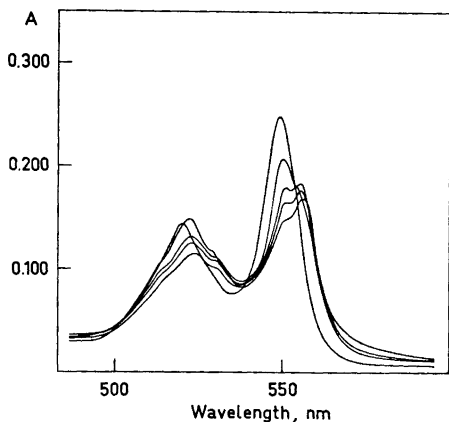
Fig. 3. Effect of pH on the spectrum of ferro-PsCCP in the visible region, $\mu = 0.1$, 25°C. The absorption curves can be distinguished at 550 nm, where the absorbance increases with increasing basicity. Sodium phosphate pH 6.0, TRIS-HCl pH 7.2, and glycine-NaOH pH 8.9 and 10.9 buffers, and 0.1 N NaOH, were used. The spectra were recorded within 5 min of the reduction of 4.3 μM PsCCP with solid sodium dithionite.
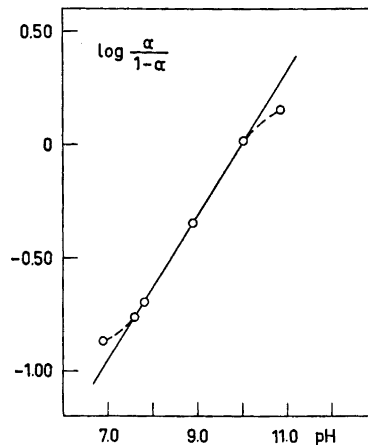
Fig. 4. Graphical determination of pK for the transition of PsCCP from the neutral to the alkaline form. The experimental conditions were identical to those in Fig. 3. $\alpha = (A_{pH\ x} - A_{pH\ 6})/(A_{pH\ 13} - A_{pH\ 6})$, where $A_{pH\ 6}$ is the absorbance at 550 nm of ferro-PsCCP at pH 6.0 (neutral form), $A_{pH\ x}$ the absorbance of ferro-PsCCP at any particular pH value and $A_{pH\ 13}$ the absorbance at pH 13.0 (alkaline form). The pK value obtained was 10.0; the slope of the line is 0.32.

Table 2. Characteristics of the difference spectra of certain compounds of *Pseudomonas* cytochrome *c* peroxidase. Measurements made in sodium phosphate buffer pH 6.0, $\mu = 0.1$, 25°C.

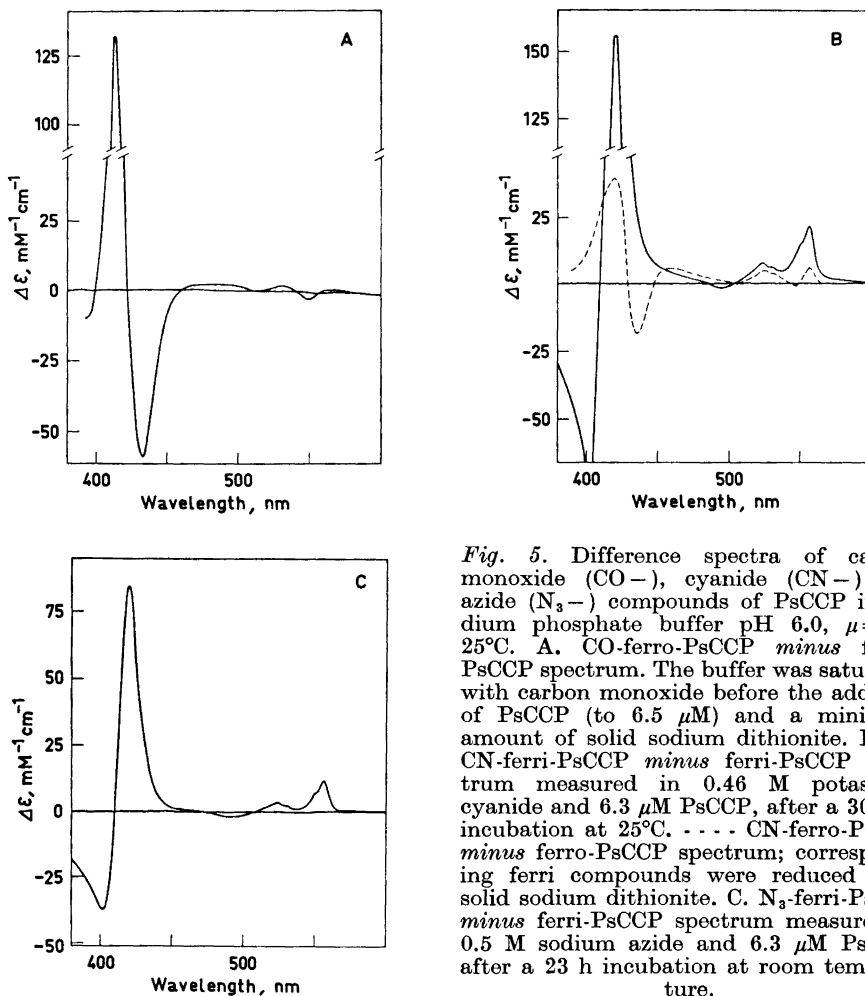| Difference spectrum	λ, nm	$\Delta\varepsilon$, mM^{-1}cm^{-1}
CO-ferro-PsCCP *minus* ferro-PsCCP	415 (maximum)	133
	433 (minimum)	59.0
CN-ferri-PsCCP *minus* ferri-PsCCP	~ 403 (minimum)	not measured
	421 (maximum)	156
CN-ferro-PsCCP *minus* ferro-PsCCP	420 (maximum)	39.6
	436 (minimum)	18.3
N$_3$-ferri-PsCCP *minus* ferri-PsCCP	402 (minimum)	35.0
	421 (maximum)	83.5

Fig. 5. Difference spectra of carbon monoxide (CO—), cyanide (CN—) and azide (N₃—) compounds of PsCCP in sodium phosphate buffer pH 6.0, $\mu = 0.1$, 25°C. A. CO-ferro-PsCCP *minus* ferro-PsCCP spectrum. The buffer was saturated with carbon monoxide before the addition of PsCCP (to 6.5 μM) and a minimum amount of solid sodium dithionite. B. — CN-ferri-PsCCP *minus* ferri-PsCCP spectrum measured in 0.46 M potassium cyanide and 6.3 μM PsCCP, after a 30 min incubation at 25°C. - - - - CN-ferro-PsCCP *minus* ferro-PsCCP spectrum; corresponding ferri compounds were reduced with solid sodium dithionite. C. N₃-ferri-PsCCP *minus* ferri-PsCCP spectrum measured in 0.5 M sodium azide and 6.3 μM PsCCP, after a 23 h incubation at room temperature.

(Table 2). In the visible region, the absorbance intensified at 520 — 540 nm and 555 — 570 nm and decreased at 550 nm. The spectrum was stable for at least 20 min.

Ferro- and ferri-PsCCP were found to react with cyanide at pH 6.0 at high cyanide concentrations (Fig. 5 B, Table 2). The spectra shown in Fig. 5 B are of 6.3 μM PsCCP in 0.46 M potassium cyanide; under these conditions the full difference spectrum of ferri-PsCCP-cyanide was attained in 8 min, and the difference in absorbance at 421 nm after 30 sec was 97 % of the maximum obtained. The difference spectrum was stable for at least 1 h after the maximum absorbances were reached. The cyanide-ferri-PsCCP/ferri-PsCCP difference spectrum shows characteristics of the spectrum of reduced PsCCP, namely the typical α- and β-bands and the shift of the Soret band to a longer wavelength,

although there are differences between the spectra of ferro-PsCCP and cyanide-ferro-PsCCP (Fig. 5 B). With 6.6 μM PsCCP in 4.6 mM potassium cyanide the spectral changes were similar, but the maximal difference absorbance at 421 nm was only about 30 % of that in the presence of 0.46 M potassium cyanide. In 0.1 mM potassium cyanide no changes in the spectrum of ferri-PsCCP were observed.

At pH 6.0 no reaction between ferri-PsCCP (5.5 μM) and azide (5 mM) could be detected during a period of 20 min. 0.5 M azide reacted slowly with ferri-PsCCP (6.3 μM) at room temperature: 20 min after mixing PsCCP and azide the difference absorbance at 421 nm was 30 % of the maximum (measured after 23 h), the proportion being 55 % after 2 h. The spectrum showed the same features of reduced hemochromogen, as was observed after the reaction of ferri-PsCCP with cyanide (Fig. 5 C, Table 2).

There was no reaction between fluoride (5 mM or 0.5 M) and ferri-PsCCP (6.3 μM), the latter being incubated for 23 h in 0.5 M fluoride.

The inhibition by cyanide and azide of the PsCCP-catalyzed peroxidatic oxidation of ferro-Ps-cyt-551 was studied in respect to H_2O_2. The concentration of ferro-Ps-cyt-551 was kept to $18.5 - 18.6$ μM, and there were two inhibitor concentrations. Sodium phosphate buffer of pH 6.0 and ionic strength 0.2 was used to avoid changes in ionic strength when the inhibitor was added; such changes affect the initial rate of the reaction.[13] Neither cyanide nor azide was observed to react with the electron donor, as judged from the spectrum of ferro-Ps-cyt-551. The effect of both ligands was determined after a short pre-
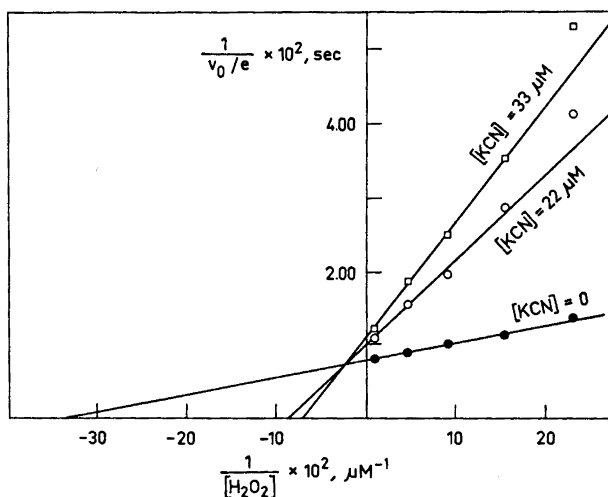

Fig. 6. Lineweaver-Burk plot of the initial rates of the peroxidatic oxidation of ferro-Ps-cyt-551 catalyzed by PsCCP in the presence and absence of cyanide in sodium phosphate buffer pH 6.0, $\mu = 0.2$, 25°C. Concentrations of reactants: 18.6 μM ferro-Ps-cyt-551, $4.4 - 110$ μM H_2O_2, 22 and 33 μM KCN and 2.73 nM PsCCP. The following values were obtained: $K_m = 3.0$ μM, $V_m/e = 125$ sec^{-1}, $K_i = 7.1$ μM and α (interaction constant for the effect of the inhibitor on the binding of substrate) = 15 from the abscissa of the intersection of the lines ($-1/\alpha$ K_m).
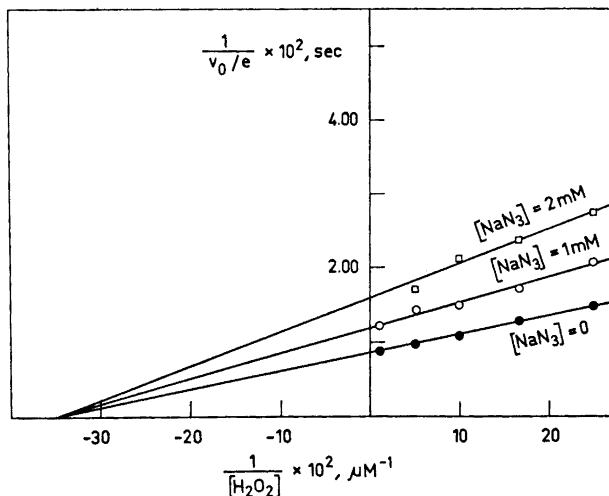
Fig. 7. Lineweaver-Burk plot of the initial rates of the peroxidatic oxidation of ferro-Ps-cyt-551 catalyzed by PsCCP in the presence and absence of azide in sodium phosphate buffer pH 6.0, $\mu=0.2$, 25°C. Concentrations of reactants: 18.5 μM ferro-Ps-cyt-551, 4 – 100 μM H_2O_2, 1 and 2 mM sodium azide, and 2.66 nm PsCCP. The following values were obtained: $K_m = 3.2$ μM, $V_m/e = 119$ sec^{-1}, and $K_i = 3.1$ mM.

incubation period because the enzyme alone is not stable, for periods of minutes, at the final dilution used in the initial rate measurements. The results are shown in Figs. 6 and 7. The mechanism of the inhibition by cyanide is of a mixed type under these conditions, that is intermediate between competitive and noncompetitive inhibition in respect to H_2O_2. The mean value of the inhibition constant K_i calculated from the slope and the $1/(v_0/e)$ intercept of the plot of $1/(v_0/e)$ against $1/[S]$ and from the $(v_0/e)/[S]$ intercept of the plot of v_0/e against $(v_0/e)/[S]$ was found to be 7.1 μM. Azide was observed to inhibit the peroxidatic oxidation of ferro-Ps-cyt-551 noncompetitively in respect to H_2O_2; the mean value for K_i was 3.2 mM obtained from the slope and the $1/(v_0/e)$ intercept of the plot of $1/(v_0/e)$ against $1/[S]$ and from the v_0/e and $(v_0/e)/[S]$ intercepts of the plot of v_0/e against $(v_0/e)/[S]$. In the presence of azide the linear portion of the reaction curve was very short, causing difficulties in the determination of the initial rates.

Reaction of ferri-PsCCP with H_2O_2. When 4.7 – 6.2 μM ferri-PsCCP was incubated with about equimolar H_2O_2 at pH 6.0 there was a decrease in the absorbance in the Soret region, without any detectable change in the wavelength of this maximum or of other features of the spectrum. The time dependence of the change in absorbance at 407 nm after the addition of H_2O_2 to PsCCP is shown in Fig. 8. Two phases of the reaction are observable. During the first minutes the absorbance decreased to a plateau (phase 1), after which there was an increase to a value somewhat below the original followed by a slow, steady decrease (phase 2). For time 0 – 2 min (first phase) the decrease in absorbance of the Soret band was 4 – 6 % of the original absorbance, and
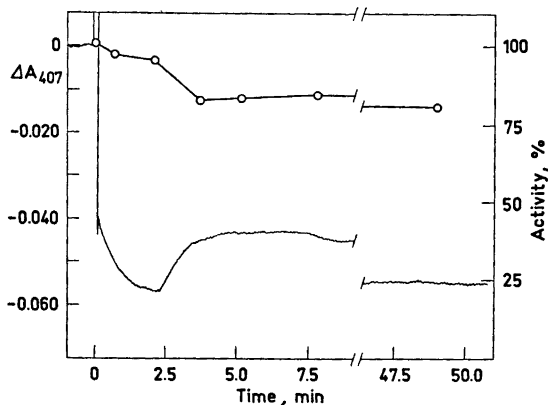
Fig. 5. Time dependence of the reaction of ferri-PsCCP with H_2O_2 in sodium phosphate buffer pH 6.0, $\mu = 0.1$, 25°C, and relative peroxidase activities during the course of the reaction. 5 μl of 1 mM H_2O_2 was added to 200 μl of 4.7 μM PsCCP and the reaction was followed by the difference in absorbance (H_2O_2/ferri-PsCCP *minus* ferri-PsCCP) at 407 nm (the solid line). The absorbance decrease of 0.028 is due to the dilution caused by the addition of H_2O_2. The peroxidase activity was measured in sodium phosphate buffer pH 6.0, $\mu = 0.01$, 25°C, as decribed in the text, and is given as a percentage of the activity of the untreated PsCCP (○).

for the time 6 – 10 min (second phase) the decrease was 2.5 – 4 %. Because H_2O_2 is known to degrade horse heart ferricytochrome c,[18],[19] the peroxidatic activity of PsCCP during incubation with H_2O_2 was measured (Fig. 8). During the second phase of the reaction of H_2O_2 with PsCCP the activity decreased from 95 % to 80 % of the original. With higher H_2O_2 concentrations the absorbance of the Soret band decreased further without a shift in the position of the absorption maximum. At molar ratios H_2O_2:PsCCP above 3 the reaction was monophasic, the absorbance decreasing almost linearly with time. At a molar ratio of 7 the peroxidase activity of H_2O_2-treated PsCCP was only 15 % of the original, the decrease of the absorbance of the Soret band being less than 5 %.

In a further study of the reaction between H_2O_2 and PsCCP, the addition of potassium ferrocyanide in slight excess after the first phase was found to give no significant increase in the absorbance, as was to be expected if the decrease in absorbance in the first phase were due to the formation of a simple complex between H_2O_2 and the iron in ferri-PsCCP.

DISCUSSION

Two cytochrome peroxidases isolated from bacteria, PsCCP and *Thiobacillus* cytochrome peroxidase, resemble the cytochromes more than the classical peroxidases in having low-spin, hemochrome-type spectra and having heme c as the prosthetic group.[4],[8] The spectra of *Thiobacillus* peroxidase deviate considerably from the spectra of PsCCP shown in Fig. 1. Characteristics of the spectrum of *Thiobacillus* peroxidase are: with ferriperoxidase Soret band 398

nm, with ferroperoxidase 415 nm, β-band 520 nm and α-band 550 nm without the shoulder characteristic of PsCCP. The functions as cytochromes of PsCCP and *Thiobacillus* peroxidase are not known.

Several bacterial cytochromes *c* with an asymmetric α-band at room temperature have been isolated: *Pseudomonas stutzeri* cytochrome *c*-552,558,[20] *Alcaligenes faecalis* cytochrome *c*-551,557,[21] *Bacillus subtilis* cytochrome *c*-550,554,[22] *Micrococcus* cytochrome *c*-548,554 (= "cytochrome b_4"),[23] *Chromatium* strain D cytochrome *c*-550,553,[24] and *Chlorobium thiosulphatophilum* cytochrome *c*-550,555.[25],[26] Common features of the bacterial "split α" type cytochromes are a high A_{Soret}(red.)/A_α (red.) ratio, a low A_α (red.)/A_β (red.) ratio, and a low extinction coefficient of the α-band as compared with that of the horse heart type cytochromes *c*.[27] PsCCP, *P. stutzeri* cytochrome *c*-552,558 and *A. faecalis* cytochrome *c*-551,557 deviate from the other bacterial "split α" hemoproteins in being of high molecular weight (43 200 − 74 000) and having two hemes per molecule; they form a sub-group of three similar proteins.

At pH values above 10 the shoulder of the α-band of ferro-PsCCP and *P. stutzeri* ferrocytochrome *c*-552,558 [20] disappears and the α-band:β-band absorbance ratio becomes equal to that of horse heart cytochrome *c* (∼ 1.7). Low molecular weight cytochromes of the "split α" type from *Micrococcus* and *Bacillus* are more stable in the alkaline range, being unchanged at pH values up to 13.0 − 13.3.[22],[23] The low-spin structure of these cytochromes, that is with internal ligands at the 5th and 6th coordination positions of the iron, are preserved after the alkaline transitions. There is evidently some degradation of PsCCP in the alkaline pH range, as shown by the unstable spectra.

Native mammalian ferrocytochrome *c* does not react with carbon monoxide in the neutral pH-range. Denaturation causes a change of reactivity towards carbon monoxide and this reaction has been widely used as a test for the denaturation of cytochromes.[28] However, several bacterial cytochromes are known to combine in the native form with carbon monoxide,[29] the denatured forms showing modified carbon monoxide spectrum. PsCCP, *P. stutzeri* cytochrome *c*-552,558 [20] and *A. faecalis* cytochrome *c*-551,557 [21] belong to this group of bacterial hemoproteins. The difference spectra of these three hemoproteins, that is of reduced carbon monoxide derivative *minus* reduced hemoprotein, are similar, with a minimum at 430 − 434 nm and a maximum at 415 nm. In contrast, the low molecular weight cytochromes of the "split α" type do not react with carbon monoxide.[22],[24] *Thiobacillus* cytochrome peroxidase also combines with carbon monoxide resulting in a shift of the Soret band of the reduced peroxidase to a shorter wavelength.[8]

Ferri- and ferro-PsCCP react with cyanide, and ferri-PsCCP slowly with azide only at high concentrations of these ligands at a neutral pH. *A. faecalis* ferrocytochrome *c*-551,557 combines with cyanide at concentrations comparable with those of our study, and does not react with azide at low concentrations.[21] The reaction of *P. stutzeri* cytochrome *c*-552,558 with cyanide and azide has not been studied.[20] Horse heart cytochrome *c* combines with such ligands in the neutral pH range only when it is in the oxidized form.[27] The reaction of external ligands with the heme iron of horse heart cytochrome *c* requires the replacement of an internal ligand in the axial position relative to the iron and conformational changes of the protein near the heme group.

PsCCP either must have a more open structure in the neighbourhood of the heme, or the 6th internal ligand of the iron must be less firmly bound than in horse heart cytochrome *c* because of the easier reactions with external ligands (carbon monoxide and ferro-PsCCP with cyanide).

Cyanide and azide may also react with secondary groups in the protein as well as with the heme iron. Cyanide can act as reductant, as has been proposed in its reaction with oxidized mammalian cytochrome oxidase; first there is a slow reaction, possibly the reduction of the enzyme at a binding site other than the heme, and then cyanide combines with the reduced heme a_3.[30] The reaction of ferri-PsCCP with cyanide may proceed in a similar way. Azide can also interact with the protein moiety, as has been shown with horseradish peroxidase; it is proposed that azide combines with a methionine residue at the active site of the enzyme.[31] The very slow reaction of ferri-PsCCP with azide may comprise secondary reactions in addition to any direct coordination with the iron.

Cyanide was observed to be an efficient inhibitor of the peroxidatic reaction catalyzed by PsCCP. The inhibition mechanism was of the mixed type, that is intermediate between competitive and noncompetitive in respect to hydrogen peroxide. This seems to indicate that several phenomena are involved in the interaction of the inhibitor with the enzyme. Cyanide competes with hydrogen peroxide, thereby interfering with the binding of the substrate, and to some extent prevents the breakdown of the active complex. The interaction constant α, which is a measure of how much cyanide effects the binding of hydrogen peroxide, was calculated from a Lineweaver-Burk plot (Fig. 6); the value indicates that the affinity of the inhibited enzyme for the substrate is only 1/15th of that of the uninhibited enzyme. The value of the inhibitor constant K_i for cyanide (7.1 μM) is close to the apparent K_m of hydrogen peroxide (6 μM).[13] Using an indirect method for measurement of activity and partially purified PsCCP, Lenhoff and Kaplan found that 0.1 mM cyanide caused a 50 % inhibition.[32] *Thiobacillus* cytochrome peroxidase also is inhibited by cyanide: 39 % inhibition is caused by 1 mM cyanide.[8]

Azide inhibited the peroxidatic reaction noncompetitively, showing that it reacts at a site different from that reacting with hydrogen peroxide. The possibility of irreversible inhibition giving a noncompetitive plot cannot be ruled out on the basis of a kinetic study only. The value of K_i for azide, 3.2 mM, is much greater than that of cyanide. Lenhoff and Kaplan observed a 84.5 % inhibition with 10 mM azide at pH 6.1, the extent of inhibition increasing with decreasing pH.[32]

Hydrogen peroxide did not form a stable, spectrally detectable compound with PsCCP, comparable with that of hydrogen peroxide and plant peroxidases,[1] yeast cytochrome *c* peroxidase,[2] catalase,[1] and myoglobin.[1] A slight decrease in absorbance of the Soret band was observed after the addition of hydrogen peroxide to PsCCP. A similar effect of hydrogen peroxide on mammalian ferricytochrome *c* has been reported by O'Brien[18] and Mochan and Degn.[19] This decrease has been shown to be due to the degradative effect of hydrogen peroxide on the tyrosine residues of the protein moiety.[18] The effect of hydrogen peroxide on PsCCP was found to be biphasic. The first phase may be due to the formation of some kind of PsCCP-hydrogen peroxide

compound, which then decomposes. The nature of the compound could not be elucidated by the present studies. The presence of ferrocyanide did not reduce the oxidizing equivalents of this compound. Such a reduction has been observed with hydrogen peroxide compounds of yeast cytochrome c peroxidase (complex ES) and horseradish peroxidase (complexes I and II).[33] This eliminates the possibility of a simple complex formation between hydrogen peroxide and the iron in PsCCP. The second phase of the reaction between hydrogen peroxide and PsCCP is evidently the degradation of PsCCP.

REFERENCES

1. Saunders, B. C. *Peroxidase*, Butterworths, London 1964.
2. Yonetani, T. and Ray, G. S. *J. Biol. Chem.* **240** (1965) 4503.
3. Carlström, A. *Acta Chem. Scand.* **23** (1969) 203.
4. Ellfolk, N. and Soininen, R. *Acta Chem. Scand.* **24** (1970) 2126.
5. Morita, Y. and Kameda, R. *Mem. Res. Inst. Food Sci. Kyoto Univ.* **12** (1957) 1.
6. Yamazaki, I., Nakajima, R., Honma, H. and Tamura, M. *Structure and Function of Cytochromes*, University Park Press, Manchester 1968, p. 552.
7. Nakajima, R., Sano, H. and Yamazaki, I. *Biochim. Biophys. Acta* **172** (1969) 578.
8. Yamanaka, T. and Okunuki, K. *Biochim. Biophys. Acta* **220** (1970) 354.
9. Soininen, R. *Acta Chem. Scand.* **26** (1972) 2535.
10. Maurer, H. R. *Disk-Elektrophorese*, Walter de Gruyter, Berlin 1968.
11. Weber, K. and Osborne, M. *J. Biol. Chem.* **244** (1969) 4406.
12. Ellfolk, N. and Soininen, R. *Acta Chem. Scand.* **25** (1971) 1535.
13. Soininen, R. and Ellfolk, N. *Acta Chem. Scand.* **26** (1972) 861.
14. Ambler, R. P. *Biochem. J.* **89** (1963) 341.
15. Yonetani, T. and Ray, G. S. *J. Biol. Chem.* **240** (1965) 3392.
16. Ellfolk, N. *Acta Chem. Scand.* **21** (1967) 175.
17. Yonetani, T. *J. Biol. Chem.* **240** (1965) 4509.
18. O'Brien, P. J. *Biochem. J.* **52** (1952) 511.
19. Mochan, E. and Degn, H. *Biochim. Biophys. Acta* **189** (1969) 354.
20. Kodama, T. and Mori, T. *J. Biochem. (Tokyo)* **65** (1969) 621.
21. Iwasaki, H. and Matsubara, T. *J. Biochem. (Tokyo)* **69** (1971) 847.
22. Miki, K. and Okunuki, K. *J. Biochem. (Tokyo)* **66** (1969) 831.
23. Hori, K. *J. Biochem. (Tokyo)* **50** (1961) 440.
24. Cusanovich, M. A. and Bartsch, R. G. *Biochim. Biophys. Acta* **189** (1969) 245.
25. Yamanaka, T. and Okunuki, K. *J. Biochem. (Tokyo)* **63** (1968) 341.
26. Meyer, T. E., Bartsch, R. G., Cusanovich, M. A. and Mathewson, J. H. *Biochim. Biophys. Acta* **153** (1968) 854.
27. Butt, W. D. and Keilin, D. *Proc. Roy. Soc. (London)* **B 156** (1962) 429.
28. Ben-Gershom, E. *Biochem. J.* **78** (1961) 218.
29. Iwasaki, H. and Shidara, S. *J. Biochem. (Tokyo)* **66** (1969) 775.
30. Antonini, E., Brunori, M., Greenwood, C., Malmström, B. G. and Rotilio, G. C. *Eur. J. Biochem.* **23** (1971) 396.
31. Brill, A. S. and Weinryb, I. *Biochemistry* **6** (1967) 3528.
32. Lenhoff, H. M. and Kaplan, N. O. *J. Biol. Chem.* **220** (1956) 967.
33. Yonetani, T. *J. Biol. Chem.* **241** (1966) 2562.