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PSEUDOMONAS CYTOCHROME *c* PEROXIDASE

INITIAL DELAY OF THE PEROXIDATIC REACTION. ELECTRON TRANSFER PROPERTIES

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Summary

A delay of some seconds is observed in the reaction of *Pseudomonas* cytochrome *c* peroxidase if the reaction is initiated by adding the enzyme to the reaction mixture containing reduced electron donor and hydrogen peroxide. This lag phase is avoided if the enzyme is incubated with the reduced electron donor and the reaction is started by adding hydrogen peroxide. The nature of the inital delay has been studied and it is shown that the peroxidase is reduced before a steady-state rate in the peroxidatic reaction is reached. The ability of the peroxidase to accept electrons from various electron donors emphasizes its cytochrome-like properties.

Introduction

Cytochrome *c* peroxidase from *Pseusomonas aeruginosa* (ferrocytochrome *c* : hydrogen peroxide oxidoreductase, EC 1.11.1.5) catalyzes the peroxidatic oxidation of *Pseudomonas c*-type cytochromes and azurin by hydrogen peroxide [1,2]. The enzyme is a low spin ferrihemoprotein that contains two heme *c* moieties [3,4]. This is in contrast to other peroxidases, e.g. yeast cytochrome *c* peroxidase, in which the prosthetic group is a protoheme [5]. The catalytic mechanism of the enzyme seems to differ from other peroxidases in not forming spectrally detectable enzyme intermediates like Compound II of horseradish peroxidase [6]. The unique properties of the *Pseudomonas* enzyme are underlined by the lag phenomenon observed when the reaction is initiated by the addition of the enzyme to the reaction mixture containing electron donor and hydrogen peroxide.

The nature of the initial delay of the peroxidatic reaction has been studied. It is shown that reduction of the enzyme has to take place before hydrogen

peroxide reacts with the enzyme and the peroxidatic oxidation of the reduced electron donor starts.

The ability of the enzyme to accept electrons from cytochrome *c*-551 and azurin, as well as from certain low molecular weight electron donors, has also been studied.

Materials and Methods

Pseudomonas cytochrome *c* peroxidase was extracted from acetone-dried cells of *P. aeruginosa* and purified as previously described [3]. The ratio A_{407nm}/A_{280nm} of the preparation was 4.5. The concentration of the enzyme was determined by using $\epsilon = 39.2\ mM^{-1} \cdot cm^{-1}$ for the reduced form and $\Delta\epsilon = \epsilon_{red.} - \epsilon_{ox.} = 25.8\ mM^{-1} \cdot cm^{-1}$ at 557 nm determined by Soininen and Ellfolk [6].

Yeast cytochrome *c* peroxidase was prepared as described previously [7]. The ratio A_{407nm}/A_{280nm} of the preparation was 1.3. The concentration of the enzyme was determined as pyridine hemochrome [8].

Horse heart cytochrome *c*, Type III of Sigma Chemical Co., was used without further purification. The concentration of the cytochrome was based on a value of $\Delta\epsilon(550\ nm)$ of $19.6\ mM^{-1} \cdot cm^{-1}$ [9].

Cytochrome *c*-551 and azurin of *P. aeruginosa* were prepared according to Ambler [10] and Ambler and Brown [11]. The purified cytochrome had an absorbance ratio $A_{551nm}(red.)/A_{280nm} = 1.17$ and azurin had a ratio $A_{625nm}(ox.)/A_{280nm} = 0.5$. The concentration of cytochrome *c*-551 was obtained by using the value $\Delta\epsilon(551\ nm) = 19.0\ mM^{-1} \cdot cm^{-1}$ [1]. Azurin concentrations were determined by using $\epsilon(625\ nm) = 5.1\ mM^{-1} \cdot cm^{-1}$ for the oxidized protein [1]. Ferrocytochrome *c*-551 was obtained by reduction of ferricytochrome with a minute excess of sodium dithionite, which was afterwards removed using anaerobic gel filtration on Sephadex G-25. Azurin was reduced with solid sodium dithionite and excess reductant was removed by dialysis.

Other bacterial cytochromes. Cytochrome *c*-550 and *c*-555 from *P. aeruginosa* were isolated according to Ambler and Taylor [12] with slight modifications. The purity ratio, $[A_{550nm}(red.) - A_{570nm}(red.)]/A_{280nm}$, of cytochrome *c*-550 was 1.3 and that of *c*-555, $[A_{555nm}(red.) - A_{570nm}(red.)]/A_{280nm}$, was 1.14. The concentrations of the cytochromes were determined using the millimolar absorptivities $\Delta\epsilon(550\ nm) = 14.9\ mM^{-1} \cdot cm^{-1}$ and $\Delta\epsilon(555\ nm) = 17.4\ mM^{-1} \cdot cm^{-1}$, respectively [2].

Cytochrome *c*-551 of *P. fluorescens* (ATCC 17 400) was isolated from acetone-dried cells according to the method of Ambler and Wynn [13]. The purity ratio $A_{551nm}(red.)/A_{280nm}$ of the preparation was 1.2. The concentration of the cytochrome was determined using $\Delta\epsilon(551\ nm) = 21.9\ mM^{-1} \cdot cm^{-1}$ [2].

The cytochromes were reduced as described above for *P. aeruginosa* cytochrome *c*-551.

Hydrogen peroxide solutions were prepared from Merck Perhydrol (30% H_2O_2) and the concentration was determined enzymatically with yeast cytochrome *c* peroxidase [9].

Static titrations. These (and all kinetic measurements) were performed using a Cary 15 spectrophotometer equipped with thermostated cell holders. The contents of the closed cuvette were purged with argon for 15—30 min before titrating with reduced azurin, which was added by means of a gas-tight microsyringe (Hamilton) through a standard gas-chromatography injection septum (Perkin-Elmer). Before recording the spectrum, 10—15 min were allowed for equilibrium to be achieved.

Measurements of reaction rates. The activity of *Pseudomonas* cytochrome *c* peroxidase was assayed spectrophotometrically by measuring the rate of peroxidatic oxidation of reduced substrates by the enzyme. Initial velocities were determined from the slope of the recorded lines and expressed in terms of mol reduced substrate oxidized per mol of enzyme and second.

Double difference spectra were measured using two pairs of matched cuvettes. One cuvette of each pair contained the peroxidase and one the cytochrome. Titrations were carried out by adding reduced cytochrome to the sample cuvette containing the enzyme. The same amount of cytochrome was added on the reference side to compensate for the absorbance of the added cytochrome. The effect of dilution was also corrected for.

Buffer. All experiments were carried out in sodium phosphate buffer, pH 6.0, $I = 0.01$ at 25°C. pH was measured with a Radiometer pH meter 27 equipped with a combination glass-calomel electrode.

Results

Kinetic analysis of the initial delay. If the reaction of *Pseudomonas* cytochrome *c* peroxidase is initiated by adding the enzyme to the reaction mixture containing the substrates, a delay of some seconds occurs before a steady-state rate is attained. In contrast, no delay is observed if the enzyme is exposed to reduced cytochrome *c*-551 or to other electron donors prior to the assay.

A graph of the rate of approach to the steady-state rate is shown in Fig. 1.

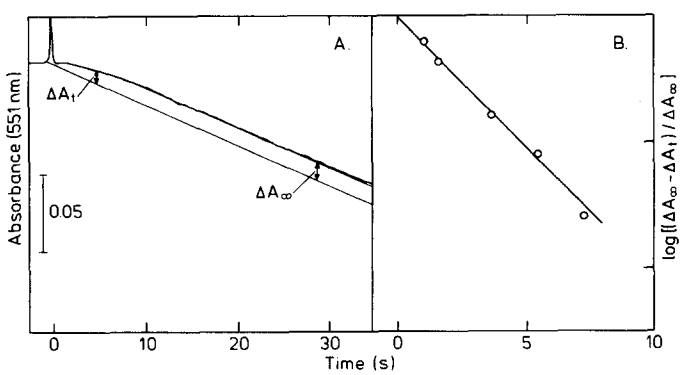

Fig. 1. Graphical determination of k_{app}. The reaction was initiated by adding the enzyme (1.5 nM) to the reaction mixture containing reduced cytochrome *c*-551 (16.3 μM) and hydrogen peroxide (84.5 μM). (A) A line was drawn through the origin of the recorder tracing parallel to the steady-state portion of the trace. The vertical distance, ΔA_t, between line and the velocity trace was measured at different times, *t*. (B) Values of $\log[(\Delta A_\infty - \Delta A_t)/\Delta A_\infty]$ from (A) were plotted against time. The rate constant for the activation of the enzyme, k_{app}, was calculated from the slope of this line.

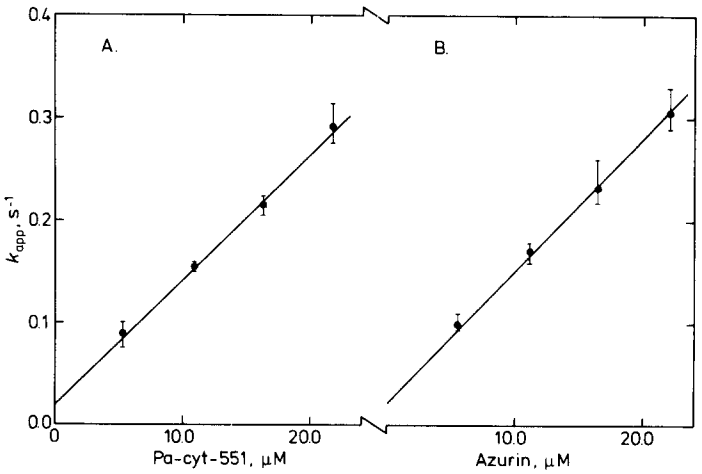

Fig. 2. The rate constant k_{app} for the activation of the enzyme versus the concentration of the electron donor. The concentration of the enzyme was 1.5 nM and that of hydrogen peroxide 84.5 μM. Values of k_{app} were determined as in Fig. 1. The points are mean values of three different measurements and the line was drawn using regression analysis. (A) Cytochrome *c*-551 (Pa-cyt-551) and (B) azurin of *P. aeruginosa*.

A straight line was drawn through A_0, the absorbance at time zero, parallel to the linear portion of the absorbance tracing. Difference in absorbance between the line and the curved region of the absorbance tracing, A_t, was evaluated at various reaction times, t. The difference after the steady-state rate was achieved was ΔA_∞, and the value of $\log[(\Delta A_\infty - \Delta A_t)/\Delta A_\infty]$ was plotted against time. Such plots were linear, and the half time ($t_{1/2}$) values were determined directly from the graph (Fig. 1B). The apparent rate constant for the activation process, k_{app}, is equal to $0.693/t_{1/2}$.

Effect of reduced substrate on rate of activation. Rate of activation of the enzyme was determined at several concentrations (5.4—21.7 μM) of cytochrome *c*-551 with a constant concentration of hydrogen peroxide (84.5 μM). The calculated k_{app} values were plotted as a function of the cytochrome concentration and a linear dependence was observed (Fig. 2A). The solid line in the figure is the least-squares line of best fit, the line having a non-zero intercept with the ordinate. The intercept is not significantly different from zero, however, and it seems justified to conclude that the rate of enzyme activation depends on cytochrome *c*-551 concentration in a straightforward manner. A linear relationship between k_{app} values and electron donor concentration was also obtained with *Pseudomonas* azurin (Fig. 2B) and horse heart cytochrome *c*. The k_{app} values calculated from representative reaction traces with different electron donors are given in Table I.

Reduction of the peroxidase by various electron donors

Titrations with azurin. Static redox titrations were carried out spectrophotometrically by making small additions of a concentrated azurin (Cu^+) solution to a solution of oxidized peroxidase. The spectra of the mixture, taken between 480 and 700 nm after each azurin addition, exhibit good isosbestic

TABLE I

THE RATE OF ACTIVATION OF *PSEUDOMONAS* CYTOCHROME *c* PEROXIDASE WITH DIFFERENT ELECTRON DONORS

Pa-cyt-551, Pa-cyt-555 and Pa-cyt-550 cytochromes *c*-551, *c*-555 and *c*-550, respectively, of *P. aeruginosa*; Pf-cyt-551, cytochrome *c*-551 of *P. fluorescens*.

Electron donor	μM	Hydrogen peroxide (μM)	Steady-state * velocity (s^{-1})	k_{app} (s^{-1})
Pa-cyt-551	13.3	84.0	58.8	0.178
Pa-cyt-555	15.8	205.5	68.5	0.151
Pa-cyt-550	10.3	49.3	72.5	0.116
Azurin	10.8	89.1	55.2	0.136
Pf-cyt-551	14.9	65.0	95.8	0.193
Horse cytochrome	15.8	84.2	0.2	0.098

* Expressed in terms of mol reduced substrate oxidized per mol of enzyme and second.

points at 503, 532, 543, 563 and 579 nm, corresponding to the isosbestic points between the oxidized and reduced peroxidase (Fig. 3). Consequently, to a good approximation the data exhibit simple behaviour and allow us to determine a unique equilibrium constant according to the equation:

$$K = \frac{[\text{peroxidase (II)}]\,[\text{azurin (II)}]}{[\text{peroxidase (III)}]\,[\text{azurin (I)}]}$$

We have after simple manipulation:

$$\frac{Az_{tot}}{((A_{557} - A_{ox})} = \frac{1}{K \cdot \Delta\epsilon_{557}} \cdot \frac{(A_{557} - A_{ox})}{(A_{\infty} - A_{557})} + \frac{1}{\Delta\epsilon_{557}}$$

where [azurin (II)] = [peroxidase (II)], by stoichiometry, and Az_{tot} = total concentration of azurin, A_{557} = absorbance observed at 557 nm, A_{ox} = absorbance of the fully oxidized peroxidase at 557 nm, and A_{∞} = absorbance of the fully reduced peroxidase at 557 nm. $\Delta\epsilon_{557}$ represents the derived difference absorption coefficient, reduced minus oxidized, of the peroxidase ($mM^{-1} \cdot cm^{-1}$) at 557 nm and K the derived equilibrium constant. A plot of this equation is shown as an inset in Fig. 3. The intercept gives $1/\Delta\epsilon$ and the equilibrium constant K may be calculated from the slope. The solid line through the points was computed from the data by linear regression analysis. An equilibrium constant of 1.1 ± 0.1 (±S.D.) was determined from five different measurements. The calculated $\Delta\epsilon_{557}$ value, 23.6 ± 2.1, is in fair agreement with that obtained from the spectra of the peroxidase, 25.8 $mM^{-1} \cdot cm^{-1}$ [6].

The method was checked by calculating in a similar manner the equilibrium constant for the reduction of oxidized *Pseudomonas* cytochrome *c*-551 by reduced azurin. The equilibrium constant obtained was 0.24 and the derived difference absorption coefficient, 19.1, is in excellent agreement with the value 19.0 $mM^{-1} \cdot cm^{-1}$ determined earlier enzymatically [1]. From our results the equilibrium constant for the reverse reaction would be 4.17, i.e. practically identical with the value 4.2 determined at pH 7 by Rosen and Pecht [14].

Reduction by cytochrome c-551 and horse heart cytochrome c. Owing to overlapping spectra, reduction of the peroxidase with cytochrome *c*-551

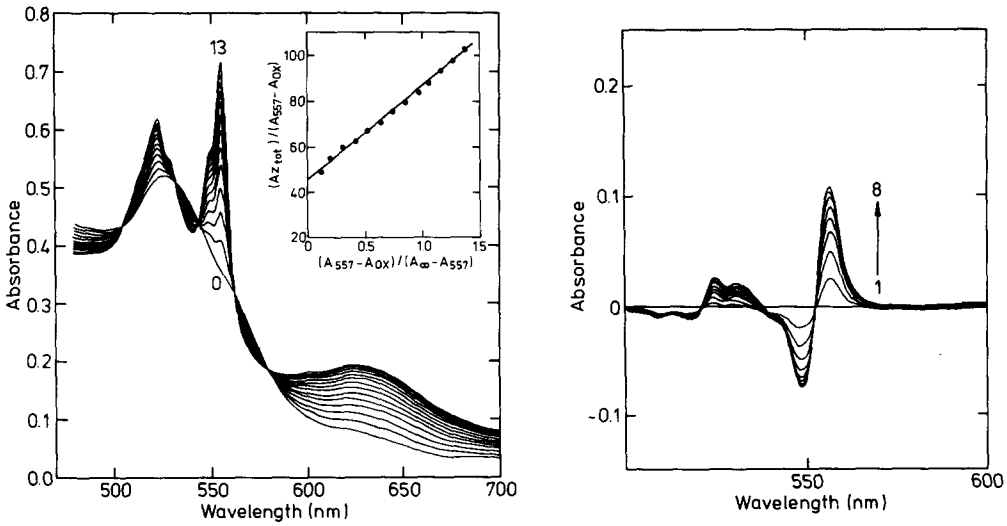

Fig. 3. Titration of oxidized cytochrome *c* peroxidase (26.0 μM) with reduced azurin. Curves are in numerical sequence which is reversed at each isosbestic point. Inset: Equilibrium data for the reduction of cytochrome *c* peroxidase with reduced azurin. A_{557} is the absorbance observed at 557 nm. A_{ox} and A_∞ are the absorbance of the fully oxidized and fully reduced peroxidase, respectively, at 557 nm. The difference absorption coefficient of the peroxidase, $\Delta\epsilon = \epsilon_{red} - \epsilon_{ox}$, was obtained from the reciprocal of the intercept and the equilibrium constant was calculated from the slope.

Fig. 4. Reduction of *Pseudomonas* cytochrome *c* peroxidase by reduced cytochrome *c*-551. Double difference spectra showed progressive reduction of the enzyme (8.8 μM) upon each addition of cytochrome *c*-551. Concentration of the cytochrome increased from 1.9 to 14.0 μM (measurements 1—8).

(*P. aeruginosa*), as well as with horse heart cytochrome *c*, was recorded by measuring double difference spectra. The spectra revealed progressive reduction of the enzyme upon addition of reduced cytochrome (Fig. 4). Because of experimental difficulties in determining the double difference absorption coefficient of the enzyme, quantitative calculation of the equilibrium constant was not attempted with these measurements.

Discussion

Pseudomonas cytochrome *c* peroxidase exhibits anomalous kinetic behaviour when the reaction is initiated with enzyme, i.e. a lag phase of some seconds occurs before a steady-state rate is achieved. However, the lag phase is not observed if the reaction is started by adding hydrogen peroxide [1].

This feature of the peroxidatic reaction seems to fit the hysteretic enzyme concept, i.e. a slow response of the enzyme to a rapid change in ligand (e.g. substrate) concentration [15].

The apparent rate constant, k_{app}, of the activation of the peroxidase, depends linearly on the concentration of the electron donor. Thus, the greater the concentration of the electron donor, the faster the activation of the enzyme and the shorter the lag phase. The reaction proceeded without delay when the enzyme was first reduced with ferrocyanide and then used to initiate the peroxidation reaction. Accordingly, the initial delay, i.e. the hysteretic

response of the peroxidase, involves reduction of the enzyme, with some changes in its conformation as shown by circular dichroism studies (unpublished results).

It is of interest to note that the k_{app} value with horse heart cytochrome *c* is of the same order of magnitude as that obtained with cytochrome *c*-551, whereas the respective steady-state velocities differ widely.

The ability of *Pseudomonas* cytochrome *c* peroxidase to accept electrons directly from certain electron donors was clearly shown by titrations with reduced azurin. Double difference spectra revealed reduction of the enzyme by cytochrome *c*-551 and horse heart cytochrome *c* as well. This change in oxidation state of the prothetic group(s) indicates that the peroxidase may also function as a cytochrome.

A comparison of the spectral and molecular properties, i.e. molecular weight [4], nature and number of the prosthetic groups [3] as well as absorbance spectrum [6], of the *Pseudomonas* peroxidase with those of cytochrome *c*-556 (*P. aeruginosa*) reported by Singh and Wharton [16] shows the two proteins to be identical. That Singh and Wharton were unable to find any function for their cytochrome is puzzling, for although the peroxidatic activity was obviously overlooked, reduction of the hemoprotein by cytochrome *c*-551 and azurin is rapid and striking.

In addition to peroxidase, *Pseudomonas* contains considerable amounts of cytochrome *cd*, which functions as cytochrome *c* oxidase. Both enzymes catalyze the oxidation of cytochrome *c*-551 and azurin. The maximal turn-over rates obtained in the peroxidase reaction are far greater [1,2] than those of the oxidase-catalyzed reaction [17]. This may be taken to indicate that peroxidase is a more efficient electron-transferring enzyme than oxidase when hydrogen peroxidase is present in the cells. Nevertheless, the possible regulatory role of the hysteretic effect of the peroxidase should be considered. Peroxidase may represent a branch point in the respiratory chain resisting a momentary flux and only responding to a continued increase in substrate concentration.

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