A quantitative model for the mechanism of action of the cytochrome *c* peroxidase of *Pseudomonas aeruginosa*

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Each of the elementary reaction steps in both the activation process and catalytic cycle of the cytochrome *c* peroxidase of *Pseudomonas aeruginosa* was characterized using stopped-flow methods. A synthesis of these data led to the establishment of a quantitative model for the action of this enzyme. Comparisons were made between experimental data and calculations over a wide range of enzyme, reductant and H₂O₂ concentrations. Close agreement was found between empirical and simulated reaction time courses from millisecond to tens of seconds time ranges, giving us confidence in the validity of the quantitative model of this enzyme's actions.

INTRODUCTION

Many organisms rid themselves of potentially harmful H₂O₂ via the intervention of a peroxidase enzyme that catalyses the general reaction:

In higher organisms the electron donor is often reduced cytochrome *c*, whereas in bacteria the blue copper protein azurin can also act as electron donor.

Having initially reacted with H₂O₂, the peroxidases of yeast, horseradish and bacteria reduce the peroxide using electrons originating from cytochrome *c* or azurin, but transferred from different intramolecular sites. Each peroxidase derives one of the two reducing electrons from a tightly bound haem group. The site from which the second reducing electron is derived is, however, unique to each of the three classes. In the case of yeast cytochrome *c* peroxidase, this electron is derived from an amino acid residue of the protein (Yonetani *et al.*, 1966). In horseradish peroxidase the electron is derived from the porphyrin ring of the haem group (Dolphin *et al.*, 1971). Only in the case of *Pseudomonas aeruginosa* cytochrome *c* peroxidase (Pccp) is the second electron obtained from a second covalently bound haem (Ellfolk & Soininen, 1971).

The relative ease with which haem redox changes can be monitored has meant that Pccp is the most thoroughly characterized of all the peroxidases. In a uniquely extensive contribution to this field, Ellfolk and his co-workers have studied most aspects of Pccp structure, function and reactivity. Many of these studies have indicated a marked inequivalence of the two haem groups (Ronnberg & Ellfolk, 1978; Ronnberg *et al.*, 1980, 1981*a,b*; Aasa *et al.*, 1981; Ellfolk *et al.*, 1983). Of particular interest has been the wide range of kinetic studies performed on the enzyme, ranging from steady-state (Soininen & Ellfolk, 1972; Ellfolk *et al.*, 1983; Ronnberg & Ellfolk, 1978), to rapid-scan stopped-flow investigations (Ronnberg *et al.*, 1981*a,b*, 1985). Many of these studies have employed azurin as reductant, so allowing a clear spectral distinction to be made between electron donor and peroxidase enzyme.

The general findings of these investigations have been that the

peroxidase enters its catalytic cycle only after pre-activation by azurin (Ronnberg & Ellfolk, 1978; Ronnberg *et al.*, 1981*a*). Although a general mechanism has been proposed for the activation process, no quantitative model for the overall action of this enzyme has previously been developed and tested against empirical data.

In the present paper we report an independent set of measurements in which we evaluate the rate constants for each of the elementary steps of a proposed mechanism. The mechanism was then investigated by using numerical integration techniques to predict the concentration-dependence and time courses of reactions under widely different conditions. A comparison is made between these theoretical data and empirical data that vindicates the model and provides a working quantitative scheme for the reaction of Pccp with H₂O₂ with reduced azurin as electron donor. This model will be of use as a tool in the study of the molecular interactions of the peroxidase with mutant azurins.

MATERIALS AND METHODS

Pseudomonas aeruginosa (N.C.T.C. 6750) was grown in 100-litre cultures from which cytochrome *c* peroxidase and azurin were purified according to the procedures of Foote *et al.* (1983, 1985).

The concentrations of cytochrome *c* peroxidase and azurin were determined by spectrophotometric measurement of the oxidized forms of the proteins by using millimolar absorbance coefficients of 237 cm⁻¹ and 5.1 cm⁻¹ at 407 nm and 625 nm respectively (Soininen & Ellfolk, 1972, 1973). The purity ratio (*A*₄₀₇/*A*₂₈₀) of the peroxidase used in these studies was 4.65.

Solutions of reduced azurin were prepared by addition of a small quantity of Na₂S₂O₄ to an anaerobic buffered solution of the protein, followed rapidly by passage down a column (25 cm × 1 cm) of Sephadex G-25. The half-reduced form of cytochrome *c* peroxidase was prepared by redox exchange with a column of immobilized reduced azurin. Azurin was first bound to CNBr-activated Sepharose 4B and the resulting gel was used to prepare a 10 cm × 0.5 cm chromatographic column. The bound azurin was then reduced with sodium ascorbate and extensively washed to remove excess reductant.

A 2 ml sample of peroxidase at the appropriate concentration was then slowly passed down the column of reduced azurin to yield a sample of the half-reduced form of the peroxidase, in the

Abbreviation used: Pccp, *Pseudomonas aeruginosa* cytochrome *c* peroxidase.

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absence of any excess reducing agent, which was found to be stable in air for at least 30 min, although all subsequent reactions were performed anaerobically in order to avoid any possible complications which might arise from reactions in the presence of O_2 .

Spectrophotometric measurements were made on a Cary 118c spectrophotometer. Rapid reactions were performed in a Gibson–Milnes stopped-flow apparatus (Gibson & Milnes, 1964), equipped with a Tracor Northern TN6500 rapid-scan spectrophotometer (Blackmore *et al.*, 1990). Digitized experimental reaction time courses were converted into ASC II files for analysis on a NEC 386SX PC computer. Reaction traces were analysed in terms of either single- or double-exponential processes, as appropriate, by using non-linear least-squares fitting programs described previously (Brittain, 1986). Numerical simulations of various model reaction systems were performed by using Gear numerical integration techniques (Gear, 1971; Blackmore *et al.*, 1990), employing previously reported algorithms (Brittain, 1990).

The buffer used in all cases was 0.1 M-phosphate, pH 6.0, containing 0.01 % (v/v) Tween 80, in order to stabilize the peroxidase (Greenwood *et al.*, 1988).

RESULTS

When oxidized Pccp was rapidly mixed with reduced azurin and the reaction was monitored at 420 nm, the reaction time courses were found to consist of the sum of two exponential processes. Each of these processes showed a hyperbolic dependence of the observed pseudo-first-order rate constant on the concentration of reduced azurin employed (Figs. 1a and 1b). Furthermore, the contribution that each process made to the overall absorbance change was also dependent on reduced azurin concentration, with the faster process making an increasing contribution as the azurin concentration was increased (Fig. 1c). Spectroscopic investigation of the reaction product showed that, subsequent to reaction with reduced azurin, the peroxidase was present in the half-reduced form, with only one of the two haems having undergone a redox change. When the half-reduced form of the peroxidase, produced by redox exchange with immobilized azurin, was rapidly mixed with a 5-fold excess of H_2O_2 and the reaction measured at 420 nm, the conversion into Compound I (see Scheme 2) was found to occur in a single exponential process with an associated second-order rate constant of $5.6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. If, however, the half-reduced protein was rapidly mixed with a 20-fold excess of H_2O_2 and a stoichiometric quantity of reduced azurin, Compound I was formed within the dead-time of the stopped-flow apparatus.

Thus only the subsequent one-electron reduction of Compound I to Compound II could be observed as a further reduction in absorbance at 420 nm. In agreement with Ronnberg *et al.* (1985), we observed that the spectrum associated with Compound II was stable for approx. 1 s. Under these conditions, a simple process was observed with a rate constant of $2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. Repeating this reaction with a 5-fold excess of reduced azurin and a stoichiometric equivalent of H_2O_2 , we expected to observe, first the conversion of Compound I into Compound II, followed by further reaction of Compound II with excess azurin, to yield once again the half-reduced species, so completing one turn of the catalytic cycle. However, under these conditions we were unable to deconvolute the two reactions and observed a single-exponential process with a rate constant of $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.

When the peroxidase reaction was studied under turnover conditions, the reaction could be initiated in two ways. If the turnover reaction was initiated by mixing oxidized enzyme with a mixture containing excess reduced azurin and excess H_2O_2 , a

Fig. 1. Reaction of oxidized peroxidase with reduced azurin

The $[Az^+]$ -dependence of the pseudo-first-order rate constants (a) and (b) and contribution of the faster process to the overall absorbance change (c) are shown, compared with the dependences predicted by Scheme 1. The experimental data were obtained at 420 nm when $0.85 \mu\text{M}$ -Pccp was rapidly mixed with excess reduced azurin at 25°C in N_2 -equilibrated 25 mM-phosphate buffer, pH 6.0. The continuous line is drawn through data points obtained as the results of simulations calculated at each $10 \mu\text{M}$ increment in azurin concentration. The simulated time courses were deconvoluted into fast and slow phases using the same computer program as was used to process the experimental time courses. Concentrations are after mixing.

distinct lag phase was observed in the oxidation of the reduced azurin as monitored at 625 nm. However, if the reaction was initiated by the addition of H_2O_2 to a mixture of peroxidase and reduced azurin, no such lag was observed. Although experiments were performed ranging from stopped-flow studies, employing micromolar concentrations of enzyme in the millisecond time range, to spectrophotometric studies using nanomolar concentrations of enzyme, in the hundreds of second time range (Figs. 2, 3 and 4), this same pattern of reactivity was maintained.

ANALYSIS AND DISCUSSION

The hyperbolic dependence, shown in Fig. 1, of the pseudo-first-order rate constant for each of the two reactions of the peroxidase on azurin concentration suggests that each reaction process might follow a scheme:

(Creutz & Sutin, 1973; Lambeth & Palmer, 1973; T. Brittain &

Fig. 2. Turnover reactions of peroxidase at high concentration

The time course of azurin oxidation observed at 625 nm (○) is compared with predictions made using Scheme 3 (—). Pccp (2.4 μM) was rapidly mixed in the stopped-flow apparatus with a mixture of 125 μM reduced azurin and 57.4 μM-H₂O₂. The reaction was performed at 25 °C in 0.1 M-phosphate buffer, pH 6.0. Concentrations are after mixing.

Fig. 3. Turnover reaction of peroxidase pre-reduced with azurin at low concentration

The time course of azurin oxidation monitored at 625 nm (○) is compared with predictions made using Scheme 3 (—). Pccp (1.6 nM) in the presence of 25 μM reduced azurin was mixed in the spectrophotometer with H₂O₂ (770 μM) at 25 °C in 0.1 M-phosphate buffer, pH 6.0. Concentrations are after mixing.

Fig. 4. Turnover reaction of peroxidase at low concentration

The time course of azurin oxidation monitored at 625 nm (○) is shown compared with predictions made using Scheme 3 (—). Pccp (1.6 nM) was mixed in the spectrophotometer with a mixture of reduced azurin (25 μM) and H₂O₂ (770 μM) at 25 °C in 0.1 M-phosphate buffer, pH 6.0. Concentrations are after mixing.

R. Blackmore, unpublished work). Double-reciprocal plots of $1/k_{\text{obs}}$ versus $1/[\text{Az}^+]$ yielded straight lines from which the equilibrium constants governing complex formation (K) and the rate-limiting electron-transfer rates (k) could be obtained from the slopes and intercepts respectively. This analysis yielded values of $K_{1,2}$ of 6.8×10^{-5} M and 4.2×10^{-6} M and $k_{1,2}$ values of 7 s^{-1} and 0.2 s^{-1} for the two processes respectively. The concentration-dependence of the contribution that each process makes to the overall absorbance change is interpreted as indicating that the two reaction processes are coupled by an equilibrium (K_e) such as shown in Scheme 1. The limiting ratio of the contributions that each reaction process makes to the total absorbance change observed at high azurin concentration gives an estimate of the value of K_e (1.25). Having established values for K_1 , K_2 , K_e , k_1 and k_2 , we iteratively varied the absolute values of k_{3-8} while maintaining their relative values at those determined by the

values of the equilibrium constants, namely $k_3/k_4 = K_1$, $k_5/k_6 = K_2$ and $k_7/k_8 = K_e$. In this way, we established a set of best-fit values for the individual rate constants for each elementary step in Scheme 1 (see Table 1) that best fit the empirical concentration-dependences of Fig. 1.

The catalytic cycle of the peroxidase appears to consist of three simple second-order sequential reactions, which can be modelled by Scheme 2. As outlined in Scheme 2, we obtained values for k_9 , k_{10} and k_{11} by variously mixing half-reduced Pccp with stoichiometric and super-stoichiometric quantities of H₂O₂ and reduced azurin. The value obtained for the peroxide reaction (k_9) was identical with that previously reported in the literature (Ronnberg *et al.*, 1985). Owing to restrictions in the time resolution of the apparatus employed in this study, we were able to investigate the conversion of Compound I into Compound II and Compound II into half-reduced Pccp only over very restricted concentration ranges. As our results are similar to those obtained by Ronnberg *et al.* (1985), but of lower precision, in subsequent

Scheme 1. The two reactions of peroxidase with azurin

$k_7/k_8 = K_e$. Pccp and Pccp represent two forms of the oxidized enzyme.

Table 1. Rate constants used in computer simulation of peroxidase reactions (see Scheme 3)

The values of the parameters marked 'Expt.' were determined experimentally. The values marked 'Fit' were obtained by iteratively fitting to Scheme 1 with the restriction that the ratios k_3/k_4 , k_5/k_6 and k_7/k_8 were fixed at the experimentally determined values of K_1 , K_2 and K_e respectively. Estimates of errors in experimentally determined rate constants are shown in parentheses.

Rate constant	Value	Values taken from the literature
k_1 (Expt.)	$7 \text{ s}^{-1} (\pm 0.05 \text{ s}^{-1})$	0.6 s^{-1*}
k_2 (Expt.)	$0.2 \text{ s}^{-1} (\pm 0.05 \text{ s}^{-1})$	0.12 s^{-1*}
k_3 (Fit)	$5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$	†
k_4 (Fit)	3.4 s^{-1}	†
k_5 (Fit)	$1 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$	†
k_6 (Fit)	4.2 s^{-1}	†
k_7 (Fit)	0.125 s^{-1}	†
k_8 (Fit)	0.1 s^{-1}	†
k_9 (Expt.)	$5.6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} (\pm 1 \times 10^7)$	$5.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1\dagger}$
k_{10} (Expt.)	$2.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} (\pm 0.5 \times 10^6)$	$2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1\dagger}$
k_{11} (Expt.)	$1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1} (\pm 0.5 \times 10^6)$	$1.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1\dagger}$

* Taken from Ronnberg *et al.* (1981a).

† Ronnberg *et al.* (1981a) reported values of $6.6 \mu\text{M}$ and $7.0 \mu\text{M}$ for K_1 and K_2 respectively, but have not evaluated individual rate constants.

‡ Taken from Ronnberg *et al.* (1985).

simulations we have employed in our quantitative simulation of the catalytic cycle the values published previously (Table 1).

In order to simulate turnover experiments, we have combined Schemes 1 and 2 to give an overall reaction scheme (Scheme 3). By setting the initial concentrations of Pccp, Az^+ , H_2O_2 and half-reduced Pccp to the appropriate concentrations, it is then possible to simulate turnover experiments employing different orders of

addition of the various species. Scheme 3 was modelled by using numerical integration techniques to obtain temporal values for the concentrations of the various species. These time courses were compared with empirical data over as wide a range of conditions as possible in order to verify the validity of the model (see Figs. 1–4).

From the above analysis it is clear that a reasonable quantitative model for the action of Pccp is given by Scheme 3. This scheme itself is essentially a combination of the two general reaction schemes previously suggested by Ronnberg *et al.* (1981a, 1985), but formerly untested against experimental data.

Although the activation reactions described in Scheme 1 are qualitatively those suggested by Ronnberg *et al.* (1981a), our measured rate constants differ significantly from those previously reported for some of the reactions. In particular, we find higher rates of intermolecular electron exchange in our preparations of the enzyme. Unfortunately, as Ronnberg *et al.* (1981a) established rate constants for these steps using only three data points in the range $5\text{--}20 \mu\text{M}$ reduced azurin, it is not clear at present if these differences represent real differences in the molecular activity of the two enzyme preparations or merely reflect differences in the precision of the two data sets. Having confirmed the existence of two forms of the resting oxidized enzyme, we have determined that these forms exist in essentially a 50/50 mixture. The question that this finding raises is, of course, about the possible origins of these two different reactivities. In this regard it is noteworthy that previous spectroscopic studies on the resting state, employing mainly e.p.r. and magnetic c.d., have clearly shown that within the resting state the high redox potential haem is in a 50/50 high-spin–low-spin equilibrium (Foote *et al.*, 1985; Greenwood *et al.*, 1988). It is thus tempting to suggest that these kinetic investigations reflect the co-ordination equilibrium that exists in the resting state, with the five- and six-co-ordinate forms of the high-potential haem exhibiting distinctly different rates of reaction.

In contrast, Scheme 3 includes only a single form of the half-reduced species, in accord with previous spectroscopic studies made on this form of the enzyme (Foote *et al.*, 1985; Greenwood *et al.*, 1988).

In terms of the catalytic-cycle part of Scheme 3, it is clear that during turnover the steady-state concentrations of the half-reduced, Compound I and Compound II forms of the enzyme will be determined by the relative concentrations of H_2O_2 and reduced azurin.

Scheme 3 in general, however, shows what now appears to be a common kinetic pattern demonstrated by a diverse group of terminal electron-transfer proteins. In the cases most thoroughly investigated to date, the terminal oxidases have two forms, a resting and a catalytically activated form. In the case of mammalian cytochrome *c* oxidase the activated or 'pulsed' form appears to be isoelectronic with the resting form but conformationally activated (Wilson *et al.*, 1981; Brunori *et al.*, 1983). In the case of the nitrite reductase of *Wolinella succinogenes*, the activated or 'redox-cycled' form appears to be conformationally activated and may be non-isoelectronic with the resting state (Blackmore *et al.*, 1990). In the case of cytochrome *c* peroxidase,

Scheme 3

the activated or 'half-reduced' state is definitely not isoelectronic with the resting state and clearly conformationally activated (Foote *et al.*, 1985; Greenwood *et al.*, 1988).

Thus in all these cases the existence of two forms of the enzyme provides a common substrate-controlled modulation of enzyme activity with switching to significant enzyme electron transfer to the acceptor only in the presence of a specific quantity of substrate. As to the question of whether this mode of operation is universal in the electron-transport proteins, the answer must await the results of kinetic studies on a greater number of these important proteins. Whatever the outcome of these studies, Scheme 3 represents the first tested quantitative description of the action of the cytochrome *c* peroxidase of *Pseudomonas aeruginosa* and will provide means of studying the nature of the molecular interactions between reduced azurin and the peroxidase, employing genetically engineered mutants of azurin.

C.G. and T.B. thank the Science and Engineering Research Council for support.

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Received 28 August 1991/24 October 1991; accepted 5 November 1991