The kinetics of the oxidation of cytochrome *c* by *Paracoccus* cytochrome *c* peroxidase

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In work that is complementary to our investigation of the spectroscopic features of the cytochrome *c* peroxidase from *Paracoccus denitrificans* [Gilmour, Goodhew, Pettigrew, Prazeres, Moura and Moura (1993) Biochem. J. **294**, 745–752], we have studied the kinetics of oxidation of cytochrome *c* by this enzyme. The enzyme, as isolated, is in the fully oxidized form and is relatively inactive. Reduction of the high-potential haem at pH 6 with ascorbate results in partial activation of the enzyme. Full activation is achieved by addition of 1 mM $CaCl_2$. Enzyme activation is associated with formation of a high-spin state at the oxidized low-potential haem. EGTA treatment of the oxidized enzyme prevents activation after reduction with ascorbate, while treatment with EGTA of the reduced, partially activated, form abolishes the activity. We conclude that the active enzyme is a mixed-valence form with the low-potential haem in a high-spin state that is stabilized by Ca^{2+}. Dilution of the enzyme results in a progressive loss of activity, the extent of which depends on the degree of dilution. Most of the activity lost upon dilution can be recovered after reconcentration. The M_r of the enzyme on molecular-exclusion chromatography is concentration-dependent, with a shift to lower values at lower concentrations. Values of M_r obtained are intermediate between those of a monomer (39565) and a dimer. We propose that the active form of the enzyme is a dimer which dissociates at high dilution to give inactive monomers. From the activity of the enzyme at different dilutions, a K_D of 0.8 μM can be calculated for the monomer-dimer equilibrium. The cytochrome *c* peroxidase oxidizes horse ferrocytochrome *c* with first-order kinetics, even at high ferrocytochrome *c* concentrations. The maximal catalytic-centre activity ('turnover number') under the assay conditions used is 62000 min^{-1}, with a half-saturating ferrocytochrome *c* concentration of 3.3 μM. The corresponding values for the *Paracoccus* cytochrome *c*-550 (presumed to be the physiological substrate) are 85000 min^{-1} and 13 μM. However, in this case, the kinetics deviate from first-order progress curves at all ferrocytochrome *c* concentrations. Consideration of the periplasmic environment in *Paracoccus denitrificans* leads us to propose that the enzyme will be present as the fully active dimer supplied with saturating ferrocytochrome *c*-550.

INTRODUCTION

Cytochrome *c* (Cyt. *c*) peroxidases have been found in yeast and several bacteria and catalyse the oxidation of ferrocytochrome *c* by H_2O_2, thus coupling two single electron transfers to a two-electron reduction

$$2\ \text{Cyt.}c\ (Fe^{II}) + 2H^+ + H_2O_2 \rightarrow 2\ \text{Cyt.}c\ (Fe^{III}) + 2H_2O$$

The yeast peroxidase is a monohaem protein of 294 amino acids (Kaput et al., 1982) found in the intermembrane space of the mitochondrion. H_2O_2 binds to the *b*-type haem of this enzyme and removes two reducing equivalents to leave Compound I which contains a ferryl(Fe^{IV}) oxene intermediate and a side-chain radical [reviewed by Bosshard et al. (1991)]. Restoration of the original enzyme is achieved by two successive one-electron transfers from ferrocytochrome *c*.

The most extensively characterized bacterial cytochrome *c* peroxidase is that from *Pseudomonas aeruginosa*. This enzyme contains two *c*-type haems (Ellfolk and Soininen, 1970; Soininen et al., 1970), one of which acts as a high-potential electron-transferring pole and is co-ordinated by methionine and histidine, and the other is a low-potential ligand-binding haem probably co-ordinated by two histidines (Ellfolk et al., 1983). The fully oxidized enzyme does not bind H_2O_2 (Araiso et al., 1980) or anionic ligands such as CN^- (Ellfolk et al., 1984). Reduction of the high-potential haem results in the low potential haem adopting a more open conformation, available for ligand binding (Araiso et al., 1980; Ellfolk et al., 1984). Foote et al. (1984) have shown that this opening up of the peroxidatic haem is associated with the conversion of the haem iron into a high-spin state. Thus, like cytochrome *c* oxidase, the bacterial peroxidase has a mechanism which prevents binding of the oxidant until the enzyme has the reducing power necessary to carry out the complete reaction.

We have recently identified a dihaem cytochrome *c* peroxidase in the periplasm of *Paracoccus denitrificans* grown under low oxygen partial pressure (Goodhew et al., 1990). The enzyme has been characterized spectroscopically and, like its *Pseudomonas* counterpart, contains a high-potential electron-transferring haem and a low-potential peroxidatic haem (Gilmour et al., 1993). The fully oxidized enzyme does not bind CN^-. Reduction of the high-potential haem results in the conversion of the low-potential haem into a high-spin form capable of binding ligands. The spin conversion is a slow process and is enhanced in rate and extent by added Ca^{2+}.

The present paper describes a kinetic characterization of the enzyme and shows that, as predicted from the spectroscopic studies, the most active form of the enzyme is that in which the high-potential haem is reduced and the low-potential haem is oxidized and high spin.

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MATERIALS AND METHODS

Growth of cells

Paracoccus denitrificans (LMD 52.44, obtained from Professor R. P. Ambler, Institute of Cellular and Molecular Biology, University of Edinburgh) was grown as described by Goodhew et al. (1990).

Purification of cytochrome *c* peroxidase

Cytochrome *c* peroxidase was purified by the method of Goodhew et al. (1990). The pure enzyme had an A_{409}/A_{280} ratio of 5.6 and gave a single band on SDS/PAGE when converted into the apoprotein (see Goodhew et al., 1990). The enzyme was stored at −20 °C in 1 mM sodium phosphate, pH 7.0, containing 10 mM NaCl, at protein concentrations of 150–350 μM.

Purification of cytochrome *c*-550

In the DEAE-cellulose chromatography of the periplasmic extract of *Paracoccus denitrificans* the cytochrome *c*-550 was eluted as the first haemoprotein at a salt concentration of 200 mM (Goodhew et al., 1990). Fractions containing this cytochrome were diluted with 4 vol. of water, re-adsorbed on to a small DEAE-cellulose column (1 cm × 2 cm) and eluted with 10 mM Tris/HCl buffer, pH 8, containing 500 mM NaCl. Molecular-exclusion chromatography was carried out on this solution on a column (82 cm × 2.5 cm) of Sephadex G-75-50 equilibrated in 20 mM Tris/HCl buffer, pH 8, containing 100 mM NaCl. Fractions containing the cytochrome *c*-550 were diluted with 4 vol. of water, again bound to the small DEAE-cellulose column and eluted with 25 mM sodium phosphate, pH 7.0, containing 500 mM NaCl. This solution was diluted with 4 vol. of water and applied to a hydroxyapatite column (Bio-Rad) (8 cm × 2 cm). The bound material was eluted with a linear gradient (600 ml) from 5 mM sodium phosphate, pH 7.0, containing 100 mM NaCl, to 200 mM sodium phosphate containing no NaCl.

The final product had a purity index (A_{410}/A_{280} ratio) of 4.4 and gave a single band on SDS/PAGE.

Assay of cytochrome *c* peroxidase activity

The *Paracoccus denitrificans* cytochrome *c* peroxidase will accept electrons from both mitochondrial cytochrome *c* and *Paracoccus* cytochrome *c*-550 (Goodhew et al., 1990) (Figure 1). The activity of the enzyme is assayed by recording the decrease in absorbance of the α-band of the ferrocytochrome *c* at 550 nm. The reaction can be initiated either by addition of the enzyme to a cuvette containing ferrocytochrome *c* and H_2O_2 or by addition of H_2O_2 to a cuvette containing ferrocytochrome *c* and enzyme. As we shall see, these two starting methods can give very different activities. The cuvette is continuously stirred using a magnetic flea so that the reaction can be monitored immediately after any additions.

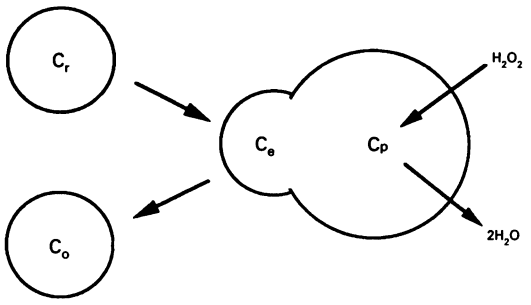


Figure 1 The nature of the assay

Cytochrome *c* peroxidase catalyses the oxidation of ferrocytochrome *c* (C_r) by H_2O_2 to release ferricytochrome *c* (C_o) and water. The enzyme has an electron-transferring haem (C_e), which accepts incoming electrons and transfers them to a peroxidatic haem (C_p).

The reaction is first-order towards horse cytochrome *c* under conditions where the reaction is rapid. Under these conditions, the initial velocity (v) can be calculated by determination of the first-order rate constant (k) and use of the relationship, $v = k[C_r]$, where $[C_r]$ is ferrocytochrome *c* concentration. When the reaction with horse cytochrome *c* is slow, and under all tested conditions with *Paracoccus* cytochrome *c*-550, the kinetics do not give good straight lines in first-order plots. This may partly be due to loss of activity of the very dilute enzyme in the cuvette. In most of these cases, linearity in the early region of the plots allowed an estimate of the rate constant. In the few cases where this was not the case, the initial velocity was determined from the gradient of the progress curve.

Measurement of formation of the high-spin state

Formation of the high-spin state at the low-potential haem after reduction of the high-potential haem was monitored using difference spectroscopy of the type described in Figure 2 of Gilmour et al. (1993). These difference spectra show a peak at 380 nm indicative of formation of a high-spin haem and are obtained by subtracting the cytochrome *c* peroxidase spectrum 2 min after reduction with ascorbate from the spectrum of the same solution 45 min after reduction.

Molecular-exclusion chromatography and the monomer–dimer equilibrium

A column of Sephadex G75-50 (1.5 cm × 90 cm) was equilibrated with 5 mM Hepes (pH 7.5) 50 mM NaCl. The *Paracoccus* cytochrome *c* peroxidase (1 ml) was applied to the column at different concentrations and in the presence of 1 mg each of BSA, ovalbumin, carbonic anhydrase, myoglobin and horse cytochrome *c*. Selected protein-containing fractions were subjected to SDS/PAGE (15 % acrylamide), stained with Coomassie Brilliant Blue R, to confirm the elution pattern of individual proteins. At low concentrations of cytochrome *c* peroxidase it was necessary to detect the enzyme after SDS/PAGE by the more sensitive haem-staining method (Goodhew et al., 1986). This stain persisted during subsequent Coomassie Blue staining, and a single photograph could then record the complete elution pattern.

Calculation of the K_D for the monomer–dimer equilibrium

The data in Figure 4 (below) can be used to provide a rough estimate of the dissociation constant for the monomer–dimer equilibrium. Extrapolation of the curves back to zero time gives an activity of approx. 45000 min^{-1}. If we assume that the monomer is inactive and that this represents the activity of the dimer, we can calculate the concentration of the dimer present at various dilutions after equilibrium has been re-established. For example, for the dilution to 0.5 μM enzyme, activity = 19250 min^{-1} (from Figure 4 below); therefore enzyme present as dimer = (19250/45000) × 0.5 μM = 0.21 μM; therefore [dimer] = 0.105 μM; therefore [monomer] = 0.29 μM; K_D = [monomer]^2/[dimer] = 0.8 μM.

K_D values of 0.6 μM and 0.8 μM are obtained for the dilutions

to 1 μM and 0.25 μM respectively. These must be regarded as rough estimates of K_D, because of the uncertainty in the value for the activity of the full dimer and because of the untested assumption that the monomer is completely inactive.

RESULTS

Effect of ascorbate reduction and Ca^{2+} on enzyme activity

In our previous work we found that ascorbate rapidly reduced only the high-potential haem of the *Paracoccus* peroxidase, and that this reduction led to a relatively slow conversion of the lower-potential haem into a high-spin state (Gilmour et al., 1993). The level of high-spin formation was found to be influenced by the pH and the presence of Ca^{2+}.

The enzyme, after purification, is in the fully oxidized state and is relatively inactive at pH 6 with a catalytic-centre activity ('turnover number') of 1920 min^{-1} (Table 1). In contrast, if the enzyme is treated with ascorbate and the electron-transfer mediator diaminodurol and left at room temperature for 45 min, the activity is enhanced 15-fold to give a catalytic-centre activity of 30000 min^{-1}. A further enhancement of activity (catalytic-centre activity 57000 min^{-1}) is observed if the ascorbate-reduced aged enzyme is treated with 1 mM $CaCl_2$ for 15 min.

These increases in activity of the enzyme at pH 6 are found to be correlated with the appearance of the high-spin state of the lower-potential haem (Figure 2). Activity and the proportion of enzyme in the high-spin state increase in parallel over approx. 45 min after reduction has occurred. Addition of 1 mM Ca^{2+} at this stage results in the further enhancement of both the activity and the proportion of the high-spin form. This result indicates that the mixed-valence high-spin form of the enzyme is the active form and that Ca^{2+} promotes this state. Since the spectroscopic changes in the first phase of activation in the absence of added Ca^{2+} are identical with those promoted by Ca^{2+} addition, the first phase of activation is probably due to residual Ca^{2+} already bound to the enzyme at this pH. The partial activation of the oxidized enzyme during the 40 s preincubation in the cuvette before initiation with H_2O_2 (catalytic-centre activity 3900 min^{-1}; Table 1) is probably due to a small proportion of high-spin formation after reduction by the ferrocytochrome *c*.

The presence of residual Ca^{2+} on the enzyme, and the importance of Ca^{2+} in promoting the active form, are confirmed by studies with EGTA. In the experiment of Figure 3(a), the enzyme shows 84% of full activity after reduction with ascorbate in the absence of added Ca^{2+}. EGTA causes complete loss of this activity with a half-time of about 60 min. Complete loss of the 380 nm band associated with the high-spin state is also observed (result not shown). Addition of Ca^{2+}, in excess over the EGTA, results in an enzyme that is as active as one to which EGTA had never been added. Thus EGTA reversibly removes endogenous Ca^{2+} in the enzyme preparation. A similar experiment is shown in Figure 3(b) for the oxidized enzyme. EGTA treatment of the oxidized enzyme gives rise to a form which is neither activated by reduction with ascorbate nor is capable of forming a 380 nm band. However, when excess Ca^{2+} is added, full activity and high-spin formation is observed.

At pH 7.5, no high-spin formation is observed at the low-potential haem after reduction of its high-potential counterpart (Gilmour et al., 1993). Correlated with this is a complete inactivity of the enzyme at pH 7.5 in both the oxidized and the mixed-valance state (Table 1). Addition of 1 mM Ca^{2+} to the mixed-valance enzyme results in formation of the same level of high spin as observed at pH 6 and a high level of enzyme activity (50% of that at pH 6). The lower activity at pH 7.5 must be due to influences other than the level of high-spin iron.

Mg^{2+} can replace Ca^{2+} in the activation of the enzyme (results not shown).

Table 1 Activity of cytochrome *c* peroxidase

The activity of the enzyme assayed under various conditions is shown. The diluted peroxidase stock was 2 μM in 5 mM Mes/5 mM Hepes, pH 6, allowed to age for 60 min to allow equilibrium between the monomer and the dimer. The enzyme was assayed at a final concentration of 1 nM in a cuvette containing 5 mM Mes, 5 mM Hepes, pH 6, 10 mM NaCl, 7 μM horse heart ferrocytochrome *c* and 18 μM H_2O_2. Reduction of the peroxidase stock was by ascorbate (1 mM), diaminodurol (DAD) (5 μM). $CaCl_2$ addition was to a final concentration of 1 mM. Assays were initiated either by the enzyme or by H_2O_2 after a 40 s preincubation of the enzyme with the ferrocytochrome *c* in the assay cuvette. 1 mM $CaCl_2$ was present in the assay mix when the Ca^{2+}-activated enzyme was assayed. To allow establishment of any high-spin–low-spin equilibrium, the ascorbate (Asc)-reduced sample was assayed after 45 min and the Ca^{2+}-treated sample after 15 min, as indicated in the Table.

		Catalytic-centre activity (min^{-1})	
pH	Enzyme pretreatment	Enzyme start	Peroxide start (40 s preincubation)
6	Oxidized, minus Ca^{2+}	1920	3900
	Asc/DAD, 45 min	30000	14500
	Asc/DAD, 45 min + Ca^{2+}, 15 min	57000	41000
7.5	Oxidized, minus Ca^{2+}	0	0
	Asc/DAD, 45 min	0	0
	Asc/DAD, 45 min + Ca^{2+}, 15 min	24900	18450

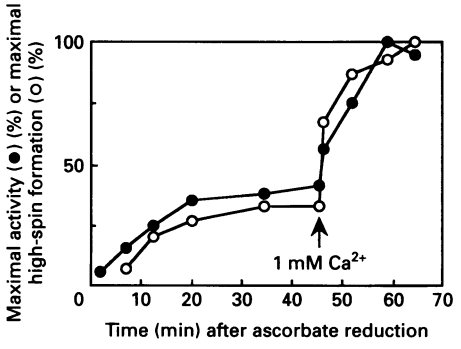


Figure 2 Enzyme activation after reduction with ascorbate

A dilution of cytochrome *c* peroxidase to 2 μM in 5 mM Mes/5 mM Hepes, pH 6, was prepared. This diluted stock was allowed to age for 60 min to allow equilibrium between the monomer and the dimer. Assays were initiated with the enzyme (final concn. 1 nM) added to a cuvette containing 5 mM Mes, 5 mM Hepes (pH 6), 10 mM NaCl, 7 μM horse heart ferrocytochrome *c* and 18 μM H_2O_2. Reduction of the diluted peroxidase stock was by 1 mM ascorbate and 5 μM diaminodurol. High-spin formation in this solution was followed spectrophotometrically by the increase in absorbance at 380 nm. Ca^{2+} activation of the diluted stock enzyme was with $CaCl_2$ to a final concentration of 1 mM. 1 mM $CaCl_2$ was present in the assay mix when the Ca^{2+}-activated enzyme was assayed.

Effect of dilution on enzyme activity and the presence of a monomer–dimer equilibrium

The enzyme has a high catalytic-centre activity and must be diluted from the purified concentrated stock solutions prior to assay. However, after dilution, the enzyme loses a proportion of its activity over a 45 min period (Figure 4). The loss of activity

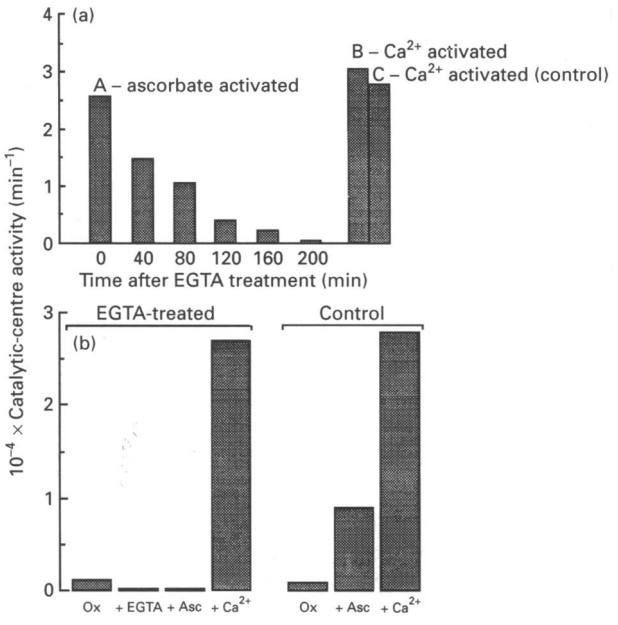


Figure 3 EGTA treatment of the reduced and oxidized enzyme

(**a**) The effect of EGTA on the enzyme after reduction with ascorbate. Cytochrome *c* peroxidase (2 μM) in 5 mM Mes/5 mM Hepes, pH 6.3, aged for 60 min to allow equilibrium between the monomer and the dimer, was reduced with 1 mM ascorbate (Asc) and 5 μM diaminodurol. After 45 min to allow the high-spin–low-spin equilibrium to be achieved, EGTA was added to 1 mM, and samples were removed for assay of activity at various time intervals (A, ascorbate-activated). Finally $CaCl_2$ was added to 2 mM, along with 2 mM NaOH (to neutralize H^+ released from the EGTA as Ca^{2+} binds) (B, Ca^{2+}-activated). This solution was assayed for activity after 15 min. A control solution was reduced with ascorbate and treated with Ca^{2+} for 15 min without EGTA addition [C, Ca^{2+}-activated (control)]. Assays were performed as described in Figure 2. (**b**) The effect of EGTA on the oxidized enzyme. A dilution of cytochrome *c* peroxidase to 2 μM in 5 mM Mes/5 mM Hepes, pH 6.2, was aged for 60 min to allow equilibrium between the monomer and the dimer and then assayed (Ox). It was then treated with EGTA (1 mM) for 2 min and assayed again (+EGTA). The enzyme was then reduced with ascorbate (1 mM) and diaminodurol (5 μM) and assayed after 45 min (+Asc). Finally Ca^{2+} was added to 2 mM, along with 2 mM NaOH. This solution was assayed after 15 min (+Ca^{2+}). A control solution was not treated with EGTA, but was otherwise treated as for the first sample. Assays were performed as described in Figure 2.

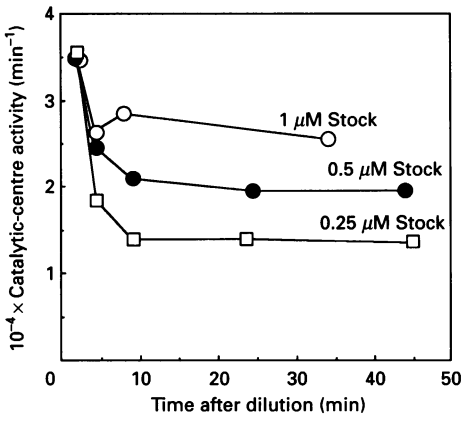


Figure 4 Effect of dilution on enzyme activity

Samples from a 100 μM peroxidase stock in 5 mM Mes/5 mM Hepes (pH 6)/1 mM $CaCl_2$ were diluted in the same buffer to 1.0 μM, 0.5 μM and 0.25 μM. Samples of each diluted stock concentration were assayed (1 nM final enzyme concn.) at various times after dilution. The assay solution contained 5 mM Mes, 5 mM Hepes, pH 6, 10 mM NaCl, 1 mM $CaCl_2$, 7 μM horse heart ferrocytochrome *c* and 18 μM H_2O_2. Assays were initiated by H_2O_2 addition following a 40 s preincubation of the enzyme with the ferrocytochrome *c* in the assay cuvette.

Table 2 Recovery of cytochrome *c* peroxidase activity by re-concentration after dilution

A 4 μM oxidized sample of peroxidase in 5 mM Mes/5 mM Hepes (pH 6)/1 mM $CaCl_2$ was allowed to age for 60 min to allow equilibrium between the monomer and dimer. It was assayed and then diluted to 0.267 μM in the same buffer. The activity was recorded at various times after dilution. After a stable activity had been reached, the enzyme was re-concentrated to 4 μM in an Amicon Centriprep-10 concentrator. The enzyme was assayed at a final concentration of 4 nM in a cuvette containing 5 mM Mes, 5 mM Hepes, pH 6, 10 mM NaCl, 1 mM $CaCl_2$, 7 μM horse heart ferrocytochrome *c* and 18 μM H_2O_2. The assays were initiated with H_2O_2 following a 40 s preincubation of the enzyme in the assay cuvette with ferrocytochrome *c*.

Conditions	Catalytic-centre activity (min^{-1})
4 μM stock	23000
Time after 15-fold dilution from 4 μM to 0.267 μM (min)	
2.5	13000
8.5	9000
15.5	8000
22	7900
34	7300
45	6300
Concentration (15-fold) from 0.267 μM to 4 μM	16500

increases with increasing dilution. Over 60% of the activity lost on dilution is recovered by re-concentration (Table 2). The inability to recover the remaining 40% of the original activity may be due to enzyme damage during the re-concentration process. We propose that the enzyme exists in a monomer–dimer equilibrium in which only the dimer is active. Dilution of the enzyme shifts the equilibrium towards the monomer and therefore activity is lost.

It is apparent from Table 1 that the ascorbate-reduced enzyme is more active when the reaction is started with enzyme rather than with H_2O_2. This is consistent with loss of activity over the 40 s preincubation period as a result of dilution of the enzyme in the cuvette. The enzyme is normally assayed at a final concentration in the cuvette of 1 nM, taken from a diluted stock of 2 μM. In the case of the oxidized enzyme, the loss of activity due to dilution in the cuvette is masked by the activation that occurs due to reduction of the high-potential haem by the substrate ferrocytochrome *c*-550 (Table 1).

Further evidence for this monomer–dimer equilibrium comes from the behaviour of the enzyme in molecular-exclusion chromatography. The enzyme has a minimum M_r (from amino acid analysis) of 39565 (Gilmour et al., 1993). However, if applied to a Sephadex G-75 column at a concentration of 28 μM, it is eluted with a M_r of approx. 66000 (Figure 5b). This value is intermediate between that of a monomer and a dimer and suggests that the enzyme exists in a rapid monomer–dimer equilibrium (Andrews, 1964). This is confirmed by the elution of a 2 μM sample with an M_r of 59000 (Figure 5a).

Substrate-dependence of cytochrome *c* peroxidase activity

(a) Horse ferrocytochrome *c* as substrate

The oxidation of horse heart ferrocytochrome *c* is first-order, even at high concentrations of substrate (Figure 6a). The first-

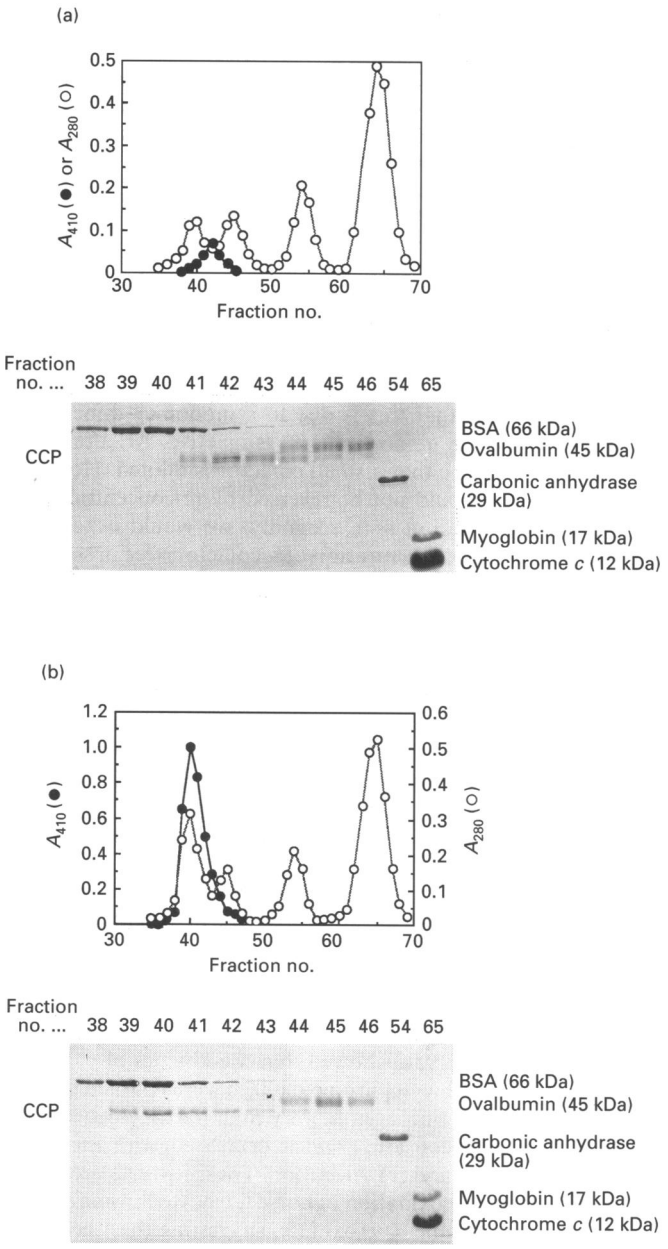


Figure 5 Molecular-exclusion chromatography and the monomer–dimer equilibrium

The *Paracoccus* cytochrome *c* peroxidase was applied to the Sephadex G-75 column as described in the Materials and methods section at concentrations of 2 μM (**a**) and 28 μM (**b**). The profile shows the absorbance at 410 nm (●) and 280 nm (○) of the eluate fractions. The identity of individual protein peaks was confirmed by subjecting the fractions indicated to SDS/PAGE (15% acrylamide) followed by Coomassie Blue staining in the case of (**b**) and haem staining followed by Coomassie Blue staining in the case of (**a**). Abbreviation: CCP, *Paracoccus* cytochrome *c* peroxidase.

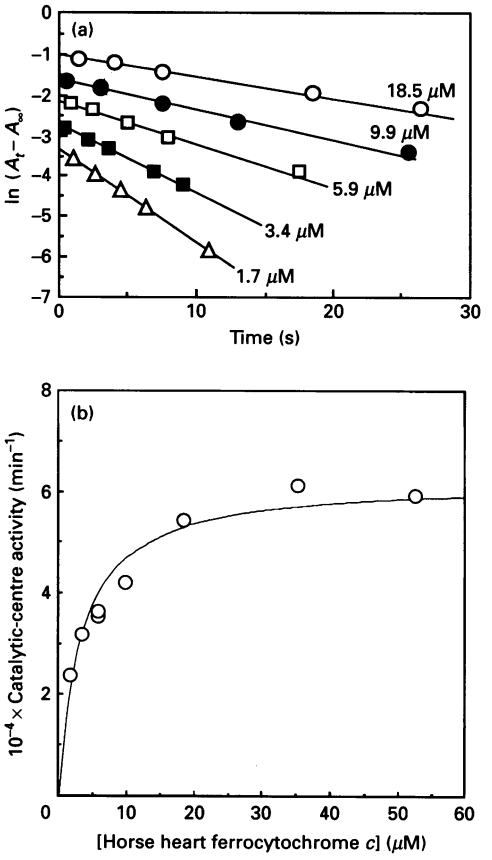


Figure 6 Effect of horse heart ferrocytochrome *c* concentration on peroxidase activity

Assays were performed on ascorbate-reduced, Ca^{2+}-activated, peroxidase. The peroxidase stock was 2 μM in 5 mM Mes/5 mM Hepes (pH 6)/1 mM ascorbate/5 μM diaminodurol/1 mM $CaCl_2$ and allowed to age for 60 min to allow equilibrium between monomer and dimer. Assays were initiated with the enzyme (final concn. 1 nM), to a cuvette containing 5 mM Mes, 5 mM Hepes, pH 6, 10 mM NaCl, horse heart ferrocytochrome *c* and 100 μM H_2O_2. The enzyme was assayed with ferrocytochrome *c* concentrations of 1.7, 3.4, 5.9, 5.93, 9.9, 18.5, 35.3 and 52.75 μM. Initial velocities were calculated from first-order plots, some of which are shown in (**a**). Catalytic-centre activities were determined by dividing the initial velocity by the enzyme concentration (**b**).

order rate constant obtained from these plots decreases with increasing ferrocytochrome *c* concentration (Figure 6a).

Although the reaction does not show zero-order kinetics, a plot of initial velocity against substrate concentration does produce a 'saturation' effect typical of Michaelis–Menten kinetics (Figure 6b). The theoretical curve shown is for a maximum catalytic-centre activity of 62000 min^{-1} and a substrate concentration which gives half-maximal turnover of 3.3 μM.

(b) Paracoccus denitrificans ferrocytochrome *c* as substrate

Semilogarithmic plots of the progress curves of the oxidation of *Paracoccus* cytochrome *c*-550 at different ferrocytochrome *c* concentrations are not linear, indicating that the reaction deviates from first-order kinetics as the oxidation progresses (Figure 7a). This may partly be due to the progressive monomerization of the enzyme in the cuvette leading to loss of activity (a problem more severe than in the case of horse cytochrome *c* because the progress of the reaction was monitored over a longer time (see Figure 7a)). The initial linear portions of the plots are less strongly influenced by substrate concentration than is the case for horse cytochrome *c*. These initial portions were used to estimate rate constants and hence initial velocities. As observed with horse cytochrome *c* these calculated velocities fit a saturation curve (Figure 7b), in this case with a maximum catalytic-centre

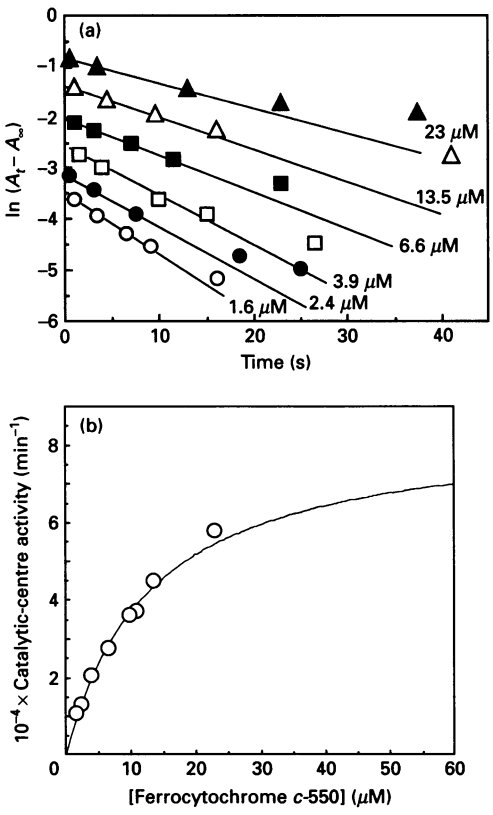


Figure 7 Effect of *Paracoccus denitrificans* ferrocytochrome *c*-550 concentration on peroxidase activity

Assays were performed on ascorbate-reduced, Ca^{2+}-activated peroxidase. The peroxidase stock was 2 μM in 5 mM Mes/5 mM Hepes (pH 6)/1 mM ascorbate/5 μM diaminodurol/1 mM $CaCl_2$, allowed to age for 60 min to allow equilibrium between monomer and dimer. Assays were initiated with the enzyme (final concn. 1 nM), to a cuvette containing 5 mM Mes, 5 mM Hepes, pH 6, 10 mM NaCl, *Paracoccus denitrificans* ferrocytochrome *c*-550 and 100 μM H_2O_2. The enzyme was assayed with ferrocytochrome *c*-550 concentrations of 1.6, 2.4, 3.9, 6.6, 10, 10.7, 13.5 and 23 μM. Initial velocities were calculated from first-order plots, some of which are shown in (**a**). Catalytic-centre activities were determined by dividing the initial velocity by the enzyme concentration (**b**).

activity of 85000 min^{-1} and a substrate concentration which gives half-maximal turnover of 13 μM.

DISCUSSION

Activation of the enzyme

The relative inactivity of the oxidized enzyme is due to the fact that, in this state, the enzyme has neither the reducing equivalents for peroxide reduction nor an accessible site for the peroxide to bind. The low activity observed when the reaction is started with H_2O_2 is due to a small amount of high-spin formation occurring in the cuvette following reduction of the enzyme by the ferrocytochrome *c*. When the oxidized enzyme is used to start the reaction, a lag effect is usually observed, again probably due to a low level of activation occurring in the cuvette. This lag effect was also observed with the *Pseudomonas aeruginosa* enzyme (Ronnberg and Ellfolk 1978).

Reduction of the high-potential haem is followed by a slow switch to a high-spin state at the lower-potential haem, making it available for ligand binding. The active enzyme that is formed has both the necessary reducing equivalents for the reaction and an available peroxide-binding site. A bound Ca^{2+} is essential for the spin-state switch, and only a proportion of the enzyme molecules, as purified, retain that Ca^{2+} at pH 6 and can be activated. Addition of 1 mM Ca^{2+} enhances the level of high-spin in the low-potential haem and therefore increases the concentration of sites available for peroxide binding. Ca^{2+} is found in other peroxidases such as horseradish peroxidase (Shiro et al., 1986) and lignin peroxidase (Poulos et al., 1993), but not in yeast cytochrome *c* peroxidase.

Monomer–dimer equilibrium and dilution of the enzyme

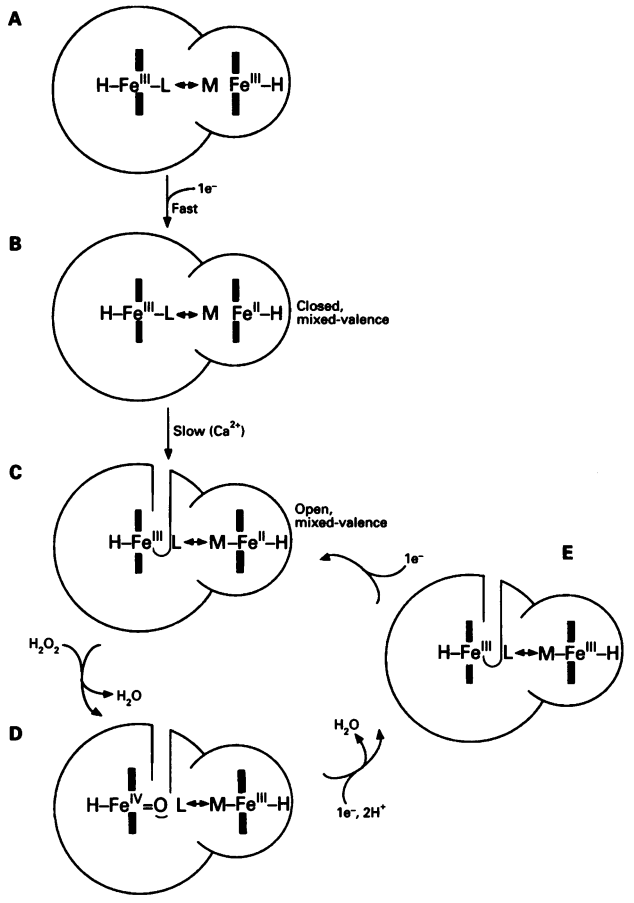
The enzyme gradually loses activity with time after dilution from concentrated solutions to concentrations that are appropriate for assay. We propose that this is due to a monomer–dimer equilibrium in which the monomer form is inactive. An alternative explanation would be that a small cofactor is diluted. However, first, such a loss should not be reversed by re-concentration of the enzyme by filtration and, secondly, we would expect that simply lowering the concentration of a cofactor, free in solution, should have an immediate, rather than a time-dependent, effect.

The data of Figure 4 can be used to provide a rough estimate for the dissociation constant for the monomer–dimer equilibrium [K_D (average) 0.74 μM] as shown in the Materials and methods section. The shift to lower values of the M_r for more dilute solutions of the enzyme in the molecular-exclusion column provide qualitative confirmation of a monomer–dimer equilibrium. A more quantitative picture cannot be obtained by this method because of the spread and dilution of the peak as it passes through the column. However the peak elution concentration of the enzyme applied to the Sephadex column at 2 μM was 0.3 μM, and for the 28 μM application it was 4 μM. Since these numbers span the proposed K_D, a detectable difference in the equilibrium position would be expected.

First-order kinetics and substrate-dependence

Smith and Conrad (1956) showed that the kinetics of oxidation of cytochrome *c* with cytochrome oxidase were characterised by (*a*) a first-order reaction even at very high [ferrocytochrome *c*], (*b*) first-order rate constants which decreased with increasing [ferrocytochrome *c*] and (*c*) saturation. These features are shared by the kinetics of the oxidation of horse ferrocytochrome *c* by the *Paracoccus* cytochrome *c* peroxidase, suggesting that the nature of the reaction is similar in the two systems. Minnaert (1961) considered a number of models which reconciled the apparent anomaly of first-order kinetics and the hyperbolic relationship between initial velocity and substrate concentration. Of these, model IV, which involves binding of equal strength of the oxidized and reduced cytochrome *c* to the oxidase, has received most experimental support.

The kinetics of oxidation of *Paracoccus* cytochrome *c*-550 deviated from first-order, and the rate constant was less affected by substrate concentration. Bolgiano et al. (1988) observed a similar lack of dependence of *k* on substrate concentration for the interaction of cytochrome *c*-550 with the *Paracoccus* cytochrome *c* oxidase. Interestingly, addition of poly-L-lysine resulted in enhanced rates with a *k* dependence similar to that seen for mitochondrial cytochromes *c*. Cytochrome *c*-550 has a very asymmetric charge distribution (Bolgiano et al., 1988; Pettigrew, 1991) with a positively charged 'front' face and a strongly negatively charged back surface. Perhaps polylysine binds to the negative back surface of the cytochrome *c*-550 conferring similar electrostatic properties to those shown by mitochondrial cytochromes *c*.



Scheme 1 Model of action of cytochrome *c* peroxidase

The model is described in the Discussion. The enzyme is represented as a two-domain structure with the smaller domain containing the electron-transferring haem with methionyl(M)/histidyl (H) co-ordination and the larger domain containing the peroxidatic haem co-ordinated by histidine (H) and a sixth ligand (L). **A–E** are referred to in the text.

Physiological activity of the enzyme

Our model of the enzyme is shown in Scheme 1 and draws on both the spectroscopic studies of Gilmour et al. (1993) and the kinetics presented here. Also it is a descendent of the models of the groups of Ellfolk and Greenwood for the *Pseudomonas aeruginosa* enzyme (although important differences exist). The oxidized enzyme is inactive and reduction at the high-potential haem is required before the reaction can be catalysed. The phase of haem-crevice opening reported by the spectroscopic studies is a slow process and clearly cannot be part of the fast enzymic cycle. Thus, although form **E** in Scheme 1 is an oxidized form of the enzyme, it cannot be the same as the oxidized state, **A**. Therefore we propose that the enzyme may relax to the inactive oxidized form only in the absence of a supply of electrons. Of course, this happens during purification and leads to the need to re-activate the enzyme before assay. It is possible that the de-activation of the enzyme in the absence of a supply of electrons is a form of protection. If H_2O_2 were able to bind to the peroxidatic haem in a fully oxidized enzyme, the single electron transfer that would follow may result in formation of the highly reactive hydroxyl radical.

This aspect of 'design' whereby the enzyme will only bind to its substrate if it has the capacity to complete its reduction to water is reminiscent of cytochrome *c* oxidase, which will only bind oxygen when it contains four transferable electrons (Palmer et al., 1976). Interestingly, cytochrome *c* oxidase is also isolated in a relatively inactive oxidized form which becomes fully active (the pulsed form) after reduction and turnover (Antonini et al., 1977).

The requirement for bivalent cations will be satisfied in the living cell by the environment of the periplasm. Mg^{2+} is an important structural component of the surface layers of Gram-negative bacteria (Costerton et al., 1974), and chelation of this ion by EDTA forms part of the protocol for spheroplast formation. It is probable that this procedure partly inactivates our enzyme by removal of intrinsic Ca^{2+} or Mg^{2+}. The very low catalytic-centre activity of the cytochrome *c* peroxidase of *Pseudomonas stutzeri* (Table 3; Villalain et al., 1984) may be due to non-optimized assay conditions and is unlikely to be a true reflection of the kinetic competence of the enzyme from this organism. It would be of interest to determine whether the enzyme from this source shows the Ca^{2+}-dependent activation in the mixed-valence state that we found for the *Paracoccus* enzyme.

From the yield of the enzyme after purification and assuming a value of 20% for the periplasmic volume (Stock et al., 1977), a physiological concentration in the periplasm of approx. 200 μM can be calculated. Our estimate of the K_D for the monomer–dimer equilibrium is 0.8 μM, and clearly the enzyme should exist as the active dimer under physiological conditions.

On the basis of similar calculations, the physiological con-

Table 3 Kinetic parameters for cytochrome *c* peroxidases

References: a, Yonetani and Ray (1966); b, Soininen and Ellfolk (1972); c, Foote et al. (1983); d, Villalain et al. (1984); e, Den Ariaz et al. (1989); f, the present study. Abbreviations: Y-cyt. *c*, baker's yeast cytochrome *c*; *c*-550, *c*-551 and *c*-552, cytochromes *c*-550, *c*-551 and *c*-552 respectively; HHC, horse heart cytochrome *c*.

Organism	Electron donor (concn.)	Catalytic-centre activity (s^{-1})	K_m (μM)	Reference
Saccharomyces cerevisiae	Y-cyt.*c* (satd.)	14000	25	a
Pseudomonas aeruginosa	*c*-551 (satd.)	710	91	b
	c-551 (12 μM)	90	–	c
Pseudomonas stutzeri	*c*-551 (16 μM)	0.38	–	d
Pseudomonas perfectomarinas (22 kDa proteolytic fragment of cyt. *c*-552)	*c*-551 (16 μM)	30	–	e
Paracoccus denitrificans	HHC (satd.)	1040	3.25	f
	c-550 (satd.)	1400	13	f

centration of the cytochrome *c*-550 is approx. 100 μM, which implies that the enzyme can work near $V_{max.}$ if the steady-state level of reduction of the cytochrome *c*-550 is high and H_2O_2 is present. However, such arguments are complicated by the fact that competitors in the use of cytochrome *c*-550 may exist in the periplasm and that the electron transfers may be localized on the cell membrane rather than dispersed through the periplasmic volume.

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