The peroxidase activity of cytochrome c-550 from Paracoccus versutus

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The peroxidase activity of cytochrome *c*-550 from *Paracoccus versutus*

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Next to their natural electron transport capacities, *c*-type cytochromes possess low peroxidase and cytochrome P-450 activities in the presence of hydrogen peroxide. These catalytic properties, in combination with their structural robustness and covalently bound cofactor make cytochromes *c* potentially useful peroxidase mimics. This study reports on the peroxidase activity of cytochrome *c*-550 from *Paracoccus versutus* and the loss of this activity in presence of H_2O_2. The rate-determining step in the peroxidase reaction of cytochrome *c*-550 is the formation of a reactive intermediate, following binding of peroxide to the haem iron. The reaction rate is very low compared to horseradish peroxidase (approximately one millionth), because of the poor accessibility of the haem iron for H_2O_2, and the lack of a base catalyst such as the distal His of the peroxidases. This is corroborated by the linear dependence of the reaction rate on the peroxide concentration up to at least 1 M H_2O_2. Steady-state conversion of a reducing substrate, guaiacol, is preceded by an activation phase, which is ascribed to the build-up of amino-acid radicals on the protein. The inactivation kinetics in the absence of reducing substrate are mono-exponential and shown to be concurrent with haem degradation up to 25 mM H_2O_2 (pH 8.0). At still higher peroxide concentrations, inactivation kinetics are biphasic, as a result of a remarkable protective effect of H_2O_2, involving the formation of superoxide and ferrocytochrome *c*-550.

Keywords: cytochrome *c*; peroxidase; protein radicals; haem; oxidation.

Peroxidases are haem containing enzymes that efficiently catalyse substrate oxidations using hydrogen peroxide [1,2]. Peroxidases can function as catalysts in a variety of oxidation reactions on a broad spectrum of substrates and their potential use is therefore considerable. This is more so because they utilize the 'clean' oxidant H_2O_2 [2]. Unfortunately, peroxidases are prone to inactivation during normal turnover. This inherent instability is poorly understood and it is important to investigate the mechanism of inactivation because it is currently the main restriction to commercial application of peroxidases and peroxidase mimics [2–4].

Peroxidase activity is inherent to many haem-proteins besides peroxidases. It has been detected in, e.g. haemoglobins and myoglobins, cytochrome *c* and microperoxidases [5–10]. The latter are small peptides derived from extensive proteolysis of cytochrome *c*, which have contained a covalently bound haem moiety [8,11]. In some cases protein modification has resulted in enhanced activity [12–14]. Understanding the peroxidase properties of *c*-type cytochromes is particularly interesting, because these are very stable proteins that remain highly soluble even under conditions of extreme heat, acidity and basicity. Importantly, the covalent linkage, via thioether bonds, of their haem prosthetic group to the protein matrix prevents dissociation of the catalytic moiety from the protein. These properties render cytochromes *c* excellent candidates to be used as peroxidase mimics.

We therefore set out to understand the peroxidase activity and inactivation kinetics of cytochrome *c*-550 from *Paracoccus versutus*. Such a study is a prerequisite to ultimately improve these properties in cytochrome *c*-550 by utilizing protein engineering and other means. Eventually, such studies might direct the way to how to suppress the H_2O_2-driven inactivation that currently hampers the application of peroxidases or peroxidase mimics as 'green chemistry catalysts'. Cytochrome *c*-550, which was selected for this study, is a member of the Class I cytochromes *c*, a class which contains also the archetypal mitochondrial cytochromes *c* and many other, bacterial cytochromes [15]. *P. versutus* cytochrome *c*-550 has a strong homology to the well-studied cytochrome c_2 from *Paracoccus denitrificans* [15–17]. *P. versutus* cytochrome *c*-550 has been studied extensively [16,18–22], and we could take advantage of an excellent heterologous expression system, allowing site-directed mutagenesis with high protein yields [22].

The present work shows that cytochrome *c*-550 has peroxidase activity. An activation phase precedes maximal rate of turnover. This activation involves an oxidative process centered on the protein. The catalytic characteristics of cytochrome *c*-550 are similar to those of the microperoxidases, although the reaction rate is more than 1000-fold slower. However, the rate of inactivation of cytochrome *c*-550 by H_2O_2 is also very low, and the protein stays active at very high peroxide concentrations. In the

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Abbreviations: ABTS, 2,2′-azinobis (3-ethylbenzo-6-thiazolinesulfonic acid); M100K cytochrome *c*-550, site-directed mutant of *P. versutus* cytochrome *c*-550 in which the iron co-ordinating methionine is replaced by lysine; K_m^{per}, apparent Michaelis constant for hydrogen peroxide; MP-8, microperoxidase-8.

Note: a website is available at http://wwwchem.leidenuniv.nl/metprot

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absence of reductants the inactivation of the protein obeys first-order kinetics, except at high concentrations of peroxide where the inactivation is biphasic.

EXPERIMENTAL PROCEDURES

Expression and purification of cytochrome *c*-550

Cytochrome *c*-550 was heterologously expressed in *P. denitrificans* strain 2131 containing the pEG400.Tv1 plasmid [22]. Typically, 3 L of culture was prepared for expression of cytochrome *c*-550. The growth medium was BHI (GibcoBRL), supplemented with 50 $\mu g \cdot mL^{-1}$ streptomycin. The cells were cultured in 2 L conical flasks (500 mL culture per flask) and shaken at 300 r.p.m. After 24 h of growth at 30 °C, the D_{660} was measured, and the cells were harvested by centrifugation at 11 300 ***g***. The cell pellet was resuspended to a calculated D_{660} of 200 in an ice-cold solution of 1 M sucrose, 1 mM EDTA, 50 mM Tris, pH 8.0 (Tes). Solid lysozyme (50 mg, Sigma) was added and the cell suspension was stirred for 20 min at room temperature and centrifuged. The cell pellet was resuspended and this procedure was repeated once. The pellet was resuspended again in ice-cold Milli Q water, using four times the volume used with Tes. After stirring for 20 min, the resultant protoplasts were centrifuged at 27 200 ***g***. The supernatants containing cytochrome *c*-550 were pooled. Further purification was performed as described [16]. The average yield of pure protein was 12 $mg \cdot L^{-1}$ culture. The protein was judged to be more than 95% pure by its UV-visible spectrum and by SDS/PAGE. The spectra of both ferrous and ferric cytochrome *c*-550 were identical to those previously published [18].

Activity assays

The assays were typically performed in 1.5 mL plastic cuvettes. The reactions were started by the addition of H_2O_2 (Merck). The H_2O_2 stock solution was freshly made and its concentration was verified spectrophotometrically ($\varepsilon_{240} = 39.4\ M^{-1} \cdot cm^{-1}$ [23]). The hydrogen donor was guaiacol (i.e. 2-methoxyphenol, Sigma), diluted from a fresh aqueous stock solution (100 mM). It was assumed that the coloured product, tetraguaiacol, is the result of four one-electron oxidations [24]. The formation of tetraguaiacol ($\varepsilon_{470} = 26.6\ mM^{-1} \cdot cm^{-1}$ [25]) was followed with a Shimadzu UVPC-200 spectrophotometer fitted with a thermostat. The reactions were performed at 25 °C in 100 mM sodium phosphate buffer, pH 8.0 (unless stated otherwise). In some cases, the reaction was monitored by using an Applied Photophysics SX.18 MV stopped-flow apparatus. Other reagents used were mannitol (Brocacef, the Netherlands), superoxide dismutase, horse heart cytochrome *c*, ABTS (all from Sigma), and M100K cytochrome *c*-550 (kindly provided by Mrs Ing. Gertrüd Warmerdam). In a number of experiments, directed at investigating the activation process preceding the steady-state reaction, a mixture was employed containing 10 mM H_2O_2 and 5 μM cytochrome *c*-550. After a fixed amount of time the H_2O_2 was removed by addition of ≈ 1200 units of buffer-equilibrated catalase attached to 4% cross-linked beaded agarose (Sigma) to 1 mL of reaction mixture. The catalase was removed after about 2 min by filtration (0.2 μm Acrodisc, Gelman Sciences). Activity was then measured by addition of H_2O_2/guaiacol and the UV/visible spectrum recorded. Controls were taken without H_2O_2, cytochrome *c*-550 or both, respectively.

Inactivation assays

Cytochrome *c*-550 was preincubated with H_2O_2 for a recorded time. The activity remaining after this time was measured by adding H_2O_2/guaiacol. The concentrations of reactants in the preincubation mix were varied, but in the end-mix the conditions were 10 mM guaiacol, and either 50 or 100 mM H_2O_2. The spectral changes of cytochrome *c*-550 upon addition of H_2O_2 in the absence of guaiacol were monitored by using the stopped-flow apparatus with a photodiode array (320–1000 nm).

RESULTS

Peroxidase activity

When cytochrome *c*-550, guaiacol and H_2O_2 were mixed together, the orange-coloured tetraguaiacol was formed. When cytochrome *c*-550 or H_2O_2 was left out, no formation of tetraguaiacol was observed. The rate of production of tetraguaiacol depends linearly on the cytochrome *c*-550 concentration (0.1–10 μM cytochrome *c*-550). When mannitol (100 mM) or when superoxide dismutase was added, no change in rate was observed. Therefore hydroxyl or superoxide radicals, respectively, are not involved in the reaction, thus excluding Haber–Weiss or Fenton chemistry. The activity was negligible after a vigorous cytochrome *c*-550 inactivation procedure, involving a long (> 60 min) preincubation with H_2O_2.

Although guaiacol can reduce ferricytochrome *c*-550, and thus is oxidized in the absence of H_2O_2, the peroxidase activity of cytochrome *c*-550 is not due to a 'pseudo-peroxidase' cycle where cytochrome *c*-550 cycles between its Fe^{2+} and Fe^{3+} oxidation states. This is concluded from the observation that, when the reaction is in steady state, ferrocytochrome *c*-550 is not observed, while the reaction between cytochrome *c*-550 and H_2O_2 is the rate-limiting step in the oxidation of guaiacol (as will be shown later). This means that ferric cytochrome *c*-550 and not ferrous cytochrome *c*-550 is the relevant reaction partner with H_2O_2. In addition, when ABTS was used as the reducing substrate, peroxidase activity is also observed (ABTS does not reduce ferricytochrome *c*-550). Finally, peroxidase activity was also observed for M100K cytochrome *c*-550. The redox potential of M100K cytochrome *c*-550 is 329 mV lower (298 K, pH 7.0) than the wild-type cytochrome *c*-550 value [20], and it is not reduced by guaiacol.

The peroxidase assay

A typical peroxidase assay with cytochrome *c*-550 and guaiacol gives a product formation curve with four phases, as depicted in Fig. 1. An initial activation phase (I in Fig. 1) is followed by a steady-state phase, represented by the straight part of the curve (II). Then the curve levels off (III) and ultimately a decrease in absorption is seen (IV). The last two features can be ascribed to the inherent instability of the reaction product (tetraguaiacol), in combination with

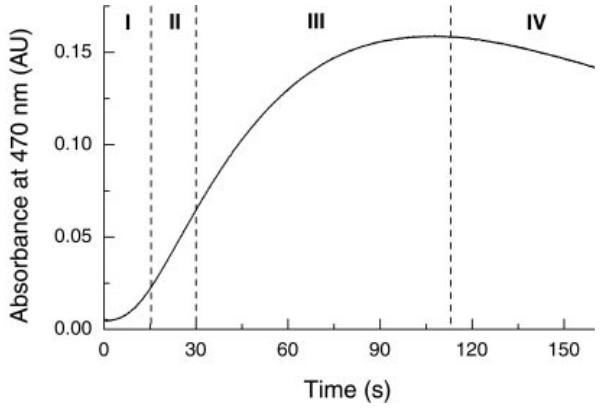

Fig. 1. A typical tetra-guaiacol formation curve. The four phases (I–IV) are described in the text. Conditions were 0.2 μM cytochrome *c*-550, 50 mM H_2O_2 and 20 mM guaiacol.

catalyst inactivation [7,25,26]. The rate of the steady-state reaction was determined by taking the maximum of the first derivative of the product formation curve. The length of the steady-state phase (II), as judged from the width of the first derivative maximum, depends strongly on conditions such as $[H_2O_2]$ and pH. Ideally, the length of the steady-state phase is > 10 s, but frequently it was shorter.

The activation phase

The presence of a lag period (phase I) suggests that cytochrome *c*-550 is activated by H_2O_2. The length of this activation phase was assessed in terms of an activation rate constant, k_{act}, which was obtained by fitting the first derivative of the product formation curve to a single exponential (Fig. 2A,B). The length of the activation phase depends on the concentration of H_2O_2 (Fig. 2A). The activation phase is also shortened by increased concentrations of cytochrome *c*-550 (Fig. 2B). When cytochrome *c*-550 is preincubated for 30 s with H_2O_2 before addition of guaiacol, the activation phase is absent (Fig. 3A). When an aliquot is removed at the end of the activation phase and transferred to a fresh mixture of substrates, no activation phase is seen. When the H_2O_2 is removed after a 30-s preincubation time by addition of agarose-immobilized catalase, the protein exhibits a diminished activation phase (Fig. 3B), and remains activated for at least 30 min. The UV/visible spectrum of the activated form is unchanged

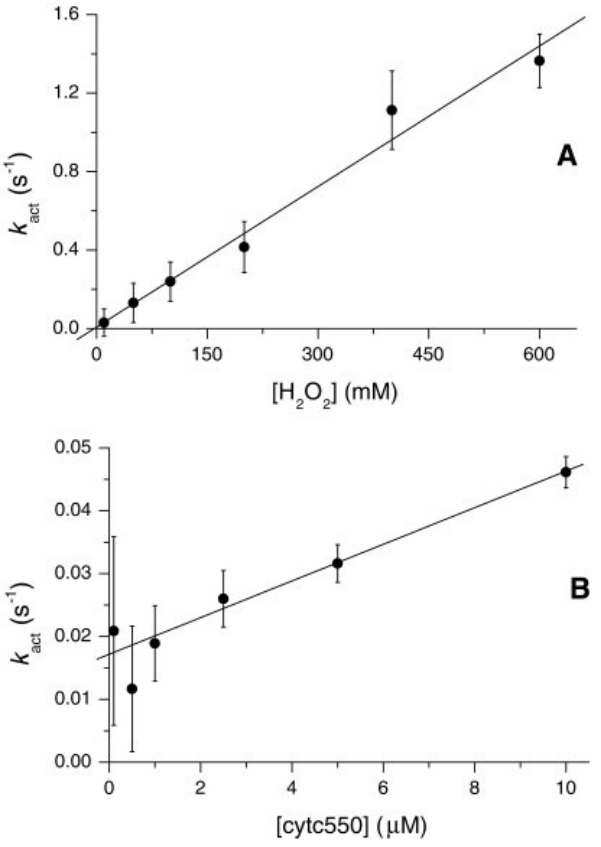

Fig. 2. Rate of activation of cytochrome *c*-550 as a function of (A) H_2O_2 and (B) cytochrome *c*-550. The value of the activation rate constant, k_{act}, was obtained by fitting the first derivative of the product formation curve to a single exponential. Conditions: (A) 25 mM guaiacol and 1 μM cytochrome *c*-550 and (B) 25 mM guaiacol 5 mM H_2O_2. cytc550, cytochrome *c*-550.

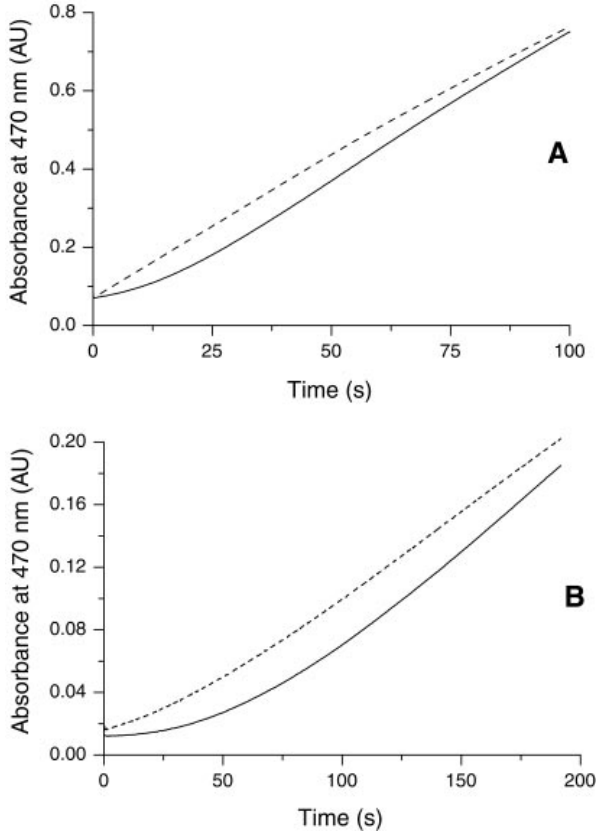

Fig. 3. Tetra-guaiacol formation without (solid line) and with (dotted line) a 30-s preincubation with 10 mM H_2O_2 (A) and tetra-guaiacol formation after a 30-s preincubation in absence of H_2O_2 (solid line) and in the presence of 10 mM H_2O_2 (dotted line) after which agarose-immobilized catalase was added (B). (A) Activity was assayed at 10 mM H_2O_2, 10 mM guaiacol and < 4 μM cytochrome *c*-550. (B) Conditions of the assay after removal of the catalase were 5 mM H_2O_2, 25 mM guaiacol and < 1 μM cytochrome *c*-550.

with respect to native ferricytochrome *c*-550. The activation appears to be partly reversible (diminished length of the activation phase) and does not originate from changes in the haem-environment or iron co-ordination, because the UV-visible spectrum is normally sensitive to such changes. The peroxidase activity of M100K cytochrome *c*-550, in which the axial Met is replaced by Lys [20], exhibits an activation phase just like the wild-type cytochrome *c*-550, corroborating the conclusion that axial co-ordination plays no role in the activation process.

Dependence on guaiacol and hydrogen peroxide

When the concentration of guaiacol is between 15 and 30 mM, the rate of the steady-state peroxidase reaction (phase II in Fig. 1) of cytochrome *c*-550 depends linearly on the concentration of H_2O_2. This linear dependence holds even up to 1 M H_2O_2 (Fig. 4). The rate also depends linearly on the concentration of cytochrome *c*-550, and therefore the oxidation rate of guaiacol due to the peroxidase activity of cytochrome *c*-550 follows the bimolecular rate law [Eqn (1)]:

$$v = k_{obs}[\text{cytochrome } c\text{-}550][H_2O_2] \quad (1)$$

The value of the bimolecular rate constant k_{obs} is $43.4 \pm 0.8\ M^{-1}\cdot s^{-1}$ (25 mM guaiacol, 100 mM sodium phosphate, pH 8.0, 25 °C). The H_2O_2 dependence of the peroxidase activity of horse heart cytochrome *c* was also probed. Similar to cytochrome *c*-550, no saturation is seen up to 1 M H_2O_2 (Fig. 4). Its bimolecular rate constant k_{obs} is $4.1 \pm 0.1\ M^{-1}\cdot s^{-1}$ (25 mM guaiacol, 100 mM sodium phosphate, pH 8.0). The complete absence of saturation by H_2O_2 is contrary to earlier reports where K_m^{per} values varying from 25 to 65 mM have been reported for horse heart cytochrome *c* [27–30]. However, in our hands it was not possible to reproduce these results, and a linear dependence of the rate on the concentration of H_2O_2 was found with both guaiacol (Fig. 4) and ABTS as reducing substrates (not shown).

The guaiacol concentration has a limited effect on the rate, even down to 50 μM guaiacol. The steady-state

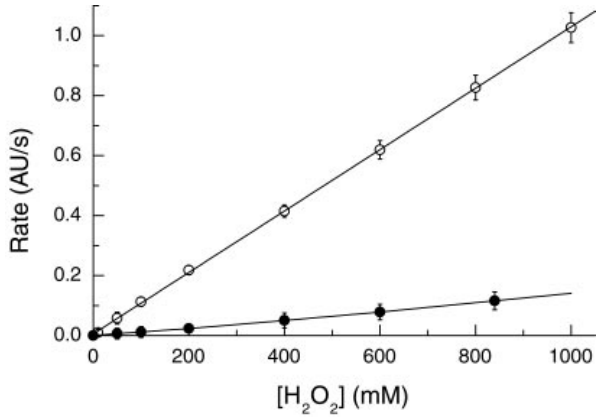

Fig. 4. Dependence of the steady-state peroxidase reaction rate as a function of H_2O_2 for cytochrome *c*-550 (○) and horse heart cytochrome *c* (●). Conditions: 5 μM cytochrome *c* and 25 mM guaiacol.

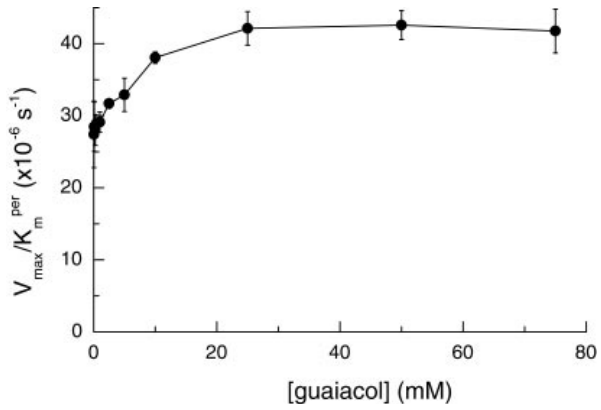

Fig. 5. Inverse slope (V_{max}/K_m^{per}) of plots of 1/rate vs. 1/[H_2O_2] as a function of [guaiacol]. [Cytochrome *c*-550] was 1 μM.

reaction rate is reduced however, by increasing concentrations of guaiacol when the H_2O_2 concentration is low. This can be attributed to substrate inhibition by guaiacol, and in fact serves as evidence for a ping-pong mechanism [31]. When the dependence of the steady-state reaction rate on [H_2O_2] is plotted in a double reciprocal plot ($1/v$ vs. 1/[H_2O_2]) at various guaiacol concentrations, parallel lines are seen above 15 mM guaiacol (not shown). Such parallel lines again are evidence for a ping-pong mechanism, in which the association with H_2O_2 is rate limiting [31]. The inverse slope of these parallel lines represents V_{max}/K_m^{per} in Michaelis–Menten formalism (which reduces to k_{obs}[cytochrome *c*-550] (Eqn 1) for $K_m^{per} \gg [H_2O_2]$). The thus calculated values of V_{max}/K_m^{per} are plotted against guaiacol concentration in Fig. 5. This shows that the value of V_{max}/K_m^{per}, or k_{obs}[cytochrome *c*-550], is constant above 15 mM guaiacol. The reduction of this value at lower guaiacol concentrations can be attributed to catalyst inactivation. When reducing substrate is lacking, inactivation can become more prevalent for peroxidases and peroxidase models [26,27,32]. This would pose no problem if true initial rates were measured, but in our assay initial rates can not be measured because cytochrome *c*-550 exhibits an activation phase in its peroxidase activity (see above). Thus the steady-state rate is measured after a certain time, and when the concentration of guaiacol is low, relatively more cytochrome *c*-550 is inactivated at the moment the rate is measured.

PH dependence of the peroxidase activity

As shown in Fig. 6, the peroxidase activity of cytochrome *c*-550 depends strongly on pH. The steady-state rate increases with increasing pH up to pH 10.0, after which a decrease is seen. Horse heart cytochrome *c* exhibits a stable rate value from pH 5.0–7.0, after which a decline in activity is seen (Fig. 6). This agrees well with earlier observations [27].

Inactivation of cytochrome *c*-550 by hydrogen peroxide

The rate of inactivation of cytochrome *c*-550 in the absence of guaiacol was determined as described in Experimental procedures. Two types of inactivation behaviour can be distinguished, depending on the concentration of H_2O_2.

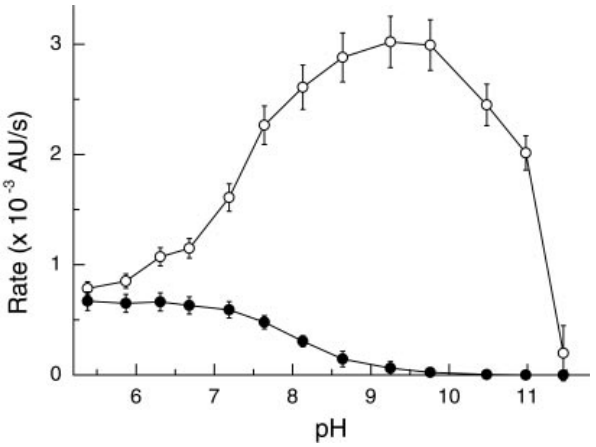

Fig. 6. Dependence of the steady-state peroxidase reaction rate as a function of pH for cytochrome *c*-550 (○) and horse heart cytochrome *c* (●). Conditions: 1 μM cytochrome *c*, 10 mM H_2O_2 and 25 mM guaiacol.

When preincubated with up to about 25 mM H_2O_2, the peroxidase activity of cytochrome *c*-550 decreases in time as a single exponential (Fig. 7, open circles). In addition, the UV-visible spectrum of cytochrome *c*-550 bleaches when exposed to up to 25 mM H_2O_2. The loss of the Soret band intensity exactly matches the decrease in activity (Fig. 7, solid line), and both can be fitted to the same single exponential function (not shown). The inactivation rate does not depend on the concentration of cytochrome *c*-550 between 0.5 and 5 μM. Up to 25 mM H_2O_2, the value of the exponential rate constant of inactivation (k_i) depends linearly on the H_2O_2 concentration. Note that with short preincubation times, the activity is somewhat increased. During this short preincubation, inactivation has relatively little effect and nearly all protein is activated at the start of the activity assay (no activation phase). In the absence of a preincubation, the protein is being activated during the activation phase but simultaneously the activated form is reduced by the substrate. In the latter case, not all the protein will become activated, but rather an equilibrium is established. This is why the activity seems to increase slightly at short incubation times.

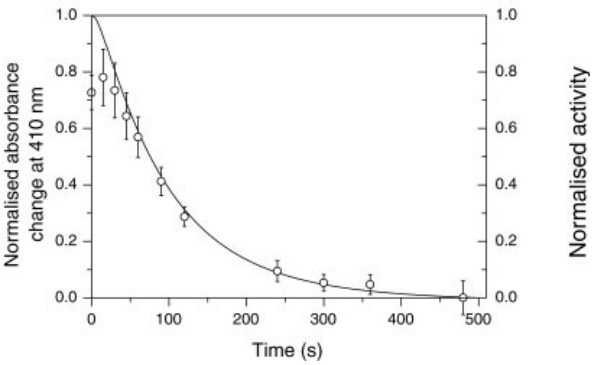

Fig. 7. The normalized absorption change at 410 nm in the presence of 20 mM H_2O_2 as a function of time (solid line) and the normalized steady-state peroxidase reaction rate of 0.5 μM cytochrome *c* as a function of preincubation time with 20 mM H_2O_2 (open circles). Normalization of the peroxidase reaction rate data was performed by taking the amplitude of a first-order exponential fit of the data into account. Activity was assayed with 10 mM H_2O_2, 10 mM guaiacol and <0.25 μM cytochrome *c*-550.

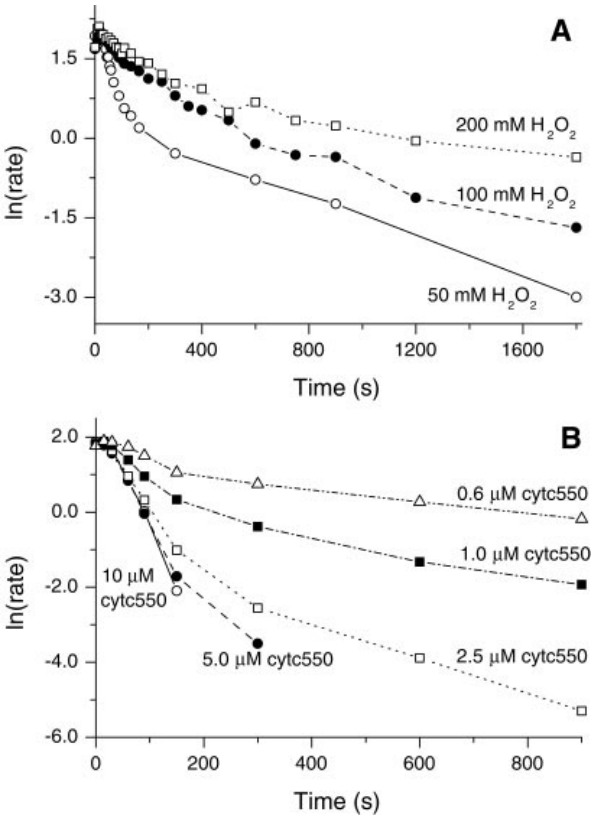

Fig. 8. Rate of the steady-state peroxidase reaction as a function of the preincubation time with H_2O_2. In (A), [cytochrome *c*-550] was 1 μM in the preincubation mix and [H_2O_2] was varied (○, 50 mM; ●, 100 mM; □, 200 (mM). In B, [H_2O_2] was 50 mM in the preincubation mix and [cytochrome *c*-550] was varied (○, 10 μM; ●, 5 μM; □, 2.5 μM; ■, 1 μM; △, 0.6 μM). Activity was assayed in 10 mM guaiacol, 50 mM H_2O_2, and <0.5 μM cytochrome *c*-550. cytc550, cytochrome *c*-550.

Above 50 mM H_2O_2, the inactivation behavior is markedly changed; the activity decreases in a biphasic fashion. A fast exponential phase is followed by a slower exponential phase (Fig. 8A,B). At even higher H_2O_2 concentrations, the second phase is reached sooner, and the rate constants of both phases are lower (Fig. 8A). The same effect is seen when the concentration of cytochrome *c*-550 is lowered in the premix (Fig. 8B). So, paradoxically, the more peroxide and the less protein present, the higher the residual activity is after a certain period of incubation. Control measurements in the presence of mannitol show that hydroxyl radicals hardly affect the inactivation. However, when superoxide dismutase is added the overall inactivation is increased (Fig. 9A). Thus, superoxide is formed when cytochrome *c*-550 is incubated with high [H_2O_2]. Superoxide is a potent reductant of cytochrome *c*-550 [33] and may be responsible for the formation of ferrocytochrome *c*-550. By using fast

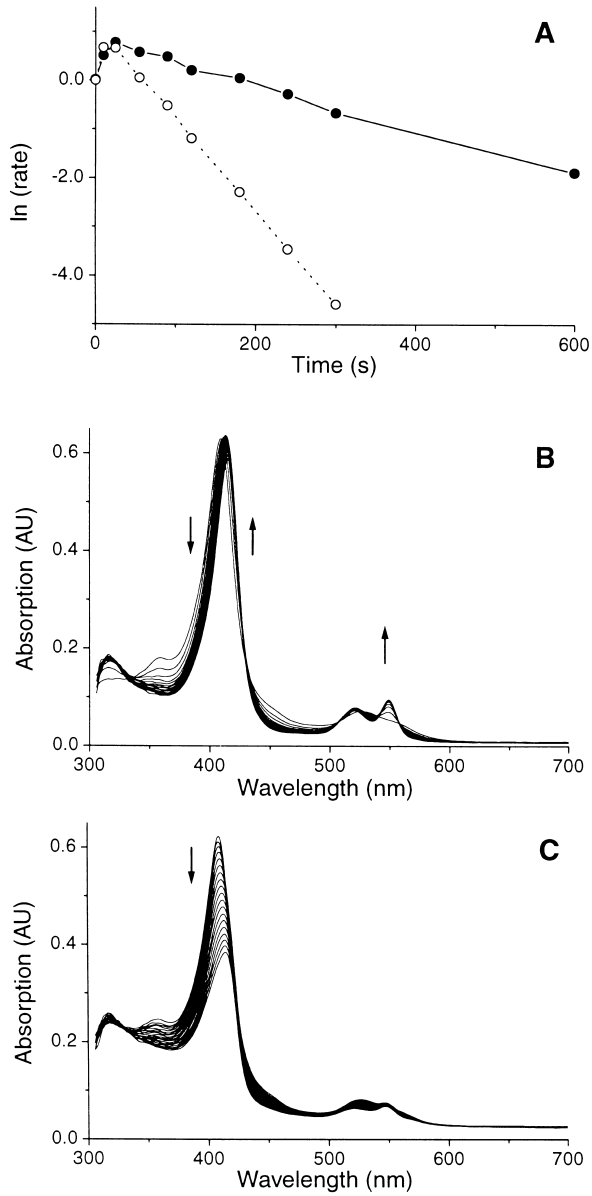

Fig. 9. The effect of superoxide dismutase on the inactivation kinetics and redox state of cytochrome *c*-550 in presence of a large excess of H_2O_2. (A) Rate of the steady-state peroxidase reaction vs. preincubation time with 100 mM H_2O_2 in the presence (○) and absence (●) of superoxide dismutase. [Cytochrome *c*-550] was 5 μM in the preincubation mix, and activity was assayed in 10 mM guaiacol, 50 mM H_2O_2, and < 2.5 μM cytochrome *c*-550. (B) Time dependence of the UV-visible spectrum of cytochrome *c*-550 (5 μM) in 100 mM H_2O_2. The reaction was monitored over 20 s and spectra are shown with a 1-s interval. The arrows highlight the conversion of ferricytochrome *c*-550 (409 nm) into ferrocytochrome *c*-550 (414 and 550 nm). (C) As in B, but in presence of superoxide dismutase. Note the limited conversion to ferrocytochrome *c*-550 and the relatively fast bleaching.

mixing in combination with a diode array, this was shown to be the case. The fraction of ferrocytochrome *c*-550 and its rate of formation both increase with higher [H_2O_2], and addition of superoxide dismutase reduces the fraction of ferrocytochrome *c*-550 formed (Fig. 9B,C).

DISCUSSION

Cytochrome *c*-550 has peroxidase activity

In presence of H_2O_2, cytochrome *c*-550 displays peroxidase activity. This is not surprising, because peroxidase activity has previously been detected for cytochromes *c* [7,27,29], and other nonperoxidase haem proteins (leghaemoglobin, haemoglobin and myoglobin) [5,6,34,35]. For peroxidase activity the H_2O_2 needs to be activated by binding to the haem iron [27,36]. Whereas for the haem iron in peroxidases and the above-mentioned oxygen-binding proteins, the sixth ligand is weak or absent, the iron in cytochrome *c*-550 is hexa-coordinated. Thus, to allow binding of H_2O_2, one of the ligands needs to be displaced. Dissociation of the axial His is not very probable because it is held in place by a hydrogen bond to Pro37, and because of its proximity to Cys12 and Cys15, which covalently link the haem to the protein. On the other hand, the ease with which exogenous ligands are known to displace the axial Met, makes the latter a likely candidate for displacement by an incoming H_2O_2 molecule [37–40].

The active species in the peroxidase reaction of cytochrome *c*-550

Once the peroxide is bound to the haem iron its O–O bond may be cleaved in a homolytic or a heterolytic fashion. Either pathway will produce the oxidizing equivalents needed to convert the reducing substrate (guaiacol). The former route produces hydroxyl radicals and other reactive oxygen species, which are capable of very fast and indiscriminate oxidation reactions. In the case of cytochrome *c*-550 radical scavengers do not influence the rate of the reaction, and thus the homolytic pathway appears not to be relevant. Heterolytic cleavage leads to a high-valent oxyferryl species, which is readily observed in peroxidases and myoglobins [1,6]. However, we were unable to confirm its occurrence spectroscopically in cytochrome *c*-550 and its presence in this protein is thus tentative.

The fact that no iron peroxo and/or oxyferryl species is observed with cytochrome *c*-550 indicates that these species are too low in concentration in the steady state to be observed. This is corroborated for the expected iron peroxo species by the observed lack of saturation of the rate by H_2O_2 (Fig. 4). In a variety of peroxidases and also in myoglobin and leghaemoglobin, the protein after heterolytic O–O bond cleavage contains one oxidizing equivalent on the oxyferryl moiety, while the other is present as an amino-acid radical [10,35,41–45]. In the case of horse heart cytochrome *c*, it has been shown that multiple protein radicals can result from the reaction with peroxides [36,46,47]. The lack of an easily observable oxyferryl species in cytochrome *c*-550 might be caused by a fast reduction of this species by an aromatic amino acid in the vicinity of the haem, as already suggested by Barr *et al*. for horse heart cytochrome *c* [36]. A similar mechanism was proposed for the autoreduction of oxyferryl myoglobin [48,49]. A second reaction with H_2O_2 may even take place, when the protein is capable of carrying more than two oxidizing equivalents. This was suggested for horse heart cytochrome *c* [36,46] and also for leghaemoglobin [50].

As in natural peroxidases, the reducing substrate might be expected to react with the (high-valent) haem. However, considering that protein radicals are so easily found in peroxidase mimics such as myoglobin and cytochrome *c* (see above), it is possible that guaiacol is oxidized via one of these radicals. Unstabilized protein radicals can be transferred easily and rapidly between different sites on a protein [51–54], and thus also to the two surface exposed tyrosines of cytochrome *c*-550 [16,17], which might be involved in guaiacol oxidation. Studies on the myoglobin/H_2O_2 catalysed oxygenation and oxidation reactions provide evidence that a protein radical is indeed the true oxidizing species in such types of reactions [54–61].

The rate-determining step in the peroxidase reaction of cytochrome *c*-550

The rate-determining step in the peroxidase activity of cytochrome *c*-550 is the formation of a high-potential intermediate. This rate-determining step is characterized by ping-pong kinetics with a high apparent Michaelis constant for H_2O_2, allowing a description of the rate in terms of a bimolecular rate-law (Eqn 1). In this respect cytochrome *c*-550 is similar to microperoxidase-8 (MP-8) [24], even though the bimolecular rate constant k_{obs} is approximately 1000-fold larger for MP-8 than for cytochrome *c*-550 [24] (this work). The latter observation seems to reflect the larger accessibility of the haem iron in MP-8 (which is fully exposed to the solvent) [8,37–39], compatible with the observed lower K_m^{per} for MP-8 [62].

The pH dependence of the peroxidase activity of cytochrome *c*-550 and horse heart cytochrome *c*

Cytochrome *c*-550 also resembles MP-8 in the pH dependence of its peroxidase activity. The pH dependence on the activity of horse heart cytochrome *c* and cytochrome *c*-550 is shown in Fig. 6. The curve of the latter compares well with that of MP-8: a steady increase in activity with pH leading to a maximum, and a drop at even higher pH [9,24]. Metal-replacement studies showed that the pH dependence of MP-8 could be explained by assuming that the pK_a of H_2O_2 is lowered by the metal [9]. The drop in rate with MP-8 at more alkaline pH has been attributed to product instability and ligation of hydroxide to the iron [8,9,24], although the latter has been disputed [63]. A similar reasoning can be used to explain the pH dependence of the rate of cytochrome *c*-550: its increase with pH is related to the pK_a of bound H_2O_2. The drop in activity above pH 10.0 coincides well with the so-called alkaline transition for cytochrome *c*-550 (pK_a 11.2 [19]), which involves exchange of the Met ligand for a Lys [64,65]. It is to be expected that the activity decreases by such a transition, because Lys is a much stronger ligand for iron than Met and thus is replaced much less easily by the incoming peroxide. The combined and counteracting effects when the pH is increased can thus account for the pH profile of the activity of cytochrome *c*-550 (Fig. 6). This could also be the case with the pH dependence on the activity of horse heart cytochrome *c* (also shown in Fig. 6), although the apparent pK_a observed in the activity profile for this protein is slightly lower than its alkaline transition determined by alternative methods (pK_a 8.9–9.5 [64]). It is important to note that, as with MP-8 [9], the protein matrix of cytochrome *c*-550 does not participate in lowering the pK_a of H_2O_2. Such a function has been shown to be the basis for the high reaction rates for the natural peroxidases [1].

The activation phase in the peroxidase reaction of cytochrome *c*-550

The steady-state oxidation of guaiacol by cytochrome *c*-550/H_2O_2 is preceded by an activation phase, meaning that the protein is activated in the course of the activity assay. As discussed in the Results section, this is due to a process that does not involve changes in the haem environment or iron co-ordination. Increasing the concentration of H_2O_2 accelerates this process, indicating an oxidative mechanism. In principle, diffusion of H_2O_2 into the protein and binding to the iron could be the reason for the activation phase. However, the activation occurs within seconds but the protein remains activated for at least 30 min after removal of all H_2O_2, suggesting that peroxide diffusion and binding cannot explain the activation phase.

It was pointed out earlier that protein radicals can accumulate on cytochrome *c* when peroxides are present. Moreover, it was suggested that protein radicals might be the true reaction partners with guaiacol. The activation process may thus represent a build up of protein radicals on the cytochrome *c*-550 molecule. Cytochrome *c*-550 contains three tyrosines and two tryptophans, the most common sites of protein radicals [54,66]. Under assay conditions, they thus compete with guaiacol for oxidizing equivalents. A number of studies have shown that, for natural peroxidases, when two substrates are present at the same time, one is preferably oxidized, and the second substrate may remain unreacted until the first substrate is completely consumed [67]. Such a competition might also be the case here between guaiacol and the aromatic residues: once the aromatic residues are 'consumed', oxidation of guaiacol proceeds via these radicals or via the (high-valent) haem.

Attempts to find support for the presence of protein radicals by EPR detection, as applied to horse heart cytochrome *c* [36], failed. Addition of 5- to 10-fold molar excess of H_2O_2 to cytochrome *c*-550 leads to reduction of the protein, which is probably related to the high protein concentration (100–500 μM) used in these experiments, necessary for EPR detection.

An alternative explanation for the observed activation phase may be that the oxidation of one of the aromatic amino acids results in structural changes, which facilitate the peroxidase reaction. In the case of F41W horseradish peroxidase, which also exhibits an activation phase, oxidation of Trp41 was suggested to be responsible for such a mechanism [68].

Inactivation of the peroxidase activity of cytochrome *c*-550 by hydrogen peroxide

As mentioned in the Results section, up to 25 mM H_2O_2 the inactivation of the peroxidase activity follows monoexponential kinetics and the inactivation rate depends linearly on the concentration of H_2O_2. The rate of inactivation does not depend on the concentration of cytochrome *c*-550. The decrease in activity occurs simultaneously with

bleaching of the UV/visible spectrum (see Fig. 7). Bleaching is common when peroxides are added to haem (proteins), and is ascribed to an oxidative attack at the haem periphery, causing ring opening, loss of iron and further degradation [69–72]. The initial degradation step might be an intramolecular attack on the haem by an activated oxygen species such as oxyferryl [73] or iron-peroxo, as seen with haem oxygenase [74]. In any case, the bleaching is an intramolecular process, in accord with the observation that it is independent of the concentration of cytochrome *c*-550. Formation of the activated oxygen species depends linearly on the concentration of H_2O_2. Therefore it is expected that the inactivation rate also depends linearly on the concentration of H_2O_2, and as mentioned above, this agrees with the observation that up to 25 mM H_2O_2, the rate of inactivation equals $k_i[H_2O_2]$ with $k_i = 0.51\ M^{-1}{\cdot}s^{-1}$.

At higher peroxide concentrations (> 50 mM H_2O_2) the inactivation characteristics of cytochrome *c*-550 are very different. The higher the concentration of H_2O_2, and the lower the concentration of cytochrome *c*-550, the slower the inactivation. Apparently H_2O_2 can protect the protein when in excess. It is interesting to see that in excess H_2O_2, ferrous cytochrome *c*-550 is readily formed (cf [75]), but that when superoxide dismutase is present this is much less (Fig. 9B,C). The latter indicates that superoxide is involved in this process. Superoxide is a potent reductant of cytochrome *c* [33], and the observation that superoxide dismutase inhibits the formation of ferrocytochrome *c*-550 suggests that $O_2^{\bullet-}$ is formed at high concentrations H_2O_2, and that this leads to the formation of ferrous cytochrome *c*-550. The appearance of ferrous cytochrome *c*-550 at excess H_2O_2 is important, because ferrous cytochrome *c* is less susceptible to H_2O_2-driven inactivation [76] or protects against it. It can thus be expected that when more cytochrome *c*-550 is in the ferrous state, the inactivation rate is less. Interesting in this respect is, that ferric cytochrome *c* catalyses its own formation from ferrous cytochrome *c* in the presence of H_2O_2 [76], which explains why cytochrome *c*-550 remains active longer when less protein is present (Fig. 8B).

CONCLUSION

Despite being a classic electron transport protein, cytochrome *c*-550 clearly possesses peroxidase activity. This activity is characterized by a poor association with H_2O_2. The protein matrix of cytochrome *c*-550 does not assist the reaction by lowering the pK_a of H_2O_2 unlike the natural peroxidases. Thus the effective concentration of oxidant is much lower for cytochrome *c*-550 than for natural peroxidases, which, in part, is responsible for the relatively low activity of cytochrome *c*-550 versus, for example, horseradish peroxidase. The activity is further lowered by limited accessibility of the haem-iron, as evidenced by the 1000-fold higher peroxidase activity of the highly accessible microperoxidases. Therefore, in order to create an artificial peroxidase out of cytochrome *c*-550, it is necessary to address the following two points: (a) the lack of a properly positioned general base catalyst to deprotonate the incoming H_2O_2 and (b) the low accessibility of the active site to H_2O_2. These topics are currently under investigation.

Interestingly, the studies on the inactivation kinetics of cytochrome *c*-550 at high and low H_2O_2 concentrations unveiled a complex accumulation of side-reactions in the absence of reducing substrate. Paradoxically, higher peroxide concentrations and lower protein concentrations increasingly protect the haem from degradation. This relative stability of cytochrome *c*-550 under such harsh conditions is remarkable, and this observation helps to identify the major determinants for peroxide-driven inactivation of haem enzymes.

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