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Ascorbate peroxidase activity of cytochrome *c*

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Abstract

The peroxidase-type reactivity of cytochrome *c* is proposed to play a role in free radical production and/or apoptosis. This study describes cytochrome *c* catalysis of peroxide consumption by ascorbate. Under conditions where the sixth coordination position at the cytochrome *c* heme iron becomes more accessible for exogenous ligands (by carboxymethylation, cardiolipin addition or by partial denaturation with guanidinium hydrochloride) this peroxidase activity is enhanced. A reaction intermediate is detected by stopped-flow UV-vis spectroscopy upon reaction of guanidine-treated cytochrome *c* with peroxide, which resembles the spectrum of globin Compound II species and is thus proposed to be a ferryl species. The ability of physiological levels of ascorbate (10–60 μM) to interact with this species may have implications for mechanisms of cell signalling or damage that are based on cytochrome *c*/peroxide interactions.

Keywords: *Cytochrome c, peroxide, ascorbate*

Introduction

Beyond its essential role in the electron transport chain, cytochrome *c* is known to be involved in apoptosis, where its release from the mitochondria is a key step [1]. Free radical reactivity and in particular interaction with peroxides has been shown for cytochrome *c* [2] and is proposed to be relevant for apoptosis [1]. However, UV-vis spectra collected upon treatment of ferric cytochrome *c* with peroxide have shown no evidence for a change in oxidation state [3], although at higher concentrations (3 mM) heme bleaching can be observed [3,4]. While this latter observation has been explained as due to the six-coordinated structure at the heme iron preventing efficient peroxide binding, it has also been shown that the sixth ligand, a methionine, can transiently dissociate under various conditions, including changes in pH or temperature, so that binding of peroxide to the heme iron is conceivable [4,5].

Indeed a range of studies have reported on peroxidase activity by cytochrome *c*. Free cytochrome *c* can induce lipid peroxidation [2], whilst immobilized cytochrome *c* has been shown to catalyse peroxide reduction by ABTS or by remazol blue, presumably via a mechanism involving peroxide binding to iron [4]. Site-directed mutagenesis has been employed to stabilize cytochrome *c* towards peroxide inactivation, enabling the detection of a protein-based radical [4]; a signal in these EPR spectra was also tentatively assigned to a Compound I species, although UV-vis evidence for Compound I or for its typically more stable cognate, Compound II, was not found [4,6–8]. Most recently lipids, especially cardiolipin, have been shown to modulate the peroxide reactivity of cytochrome *c* [9], at least in part by displacement of the methionine ligand.

Haemoglobin and myoglobin are similar low activity peroxidases. We recently characterized kinetically

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the ascorbate peroxidase enzymatic activity for these globins and demonstrated a remarkably high affinity for ascorbate [10]; subsequently, a stable haemoglobin–ascorbate adduct was directly observed with NMR spectroscopy [11]. The physiological implications of these data were discussed in light of the fact that ascorbate is present in plasma and red blood cells at concentrations exceeding its apparent K_m for haemoglobin and that the peroxide–haemoglobin interaction occurs in normal human blood and is exacerbated in certain pathological conditions [10,11].

Peroxidases have the ability to oxidize a wide range of substrates. These can include the normal antioxidant defenses of the cell. Glutathione, ascorbate and NADH were shown to affect the cytochrome *c* catalysed peroxidation of 2,7-dichlorofluorescin [8,12]. We therefore decided to characterize the ascorbate peroxidase activity of cytochrome *c*. We demonstrated a high affinity activity, evidence for a mechanism involving peroxide binding to the iron and detection of a reaction intermediate proposed to be a species akin to the classical Compound II ferryl intermediate.

Materials and methods

Carboxymethylated cytochrome *c* was a kind gift from Professor M. T. Wilson (University of Essex). Cytochrome *c* from horse heart and yeast (Sigma-Aldrich, Munich, Germany) were used as received without further purification. Bovine haemoglobin was purified from bovine blood as previously described [13]. Where needed, guanidinium hydrochloride was added to reaction mixtures from 6 M stock solutions.

UV-vis spectra were recorded on Agilent 8453 (Agilent, Inc., Foster City, CA, USA) and Cary 50 (Varian, Inc., Foster City, CA, USA) instruments. Stopped-flow spectra were collected on a Biologic SFM-300 system equipped with three syringes and capable of sequential mixing, with a high-speed diode array detector. Stopped-flow data were analysed within the SPECFIT32 software package (BioLogic system, Claix, France) using Singular Value Decomposition (SVD) and global multi-exponential fitting of the SVD treated data, with the spectra fitted to simple kinetic models using Levenberg-Marquardt or Simplex algorithms.

Ascorbate peroxidase assays were conducted following protocols previously described [10]. Thus, the reaction was monitored in 3-mL quartz cuvettes monitoring the UV absorbance at 290 nm, where all absorbance changes are due to ascorbate, with an extinction coefficient of 2000 $cm^{-1}M^{-1}$. The order of reagent addition to the reaction mixture was buffer, then ascorbate, then peroxide, then cytochrome *c*—at concentrations indicated in Figure legends; in the absence of cytochrome *c* the background ascorbate consumption was very small (f any) and the numbers reported in the Figures are obtained taking this into account (background ascorbate consumption was always subtracted before preparing Michaelis-Menten plots). Plots showing measurements of reaction rates as a function of substrate concentrations were fit to a simple Michaelis-Menten equation, using a least-squares procedure and the Solver module within the Microsoft Excel software package.

For EPR spectra, a Bruker EMX Micro spectrometer with a liquid nitrogen cooling system was employed. Instrument conditions: microwave frequency 9.43 GHz, microwave power 15.89 mW, modulation frequency 100 kHz, modulation amplitude 3 G, sweep rate 22.6 G/s; time constant 81.92 ms, average of three sweeps for each spectrum, temperature 100 K.

Results and discussion

Figure 1 shows that native cytochrome *c* (cytc) from yeast and from horse heart both catalyse ascorbate oxidation by peroxide. The k_{cat} values of 3 and 3.7 min^{-1}, respectively, are of similar magnitude to that measured for haemoglobin under similar conditions [10]. On the other hand, the peroxide K_m of cytc, also illustrated in Figure 1, appears much weaker than the ~ 1 mM previously estimated for haemoglobin [10].

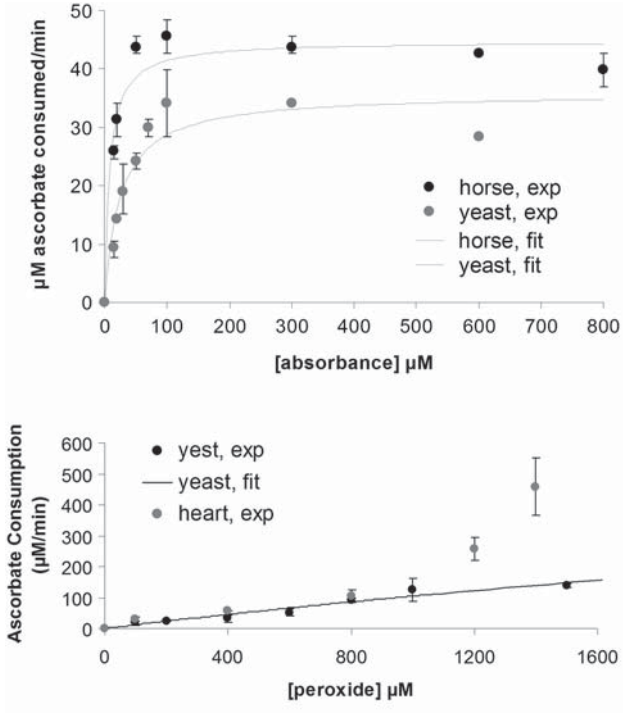

Figure 1. Top panel: dependence on ascorbate concentration for the ascorbate peroxidase activity of yeast and horse heart ferric cytochrome *c*. Conditions: room temperature, 50 mM acetate, pH 5, 800 µM H_2O_2, 12 µM cytc. Bottom panel: dependence on peroxide concentration for the ascorbate peroxidase activity of yeast and horse heart ferric cytochrome *c*. Conditions: room temperature, 50 mM acetate, pH 5, 350 µM ascorbate, 12 µM cytc. The curves show fits of experimental data to Michaelis-Menten kinetics.

The tentative fit shown in Figure 1 for peroxide may be taken to indicate only that the K_m is >> 5 mM, with the upper limit of its value remaining unknown. Therefore the concentration dependence of ascorbate activity could not be carried out under conditions where the system is saturated in peroxide. Nevertheless the estimated K_m values for ascorbate are 8 μM for horse heart cytc and 25 μM for yeast cytc—remarkably low and reminiscent in this way of the values measured for haemoglobin under similar conditions [10].

As the sixth coordination position on iron is occupied in native cytochrome *c* but is liberated upon carboxymethylation of the sixth ligand, methionine 80, it would be expected that carboxymethylated cytochrome *c* would exhibit distinctly stronger peroxidase activity. Indeed, this modified cytochrome *c* did display an increase in peroxidase activity (as measured at pH 5, 350 μM ascorbate, 800 μM peroxide), although the effect was rather modest (1.7-fold increase). Cardiolpin binding is also able to displace the methionine 80 ligand. At pH 5 the complex precipitates, so it was not possible to do a direct comparison using the conditions of Figure 1. However, at pH 7.4 there is a significant enhancement of ascorbate peroxidase activity when cardiolipin binds to cytochrome *c* (Supplementary Figure S1).

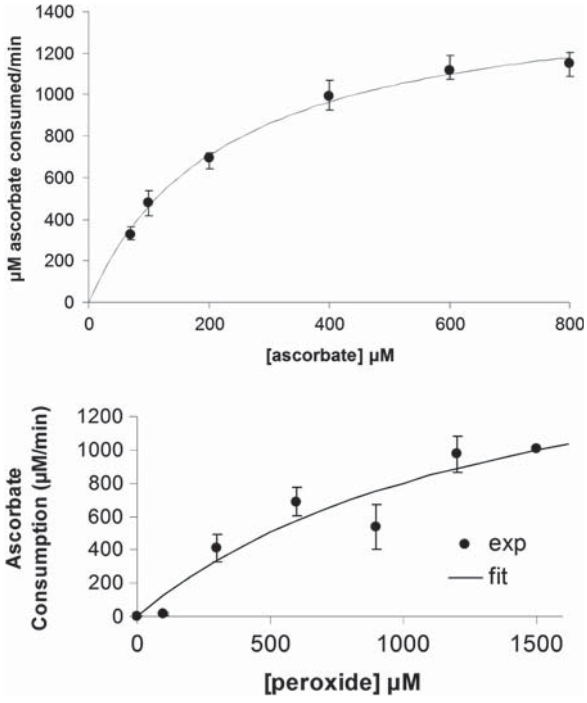

Figure 2. Top panel: dependence on ascorbate concentration for the ascorbate peroxidase activity of horse heart ferric cytochrome *c*. Conditions: room temperature, 50 mM acetate, pH 5, 800 μM H_2O_2, 4 μM cytc, 5 M guanidinium hydrochloride. Bottom panel: dependence on peroxide concentration for the ascorbate peroxidase activity of horse heart ferric cytochrome *c*. Conditions: room temperature, 50 mM acetate, pH 5, 350 μM ascorbate, 12 μM cytc. The curves show fits of experimental data to Michaelis-Menten kinetics.

Guanidinium hydrochloride treatment also takes the cytochrome towards a penta-coordinated high-spin form akin to microperoxidases [14–16]—as witnessed by an increase in Soret band (Supplementary Figure S2). This is in contrast to the situation with heme *b* proteins, such as haemoglobin, where guanidinium hydrochloride induces complete denaturation within the range of concentrations employed here, manifested by a marked widening and decrease in intensity of the Soret band. The difference between Hb and cytc can be traced back to the fact that in cytc *c* the proximal histidine ligand is connected to the edge of the *c* heme covalently by a short peptide stretch, imparting more structural stability. The possibility to attain penta-coordination at the cytochrome *c* heme using denaturing agents and the physical properties of the polypeptide under these conditions were characterized extensively elsewhere [17–24]. Circular dichroism data confirm that 5M is sufficient for the conformational change to be complete (Supplementary Figure S3).

Figure 2 shows that guanidinium hydrochloride treatment of cytc leads to a strong increase in peroxidase reactivity, manifested in larger k_{cat} (380 min^{-1}, which is ~ 100-times larger than for the native protein). An increase in the apparent ascorbate K_m is seen (to 230 μM), which may suggest that the binding site is at least partially disturbed by guanidine treatment. The affinity for peroxide appears to be larger than in the native protein and a K_m may be computed, at 1.4 mM; this is in line with the UV-vis spectra suggesting that guanidine treatment improves access of

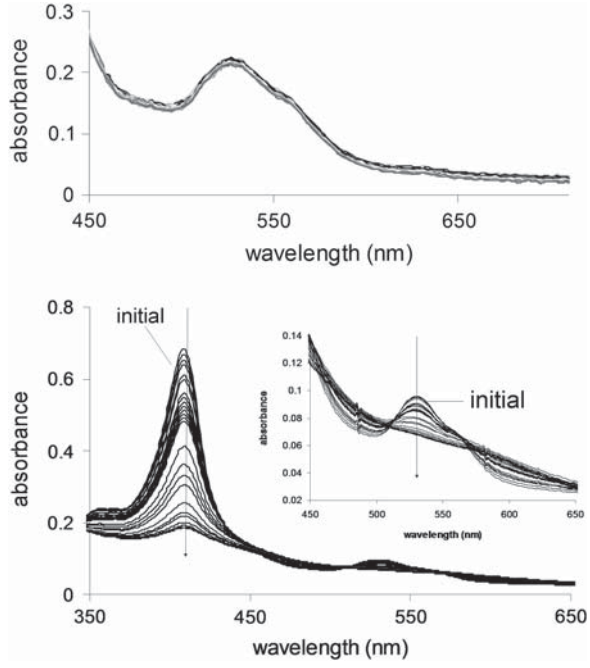

Figure 3. UV-vis spectra of ferric cytochrome *c* in the absence and presence (upper and lower panels, respectively) of 5 M guanidinium hydrochloride following addition of 5 mM hydrogen peroxide.

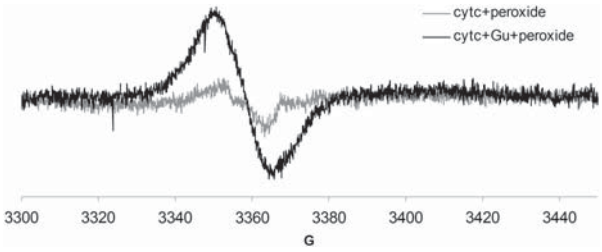

Figure 4. EPR spectra recorded at 100 K for ferric cytochrome *c* (400 μM) mixed with hydrogen peroxide (1 mM) in 50 mM acetate pH 5, in the presence or absence of 5 M guanidium hydrochloride; samples were frozen in liquid nitrogen 30 s after mixing.

exogenous ligands (water, peroxide) to the sixth coordination position of the heme.

In agreement with previous data [4], hydrogen peroxide treatment of native cytochrome *c* does not induce any changes in the UV-vis spectrum (Figure 3, upper panel). On the other hand, hydrogen peroxide does efficiently bleach the spectrum of the high-spin form of cytochrome *c* generated upon guanidine hydrochloride treatment (Figure 3, lower panel), further suggesting a peroxidase mechanism wherein peroxide binds to the iron to generate highly-reactive intermediates. Such intermediates typically involve Compound I (where the iron is formally Fe(IV), coupled to a cation radical located on the porphyrin), Compound II (similar to compound II but lacking the cation radical) and protein-located free radicals generated by oxidative decay of Compounds I and II [25]. Evidence for such intermediates is shown in Figure 4: electron paramagnetic resonance spectra of hydrogen peroxide-treated cytochrome *c* in plain buffer yield only very small amounts of free radical, while at guanidine concentrations identical to those of the UV-vis experiments of Figures 2 and 3, the amount of free radical is decidedly larger. When such free radicals are formed in ferric haemoproteins in the presence of hydrogen peroxide, the only mechanism that can be invoked is the peroxidatic one, where the ferric heme reacts with peroxide to form a Compound I, whose decay via two successive one-electron reduction processes leads to protein-located free radicals [25].

Although no high-valent iron intermediate was directly detectable here in manual-mixing experiments with cytc and peroxide, spectra collected on a stopped-flow instrument within 1 s after mixing the two reagents (Figure 5A) suggest a short-lived intermediate. Indeed, the time course of the absorbances was reasonably fit with a minimal reaction scheme A→B→C (cf. Figure 5B), where A is the starting ferric species, B is (as detailed below) a Compound II-like species and C is proposed to be a ferric species different from A.

The computed spectrum (cf. Figure 5C) for species A resembles the ferric form of cytochrome *c*, the spectrum of the short-lived species B resembles that of the

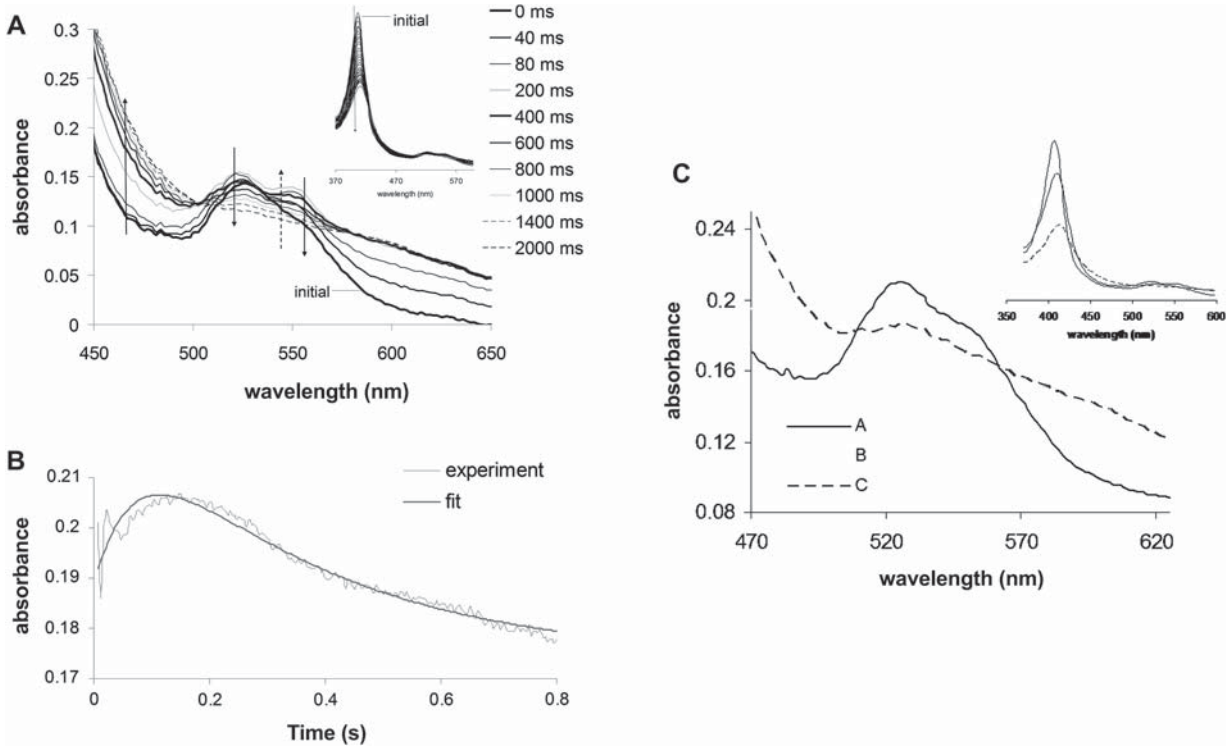

Figure 5. (A) UV-vis spectra collected within 2 s after mixing of 18 μM ferric cytochrome *c* with 5 mM hydrogen peroxide, in 5 M guanidinium hydrochloride; times of spectra collection are indicated; arrows illustrate trends in absorbance (dashed arrow indicates initial rise in absorbance, followed by a decrease at longer reaction times). (B) Fit to an A→B→C reaction scheme (showing the 523 nm trace), with k_1 = 13.9 s^{-1} and k_2 = 2.3 s^{-1}. (C) Computed spectra for the fit shown in (B).

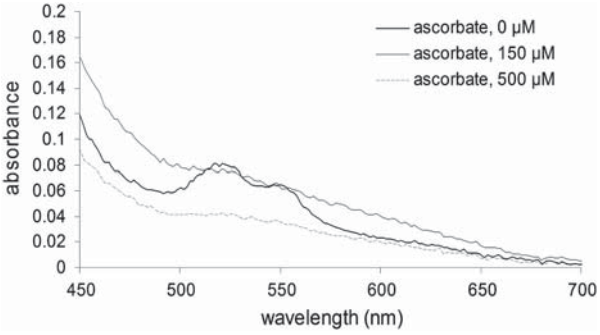

Figure 6. The first UV-vis spectra collected after stopped-flow mixing of species B (cf. Figure 5) with ascorbate, at concentrations indicated. Species B was prepared in the stopped-flow instrument as shown in Figure 5; at 150 ms after its formation, it was mixed with a solution of ascorbate in 5 M guanidine (ascorbate concentrations after mixing are indicated in the Figure).

Compound II (ferryl) form of other haemoproteins, such as globins [26] and species C represents an altered (probably damaged) ferric form of the protein. Experience with most other heme proteins indicates that if a reaction intermediate is to be detected upon mixing with peroxide, Compound II would be the most likely candidate, as it is more stable than Compound I (formally Fe(V)) or Compound 0 (formally ferric-hydroperoxo) [26–29]. Moreover, peroxide treatment of microperoxidase 8 (MP8), a heme-octapeptide adduct obtained by proteolysis of cytochrome *c* retaining the proximal histidine as ligand, have also shown evidence for a Compound II-type of species whose kinetic properties suggested a Fe(III)-peroxide adduct as a required reaction intermediate *en route* to the ferryl [15].

Preliminary data indicate that the B→C process in our stopped-flow experiment is drastically accelerated by ascorbate, further supporting the concept of B being a high-valent form akin to the ferryl seen in globins and other haemoproteins; thus, in sequential stopped-flow experiments where cytochrome *c* was mixed with peroxide in guanidine to generate species B, which was then in turn mixed with ascorbic acid solution (150–2000 mM), species B was found to already disappear during mixing (Figure 6), in a manner similar to those previously reported for globin ferryl species [26].

In this brief communication we have demonstrated that, like haemoglobin and myoglobin, cytochrome *c* exhibits a genuine catalytic ascorbate peroxidase activity, with a similar ferryl intermediate. The ability of physiological levels of ascorbate to reduce this intermediate makes it unlikely that under normal cellular conditions peroxide interactions with cytochrome *c* create significant levels of free radicals. Modifications of cytochrome *c* by lipids such as cardiolipin must take this ascorbate reactivity into account if they are to exert their proposed physiological signalling roles [8,9,12,30–32]. Two mechanisms seem possible. One is a structural alteration limiting ascorbate accessibility akin to what we show following guanidinium hydrochloride treatment. Alternatively the modified cytochrome *c* may react with lipid peroxides in cellular environments that are ascorbate-inaccessible.

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Declaration of interest

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Supplementary material available online

Figures S1 to S3.

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