A cytochrome c variant resistant to heme degradation by hydrogen peroxide

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Background: Cytochrome c has peroxidase-like activity and can catalyze the oxidation of a variety of organic substrates, including aromatic, organosulfur and lipid compounds. Like peroxidases, cytochrome c is inactivated by hydrogen peroxide. During this inactivation the heme prosthetic group is destroyed.

Results: Variants of the *iso*-1-cytochrome c were constructed by site-directed mutagenesis and were found to be more stable in the presence of hydrogen peroxide than the wild type. No heme destruction was detected in a triple variant (Tyr67→Phe/Asn52→Ile/Cys102→Thr) with the catalytic hydrogen peroxide concentration of 1 mM, even following the loss of catalytic activity, whereas both double variants Tyr67→Phe/Cys102→Thr and Asn52→Ile/Cys102→Thr showed a greater rate of peroxide-induced heme destruction than observed with the wild-type protein.

Conclusions: Heme destruction and catalytic inactivation are two independent processes. An internal water molecule (Wat166) is shown to be important in the heme destruction process. The absence of a protein radical in the resistant variant suggests that the protein radical is necessary in the heme destruction process, but presumably is not involved in the reactions leading up to the protein inactivation.

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Introduction

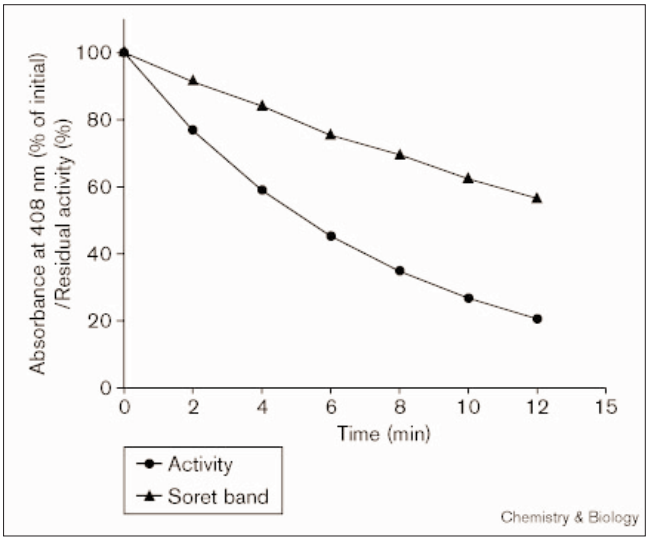
Cytochromes c, which are involved in the electron transport system, can catalyze peroxidase-like reactions *in vitro* [1]. As observed for peroxidases, the heme prosthetic group of cytochrome c is activated by an electron acceptor, such as hydrogen peroxide or an organic hydroperoxide. No catalytic activity of cytochrome c has been described in living systems. More than 45 years ago, however, the ability of cytochrome c to induce lipid peroxidation, as well as to promote hydroperoxide cleavage, was reported [2]. Cytochrome c has subsequently been reported to catalyze the hydroxylation of 4-nitrophenol [3], the oxidation of 2-keto-4-thiomethyl butyric acid [4], the oxidation of (2-2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) diammonium salt), and the oxidation of 4-aminoantipyrine [5]. Cytochrome-P450-like oxidative reactions with free and immobilized cytochrome c (e.g., *N*- and *O*-demethylation, *S*-oxidation and epoxidation of olefins) are performed in the presence of hydrogen peroxide or organic hydroperoxides [6]. These activities have led to the use of cytochrome c as a biocatalyst in the oxidation of thiophenes and organosulfides to form sulfoxides [7,8] and aromatic compounds [9,10].

As with horseradish peroxidase [11], lignin peroxidase [12], manganese peroxidase [13], lactoperoxidase [14], and other peroxidases, cytochrome c is inactivated by an excess of hydrogen peroxide or organic hydroperoxides in the absence of reducing substrate [8,15]. The substrate inactivation of peroxidases leads to the modification of the heme prosthetic group and probably to the formation of a verdohemoprotein as a final product [16]. The inactivation mechanism of peroxidases, however, has not yet been clearly elucidated. Recently we have reported that variants of *iso*-1-cytochrome c have differing stability in the presence of hydrogen peroxide when compared with the wild-type protein [1,10]. We are currently using site-directed mutagenesis to study the inactivation mechanism of cytochrome c and for the rational design of a new and more stable biocatalyst. In this study, we report a triple variant in which the heme prosthetic group is not degraded by and catalytic concentrations of hydrogen peroxide. The differentiation of the inactivation and the heme destruction processes in cytochrome c exposed to peroxide are discussed.

Results

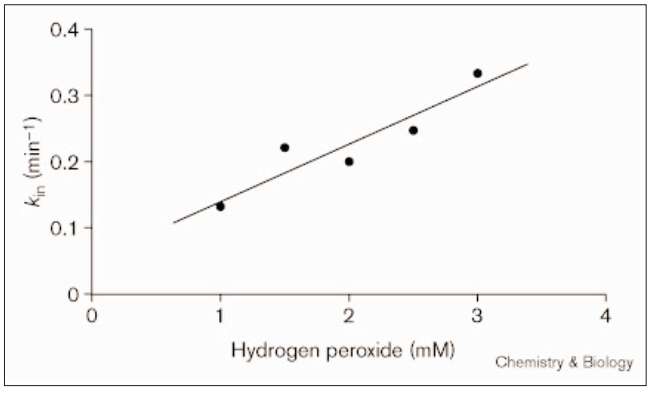
During catalytic turnover, *iso*-1-cytochrome c is inactivated by hydrogen peroxide. The stability of cytochrome c was determined by incubating the protein with 1 mM H_2O_2, which is the concentration of peroxide used in the biocatalytic activity assay (see the Materials and methods section). After 10 minutes, the cytochrome lost 75% of its activity (Figure 1), with an inactivation rate constant (k_{in}) of 0.132 min^{-1} determined from fitting the data to a first-order kinetic model. Comparing the inactivation constants

Figure 1



Inactivation and heme destruction of *iso*-1-cytochrome c in the presence of 1 mM H_2O_2.

Figure 2



The effect of hydrogen peroxide concentration on the rate constant for inactivation of *iso*-1-cytochrome c.

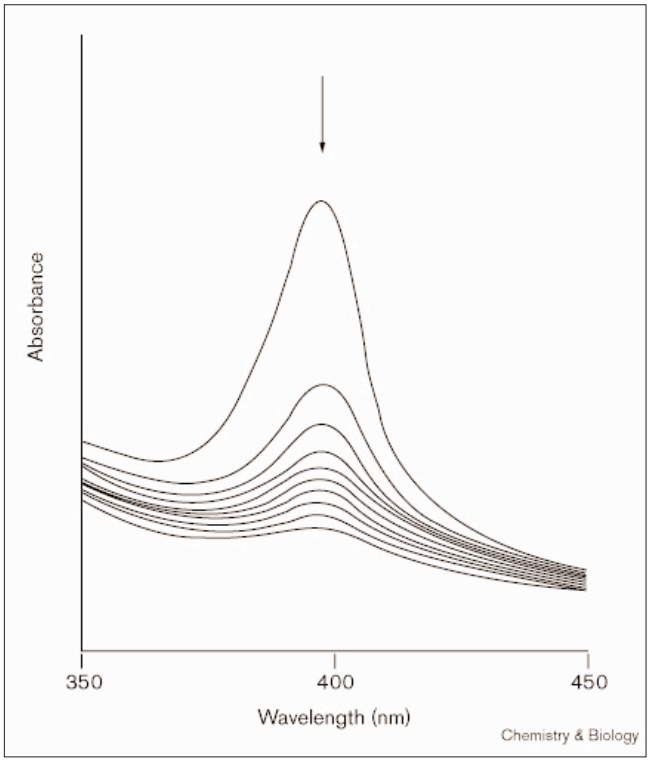
at 1 mM H_2O_2 showed that yeast cytochrome c is less stable than peroxidases such as chloroperoxidase from *Caldariomyces fumago* (k_{in} = 0.006 min^{-1}), horseradish peroxidase (k_{in} = 0.021 min^{-1}), lactoperoxidase (k_{in} = 0.034 min^{-1}) and lignin peroxidase (k_{in} = 0.111 min^{-1}), and manganese peroxidase (k_{in} = 0.082 min^{-1}) from *Phanerochaete chrysosporium*. The cytochrome c inactivation constant increases with increasing hydrogen peroxide concentration, as is observed for the peroxidases (Figure 2).

During the inactivation process, the heme prosthetic group is degraded, as indicated by the diminished absorbance in the Soret region (Figure 3). Both heme destruction and the loss of catalytic activity exhibit first-order kinetics, but the rate constants for the two processes differ (Figure 1). After eight minutes of incubation with hydrogen peroxide, the cytochrome lost 64% of its activity, whereas only 30% of the heme has been destroyed, according the Soret band (Figure 1). The inactivation process therefore appears to precede the heme destruction process. To corroborate the heme destruction results, the rate of iron release was measured using atomic absorption spectroscopy. After 10 minutes incubation in the presence of 1 mM H_2O_2, 63% of iron present in the protein is released and can be detected as protein-free iron in the supernatant solution, after protein precipitation. No soluble iron was detected in control samples incubated without hydrogen peroxide and extracted in the same manner as the experimental samples.

Amino acid analyses were carried out before and after 8 minutes incubation with hydrogen peroxide to determine whether proteic moiety also undergoes modification during inactivation. Some changes in the amino acid composition were found after incubation (Figure 4). All five tyrosine residues and three cysteine residues disappeared after the 8 minute treatment. The amino acid composition of untreated protein, however, showed that only four tyrosines and only one cysteine could be detected because the other two cysteines are covalently bonded to heme. In addition, one methionine and one lysine appear to be transformed. No significant changes could be detected in the amounts of the other amino acids following inactivation of the cytochrome with hydrogen peroxide.

From these results, it appears that modification of the protein structure could be the origin of the inactivation. In the presence of hydrogen peroxide, heme proteins are known to form protein radicals located primarily on tyrosine residues [17]. Tyrosine radical formation is involved in the peroxide-based dimerization [18,19] of myoglobin and in the formation of a covalent bond between apo-myoglobin and the heme prosthetic group [20]. For horse heart cytochrome c, it has been suggested that initial oxidation of the heme by hydrogen peroxide results in a ferryl (Fe^{IV} = O) species. The radical might then undergo an intramolecular transfer to a tyrosyl residue [21]. To explore the involvement of tyrosine free radicals following reaction of cytochrome c with peroxide, a variant of *iso*-1-cytochrome c devoid of tyrosine (Y–) was prepared using the polymerase chain reaction (PCR) with various amplifications steps. After purification, the stability of the multiple variant in the presence of 1 mM H_2O_2 was assayed, and a k_{in} of the variant was 0.1139 min^{-1}— a value very similar to that observed for the wild-type protein (k_{in} = 0.132 min^{-1}). Because our main interest in cytochrome biocatalysis is polycyclic aromatic hydrocarbon oxidation [1,10], we have tested the different variants for pyrene oxidation. The variant that did not contain tyrosine also showed reduced specific activity for pyrene oxidation (1.45 min^{-1}), relative to that of the wild-type protein (2.8 min^{-1}).

Figure 3



Heme prosthetic group destruction as indicated by disappearance of the Soret band in the presence of 1 mM H_2O_2. Spectra were recorded every 2 minutes, and the arrow indicates the direction of change.

Eight yeast cytochrome variants were assayed for stability at varying hydrogen peroxide concentrations (Table 1). The double variants Phe82→Ala/Cys102→Thr (F82A/C102T), Lys72→Ala/Cys102→Thr (K72A/C102T), and Lys79→Ala/Cys102→Thr (K79A/C102T) were less stable than the wild-type cytochrome in the presence of 1 mM H_2O_2, whereas the Lys73→Ala/Cys102→Thr (K73A/C102T), Lys87→Ala/Cys102→Thr (K87A/C102T) and Asn52→Ile/C102T (N52I/C102T) variants were significantly more stable. In the case of the Tyr67→Phe/C102T (Y67F/C102T) variant, stability in the presence of 1 mM H_2O_2 is similar to that of the wild-type protein. Nevertheless, this variant is less sensitive to increased hydrogen peroxide concentration (Table 1). All these variants were catalytically active, and they showed specific activities for the pyrene oxidation of 1.3–3.3 min^{-1}, whereas the specific activity of the wild-type cytochrome was of 2.8 min^{-1}. In our approach, stability was prioritized over activity and thus we have selected the variants on the basis of stability, although higher activity was achieved in some of the cytochrome variants.

A variant was produced that contained two new substitutions, each of which individually stabilizes cytochrome c: Asn52→Ile (decreased k_{in} at 1 mM H_2O_2) and Tyr67→Phe (decreased response to increased hydrogen peroxide concentration). The resulting triple variant, N52I/Y67F/C102T, had a slightly lower stability than the wild-type protein (Table 2). It is significant, however, that the heme group of this variant was highly resistant to heme destruction when

Figure 4

Amino acid composition of *iso*-1-cytochrome c before and after incubation for 8 minutes in 1 mM H_2O_2.

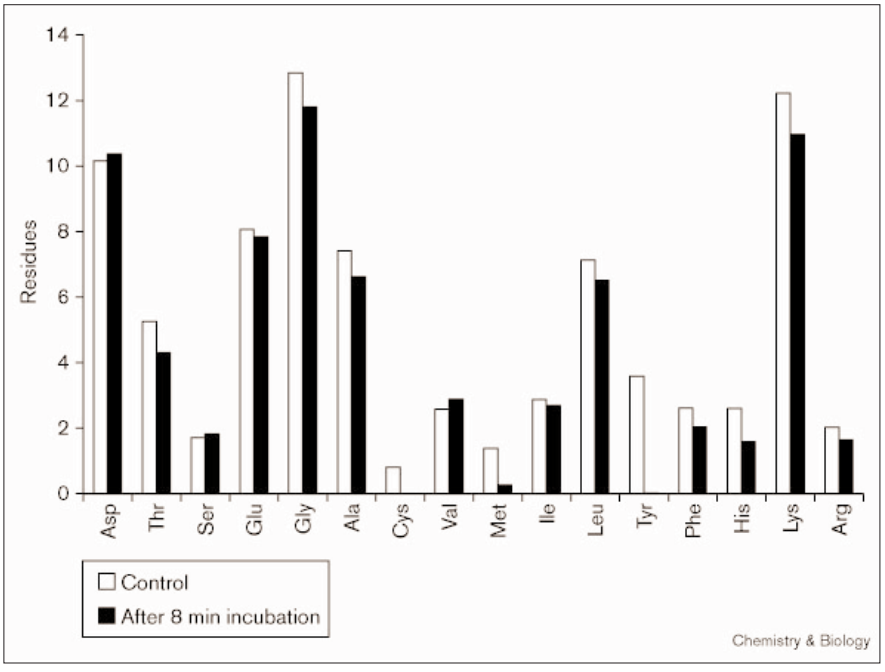


Table 1

Inactivation constants of *iso*-1-cytochrome c variants in the presence of different concentrations of hydrogen peroxide.

		k_{in} (min^{-1}) in the presence of hydrogen peroxide		
Variant	Specific activity (min^{-1})*	1 mM	2 mM	3 mM
N52I/C102T	1.9	0.060	0.095	0.110
K73A/C102T	2.0	0.068	0.167	0.192
K87A/C102T	3.3	0.084	0.085	0.186
Wild type	2.8	0.132	0.200	0.333
Y67F/C102T	1.2	0.149	0.166	0.183
K72A/C102T	1.3	0.216	0.370	0.445
K79A/C102T	1.7	0.228	0.464	ND
F82A/C102T	ND	0.347	0.482	1.036

*Specific activity for pyrene oxidation. ND, not determined.

incubated in 1 mM H_2O_2 (Figure 5). After a 22 minute incubation with 1 mM H_2O_2, the intensity of the Soret band had decreased <10% for this variant, whereas under the same conditions, the wild-type protein showed a 90% decrease in the Soret band absorbance. Interestingly, the turnover of the variant N52I/Y67F/C102T for pinacyanol oxidation was 6.47 (±0.37) times higher than the native protein in hydrogen peroxide concentrations of 1–5 mM. Among the *iso*-1-cytochrome c variants studied here, no correlation could be found between the rate constants for inactivation (Table 1) and heme destruction (Figure 5).

It has been demonstrated that cytochrome c can produce free radicals after reacting with hydrogen peroxide [21,22], and it is very likely that these radicals are involved in the inactivation process of this protein. To test this possibility we performed electron spin resonance (ESR) spectroscopy experiments at a low temperature (4°) using the same reaction conditions as we used before to see whether we were able to identify any radical. As shown in Figure 6, a radical was produced in the reaction of wild-type cytochrome c, but not in the case of the triple variant, even under various protein concentrations and incubation times.

The signal obtained for the wild-type protein has a g value of 2.006 and a line width of 13.6 G. This signal is highly temperature dependent as can be seen in Figure 6 (inset). The intensity suffers a significant decrease after changing the temperature from 4° to 40°; however, the signal intensity is recovered after taking the temperature back to 4° (data not shown). To establish the saturation profile of the signal we obtained a saturation curve from 10 μW to 80 mW (data not shown) The signal was saturated at 1 mW. To establish the identity and number of species involved, we tried to obtain an hyperfine structure of the radical by using very low modulation amplitude and adjusting the other parameters but we could not distinguish any hyperfine coupling at a modulation as low as 0.7 G.

Table 2

Inactivation constants (k_{in}) of wild-type and variant N52I/Y67F/C102T forms of *iso*-cytochrome c at different hydrogen peroxide concentrations.

	k_{in} (min^{-1})	
H_2O_2 (mM)	Wild type	N52I/Y67F/C102T
1.0	0.13	0.18
1.5	0.20	0.30
2.0	0.22	0.36
2.5	0.25	0.38
3.0	0.33	0.41

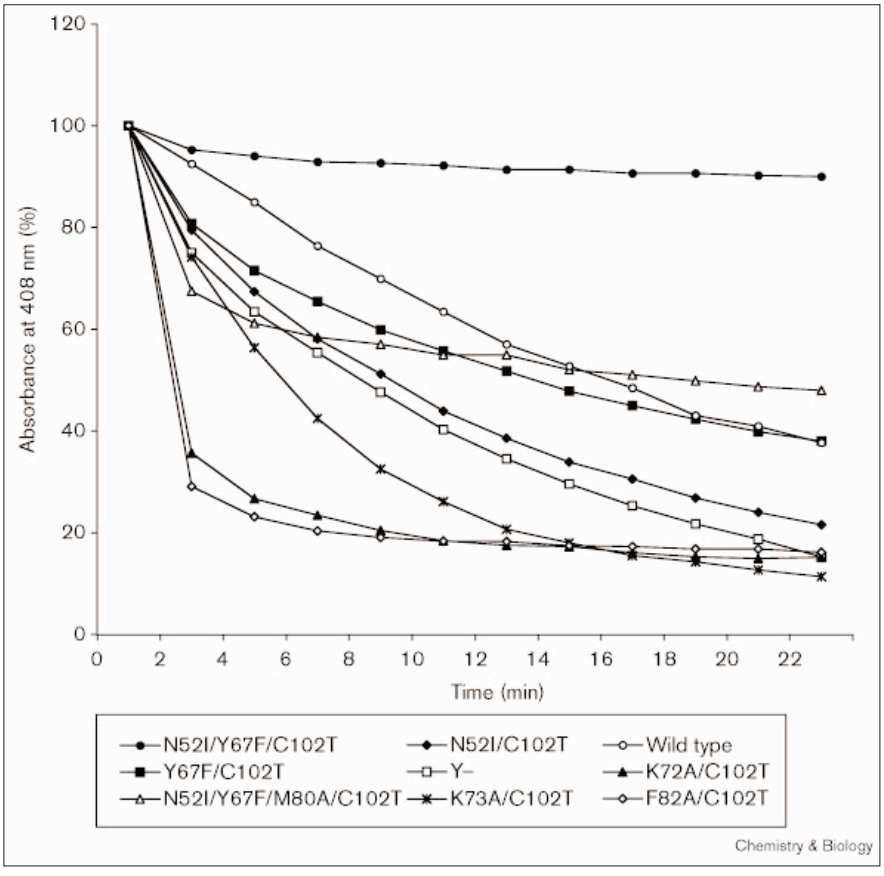
Discussion

Cytochrome c can catalyze several reactions and could be considered a biocatalyst [1]. Particular attention has been paid to ability of this protein to catalyze the oxidation of aromatic substrates, including polycyclic aromatic hydrocarbons [9,10]. The catalytic mechanism for free radical production by cytochrome c in the presence of organic peroxides has been studied [22], and peroxyl and alkoxyl radicals have been detected using EPR and spin trapping techniques [23]. By increasing the trapping agent concentration, Barr and Manson [22] demonstrated that the alkoxyl radical is formed initially. As for peroxidases, an initial activation of cytochrome c by hydrogen peroxide mediates the conversion of the protein to a catalytically more active, oxoheme complex [5,21]. Superoxide anion radical ($O_2^{-\bullet}$) and hydroxyl radical ($HO^{\bullet}$) do not appear to be involved in this catalytic activity because adding superoxide dismutase or mannitol does not inhibit the reaction [5].

This study was undertaken to improve the stability of cytochrome c during catalytic turnover, using site-directed mutagenesis. Over 20 years ago, Brown [24] analyzed the biliverdin isomers produced by the hydrogen peroxide oxidation of various hemoproteins and suggested that the amino acid residues adjacent to the methylene bridges of the porphyrin ring might protect these sites against hydrogen peroxide oxidation. As shown in Figures 1 and 2,

Figure 5

Absorbance decrease of the Soret band (408 nm) of cytochrome c variants in the presence of 1 mM H_2O_2. Y– is a protein devoid of tyrosine which was prepared by PCR with various amplifications steps.

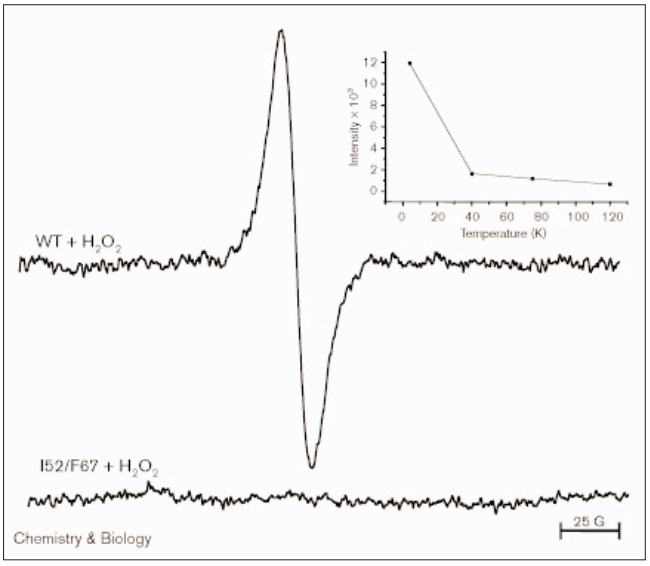


cytochrome c is inactivated by treatment with hydrogen peroxide, and the heme prosthetic group is degraded. In the case of peroxidases, hydrogen peroxide inactivation leads to the modification of the heme prosthetic group to form a verdohaemoprotein as a final product [12]. Two principal mechanisms have been proposed for the inactivation of peroxidases by hydrogen peroxide. One mechanism involves the reaction of Compound II with hydrogen peroxide in the absence of a reducing substrate to form Compound III. If Compound III is a peroxyiron(III)porphyrin free radical, it should be considered a highly reactive intermediate. Because of the proximity of the uncoupled electron to the porphyrin ring, any electron transfer from the ferrous state to an extra hydrogen peroxide moiety would generate a hydroxyl radical, which could in turn react with the heme group to produce irreversible inactivation. An alternative mechanism involves a reaction of Compound I with an excess of hydrogen peroxide in the absence of reducing substrate to form an irreversibly inactivated verdohaemoprotein. In this mechanism, Compound III is proposed to be a superoxide-anion-generating system that has a protective effect against inactivation. The inactivation mechanism for peroxidases, however, has not been clearly elucidated at present [11,12,14, 25–29].

Tyrosine residues of *iso*-1-cytochrome c are modified in the presence of hydrogen peroxide (Figure 4). Tyrosine free-radicals are involved in the dimerization of cytochrome in the presence of hydrogen peroxide [20], or they could participate in an intramolecular modification, as in the case of the myoglobin [18,19]. In addition, tyrosine radicals generated by hydrogen peroxide near the porphyrin ring might react to form a covalent bond with the prosthetic group [18,19,30]. Nevertheless, these results demonstrate that a variant containing no tyrosyl residues undergoes peroxide-induced inactivation and heme destruction, indicating that tyrosines are not required for inactivation or heme destruction that occurs during turnover. The Y67F/C102T variant, however, was found to be less sensitive than the wild-type protein to increased hydrogen peroxide concentration (Table 1). The Tyr67 residue is highly conserved among 96 eukaryotic cytochromes c with only the protein from the algae *Euglena gracilis* having a phenylalanine residue at this position instead of tyrosine.

In this work, we have shown that turnover-induced inactivation of yeast *iso*-1-cytochrome c and peroxide-induced heme destruction are two distinct processes. Inactivation

Figure 6



Direct ESR spectra obtained from the reaction of the wild-type (WT) yeast cytochrome c or the mutant Ile52→Phe67 with hydrogen peroxide. The reaction was performed with 250 μM cytochrome c and 0.25 M hydrogen peroxide (1:1000 ratio) in 20 mM phosphate buffer pH 7.2. After the addition of the peroxide, the reaction mixture was frozen in liquid nitrogen (within 15 seconds). The spectra were recorded at 4° under the following parameters: frequency 9.45 GHz; microwave power 0.5 mW; receiver gain 1×10^5; modulation frequency 100 KHz; modulation amplitude 3.89 G . The inset shows the effect of increasing temperature on the signal intensity.

appears to precede heme destruction (Figure 1), and it is possible to minimize heme destruction through appropriate substitutions for residues Asn52 and Tyr67. The triple variant Ile52/Phe67/Thr102 of *iso*-1-cytochrome c is inactivated with a rate constant comparable to that for the wild-type protein (Table *2*), but it was significantly less sensitive to heme destruction by hydrogen peroxide (Figure 5). This property is shown only by the triple variant; both the Ile52/Thr102 and Phe67/The102 variants undergo slow but significant rates of heme destruction (Figure 5). It has been reported that Tyr67, Asn52, Trp78 and the heme propionate form a hydrogen-bond network that includes a water molecule (Wat166) [31]. This Wat166 molecule is retained in the Y67F variant, whereas it is absent in the N52I variant [32]. When two of the three groups forming hydrogen bonds with Wat166 are changed, however, this molecule could be lost. In the oxidized wild-type protein, the dipole of Wat166 is reoriented to stabilize the positive charge residing on the heme iron atom [31]. This water molecule also appears to be critical for modulating the flexibility of nearby polypeptide chain segments between oxidation states, a feature that disappears when the hydrogen-bond network is disrupted. In contrast, Asn52 destabilizes a positive charge on the heme group to a greater extent than isoleucine does at this position, so this interaction does not appear to be completely compensated for by the rearrangement of the rest of the protein and water system [32]. In addition, the absence of the hydrogen-bond network, which involves the pyrrole A propionate, might result in a modification of the delocalized π-electron system on the porphyrin ring. These delocalized π-electrons in the porphyrin ring could have a role in the heme destruction process.

In agreement with the fact that the properties of single variants are not additive [33,34], the triple variant was less resistant to the presence of hydrogen peroxide (Figure 5). The direct EPR spectrum of the reaction of horse heart cytochrome c and hydrogen peroxide has been reported previously [35]. The radical formed in the reactionwas identified as a tyrosine radical by comparing its hyperfine structure after proteolysis in the presence of MNP with the coupling of a known tyrosine radical. In this case, besides using a different cytochrome c, the signal we obtained showed a strong temperature dependence as it was only evident at low temperatures (Figure 6, inset). Moreover, the signal was recorded immediately after starting the reaction, in contrast to the radical reported in horse heart cytochrome c, where the reaction was allowed to proceed for 3 minutes.

Because of the lack of coupling constants of this radical, even at low modulation amplitude, further investigation is needed to establish the identity and the number of species involved. On the basis of the characteristics of the signal, it is likely that the radical we observed is not a tyrosine radical but another oxidizable residue which is either close to or directly involved in the hydrogen network discussed above. One possibility is the only tryptophan in yeast cytochrome c because it is close to the heme and the hydrogen network, and is located on the same side of the heme plane. Furthermore, it has been reported that a tryptophan radical is produced in the reaction of myoglobin with hydrogen peroxide [36] and the radical was firmly identified using mass spectrometry [37].

One of the most relevant results from the study reported here is the absence of the radical from the mutant reaction. The triple variant shows no heme destruction but it is still inactivated, suggesting that the radical could be necessary for the heme destruction process; therefore, it cannot be involved in the set of reactions that lead to the direct inactivation of the protein, which probably starts before the heme begins to be destroyed. Nevertheless, after these results we conclude that for *iso*-1-cytochrome c the loss of catalytic activity (inactivation) and the heme degradation are two different and independent consequences of the presence of hydrogen peroxide. At first, therefore inactivation mechanism is due to a chemical modification of specific residues in the peptide chain followed by the destruction of the heme prosthetic group. In contrast, a hydrogen-bond network formed by the Tyr67, Asn52, Thr78 and pyrrole A

propionate, in which Wat166 is involved, appears to be necessary for the heme destruction process. The triple variant Y67F/N52I/C102T proved to be resistant to a catalytic concentration of hydrogen peroxide (1 mM) when compared with the wild-type protein. This protein variant represents a good model for the study of cytochrome inactivation because the participation of the heme destruction process was eliminated. Rational site-directed mutagenesis is currently being carried out in our laboratory to obtain a more stable *iso*-1-cytochrome c biocatalyst.

Significance

Cytochrome c contains a heme prosthetic group and has peroxidase-like activity, with the accompanying peroxide-mediated inactivation demonstrated by all peroxidases. In this study, some *iso*-1-cytochrome c variants constructed by site-directed mutagenesis showed higher specific activity and were more stable in the presence of hydrogen peroxide than the wild-type protein. One triple mutant Asn52→Ile/Tyr67→Phe/Cys102→Thr) has a slightly lower inactivation rate than the wild type, but has a higher turnover rate than the native protein and is almost completely resistant to heme destruction in the peroxide concentrations of interest. Heme destruction and catalytic inactivation appear to be independent processes. By performing electron spin resonance spectroscopy experiments at low temperatures, we were able to distinguish radical formation during the reaction with the wild-type protein, but not in the case of the triple variant. We therefore conclude that the heme degradation process is a result of peroxide-mediated radical formation. Free radicals are known to be generated from tyrosine residues in hemoproteins but a variant containing no tyrosine residues still undergoes peroxidase-induced inactivation and heme destruction. Residues Asn52 and Tyr67 (in addition to Trp78), however, are known to be bonded to an internal water molecule, and it appears that the hydrogen bonding to this water molecule is also important for the heme destruction process. The triple mutant could be useful for studying cytochrome inactivation without the interference of the heme destruction.

Materials and methods

Chemicals

Yeast cytochrome c, pinacyanol chloride and electrophoresis reagents were obtained from Sigma Chemical Company (St. Louis, MO). Buffer salts in ultrapure grade were purchased from J.T. Baker (Phillipsburg, NJ). Hydrogen peroxide 30% solution was obtained from Aldrich (Milwaukee, WI). Restriction and modification enzymes were purchased from Life Technologies (Gaithersburg, MD) and from Boehringer Mannheim (Mannheim, Germany). *Escherichia coli* strains expressing *iso*-1-cytochrome c variants were constructed as previously reported [38]

Mutagenesis

Site-directed mutagenesis was performed using a mutagenic oligonucleotide expression system for *E. coli* in which the genes for *iso*-1-cytochrome c and cytochrome c heme lyase from the yeast *Saccharomyces cerevisiae* are expressed in parallel [38]. Recombinant clones were identified by a PCR reaction eliminating a restriction site. The mutations were confirmed by DNA sequence determination using the chain terminator method [39].

Protein purification

Variant proteins were isolated and purified with a modified method reported previously [38]. The modification in the final purification step involved use of a weakly acidic cation exchanger, Econo–Pack CM cartridge (Bio-Rad Laboratories, Hercules, CA), in a Perkin-Elmer HPLC system (Perkin–Elmer series 200) using a 25 min gradient from phosphate buffer 20 mM pH 6.0 to 100% 0.5 M NaCl in the same buffer. The identity of the collected fractions was determined with a Beckman DU 650 spectrophotometer. All spectrophotometric measurements were performed at 25°C.

Biocatalytic activity and chemical stability

Biocatalytic activity of the wild-type and the variant forms of *iso*-1-cytochrome c were estimated by the oxidation of pinacyanol chloride (5 μg/ml) in 60 mM phosphate buffer (pH 6.1) following addition of hydrogen peroxide (1 mM) [15]. Stability against hydrogen peroxide was estimated as residual activity after incubation with varying concentrations of hydrogen peroxide. The inactivation constant (k_{in}) was calculated from a first order equation ($A = A_o\, e^{-kt}$) obtained with the residual activity versus time. All experiments were carried out in triplicate.

Analytical procedures

The catalytic activity was monitored as the decolorization of pinacyanol chloride at 630 nm in a Beckman DU 650 Spectrophotometer. The specific activity was estimated from an extinction coefficient for pinacyanol chloride of 82,350 $M^{-1}cm^{-1}$ [40]. Heme destruction was monitored spectrophotometrically by scanning the absorbance spectrum of a solution containing *iso*-1-cytochrome c and hydrogen peroxide at two minute intervals. Protein concentration was measured with the Bio-Rad protein reagent (Bio-Laboratories, Hercules CA) using yeast cytochrome c as standard, and from the absorbance of the α band (550 nm) of reduced cytochrome c ($\varepsilon = 28{,}000\ M^{-1}cm^{-1}$). Amino acid analyses of yeast cytochrome c (8 nmols) were performed with a Beckman 6300 amino acid analyzer.

To determine the extent of iron release during the inactivation reaction, *iso*-1-cytochrome c (4 ml of a 52 nM in 4 ml, phosphate buffer, pH 6.0) was oxidized with H_2O_2 (1 mM) for 10 min. The reaction was stopped by the addition of sodium dithionite. Protein was then precipitated by the addition of trichloroacetic acid to a final concentration of 20% on ice. After centrifugation, the supernatant fluid was collected and assayed for free-iron content with an atomic absorption spectrophotometer equipped with a graphite furnace. Control samples were prepared as described but without addition of protein.

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