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Cytochrome *c* as a biocatalyst

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Abstract

Type *c* cytochromes, which are involved in the electron transport system, are also able to catalyze peroxidase-like reactions in the presence of an electron acceptor, such as hydrogen peroxide or an organic hydroperoxide. This work reviews the catalytic activity of cytochrome *c*, and the potential design by site-directed mutagenesis and chemical modification of new biocatalysts for environmental purposes. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Cytochrome; Biocatalyst; Hemoprotein; Peroxidase; PAH; Thermostability; Stability

1. Introduction

The *c* type cytochromes are probably among the most popular proteins for biochemical studies and have been the subject of several review books [1–3]. In in vivo conditions, cytochromes *c* are part of the energy-conserving electron transport system. They have a protoheme prosthetic group covalently attached by two thioether bridges between the cysteine residues of the protein and the vinyl side chains of the heme. Mammalian peroxidases [4] and type *c* cytochromes are the only hemoproteins with a covalently bound heme group. The location and role of mitochondrial cytochrome *c* are well known but the means by which cytochrome *c* conducts electrons between its membrane reductase and oxidase remains controversial. However, arguments have been put forward for the diffusion of cytochrome *c* across the membrane surface to interact separately with its reductase and oxidase.

Cytochrome *c* is a protein ubiquitous to all eukaryotic organisms and the sequence of many such proteins have been determined. This intensive inventory of sequences has been used to trace the phylogeny of the eukaryotes [5]. Comparison of these sequences shows that cytochromes *c* in eukaryotic organisms are highly conserved. High-resolution structures of five eukaryotic and six bacterial cytochromes *c* have been completed to date. The high degree of sequence homology expresses itself in a high degree of structural conservation among the these proteins.

2. Biocatalysis

In living systems no catalytic activity of cytochrome *c* has been described. However, forty years ago the ability of cytochrome *c* to induce lipid peroxidation as well as its involvement in

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hydroperoxide cleavage were reported [6]. Cytochrome *c* participates in the hydroxylation of 4-nitrophenol [7] and in the oxidation of 2-keto-4-thiomethyl butyric acid [8] in the presence of hydrogen peroxide. The peroxidase activity of cytochrome *c* has been demonstrated by the oxidation of various electron donors including ABTS (2-2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 4-aminoantipyrine [9]. This peroxidase activity is greatly enhanced by binding cytochrome *c* to a phospholipid bilayer membrane [10], and the enhancement of guaiacol oxidation in this system can be attributed to the interaction of the negative charges of mem-

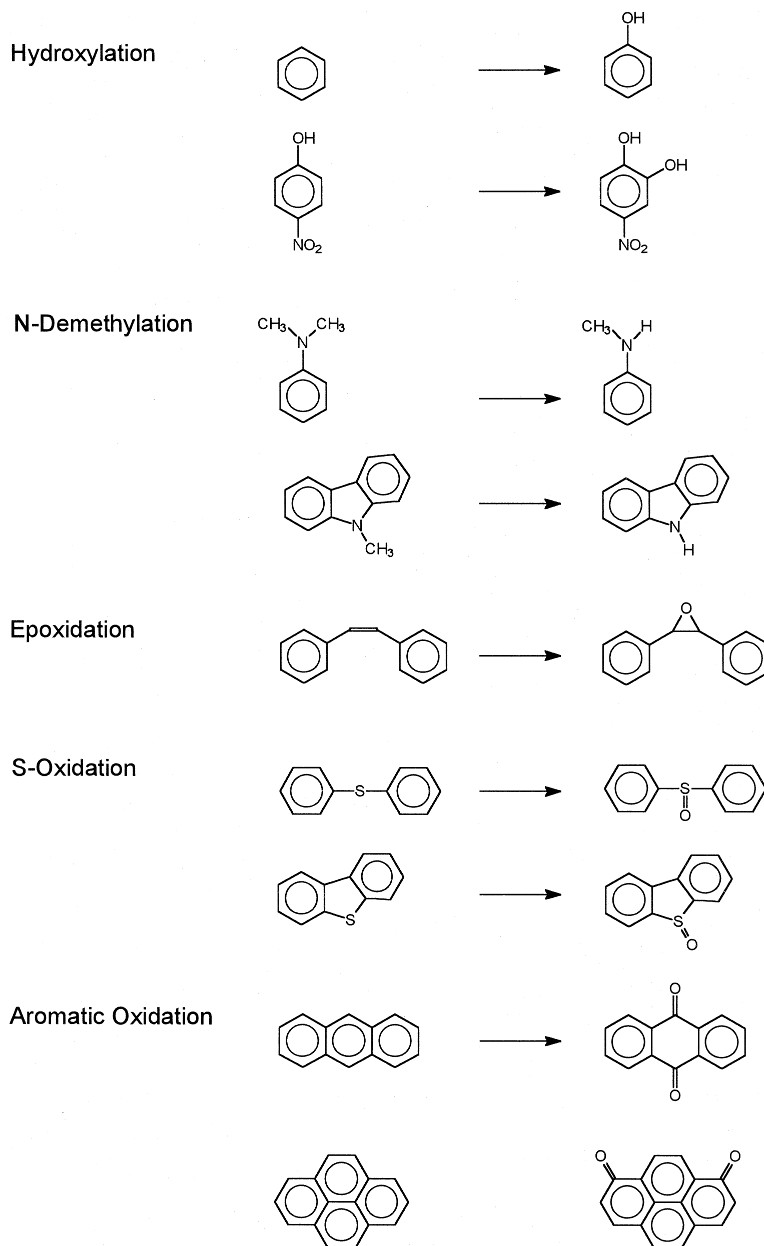


Fig. 1. Most interesting biotransformations catalyzed by cytochrome *c*.

Table 1
Substrates and products of cytochrome *c* biocatalytic reactions

Substrate	Product	Refs.
Aromatic hydrocarbons		
Anthracene	9,10-Anthraquinone	[14]
Benzene	Phenol	[15]
Benzo(*a*)pyrene	1,6-Benzo(*a*)pyrenodione	[19]
Pyrene	1,8-Pyrenodione	[14]
Organosulfur and heterocyclic compounds		
Benzothiophene	Benzothiophene sulfoxide	[12]
Carbazole	Unknown	[14]
Dibenzothiophene	Dibenzothiophene sulfoxide	[12]
Dibenzyl sulfide	Dibenzyl sulfoxide	[12]
Diphenyl sulfide	Diphenyl sulfoxide	[12]
N-Methylcarbazole	*N*-Hydroxymethylcarbazole	[11]
Thianthrene	Thianthrene disulfoxide	[12]
Thioanisole	Methylphenylsulfoxide	[11]
Other substrates		
ABTS	ABTS cation radicals	[9]
Guaiacol	Tetraguaiacol	[10]
Linolenic acid	Linoleate peroxide	[6]
Luminol	Chemiluminescense	[9]
Methionine	Ethylene	[8]
Stilbene	Stilbene epoxide	[11]

brane with the positive charges on the protein surface. Oxidative reactions with free and immobilized cytochrome *c* such as *N*- and *O*-demethylations, *S*-oxidations and epoxidation of oleofins, which are cytochrome P450-like reactions, are performed in the presence of hydrogen peroxide or other organic hydroperoxides [11]. As with cytochrome P450, cytochrome *c* has been used as biocatalyst in the oxidation of thiophenes and organosulfides to form sulfoxides [12,13]. Aromatic substrates of cytochrome *c* interact with the heme group as ligand rather than as a substrate [12,14]. Some of the biotransformations catalyzed by cytochrome *c* in the presence of peroxide are shown in Fig. 1.

The first oxidation of an aromatic hydrocarbon with cytochrome *c* in the presence of hydroperoxide was reported by Akasaka et al. [15]. Hydroxylation of benzene was carried out in organic solvent with less than 5% water, and with immobilized protein. Free cytochrome *c* was unable to perform this reaction. Using ^{18}O label the authors showed that two thirds of the oxygen in the produced phenol was derived from hydroperoxide and the other third from molecular oxygen. Recently we have reported that yeast cytochrome *c* can perform oxidations of polycyclic aromatic hydrocarbons (PAHs) in a system containing 10% acetonitrile and 1 mM hydrogen peroxide [14]. Table 1 shows some substrates and products of cytochrome *c* biocatalysis. The catalytic activity has been also detected in cytochromes from different sources, such as animal, yeast and bacterial cells (Table 2).

3. Cytochrome c a strong protein

Cytochrome *c* presents several advantages for use as a biocatalyst.

(a) The heme prosthetic group is covalently bound to protein. This property may be important for catalysis in the presence of organic

Table 2
Kinetic constants of different type *c* cytochromes and modified horse heart cytochrome *c* in 90% of tetrahydrofuran. The oxidation rates were determined for the decoloration of pinacyanol chloride [16]

Type *c* cytochrome	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
Horse heart cytochrome *c*	52.2	12.5	4.1
Acetylated cytochrome *c*	48.6	1.7	27.7
Benzyl cytochrome *c*	10.9	2.0	5.5
Poly(ethylene glycol) cytochrome *c*	210.1	1.6	132.6
Polymeric cytochrome *c*	137.2	9.2	14.9
Saccharomyces cerevisiae cytochrome *c*	7.3	5.7	1.3
Pseudomonas aeruginosa cytochrome c_{551}	8.5	1.6	5.2
Desulfovibrio vulgaris cytochrome c_3	29.1	3.0	9.8

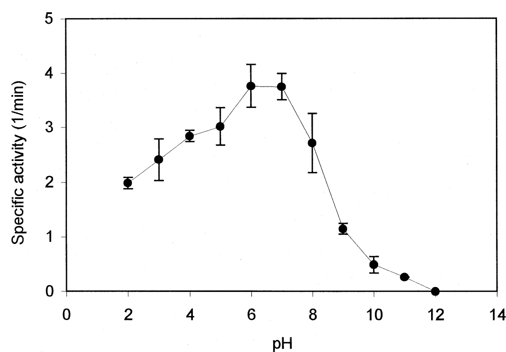


Fig. 2. Effect of pH on the biocatalytic oxidation of dibenzothiophene by cytochrome *c* in the presence of 1 mM hydrogen peroxide [12].

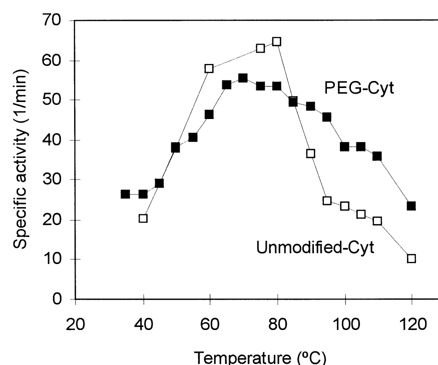


Fig. 3. Effect of temperature on the biocatalytic oxidation of pinacyanol chloride by poly(ethylene)glycol-modified (PEG-Cyt) and unmodified (Cyt) cytochrome *c*.

solvents, cytochrome *c* does not lose its heme catalytic group in these systems, while peroxidases do.

(b) Cytochrome *c* is active over a wide range of pH [12]. Fig. 2 shows that oxidation of dibenzothiophene is performed from pH 2 to pH 11. To our knowledge, no other enzyme is active over such a pH range.

(c) Biocatalytic activity was found at high concentration of organic solvents. In a system containing 90% of tetrahydrofuran, horse heart cytochrome *c* still showed 18% of its maximum activity [16]. Cytochromes *c* from different sources showed high catalytic activity in 90% tetrahydrofuran (Table 2).

(d) Horse heart cytochrome *c* is able to perform biocatalytic reactions at higher temperatures than 120°C, with a maximum activity at 80°C (Fig. 3). In addition, after chemical modification its thermostability is greatly increased (Fig. 4).

(e) Finally, cytochrome *c* is inexpensive. Cost and stability are the two main factors for biocatalysis in a large scale.

4. Biocatalyst design by genetic engineering

The development of molecular genetics and recombinant DNA procedures has allowed expression in yeast cultures of mutant cytochromes *c* bearing any desired amino acid change [17]. Recently, directed random mutagenesis of iso-1-cytochrome *c*, using PCR techniques and transformation into *E. coli*, can be used for the screening of a large number of protein variants [18]. We have performed site-directed mutagenesis on yeast cytochrome *c* and it is seems that the Phe82 substitution significantly altered the kinetic behaviour of the protein (Table 3). The Gly82:Thr102 variant showed 10 times more catalytic activity and a 10-fold increased catalytic efficiency over the wild-type iso-1-cytochrome *c* [14]. For oxida-

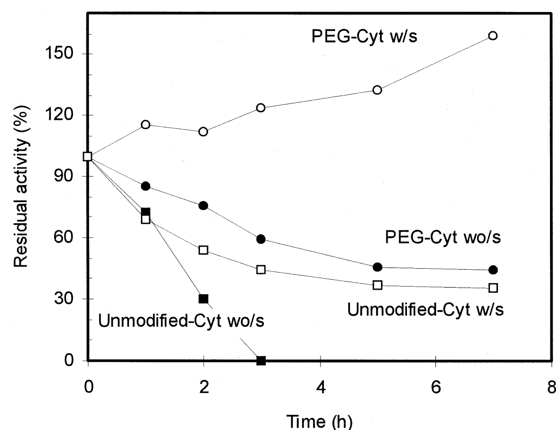


Fig. 4. Thermostability of poly(ethylene)glycol-modified (PEG-Cyt) and unmodified (Cyt) cytochrome *c*. The protein was incubated at 80°C during different times with (w) and without (wo) the presence of substrate (pinacyanol chloride). The reaction was started by the addition of hydrogen peroxide, and the remaining activity was measured.

Table 3
Kinetic constants of wild-type and variants of yeast iso-1-cytochrome *c* in pinacyanol chloride [14] and pyrene [20] oxidations

Variant	k_{cat} (s^{-1})	$K_{M,app}$ (mM)	$k_{cat}/K_{M,app}$ (s^{-1} M^{-1})
Pinacyanol oxidation			
F82G;C102T	33.93	17.1	1959
F82Y;C102T	13.86	11.7	1087
F82S;C102T	8.69	4.6	1874
F82W;C102T	5.65	8.5	662
F82A;C102T	4.77	11.2	425
Wild-type	2.97	10.5	284
C102T	2.31	12.0	192
Pyrene oxidation			
K79A;C102T	3.28	101.8	32
K87A;C102T	0.39	3.9	99
Wild-type	0.31	9.7	32
K73A;C102T	0.28	7.5	38
N52A;C102T	0.18	4.7	39
K86A;C102T	0.17	4.0	33
X72A;C102T	0.13	4.0	33
Y67F;C102T	0.10	3.3	32

tion of pyrene and pinacyanol chloride, these different variants of yeast cytochrome *c* have shown different catalytic constants (Table 3). On the other hand, Lysine 79 residue is placed at the edge of the solvent access to the heme group, and its substitution by alanine produced a protein with a higher k_{cat} but also a higher K_M, resulting in similar catalytic efficiency.

Protein design by site-directed mutagenesis is a promising biotechnological field. As demonstrated with cytochrome *c*, protein engineering may be able to solve some application problems by changing specific properties of the protein. Protein activity may be improved by the modification of the active site pocket. This modification could be rational with the aim to open the substrate access or to change the substrate affinity. On the other hand, even if cytochromes *c* are very stable proteins, their stability may be improved. Thermostability may be improved by promoting the disulfide links formation with the introduction of cysteine residues in specific sites of the protein. In addition, as discussed afterwards, inactivation of cytochromes by peroxidases by the presence of hydrogen peroxide is a limiting factor for the biocatalysts application at large scale. This problem could be also addressed by using site-directed mutagenesis. When specific amino acid residues are involved in the inactivation process, the change of these sensitive residues for others less susceptible to be oxidized or to form protein radicals, can leads to more stable biocatalysts. These results show that site-directed mutagenesis can be a tool for the design of a better biocatalyst for the oxidation of PAHs.

5. Biocatalyst design by chemical modification

In addition to genetic techniques, chemical modification has been performed on horse heart cytochrome *c* [11,16,19]. As mentioned above, cytochromes are very stable proteins (Figs. 2 and 3), and it is possible to perform a large variety of chemical reactions without affecting their activity. Chemical modification of free amino groups with different reagents has significantly altered the kinetic constants of cytochrome *c* (Table 2). Poly(ethylene)glycol modifications increased both the biocatalytic activity and efficiency in a system containing 90% tetrahydrofuran [15].

Free amino and carboxylic groups of horse heart cytochrome *c* have been modified by reactions with methyl, trimethylsilyl (TMS), and poly(ethylene)glycol (PEG) moieties [19]. As a consequence of these modifications the heme environment (active site) was altered. The kinetic constants and substrate specificities have been determined for these differently modified cytochromes. Cytochrome *c* can be prepared with a double modification; that is with PEG on free amino groups and methyl esters on carboxylic groups (including propionates of heme). Such a preparation was able to oxidize 17 aromatic compounds from 20 tested, while the unmodified protein was only able to oxidize eight compounds (Table 4).

While, unmodified cytochrome *c* was able to perform oxidation reactions up to 120°C with a

Table 4
Oxidation of polycyclic aromatic hydrocarbon by unmodified-cytochrome *c* and methylated poly(ethylene)glycol-modified-cytochrome *c* [19]

Aromatic compound	Specific activity (min^{-1})	
	Unmodified	PEG-Cyt-Met
7,12-Dimethylbenzanthracene	24.59 ($\pm$1.52)	80.33 ($\pm$3.83)
1,2:3,4-Dibenzanthracene	NR	16.60 ($\pm$2.24)
Azulene	2.26 ($\pm$0.29)	14.32 ($\pm$0.57)
3-Methylcholanthrene	1.88 ($\pm$0.07)	10.96 ($\pm$0.54)
7-Methylbenzo(*a*)pyrene	NR	7.56 ($\pm$0.42)
1,2:5,6-Dibenzanthracene	NR	5.70 ($\pm$0.31)
Triphenylene	NR	5.27 ($\pm$1.05)
Dibenzothiophene	0.67 ($\pm$0.06)	4.73 ($\pm$0.05)
Anthracene	0.33 ($\pm$0.06)	3.09 ($\pm$0.32)
Thianthrene	0.49 ($\pm$0.06)	1.41 ($\pm$0.08)
Pyrene	0.51 ($\pm$0.05)	0.97 ($\pm$0.03)
Fluoranthene	NR	0.65 ($\pm$0.09)
Acenaphthene	NR	0.40 ($\pm$0.01)
Benzo(*a*)pyrene	0.22 ($\pm$0.02)	0.39 ($\pm$0.06)
Fluorene	NR	0.22 ($\pm$0.01)
Phenanthrene	NR	0.17 ($\pm$0.02)
Chrysene	NR	NR
9,10-Dimethylanthracene	NR	NR
Naphthalene	NR	NR
Biphenyl	NR	NR

NR = no reaction detected.

maximum activity at 80°C (Fig. 3). Chemical modification with poly(ethylene)glycol greatly improved the thermostability of cytochrome *c* (Fig. 4). Unmodified cytochrome *c* was totally inactivated after 3 h at 80°C, while the poly(ethylene)glycol-modified cytochrome showed 40% of the initial activity after 7 h incubation at 80°C. This stabilization effect of the chemical modification was greater when the protein was incubated in the presence of the substrate, and in this case more than 100% of the initial activity was found after 7 h incubation at 80°C (Fig. 4).

On the opposite to site-directed mutagenesis, chemical modification is not a site-specific technique. However, chemical modification of the free amino groups on the proteins surface can change drastically the solubility properties of the molecule. In this way, it is possible to obtain biocatalytic proteins fully soluble in organic solvents and to reduce the mass transfer limitations in reaction systems containing hydrophobic substrates. With cytochrome *c* has been demonstrated that chemical modification of internal reactive groups altered both activity and substrate specificity. New substrates were transformed with chemically modified proteins. Thus, chemical modification of the biocatalyst can be also a tool for the design of new biocatalyst with environmental proposes.

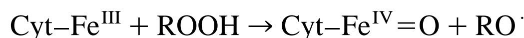
6. Reaction mechanism

Hemoproteins show structural differences in both the heme and polypeptide moieties. However, it is generally accepted that the generation of a high-valent oxoiron(IV)porphyrin radical cation by hydrogen peroxide is the first active intermediate in catalytic cycle of hemoproteins. As for peroxidases, the generation of a high-valent oxoiron(IV)porphyrin radical cation from cytochrome *c* by the action of one molecule of hydrogen peroxide has been suggested [21]. This radical cation is called Compound I. The presence of 0.5 equivalent of a two-electron reducing agent, such as an aromatic compound, generates the Compound II, which is oxoiron-(IV)porphyrin without the associated porphyrin π-radical cation. Compound II oxidizes a second molecule of substrate using a peroxidase shortcut to form the resting state iron(III)-porphyrin. In the absence of reducing substrate, excess hydrogen peroxide can react with Compound I as an electron donor to form Compound III. This peroxyiron(III)porphyrin free radical is a strong oxidizing agent, and located close to the porphyrin ring. Because of this proximity, any electron transfer from the ferrous state to the extra hydrogen peroxide moiety would generate a hydroxyl radical, which could reach the tetrapyrrol structure and lead to an irreversible inactivation of the enzyme. All peroxidases are inactivated by an excess of hydrogen peroxide or in the absence of an oxidizable organic substrate, nevertheless the role of Compound III as

an inactivating intermediate remains controversial, as discussed below.

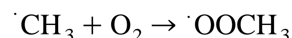
The catalytic mechanism for free radical production of cytochrome *c* has been elucidated [22]. Ferricytochrome *c* alone showed low-level chemiluminescence in the presence of hydroperoxides [23]. The requirements for chemiluminescence were oxidized cytochrome *c*, organic hydroperoxide and O_2. The light-emission that arises from the reaction can be understood in terms of a process that includes a catalyzed scission of hydroperoxide by cytochrome *c*, a free-radical oxidation process, and the generation of a chemiluminescent singlet oxygen. As in the case of peroxidases, an initial activation of cytochrome *c* by hydrogen peroxide mediates the reaction to a catalytically more active species, an oxo-heme complex which has a high oxidation state. Superoxide (O_2^-) and hydroxyl radical ($HO^{\cdot}$) are not involved in this catalytic activity, since it is not sensitive to superoxide dismutase and mannitol [9].

Peroxyl and alkoxyl radicals have been detected by electron spin resonance spectroscopy (EPR), and using trapping techniques [24]. In the peroxidase mechanism, the ferric form of cytochrome *c* could heterolytically cleave the peroxide O–O bond, and reduce the organic hydroperoxide to its corresponding alcohol. Also, a ferryl cation-radical from cytochrome *c* (Compound I) could take one hydrogen from another molecule of hydroperoxide, producing a peroxyl radical. Alternatively, ferric cytochrome *c* could cleave the peroxide O–O bond homolytically which would produce an alkoxyl radical. Nevertheless, the main question whether there was, heterolytic or homolytic cleavage of hydroperoxide, remained unsolved until the elegant work of Barr and Mason [22]. In this study, ESR spin trapping with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used to detect the formation of peroxyl, alkoxyl, and methyl radicals from organic hydroperoxides and cytochrome *c*. By increasing the DMPO concentration the authors demonstrated that the alkoxyl radical is the initial radical produced. Thus, cytochrome *c* produces a homolytic cleavage of hydroperoxide, in contrast to previous proposed mechanisms. The peroxyl radicals are secondary products from the reaction of oxygen with methyl radicals, which are formed from the β-scission of alkoxyl radicals. To explain how all three of these radical adducts were observed in the ESR spin trapping experiments, the following free-radical reactions should be considered:



$$\text{Cyt–Fe}^{III} + \text{ROOH} \rightarrow \text{Cyt–Fe}^{IV}\text{=O} + \text{RO}^{\cdot}$$

$$\text{RO}^{\cdot} \rightarrow {}^{\cdot}\text{CH}_3 + \text{ketone}$$



$${}^{\cdot}\text{CH}_3 + \text{O}_2 \rightarrow {}^{\cdot}\text{OOCH}_3$$

7. Peroxide inactivation, an unsolved problem

As in the cases of horseradish peroxidase [25], lignin peroxidase [26], manganese peroxidase [27], lactoperoxidase [28], and other peroxidases, cytochrome *c* is inactivated by the presence of an excess of hydrogen peroxide or of an organic hydroperoxide. This substrate inactivation leads to the modification of the heme prosthetic group and probably, as for peroxidases, to the formation of a verdohaemoprotein as a final product [27]. So far, the inactivation mechanism has not been clearly elucidated [25–30]. Two main ways have been proposed for the inactivation process of peroxidases by hydrogen peroxide. One in which the Compound II reacts with hydrogen peroxide in the absence of a reductant substrate to form the Compound III. If the Compound III is a peroxyiron(III)porphyrin free radical, it should be considered as a very aggressive intermediate, and 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 reach the tetrapyrrol structure leading to an irreversible inactivation. The second mechanism includes a reaction of Compound I with an

excess of hydrogen peroxide in the absence of reductant substrate to form an irreversibly inactivated verdohaemoprotein. In this mechanism the Compound III is proposed as a superoxide anion generating system that has a protective effect against the inactivation.

Cytochrome *c* inactivation by hydrogen peroxide seems to be correlated with an absorbance decrease of the Soret peak (Fig. 5). The stability of eight variants, produced by site-directed mutagenesis on the Phe82 residue of yeast cytochrome *c*, was determined by incubating the protein with 1 mM hydrogen peroxide [14]. The mutations at position 82 significantly affected stability, and these stability changes were correlated to the accessibility of hydrogen peroxide into the active site [14]. Several variants from site-directed mutagenesis of iso-1-cytochrome *c* were tested for stability, with the aim of producing a more stable biocatalyst against inactivation with hydrogen peroxide (Table 5). As in the case of horseradish peroxidase [29], the yeast cytochrome variants showed different inactivation constants, and some were significantly more stable than the wild type cytochrome. So far, no clear relationship between stability and structure changes has been elucidated and studies are currently under way to find structural justifications to these results. However, these results show that site-directed mutagenesis can be a biotechnological tool for the design of more stable biocatalysts.

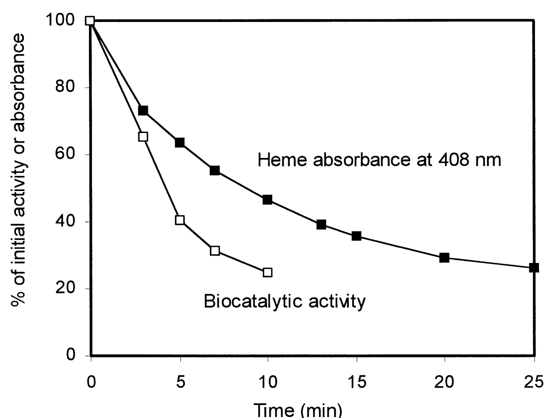


Fig. 5. Cytochrome inactivation and decrease of heme absorbance by the presence of 1 mM hydrogen peroxide.

Table 5
Inactivation constants of wild-type and variants of yeast iso-1-cytochrome *c* in the presence of 1 mM hydrogen peroxide

Variant	k_{in} (min^{-1})
F82A;C102T	0.3471
K72A;C102T	0.216
Y67F;C102T	0.1494
Wild-type	0.1319
K79A;C102T	0.0839
K87A;C102T	0.0839
K73A;C102T	0.0675
N52A;C102T	0.0596

Hydrogen peroxide inactivation was measured by incubating the protein in the presence of 1 mM hydrogen peroxide, for different times. The reaction was started by adding 5 μg of pinacyanol.The inactivation constant was determined by fitting the data to the first-order equation.

8. Conclusions

Hemoproteins exhibit an impressive range of biological functions. These include: simple electron transfer reactions, oxygen transport and storage, oxygen reduction to the level of hydrogen peroxide or water, oxygenation of organic substrates, and reduction of peroxides. This versatility in functions is made possible by a combination of differences in both the polypeptide and heme constituents of the various hemoproteins. Type *c* cytochromes are generally involved in the energy-conserving electron transport system. However, their catalytic site is able to perform peroxidase-like reactions. A large range of substrates can be modified by cytochrome *c* in the presence of hydrogen peroxide or other organic hydroperoxides. Oxidation of substituted-phenols, organic sulfurs, and thiophenes can be catalyzed by type *c* cytochromes. In addition to ABTS, guaiacol and luminol reactions, cytochrome *c* is able to perform lipid peroxidation and *N*-*O*-demethylations. Oxidation of polycyclic aromatic hydrocarbons (PAHs) by type *c* cytochromes has introduced a

new biocatalyst for environmental purposes: PAH's are considered to be a potential health risk because of their possible carcinogenic and mutagenic activities.

On the other hand, cytochrome *c* is able to perform enzymatic reactions in extreme conditions: pH from 2 to 11, temperatures higher than 120°C, and organic solvent concentrations up to 90%, and it is cheap. In our knowledge, no protein has better range of reaction conditions than cytochrome *c*. In addition, site-directed mutagenesis and chemical modifications have been shown to be useful tools for the design of a new biocatalyst with improved properties, such as substrate specificity and protein stability.

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