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Biocatalytic chlorination of aromatic hydrocarbons by chloroperoxidase of *Caldariomyces fumago*

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Abstract

Chloroperoxidase from *Caldariomyces fumago* was able to chlorinate 17 of 20 aromatic hydrocarbons assayed in the presence of hydrogen peroxide and chloride ions. Reaction rates varied from 0.6 min^{-1} for naphthalene to 758 min^{-1} for 9-methylanthracene. Mono-, di- and tri-chlorinated compounds were obtained from the chloroperoxidase-mediated reaction on aromatic compounds. Dichloroacenaphthene, trichloroacenaphthene, 9,10-dichloroanthracene, chloropyrene, dichloropyrene, dichlorobiphenylene and trichlorobiphenylene were identified by mass spectral analyses as products from acenaphthene, anthracene, pyrene and biophenylene respectively. Polycyclic aromatic hydrocarbons with 5 and 6 aromatic rings were also substrates for the chloroperoxidase reaction. The importance of the microbial chlorination of aromatic pollutants and its potential environmental impact are discussed.

Keywords: Fungal chloroperoxidase; Enzymatic chlorination; Polycyclic aromatic hydrocarbons

1. Introduction

Organochlorine industrial compounds, chlorinated pesticides and polychlorinated biphenyls (PCBs) are considered among the most important pollutant xenobiotics. Their toxic effects have been extensively studied (Evangelista de Duffard and Duffard, 1996; Giesy and Kannan 1998; Tilson and Kodavanti, 1998). Because of their potential public health risk, some organochlorine compounds, such as PCBs, have been banned in western countries, but many are still manufactured and used as pesticides, plasticizers, paint and printing-ink components, adhesives, flame retardants, hydraulic and heat transfer fluids, refrigerants, solvents, additives for cutting oils, and textile auxiliaries. Thus, contamination with organochlorine compounds still occurs and this is of great public concern due to potential toxicity to humans and wildlife. Microbiological studies have been almost entirely focused on the degradation of these toxic compounds (Robinson, 1998; Wiegel and Wu, 2000; Chaudhry and Chapalamadugu, 1991).

In spite of the microbial capacity to produce halogenated compounds (Neidleman, 1975; de Jong and Field, 1997), information is scarce on the microbial production of toxic organochlorine compounds. In addition to de novo synthesis of chlorinated compounds, microorganisms transform some non-halogenated xenobiotics into organochlorine compounds with a possible increase in their toxicity. In this work, we show the capacity of chloroperoxidase from the fungus *Caldariomyces fumago* to chlorinate aromatic hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs). PAHs are widely dispersed in the environment, and they are considered to be a potential health risk because of their possible carcinogenic and mutagenic activities. Chloroperoxidase (CPO) is a 42,000 Da extracellular heme glycoenzyme containing ferriprotoporphyrin IX as the prosthetic group (Sundaramoorthy et al., 1995). CPO exhibits a broad spectrum of chemical reactivities, it is a peroxide-dependent chlorinating enzyme and it also catalyzes peroxidase-, catalase- and cytochrome P450-type reactions of dehydrogenation, H_2O_2 decomposition and oxygen insertion, respectively (Yi et al., 1999). CPO is only one of a variety of halogenase enzymes that can be found in nature, other enzymes such as vanadium and non-heme halogenases (van Pee et al., 2000; Vollenbroek et al., 1995) are also potentially able to chlorinate organic pollutants.

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2. Results and discussion

Chloroperoxidase was able to utilize 17 of the 20 aromatic hydrocarbons assayed as substrate (Table 1). Only biphenyl, and the oxygen-containing dibenzofuran and anthrone, were not substrates for CPO under our reaction conditions. The specific activity values were from 0.6 min^{-1} for naphthalene to 758 min^{-1} for 9-methylanthracene. Interestingly, recalcitrant and carcinogenic 5- and 6-aromatic rings PAHs were substrates for chloroperoxidase in the presence of hydrogen peroxide and chloride ions. Chloroperoxidase is able to perform a broad range of reactions, like the enantioselective epoxidation of alkenes (Zaks and Dodds, 1995), oxidation of phenolic pollutants (Aitken et al., 1994; Carmichael et al., 1983), oxygenation of sulfides (Colonna et al., 1990), oxidation of organophosphorus pesticides (Hernandez et al., 1998), and the PAH–DNA adduct formation (Marquez-Rocha et al., 1997), to give only a few examples. In addition, CPO is able to oxidize very complex molecules, such as asphaltenes (Fedorak et al., 1993) and complex mixtures such as petroleum distillates (Ayala et al., 1998). Thus this halogenating enzyme is the most versatile enzymatic hemoprotein. Other halogenases are also able to perform halogenations, mainly chlorination, on a variety of substrates (Neidleman, 1975; Vollenbroek et al., 1995).

Table 1
Specific activity of chloroperoxidase from *Caldariomyces fumago* against aromatic compounds

Aromatic compound	Specific activity (min^{-1})
9-Methylanthracene	758 (±27)
Azulene	676 (±34)
Anthracene	134 (±14)
2-Methylanthracene	107 (±8)
7,12-Dimethylbenzanthracene	87 (±8)
Benzo[a]pyrene	84 (±6)
7-Methylbenzo[a]pyrene	81 (±7)
Acenaphthene	65 (±8)
Pyrene	53 (±6)
Benzo[ghi]perylene	45 (±7)
Perylene	25 (±10)
Biphenylene	10 (±0.5)
Phenanthrene	7 (±0.1)
Fluoranthene	3 (±0.2)
Fluorene	1.9 (±0.13)
Triphenylene	0.8 (±0.09)
Naphthalene	0.6 (±0.01)
Biphenyl	NR[a]
Dibenzofuran	NR[a]
Anthrone	NR[a]

[a] NR, no reaction detected.

The chemical nature of the reaction products was determined by gas chromatography–mass spectrometry (GC–MS). The mass spectra of the products from the enzymatic reaction with acenaphthene, anthracene, biphenylene, fluorene, phenanthrene, pyrene, and triphenylene are shown in Table 2. Monochlorinated, dichlorinated and trichlorinated compounds were found (Fig. 1), and they showed major ions at $m/z = [M^+]$-35, $m/z = [M^+]$-70, and $m/z = [M^+]$-105, respectively. No oxygen incorporation was detected in any of the products from the CPO-mediated reactions. In addition, no reaction could be detected with any PAH tested under peroxidase activity conditions (pH 5.0 and in the absence of chlorine ions). Significant information is available on the toxicity of chlorinated aromatic compounds, such as polychlorophenols (International

Table 2
Mass spectral data of the products from the chloroperoxidase-mediated reaction on aromatic compounds

Substrate	Product	Mass spectral ions (m/z)[a,b]
Acenaphthene	Dichloroacenaphthene	224 (39), 222 (64) [M^+], 187 (100), 152 (95), 93 (17), 75 (24).
	Trichloroacenaphthene	258 (57), 256 (81) (M^+], 221 (66), 186 (100), 150 (50), 110 (27), 98 (18), 75 (23).
Anthracene	9,10-Dichloroanthracene	248 (68), 246 (100) [M^+], 176 (43), 87 (10).
Biphenylene	Dichlorobiphenylene	222 (64), 220 (100) [M^+], 185 (17), 150 (45), 75 (11).
	Trichlorobiphenylene	258 (30), 256 (93), 254 (100) [M^+], 219 (13), 184 (49), 149 (14), 74 (10).
Fluorene	Dichlorofluorene	238 (25), 237 (7), 236 (40) [M^+], 201 (31), 199 (18), 166 (63), 165 (100), 164 (17), 163 (24), 100 (11), 82 (35).
Phenanthrene	Chlorophenanthrene	214 (32), 213 (16) [M^+], 212 (100), 177 (20), 176 (55), 175 (11), 174 (10), 151 (14), 150 (14), 106 (17), 88 (33), 87 (11), 75 (13).
Pyrene	Chloropyrene	238 (31), 236 (100) [M^+], 200 (34), 100 (12).
	Dichloropyrene	272 (62), 270 (100) [M^+], 235 (11), 200 (53), 135 (12), 100 (23).
Triphenylene	Chlorotriphenylene	265 (7), 264 (34), 263 (21) [M^+], 262 (100), 227 (14), 226 (63), 225 (16), 224 (23), 200 (11), 132 (9), 131 (20), 113 (56), 112 (43), 100 (21), 99 (12), 87 (9).

[a] Values in parentheses are relative abundances.
[b] [M^+] molecular ion.

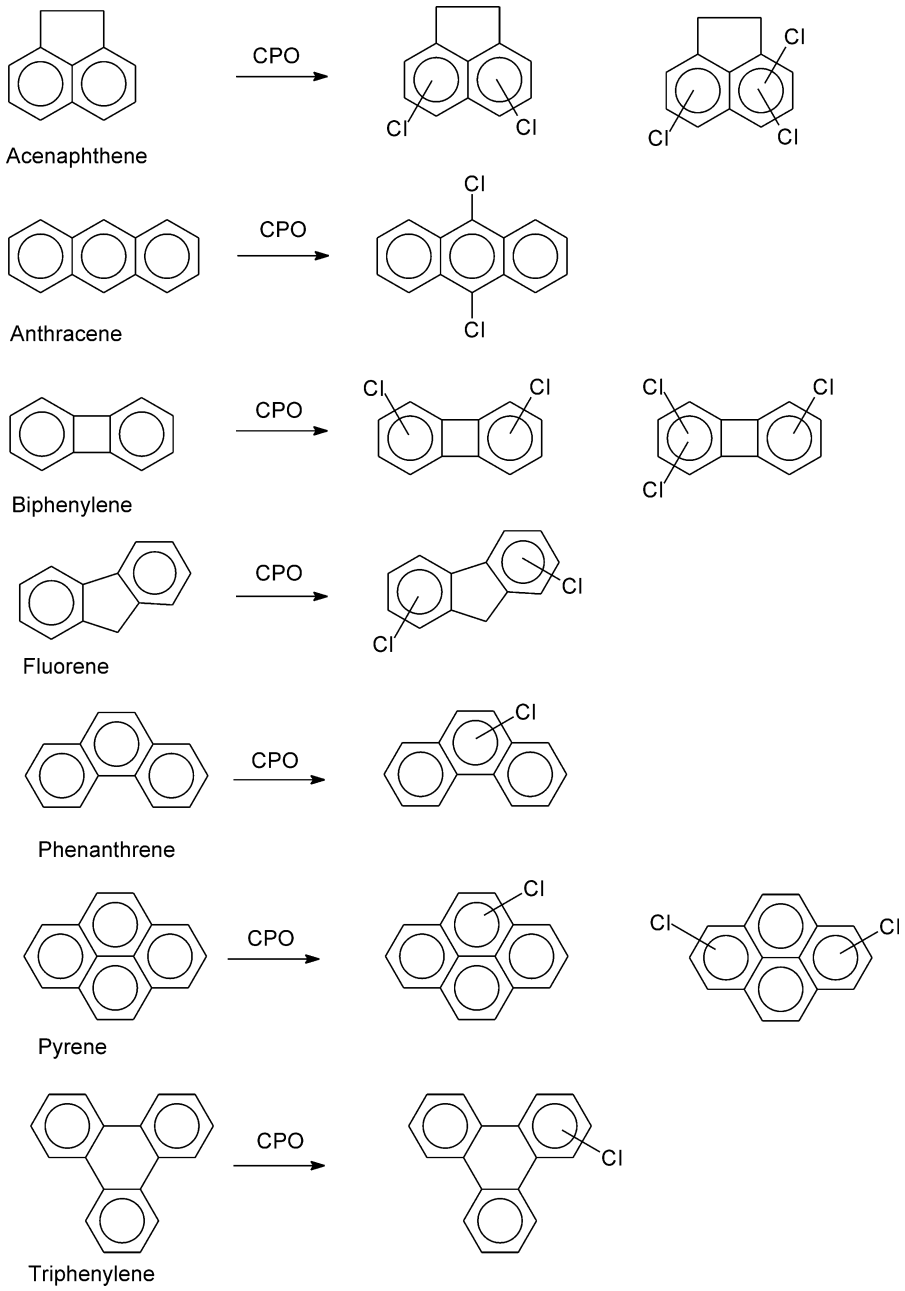

Fig 1. Chlorinated products from the enzymatic halogenation of aromatic compounds by chloroperoxidase in the presence of hydrogen peroxide and chloride ions.

Agency for Research on Cancer, 1999) and PCBs (Robinson, 1998; Wiegel and Wu, 2000), and to a less extent, on the toxicity of chlorinated derivatives of polycyclic aromatic hydrocarbons. Chlorinated PAHs have been tested for mutagenic activity by the Ames test on *Salmonella typhimurium* (Colmsjo et al., 1984; Johnsen et al., 1989). Chlorinated fluorene, flouranthene and benzo(a)pyrene acted as strong mutagens both in the presence and in the absence of metabolic activation, while only benzo(a)pyrene showed mutagenic activity as parent hydrocarbon. Mono- and di-chloropyrene isomers showed from 40 to 4000 times higher mutagenic activity than 1-nitropyrene and pyrenoquinones, respectively. On the other hand, a mixture of chlorinated chrysene isomers was considerably more potent than the parent hydrocarbon in terms of embryolethality and cytochrome P450 induction (7-ethoxyresorufin-*O*-deethylase and aryl hydrocarbon hydroxylase) (Gustafsson et al., 1994). The chlorinated chrysene caused anomalies, including edema and beak defects, similar to those reported after treatment of chick embryos with coplanar PCBs. These effects of the chlorinated mixture were mainly accounted for by 6-chlorochrysene and 6,12-dichlorochrysene. Chloronaphthalene was 5000-times

more potent than naphthalene for the inhibition of mitochondrial respiration in molar basis (Beach and Harmon, 1992). Monochloronaphthalene represent a risk for human health (Tsunenar et al., 1982) and for aquatic organisms (Ward et al., 1981).

3. Conclusions

Chloroperoxidase from the imperfect fungus *Caldariomyces fumago* is the most versatile enzyme in the hemoprotein family (Yi et al., 1999). CPO performs halogenase, peroxidase, catalase and cytochrome P450-like reactions. However, under our reaction conditions and with PAHs as substrates, CPO only acts as halogenase and no oxygenated products could be detected. In contrast, peroxidase activity on aromatic compounds produces mainly quinones, such as in the case of lignin peroxidase (Hammel et al., 1986; Vazquez-Duhalt et al., 1994) and manganese peroxidase (Bogan et al., 1996).

Caldariomyces fumago has been isolated from damp sites and also has been reported as a marine fungus (Dawson and Sono, 1987; Colonna et al., 1999). Chloroperoxidase, as other halogenases, is an extracellular enzyme that can react with a variety of substrates in the microbial environment. This enzyme is able to catalyze PAH-DNA adduct formation in vitro (Marquez-Rocha et al., 1997), suggesting the production of genotoxic aromatic intermediates. The present work shows that the transformation of aromatic pollutants into chlorinated derivatives by microbial enzymes may occur in polluted sites. This biocatalytic process should be considered because the toxicity and environmental impact of aromatic compounds may be increased.

4. Experimental

4.1. Chemicals

Purified CPO [EC 1.11.1.10] from *C. fumago* 89362 (Commonwealth Mycological Institute, Kew, Surrey, UK) was produced in a fructose medium and purified according to Pickard et al. (1991); all preparations used in this study had an $Rz = 1.36$, which corresponds to 95% purity. Hydrogen peroxide and buffer salts were obtained from J.T. Baker (Phillisburg, NJ). Polycyclic aromatic hydrocarbons were purchased from Aldrich Chemical (Milwaukee, WI). HPLC-grade acetonitrile and methylene chloride were purchased from Fisher Scientific (Springfield, NJ).

4.2. Reaction rate measurements

Reactions were carried out in a 1-ml reaction mixture containing 20 μM substrate and 15% acetonitrile in a 60 mM acetate buffer, pH 3.0, with 20 mM KCl at room temperature. From 0.4 pmol to 0.2 nmol of the purified enzyme were used in the mixtures. Reactions were started by addition of 1 mM H_2O_2 and monitored by HPLC. Reaction rates were measured from the differences in peak area after 10 min and referred to the purified protein concentration for specific activity calculations. Reported values are the mean of three replicates. Specific reaction rates are given as mol of substrate converted per mol of enzyme per minute or simply in min^{-1}. Reactions for 5 and 6 aromatic rings PAHs 7,12-dimethylbenzanthracene, benzo[a]pyrene, 7-methylbenzo[a]pyrene, benzo[ghi]perylene and perylene were also monitored by fluorescence spectrum, with an excitation at 300 nm, in a Luminescence spectrometer, Perkin-Elmer, Model LS 50, during 2 min at 25 °C. The CPO activity was measured as the disappearance of the respective maximum emission peak for each PAH over a two minute reaction.

4.3. Analytical methods

Substrate concentration was measured in a Perkin-Elmer (series 200) HPLC system, using a C_{18} Hypersyl 5 μm Hewlett-Packard column eluted with an acetonitrile–water (70:30 v/v) solvent mixture. Substrate and product detection was carried out using a diode array detector coupled to the HPLC system. Product identification was performed in a Hewlett-Packard GC (model 6890)-MS (model 5972) equipped with a SPB-20 column (30 m×0.25 mm, Supelco). The temperature program started at 100 °C for 2 min; the temperature was raised to 290 °C at a rate of 8 °C/min and kept at 290 °C for 10 min.

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