

Substrate Specificity and Ionization Potential in Chloroperoxidase-Catalyzed Oxidation of Diesel Fuel

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Straight-run diesel fuel containing 1.6% of sulfur was enzymatically oxidized with chloroperoxidase from *Caldariomyces fumago*. Most organosulfides and thiophenes were transformed to form sulfoxides and sulfones. The oxidized organosulfur compounds can be effectively removed by distillation. The resulting fraction after distillation contained only 0.27% sulfur, while the untreated straight-run diesel fuel after the same distillation process still showed 1.27% sulfur. To know the chemical nature of the products, nine organosulfur compounds and 12 polycyclic aromatic compounds (PACs) were transformed by chloroperoxidase in the presence of chloride and hydrogen peroxide. Organosulfur compounds were only oxidized to form sulfoxides and sulfones, and no chlorinated derivatives were detected, except for bithiophene. In contrast, PACs were exclusively chlorinated, and no oxidized derivatives could be found. No enzymatic activity was detected on PACs with an ionization potential higher than 8.52 eV, while in the lower region it was found that the higher the ionization potential of the PAC the lower the specific activity. On the other hand, the substrate ionization potential did not seem to influence chloroperoxidase activity in the oxidation of organosulfur compounds. All organosulfur compounds tested were oxidized by chloroperoxidase. From double-substrate experiments, it appears that organosulfur compounds are oxidized by both compound I and compound X enzyme intermediates, while PACs react only with the halogenating intermediate, compound X.

Introduction

The environmental driver for diesel sulfur reduction is well-established. Meeting sulfur regulations on petroleum products is driving up the cost of refining, because conventional hydrodesulfurization becomes increasingly expensive and less efficient in handling sulfur removal as lower and lower sulfur levels are reached (1). In the United States, there are plans to greatly reduce motor-vehicle emissions and sulfur content

in gasoline (2). The plan would cut the average sulfur level in gasoline by 90%, from an average of about 330 ppm to 30 ppm, by 2004.

Microbial desulfurization of fossil fuels has been under active investigation for several decades and has been recently reviewed (3–5). Research groups and companies worldwide are developing the technology for fuel biodesulfurization, the most successful being a unique refinery process using bacteria to selectively remove sulfur from diesel. The patented bacteria, *Rhodococcus* IGTS8, has been genetically engineered to increase both activity and stability (6).

Recently, we have described an enzymatic method for fuel desulfurization (7). The method includes the steps of biocatalytic oxidation of organosulfur compounds contained in straight-run diesel fuel by chloroperoxidase from *Caldariomyces fumago*, followed by a distillation process in which the oxidized compounds are removed. Chloroperoxidase from *Caldariomyces fumago* (CPO) (EC 1.11.1.10) is a versatile heme enzyme because of its catalytic diversity. CPO is a peroxide-dependent chlorinating enzyme, and it also catalyzes peroxidase-, catalase-, and cytochrome P450-type reactions of dehydrogenation, H₂O₂ decomposition, and oxygen insertion, respectively. This unusual combination of enzymatic activities is the origin of a number of studies involving CPO as a catalyst with potential applications, including the petroleum industry. It has been demonstrated that CPO is able to remove nickel and vanadium from asphaltene fractions (8). CPO is also able to perform interesting reactions, like the enantioselective epoxidation of alkenes (9), oxidation of phenolic pollutants (10, 11), oxygenation of sulfides (7, 12), oxidation of organophosphorus pesticides (13), and the determination of genotoxic potential of pollutants (14), to give only a few examples. However, the catalytic mechanism of CPO has not been completely established, and the exact role of chloride and the identity of the halogenating species remains a subject of controversy (15–18).

The ability of fungal peroxidases to biotransform petroleum compounds, such as polyaromatic hydrocarbons (PAHs), has been investigated before, specially lignin peroxidase (LiP) and manganese peroxidase (MnP) from *Phanerochaete chrysosporium*. These nonspecific extracellular enzymes are believed to be involved in pollutant biotransformation. Interestingly, the activity of LiP and MnP correlates with the ionization potential (IP) of the PAHs. A threshold IP value was found for each enzyme. LiP oxidizes PAHs with IP ≤ 7.55 eV as well as some heterocyclic compounds with IP ≤ 8 eV (19, 20), while MnP oxidizes PAHs with IPs as high as 8.1 eV (21, 22). With this evidence it was possible to distinguish whether a substrate was transformed via an electron subtraction process.

Considering that both organosulfur and PACs are contained in diesel fuel, in the present work the enzymatic activity of CPO toward a group of several organosulfur compounds (thiophenes and organic sulfides) and PACs was determined. The chemical nature of reaction products and the role of the substrate ionization potential were analyzed.

Experimental Section

Chemicals. Purified CPO from *Caldariomyces fumago* was produced in a fructose medium and purified according to Pickard (23); all preparations used in this study had an R_z = 1.36, which corresponds to 95% purity. Hydrogen peroxide and buffer salts were obtained from J. T. Baker (Phillisburg, NJ). Polycyclic aromatic compounds and aromatic thiophenes and sulfides were purchased from Aldrich

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TABLE 1. Sulfur Content of Straight-Run Diesel Fuel after Enzymatic Oxidation with Chloroperoxidase from *Caldariomyces fumago* Followed by a Distillation to 325 °C as Final Distillation Point

	distillation		enzymatic + distillation	
	TPH ^a (%)	sulfur (%)	TPH (%)	sulfur (%)
distillate	83	1.27	71	0.27
residue	17	3.21	29	5.51

^a Total petroleum hydrocarbons.

Chemical (Milwaukee, WI). HPLC-grade acetonitrile and methylene chloride were purchased from Fisher Scientific (Springfield, NJ).

Reaction Conditions. Diesel fuel oxidations with chloroperoxidase were carried as previously reported (7). Oxidation reactions of individual organosulfur and aromatic compounds 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 or without 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 H₂O₂. Reaction rates were estimated by monitoring the substrate peak in a HPLC system equipped with a diode array detector. Enzyme activities were obtained from the differences in peak area after 10 min of reaction, transformed by a standard curve, and adjusted for protein concentration. 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. For products identification, 10-mL reactions were performed; after 1 h, the mixture was acidified and extracted with methylene chloride, and the extract was reduced under nitrogen, before being analyzed by GC-MS.

Two-Substrate Reactions. Reaction mixtures contained either 20 μM thianthrene or 30 μM pyrene and 100 μM monochlorodimedone (MCD) in 15% acetonitrile in a 20 mM KCl, 60 mM acetate buffer pH 3.0. The reaction was started by addition of 0.25 mM H₂O₂ and monitored spectrophoto-

metrically at 288 nm (MCD) and either 254 nm (thianthrene) or 335 nm (pyrene).

Kinetic Constants Determination. Reactions were performed in 1 mL of 60 mM acetate buffer pH 3.0, 20 mM KCl and either 15% for thianthrene or 20% acetonitrile for MCD and pyrene. Reaction was started by addition of 1 mM H₂O₂. The initial reaction rates were obtained by following the decrease in absorbance at 254 nm for thianthrene ($\epsilon = 35 \text{ mM}^{-1} \text{ cm}^{-1}$) and at 335 nm for pyrene ($\epsilon = 32.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

Analytical Methods. Substrate concentration was measured in a Perkin Elmer (series 200) HPLC system, using a C₁₈ Hypersyl 5 μm Hewlett-Packard column and eluted with an acetonitrile–water (70:30 v/v) solvent mixture. Substrate and products detection was carried out using a diode array detector coupled to the HPLC system. The used wavelengths for detection (λ_{det}) are listed in Tables 3 and 4. Other UV measurements were made in a Beckman Spectrophotometer (DU 530). Product identification was performed in a Hewlett-Packard GC (model 6890)-MS (model 5972) equipped with a SPB-20 column (30m × 0.25 mm, Supelco). The GC system was coupled to both a flame ionization detector (FID, general detector) and a flame photometric detector (FPD, specific sulfur detector). 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.

Microdistillations were carried out according to the standard test for boiling range distribution of petroleum fractions by gas chromatography, ASTM D 2887-89. Organic sulfur content on diesel fuel were determined by X-ray fluorimetry in a Horiba X-ray fluorimeter. Total petroleum hydrocarbons (TPH) were estimated by the USEPA 8015 method (modified).

Results

Straight-run diesel fuel, obtained from primary distillation and containing 1.6% sulfur, was oxidized with chloroperoxidase in the presence of 20 mM KCl and 1 mM hydrogen peroxide. The gas chromatographic analysis with both flame

TABLE 2. Mass Spectral Data of Products^a

substrate	product	mass spectral ions (m/z)
benzothiophene	benzothiophene sulfone	166 (42) [M ⁺], 138 (9), 137 (100), 118 (15), 109 (48), 90 (14), 89 (16), 76 (15), 75 (15), 74 (14), 65 (9), 63 (13)
diphenyl sulfide	diphenyl sulfone	218 (27) [M ⁺], 125 (100), 97 (26), 77 (53), 51 (47), 50 (16)
dibenzothiophene	dibenzothiophene sulfone	216 (100) [M ⁺], 187 (46), 160 (31), 150 (16), 139 (30)
thianthrene	5-thianthrene oxide	232 (16) [M ⁺], 184 (100), 171 (15), 139 (14), 69 (14)
	5,10-thianthrene dioxide	248 (77) [M ⁺], 200 (86), 184 (84), 171 (100), 168 (23), 139 (30), 108 (24), 69 (36)
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)
bithiophene	dichlorobithiophene	238(15), 236 (72) [M ⁺], 234 (100), 201 (36), 164 (45), 157 (28), 155 (76), 142 (10), 119 (21), 93 (17), 82 (19), 79 (14), 69 (30)
	trichlorobithiophene	272 (37), 271 (11), 270 (100) [M ⁺], 233 (58), 198 (81), 191 (53), 189 (82), 163 (15), 154 (33), 135 (18), 119 (37), 103 (19), 93 (39), 81 (46), 79 (58), 69 (36), 58 (12)
	tetrachlorobithiophene	308 (13), 306 (49), 304 (96) [M ⁺], 302 (71), 267 (52), 232 (44), 223 (39), 197 (22), 188 (30), 162 (12), 153 (76), 117 (72), 98 (11), 93 (36), 81 (74), 79 (100), 69 (26)

^a Values in parentheses are relative abundances.

TABLE 3. Specific Activity of CPO with Organosulfur Compounds

		λ_{det} (nm)	IP (eV)	specific activity (min^{-1})
1	thianthrene	254	7.80	1310 (\pm 132)
2	2,2'-bithiophene	300	7.83 ^a	840 (\pm 8)
3	diphenyl sulfide	248	7.88	831 (\pm 32)
4	dibenzothiophene	232	8.39	126 (\pm 9)
5	benzothiophene	226	8.73	557 (\pm 42)
6	ethyl phenyl sulfide	254	8.80	1725 (\pm 145)
7	benzenethiol	238	8.90	116 (\pm 5)
8	thioanisole	256	8.95	2917 (\pm 58)
9	diphenyl disulfide	240	9.40	352 (\pm 10)

^a IP measured by charge transfer (25).

TABLE 4. Specific Activity of CPO with Aromatic Compounds

		λ_{det} (nm)	IP (eV)	specific activity (min^{-1})
1	azulene	270	7.43 ^a	676 (\pm 34)
2	9-methylanthracene	254	7.46	758 (\pm 27)
3	anthracene	250	7.51	134 (\pm 14)
4	biphenylene	248	7.56 ^a	10 (\pm 0.5)
5	2-methylanthracene	248	7.70	107 (\pm 8)
6	pyrene	236	7.72	53 (\pm 6)
7	acenaphthene	226	7.73 ^a	65 (\pm 8)
8	fluorene	260	7.91	1.9 (\pm 0.13)
9	fluoranthene	236	7.95 ^a	3 (\pm 0.2)
10	phenanthrene	250	8.07	7 (\pm 0.1)
11	triphenylene	256	8.10	0.8 (\pm 0.09)
12	naphthalene	220	8.18	0.6 (\pm 0.01)
13	biphenyl	250	8.64	NR ^b
14	dibenzofuran	280	8.77	NR ^b
15	anthrone	260	9.43	NR ^b

^a IP measured by photoelectron spectroscopy (25). ^b NR: no reaction detected.

ionization (FID) and flame photometric (FPD) detectors showed that chloroperoxidase was able to oxidize most of organosulfur compounds contained in the diesel fuel. The oxidation was detected by the increase of boiling point (retention time) of these compounds on the gas chromatogram monitored by the sulfur selective detector (FPD). Microdistillation of both chloroperoxidase-oxidized and untreated diesel fuels monitored by FID (general detection) and FPD (sulfur selective detection) (Figure 1) shows that the hydrocarbon distillation profile changes slightly after enzymatic treatment. In contrast, the specific sulfur detector (FPD) shows a significant change of the distillation profile, in which most of organosulfur compounds were effectively oxidized and their boiling points increased after enzymatic treatment.

Oxidized sulfur compounds can be removed by a distillation process (Table 1). After distillation, the sulfur content in the enzymatically oxidized diesel fuel is only 0.27%, while for the untreated fuel is 1.27%. The distillation of the straight-run diesel fuel (1.6% sulfur) to a final distillation point of 325 °C produced a distillate containing 66% of the total sulfur, while if the diesel fuel is previously oxidized with chloroperoxidase, the obtained distillate contained only 12% of the total sulfur. Thus, by using an enzymatic oxidation with chloroperoxidase coupled with a distillation process it is possible to obtain a diesel fuel with six times lower sulfur concentration than straight-run diesel fuel. Few hydrocarbons are also transformed during the enzymatic treatment, and after distillation an additional 12% of them remain in the residue (Table 1).

To know the chemical nature of the products from the enzymatic reaction, nine organosulfur compounds, including

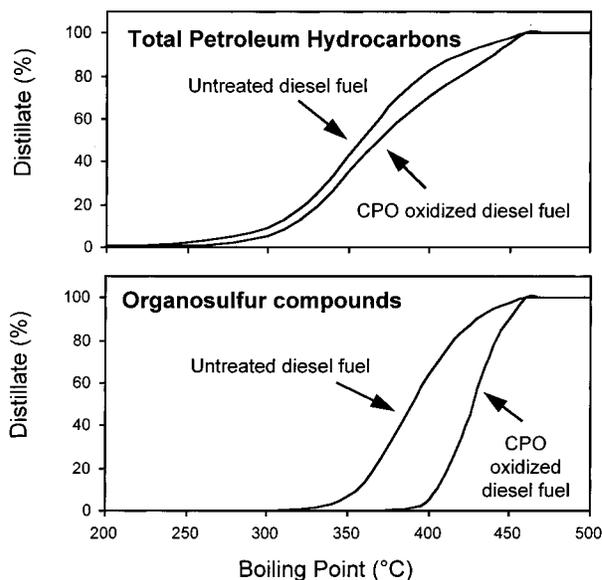


FIGURE 1. Microdistillation of untreated and chloroperoxidase-oxidized straight-run diesel fuel: FID, flame ionization detector (general detector) and FPD, flame photometric detector (sulfur selective detector).

thiophenes, organic sulfides, and thiols, and 15 aromatic compounds were tested for chloroperoxidase transformation. Table 2 shows the products identified by GC-MS. Products from all the organosulfur compounds were their respective sulfoxides and sulfones, except for biothiophene for which chlorinated derivatives were detected. Sulfones are the final product of CPO reactions; successive additions of both enzyme and H_2O_2 to complete substrate modification did not change the chemical nature of the products. In addition, sulfone standard compounds were not substrate for CPO as determined by GC and HPLC methods.

Aromatic hydrocarbons are also important constituents of diesel fuel. It is well known that chloroperoxidase is able to transform some PAHs (14, 24). Twelve of the 15 PACs tested were transformed by CPO in the presence of 1 mM H_2O_2 and 20 mM KCl, as monitored by HPLC. GC-MS analysis of the reaction products showed that the substrates were exclusively chlorinated during the reaction (Table 2). Furthermore, in the absence of chloride there was not observable reaction.

Tables 3 and 4 show the specific activity of CPO and IP values for the organosulfur and PACs compounds assayed. The ionization potentials (IP) taken are measured by electron impact, except for azulene, biphenylene, fluoranthene, and bithiophene (25). Figure 2 shows the correlation between IP values and specific activity for PACs and organosulfur compounds.

To determine the effect of the presence of a good substrate for halogenation, such as monochlorodimedone (MCD), thianthrene oxidation and pyrene halogenation reactions were performed in the presence of 0.1 mM MCD (Figure 3). Under these conditions, thianthrene was initially oxidized to form a sulfoxide with a significantly low rate (Table 3). Once MCD was exhausted, thianthrene oxidation rate became similar to that found in the absence of MCD (Figure 3a). In the case of pyrene, halogenation did not start until all MCD was halogenated, suggesting a strong affinity of MCD for the enzyme (Figure 3b). Under our experimental conditions, the specific activity of halogenation of MCD is 3480 min^{-1} . The specific reaction rate is only slightly affected in the presence of pyrene (3200 min^{-1}). On the other hand, the presence of thianthrene decreases the specific reaction rate for halogenation of MCD (2830 min^{-1}), while the initial rate for

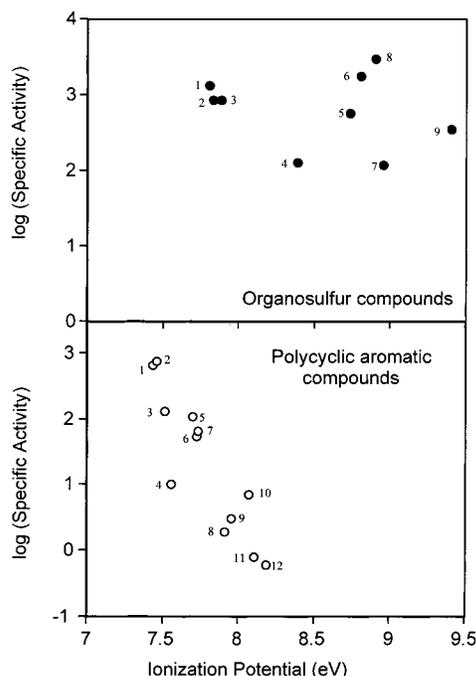


FIGURE 2. Influence of the substrate ionization potential on the specific activity of CPO. Substrates are organosulfur compounds (●) and polycyclic aromatic compounds (○). Numbers in superior and inferior panels correspond to those in Tables 3 and 4, respectively.

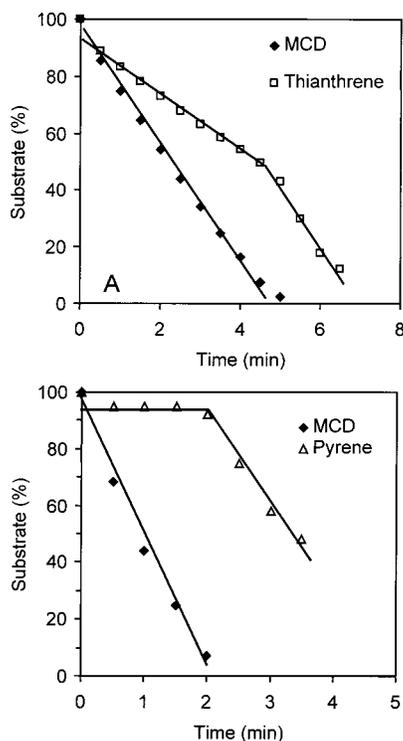


FIGURE 3. Competition between (A) MCD and thianthrene and (B) MCD and pyrene.

thianthrene oxidation is 300 min^{-1} , until MCD is exhausted. The addition of the MCD halogenation and thianthrene oxidation rates results in a value close to that obtained with MCD alone.

Kinetic constants for pyrene halogenation and thianthrene oxidation were determined (Table 5). Chloroperoxidase is a more efficient catalyst in the reaction of oxidation of thianthrene than in the reaction of halogenation of pyrene,

TABLE 5. Kinetic Constants for Thianthrene Oxidation and Pyrene Halogenation

substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\mu\text{M}^{-1} \text{s}^{-1}$)
MCD	94	1.4	67
thianthrene	64	1.5	44
pyrene	37	32	1.2

as can be seen from the catalytic efficiencies k_{cat}/K_M . Though both the affinity and the catalytic constant are higher for thianthrene, the main effect comes from the affinity of the enzyme for the substrate, which is 1 order of magnitude lower for pyrene.

Discussion

Enzymatic oxidation of diesel fuel allows the organosulfur compounds to be separated by a single distillation process. Chloroperoxidase from *C. fumago* is a very active enzyme able to perform transformation of complex oil fractions, such as diesel (7) and asphaltenes (8). Chloroperoxidase shows three different catalytic activities: halogenase, peroxidase, and catalase (26–28). In addition, some reports have claimed that chloroperoxidase catalyzes two-electron reactions (peroxygenase), which could be considered a kind of monooxygenase activity (29–32). Nevertheless, when organosulfur compounds such as thiophenes and organosulfides are substrates, mainly sulfoxides are formed by the peroxidase activity (Table 2 and Figure 1). All nine organosulfur compounds tested were oxidized by chloroperoxidase, even when the reaction system contained 20 mM KCl (Table 3), except for 2,2'-bithiophene from which halogenated derivatives were detected. These results are in agreement with previous work reporting that sulfoxides are produced from chloroperoxidase activity (30, 32, 33).

On the other hand, polycyclic aromatic compounds (PACs) are halogenated (Table 2). Other peroxidases, such as lignin peroxidase (19, 20) and manganese peroxidase (21, 22) and even hemoproteins with peroxidase activity (24, 34), produce mainly quinones from PAHs oxidation. Specific activity of chloroperoxidase on PACs halogenation shows a clear correlation with the substrate ionization potential (Figure 2). Because ionization potential could be defined as the energy involved in taking out one electron from the substrate molecule, this correlation suggests a one-electron mechanism with a free radical-mediated reaction. Only PACs with ionization potential lower than 8.52 eV were halogenated (Table 4). In general, the lower the ionization potential of the PAC, the higher the specific activity of the chloroperoxidase for that substrate (Figure 2). The ionization potential value of 8.52 eV appears to be a threshold, as none of the compounds tested having higher ionization potentials were transformed by chloroperoxidase. This threshold value is significantly higher than those reported for other peroxidases. Lignin peroxidase is able to oxidize PAHs and form quinones up to a PAHs ionization potential of 8.0 eV (20), and manganese peroxidase from *P. chrysosporium* shows a threshold value for PAHs substrates of 8.1 eV (22). Interestingly, no clear correlation could be found between the ionization potential and the specific activity for organosulfur compounds (Figure 2). In fact, we were not able to find a single organosulfur compound, thiophene or sulfide, which is not transformed by chloroperoxidase.

A possible production of chlorinated derivatives from PAHs by CPO reactions is an undesirable side of the process. However, as shown in Tables 3 and 4 (enzyme activity on single substrates) and in Table 5 (affinity constants, K_M , for thianthrene and pyrene), organosulfur compounds are better substrates and therefore can compete favorably with PAHs. This means that in a mixture containing both types of

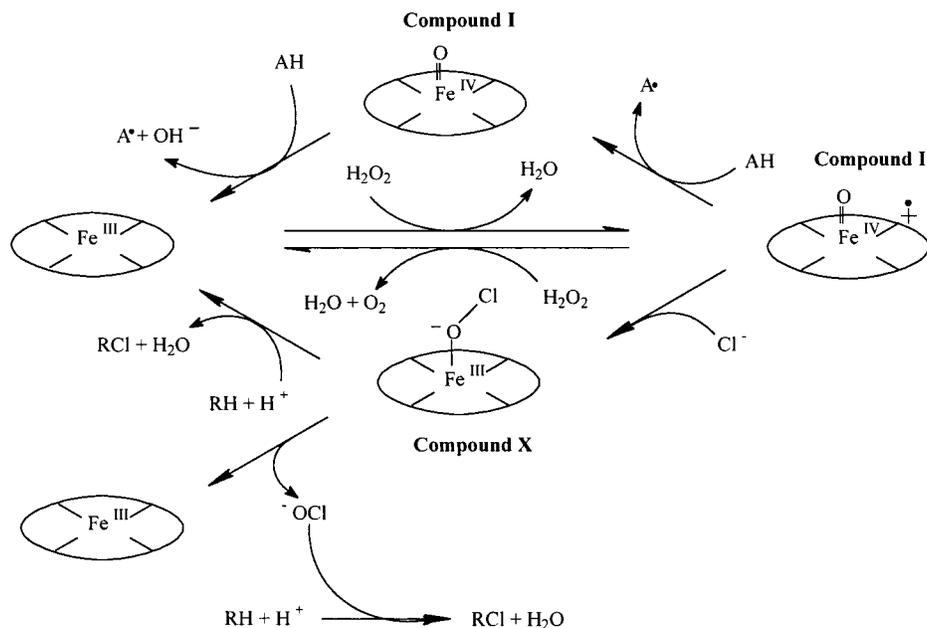


FIGURE 4. Proposed catalytic cycle of chloroperoxidase.

compounds, the sulfur compounds would be preferentially oxidized by CPO. Nevertheless, the reaction conditions and the biocatalyst preparation should be designed to minimize the halogenation reactions because of the environmental implications of chlorinated aromatic compounds.

So far, the catalytic cycle of chloroperoxidase is not completely elucidated (15, 16, 18, 35). The proposed mechanism (Figure 4) includes a first activation step, in which hydrogen peroxide transforms the (Fe^{III})porphyrin group (native state) to oxo(Fe^{IV})porphyrin radical cation (compound I). Then compound I can follow two ways: the oxidation of a substrate molecule to form an oxo(Fe^{IV})porphyrin without the associated porphyrin π -radical cation (compound II) or the reaction with a chlorine ion to form a ClO(Fe^{III})porphyrin group, called compound X, which is the only responsible for the enzymatic reaction of halogenation. In addition, this compound X seems also to be able to perform oxidation reactions liberating a chlorine ion. After both reactions, compound X returns to the native (Fe^{III})porphyrin state. From this proposed mechanism, it seems that the organosulfur compounds are able to react with both compound I and compound X, while PACs are only reactive to compound X. This is in agreement with our results, as when a high affinity halogenation substrate (MCD) is present in the medium, a slow thianthrene oxidation is found (Figure 3a). The oxidation rate is lower because most of compound I is rapidly transformed to compound X, due to rapid compound X turnover by MCD reaction. Thus the observed thianthrene oxidation is mainly mediated by compound X. When MCD is exhausted, thianthrene competes more favorably with the chlorine ions for compound I, its transformation involving both forms: compound I and compound X. This competition between a halogenation substrate (MCD) and a peroxidase substrate (catechol) has been previously reported (35). In this case, MCD quantitatively replaces catechol as a substrate for part of the enzymatic reaction. In contrast, and as expected, chloroperoxidase is not able to react with pyrene when MCD is present in the medium (Figure 3b), a situation that can be explained by the significant differences between the catalytic efficiencies of MCD ($k_{cat}/K_M = 55 \mu\text{M}^{-1} \text{s}^{-1}$) and pyrene ($k_{cat}/K_M = 1.2 \mu\text{M}^{-1} \text{s}^{-1}$). The main effect comes from the different affinity of chloroperoxidase for the substrates, whereas for MCD $K_M = 1.2 \mu\text{M}$, and for pyrene $K_M = 32 \mu\text{M}$,

1 order of magnitude lower. Under these conditions the available halogenating active sites are readily saturated by MCD; pyrene, which is unable to compete for compound X, is transformed only until MCD is exhausted.

Chloroperoxidase from *C. fumago* catalyzes the oxidation of most of organosulfur compound found in straight-run diesel fuel. This oxidation allows the desulfurization of diesel fuel by distillation. Sulfoxides and sulfones are the main products from CPO reaction on organosulfur compounds, while halogenated aromatic compounds are the only products from PACs reactions. Furthermore, PACs halogenation by chloroperoxidase seems to be dependent on the substrate ionization potential. In general, PACs with an ionization potential of $< 8.52 \text{ eV}$ were halogenated. Our results support a free radical mechanism for enzymatic halogenation and a catalytic cycle in which compound X [ClO(Fe^{III})porphyrin] could be responsible for both substrate halogenation and oxidation in a chlorine-dependent process.

The broad specificity and high activity of chloroperoxidase encourage further investigation in the use of this enzyme as an efficient catalyst in a desulfurization process, including an enzymatic treatment followed by a fractional distillation step. Sulfur removal from a very complex mixture, such as petroleum fractions, is far from being accomplished. Conventional hydrodesulfurization becomes expensive and less efficient as lower and lower sulfur levels are reached. The biotechnological process could be applied after a conventional desulfurization process in order to reach these new regulatory low-levels for sulfur content in fuels. At the moment, the use of chloride as an activator in this process seems unavoidable, since in this hydrophobic medium, chloroperoxidase presents very low activity and chloride greatly improves the reaction rate. Unfortunately, the presence of halogens would yield some environmentally undesirable products. Our research is currently focused on the protein engineering of chloroperoxidase in order to reduce its halogenase activity, maintaining or increasing the peroxidase activity. In addition, different approaches to improve the stability of chloroperoxidase are under research, such as genetic engineering and cross-linking of enzyme crystals. The stabilization of enzymes in non-conventional low-water content medium is a priority for the successful development of industrial enzymatic processes.

Acknowledgments

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