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CHLOROPEROXIDASE AND HYDROGEN PEROXIDE: AN EFFICIENT SYSTEM FOR ENZYMATIC ENANTIOSELECTIVE SULFOXIDATIONS.

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ABSTRACT. High enantioselectivities were obtained in chloroperoxidase catalyzed oxidation of organic sulfides (99% ee in the case of methyl 2-pyridyl sulfide) with H_2O_2 in aqueous buffer solution, pH 5, at 25°C. The kinetic parameters in the oxidation of a series of sulfides both with H_2O_2 and *tert*-butyl hydroperoxide were determined and the data are consistent with enzymatic oxidation involving presumably a ternary complex. In all cases the reaction afforded the (R) sulfoxide as predominant or exclusive enantiomer.

INTRODUCTION

Enantiomerically pure sulfoxides are important synthons for the asymmetric synthesis of natural products and rank among the most powerful stereodirecting groups. For these reasons numerous studies have been devoted at chemical methodologies leading to this structural unit and good to excellent stereoselectivities have been reported for the oxidation of organic sulfides to the corresponding sulfoxides. [2] On the other hand, the enzymatic oxidation of organic sulfides is an important process in the mammalian detoxification of xenobiotics; however relatively little information is available on this matter.[3]

In general, organic sulfides are oxidized both in vitro and in vivo by heme monoxygenases of the cytochrome P-450 type.[4a] Alternatively, flavoenzyme monooxygenases can be used not only for the oxidation of alkyl sulfides,[4b] but also of 1,3 dithiolanes and 1,3-oxathiolanes.[4c] Sulfides are also oxidized by a large group of non-heme metalloenzymes, whose oxygenation involves iron or copper as an essential active site

metal. [5] While cytochrome P-450 and flavin dependent monoxygenases have been intensively studied at the molecular level, the understanding of the catalytic behaviour of non-heme metalloenzymes is poor.

Horseradish peroxidase (HRP) has been the most investigated heme-containing peroxidase. According to Nakano and co-workers [6] HRP shows no stereoselectivity in the oxidation of *p*-tolyl methyl sulfide with H_2O_2 to the corresponding sulfoxide. More recently Blee and Schuber [7] have examined the stereochemical course of the enzymatic oxygenation of *p*-tolyl methyl sulfide catalyzed by a soybean hydroperoxide-dependent oxygenase.

Chloroperoxidase (CPO) (chloride peroxidase: hydrogen peroxide oxidoreductase, EC 1.11.1.10), a monomeric glycoprotein isolated from the mold *Caldariomyces fumago*, [8] is a heme protein possessing several diverse catalytic abilities. It catalyzes the peroxidative formation of carbon-halogen bonds using hydrogen peroxide as oxidant [9] and a number of reactions peculiar to catalases [10] and peroxidases. [11] Very recently it has been used by us [12] and by others [6] in the peroxide dependent enantioselective oxidation of organic sulfides to the corresponding sulfoxides.

The effects of variation of substrates, oxidants, pH of the aqueous buffer solution and other factors controlling the enantioselectivity have been studied.[12] Our previous results indicated that at 4 °C *t*-BuOOH was the most stereoselective of oxidants tested. Substrate steric effects were important. For instance, *p*-substituted phenyl alkyl sulfides are oxidized in higher enantioselectivity and higher yield than *o*-substituted phenyl alkyl sulfides. In all cases examined the prevailing sulfoxide had the (R) absolute configuration.

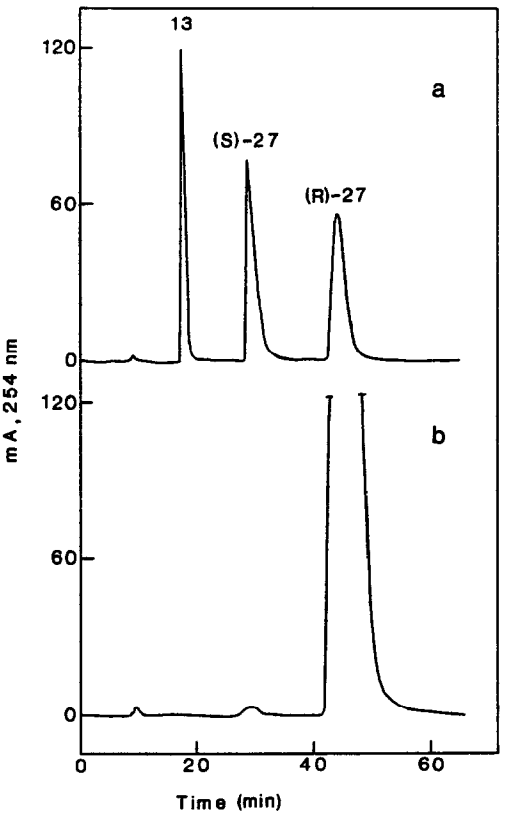
In this paper we investigate in more detail the use of hydrogen peroxide as the oxidant, and the relative contribution of the enzymatic and the spontaneous oxidation in a series of sulfides both with H_2O_2 and with *t*-BuOOH at 25°C. We also determined the kinetic parameters for these reactions

$$\underset{(1)\text{-}(14)}{(R')\text{-}Ar\text{-}S\text{-}R} \xrightarrow[CPO]{[\,O\,]} \underset{(15)\text{-}(28)}{(R')\text{-}Ar\text{-}SO\text{-}R}$$

RESULTS

Stereochemical studies. The oxidation of several sulfides by

hydrogen peroxide and *tert*-butyl hydroperoxide, in the presence of chloroperoxidase was examined in buffered aqueous solution, pH 5, at 25° C. The crude products were purified by column chromatography and the ee was determined by optical rotation measurement, ^{1}H NMR spectroscopy and/or by HPLC analysis. All the sulfides and the corresponding sulfoxides were base-line separated by chiral HPLC. A representative chromatogram is shown in Figure. On Chiralcel OB the (S)-sulfoxides were eluted first, whereas on Chiralcel OD the elution order was the opposite.



Chiralcel OB chromatography of methyl 2-pyridyl sulfide (*13*) and of racemic methyl 2-pyridyl sulfoxide (*27*) (a), and of the reaction product obtained from CLP-catalyzed oxidation of *13* (b). Mobile phase: 85/15 *n*-hexane/2-propanol. Flow rate: 0.5 mL/min.

The comparison of the results reported in Table I with those previously described by some of us [12] and by others shows new aspects of the CPO-dependent S-oxygenation. Not only is the choice of oxidant crucial for the chemical yield and for the enantiomeric excess, but also the experimental procedure, particularly in the case

TABLE I: Chloroperoxidase Catalyzed Oxidation of Sulfides to Sulfoxides at 25 °C Using H_2O_2 or *t*-BuOOH as Oxidizing Agents.

	sulfide	sulfoxides	oxidant	yield(%)	ee(%)	control reaction, yield (%)
1	*p*-CH_3-C_6H_4-S-CH_3	*15*	H_2O_2	98	91	18
			t-BuOOH	80	70	24
2	*o*-CH_3-C_6H_4-S-CH_3	*16*	H_2O_2	27	33	13
			t-BuOOH	56	43	33
3	*p*-CH_3O-C_6H_4-S-CH_3	*17*	H_2O_2	72	90	16
			t-BuOOH	70	61	31
4	*o*-CH_3O-C_6H_4-S-CH_3	*18*	H_2O_2	24	27	23
			t-BuOOH	30	37	18
5	C_6H_5-S-CH_3	*19*	H_2O_2	100	98	30
			t-BuOOH	90	80	65
6	*p*-Cl-C_6H_4-S-CH_3	*20*	H_2O_2	77	90	13
			t-BuOOH	60	70	20
7	*o*-Cl-C_6H_4-S-CH_3	*21*	H_2O_2	33	85	13
			t-BuOOH	17	45	7
8	*p*-NO_2-C_6H_4-S-CH_3	*22*	H_2O_2	10	80	2
			t-BuOOH	16	80	2
9	*p*-CH_3-C_6H_4-S-C_2H_5	*23*	H_2O_2	50	68	15
			t-BuOOH	50	68	20
10	*p*-$CH_3C_6H_4$-S-C_3H_7	*24*	H_2O_2	53	5	22
			t-BuOOH	30	5	28
11	*p*-CH_3-CO-NH-C_6H_4-S-CH_3	*25*	H_2O_2	86	67	23
			t-BuOOH	86	70[b]	33
12	C_6H_5-CH_2-S-CH_3	*26*	H_2O_2	100	90	33
			t-BuOOH	73	55	50
13	2-pyridyl-S-CH_3	*27*	H_2O_2	100	99	26
			t-BuOOH	61	89	16
14	*p*-F-C_6H_4-S-CH_3	*28*	H_2O_2	100	97	27
			t-BuOOH	90	70	29

[a] The absolute configuration of all the sulfoxides was (R). [b] The ee=84% for the same reaction performed at 4°C was erroneously reported equal to zero in our previous paper. [12]

of H_2O_2. In fact, when hydrogen peroxide was added all at once, isolation of the product (without quenching with Na_2SO_3) after 12 h reaction time at 4°C afforded (R)-(+)-methyl *p*-tolyl sulfoxide in 48% chemical yield and 35% ee. Kobayashi et al.[6] for the same reaction performed in similar conditions at 27° C, found that (R)-sulfoxide *15* was formed in 12.7% ee. However, when the oxidant was added gradually at 4° C and the reaction quenched with sodium sulfite, the enantioselectivity increased to 89% ee (datum not reported in Table I). Even more important is the fact that by our new procedure a very high level of asymmetric induction and chemical conversion is obtained in the CPO catalyzed oxidation of methyl *p*-tolyl sulfide at 25 °C. Hydrogen peroxide as the oxidant was generally more efficient than *t*-BuOOH in terms of chemical conversion and of enantiomeric excess.

The controlled addition of H_2O_2 at 25°C also resulted in improved chemical and optical yield for the hindered sulfides, previously studied at 4°C.[12] In fact, not only ethyl *p*-tolyl sulfoxide *23*, but also *o*-substituted methyl phenyl sulfoxides *16*, *18* and *21* were obtained in higher chemical and optical yields. Even so almost no stereoselection was observed with the bulkier isopropyl *p*-tolyl sulfide *10* as substrate. This tallies with kinetic studies which showed that this compound is a poor substrate for chloroperoxidase. Electronic effects of the substituents in the phenyl ring remain relevant; electron donating or poor electron withdrawing groups gave sulfoxides in high ee. On the other hand a strong withdrawing group, such as the nitro group, markedly decreased the chemical conversion in the formation of the sulfoxide *22*, without affecting the enantioselectivity.

In all cases the chloroperoxidase catalyzed oxidation is in competition with the spontaneous oxidation of the sulfides by the oxidants, which leads to racemic sulfoxides at a rate dependent on the concentration and nature of the substrate and the oxidant. The data relative to the catalyzed and spontaneous oxidations for all the cases examined, determined by ^1H NMR spectroscopy and confirmed by HPLC analysis, are reported in Table I. The ee reported for the catalyzed reactions thus refer to the sulfoxides resulting from the two competitive processes, the enzymatic oxidation and the spontaneous one, this latter leading to racemic sulfoxides. When considering the contribution of the non enzymatic process, it must be remembered that the stereoselectivity for the chloroperoxidase catalyzed oxygenation could be very high. Unfortunately this contribution cannot be determined accurately because the actual

concentration of the oxidant is different for the catalyzed and the uncatalyzed reactions. In fact, in the presence of CPO the oxidant is used by the enzyme not only for sulfide oxidation but it is also consumed in a disproportionation reaction of a catalase type.[13a] Therefore, it is likely that the average concentration of the oxidant in the catalyzed reaction is much lower than in the uncatalyzed one. This explains why very high ee values can be obtained with CPO in spite of the significant spontaneous conversions observed in control experiments performed under apparently comparable conditions.

The exceptionally high reactivity of methyl phenyl sulfide *5* with *t*-BuOOH in the absence of the enzyme is difficult to account for and may be due to solubility factors.

Kinetic studies. The kinetic parameters obtained in the oxidation of several sulfides by H_2O_2 or *t*-BuOOH in the presence of chloroperoxidase are shown in Table II. For both oxidants, the k_{cat} values do not vary markedly in the series, with the exception of *11* (and also of *2* and *4* with H_2O_2), whereas significant differences are found in the K_m values. With the *o*-substituted sulfides *2* and *4* it was impossible to determine reliable K_m values due to their extremely high apparent affinity for the enzyme. In fact, at 1 μM concentration of either substrate the activity of chloroperoxidase was still maximal with both oxidants. It should be emphasized that the spontaneous oxidation of sulfides in the conditions used for kinetic experiments was low because their concentration in the medium (3-400 μM) was much lower than that employed in the preparative-scale experiments (9 mM). The k_{cat}/K_m values reported in Table II are in good agreement with the data previously reported by Kobayashi et al.[6] for *p*-substituted alkyl phenyl sulfides and H_2O_2.

The k_{cat} and K_m values for sulfides were higher with H_2O_2 than with *t*-BuOOH. However, if we consider k_{cat}/K_m, i.e. the second order rate constant for the enzyme-substrate reaction at substrate concentration much lower than K_m, the values with *t*-BuOOH are slightly higher than those with H_2O_2 even though the concentration of *t*-BuOOH employed was not saturating. In fact, the K_m value of chloroperoxidase for H_2O_2 was found to be in the 25-33 μM range depending on the sulfide, thus in the conditions used for kinetic studies the concentration of H_2O_2 (400 μM) was saturating. In contrast, the K_m value for *t*-BuOOH was 54-70 mM and therefore, because of the interfering effect of high concentrations of oxidant on spectrophotometric measurements, non-saturating concentrations of *t*-BuOOH (26 mM) had to be used. It can be calculated that for *t*-BuOOH

the real k_{cat} (and k_{cat}/K_m) values are approximately 4 times higher than those reported in Table II.

In the case of sulfide *1*, the effect of the product on the reaction rate was also studied. No inhibition was found for product concentrations 2-10 times the K_m value of *1*. This result is important from the practical point of view as it means that the accumulating product does not decrease the rate of the asymmetric reaction in preparative transformations.

Table II: Kinetic Parameters for Chloroperoxidase Catalyzed Oxidation of Sulfides by H_2O_2 and *t*-BuOOH.

sulfide	K_m (μM)		k_{cat} (s^{-1})		k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	
	H_2O_2	*t*-BuOOH	H_2O_2	*t*-BuOOH	H_2O_2	*t*-BuOOH
1	210	12	80	15	0.86	1.25
2	<1	<1	22	17		
3	700	32	105	8	0.15	0.25
4			18	16		
5	760	40	220	19	0.29	0.47
6	300	18	171	16	0.57	0.89
8	1200	98	107	10	0.09	0.10
9	2300	260	145	11	0.06	0.04
11	950	97	41	3	0.04	0.03
14	290	10	187	19	0.65	1.92

DISCUSSION

Chloroperoxidase is a rather versatile enzyme since it can catalyze the oxidative transformation of a variety of organic substrates and is unique in the ability to catalyze the chloride ion dependent chlorination of nucleophilic substrates.[13] During catalysis, chloroperoxidase can form the classical peroxidase intermediates, compounds I and II, which represent Fe(V) and Fe(IV) enzyme states.[14] Compound I has the spectral properties of an oxyferryl cation radical;[15] in the halogenation and catalase reactions it receives two electrons from the substrate (halide ion, H_2O_2 or ethanol) and is reduced to the ferric state.[9b,16] Alternatively, compound I can react with

one-electron donor substrates to form compound II, which returns to the ferric state by reaction with a second substrate molecule .[16] In the case of the S-oxygenation the actual mechanism of the reaction is not fully understood. While experiments with ^{18}O-labeled H_2O_2 show oxygen incorporation into the product,[6] it is not known whether ferryl oxygen transfer involves compound I or II. Kinetic studies performed on the CPO-catalyzed oxidation of thioanisole, benzyl methyl sulfide and thiobenzamide by H_2O_2 showed that the enzymatic reactivity is essentially independent of the oxidation potential and the nucleophilicity of the substrates.[17] This is consistent with, but does not prove the direct involvement of a highly reactive compound I intermediate in the S-oxygenation. Indeed compound II could be the ultimate oxygen donor in a process too fast for the sulfenium radical cation to isomerize.[6]

Although, as shown in a separate investigation (Casella et al., in preparation), the sulfides bind to chloroperoxidase near the heme active site, it is clear from the data in Table II that the nature of the oxidant has a marked effect on the apparent affinity of these substrates for CPO, and that the affinities are higher with the more lipophilic *t*-BuOOH. Reciprocally, the type of substrate influences the apparent affinity of the oxidant for the enzyme, as shown by comparison of the K_m data of H_2O_2 for CPO when using a sulfide (30 μM), pyrogallol (4.3 mM),[18] or monochlorodimedone (0.8 mM)[9a] as the substrate. Thus, a ternary complex between CPO, the oxidant and the sulfide is implicated in one of the key steps of the catalytic cycle. Subsequent steps lead to oxygen incorporation in the substrate. The nature of the peroxide substituent R has some effect on the reactivity, but little effect on the stereoselectivity. In analogy to other peroxidases[19] it is expected that formation of CPO compound I would be slower from *t*-BuOOH, because of its higher pK_a and lipophilicity, than from H_2O_2. On the other hand, the stereoselectivity could be generated in the oxygen transfer step and, therefore, substrate immobilization by the protein chain is probably the most important parameter controlling this aspect of the reaction.

The stereochemical course of the CPO-promoted sulfoxidations provides an additional argument in favor of a ferryl oxygen transfer to sulfur. In all cases examined chloroperoxidase led to the preferential formation of the sulfoxide having the (R) absolute configuration. The extent of asymmetric induction for a large series of alkyl aryl, dialkyl and heterocyclic sulfides indicates

that the ferryl group is a likely candidate as the oxidant species. Steric factors could prevent the more hindered sulfides from getting close enough to the oxoiron group of the enzyme to permit a direct oxygen transfer. This interpretation is in line with the mechanism proposed by Ortiz de Montellano for the CPO-dependent epoxidation of trans-[1-^{2}H]styrene, which proceeds by oxo transfer without any detectable loss of stereoselectivity. [20]

It is interesting to note that the same stereochemical course observed with CPO was found in the biotransformations of sulfides by rabbit liver microsomal P-450.[4a] However, a parallel between the sense of asymmetric induction in the oxidation at sulfur by chloroperoxidase and cytochrome P-450 should be considered with some caution. In fact, not only is the stereoselectivity found in the rabbit liver microsomal P-450 catalyzed reaction rather low, but (more important) also the preference for the (R) enantiomer could be influenced by the presence of several isoenzymes. [21]

Subtle changes in the reaction mechanism of the sulfoxidation reaction catalyzed by different heme monoxygenases and also differences in the active site environment, which controls the mode of binding of the sulfides, could have dramatic consequences on the stereochemistry of the process. Therefore, it is not surprising that the soybean sulfoxidase [7] catalyzes enantioselective oxygen transfer to *p*-substituted thioanisoles, leading preferentially to the corresponding (S) sulfoxides, i.e. the enantiomers of those obtained with chloroperoxidase.

In conclusion, our results show that the enantioselectivity in the chloroperoxidase oxidation reaction in aqueous buffer solution at pH 5 can be very high, even at room temperature, especially if hydrogen peroxide is used as the oxidant. The reaction is sensitive to steric and electronic factors and leads predominantly to the (R) sulfoxides. A ferryl transfer from compound I to sulfur is consistent with our results, but our data do not rule out the formation of a radical cation intermediate.

The asymmetric oxidations by chloroperoxidase compare favorably in terms of enantioselectivity with the same reactions performed by other heme monoxygenases. Presently, there is only one chemical oxidation method which leads to high ee in alkyl aryl sulfoxides.[2] However, it makes use of 50% molar equivalents of chiral catalyst.

EXPERIMENTAL SECTION

Materials. The sulfides used were methyl *p*-tolyl sulfide (*1*), methyl *o*-tolyl sulfide (*2*), *p*-methoxyphenyl methyl sulfide (*3*), *o*-methoxyphenyl methyl sulfide (*4*), methylphenyl sulfide (*5*), *p*-chlorophenyl methyl sulfide (*6*), *o*-chlorophenyl methyl sulfide (*7*), methyl *p*-nitrophenyl sulfide (*8*), ethyl *p*-tolyl sulfide (*9*), isopropyl *p*-tolyl sulfide (*10*), *p*-acetamidophenyl methyl sulfide (*11*), benzyl methyl sulfide (*12*), methyl 2-pyridyl sulfide (*13*), and *p*-fluorophenyl methyl sulfide (*14*).

Sulfides (*1*), (*3*), (*5*), (*6*), (*7*) and (*11*) were prepared according to Kobayashi et al.,[6] sulfides (*8*)-(*10*) and (*12*) according to Sugimoto et al.,[22] sulfides (*4*) and (*13*) according to Pitchen et al.,[23] sulfide (*2*) according to Vowinkel,[24] and sulfide (*14*) according to Holland and Carter.[25]

Chloroperoxidase from *Caldariomyces fumago* (RZ 0.6) was obtained from Sigma.

General Methods. The optical rotations were determined with a Perkin Elmer R241 polarimeter. The ^{1}H NMR spectra of the products were recorded in $CDCl_3$ on a Varian 390 instrument. Enantiomeric excesses were determined by ^{1}H NMR with the aid of $Eu(hfc)_3$ as a chiral shift reagent on a Varian XL 200 instrument or by chiral HPLC analyses.

Enzymatic Oxidations: Typical Procedure. The sulfide (0.42 mmol) and CPO (6.7×10^{-6} mmol) were magnetically stirred in 42 mL of aqueous citrate buffer solution, pH 5 at 25 ° C for 5 min. In the case of H_2O_2, the oxidant (0.84 mmol) in 4.3 mL of buffer solution, pH 5, was added in 1 h in 13 aliquots of 330 μL at 5 min intervals and the reaction continued for 5 min. When *t*-BuOOH was used, the oxidant was added at once and the reaction carried out for 22 h. Both reactions were finally quenched with sodium sulfite. Extraction with 4 portions (100 mL each) of diethyl ether and with 4 portions (100 mL each) of methylene chloride, followed by drying and evaporation of the organic solvents collected together, gave the crude product. When necessary the sulfoxide was purified by flash chromatography (SiO_2) with mixtures of diethyl ether and methanol as eluents.

Characterisation of the Sulfoxides. Sulfoxides were all known in the optically active form, with the exception of sulfoxide *25*, and the physical properties of our specimens were in agreement with those reported.[23,26]

HPLC Analyses. HPLC analyses were performed on Chiralcel OB (compounds *15-24*, *26-28*) or OD (compound *25*) columns employing a chiral stationary phase (Daicel), on a Jasco HPLC instrument (model 880-PU

pump, model 870-UV detector) using *n*-hexane/2- propanol 85/15 (75/25 for compound *22*) as the mobile phase. The flow rate was 0.5 mL/min and readings were made at 254 nm.The data were computed by a HP-3390A integrator

Kinetics. The kinetic experiments were carried out in 0.05 M citrate buffer, pH 5, at 25 °C, in 3 mL cuvettes, 1 cm path length. The reaction mixtures contained 2-10 nM chloroperoxidase, 400 μM H_2O_2 or 26 mM *t*-BuOOH and 3-400 μM sulfide. The oxidation of sulfides to sulfoxides was spectrophotometrically monitored using the following ε values (M^{-1} cm^{-1}): *1*, 8040 at 253 nm; *2*, 8350 at 253 nm; *3*, 8150 at 256 nm; *4*, 8220 at 256 nm; *5*, 5875 at 272 nm; *6*, 8650 at 256 nm; *8*, 10710 at 338 nm; *9*, 7720 at 253 nm; *11*, 8310 at 268 nm; *14*, 7810 at 260 nm. The K_m and V_{max} values were obtained from the initial rate measurements, using the equations of Wilkinson, [27] programmed on an Apple IIc computer. [28] The enzymatic rates were corrected for the small oxygenation rates in the presence of the oxidant alone.

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