Sulphoxidation reaction catalysed by myeloperoxidase from human leucocytes

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The oxidation of alkyl aryl sulphides by myeloperoxidase (MPO) at the expense of hydrogen peroxide was investigated under steady-state conditions. The sulphide concentration effect was studied under saturating H_2O_2 concentrations at pH 5.0 and 20 °C. The kinetic constants, k_{cat} and K_m , of the different substrates were determined and the values were in the 1–10 s^{-1} range and around $43 \pm 26 \mu\text{M}$ respectively, whatever the sulphide considered. In the case of *p*-substituted thioanisoles, the oxidation rate was dependent upon the substituent effect. The correlation of $\log(k_{\text{cat}})$ with the substituent constants (σ^+ values) (Hammett equation) could be explained by a reaction mechanism involving the enzyme compound II and a sulphenium radical cation. This conclusion was also supported by spectrophotometric analysis of catalytic intermediates of the enzyme, showing the accumulation

of compound II. Moreover, chiral HPLC analyses showed that MPO oxidation of alkyl aryl sulphides produced the corresponding (*R*)-sulphoxides with a low enantioselectivity (4–8 %). Chloride ion effects on the MPO-catalysed oxygenation of sulphides were also studied. Chloride acted as a substrate for MPO and as an activator in MPO-catalysed sulphoxidation. Inhibition occurred at chloride concentrations above 120 mM, whereas below 120 mM, chloride increased the reaction rate when using *p*-tolyl methyl sulphide as the substrate. In the presence of 100 mM chloride the catalytic efficiency (k_{cat}/K_m) of MPO increased 3–4-fold, whatever the sulphide considered, but racemic products were obtained. These data have been interpreted in the light of known structural information on the accessibility of the distal haem cavity.

INTRODUCTION

Among xenobiotics, organosulphur compounds are an important class of environmental chemicals the activity of which is often modified by oxidative metabolism. The enzymic sulphoxidation of organic sulphides is an important process in the mammalian detoxification of xenobiotics [1]. Enzymic sulphoxidation produces metabolites that show increased water solubility, leading to enhanced excretion. Sulphoxidation can also produce species that are chemically more reactive than the starting substrate and which are responsible for direct cytotoxicity or for toxicity elicited by binding to a protein resulting in induction of an immune response [2,3]. However, little information is available on the reaction mechanisms of these processes.

Peroxidases are haem-containing enzymes that can catalyse the oxygenation of organosulphur compounds at the expense of hydrogen peroxide, as shown in *in vitro* studies performed by many research groups with horseradish peroxidase (HRP) [4–11], chloroperoxidase (CPO) [5,12] and lactoperoxidase (LPO) [13,14]. The catalytic cycle of peroxidases involves two intermediate forms of the enzyme, compounds I and II (CI and CII), which represent states that are more oxidized by two and one electrons respectively than the native ferric form. Peroxidases catalyse the S-oxygenation of alkyl aryl sulphides with sometimes remarkable enantioselectivities which result from the interaction of sulphides with a chiral environment near the haem active site [15]. Indeed, peroxidases produce chiral sulphoxides to a degree

that is dependent on the structure of the sulphides and on the active site, which controls the mode of binding of the sulphides [8]. The prevailing sulphoxide has the *R* absolute configuration on reaction with CPO [6] and LPO [14], and the *S* configuration with HRP [7,9,10], soybean peroxxygenase [16] and microperoxidase [17].

There is evidence from ^{18}O labelling studies that quantitative oxygen atom transfer from the peroxide to the sulphide is catalysed by HRP [4,5], CPO [4,5,18] and LPO [13]. Two mechanisms, called oxene and rebound, are to be considered [5]. The first mechanism involves direct oxygen transfer from CI to the sulphur atom by an oxene process in which CI undergoes a two-electron reduction:

where MPO is myeloperoxidase and Ph denotes the benzene ring. The rebound mechanism is a two-step mechanism: a single-electron transfer to CI, with concomitant formation of a sulphur radical cation ($\text{S}^{\cdot+}$) and CII, is followed by the recombination associated with oxygen transfer:

The rebound mechanism has been proposed for S-oxygenation by cytochrome *P*-450 [19], HRP [8–11,20] and soybean peroxxygenase [16], while direct oxygen transfer from CI was hypo-

Abbreviations used: HRP, horseradish peroxidase; CPO, chloroperoxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; CI, compound I; CII, compound II; CIII, compound III; Ph, benzene ring.

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thesized for the S-oxygenation of alkyl aryl sulphides by LPO [13], CPO [8] and prostaglandin H synthase [21]. Thus the differences in the mechanisms of sulphoxidation reactions catalysed by peroxidases and related haemo-enzymes most probably reflect differences in the active-site environment, which controls the mode of binding of the sulphides and the stereochemistry of the process.

In vivo, organic sulphides are in general oxidized by haem-containing monooxygenases of the cytochrome *P*-450 type [22] and by flavin mono-oxygenases [23]. MPO from activated leucocytes has been implicated in the metabolism of various drugs and xenobiotics, including those containing sulphur functional groups, e.g. methimidazole, propylthiouracil and tenoxicam [3]. Indeed, MPO shares similar properties with the classical haem peroxidases that catalyse H_2O_2 -dependent one-electron oxidation of organic and inorganic substrates, generating radical species. MPO differs from HRP in that Cl is a strong oxidant and can react with chloride ions to produce HOCl, a potent bactericidal agent, and regenerate native enzyme by a one-step oxygen transfer [2,24]. Moreover, the three-dimensional structure of MPO is known at 2.3 Å (0.23 nm) resolution [25,26] and its gene has been cloned, allowing site-directed mutagenesis studies to be carried out [27].

To compare the catalytic mechanisms of peroxidases, the present paper aims to shed light on the mechanism of S-oxygenation of organic sulphur compounds catalysed by MPO purified from human neutrophils. The problem has been addressed by determining the kinetic parameters (k_{cat} and K_m) for the oxidation of a series of alkyl aryl sulphides. The effects of substituents on the rates of S-oxygenation of *p*-substituted thioanisoles catalysed by MPO and the stereochemistry of the sulphoxidation reaction were also studied. In an attempt to identify the catalytic species of MPO involved in the oxidation of sulphide, spectral studies monitoring the changes in the enzyme state during the reaction were conducted. The whole set of results suggests a two-step oxygen transfer mechanism, with the formation of a sulphenium radical cation intermediate. Moreover, the effects of chloride ions on the kinetic parameters of MPO-catalysed sulphoxidation and on the stereochemistry of this oxidation were investigated.

MATERIALS AND METHODS

Chemicals

Alkyl aryl sulphides were obtained from Aldrich Chemical Co., and 20 mM stock solutions were prepared daily in 98 % (v/v) ethanol. Ethanol is necessary to solubilize the sulphides in the aqueous reaction mixture. Corresponding sulfoxides were prepared by reaction with $NaIO_4$ in aqueous methanol solution as described in [28] and were isolated by column chromatography on silica gel. The purity of these compounds was checked by HPLC and they were identified by NMR spectrometry. Hydrogen peroxide, obtained as 30 % solution from Sigma, was freshly diluted and the concentration determined by absorbance at 240 nm using a molar absorption coefficient of $43.6\text{ M}^{-1}\cdot\text{cm}^{-1}$ [29]. For the buffer solutions, the chemicals were used without further purification. Aqueous solutions were prepared using water purified through the Milli-Q system (Millipore Corp.). All other reagents were of suitable purity and grade.

Enzyme

Human neutrophil MPO was isolated as described in [30] and purified using a combination of modified procedures [30–32]; C. Capeillère-Blandin, unpublished work (details available on ap-

Table 1 k_{cat} and K_m values determined from fits of the data for sulphoxidation of alkyl phenyl thioethers by MPO

Data were obtained under the experimental conditions described in the legend to Figure 2 (curve a).

Sulphide		k_{cat} (s^{-1})	K_m (μM)	$10^5 \times k_{cat}/K_m$ ($\text{M}^{-1}\cdot\text{s}^{-1}$)
Number	Formula/name			
1	<i>p</i> -NH ₂ -Ph-S-CH ₃	10	57	1.7
2	<i>p</i> -CH ₃ O-Ph-S-CH ₃	0.43	12	0.4
3	<i>p</i> -CH ₃ -Ph-S-CH ₃	3.0	32	0.9
4	Ph-S-CH ₃	4.0	20	2
5	<i>p</i> -Cl-Ph-S-CH ₃	5.0	27	1.8
6	<i>p</i> -CH ₃ CO-Ph-S-CH ₃	4.2	16	2.6
7	<i>p</i> -NO ₂ -Ph-S-CH ₃	1.8	66	0.3
8	Ph-CH ₂ -S-CH ₃	1.0	83	0.12
9	Ph-S-CH(CH ₃) ₂	7.3	41	1.8
10	Benzothiophene	1.0	81	0.12

plication to the author)). The enzyme preparation used in this study exhibited a purity index (A_{428}/A_{279}) equal to or higher than 0.8. The concentration of isolated MPO was determined spectrophotometrically at 428 nm using a molar absorption coefficient of $89\text{ mM}^{-1}\cdot\text{cm}^{-1}$ per haem subunit [31].

MPO-catalysed oxidation of sulphides

To determine k_{cat} and K_m values for sulphoxidation, kinetic experiments were carried out in 50 mM acetate buffer, pH 5.0, and at 20 °C. The reaction mixture contained 0.2 μM MPO, 150 μM H_2O_2 and sulphide (0, 10, 20, 40, 60, 80, 100, 120 or 150 μM) in a 400 μl cuvette with a 2 mm optical path. The reaction was initiated by the addition of H_2O_2 and the rate of S-oxidation was followed by monitoring the decrease in the absorbance of the sulphides recorded over 5 min. The rate of oxidation in the absence of MPO was negligible. Initial rates were calculated by using the differences in the millimolar absorption coefficients ($\Delta\epsilon$) between the sulphides and the corresponding sulfoxides. $\Delta\epsilon$ values ($\text{mM}^{-1}\cdot\text{cm}^{-1}$) and the monitored wavelengths (nm) were as follows: **1**, 8.57 (254); **2**, 8.15 (256); **3**, 8.04 (253); **4**, 7.87 (254); **5**, 8.65 (256); **6**, 8.5 (296); **7**, 10.7 (338); **8**, 3.1 (225); **9**, 6.4 (245); **10**, 8.0 (256). Bold numbers refer to sulphide molecules listed in Table 1. All experiments were repeated at least three times. The steady-state kinetic parameters, k_{cat} and K_m , were determined by direct fit of the data to the Michaelis–Menten equation using a non-linear regression analysis program (Kaleidagraph).

Stereochemistry of sulphoxidation

To a 10 ml solution of MPO (0.2 μM) and sulphide (80 μM) in 50 mM acetate buffer (pH 5.0) was added H_2O_2 in 10 equal aliquots of 90 μl (final concentration 300 μM) over 30 min. After a total incubation time of 1 h, the solution was extracted with dichloromethane and the extracts were concentrated nearly to dryness under a stream of argon. The residue was applied on chiral OB and OD columns installed on an HPLC apparatus and eluted isocratically at a flow rate of $1\text{ ml}\cdot\text{min}^{-1}$, as in [12]. The HPLC effluent was monitored at 254 nm. On the OB column the retention times were 13.3 and 24.3 min for (*S*)- and (*R*)-*p*-Cl-Ph-S-CH₃ respectively, and 11.7 and 30.5 min for (*S*)- and (*R*)-*p*-CH₃-Ph-S-CH₃ respectively. On the OD column the retention times were 9.5 and 11.8 min for (*R*)- and (*S*)-*p*-Cl-Ph-S-CH₃

respectively, and 20.5 and 22.4 min for (*R*)- and (*S*)-*p*-CH₃-Ph-S-CH₃ respectively. Enantiomers were baseline-separated on both columns.

Absorbance spectra

Repetitive scans were taken every 0.5 min in the range 700–200 nm using a Kontron Uvicon 941 spectrophotometer during the steady-state sulfoxidation reaction occurring in the presence of 1 μ M MPO, 110 μ M *p*-tolyl methyl sulphide and 130 μ M H₂O₂ in 50 mM acetate buffer at pH 5.0 and 20 °C. The reaction was started by the addition of H₂O₂. Control measurements were performed without sulphide and/or in the presence of 100 mM NaCl.

RESULTS

Substrate effects on the sulfoxidation reaction

The effect of H₂O₂ concentration on the sulfoxidation of *p*-tolyl methyl sulphide catalysed by MPO has been examined at two different pH values in order to determine appropriate experimental conditions. Steady-state analyses with H₂O₂ as the variable substrate indicated that the maximal activity of MPO was shifted from 1 s⁻¹ at pH 7.0 to 3 s⁻¹ at pH 5.0 (Figure 1). Of additional interest was the finding that the *K_m* value determined for H₂O₂ was independent of pH and equal to 70 μ M, as previously demonstrated [33]. However, at pH 5.0, H₂O₂ at high concentrations inhibited the enzyme. The rate of sulfoxide formation increased as the H₂O₂ concentration increased up to 200 μ M, while a further increase in concentration resulted in a decrease in the rate of sulfoxide formation; in particular, 40 % inhibition was observed at 1 mM H₂O₂. To prevent inhibition of the sulfoxidation reaction, the concentration of H₂O₂ was kept at 150 μ M for further sulfoxidation studies performed at pH 5.0.

The next set of steady-state experiments was performed by varying the concentration of sulphide systematically. Figure 2 (curve a) illustrates the results obtained with thioanisole. Classical Michaelis–Menten kinetics were obtained for the variation of the initial rate of sulfoxidation with concentration in the range 0–240 μ M. Apparent *k_{cat}* and *K_m* values were deduced by non-linear least-squares fitting, and were 4 s⁻¹ and 20 μ M respectively

Figure 1 Effect of H₂O₂ concentration on the initial rate (*V/E*) of *p*-tolyl methyl sulphide oxidation at pH 5 and pH 7

Reaction mixtures contained 0.2 μ M MPO, 150 μ M sulphide and various concentrations of H₂O₂ in 50 mM acetate buffer, pH 5.0, or 50 mM phosphate buffer, pH 7.0.

Figure 2 Steady-state kinetics for the oxidation of thioanisole by MPO

The dependence of the initial rate of formation of sulfoxide (*V/E*) on thioanisole concentration is shown in the absence (curve a) and in the presence (curve b) of 100 mM NaCl. The lines through the data points were obtained by fitting the data to the Michaelis–Menten equation, and *k_{cat}* and *K_m* values corresponded to 4 s⁻¹/20 μ M and 12 s⁻¹/18 μ M in absence and presence of 100 mM NaCl respectively. Reaction mixtures contained 0.2 μ M MPO and 150 μ M H₂O₂ in 50 mM acetate buffer, pH 5.0, at 20 °C.

at pH 5.0 in the presence of 150 μ M H₂O₂. At high sulphide concentrations, enzymic inhibition was observed above 250 μ M, which was possibly a consequence of a substrate stacking effect. For example, 20 % inhibition was seen at 300 μ M sulphide.

The kinetic parameters *k_{cat}* and *K_m*, determined for the sulfoxidation of several *p*-substituted phenyl alkyl sulphides from Michaelis plots by considering the non-inhibited part, are given in Table 1. The sulfoxidation reaction was efficiently catalysed by MPO, as shown by the *k_{cat}* values, which were in the range 1–10 s⁻¹, depending on the sulphide. These values are 10-fold and 100-fold greater than those obtained with HRP and LPO respectively under similar pH conditions [7,14]. Interestingly, *K_m* values determined with MPO were found to be around 43 ± 26 μ M, whatever the sulphide considered. These values are unusually low compared with the values reported for halide substrates. A *K_m* of 25 mM was determined for chloride ions under the same conditions of pH and H₂O₂ concentration [34,35], and a value of 12.5 mM was reported for thiocyanate [36]. However, low *K_m* values (between 67 and 177 μ M) were reported for phenolic compounds at pH 7.4 [37], together with a *K_m* of 300 μ M for thiocyanate [38]. Finally, corresponding second-order rate constants with a mean value of (1.2 ± 0.9) × 10⁵ M⁻¹·s⁻¹ were similar to those obtained for CPO-catalysed sulfoxidation [12].

Interestingly, a relationship is seen between the *k_{cat}* values for oxidation by MPO and the electron-donating activity of the *p*-substituent of the phenyl ring. With the exception of methoxy-thioanisole, Hammet plots [39] relating the log of *k_{cat}*(i) for a *p*-substituted thioanisole over *k_{cat}*(h) determined for thioanisole itself [where (h) denotes a hydrogen atom as substituent and (i) denotes any other type of substituent] to the electron-releasing ability of the *p*-substituent showed a better linear correlation (*r* = 0.88) for the substituent constant σ^+ with a slope value (ρ) of −0.32 than for σ_p (*r* = 0.82) (results not shown). The negative ρ value was similar to that obtained for the reaction catalysed by HRP (ρ = −0.35) [10]. This correlation is consistent with a mechanism in which the substrate undergoes one-electron abstraction to generate a transient sulphenium radical cation intermediate [40] stabilized by an electron-releasing substituent. The slope factor ρ is a measure of the extent of charge

Figure 3 Spectral changes observed after mixing H_2O_2 with MPO and *p*-tolyl methyl sulphide

Upper panel, spectra recorded over 500 nm; lower panel, detail in the region of the α - and γ -bands. Final concentrations were: $0.9 \mu\text{M}$ MPO, $110 \mu\text{M}$ sulphide and $130 \mu\text{M}$ H_2O_2 in 50 mM acetate buffer at pH 5.0. Spectra were recorded before the addition of H_2O_2 (curve 1), after H_2O_2 addition (curve 2), and then every 0.5 min: curve 3, $t = 0.5$ min; curve 4, $t = 1$ min; curve 5, $t = 1.5$ min; curve 6, $t = 2$ min. Arrows indicate the direction of spectral changes.

Table 2 MPO complexes detected during the oxidation of *p*-tolyl methyl sulphide

The extent of the reaction was deduced from absorbance changes at 251 nm (see Figure 3, upper panel). The ratio of spectral changes at 625 nm and 454 nm, detected during the course of the sulphoxidation over 2.3 min (Figure 3, lower panel), allowed estimation of the relative amounts of CII and CIII. This ratio is equal to 0.20 for CII and 0.43 for CIII [41].

Spectrum no.	Reaction extent (%)	CIII (%)	CII (%)
1	0	0	0
2	45	0	100
3	58	7	93
4	68	20	80
5	94	25	75
6	100	30	70

development in the transition state, and the negative value is consistent with an electron demand on the substrate forming the sulphenium radical cation, as would be expected from eqn. (3).

Figure 4 Spectral changes recorded in the presence and absence of sulphide and NaCl

The conditions and curve numbering are as described in the legend to Figure 3. Top panel, reaction between $1 \mu\text{M}$ MPO and $150 \mu\text{M}$ H_2O_2 (arrows indicate the direction of spectral changes); middle panel, reaction between $1 \mu\text{M}$ MPO, $150 \mu\text{M}$ H_2O_2 and 100 mM NaCl; bottom panel, same reaction with the addition of $150 \mu\text{M}$ *p*-tolyl methyl sulphide.

Spectral changes during turnover

As shown in Figure 3, the addition of H_2O_2 to a mixture of MPO and methyl *p*-tolyl sulphide produced an initial shift in the γ -band enzyme absorbance spectrum from 430 nm to 454 nm, indicating that CII was by far the major form of the enzyme during turnover. Its presence was synchronized with spectral changes undergone by the sulphide at 251 nm, which allowed us to monitor the extent of sulphoxidation. Once 50 % of the

Table 3 Stereochemistry for the sulfoxidation of alkyl phenyl sulphides by MPO

Results are from quadruplicate experiments. The intra-assay S.D. of the HPLC system, obtained with samples with e.e. (enantiomeric excess) values of 4 and 8%, was between 0.8 and 1.0 for both columns.

Sulphide	NaCl (mM)	Yield (%)	e.e. (%)	Configuration
<i>p</i> -Cl-Ph-S-CH ₃	0	90	4.3 ± 1.3	<i>R</i>
<i>p</i> -CH ₃ -Ph-S-CH ₃	0	95	7.9 ± 1.2	<i>R</i>
<i>p</i> -CH ₃ -Ph-S-CH ₃	100	99	0	Racemic

reaction had occurred, CII appeared to become contaminated by the formation of CIII, mainly detectable due to a slight shift of the γ -band to 452 nm and to the development of a narrower α -band (Figure 3, lower panel). The A_{625}/A_{454} ratio was used to quantify the relative amounts of CII and CIII [41], as illustrated in Table 2. At the end of the reaction the absorbance ratio indicated that 30% of the enzyme had been converted into CIII. When the changes were stopped after a 2.3 min reaction, the slow return of the enzyme to the native state was observed over 6 min; 80% of the initial MPO was recovered. Thus sulphide and H₂O₂ partly converted MPO into CIII, which subsequently lost haem absorbance. Similar modifications were recently reported for the inactivation of MPO by 4-aminobenzoic acid hydrazide [42].

In order to confirm that sulphide was responsible for converting MPO into CII, control experiments were performed. Repetitive scans recorded during the reaction of MPO with H₂O₂ alone showed a transient formation of CII corresponding to 80% conversion, which occurred at the first scan after H₂O₂ addition and before the return of MPO to the native state (Figure 4, top panel). In contrast, when monitoring spectral changes in the presence of chloride, no CII was seen in the absence or in the presence of sulphide (Figure 4, middle and bottom panels).

Stereochemistry of sulfoxidation

The kinetic parameters of the oxidation of thioanisole and *p*-substituted derivatives by MPO are reported in Table 1. The enantiomeric excess of the products and their stereochemistry were established by HPLC comparisons with authentic sulphoxide standards using chiral OB and OD columns that afford excellent resolution of the sulphoxide enantiomers. We observed low enantioselectivity whatever the substituent considered (Table 3). Over an incubation time of 1 h, the reaction yield was greater than 90%. The enantiomeric excess ($R - S/R + S$) was found to be between 4 and 8%, and the sulphoxide formed in excess had an *R* absolute configuration. Enzyme enantioselectivity, though low, is real because the values were confirmed, for each sample, on two chiral columns with opposite elution orders: with the OB column the *S*-enantiomers were eluted before the *R*-enantiomers, while with the OD column the opposite was true. This should also rule out the possibility that the results were affected by the presence of some impurities that incidentally co-eluted with one of the enantiomers.

Effects of chloride on the sulfoxidation reaction

The effect of NaCl on sulphide oxidation by MPO was investigated. The rate of *p*-tolyl methyl sulphide oxidation was studied by varying the concentrations of chloride ions and sulphide systematically in the presence of a saturating concentration of hydroperoxide. The addition of chloride to the system containing

Figure 5 Activation of MPO-catalysed *p*-tolyl methyl sulphide oxidation at various concentrations of chloride

Reaction conditions were as follows: 50 mM citrate buffer, pH 5.0, 150 μ M H₂O₂, 0.2 μ M MPO and 10, 20, 40, 60, 80, 100, 120, 150, 200 or 300 μ M sulphide, with 0 mM (curve 1), 20 mM (curve 2), 80 mM (curve 3), 150 mM (curve 4) or 300 mM (curve 5) NaCl. The initial rates of sulphide oxidation (V/E) were measured by varying the sulphide concentration in the presence of NaCl.

Figure 6 Effect of chloride concentration on k_{cat}

Values for k_{cat} were determined by fitting the data in the non-inhibited section of the plots illustrated in Figure 5 (i.e. below 200 μ M sulphide) to the Michaelis–Menten equation. The line through the data points was obtained by fitting the data to a saturation curve with best-fit parameters of $k_{max} = 25 \text{ s}^{-1}$ and $K_m(\text{Cl}) = 22 \text{ mM}$.

MPO, H₂O₂ and sulphide resulted in an increase in the rate of sulphide oxidation (Figure 5). The rate enhancement was about 8-fold at 120 mM NaCl. The rate then began to level off as the chloride concentration was increased further. Fits of the data to the Michaelis equation, when considering the non-inhibited part of the plot, indicated that the K_m value was less affected by the chloride concentration. Although slight variations were noted in the 20–40 μ M range, with a mean of $28 \pm 6 \mu$ M, all apparent K_m values were equivalent within experimental error. Variations of the apparent k_{cat} values with NaCl concentration yielded a saturation plot with a half-maximal effect corresponding to a K_m for chloride of 22 mM (Figure 6). This value is close to the K_m value of 25 mM reported in the reaction with monochlorodimedon at pH 5.0 [35].

Table 4 summarizes the k_{cat} and K_m values determined in the presence of 100 mM NaCl for different sulphides. K_m values did not vary markedly, whereas significant differences were found in

Table 4 Effect of 100 mM NaCl on the kinetic parameters k_{cat} and K_m for sulphoxidation by MPO

Data were obtained under the experimental conditions described in the legend to Figure 2 (curve b).

Sulphide		k_{cat} (s^{-1})	K_m (μM)	$10^5 \times k_{\text{cat}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Number	Formula/name			
1	$p\text{-NH}_2\text{-Ph-S-CH}_3$	40	104	3.8
2	$p\text{-CH}_3\text{O-Ph-S-CH}_3$	6	30	2.0
3	$p\text{-CH}_3\text{-Ph-S-CH}_3$	20	40	5.0
4	Ph-S-CH_3	12	18	6.7
5	$p\text{-Cl-Ph-S-CH}_3$	35	67	5.2
6	$p\text{-CH}_3\text{-CO-Ph-S-CH}_3$	56	39	14
7	$p\text{-NO}_2\text{-Ph-S-CH}_3$	5.5	76	0.7
8	$\text{Ph-CH}_2\text{-S-CH}_3$	8	50	1.6
9	$\text{Ph-S-CH(CH}_3)_2$	14	26	5.4
10	Benzothiophene	1.6	72	0.2

the k_{cat} values. Finally, the catalytic efficiency (k_{cat}/K_m) of MPO for the sulphoxidation reaction increased by 3–4-fold, indicative of an activator effect of NaCl, whatever the sulphide considered.

DISCUSSION

Information on S-oxygenation, which is available for several other peroxidases, is reported here for the first time for MPO. Sulphoxidations of alkyl aryl sulphides are well established reactions catalysed by haemoproteins [15,43]. The two oxidation equivalents required for this reaction are stored in the well characterized CII, as an Fe(IV)=O porphyrin radical cation, and they must be recruited into a concerted process to catalyse the sulphoxidation, as illustrated by eqns. (1) and (2). However, the present data on the oxygenation of alkyl aryl sulphides by MPO provide kinetic evidence for a two-step oxygen-transfer mechanism involving a radical cation intermediate (eqns. 3 and 4). Indeed the predominant catalytic species is CII, as shown by monitoring the changes in absorbance spectra during the sulphoxidation of p -tolyl methyl sulphide. Support for this mechanism is provided by the Hammett plot correlation against σ^+ , with a ρ value of -0.32 indicative of the transient involvement of a sulphur radical species bearing a significant amount of positive charge.

Stereochemical results shed light on the subsequent step generating sulphoxide and native MPO (eqn. 4). The sulphoxides could arise either from a ferryl oxo species or from an oxidized halogen intermediate (Scheme 1). The existence of saturation kinetics with respect to sulphide concentration would be in

favour of a reaction between enzyme and sulphide, with a direct transfer of oxygen from CII to the substrate. However, as pointed out by Libby et al. [44] in their study of the CPO-catalysed oxidation of catechol and trimethylphenol in the presence of chloride: 'Saturation kinetics can be produced in a mechanism where the substrate reacts with an enzyme-generated freely dissociable intermediate'. Moreover, it should be taken into account that HOCl is a metastable species which, upon dissociation from the enzyme, can react non-enzymically with the sulphide. Considering the stereochemistry of oxidation, we found that, in the presence of 100 mM NaCl, a racemic sulphoxide was formed, whereas in its absence low but reliable enantioselectivity was observed. These results can be rationalized by assuming that the main reaction pathway involves the formation of hypochlorous acid, thus leading to racemic sulphoxides (Scheme 1). In this respect, Harrison and Schultz [45] have demonstrated conclusively that the immediate product of MPO-catalysed chloride ion peroxidation, HOCl, can diffuse freely from the enzyme active site. Indeed, Scheme 1 is consistent with the absence of spectral changes due to CII during the reaction in the presence of NaCl. The formation of sulphoxides with low enantiomeric excess could be due to a process involving oxygen transfer from CII to the sulphenium radical cation. Although MPO is a much better sulphoxidation catalyst than is HRP, and is similar to CPO, its enantioselectivity is lower than that of these two enzymes in the absence of NaCl [7,12]. Substrates to be oxidized by oxygen transfer must have direct access to the oxo-ligand of CII. The low stereospecificity in sulphoxidation reactions catalysed by MPO might suggest that the radical cation does not remain tightly bound to CII throughout the coupling process.

The mode of binding of sulphides is important to explain the origin of the low enantioselectivity observed in the oxygenation reactions. It seems likely that MPO has an active-site structure that can accommodate an aromatic group to give a complex [46,47]. Model building based on the three-dimensional structure of the enzyme [25,26] revealed the presence of a hydrophobic pocket at the entrance to the distal haem cavity, which suggested the possibility of favourable interactions with aromatic molecules [46]. In particular, the model proposed for the MPO-salicylhydroxamic acid complex [47] suggested that the aromatic ring of salicylhydroxamic acid is almost parallel to the haem plane, rather than having the perpendicular orientation indicated by NMR studies of HRP and LPO complexes [48,49]. The existence of an open active site in MPO could explain the high reactivity of MPO with the bulkier cyclopropyl phenyl sulphide, which can be accommodated by the hydrophobic binding site.

In conclusion, we have shown in the present study that NaCl increases the rate of the MPO-catalysed sulphoxidation reaction and that sulphoxides are produced mainly via the intermediate

Scheme 1 Proposed mechanism for MPO-catalysed sulphoxidation

formation of free hypochlorite. This mechanism is consistent with there being no direct interaction between the binding sites for the sulphide and enzyme-bound chlorinating intermediate, and implies only a diffusion phenomenon, as already found for CPO- and LPO-catalysed sulfoxidation [14,18].

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