The Action of Chloride Peroxidase on 4-Chloroaniline

N-OXIDATION AND RING HALOGENATION

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Chloride peroxidase catalyses both the ring halogenation and *N*-oxidation reactions of 4-chloroaniline by H_2O_2 and either KCl or KBr. In the absence of any halide salt only the *N*-oxidation reaction was observed, with the resulting conversion of 4-chloroaniline into 4-chloronitrosobenzene. The *N*-oxidation reaction proceeded even more rapidly in the presence of Cl^- or Br^- , in spite of the fact that ring halogenation was also a rapid reaction. The enhancement of *N*-oxidation was highly dependent on the pH of the media and displayed an optimum in the region of pH 3.5–4.0. No rate enhancement was observed above pH 5.5. KF partially inhibited the rate of *N*-oxidation in a pH-dependent manner. On the basis of calculated catalytic-centre activity the *N*-oxidation reaction was the major reaction at pH 3.5 or higher, whereas the ring-halogenation reaction became the major reaction below pH 3.5. In the presence of high concentrations of 4-chloroaniline relative to H_2O_2 the reaction intermediate, 4-chlorophenylhydroxylamine, was detected for the first time in a chloride peroxidase-catalysed reaction with this arylamine substrate. These findings were interpreted on the basis of current knowledge concerning the mechanism of action of chloride peroxidase.

Chloride peroxidase ('chloroperoxidase'; chloride–hydrogen peroxide oxidoreductase; EC 1.11.1.10) is a unique fungal enzyme, the properties of which have been well characterized by Hager and his co-workers (Shaw & Hager, 1961; Morris & Hager, 1966). A most unusual property is the ability of chloride peroxidase to introduce Cl^- , Br^- and I^- into a large number of organic substrates. The great abundance of halogenated natural products in numerous marine organisms has prompted recent investigations into the nature of the enzymes responsible for such transformations. Particularly abundant are brominated natural products; and the bromoperoxidases are currently a subject of great interest (Pederson, 1976; Theiler *et al.*, 1978; Baden & Corbett, 1980). Such enzymes are quite possibly very similar to chloride peroxidase (Fenical, 1975; Theiler *et al.*, 1978).

Our interest in chloride peroxidase and related enzymes resulted from our discovery that this enzyme possesses a rather unique ability to catalyse H_2O_2 oxidation of arylamines to aromatic nitroso compounds (Scheme 1; Corbett *et al.*, 1978, 1979). Before this finding no other enzyme system had been

known to effect this conversion in such a highly specific manner. The microsomal oxidase system in mammals is known to effect similar *N*-oxidations, although it lacks the high specificity for nitroso production that we observed for chloride peroxidase. Nevertheless, the strong similarity between chloride peroxidase and cytochrome *P*-450 with respect to certain physicochemical properties (Corbett *et al.*, 1978; Remba *et al.*, 1979) has prompted us to further investigate the *N*-oxidation activity of chloride peroxidase as a potential model for *N*-oxidation by microsomal oxidases.

Scheme 1. Conversion of 4-chloroaniline (I) and 4-chlorophenylhydroxylamine (II) into 4-chloronitrosobenzene (III)

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

In a previous study we reported that *N*-oxidation proceeded in the absence of any halide anion, but that the addition of KCl or KBr facilitated the reaction (Corbett *et al.*, 1978). Through the use of h.p.l.c. we have since discovered that the interaction of chloride peroxidase with arylamines in the presence of Cl⁻ or Br⁻ is more complex than originally reported. We have found that in addition to facilitation of *N*-oxidation by Cl⁻ or Br⁻, chloride peroxidase also catalyses ring halogenation of 4-chloroaniline in a process independent of and in competition with *N*-oxidation. The major variable that controls these two enzymic reactions of chloride peroxidase is pH.

Experimental

Chloride peroxidase (purified grade) was obtained from Sigma Chemical Co. and had a specific activity (Morris & Hager, 1966) of about 1500 units/mg of protein. Chemicals obtained from commercial sources were 4-chloroaniline, 2,4-dichloroaniline and 2,4,6-trichloroaniline, which were purified by re-crystallization from ethanol before use. Authentic 4-chloronitrosobenzene and other aromatic nitroso compounds (Table 1) were prepared by a previously described method (Lutz & Lytton, 1938) and purified as previously described (Corbett *et al.*, 1979). A stock solution of 30% H₂O₂ was assayed by the thiosulphate oxidation method (Pietrzyk & Frank, 1974). Solvents for chromatography were obtained from Burdick and Jackson Co. (Muskegon, MI, U.S.A.). All other reagents were of

A.C.S. analytical-reagent-grade purity. Silica gel for gravity-column chromatography was EM Silica Gel 60 (Brinkman Instruments, Westbury, NY, U.S.A.). H.p.l.c. was carried out on a system composed of a Waters Associates (Milford, MA, U.S.A.) model U6K septumless injector, model 6000A solvent pump and model 440 spectrophotometric detector. Prepacked h.p.l.c. columns and packing materials for guard columns were also obtained from Waters Associates. U.v. spectrophotometry was carried out with a Beckman model 24 spectrophotometer. N.m.r. spectra were obtained from a JEOL C-60 HL spectrometer, and i.r. spectra were obtained from a Perkin-Elmer Infracord. M.p. determinations were made with a calibrated Thomas-Hoover melting-point apparatus. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN, U.S.A.).

Synthesis of 2-bromo-4-chloroaniline

To a rapidly stirred solution of 12.8 g (0.1 mol) of 4-chloroaniline in 400 ml of 50% ethanol was added, in the course of 10 min, 11.2 g (0.07 mol) of Br₂ in 600 ml of water. After stirring for 15 min the product was collected by filtration, then chromatographed on 400 g of silica gel with chloroform. Re-chromatography of appropriate combined and evaporated fractions on 200 g of silica gel with hexane gave the desired product free from the dibrominated product. Re-crystallization from ethanol gave pale-pink needles with m.p. 64.5–65.0°C (literature value 65–67°C; Carlin & Larson, 1957). H.p.l.c. and elemental analysis for C₆H₅NBrCl indicated that the product was pure. The product showed the expected AMX pattern for the aromatic protons of 2-bromo-4-chloroaniline in ¹H n.m.r. spectroscopy (H at C-3 = 7.45 p.p.m., H at C-5 = 7.25 p.p.m. and H at C-6 = 6.70 p.p.m. downfield from tetramethylsilane; J₃₋₅ 2.0 Hz, J₅₋₆ 9.0 Hz).

Synthesis of 2,6-dibromo-4-chloroaniline

To a rapidly stirred suspension of 32 g (0.2 mol) of Br₂ in 1.0 litre of water was added 12.8 g (0.1 mol) of 4-chloroaniline in 200 ml of water. Stirring was continued for 15 min, then the precipitate was collected by filtration and partitioned between 200 ml of diethyl ether and water. After removal of solvent the residue was chromatographed on 200 g of silica gel with hexane as eluent. Fractions were combined on the basis of h.p.l.c. analysis and evaporated to give a residue that was re-crystallized twice from ethanol to give fine off-white-coloured needles with m.p. 94.0–94.5°C (literature value 92.9°C; Gilbert, 1926). H.p.l.c. and elemental analysis for C₆H₄NBr₂Cl indicated that the product was pure. The product showed the expected sharp singlet for the two equivalent aromatic protons of 2,6-dibromo-4-chloroaniline in ¹H n.m.r. spectro-

Table 1. Retention times for authentic standards in two h.p.l.c. systems

H.p.l.c. system 1 was a μ Bondapak C₁₈ column (30 cm $\times$ 3.9 mm internal diam.) with 70% methanol as eluant at a flow rate of 1.5 ml/min. H.p.l.c. system 2 was a μ Porasil column (30 cm $\times$ 3.9 mm internal diam.) with 0.05% methanol in iso-octane as eluent at a flow rate of 1.5 ml/min. Standard compounds for analysis with system 1 were dissolved in methanol, and standard compounds for analysis with system 2 were dissolved in iso-octane or hexane.

Compound	Retention time (min)	
	System 1	System 2
4-Chloroaniline	3.0	25
4-Chloronitrosobenzene	4.7	3.1
2-Bromo-4-chloroaniline	4.8	11.9
2,6-Dibromo-4-chloroaniline	9.2	3.9
2,4-Dichloroaniline	4.4	19.2
2,4,6-Trichloroaniline	7.7	2.7
2,4-Dichloronitrosobenzene	6.7	3.0
3,4-Dichloroaniline	3.8	18.0
3,4-Dichloronitrosobenzene	8.7	3.0

scopy (7.45 p.p.m. downfield from tetramethylsilane).

Incubation procedure

A solution of 4-chloroaniline and H_2O_2 with or without a potassium halide salt was prepared at twice the desired reaction concentration for each component in 0.05 M-potassium phosphate buffer of the desired pH. A solution of about $1\text{ }\mu\text{g}$ of chloride peroxidase/ml in the same phosphate buffer was prepared by appropriate dilution of an enzyme suspension to give approx. 1–2 units of enzyme activity/ml. An accurate determination of chloride peroxidase activity in such solutions was made by the standard method (Morris & Hager, 1966). After equilibration of the solutions to 22°C in a water bath, the reaction was initiated by combining equal volumes of the two solutions. The reaction was stopped at the desired time by dilution of a 1.0 ml portion with 1.0 ml of methanol precooled to -20°C . The quenched portion was kept at -20°C until h.p.l.c. analysis was performed.

H.p.l.c. analysis

Analyses were performed by injection of $10\text{ }\mu\text{l}$ of each quenched portion on to a system composed of a $\mu\text{Bondapak C}_{18}$ column ($30\text{ cm} \times 3.9\text{ mm}$ internal diam.) protected by a guard column of C_{18} -Corasil and with methanol/water (7:3, v/v) as eluent at a flow rate of 1.5 ml/min and with detection at 313 nm. A second h.p.l.c. system employed was a $\mu\text{Porasil}$ column ($30\text{ cm} \times 3.9\text{ mm}$ internal diam.) with a guard column of Corasil II, and with 0.05% methanol in iso-octane as the eluent at a flow rate of 1.5 ml/min and with detection at 313 nm. Before analysis on the second h.p.l.c. system, each quenched sample was extracted with an equal volume of iso-octane (2,2,4-trimethylpentane). Analysis was then made by injection of $10\text{ }\mu\text{l}$ of the iso-octane extract.

Identification of incubation products

Incubations carried out with a low concentration (0.25 mM) of 4-chloroaniline in the presence of 2.0 mM- H_2O_2 and either 20 mM-KCl or 20 mM-KBr were analysed on completion of the enzymic reaction, which was usually a period of 5 min. H.p.l.c. analysis by two chromatographic systems along with authentic standards (Table 1) provided presumptive evidence on the identity of the final products. Confirmation of each final product was achieved by u.v. spectrophotometry on incubations carried out under conditions that gave rise to only a single product. The quenched portions were extracted with an equal volume of iso-octane for u.v. analysis. Those incubations carried out at pH 6.0 or higher gave u.v. spectra identical with that of authentic 4-chloronitrosobenzene: λ_{max} . (iso-octane) 310 (ϵ 9900) and 288 nm (ϵ 11500). Incubations

carried out at pH 3.0 or lower in the presence of KCl gave u.v. spectra identical with that of 2,4,6-trichloroaniline: λ_{max} . (iso-octane) 304 nm (ϵ 3300), with a shoulder at 307 nm (ϵ 3200), and 241 nm (ϵ 9700). Incubations carried out at pH 3.0 or lower in the presence of KBr gave spectra identical with that of 2,6-dibromo-4-chloroaniline: λ_{max} . (iso-octane) 307–313 (ϵ 3600) and 243 nm (ϵ 10200).

A special large-scale incubation was carried out to prove the identity of 2-bromo-4-chloroaniline as the intermediate product of chloride peroxidase action on 4-chloroaniline in the presence of KBr. A solution of 0.4 mmol of 4-chloroaniline, 0.2 mmol of H_2O_2 and 2.0 mmol of KBr in 50 ml of 0.05 M-potassium phosphate buffer, pH 2.75, was mixed with 50 ml of the same buffer containing 100 units of chloride peroxidase activity. After 1 min reaction time the incubation mixture was extracted with 100 ml of diethyl ether, and the organic layer was treated with 10 ml of hexane, dried over Na_2SO_4 and reduced *in vacuo* to a volume of 2 ml. This extract was chromatographed on a silica gel column ($18\text{ cm} \times 1.1\text{ cm}$ bed volume) with 300 ml of hexane followed by 300 ml of chloroform/hexane (1:19, v/v), with 20 ml fractions being collected. Those fractions containing the suspected intermediate, as indicated by h.p.l.c., were combined. The u.v. spectrum of the combined fractions was identical with that of authentic 2-bromo-4-chloroaniline: λ_{max} . (hexane) 303 (ϵ 2900) and 247 nm (ϵ 11000). Evaporation *in vacuo* gave an oil that crystallized from 30% ethanol to give 5 mg of product with m.p. $64\text{--}65^\circ\text{C}$ (literature value $65\text{--}67^\circ\text{C}$; Carlin & Larson, 1957). The i.r. spectrum (KBr pellet) was superimposable with that of authentic 2-bromo-4-chloroaniline. A nearly identical procedure, but employing KCl in place of KBr, led to the isolation of 3 mg of 2,4-dichloroaniline with m.p. $60\text{--}61^\circ\text{C}$; λ_{max} . (hexane) 302 (ϵ 2800) and 242 nm (ϵ 10900). The i.r. spectrum (KBr pellet) of the isolated product was identical with that of authentic 2,4-dichloroaniline.

Detection of 4-chlorophenylhydroxylamine

Incubations of 8.0 mM-4-chloroaniline and either 0.25 mM- or 0.10 mM- H_2O_2 with 2 units of chloride peroxidase activity/ml in 0.05 M-potassium phosphate buffer, pH 4.5, with and without 20 mM-KBr, were analysed by h.p.l.c. after 30 s incubation periods and the usual quenching procedure. Portions ($10\text{ }\mu\text{l}$) were analysed on $\mu\text{Bondapak C}_{18}$ with 30, 40 and 50% methanol as eluents and with detection at 254 nm. Simultaneous analyses for retention times were made on freshly prepared solutions of authentic 4-chlorophenylhydroxylamine. Co-injections of incubation portions with authentic 4-chlorophenylhydroxylamine were also made to determine identical chromatographic

behaviour in the three solvents. A 10ml reaction portion was extracted with diethyl ether, and the diethyl ether was evaporated with a stream of N_2 . The residue was dissolved in 10ml of methanol, a portion of which was analysed by h.p.l.c., and the remainder was treated with 1.0ml of 0.1% $FeCl_3$ in methanol, which was then reanalysed by h.p.l.c., with 50% methanol as eluent and detection at 313nm. Identical experiments were carried out at pH 3.0 with 20mM-KBr in an attempt to detect a possible intermediate *N*-bromo-4-chloroaniline, in addition to 4-chlorophenylhydroxylamine.

Results

The incubation of 4-chloroaniline with chloride peroxidase and excess H_2O_2 resulted in the production of 4-chloronitrosobenzene and, depending on whether either Br^- or Cl^- was present, 2,6-dibromo-4-chloroaniline or 2,4,6-trichloroaniline. Under certain conditions, such as excess 4-chloroaniline or short reaction periods, 2-bromo-4-chloroaniline and 2,4-dichloroaniline were demonstrated to be present in the enzymic reaction mixtures. In the absence of either chloride peroxidase or H_2O_2 no reaction occurred. Elemental Br_2 did not convert 4-chloroaniline into 4-chloronitrosobenzene under identical conditions in the absence of enzyme, but did effect ring halogenation by the expected chemical reaction to give a mixture of 2-bromo-4-chloroaniline and 2,6-dibromo-4-chloroaniline. Because of the multiplicity of products when the enzymic reaction was carried out in the presence of Cl^- or Br^- , u.v. spectrophotometry could not be used to study the kinetics of the competing reactions.

The products resulting from the action of chloride peroxidase and H_2O_2 on 4-chloroaniline were determined quantitatively by using h.p.l.c. All major products were individually measured by using either an adsorption-mode h.p.l.c. system or a reversed-phase h.p.l.c. system. The retention times of authentic standard compounds in these two h.p.l.c. systems are recorded in Table 1. A typical h.p.l.c. chromatogram employing the adsorption system is illustrated in Fig. 1. Quantitative determinations were achieved by measurement of peak heights and comparison with peak-height curves prepared from authentic standards.

The assignment of peaks in a chromatogram to a specific compound was made on the basis of retention times in the two different h.p.l.c. systems, peak enhancement on co-injection of the sample with an authentic standard and by spectrophotometric methods. U.v. spectra were readily obtained for the final products of the enzymic incubations, and were found to be identical with the authentic compounds when the incubation was conducted at a pH that favoured the formation of a single final product (Fig.

Fig. 1. Representative h.p.l.c. chromatogram of a portion from chloride peroxidase incubation with 4-chloroaniline, H_2O_2 and Br^- .

H.p.l.c. conditions consisted of a μ Porasil column (30cm $\times$ 3.9mm internal diam.) with 0.05% methanol in iso-octane as eluent at a flow rate of 1.5ml/min. Before analysis the incubation portion taken after 2min reaction was quenched with an equal volume of methanol, and the aqueous methanol solution was then extracted with an equal volume of iso-octane. The injection of 10 μ l of the iso-octane layer resulted in the chromatogram shown when the detector wavelength was set for 313nm. Conversion of peak heights into total amount of each compound injected was achieved through use of a calibration curve prepared daily. An attenuation change was made at $t = 6$ min to increase detector sensitivity 5-fold. (a) 4-chloronitrosobenzene (86 ng); (b) 2,6-dibromo-4-chloroaniline (236 ng); (c) 2-bromo-4-chloroaniline (147 ng).

2). Thus the u.v. spectrum of the 5 min incubation of 4-chloroaniline with excess H_2O_2 and Br^- in the presence of chloride peroxidase at pH 6.0 or above was identical with that of 4-chloronitrosobenzene. The measurement of optical absorption at 310 and 288nm for this product indicated that 78% of the substrate had been converted into 4-chloronitrosobenzene, which was the same value for percentage conversion as determined by h.p.l.c. analysis (Fig. 2). Likewise, the 5 min incubation carried out at pH 3.0 gave a u.v. spectrum identical with that of 2,6-dibromo-4-chloroaniline and also indicated a 95% yield of this product on the basis of optical absorption measured at 313 and 243nm. The intermediate product, 2-bromo-4-chloroaniline, was isolated from a large-scale reaction and identified on

the basis of i.r. and u.v. spectrophotometry. In addition the chromatogram peak suspected to be this intermediate product was found to chromatograph in a manner identical with authentic 2-bromo-4-chloroaniline. The incubation of 2-bromo-4-chloroaniline under the usual conditions in the presence of Br^- gave 2,6-dibromo-4-chloroaniline as the exclusive product. Thus 2-bromo-4-chloroaniline is a discrete intermediate in the conversion of 4-chloroaniline into 2,6-dibromo-4-chloroaniline. For incubations carried out in the presence of Cl^- , 2,4-dichloroaniline was conclusively identified and also shown to be an intermediate in the conversion of 4-chloroaniline into 2,4,6-trichloroaniline.

Fig. 2. Effect of pH on final product composition after chloride peroxidase incubation with 4-chloroaniline in the presence of H_2O_2 and Br^-

Incubations were conducted at each pH in 3.0 ml of 0.05 M-potassium phosphate buffer containing 0.05 mM-4-chloroaniline, 2.0 mM- H_2O_2 , 20 mM-KBr and a total of 2.9 units of chloride peroxidase activity. A 1.0 ml portion was taken after 5 min and quenched with 1.0 ml of methanol, followed by extraction with 2.0 ml of iso-octane. Analysis of 10 μl portions of the iso-octane extract on a $\mu\text{Porasil}$ column with 0.05% methanol in iso-octane as eluent resulted in the detection of the two products 4-chloronitrosobenzene (●) and 2,6-dibromo-4-chloroaniline (○). The percentage conversion into each product was based on the concentration of 4-chloroaniline present at the start of the incubation.

Fig. 2 illustrates the effect of pH on the final-product composition when 4-chloroaniline was incubated with an excess of H_2O_2 and Br^- in the presence of chloride peroxidase. At pH 6.0 or above, *N*-oxidation to give 4-chloronitrosobenzene was the only reaction observed. At pH 3.0 or below only dibromination was observed, and no significant *N*-oxidation was evident at the low substrate concentration that was used. On the other hand, h.p.l.c. analysis of incubations after only 30 s reaction time clearly demonstrated that the monobromination product (2-bromo-4-chloroaniline) was also present in incubations carried out below pH 6.0 (Fig. 3). In the presence of excess H_2O_2 and Br^- , 2-bromo-4-chloroaniline was rapidly converted into the dibrominated product. This sequence of monobrominated, then dibrominated, product is also well represented by the time-course study presented in Fig. 4. The good material balance, as shown in Fig. 4, is a further indication of the identity of the intermediate and final products for chloride peroxidase action on 4-chloroaniline. The results and material balance illustrated by Fig. 4 also supported our conclusion that no other unidentified products

Fig. 3. Effect of pH on product composition after 30 s incubation of chloride peroxidase with 4-chloroaniline in the presence of H_2O_2 and Br^-

The incubation conditions and analytical procedure at each pH were identical with those described for Fig. 2, except that reaction time was only 30 s. Symbols: ●, 4-chloronitrosobenzene; ○, 2,6-dibromo-4-chloroaniline; ▲, 2-bromo-4-chloroaniline.

were produced in significant amounts during the course of this enzymic reaction.

The possibility that 2-bromo-4-chloroaniline might be converted into 2-bromo-4-chloronitrosobenzene was carefully investigated. Throughout the pH range of 2.5–7.0, no *N*-oxidation of 2-bromo-4-chloroaniline was detected when Br^- was present. Instead, conversion into 2,6-dibromo-4-chloroaniline was the only reaction observed. In the absence of Br^- , 2-bromo-4-chloroaniline was very slowly oxidized to the nitroso compound. The rate of *N*-oxidation of 2-bromo-4-chloroaniline was not determined since it was obviously too slow to compete with the bromination process that occurred rapidly when Br^- was present in the reaction mixture. The inability of chloride peroxidase to effect oxidation of 2-bromo-4-chloroaniline at a significant rate is in agreement with our finding that *N*-oxidation of arylamines is highly sensitive to steric

factors (Corbett *et al.*, 1979). As expected, 2,6-dibromo-4-chloroaniline was totally inert to *N*-oxidation or any other reaction catalysed by chloride peroxidase. On the other hand, 4-chloroaniline was readily oxidized to 4-chloronitrosobenzene by chloride peroxidase, since this arylamine substrate is not sterically crowded.

It was found that 2,4-dichloroaniline was partially converted into 2,4-dichloronitrosobenzene even when Cl^- was present in the reaction mixture; however, the production of 2,4-dichloronitrosobenzene during incubations of 4-chloroaniline with H_2O_2 and Cl^- in the presence of chloride peroxidase was undetectable in the rate studies that employed an excess of 4-chloroaniline and short reaction times. The more facile *N*-oxidation of 2,4-dichloroaniline compared with 2-bromo-4-chloroaniline is readily explained on the basis of steric factors.

It was also found that 4-chloronitrosobenzene was not halogenated by chloride peroxidase in the presence of Br^- or Cl^- . This was a reasonable observation in view of the low reactivity of this substrate towards electrophilic substitution, a feature that contrasts greatly with the high reactivity of arylamines towards electrophilic aromatic substitution.

The competing chloride peroxidase-catalysed reactions of halogenation and *N*-oxidation were found to be highly pH-dependent. Maximal reaction rates for each process were determined by using a saturating concentration of 4-chloroaniline and H_2O_2 in the presence of either Br^- or Cl^- over a broad pH range. The maximal rates of bromination and *N*-oxidation as a function of pH are illustrated in Fig. 5. The pH optimum for bromination occurred at 2.75, a finding in agreement with other reported halogenation reactions catalysed by chloride peroxidase (Thomas *et al.*, 1970a). The rate of bromination decreased rapidly as the pH was increased above 3.0, whereas the rate of *N*-oxidation was found to increase with increasing pH. The pH optimum for *N*-oxidation in the presence of KBr was found to be from 4.0 to 4.5, with further increases in pH resulting in a slow decline in the rate of *N*-oxidation. Bromination was not a detectable process at pH 5.5 and above, yet *N*-oxidation still occurred at the highest pH investigated of 7.0. A nearly identical pH dependency was found for incubations carried out with KCl. The major difference between incubations carried out with Cl^- or Br^- was the finding that both the rate of halogenation and of *N*-oxidation was greater at nearly all acidic pH values in the presence of Br^- . This trend is also in agreement with the reported pH dependency of group 1 and group 2 processes effected by chloride peroxidase (Thomas *et al.*, 1970b). The effect of halide on the rate of *N*-oxidation is illustrated in Fig. 6. As previously

Fig. 4. The action of chloride peroxidase on 4-chloroaniline in the presence of H_2O_2 and Br^- at pH 4.5 as a function of time

The incubation was carried out in 20 ml of 0.05 M-potassium phosphate buffer, pH 4.5, containing 0.25 mM-4-chloroaniline, 0.5 mM- H_2O_2 , 20 mM-KBr and a total of 5.0 units of chloride peroxidase activity. Portions (1.0 ml) were taken at the indicated time intervals and quenched with 1.0 ml of cold methanol. A 1.0 ml portion of the quenched solution was extracted with 1.0 ml of iso-octane. The quantitative determination of unreacted 4-chloroaniline (Δ) was achieved by analysis of 10 μl portions of the aqueous methanol samples on a $\mu\text{Bondapak C}_{18}$ column with 70% methanol as eluent. The quantitative determination of 4-chloronitrosobenzene ($\bullet$), 2,6-dibromo-4-chloroaniline ($\circ$) and 2-bromo-4-chloroaniline ($\blacktriangle$) was achieved by analysis of 10 μl portions of the iso-octane samples on a $\mu\text{Porasil}$ column with 0.05% methanol in iso-octane as eluent. The molar sum of 4-chloroaniline and all detectable products is indicated ($\square$).

Fig. 5. Effect of pH on rates of halogenation and *N*-oxidation of 4-chloroaniline by chloride peroxidase in the presence of Br^-

Incubations were conducted at each pH in 2.0 ml of 0.05 M-potassium phosphate buffer containing 2.0 mM-4-chloroaniline, 4.0 mM- H_2O_2 , 20 mM-KBr and a total of 0.92 unit of chloride peroxidase activity. Incubations were quenched with cold methanol after 30 s reaction time, then analysed in the manner described in the legend to Fig. 2. Symbols: ●, 4-chloronitrosobenzene; ○, 2-bromo-4-chloroaniline; □, 2,6-dibromo-4-chloroaniline.

Fig. 6. Comparative rates of *N*-oxidation of 4-chloroaniline by chloride peroxidase as a function of pH and halide ion type

Incubations were conducted at each pH in 2.0 ml of 0.05 M-potassium phosphate buffer containing 2.0 mM-4-chloroaniline, 4.0 mM- H_2O_2 , a total of 0.92 unit of chloride peroxidase activity, and either no halide (●), 20 mM-KCl (□), 20 mM-KBr (○) or 20 mM-KF (▲). Incubations were quenched with cold methanol after 30 s reaction and then analysed for 4-chloronitrosobenzene as described in the legend to Fig. 2.

reported (Corbett *et al.*, 1978), the conversion of 4-chloroaniline into 4-chloronitrosobenzene is the only reaction that occurred in the absence of halide ions, and this reaction occurred over a broad pH range with an optimum of about 4.5. The addition of Br^- to the enzymic incubations resulted in a pronounced increase in the rate of *N*-oxidation, but only in the pH range 3.5–5.5. The effect of Cl^- was much less and limited to the pH range 3.5–4.5. Such a rate-enhancing effect is typical for group 2 substrates of chloride peroxidase (Thomas *et al.*, 1970b). Fluoride ion caused a strong inhibition of *N*-oxidation below pH 5.0. No ring fluorination occurred since peroxidases cannot oxidize F^- (Hager *et al.*, 1966).

The catalytic-centre activities for the halogenation reactions and for the *N*-oxidation reaction both in the presence and absence of halide at selected pH values are summarized in Table 2. At pH 6.0 no halogenation products were detected when the incubations were conducted with either Cl^- or Br^- . The rates of production of the nitroso compound were nearly identical in the presence or absence of halide at pH 6.0. At pH 4.0 in the presence of Cl^- or Br^- , *N*-oxidation was the predominant reaction, and occurred nearly twice as fast as did the simultaneous halogenation reactions. Most notable at pH 4.0 was a nearly 3-fold increase in the rate of *N*-oxidation in the presence of Br^- compared with the reaction carried out in the absence of halide. At pH 2.75 chlorination and bromination proceeded at rates 20 and 60 times faster than did the *N*-oxidation reaction. The bromination rate of 4-chloroaniline at pH 2.75 was 2.5 times faster than chlorination, and had an unusually high catalytic-centre activity of 1185. *N*-Oxidation was a minor, yet detectable, reaction at pH 2.75.

An attempt was made to determine relative K_m values for the competing reactions of *N*-oxidation and bromination at pH 4.0 by utilizing h.p.l.c. U.v. spectrophotometry could not be used for this purpose, since the products of the two reactions display optical absorbance over the same wavelengths. The effect of 4-chloroaniline concentration on the individual reactions was studied over the concentration range 0.125–3.0 mM. A complicated dependency of these two processes on substrate concentration was observed, and no attempt was made to calculate K_m values. It was possible to estimate that substrate saturation of the bromination reaction of chloride peroxidase occurred near 0.5 mM, whereas saturation for *N*-oxidation was about 2.0 mM.

4-Chlorophenylhydroxylamine was not detected in incubations carried out with excess H_2O_2 , or even when 4-chloroaniline was in severalfold excess of H_2O_2 . Special incubations that employed large ratios of 4-chloroaniline to H_2O_2 did result in the obser-

vation of a distinct peak in h.p.l.c. chromatograms that is most probably due to the intermediate production of the hydroxylamine. At pH 4.5, in the absence of KBr and at a ratio of 4-chloroaniline/ H_2O_2 of 32, a 71% production of 4-chlorophenylhydroxylamine (on the basis of available H_2O_2) resulted; and a ratio of 4-chloroaniline/ H_2O_2 of 80 resulted in a 77% production of this intermediate. A repetition of the latter incubation in the presence of 20mM-KBr gave nearly the same production (72%) of the hydroxylamine. The retention times of the reaction intermediate and authentic 4-chlorophenylhydroxylamine were identical in the three solvents employed: 5.0min in 50% methanol, 7.2min in 40% methanol and 11.8min in 30% methanol. This is considered to be very strong evidence that the hydroxylamine is a reaction intermediate for the conversion of 4-chloroaniline into 4-chloronitrosobenzene by chloride peroxidase catalysis. Confirmation of the hydroxylamine structure by direct spectral methods was not feasible, owing to the high concentration of unreacted 4-chloroaniline; however, FeCl_3 treatment resulted in the disappearance of the peak, thought to be 4-chlorophenylhydroxylamine, and the appearance of an h.p.l.c. peak identical with that of 4-chloronitrosobenzene.

Attempts to detect other possible short-lived intermediates, such as the *N*-halo-4-chloroanilines, were unsuccessful. At pH 3.0 in the presence of 20mM-KBr and a concentration ratio of 4-chloroaniline/ H_2O_2 of 80, neither 4-chlorophenylhydroxylamine nor a peak that might have indicated the presence of *N*-bromo-4-chloroaniline was observed by h.p.l.c. methods; however, a conversion of nearly 70% (based on H_2O_2) into 2-bromo-4-chloroaniline was observed with only a 15s incubation period.

Discussion

Chloride peroxidase catalyses two peroxidatic reactions on the model substrate 4-chloroaniline. Halogenation occurs in the presence of Cl^- or Br^- and *N*-oxidation occurs in either the presence or absence of these two halides. In view of the reactivity of arylamines towards electrophilic aromatic substitution, ring halogenation is an expected reaction, even though the halogenation of an arylamine by an enzymic process has never been clearly documented. Halogenation of phenols by chloride peroxidase has been reported (Brown & Hager, 1967) and the use of chloride peroxidase to label the aromatic amino acids of proteins is a well known application of this enzyme (Hager, 1974). We previously reported on the ability of chloride peroxidase to catalyse *N*-oxidation of arylamines, but those early kinetic studies were conducted

in the absence of added halide (Corbett *et al.*, 1978, 1979). The present study clearly demonstrated that the *N*-oxidation process also occurs quite well in the presence of Cl^- and Br^- salts. Our initial expectation that the halogenation process would occur so rapidly as to exclude the chance for *N*-oxidation proved to be unfounded.

Two additional reactions catalysed by chloride peroxidase might conceivably occur with arylamine substrates. These reactions are ring hydroxylation and *N*-halogenation to produce phenols and *N*-haloamines respectively. We were unable to detect such metabolites for 4-chloroaniline, although the *N*-haloamines might be too unstable to allow for their detection. The possibility that the observed ring-halogenated products might result from a rapid $\text{N} \rightarrow \text{C}$ migration of halogen after initial production of an *N*-haloamine could not be disproved in this study, even though we were unable to detect such an intermediate by h.p.l.c. in a biased reaction that would have favoured its accumulation.

In spite of our observation that Cl^- and Br^- allow for the competing and very facile process of ring halogenation, an enhancement of the rate of *N*-oxidation was observed to occur in the presence of these halides between pH 3.0 and 5.5. Under no conditions was the presence of Cl^- or Br^- found to inhibit *N*-oxidation (Fig. 6). Fluoride was a fairly strong inhibitor of *N*-oxidation below pH 5.5, a finding in agreement with a previous study on the effect of F^- on chloride peroxidase-catalysed halogenation reactions (Hager *et al.*, 1966). According to the chloride peroxidase substrate-classification scheme of Thomas *et al.* (1970b), the *N*-oxidation reaction of 4-chloroaniline and other arylamines is probably best considered to be a group 2 reaction. Group 2 substrates are those that undergo more classical peroxidatic reactions, but which display reaction-rate enhancement in the presence of halides (excluding F^-). The halogenation reaction of arylamines reported in the present paper is obviously a group 1 reaction.

Group 2 reactions normally have a pH optimum of about 2.8, yet halide rate enhancement of *N*-oxidation of 4-chloroaniline was found to be maximal at pH 3.5–4.0 depending on the halide (Fig. 6). This observed pH dependency is most likely an anomaly due to the competing reaction of halogenation, which becomes very rapid as the pH is decreased (Fig. 5). If it was possible to selectively inhibit the halogenation reaction of chloride peroxidase, then a pH profile more typical of group 2 reactions would probably result for the *N*-oxidation process.

The catalytic-centre activity for the conversion of 4-chloroaniline into 4-chloronitrosobenzene at pH 4.0 was 425 in the presence of Br^- (Table 2). Reported catalytic-centre activities for other group 2

Table 2. *Catalytic-centre activities for chloride peroxidase-catalysed reactions on 4-chloroaniline*

Catalytic-centre activities were calculated from the amount of each product produced after 30s incubation. The molecular weight of chloride peroxidase was assumed to be 42000 (Morris & Hager, 1966), and the specific activity of pure chloride peroxidase was assumed to be 2100 units/mg (Hollenberg & Hager, 1973). Incubations under each condition were carried out in triplicate in 2.0ml of 0.05 M-potassium phosphate buffer at the indicated pH and contained 2.0 mM-4-chloroaniline, 4.0 mM- H_2O_2 , either no halide, 20 mM-KCl or 20 mM-KBr and a total of 1.02 units of chloride peroxidase activity.

pH	Halide	Catalytic-centre activities for production of:		
		4-Chloronitrosobenzene	2-Bromo-4-chloroaniline	2,4-Dichloroaniline
2.75	None	47	—	—
2.75	KCl	28	—	478
2.75	KBr	22	1185	—
4.0	None	157	—	—
4.0	KCl	182	—	113
4.0	KBr	425	243	—
6.0	None	150	—	—
6.0	KCl	150	—	0.0
6.0	KBr	158	0.0	—

substrates, such as thiourea and uric acid, are 834 at pH 2.75 (Thomas *et al.*, 1970b). In view of the fact that the latter values were obtained at the pH optimum for group 2 reactions, 4-chloroaniline appears to be a relatively good substrate for chloride peroxidase. This conclusion becomes even more obvious on consideration of the stoichiometry for the conversion of 4-chloroaniline into 4-chloronitrosobenzene. The oxidation of 4-chloroaniline to 4-chloronitrosobenzene is a 4e process (Scheme 1), and requires two oxidation cycles of chloride peroxidase (Corbett *et al.*, 1978); thus, the real catalytic-centre activity in terms of active-site processing of substrate molecules becomes 851 for 4-chloroaniline at pH 4.0 for the *N*-oxidation reaction alone.

A major accomplishment of the present study was the detection of the suspected intermediate, 4-chlorophenylhydroxylamine, in the oxidation of 4-chloroaniline to 4-chloronitrosobenzene. Under the usual reaction conditions this intermediate could not be detected even by a highly sensitive h.p.l.c. method. We had previously demonstrated that 4-chlorophenylhydroxylamine was oxidized to 4-chloronitrosobenzene by chloride peroxidase even more rapidly than 4-chloroaniline (Corbett *et al.*, 1978), which readily explains the difficulty in detecting such an intermediate. By using a large excess of 4-chloroaniline relative to H_2O_2 we were successful in preventing further oxidation of the hydroxylamine intermediate to 4-chloronitrosobenzene. Thus, the oxidation of 4-chloroaniline to 4-chloronitrosobenzene occurs through two discrete 2e transfer reactions (Scheme 1). This conclusion is consistent with the concept that chloride peroxidase effects 2e oxidations, unlike other peroxidases, which carry out discrete 1e oxidations.

The most unusual aspect of the *N*-oxidation

reaction catalysed by chloride peroxidase is the high degree of product specificity of this process. Direct comparison with other enzymic processes is not possible as no biochemical system has ever previously been reported to convert an arylamine into a nitrosoaromatic compound in a well-defined manner. Horseradish peroxidase oxidizes arylamines in a highly non-specific manner to give a complex reaction mixture (Saunders *et al.*, 1964). Chemical oxidations on an arylamine nitrogen atom typically give rise to numerous products (Boyer, 1969). Thus, it is difficult to draw a direct analogy between an enzyme-bound reaction intermediate and postulated reaction intermediates of purely chemical reactions. In some manner chloride peroxidase so closely controls arylamine oxidation as to virtually prevent the formation of any side products. Such control would explain why competing ring hydroxylation, which occurs during chemical oxidation as an expected result of charge delocalization, is not observed during this enzymic *N*-oxidation. Although we cannot explain the reason for such specificity, we have proposed a mechanism that is consistent with what is currently known concerning the active-site chemistry of chloride peroxidase.

It has been proposed that the major resonant form of oxidized chloride peroxidase (Compound I) is a tetravalent iron porphyrin π -cation radical (IV), which is produced by the 2e oxidation of the native enzyme (Hager *et al.*, 1975; DiNello *et al.*, 1975). This structure for Compound I is analogous to that for the 'active oxygen' of cytochrome P-450 (Ullrich, 1977). Although this tetravalent iron form of Compound I can explain much of the spectral characteristics of oxidized peroxidases, there is considerable question concerning its stability (Jones & Dunford, 1977). The oxene resonant form of chloride peroxidase is depicted as structure (V) in

Scheme 2. *Proposed mechanism for halide-independent N-oxidation*

(IV), Tetravalent iron porphyrin π -cation radical resonant form of chloride peroxidase Compound I; (V), trivalent iron oxene resonant form of chloride peroxidase Compound I; (VI), arylhydroxylamine ligand of Compound I. P represents the sixth axial ligand of chloride peroxidase. Abbreviation: Ar, aryl.

Scheme 2. A rational mechanism for the sequential oxidation of an arylamine to the hydroxylamine and finally to the nitroso oxidation state is much more readily apparent by employing this resonant form rather than the tetravalent iron π cation radical resonant form, which has a high negative charge density on the ligand oxygen. We propose that a nucleophilic attack by the aniline nitrogen on the oxygen ligand, followed by a prototropic shift, will result in the arylhydroxylamine linked in a coordinate bond to the haem iron (structure VI). Dissociation gives the free hydroxylamine and native enzyme. A similar sequence would account for subsequent oxidation of the observed free intermediate hydroxylamine to the nitroso compound. Further oxidation of the nitroso compound is not observed, a feature that is readily explained by the extremely poor nucleophilicity of the nitroso group. Our preference for the trivalent iron resonant form for chloride peroxidase Compound I is even more necessary to account for *N*-oxidation in the presence of Br^- or Cl^- . A halogenating form of chloride peroxidase is difficult to envisage with the tetravalent iron resonant form, which is probably why the halogenating form of chloride peroxidase has been proposed to consist of the hypohalite ligand to the trivalent haem iron, as illustrated by structure (VII) in Scheme 3 (Hollenberg *et al.*, 1974). The process depicted in Scheme 3 is nearly identical with that of Scheme 2, the only difference being the participation of the halide ion as required by our observation of rate enhancement by Br^- and Cl^- . Two possible ways in which halide could facilitate *N*-oxidation are either by increasing the contribution of the trivalent iron resonant form to the structure of Compound I, or by facilitating the nucleophilic attack of the arylamine on the oxygen ligand. Ring halogenation is also proposed to result from the interaction of the arylamine with structure (VII). To explain this competing reaction, it is proposed that nucleophilic attack at halide would give the halogenated product and native chloride peroxidase.

Scheme 3. *Proposed mechanism for halide-dependent N-oxidation*

(VII), Hypohalite ligand of chloride peroxidase Compound I.

The two closely related mechanisms for *N*-oxidation both imply incorporation of the ligand oxygen into the nitroso product. A study of the catalase-like action of chloride peroxidase by Hager *et al.* (1972) suggests that the incorporation of the oxene oxygen atom into the arylhydroxylamine intermediate and nitroso product would result from chloride peroxidase-catalysed oxidation of the arylamine. However, this feature of the *N*-oxidation reaction has not yet been proven. The incorporation of an oxygen atom from a peroxide oxidant into the product of a peroxidase-catalysed reaction has only recently been demonstrated (Ishimaru & Yamazaki, 1977). This peroxidase, present in pea seeds, was termed a 'peroxygenase'. Most interesting was the discovery that pea seed 'peroxygenase' converted aniline into phenylhydroxylamine and indole into indoxyl (3-hydroxyindole); although we discovered that chloride peroxidase oxidizes indole exclusively to oxindole (indol-2-one; Corbett & Chipko, 1979). The probability that chloride peroxidase also functions as a 'peroxygenase' in its non-halogenating reactions should be investigated, especially since such a finding would further clarify the functional relationship between chloride peroxidase and cytochrome P-450.

Other investigators have reported on the similarity of chloride peroxidase and cytochrome *P*-450, especially with regard to the spectral properties of these haemoproteins (Morrison & Schonbaum, 1976; Williams *et al.*, 1977; Remba *et al.*, 1979). Most evidence now suggests that the 'active oxygen' of both are nearly identical in structure and reactivity (Makino *et al.*, 1976; Remba *et al.*, 1979). The ability of chloride peroxidase to effect *N*-oxidations of arylamines is another similarity with cytochrome *P*-450, although chloride peroxidase is much more efficient at this conversion than is cytochrome *P*-450. As more information becomes available on the multiple forms of cytochrome *P*-450, especially information concerning the types and specificity of oxidative reactions catalysed by the various cytochromes *P*-450, then a comparison between the oxidative reactions catalysed by chloride peroxidase and the various types of cytochrome *P*-450 should be most interesting.

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