Peroxide Oxidation of Indole to Oxindole by Chloroperoxidase Catalysis

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In the presence of chloroperoxidase, indole was oxidized by H_2O_2 to give oxindole as the major product. Under most conditions oxindole was the only product formed, and under optimal conditions the conversion was quantitative. This reaction displayed maximal activity at pH 4.6, although appreciable activity was observed throughout the entire pH range investigated, namely pH 2.5–6.0. Enzyme saturation by indole could not be demonstrated, up to the limit of indole solubility in the buffer. The oxidation kinetics were first-order with respect to indole up to 8 mM, which was the highest concentration of indole that could be investigated. On the other hand, 2-methylindole was not affected by H_2O_2 and chloroperoxidase, but was a strong inhibitor of indole oxidation. The isomer 1-methylindole was a poor substrate for chloroperoxidase oxidation, and a weak inhibitor of indole oxidation. These results suggest the possibility that chloroperoxidase oxidation of the carbon atom adjacent to the nitrogen atom in part results from hydrogen-bonding of the substrate N–H group to the enzyme active site.

We recently demonstrated the ability of the fungal enzyme chloroperoxidase (chloride–hydrogen peroxide oxidoreductase, EC 1.11.1.10) to effect *N*-oxidation of arylamines, a process resulting in a highly efficient conversion of arylamines into the corresponding nitroso compounds (Corbett *et al.*, 1978, 1979). This high degree of reaction specificity for *N*-oxidation prompted us to investigate the possibility that chloroperoxidase might effect *N*-oxidation of indole substrates. *N*-Hydroxylated derivatives of indole have long been interesting natural products (Acheson *et al.*, 1974, 1978), but little is known about the enzymology of biochemical reactions that give rise to such compounds. Recently the *N*-oxidation of certain indole derivatives was reported as a minor reaction of a liver microsomal fraction (Jaccarini & Felice, 1978). *N*-Hydroxylation of 2-phenylindole gave *N*-hydroxy-2-phenylindole, which is a highly stable derivative of *N*-hydroxyindole.

Preliminary studies by us indicated that indole was rapidly oxidized by chloroperoxidase catalysis in the presence of H_2O_2 to give a single major product. We now report that this product is oxindole (Scheme 1),

Scheme 1. Chloroperoxidase-catalysed oxidation of indole to oxindole

which itself is an unusual metabolite of indole in view of the work of other investigators. Although we failed to observe *N*-oxidation of indole by chloroperoxidase catalysis, our results do further establish chloroperoxidase as a unique oxidative enzyme.

Materials and Methods

Indole was obtained from Fisher Scientific Co. (Pittsburgh, PA, U.S.A.) and purified by recrystallization from 95% ethanol. Oxindole and 1-methylindole were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Indoxyl β -D-glucoside, 5-hydroxyindole, β -glucosidase and horseradish peroxidase (type II) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Indoxyl was generated *in situ* by the action of β -glucosidase on indoxyl β -D-glucoside in 0.05 M-potassium phosphate buffer, pH 5.0, at 25°C. Chloroperoxidase (purified grade) was obtained from Sigma Chemical Co. and diluted with 10 ml of 0.05 M-potassium phosphate buffer, pH 4.4. As needed, dilutions were made of 1.0 ml of this enzyme solution with 50 ml of 0.05 M-potassium phosphate buffer of the desired pH. Determinations of enzyme activity were made as previously described (Corbett *et al.*, 1978).

H.p.l.c. was conducted on a system composed of Waters Associates (Milford, MA, U.S.A.) model U6K septumless injector, model 6000A solvent-delivery system and model 440 absorbance detector. The h.p.l.c. column was Waters Associates μ Bondapak C₁₈. Silica gel for gravity column chromatography was EM-Silica Gel 60 (70–230 mesh; Brinkmann Instruments, Westbury, NY, U.S.A.).

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

Incubation procedure

The general procedure to initiate the enzymic reaction consisted of combining a solution of substrate and H_2O_2 at twice their desired reaction concentrations in 0.05M-potassium phosphate buffer at the appropriate pH with an equal volume of a solution of chloroperoxidase (2.15 units/ml) in the same buffer. Both solutions were pre-equilibrated to 25°C before use. Most often, total reaction volumes of 10.0ml were employed with H_2O_2 concentrations ranging from 0.05 to 5.0mM, and indole concentrations ranging from 0.25 to 4.0mM. A study of the effect of pH on the initial reaction velocity was made in the pH range 2.5–6.0 with an indole concentration of 0.5mM and an H_2O_2 concentration of 2.0mM. A study of the effect of H_2O_2 concentration was made at pH4.2 with indole concentrations of both 0.25 and 0.50mM. A study of the course of the reaction with time was conducted at pH4.2 with an indole concentration of 0.5mM and an H_2O_2 concentration of 2.0mM. A special incubation procedure was employed in a study of the effect of indole concentration on the initial rate of oxidation and was necessary for the higher concentrations of indole employed in the study, since indole solubility becomes limiting at about 9mM. In this special procedure, sufficient indole and H_2O_2 were dissolved in a 9.8ml volume of buffer, pH4.2, such that eventual dilution to 10.0ml would give the desired indole concentration of from 0.5 to 8.0mM with an H_2O_2 concentration of 4.0mM. To initiate the reaction, 0.20ml of buffer containing 10.8 units of chloroperoxidase was added. For all incubations, timed samples were taken and diluted with an equal volume of cold methanol. The quenched samples were kept at –20°C until analyses could be made.

Controls included a study of the effect of chloroperoxidase on indole in the absence of H_2O_2 , a study employing boiled chloroperoxidase, and a study of the action of H_2O_2 on indole and 2-methylindole in the absence of chloroperoxidase.

The effect of chloroperoxidase and H_2O_2 on 1-methylindole, 2-methylindole and oxindole was investigated in a manner identical with that for studies on indole, except that the concentrations of the methylindoles and oxindole were 0.5mM with an H_2O_2 concentration of 2mM.

Studies were conducted on the inhibition by both 2-methylindole and 1-methylindole of chloroperoxidase-catalysed indole oxidation. The indole concentration was either 0.5 or 1.0mM with 2mM- H_2O_2 , and the concentrations of 2-methylindole and 1-methylindole were varied from 0.125 to 1.0mM.

Analytical method

Samples quenched with methanol were analysed by injection of 10 μl samples on to a 30cm $\times$ 3.9mm

(internal diam.) $\mu\text{Bondapak C}_{18}$ column and chromatographed isocratically with 50% (v/v) methanol at a flow rate of 1.5ml/min. Quantitative detection of indole, oxindole and 2-methylindole was achieved by use of the 254nm spectrophotometric detector and a strip-chart recorder. A linear relationship between peak heights and amount of each compound injected allowed for quantitative determination of each compound by the peak-height method (Snyder & Kirkland, 1974). Detector attenuation was adjusted as needed to maintain convenient peak heights.

Isolation of oxidation product

A large-scale incubation was conducted at 25°C by dissolving 234mg (2mmol) of indole in 500ml of 0.05M-potassium phosphate buffer, pH4.5. To this solution was added 10ml (1.0mmol) of an aq. 0.3% solution of H_2O_2 , followed by 100ml (300 units) of a solution of chloroperoxidase in buffer. The addition of H_2O_2 and chloroperoxidase was repeated every 5min until a total of 4mmol of H_2O_2 and 1200 units of chloroperoxidase had been added. The reaction mixture was allowed to stand for 15min, and then it was extracted twice with 400ml of ethyl acetate. The combined ethyl acetate extracts were dried (over Na_2SO_4) and evaporated *in vacuo* at 40°C to give 0.3g of a brown oil. The oil was chromatographed on a 16cm $\times$ 1.1cm (internal diam.) column of silica gel with methanol/chloroform (1:49, v/v). Fractions were monitored for the presence of the oxidation product by h.p.l.c. Those fractions containing the product were combined and evaporated to give 150mg of a brown solid. Rechromatography of this residue on silica gel with chloroform effected the removal of trace amounts of pigmented contaminants to give 110mg (41% yield) of pale yellow crystals identified as oxindole on the basis of identical i.r., ^{13}C n.m.r. and u.v. spectra and h.p.l.c. retention times.

Incubation of indole with H_2O_2 and horseradish peroxidase

Incubations of 0.5mM-indole with 2mM- H_2O_2 and 2 μg of horseradish peroxidase/ml were carried out at 25°C in 0.05M-potassium phosphate buffer at both pH3.5 and 4.5. Timed samples were taken and analysed by h.p.l.c. for the presence of oxindole and for the disappearance of indole.

Results

The incubation of indole with H_2O_2 and chloroperoxidase in acidic buffers gave rise to only a single product as indicated by h.p.l.c. analysis. Comparison of h.p.l.c. retention-time data (Table 1) for this single oxidation product with those for indoxyl

and 5-hydroxyindole indicated that neither of these possible indole derivatives was the product produced in this enzymic oxidation. H.p.l.c. analysis did suggest the possibility that the product might be oxindole. To prove the identity of the oxidation product, a large-scale incubation of indole with H_2O_2 and chloroperoxidase was conducted. This procedure included the sequential addition of both H_2O_2 and enzyme to avoid the known H_2O_2 -induced inactivation of chloroperoxidase (Shaw & Hager, 1961). The oxidation product was isolated in pure form from the preparative reaction in 41% overall yield. The isolated product displayed h.p.l.c. retention identical with that of the product observed in small-scale incubations, and that had not been subjected to the isolation processes. This finding, coupled with the identical u.v.-absorption spectra displayed by the isolated and non-isolated products, proved that no changes in molecular structure were induced by the physical processes employed to isolate the product in pure form. A comparison of i.r., ^{13}C n.m.r. and u.v. spectral data for the isolated oxidation product and an authentic sample of oxindole conclusively proved that oxindole was the product of this indole oxidation.

During the purification of oxindole from the large-scale enzymic reaction it was obvious that other oxidation products were present in trace amounts. Most obvious were trace amounts of intensely coloured pigments that included indigo. The total production of these pigments was estimated to be less than 2% on a weight basis. Unchanged indole was recovered to the extent of 10% of the original amount employed in the preparative incubation. Most of the remaining product unaccounted for consisted of oxindole contaminated with trace pigments.

H.p.l.c. proved to be a convenient method with which to investigate indole oxidation by chloroperoxidase. By use of h.p.l.c. the amounts of both product formed and substrate consumed could be readily determined quantitatively at any sampling time during the reaction. Such an accomplishment would have been most difficult by the more conventional spectrophotometric methods, since both

indole and oxindole display considerable optical absorption at the absorption maxima of the other. The successful use of h.p.l.c. in the present investigation also depended on the ability to quench the enzymic reaction rapidly with methanol, and on the prolonged stability of the product under the conditions of the incubation and in the quenched samples. A typical analysis of a timed sample taken from this enzymic reaction is illustrated in Fig. 1. The assignment of peaks in the chromatogram was made by reference to retention times determined for authentic compounds (Table 1), and by the observation of peak enhancement when an authentic compound was co-injected with the sample. Both indole and oxindole were readily determined quantitatively by peak-height measurements. Repetitive analyses indicated that the precision of the h.p.l.c. method was about $\pm 2\%$.

Especially noteworthy in Fig. 1 is the absence of any significant secondary peaks that would be suggestive of alternative products. This same observation was also true for nearly all analyses made in the present study. Variation of solvent composition from 50% to 90% methanol did not indicate any additional products that might not have been chromatographed in the usual 50%-methanol solvent. These h.p.l.c. observations, and the fact that the sum of oxindole and indole present in any given sample usually exceeding 90% of the starting amount of indole, indicated the

Fig. 1. Representative h.p.l.c. chromatogram of indole oxidation samples

A $10\mu\text{l}$ volume of the 5 min quenched sample taken from the reaction of 0.5 mM-indole with 2 mM- H_2O_2 in the presence of chloroperoxidase at pH 4.2 was analysed by h.p.l.c. on $\mu\text{Bondapak C}_{18}$ with 50% (v/v) methanol at a flow rate of 1.5 ml/min. Detection was at 254 nm with attenuation set at 0.05 A_{254} unit full scale: a, injection solvent; b, oxindole; c, indole.

Table 1. H.p.l.c. retention times for indole and derivatives
Authentic samples were chromatographed on a 30 cm $\times$ 3.9 mm (internal diam.) $\mu\text{Bondapak C}_{18}$ column with 50% (v/v) methanol at a flow rate of 1.5 ml/min.

Compound	Retention time (min)
5-Hydroxyindole	2.8
3-Hydroxyindole (indoxyl)	3.3
Oxindole	3.8
Indole	5.9
2-Methylindole	10.2
1-Methylindole	12.5

high reaction specificity of this enzymic process. On the other hand, horseradish peroxidase produced a mixture of coloured products and only traces of oxindole.

The requirements for both H_2O_2 and chloroperoxidase in order to observe conversion of indole into oxindole were readily shown. The use of heat-inactivated chloroperoxidase also resulted in the failure to detect oxindole production. Oxindole was found to be completely stable with respect to chloroperoxidase oxidation under the conditions employed for indole oxidation.

No reaction occurred when 2-methylindole was employed in this reaction, a further indication of the high specificity for oxidation by chloroperoxidase at the 2-position of indole. We had thought that, if a methyl group at C-2 prevented oxidation at that position, then possibly chloroperoxidase would effect oxidation at another position, the most likely being C-3, which has the highest electron density (Paquette, 1968). No alternative products were observed, and h.p.l.c. indicated no change in the amount of 2-methylindole present even on prolonged incubation with chloroperoxidase. A similar investigation of the action of chloroperoxidase and H_2O_2 on 1-methylindole indicated that this substrate was oxidized by chloroperoxidase, but at a much lower rate than was indole. Although the products were not conclusively identified, h.p.l.c. evidence suggests that *N*-methyl-oxindole was the major oxidation product.

A study of the effect of pH on the initial rate of reaction indicated substantial activity throughout the investigated pH range 2.5–6.0 (Fig. 2). The optimal pH was 4.6, although activity was nearly optimal from pH 4.0 to 4.8. This pH effect is similar to that found for arylamine oxidation (Corbett *et al.*, 1978) and is consistent with other non-halogenating oxidation reactions of chloroperoxidase (Thomas *et al.*, 1970).

The H_2O_2 concentration that gave the maximal initial reaction velocity was found to be 0.50 mM (Fig. 3). This is markedly lower than the optimal H_2O_2 concentration of 4.5 mM found for arylamine oxidation (Corbett *et al.*, 1979). This low optimal H_2O_2 concentration required us to investigate the kinetics of this enzymic reaction at a H_2O_2 concentration much higher than optimal, since preliminary experiments had indicated to us that enzyme saturation by indole would occur at concentrations greater than 4 mM. The H_2O_2 concentration employed was 4 mM, which was sufficiently high that its depletion within a required 30 s reaction period was minimal (Segel, 1976).

The substrate-concentration experiment was conducted with the indole concentration approaching that of its solubility in the buffer. The results of this study are presented in Fig. 4. It is obvious that substrate saturation of the enzyme cannot be achieved before the solubility limit of indole is reached. The oxidation of indole by chloroperoxidase displayed

Fig. 2. Effect of pH on the initial rate of oxidation of indole by chloroperoxidase

Each incubation mixture contained 5.0 μmol of indole, 20 μmol of H_2O_2 and 10.8 units of chloroperoxidase in 10.0 ml of 0.05 M-potassium phosphate buffer. The pH of the buffer was varied from 2.5 to 6.0. The initial rate of the reaction was computed from the amount of oxindole formed after a 30 s incubation period as determined by h.p.l.c. analysis.

Fig. 3. Effect of H_2O_2 concentration on the initial rate of oxidation of indole by chloroperoxidase

Each incubation mixture contained 2.5 μmol of indole and 10.8 units of chloroperoxidase along with H_2O_2 to give the indicated concentration in 10.0 ml of 0.05 M-potassium phosphate buffer, pH 4.2. The initial rate of the reaction was computed from the amount of oxindole formed after a 30 s incubation period as determined by h.p.l.c. analysis.

first-order kinetics with respect to indole. The apparent second-order rate constant under the conditions of 4 mM- H_2O_2 , pH 4.2 and 25°C was computed to be $7.0 \times 10^{-4} \text{ ml} \cdot \text{unit}^{-1} \cdot \text{s}^{-1}$ or $6.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ given the molecular weight and specific

Fig. 4. Effect of indole concentration on the initial rate of oxidation of indole by chloroperoxidase

Each incubation mixture contained $40\mu\text{mol}$ of H_2O_2 and 10.8 units of chloroperoxidase along with indole to give the indicated concentration in 10.0ml of 0.05 M-potassium phosphate buffer, pH 4.2. Details on the initiation of the incubation mixtures are presented in the Materials and Methods section. The initial rate of the reaction was computed from the amount of oxindole formed after a 30s incubation period as determined by h.p.l.c. analysis. Each data point is the average of three individual incubations, and the precision is indicated on the graph as $\pm$ s.d. The straight line plotted was the best visual fit to the data and has a slope of 0.0454min^{-1} .

activity of pure chloroperoxidase (Hollenberg & Hager, 1973). At the lower and optimal H_2O_2 concentration of 0.50mM, the apparent second-order rate constant increases to $1.7 \times 10^{-3}\text{ml} \cdot \text{unit}^{-1} \cdot \text{s}^{-1}$ or $1.5 \times 10^5\text{M}^{-1} \cdot \text{s}^{-1}$. The first-order kinetics with respect to indole concentration could not be confidently tested beyond a concentration of 2mM owing to the rapid depletion of available H_2O_2 when starting at 0.50mM- H_2O_2 .

Both 2-methylindole and 1-methylindole were found to inhibit the rate of oxidation of indole by chloroperoxidase, and the degree of inhibition was a function of the concentration of the inhibitor (Fig. 5). Inhibition was much stronger by 2-methylindole than by 1-methylindole. The enzymic incubation of indole in the presence of 2-methylindole, at both 0.5 and 1.0mM concentration, resulted in a rate of indole oxidation that was 31% that of the uninhibited rate. On the other hand, 1-methylindole only lowered the indole oxidation rate to 90% that of the uninhibited rate, when both compounds were present at

Fig. 5. Effect of 1-methylindole and 2-methylindole on indole oxidation by chloroperoxidase

Each incubation mixture contained $4.0\mu\text{mol}$ of H_2O_2 , 1.8 units of chloroperoxidase, either 1.0 or $2.0\mu\text{mol}$ of indole, and sufficient 1-methylindole or 2-methylindole to give the indicated concentration in 2.0ml of 0.05 M-potassium phosphate buffer, pH 4.2. Samples (1.0ml each) were taken at 30s and quenched by addition to 1.0ml of cold methanol. The amount of oxindole in each sample was determined by h.p.l.c. analysis, and compared with the amount of oxindole in an identical incubation mixture lacking either methylindole. Δ , 1.0mM-Indole and 1-methylindole; $\blacktriangle$, 0.5mM-indole and 1-methylindole; $\circ$, 1.0mM-indole and 2-methylindole; $\bullet$, 0.5mM-indole and 2-methylindole.

1.0mM concentrations. Unlike 2-methylindole, a constant ratio of 1-methylindole to indole did not result in a constant percentage inhibition.

A study of the course of the chloroperoxidase-catalysed oxidation of indole is illustrated in Fig. 6. The curves in Fig. 6 are typical curves for enzymic reactions in which available substrate is being exhausted. Indole utilization was balanced by oxindole production, indicative of the specificity of the oxidative process. The sum of indole and oxindole at any time was generally about 95% of the initial amount of substrate.

Subsequent to our observations of nearly quantitative conversions of indole into oxindole, we further investigated the parameters that would be expected to affect this quantitative conversion. The h.p.l.c. analysis of the terminal reaction products resulting

Fig. 6. Oxidation of indole by chloroperoxidase as a function of time

This incubation mixture contained 5.0 μ mol of indole, 20 μ mol of H_2O_2 and 10.8 units of chloroperoxidase in 10.0 ml of 0.05 M-potassium phosphate buffer, pH 4.2. Samples (1.0 ml each) were taken at the indicated times after initiation of the reaction and quenched by addition to 1.0 ml of cold methanol. The amounts of both indole and oxindole in each sample were determined by h.p.l.c. analysis. The percentage of either indole (▲) or oxindole (●) indicated is based on the molar percentage of the indole present at the start of the reaction.

after 2 h or more of incubation under various conditions yielded considerable information about this enzymic oxidation.

The nature of terminal reaction products as a function of pH is illustrated in Fig. 7. At about pH 4 the production of oxindole was nearly quantitative. As the pH exceeded this optimal value the decreased production of oxindole was balanced on a molar basis by the presence of unchanged indole. At more acidic pH values than the optimal, the terminal product was somewhat different in that not all the substrate could be accounted for by oxindole and unchanged indole. At these more acidic pH values, the final incubation mixtures were visibly yellowish, indicative of other reactions becoming important. The nature of these by-products has not been determined, but very possibly they are similar to the products characteristic of the horseradish-peroxidase-catalysed oxidation of indole (Holmes-Siedle & Saunders, 1957). Thus the high degree of reaction specificity for the chloroperoxidase-catalysed oxidation of indole occurs only at

Fig. 7. Effect of pH on the composition of terminal reaction products resulting from the oxidation of indole by chloroperoxidase

Each incubation mixture contained 5.0 μ mol of indole, 20 μ mol of H_2O_2 and 10.8 units of chloroperoxidase in 10.0 ml of 0.05 M-potassium phosphate buffer at the indicated pH. After reaction at 25°C for at least 2 h, 10 μ l samples were directly analysed by h.p.l.c. The percentage of indole (▲) or oxindole (●) indicated is based on the molar percentage of the indole present at the start of the reaction.

pH values of 4 or higher, although oxindole is still the major product at pH values down to at least 2.5.

On the basis of the pH study, an investigation of the terminal reaction products as a function of H_2O_2 concentration was made at pH 4.2 with an indole concentration of 0.25 mM. The optimal H_2O_2 concentration for oxindole formation was found to be 1.0–2.0 mM (Fig. 8). Higher concentrations of H_2O_2 probably resulted in lower conversion into oxindole, as a result of enzyme inactivation. The H_2O_2 -induced inactivation of chloroperoxidase has been previously reported, and becomes very pronounced as the H_2O_2 concentration exceeds 2 mM (Corbett *et al.*, 1978; Shaw & Hager, 1961).

Discussion

Our discovery that chloroperoxidase catalyses the oxidation of indole to oxindole is quite interesting in view of current knowledge concerning enzymic oxidations of this compound. The most interesting aspect of this reaction is our finding that this reaction is highly specific, in that only oxindole is produced in significant amounts under most conditions. This

Fig. 8. Effect of H_2O_2 concentration on the composition of terminal reaction products resulting from the oxidation of indole by chloroperoxidase

Each incubation mixture contained 2.5 μ mol of indole and 10.8 units of chloroperoxidase in 10.0 ml of 0.05 M-potassium phosphate buffer, pH 4.2, along with H_2O_2 to give the indicated concentration. After reaction at 25°C for at least 2 h, 10 μ l samples were directly analysed by h.p.l.c. The percentage of indole (▲) or oxindole (●) indicated is based on the molar percentage of the indole present at the start of the reaction.

finding, coupled with the detection of only minute traces of indigo, suggestive of initial indoxyl production, demonstrates that oxidation at the 3-position of indole is not a significant reaction for chloroperoxidase. This is most unusual, since the 3-position of indole possesses the highest electron density and is the position most susceptible to attack by oxidizing agents and by other electrophilic reagents (Acheson, 1967; Paquette, 1968).

Recently we reported that chloroperoxidase can bring about *N*-demethylation of 4-chloro-*N*-methylaniline (Corbett & Chipko, 1979); however, this was no basis on which to suspect that chloroperoxidase would oxidize the carbon atom adjacent to the nitrogen atom in indole. Horseradish peroxidase has been reported to effect dealkylations of anilines (Griffin, 1978), but not oxindole production from indole. Furthermore, the carbon atom at the 2-position of indole is of the sp^2 hybrid type, whereas the methyl carbon atom of 4-chloro-*N*-methylaniline is of the sp^3 hybrid type. Thus α -carbon atom oxidation by chloroperoxidase on these two totally different chemical substrates can only be considered coincidental at this time.

Our studies also demonstrated that even the blocking of the 2-position, as in 2-methylindole, fails to divert the oxidation process to the 3-position. Arguments that the 2-methyl substituent might hinder

attack at the 3-position for steric reasons are discredited by our demonstration that 1-methylindole is not oxidized at the 3-position either. The factor controlling this specificity of chloroperoxidase for the 2-position of indole is not known. It was obvious to suspect the indolic N-H group as the feature controlling the position of oxidation, even though indole is an extremely weak base (Acheson, 1967). Our observation that 1-methylindole was oxidized at the 2-position at a rate almost negligible compared with that of indole led us to propose that hydrogen-bonding between the indole N-H group and an unknown group at the chloroperoxidase active site is the most likely explanation for the observed reaction specificity. Such a proposal is strongly supported by our observation that 2-methylindole, which is not oxidized by chloroperoxidase, was a strong inhibitor of indole oxidation by chloroperoxidase. 2-Methylindole possesses an N-H group for the proposed hydrogen-bonding to chloroperoxidase, yet is not oxidized, as a result of the blocking effect of the 2-methyl substituent. On the other hand, the fact that 1-methylindole also inhibits indole oxidation, although it is a much weaker inhibitor than 2-methylindole, suggests that hydrogen-bonding alone cannot explain the reaction specificity of chloroperoxidase.

There are surprisingly few reports in the literature dealing with enzymic oxidation of indole and its simple derivatives. Horseradish peroxidase produces a much more complex product mixture from indole than does chloroperoxidase. The major product of indole oxidation by horseradish peroxidase is a yellow trimer, which results by oxidative coupling of indole nuclei at their 3-positions (Holmes-Siedle & Saunders, 1957). Our analysis of the horseradish-peroxidase-catalysed oxidation product of indole by h.p.l.c. indicated only the possible trace formation of oxindole, and no other significant monomeric products were found to accumulate. We conclude that there is no similarity between horseradish peroxidase and chloroperoxidase with regard to indole oxidation. This same conclusion was made from our study of arylamine oxidation by these two peroxidases (Corbett *et al.*, 1978).

A newly discovered pea-seed microsomal enzyme, which has been termed a peroxygenase, was reported to convert aniline into phenylhydroxylamine, which was actually detected as nitrosobenzene (Ishimaru & Yamazaki, 1977). This close similarity to our reports on chloroperoxidase activity on anilines (Corbett *et al.*, 1978, 1979) suggests a possible similarity between the two enzymes. On the other hand, pea-seed peroxygenase reportedly oxidizes indole only at the 3-position. Thus pea-seed peroxygenase is quite different from chloroperoxidase in terms of indole oxidation.

Most reports on the biochemical production of oxindole have dealt with studies on whole organisms,

including humans (Gupta *et al.*, 1972), or crude enzyme preparations. In all of these cases, oxindole was a minor product resulting from indole metabolism. Beckett & Morton (1966) reported that a rat liver microsomal fraction converted indole into oxindole, although quantitative data were not presented. This contrasts with an earlier report that indole was metabolized to indigo via indoxyl when incubated with a rabbit liver microsomal fraction (Posner *et al.*, 1961). King *et al.* (1966) also reported oxindole formation along with the indoxyl pathway by a rat liver microsomal fraction. The most interesting finding in this work (King *et al.*, 1966) was that production of oxindole mediated by the microsomal fraction would also proceed under anaerobic conditions. The physicochemical similarities between chloroperoxidase and cytochrome *P*-450 have been previously reported (Hollenberg & Hager, 1973; Champion *et al.*, 1976; Makino *et al.*, 1976; Estabrook & Werringloer, 1977). It is possible that the reactions catalysed by chloroperoxidase and cytochrome *P*-450 become very similar when the latter is subjected to anaerobic conditions. Under anaerobic conditions peroxidatic reactions catalysed by cytochrome *P*-450 become important (Rahimtula & O'Brien, 1977).

Chloroperoxidase has proved to be an extremely interesting enzyme in terms of both the types of reactions that it catalyses and the simplicity of the resulting products. In comparison with horseradish peroxidase and microsomal enzymes, chloroperoxidase is a much easier enzyme with which to investigate certain biochemical oxidations because of its observed tendency to yield only a single product from a given substrate. A complete understanding of the chemical and physical processes occurring at the active site of this enzyme would be most useful in attempts to elucidate similar processes in other haem-containing enzymes.

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