Biochemical Pharmacology, Vol. 52, pp. 1169–1175, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00453-4

Lactoperoxidase-Catalysed Oxidation of Indomethacin, a Nonsteroidal Antiinflammatory Drug, Through the Formation of a Free Radical

*Ratna Chatterjee, Uday Bandyopadhyay, Abhijit Mazumdar and Ranajit K. Banerjee**
DEPARTMENT OF PHYSIOLOGY, INDIAN INSTITUTE OF CHEMICAL BIOLOGY, 4 RAJA S C MULLICK ROAD, CALCUTTA, 700032, INDIA

ABSTRACT. Lactoperoxidase (LPO, EC 1.11.1.7; donor-H_2O_2 oxidoreductase) catalyses the oxidation of indomethacin, a nonsteroidal antiinflammatory drug by H_2O_2 as measured by time-dependent decay of indomethacin extinction at 280 nm and concurrent appearance of stable oxidation product(s) at 412 nm. From a plot of log V_{max} against varying pH of indomethacin oxidation, involvement of an ionizable group of the enzyme having pka = 5.7 could be ascertained for controlling the oxidation process. Spectral studies revealed that LPO-compound II oxidises indomethacin through one-electron transfer and is reduced to the native ferric state as shown by its spectral shift from 430 nm to 412 nm through an isosbestic point at 421 nm. The one-electron oxidation product is a nitrogen-centered free radical detected as a 5,5-dimethyl-l-pyrroline N-oxide (DMPO) adduct (a_N = 15 G, a_β^H = 16 G) in electron spin resonance spectroscopy. The free radical is scavenged by reaction with O_2 as shown by O_2 consumption sensitive to the free-radical trap, DMPO. Binding studies by optical difference spectroscopy indicate that indomethacin binds to LPO with an apparent K_D value of 24.5 μM. The free energy change, ΔG′, for the binding is −26.3 KJ mol^{-1}, suggesting that the interaction is favourable for oxidation. Indomethacin binding remains unaltered by a change of pH from 5.25 to 7.5, presumably because of hydrophobic interaction. The binding is competitive with resorcinol, an aromatic electron donor, showing the K_D value to be as high as 100 μM. We suggest that indomethacin interacts at the aromatic donor binding site and is oxidised by one-electron transfer by LPO catalytic intermediates to stable oxidation product(s) through the formation of a free radical. BIOCHEM PHARMACOL 52;8:1169–1175, 1996.

KEY WORDS. lactoperoxidase; indomethacin; nonsteroidal antiinflammatory drug; indomethacin oxidation; indomethacin free radical; lactoperoxidase-catalyzed indomethacin oxidation

IMN,† a well-known nonsteroidal antiinflammatory drug, has widespread clinical application for the treatment of inflammatory diseases including arthritis and gout [1]. The pharmacological target of this drug is the cyclooxygenase part of prostaglandin synthase [2, 3], which binds it tightly but noncovalently, causing a conformational change to inhibit prostaglandin biosynthesis [4, 5]. The drug also inhibits neutrophil responses such as aggregation and activation [6] and the defence mechanism by lymphocytes, monocytes, and neutrophils [7, 8]. The drug also causes gastric damage leading to gastric ulceration [9]. It is not clear yet whether these effects are caused by the drug or its active metabolites. A concept is emerging that many drugs are activated *in vivo* to a free radical derivative that, being highly reactive, interacts with the cellular macromolecules to cause toxicity and carcinogenicity. Although the oxidation of drugs and biochemicals by cytochrome P-450-dependent monooxygenases [10], alcohol, and aldehyde dehydrogenases [11], and flavin monooxygenases [12] is established, current interest is now being focussed on the role of peroxidases in the oxidative metabolism of drugs and chemical carcinogens [13–17]. Peroxidases catalyse the oxidation of a wide variety of organic molecules with H_2O_2 by two one-electron transfers that produce free radicals, which are stabilized by coupling, disproportionation, and/or reaction with molecular oxygen [18]. Among the peroxidases extensively studied are HRP and LPO (EC 1.11.1.7; donor-H_2O_2 oxidoreductase) [18–20] and LPO has been proposed to act as a model for the reaction of most mammalian peroxidases with xenobiotics [14–21].

LPO, a hemoprotein of iron porphyrin thiol [22, 23], most abundant in milk, saliva, and tears, oxidises SCN^- by H_2O_2 to produce hypothiocyanous acid, which has potent bactericidal activity [24, 25] and thus takes part in host defence mechanism. We reported earlier that IMN competitively inhibits gastric peroxidase [26] and intestinal eosinophil peroxidase [27] activity when iodide or guaiacol is

* Corresponding author: TEL: 91-33-4733491; FAX: 91-33-4730284, 91-33-4735197.

† *Abbreviations:* LPO, lactoperoxidase; HRP, horseradish peroxidase; IMN, indomethacin; DMPO, 5,5-dimethyl-l-pyrroline N-oxide; DETAPAC, diethylene triaminepentaacetic acid; ESR, electron spin resonance.

Received 18 December 1995; accepted 3 June 1996.

used as an electron donor. It also competitively inhibits the activity of myeloperoxidase [8] and thyroid peroxidase [28]. These observations raise the possibility that IMN may be oxidized by peroxidases. However the mechanism of oxidation by any of these peroxidases is not clear. Because LPO and related enzymes are widely distributed throughout the human and animal systems [24], it is more likely that the drug is oxidized by this enzyme. In this paper, we present evidence to show that the drug is primarily oxidized by LPO–H_2O_2 system through a one-electron transfer mechanism that generates a free radical, which reacts with molecular oxygen to form stable oxidation product(s). The possible site of interaction of this drug with respect to heme iron, aromatic donor, and SCN^- binding sites of LPO has been suggested.

MATERIALS AND METHODS

IMN, resorcinol, DETAPAC, and bovine milk LPO (RZ = 0.88) were obtained from Sigma. DMPO was purchased from Aldrich. All other chemicals were of analytical grade.

Enzyme Assays

All kinetic and spectral studies were carried out at room temperature (25 ± 1°C) in a Shimadzu UV-2201 computerized spectrophotometer using quartz cuvettes of 1-cm path of light. The concentration of LPO was calculated from its extinction coefficient of 112 $mM^{-1}cm^{-1}$ at 412 nm [29]. LPO-catalysed IMN oxidation was assayed by the increase in absorbance of its oxidation product(s) at 412 nm as described earlier [27]. The activity was expressed as ΔA_{412} min^{-1} after correction for the nonenzymatic rate, if any. Oxygen consumption during IMN oxidation was recorded in a Gilson oxygraph fitted with a Clark electrode. The incubation mixture contained, in a final volume of 2 mL, 100 mM sodium phosphate buffer, pH 5.5, 75 μM IMN, and 0.1–0.2 μM LPO in the absence or presence of 4.5 mM DMPO. Reaction was started with 100 μM H_2O_2.

Detection of Free Radical

ESR spectra were obtained in a Varian E-112 ESR spectrometer operating at 9.5 GHz, with a 100-KHz modulation frequency, and equipped with a TM 110 cavity. The incubation system contained 1 mM DETAPAC, 10 mM IMN, 100 mM DMPO, 300 μM H_2O_2, and 5 μM LPO in 100 mM phosphate buffer, pH 5.5.

Binding Studies

The binding of indomethacin to LPO was studied by optical difference spectroscopy [30, 31]. The sample and reference cuvettes were filled with 1 mL enzyme solution (2.5 μM) in 100 mM sodium phosphate buffer, pH 5.75, and corrected for the base line. A small volume (2–10 μL) of the drug was then added to the sample cuvette, with concomitant addition of the same volume of buffer into the reference cuvette. The contents of both cuvettes were stirred with a plastic rod, and the spectrum was recorded. From the resulting difference spectra, the apparent equilibrium dissociation constant (K_D) for the complex formation was obtained from the following expression [31]:

$$1/\Delta A = \frac{K_D}{\Delta A_\alpha}\frac{1}{[S]} + \frac{1}{\Delta A} \quad (1)$$

where K_D is the dissociation constant of the enzyme-drug complex at the drug concentration [S], ΔA is the observed absorption change at a particular wavelength, and ΔA_α is the absorption change at saturating concentration of the drug. The thermodynamic parameter, free energy change ($\Delta G'$) for the binding of the drug to LPO, was calculated from the K_D value according to the following expression [32]:

$$\Delta G' = RT\ln K_D \quad (2)$$

where R is the universal gas constant and T is the temperature in degrees Kelvin.

RESULTS

LPO-Catalysed IMN Oxidation

When IMN was added to a system containing LPO and H_2O_2, there was a time-dependent decay of its absorbance at 280 nm with concurrent increase in extinction of its oxidation product(s) at 412 nm (Fig. 1). The rate of formation of the product(s) varied with the enzyme concentrations and could be inhibited by the peroxidase inhibitor azide (Fig. 1A, inset). IMN oxidation, as measured by product(s) formation at 412 nm, is also dependent on IMN concentration showing substrate saturation (Fig. 1B, inset). The pH dependence of IMN oxidation by LPO was examined over the pH range of 5 to 7.2. IMN is optimally oxidised at a slightly acid pH with a broad pH optimum between 5 to 5.5 (not shown). When log V_{max} values obtained from the Lineweaver-Burk plot were plotted against

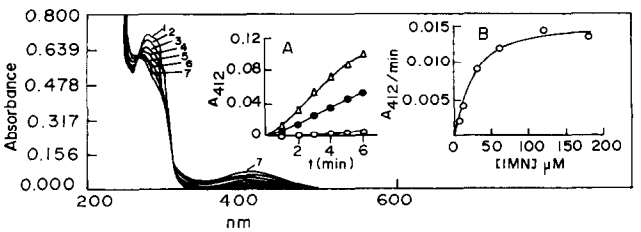

FIG. 1. Kinetics of the oxidation of IMN by lactoperoxidase. Assay system contained in a final volume of 1 mL: 100 mM sodium phosphate buffer, pH 5.5, 75 μM IMN, and 0.2 μM LPO. Reaction was started by adding 200 μM H_2O_2, and the scan was taken at 1-min intervals (scans 1–7). (A) Increase in A_{412} with time with 0.1 μM LPO (solid circles), 0.2 μM LPO (triangle), and 0.2 μM LPO plus 100 μM azide (open circle). (B) The product formation as a function of IMN concentrations.

pH, a curve resulted (Fig. 2) from which involvement of an ionizable residue on the enzyme [33] having pka value of 5.7 could be ascertained for controlling the oxidation of IMN.

Spectral Evidence for IMN Oxidation by LPO Compound II

Spectral studies were performed to study the oxidation of IMN with LPO in presence of H_2O_2. Trace 1 (Fig. 3A) is the Soret spectrum of the native ferric enzyme absorbing at 412 nm. On addition of 5 equivalents of H_2O_2, the Soret peak shifted to 430 nm (trace 2) due to formation of catalytically active LPO–compound II [14]. Upon subsequent addition of IMN, compound II is immediately reduced to the ferric enzyme at 412 nm through an isosbestic point at 421 nm, suggesting one-electron oxidation of IMN by catalytically active LPO. However, LPO–compound II is stable for up to 10 min in the absence of any electron donor (Fig. 3B) and slowly returns almost to its native state within 50 min.

Oxygen Consumption During Indomethacin Oxidation

When IMN was oxidized by LPO in presence of H_2O_2, a time-dependent consumption of oxygen was observed, the rate of which was dependent on the concentration of LPO and inhibited by the peroxidase inhibitor azide (Fig. 4A). Superoxide was not produced because no reduction of ferricytochrome c was observed under identical conditions. Oxygen consumption was also prevented by DMPO (Fig. 4B), the free radical trap, indicating that IMN is oxidized to a free radical by one-electron transfer and the resulting free radical is scavenged by reaction with molecular oxygen.

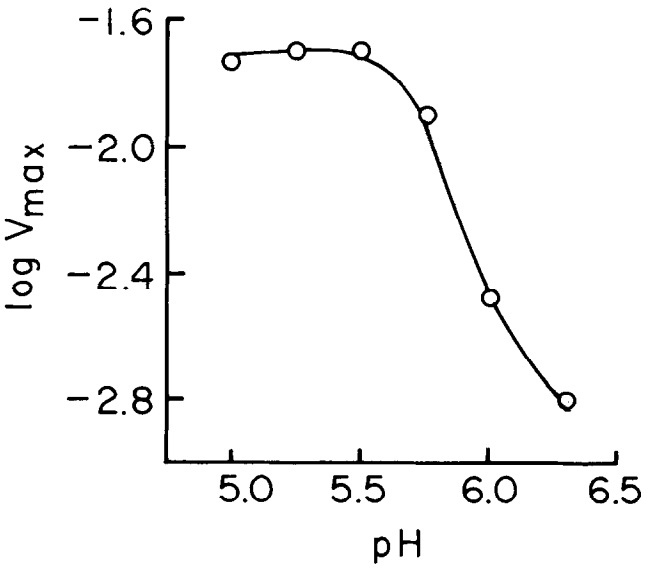

FIG. 2. pH dependence of IMN oxidation by LPO. V_{max} values for IMN oxidation by LPO at different pH were determined from initial plot of I/v against I/[IMN] (not shown), and log V_{max} values were then plotted against pH. The data are the mean of three experiments.

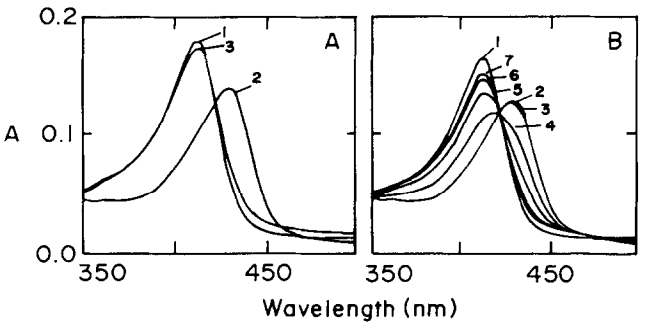

FIG. 3. Spectral evidence for the oxidation of IMN by LPO–compound II. (A) LPO (1.6 μM, trace 1) plus 8 μM H_2O_2 (compound II, trace 2) plus 16 μM IMN (trace 3) in 1 mL of 100 mM sodium phosphate buffer, pH 5.5. (B) Stability of LPO–compound II. LPO (1.6 μM, trace 1) plus 8 μM H_2O_2 (compound II, trace 2); scan repeated after 10-min intervals (traces 3–7).

Detection of IMN Free Radical by ESR Studies

When LPO-catalysed IMN oxidation by H_2O_2 was studied in the presence of DMPO, an ESR spectrum was obtained for DMPO-indomethacin free radical adduct, having splitting constants of a^N = 15 G and a^H_β = 16 G as shown in Fig. 5A. The spectral intensity was dependent on the enzyme concentration (not shown). The signal was not observed in absence of H_2O_2 or IMN (Fig. 5B,C). The signals characteristic of $OH^\cdot$ or O_2^- [34] were not detected.

Binding of IMN to LPO as Probed by Optical Difference Spectroscopy

Because binding is a prerequisite for oxidation, IMN binding to LPO was studied with optical difference spectroscopy [30–32]. The difference spectra of the LPO–IMN complex versus LPO at different IMN concentrations are shown in Fig. 6. The complex has the maximum at 430 nm and the minimum at 406 nm. The apparent K_D for the binding of IMN to LPO as calculated from the plot of $1/\Delta A$ against 1/[IMN] (Fig. 6, inset) using Eqn 1 was 24.5 μM. The free

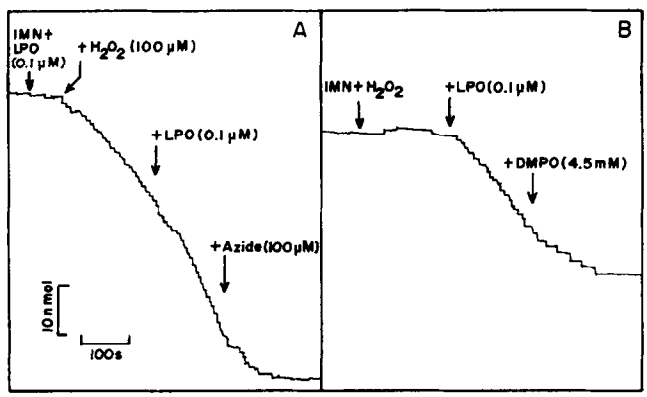

FIG. 4. Oxygen consumption during LPO-catalysed IMN oxidation. Arrows indicate the addition of the components shown in A and B with the concentration as indicated.

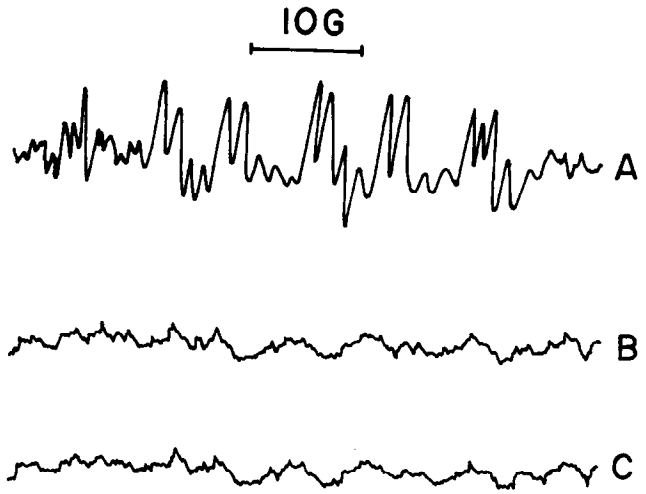

FIG. 5. ESR spectrum of the DMPO-IMN free radical adduct. (A) Incubation system as described in Materials and Methods. (B) Incubation in the absence of H_2O_2. (C) Incubation in absence of IMN. Spectrometer settings: scan range = 100 G, time constant = ls, microwave power = 20 mW, modulation amplitude = 10 G, gain = 5 × 10^4, and scan rate = 12.5 G/min.

energy change $\Delta G'$ calculated from the K_D value (Eqn 2) was −26.3 KJ mol^{-1}, indicating that IMN binding is favourable for oxidation by LPO.

Dependence of IMN Binding on pH

The pH dependence of IMN binding to LPO was studied over the pH range of 5 to 7.5. The apparent K_D values at different pH were calculated from the difference spectra, and the log K_D values were plotted against different pH values (Fig. 7). The resulting straight line indicates that the binding is not affected by change of pH, suggesting that the enzyme ionisable group does not control IMN binding.

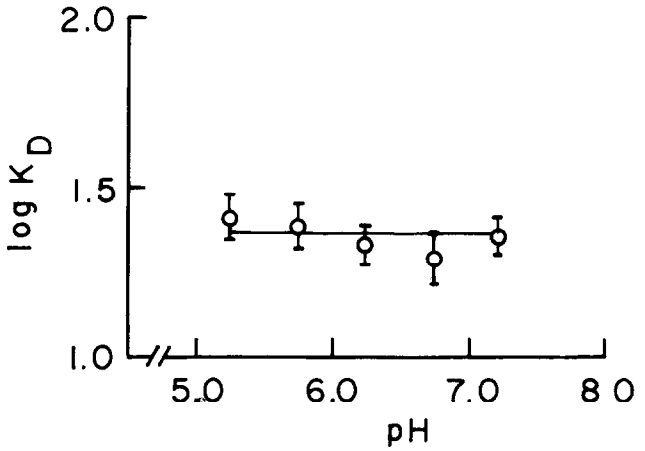

FIG. 7. pH-dependence of the binding of IMN to LPO. K_D values for the formation of the LPO–IMN complex were determined from the difference spectra at different pH values as indicated. The data presented are mean ± SEM for three experiments.

Location of IMN Binding Site in LPO

The possible binding site of IMN in LPO with respect to heme-iron, aromatic donor, and SCN^- binding sites was studied by optical difference spectroscopy. IMN also binds to the LPO–CN (cyanide) complex with a K_D value of 23 μM, showing a difference spectrum having minimum at 430 nm (not shown). This indicates that IMN binds at a site away from the heme-iron centre. IMN binding was also studied in presence of resorcinol, an aromatic electron donor that binds at the heme edge through hydrophobic interaction [30]. The result shown in Fig. 8 indicates that the K_D value of IMN (22 μM) was increased to 100 μM in the presence of 100 μM resorcinol. The plot further indicates that IMN competes with resorcinol for binding at the same site. However, no difference spectrum of the LPO–IMN complex was evident in the presence of SCN^-.

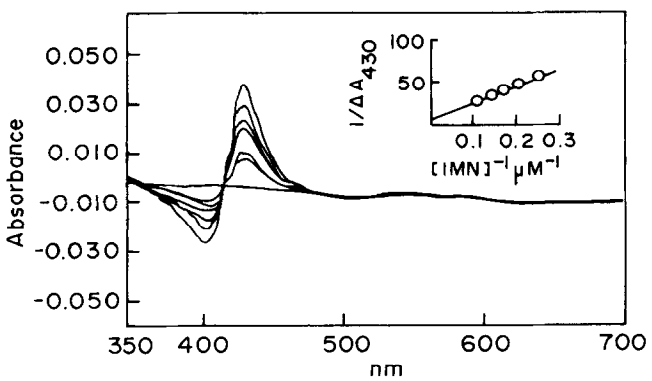

FIG. 6. Optical difference spectra for the binding of IMN to LPO. Difference spectra of the LPO–IMN complex versus LPO were obtained at pH 5.75 at different IMN concentrations. Inset represents the plot of $1/\Delta A$ against 1/[IMN] to calculate the K_D. ΔA was measured at 430 nm. Each trace was repeated at 1-min intervals after adding IMN to check the stability of the complex.

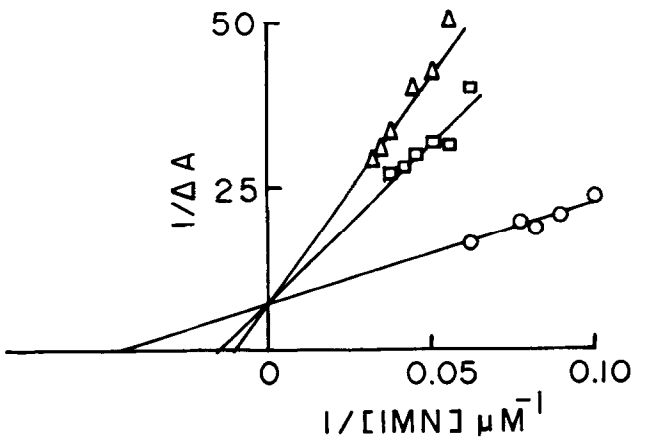

FIG. 8. Competitive binding of IMN to LPO with different concentrations of resorcinol. Difference spectra of the LPO–IMN complex versus LPO were obtained in presence of 0 (circle), 50 (square), and 100 (triangle) μM resorcinol, and data were analysed using Eqn 1.

DISCUSSION

The present study indicates that LPO can oxidise IMN through a free radical intermediate to product(s) having extinction at 412 nm. The low K_D value (24.5 μm) for the LPO–IMN complex indicates that LPO oxidises IMN with a high affinity of binding. The pH dependence of IMN oxidation suggests that protonation of an ionisable residue of LPO having pKa = 5.7 favours the oxidation process. The group is presumably contributed by the distal histidine having a pKa value of approximately 6, which is implicated in controlling the binding of iodide, thiocyanate, and aromatic donors as well as the electron transfer from the donor group to the heme-ferryl group [35–37]. IMN oxidation can be dissociated into (i) IMN binding to LPO and (ii) electron transfer from bound IMN to the heme-ferryl group. The K_D of the LPO–IMN complex was found to be constant in the pH range of 5–7.5. This indicates that binding of IMN is not influenced by any ionisable group of the enzyme at this pH range. Rather, a hydrophobic interaction similar to the binding of aromatic donor [30] may be involved in the process. Thus, the ionisable group of pKa = 5.7 presumably contributed by the distal histidine is probably required for controlling the electron transfer from bound IMN to the heme-ferryl group, which is favoured by its protonation. Binding of IMN in the LPO–CN complex with a K_D value (23 μM) similar to that of the native LPO (24.5 μM) indicates that IMN binds at a site away from the heme-iron centre. IMN binding was found to be competitive with resorcinol, with the K_D value being fivefold higher than in its absence. This finding suggests that IMN interacts at a site where aromatic donors bind. Hosoya et al. [30] suggested that aromatic donors may bind to the heme edge mainly through hydrophobic interaction at a specific site that is close to the iodide binding site [35]. In HRP also, aromatic donor binding site (see note added in proof) is presumably constituted by a hydrophobic pocket made by 8-CH_3, Tyr-185, and Arg-183 residues [38, 39], and this site appears to be different from the iodide binding site [40]. Modi et al. [37], however, suggested that aromatic donors and SCN^- occupy the same site in LPO. Because IMN binding is pH independent and competitive with resorcinol, it is logical to suggest that IMN may bind through hydrophobic interaction to the aromatic donor binding site. The complete loss of formation of the LPO–IMN complex in presence of SCN^- indicates that IMN, resorcinol, and SCN^- bind at the same site or that SCN^- binding at a nearby site causes steric hindrance to prevent IMN binding.

IMN can reduce LPO–compound II to the native ferric state as shown by the spectral shift from 430 nm to 412 nm through an isosbestic point at 421 nm (compound II/native), suggesting that oxidation occurs by a one-electron transfer mechanism [14]. This one-electron oxidation generates the free radical of IMN that is detected by ESR spectroscopy. From the splitting constants (a_N = 15 G and a_β^H = 16 G), the free radical appears to be a nitrogen-centred radical species [17]. Because no signal appears in the absence of IMN, an assignment of the spectrum to a nitrogen-centred DMPO radical can be excluded. However, IMN has some structural similarity with indoleacetic acid, which is oxidized by HRP to indole-3-carbinol through the formation of a carbon-centred skatole radical after decarboxylation from the acetate group [41]. The skatole radical forms a DMPO adduct with splitting constants of a_N = 16 G and a_β^H = 22.4 G [42]. Although IMN is likely to form a similar carbon-centred radical species resulting from decarboxylation of its acetate group, more studies are required for conclusive characterisation of the radical intermediate formed during oxidation. Moreover, decarboxylation of indoleacetic acid or its 5-methoxy analogue, which is structurally close to IMN, has a pKa value of 5.1 and 5.5, respectively [43]. This is close to the pKa value of 5.7 obtained for the LPO-catalysed IMN oxidation. Because N-I of IMN cannot deprotonate, this pKa value could be assigned to the ionisable group of LPO, presumably to its distal histidine residue, as already discussed. Substrate free radicals are usually stabilised by coupling, disproportionation, and/or reaction with molecular oxygen [18]. Oxygen uptake data indicate that the free radical, being highly reactive, is stabilised to a product after reaction with molecular oxygen. This reaction should generate O_2^-, which is usually detected by reduction of ferricytochrome c [44]. However, no cytochrome reduction was evident in our system. This finding is in contrast with the observation [44] that the free radical generated during oxidation of indoleacetic acid by turnip peroxidase reduces ferricytochrome c. However, oxygen uptake could also occur, if the generated free radical traps molecular oxygen, as suggested by Hinman and Lang [45] and Candeias et al. [41] in HRP-catalysed oxidation of indoleacetic acid. Therefore, it is more likely that IMN free radical incorporates O_2 without producing O_2^- before being converted into stable oxidation product(s). The following reaction mechanism may thus be proposed for LPO-catalysed IMN oxidation:

$$\text{LPO} + H_2O_2 \rightarrow \text{compound I} \quad (3)$$

$$\text{compound I} + \text{IMN} \rightarrow \text{compound II} + \text{IMN}^{\cdot+} \quad (4)$$

$$\text{compound II} + \text{IMN} \rightarrow \text{LPO} + \text{IMN}^{\cdot+} \quad (5)$$

$$\text{IMN}^{\cdot+} + O_2 \rightarrow \text{IMN oxidation product(s)} \quad (6)$$

Therefore, we conclude that IMN binds at the aromatic donor binding site and is oxidized by the LPO–H_2O_2 system through one-electron transfer to form a free radical that incorporates O_2 to form stable oxidation product(s). The pH dependence of oxidation suggests that IMN is oxidized at a reduced rate at physiological pH by LPO or LPO-like peroxidases, which may be the extrahepatic route of its metabolism, the primary route being the hepatic cytochrome P-450 system [10]. However, higher rate of oxidation at acid pH has some relevance in inflammatory reactions, where phagocytic cells such as neutrophils are recruited at the inflammed site and get activated with

subsequent decrease in the phagolysosomal pH to nearly 5 [46]. The more reactive IMN radical likely to be generated at a higher rate at acid pH through oxidation by myeloperoxidase system [8] may take part in the inflammatory reaction or may be toxic to the neutrophil, leading to neutropenia often observed after therapeutic doses of IMN [1]. Because IMN also inhibits eosinophil peroxidase activity [27], eosinophil-mediated antibacterial function is also expected to be deranged by this antiinflammatory drug. It is worth investigating whether the toxic reactions caused by IMN [1, 6–8] are mediated through its highly reactive free radical intermediates.

R. Chatterjee gratefully acknowledges the receipt of Research Associateship from the Council of Scientific and Industrial Research (CSIR), New Delhi, during this work.

Note added in proof: Recently arginine-38 has been shown to be involved in aromatic donor binding site in HRP (Rodriguez-Lopez JN, Smith AT and Thorneley NF, Role of arginine 38 in horseradish peroxidase. *J Biol Chem* **271:** 4023–4030, 1996).

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