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AROMATIC HYDROXYLATION DURING THE MYELOPEROXIDASE-OXIDASE OXIDATION OF HYDRAZINES

BEN J. VAN DER WALT,* JOHANN M. VAN ZYL and ANDRÉ KRIEGLER

Department of Pharmacology, Medical School, University of Stellenbosch, Tygerberg 7505, South Africa

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Abstract—Benzoic acid was found to be hydroxylated by a mixture of myeloperoxidase (MPO) and the mycobactericidal drug, isoniazid. Aromatic hydroxylation and formation of compound III (oxyperoxidase) were coincident during the MPO-oxidase oxidation of isoniazid which proceeded without augmentation from the reagent hydrogen peroxide. An intermediate of isoniazid reduced ferric MPO to ferrous MPO which associated with dioxygen to form compound III. Aromatic hydroxylation also occurred in a mixture of isoniazid (or phenylhydrazine) and a ferric salt. Hydroxylations in both the enzymatic and nonenzymatic reaction systems were inhibited by the iron chelator, desferal, as well as by the specific hydroxyl radical scavenger, mannitol. To distinguish between the hydroxylating intermediates in the different reaction systems, the unique properties of the natural antioxidant, phytic acid, were exploited. Phytic acid inhibited aromatic hydroxylation in the Fe^{3+}–INH system, which is in accordance with its known properties as a powerful inhibitor of iron-driven reactions ($^{\cdot}OH$ formation). By contrast, phytic acid stimulated hydroxylation in the enzymatic system which was accompanied by a concomitant stimulation in the rate of compound III formation. These events were, however, not directly related to each other. Phytic acid had a direct effect on the redox transformation of isoniazid by stimulating superoxide generation during auto-oxidation of the drug. In addition, phytic acid also facilitated compound III decay in the absence of isoniazid, suggesting that it may also regulate the oxygen affinity of MPO, similar to its effect on the oxygenation of haemoglobin. The data on aromatic hydroxylation in the MPO-isoniazid system do not support a role for $^{\cdot}OH$ in the reaction and may fit the model for the P450 mixed oxidase system.

Key words: myeloperoxidase; aromatic hydroxylation; hydrazine oxidation

Hydrazines are widely used in industry and medicine. These compounds can penetrate to the O_2-binding site of haemoglobin, and react with it [1]. Phenylhydrazine is one of the most studied derivatives of this group and, like other hydrazines, its oxidation is catalysed by transition-metal ions as well as by haemoglobin. Oxidation products thus formed can react with O_2 to form superoxide [2]. The metabolism of the anti-tubercular drug, INH†, has also been reported to produce hydrazine derivatives and free radicals which might contribute to the mycobactericidal activity of the drug or to its side-effects [3, 4].

Hydrazines can be oxidized by MPO [4, 5] and other peroxidases [6–8]. The MPO system is a critical component of oxygen-dependent neutrophil-mediated host defence against infection, but is also involved in the initiation and promotion of inflammatory conditions. MPO forms three distinct complexes on reaction with H_2O_2; compounds I, II and III. Relatively low (equimolar) concentrations of H_2O_2 react with the iron of MPO to form compound I, which is at an oxidized level two equivalents above that of the resting ferric enzyme. Compound I is believed to be the primary catalytic intermediate of MPO and is involved in the oxidation of Cl^- to form the highly reactive chlorinating oxidant, hypochlorous acid (HOCl). In the absence of a suitable electron donor, compound II is formed which is at an oxidized level one equivalent above that of the resting enzyme. Compound II is an inactive form of MPO in respect to the oxidation of Cl^- [9].

Compound III is an oxyperoxidase which, like oxygenated haemoglobin, has oxygen attached to the haem iron. It can be formed in the following reactions: (1) Compound II with H_2O_2 [10]; (2) ferrous MPO with molecular oxygen [10]; (3) native ferric MPO with superoxide [10, 11]; (4) during the MPO-oxidase oxidation of hydrogen donors, such as NADH [10], dihydroxyfumarate [12], various aminothiols [13] and INH [5]. When neutrophils are activated, extracellularly released MPO may be exposed to a high flux of superoxide and spectral evidence suggesting the *in vivo* formation of compound III has been presented [14].

Aromatic hydroxylation can take place via the hydroxyl radical ($^{\cdot}OH$), which can be generated in an iron-catalysed Haber–Weiss reaction [15]. Evidence has also been presented that $^{\cdot}OH$ can be generated in the activated neutrophil via the MPO system in a transition-metal independent fashion [16]. Hydroxylations of phenylbutazone [17] and

* Corresponding author. Tel. (27) 21 931-3131; FAX (27) 21 931 7810.

† Abbreviations: GO, glucose oxidase; INH, isonicotinic acid hydrazide (isoniazid); MPO, myeloperoxidase; NBT, nitroblue tetrazolium; SOD, superoxide dismutase.

salicylate [18] by activated neutrophils have been reported. While MPO was implicated in the hydroxylation of phenylbutazone, studies with salicylate did not support a role for MPO or ·OH in the hydroxylation reaction. MPO also catalysed hydroxylation of diclofenac by an unknown reaction pathway [19]. In one study, spin trapping evidence suggested the generation of ·OH during horseradish peroxidase-catalysed oxidation of INH [7], while another study failed to detect ·OH in a similar system, although the formation of a radical species equivalent to ·OH has been postulated [8].

The extensively studied mixed oxidase cytochrome P450 system is known to catalyse aromatic hydroxylation reactions, apparently independently of the hydroxyl radical [20]. Haemoglobin can replace cytochrome P450 with comparable efficacy in this system [21].

In this communication, evidence will be presented that compound III, generated during the oxidation of hydrazines, is involved in aromatic hydroxylation. To distinguish between iron-driven and MPO-catalysed hydroxylation reactions, we have exploited the unique properties of the natural anti-oxidant and potent iron chelator, phytic acid [22].

MATERIALS AND METHODS

Reagents. MPO (donor H_2O_2 oxidoreductase, EC 1.11.1.7) from human neutrophils was prepared as described [23] and enzyme with a purity index [RZ = A_{423}/A_{280} (nm)] of at least 0.73 was used. Phenylhydrazine hydrochloride, INH and phytic acid (dodecasodium salt hydrate) were products from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). GO was from Boehringer Mannheim (Mannheim, F.R.G.); SOD and NBT from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and benzoic acid GR from E. Merck (Darmstadt, F.R.G.).

Spectroscopic analyses. UV absorption analyses were performed on a Beckman DU 640 spectrophotometric system and fluorescence measurements made with a Perkin Elmer MPF-44A fluorescence spectrometer.

Iron-catalysed hydroxylation. Hydrazines (200 μM in reaction) were added to mixtures containing 200 μM ferric chloride, 1 mM citrate (to keep the iron ions in solution), 1 mM benzoic acid and increasing concentrations of phytic acid in 100 mM phosphate buffer at different pH values. Reaction mixtures were incubated at room temperature for 2 hr after which fluorescence of the hydroxylated benzoate products were measured (excitation and emission wavelengths at 305 nm and 407 nm, respectively) [24].

Compound III formation induced by the hydrazines and aromatic hydroxylation. Reactions were initiated by adding the hydrazine (200 μM in reaction) to mixtures containing 300 nM MPO, 1 mM benzoic acid and increasing concentrations of phytic acid in 100 mM phosphate buffer (pH 7.4 or pH 8). Formation of compound III was monitored at 456 nm. The presence of benzoic acid had no measurable effect on the generation or decay of compound III. Formation of fluorescent hydroxylated benzoic acid derivatives was measured after 2 hr on aliquots of the reaction mixtures used for monitoring compound III formation. In some experiments, SOD or a GO/glucose H_2O_2-generating system was used to determine the effect of H_2O_2 on INH-induced compound III generation.

Generation of compound III as the ferro–dioxygen complex. MPO (33 μM) in 100 mM phosphate buffer (pH 7.4) was reduced with a few crystals of sodium dithionite, and 500 μL of the solution was applied to a 4 × 1 cm-diameter column of Sephadex G-10 at 4°. The ferrous haem protein separates from the dithionite and reacts with O_2 dissolved in the buffer. The decay of compound III was monitored at 456 nm in the absence and presence of 2 mM phytic acid.

NBT reduction. The reaction mixtures consisted of 0.2 mM NBT in 100 mM Na_2HPO_4 (pH 9) and different concentrations of phytic acid. INH (2.5 mM in reaction) were added at zero time and change in A_{560} (nm) was recorded continuously.

Anaerobic reduction of MPO. A solution of 1.3 μM MPO and 2 mM phytic acid was bubbled with oxygen-free nitrogen for at least 5 min to remove oxygen from the reaction system and cuvettes were sealed with parafilm. The reaction was started with INH (200 μM in reaction) and overlay scans recorded at different time intervals.

RESULTS

Although all results illustrated employed INH as prototype hydrazine, qualitatively similar results were obtained with phenylhydrazine.

Non-enzymatic iron-catalysed hydroxylation

Monohydroxylation of benzoic acid forms highly fluorescent products [24]. Figure 1 shows the effect of phytic acid on the hydroxylation of benzoic acid (expressed as relative fluorescence units) in reaction mixtures containing Fe^{3+} and INH at different pH values. It is evident that phytic acid inhibited hydroxylation, which may be expected from its known properties as an inhibitor of iron-driven oxidation reactions [22]. Furthermore, phytic acid was apparently more potent at pH 8 than at the lower pH values. At alkaline pH, the phosphate groups on the phytic acid molecule should be negatively charged and consequently its binding affinity for the positively charged iron ions will be increased. This may explain the higher potency of phytic acid at alkaline pH. Hydroxylation was also inhibited by another iron chelator, desferal, and by the ·OH scavenger, mannitol.

MPO-catalysed aromatic hydroxylation

The excitation and emission scans of benzoate hydroxylation, products generated in the MPO–INH system, are shown in Fig. 2. In contrast to the effect of phytic acid in the iron-driven reaction (Fig. 1), a 5-fold stimulation of hydroxylation was observed at 1.5 mM phytic acid (Fig. 2; scan 1 em). Hydroxylation in the presence of 1 mM desferal (scan 4 em) was similar to that of unhydroxylated benzoic acid which is only weakly fluorescent under the conditions described, while 1 mM mannitol also had an inhibiting effect (scan 3 em).

Figure 3 shows fluorescence yields of hydroxylated

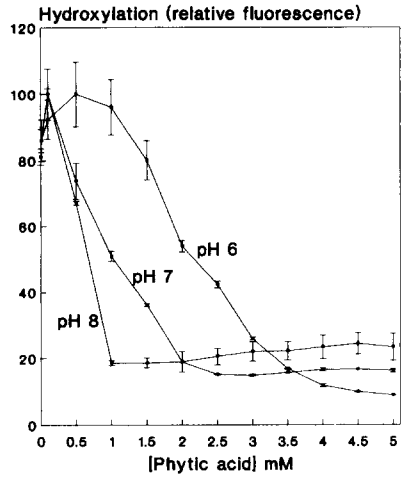

Fig. 1. Inhibitory effect of phytic acid on benzoate hydroxylation in the Fe^{3+}–INH system. INH (200 μM in reaction) was added to solutions containing 200 μM $FeCl_3$, 1 mM citrate, 1 mM benzoic acid and increasing concentrations of phytic acid in 0.01 M phosphate buffer at different pH values. Reaction mixtures were incubated for 2 hr at room temperature after which fluorescence of the hydroxylated benzoate products (excitation at 305 nm and emission at 407 nm) was measured. Each data point represents the mean ± SD of four analyses. Mean values were normalized to the same maximum at each pH value.

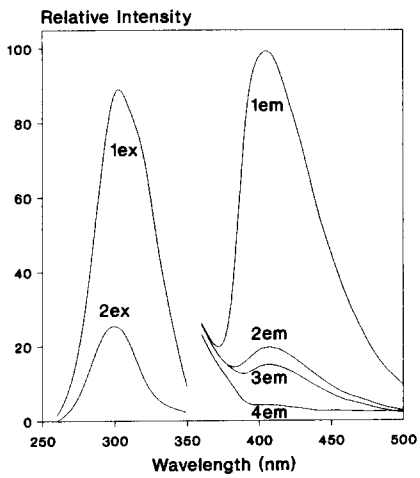

Fig. 2. Fluorescence scans of benzoate hydroxylation products formed in the MPO–INH reaction mixture. INH (200 μM in reaction) was added to mixtures containing 300 nM MPO, 1 mM benzoic acid in 0.01 M phosphate buffer (pH 7.4) in the absence and presence of phytic acid, desferal or mannitol. Fluorescence was recorded after 2 hr. Scans (ex = excitation and em = emission): 2, control (no additional reagent); 1, with 1.5 mM phytic acid; 3, with 1 mM mannitol; 4, with 1 mM desferal.

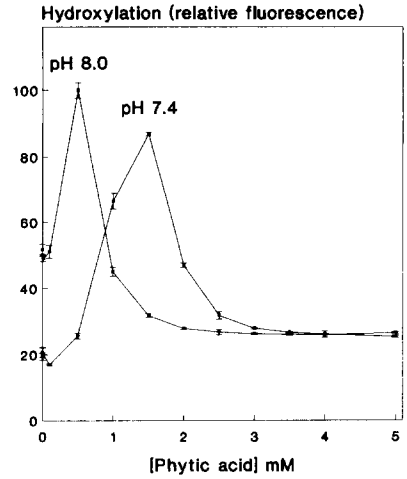

Fig. 3. Stimulating effect of phytic acid on benzoate hydroxylation in the MPO–INH system. INH (200 μM in reaction) was added to mixtures containing 300 nM MPO, 1 mM benzoic acid in 0.01 M phosphate buffer (pH 7.4 and 8). Fluorescence was measured after 2 hr (excitation at 305 nm and emission at 407 nm). Each data point represents the mean ± SD of three analyses.

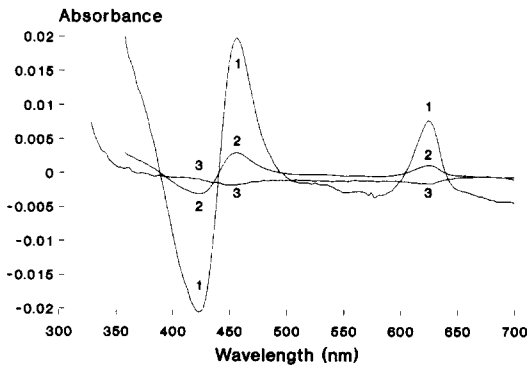

Fig. 4. Compound III formation during the MPO-oxidase oxidation of INH. Both sample and reference cuvettes contained 300 nM MPO in 0.01 M phosphate buffer (pH 7.4) in the absence or presence of phytic acid or desferal. Scans were made 1 min after adding INH (200 μM in reaction) to the sample cuvettes. Difference scans: 1, with 1.5 mM phytic acid; 2, control (no additional reagent); 3, with 1 mM desferal.

benzoic acid, products in MPO–INH reaction mixtures as a function of phytic acid concentration. Maximum hydroxylation occurred at pH 7.4 in the presence of 1.5 mM phytic acid, and at pH 8 in the presence of 0.5 mM phytic acid. At phytic acid concentrations above these maxima, the fluorescent yields decreased again.

When phenylhydrazine was substituted for INH in these experiments, the fluorescence yields were significantly lower. In addition, phenylhydrazine had a greater potency to damage the MPO haem.

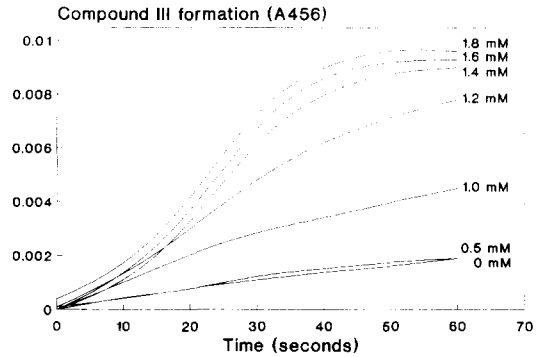

Fig. 5. Effect of phytic acid on the kinetics of compound III formation. INH (200 μM in reaction) was added to mixtures of 300 nM MPO and different concentrations of phytic acid (indicated against each curve) in 0.01 M phosphate buffer (pH 7.4). Recordings at 456 nm were started immediately after initiating the reaction with INH.

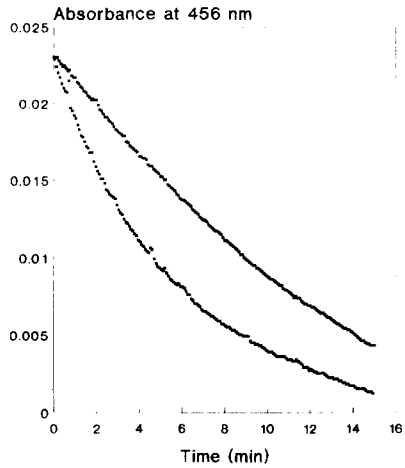

Fig. 7. Effect of phytic acid on compound III decay. MPO (33 μM) in 50 mM phosphate buffer was reduced with dithionite and applied to a Sephadex G-10 column at 4° to remove excess reducing agent. Compound III decay was monitored at 456 nm in the absence (top curve) and presence of 2 mM phytic acid (bottom curve).

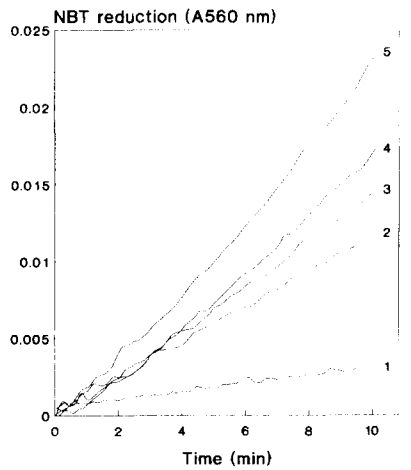

Fig. 6. NBT reduction during INH auto-oxidation. Reaction mixtures contained 0.2 mM NBT in 100 mM Na_2HPO_4 (pH 9). INH (2.5 mM in reaction) was added at zero time and change in A_{560} (nm) was recorded. Curves: 1, in the presence of SOD; 2, control—no additional reagent; 3, 0.5 mM phytate; 4, 1 mM phytate; 5, 2 mM phytate.

Generation of compound III

Figure 4 depicts difference scans of compound III formed during INH oxidation in the presence (spectrum 1) and absence (spectrum 2) of 1.5 mM phytic acid, 1 min after adding INH to MPO at pH 7.4. The characteristic compound III wavelength maxima at 456 nm and 625 nm, as well as the A_{625}/A_{456} ratio of about 0.5 [25], are evident. In the presence of 1 mM desferal, compound III was not formed (spectrum 3). Mannitol (1 mM) had a slight inhibitory action on compound III generation (not shown).

The stimulatory effect of phytic acid on the kinetics of compound III formation at pH 7.4, is shown in Fig. 5. Auto-oxidation of INH was induced at alkaline pH and the reduction of NBT monitored (Fig. 6). It is evident that phytic acid stimulated NBT reduction, in this system (curves 2–5). The inhibitory effect of SOD (curve 1) suggests that most of the NBT reduction was induced by superoxide. Figure 7 shows that phytic acid had a direct effect on the MPO molecule, independently of its effect on the redox transformation of INH. When compound III was prepared as the ferrous–dioxygen complex (in the absence of INH), phytic acid clearly stimulated compound III decay.

Formation of ferrous MPO and the effect of hydrogen peroxide

Figure 8 shows that INH can directly reduce the haem iron of MPO in the presence of phytic acid. When INH was added to a solution of MPO and 2 mM phytic acid under anaerobic conditions, a gradual conversion to ferro-MPO occurred (curves 1–6). Curves 4–6 have a shoulder at 456 nm indicating compound III formation due to traces of oxygen still present in the solution. Curve 7 shows the spectrum of ferro-MPO (peak at 472 nm) which was formed by adding a few crystals of dithionite to a MPO solution. In the absence of phytic acid, INH could not reduce MPO, suggesting that a triggering mechanism was necessary to activate the MPO–INH complex.

Figure 9 shows that H_2O_2 is required for INH-induced compound III formation. In Fig. 9A, SOD stimulated the formation of compound III (curve 3). Catalase, however, did not have any significant effect (curve 2). Catalase is known to be inefficient at the low H_2O_2 concentrations which might be encountered in this system. When a GO H_2O_2-generating system was present in the MPO–INH mixture (without

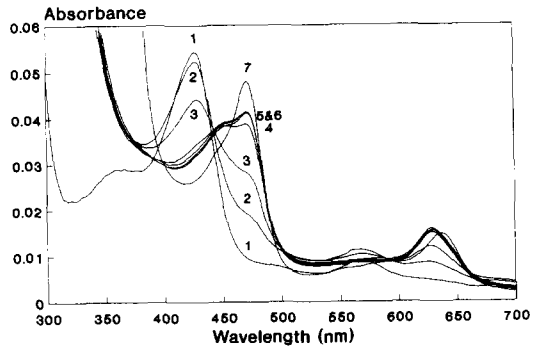

Fig. 8. Anaerobic reduction of MPO. A solution of 1.3 μM MPO and 2 mM phytic acid in 50 mM phosphate buffer (pH 7.4) was bubbled with oxygen-free nitrogen. The reaction was started with INH (200 μM in solution) and, overlay scans recorded. Scans: 1, control MPO; 2, 1 min after INH addition; 3–6, at 2 min intervals; 7, a few crystals of sodium dithionite were added to a solution of 1.3 μM MPO.

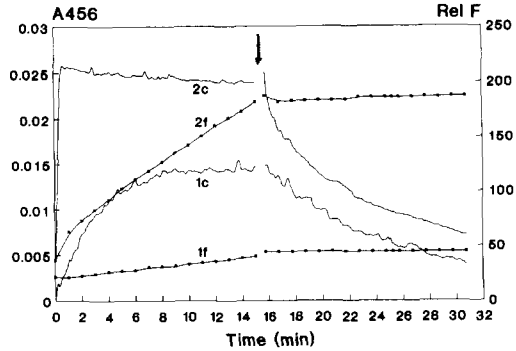

Fig. 10. Relationship between compound III formation and aromatic hydroxylation. Compound III was monitored at 456 nm after adding INH (200 μM in reaction) to solutions containing 1.3 μM MPO and 1 mM benzoic acid in 100 mM phosphate buffer (pH 7.4) in the absence (curve 1c) and presence of 1.5 mM phytic acid (curve 2c). Fluorescence measurements (excitation 305 nm; emission 407 nm) were made on aliquots of the same samples used for monitoring compound III formation (curve 1f, without phytic acid; curve 2f, with phytic acid). After 15 min of reaction, desferal (1 mM in solution) was added to the reaction mixtures (indicated by the arrow).

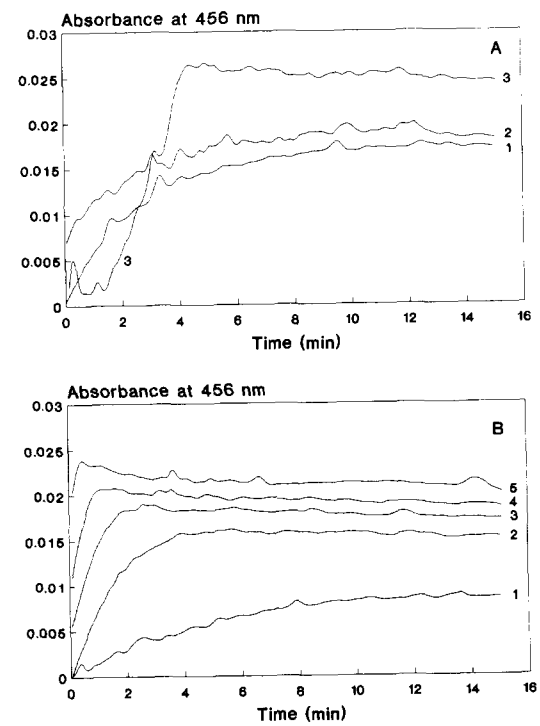

Fig. 9. H_2O_2 requirement for compound III formation. (A) Compound III generated by adding INH (200 μM in solution) to 1.3 μM MPO in 50 mM phosphate buffer (pH 7.4). Curves: 1, control—no additional reagent; 2, in the presence of catalase; 3, in the presence of SOD. (B) Compound III was generated as in A. Curves: 1, control—no additional reagent; 2–5, in the presence of, respectively, 0.8, 1.5, 3.75 and 7.5 U of a glucose–glucose oxidase H_2O_2 generating system.

SOD), formation of compound III was likewise stimulated (Fig. 9B).

Relationship between compound III generation and aromatic hydroxylation

The relationship between compound III formation and aromatic hydroxylation is shown in Fig. 10. In the absence of phytic acid, steady-state compound III concentrations were reached within about 9 min (curve 1c). In the presence of 1.5 mM phytic acid, compound III formation reached a maximum within about 30 sec (curve 2c) and then slowly declined to reach a plateau level after about, 1 hr (not shown). Addition of desferal after 15 min (indicated by the arrow), led to a gradual decay of compound III. The corresponding hydroxylation curves are denoted 1f (without phytic acid) and 2f (with 1.5 mM phytic acid). Addition of desferal led to an inhibition of further hydroxylation. Without desferal, hydroxylation proceeded for at least 6 hr in both cases. Furthermore, the rates of hydroxylation were proportionally higher in the presence of phytic acid (relative to compound III concentration) than in the absence of phytic acid.

DISCUSSION

Hydroxylation of the aromatic nucleus does not occur during normal peroxidatic oxidations in which peroxidase catalyses the transfer of electrons from donor to hydrogen peroxide. Thus hydroxylation reactions occurring in these systems are often thought to be due to hydroxyl radicals. It is difficult to prepare metal-free buffer solutions and also interactions of compounds such as hydrazines with haem-containing proteins may result in haem splitting [5, 8, 26]. Iron thus released [26] and other "free"

transition metal ions could be involved in Fenton-type $^{\cdot}OH$-generating reactions.

Hydrazines slowly oxidize in aqueous solution to form $O_2^{\cdot-}$ and H_2O_2, a reaction which is catalysed by transition-metal ions (e.g. Fe^{3+}). The initial stages of the reaction may be represented by the following equations [15], taking INH as an example (R = isonicotinyl):

$$R—CO—NH—NH_2 + Fe^{3+} \rightarrow H^+ + R—CO—NH—NH^{\cdot} + Fe^{2+} \quad (1)$$

$$R—CO—NH—NH^{\cdot} + O_2 \rightarrow H^+ + O_2^{\cdot-} + R—CO—N{=}NH. \quad (2)$$

Superoxide can dismutate to form H_2O_2 or may recycle Fe^{3+}:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2. \quad (4)$$

Hydroxyl radical generation in a Fenton reaction is thus possible:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-. \quad (5)$$

Formation of $^{\cdot}OH$ in the Fe^{3+}-INH nonenzymatic system was suggested, since the iron chelators, desferal and phytic acid, and the scavenger, mannitol, inhibited hydroxylation of benzoate.

The principal features of peroxidase-oxidase reactions have been shown to be auto-oxidation of the substrate to yield $O_2^{\cdot-}$ (eqns 1 and 2). The dismutation product of $O_2^{\cdot-}$, H_2O_2, can then be used in a classical peroxidase cycle to oxidize the substrate to an intermediate (the isonicotinyl radical in the case of INH) [27, 28]. Dismutation of $O_2^{\cdot-}$ to form H_2O_2 may also be catalysed by MPO [29]:

$$MPO\ (Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} \quad (6)$$

$$\text{compound I} + R—CO—NH—NH_2 \rightarrow H^+ + R—CO—NH—NH^{\cdot} + \text{compound II} \quad (7)$$

$$\text{compound II} + R—CO—NH—NH_2 \rightarrow H^+ + R—CO—NH—NH^{\cdot} + MPO\ (Fe^{3+}). \quad (8)$$

Superoxide, generated via the isonicotinyl radical (eqn 2), can react with MPO to form compound III (oxyperoxidase):

$$MPO\ (Fe^{3+}) + O_2^{\cdot-} \rightarrow MPO\ (Fe^{3+}O_2^{\cdot-} \leftrightarrow Fe^{2+}O_2). \quad (9)$$

Equation 9 represents the intermediate structure of compound III which can also be formed by dioxygen binding to reduced MPO. This pathway for compound III formation has been observed during the oxidation of hydroquinone by MPO [30]. Since hydrazines form complexes with haem proteins and are oxidized to intermediates which can alkylate the prosthetic haem or the non-haem portion at a position close to the active site [20, 31, 32], a mechanism for compound III formation which is not reliant on superoxide seems possible. Under "anaerobic" conditions in the presence of phytic acid, INH was able to reduce the haem iron of MPO (Fig. 8). The reaction mixture, however, still contained traces of oxygen, since compound III was also formed. Steady state concentrations of ferro MPO were reached only after about 10 min, while under aerobic conditions (Fig. 10), maximum compound III concentrations (in the presence of 1.5 mM phytic acid) were reached within only 30 sec. Furthermore, under conditions of limited oxygen supply in the absence of phytic acid, the spectrum of MPO remained unaltered. The data thus suggest that oxygen is involved in the reduction of the MPO haem and that phytic acid acts as a catalyst. In fact, phytic acid had a direct effect on the redox transformation of INH by stimulating its auto-oxidation at alkaline pH (Fig. 6). Under aerobic conditions, SOD (Fig. 9A) or a H_2O_2-generating system (Fig. 9B), stimulated compound III generation in the presence of INH, suggesting that H_2O_2 is involved in the reaction. Taken together, these data indicate that INH is oxidized to a reactive intermediate in a classical peroxidase cycle (eqns 6–8). This reactive intermediate (possibly an isonicotinyl radical) is able to reduce ferric MPO to ferrous MPO, which can combine with dioxygen to form compound III.

To evaluate the role of $^{\cdot}OH$ in the hydroxylation of benzoic acid, we have exploited the properties of phytic acid. Phytic acid forms a unique iron chelate which makes it a potent inhibitor of iron-catalysed reactions [22]. In the non-enzymatic Fe^{3+}–INH system, phytic acid clearly acted as an antioxidant by inhibiting benzoate hydroxylation (Fig. 1). By contrast, phytic acid had the opposite effect in the enzymatic system; hydroxylation was first stimulated and then decreased at higher phytic acid concentrations (Fig. 3). Stimulation of hydroxylation can be explained by the fact that phytic acid accelerated the auto-oxidation of INH (Fig. 6). This increase in production of INH derivatives and reactive oxygen species resulted in considerable MPO haem damage (results not shown) which was accompanied by a decrease in hydroxylation yield (Fig. 3). This suggests that an intact haem was important for aromatic hydroxylation. Any iron released during haem splitting would have been inactivated by phytic acid; thus eliminating iron-driven hydroxyl radical generation in the enzymatic system. Furthermore, if $^{\cdot}OH$ were produced in an iron-catalysed Haber–Weiss reaction according to reactions 1–5, then phenylhydrazine, which auto-oxidizes more readily than INH and consequently has a greater potential to release iron from the MPO haem, should be a more potent inducer of aromatic hydroxylation. The converse, however, was true; enzymatic phenylhydrazine-driven aromatic hydroxylation (in the absence of phytic acid) was considerably less than INH-driven hydroxylation under the same experimental conditions (results not shown).

If $^{\cdot}OH$ were not involved in MPO mediated hydroxylation, why were desferal and to a lesser extent, mannitol, inhibitors of hydroxylation? In general, compound III formation and hydroxylation were coincident. This has also been recognized in a horseradish peroxidase-dihydroxyfumarate system [12, 33]. When desferal was present in our reaction mixture before addition of INH, no hydroxylation occurred (Fig. 2) and compound III was not generated (Fig. 4). On the other hand, when desferal was added after compound III had been formed,

decay of compound III resulted and hydroxylation was inhibited (Fig. 10). Inhibition of compound III formation is consistent with desferal acting as an electron donor and a scavenger of O_2^- [34, 35]. Mannitol had some inhibitory action on both hydroxylation (Fig. 2) and compound III formation (not shown). Various agents, such as alcohols, ethers and ketones coordinate weakly via their oxygen atoms to the haem iron of cytochrome P450, resulting in inhibition of the enzyme (page 275 of Ref. 20). It is reasonable to expect that mannitol, which has been reported to be a weak iron chelator [36], should have a similar effect on the MPO haem iron.

Our data on MPO-catalysed hydroxylation may fit the model for the P450 mixed oxidase system [20]: (1) The first steps in the P450 catalytic cycle involve one-electron reduction of its iron to the Fe^{2+} state and association with dioxygen. Reduction of MPO haem by INH under oxygen-poor conditions, suggests that compound III can also be generated by a P450-like mechanism (Fig. 8). Alternatively, compound III can be formed as the superoxide-ferric adduct of MPO.

(2) The next steps of the P450 mechanism involve further reduction to form the iron peroxo species ($Fe^{3+}O_2^-$) and heterolysis of the O–O bond. The observations that compound III can act as a SOD [29], that H_2O_2 is a product of the auto-oxidation of compound III [37], and that oxyferroperoxidase (compound III) decayed to ferric peroxidase without the formation of ferroperoxidase as an intermediate, are consistent with the formation of a peroxo–MPO complex. Addition of an electron to compound III to yield a short-lived highly reactive species has been suggested [37, 38].

Phytic acid has a high affinity for the 2,3-diphosphoglycerate site in haemoglobin [22] and, like diphosphoglycerate, it facilitates dissociation of oxygen from haemoglobin. It may be that phytic acid increases the rate of compound III recycling by lowering the oxygen affinity of MPO. The observation that phytic acid accelerated compound III decay suggests such a mechanism (Fig. 7). Although compound III formation and hydroxylation were coincident (Fig. 10), the two events are apparently not directly related to each other. Phytic acid appeared to facilitate hydroxylation without increasing compound III concentrations proportionally.

(3) The final step in P450 catalysis is a two-electron oxidation of substrate to produce the oxygenated substrate with regeneration of the ferric resting state of the enzyme. In our system, hydroxylation of benzoate was monitored, and if ferric MPO were formed, it would have been immediately reconverted to compound III.

In conclusion, a mechanism for MPO-catalysed aromatic hydroxylation has been described which is independent of $^{\cdot}OH$ and apparently similar to that of the cytochrome P450 mono-oxygenase system. Certain drugs are hydroxylated by the stimulated neutrophil, but consensus on the involvement of MPO does not exist [17–19]. Since O_2^- is generated during the oxidative burst of neutrophils, compound III may indeed be generated *in vivo* [14] and involved in oxidation reactions not normally associated with peroxidase catalysis.

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