Superoxide-dependent Oxidation of Melatonin by Myeloperoxidase*

Valdecir F. Ximenes‡, Sueli de O. Silva§, Maria R. Rodrigues‡, Luiz H. Catalani§, Ghassan J. Maghzal¶, and Ana Campa†.

Myeloperoxidase uses hydrogen peroxide to oxidize numerous substrates to hypohalous acids or reactive free radicals. Here we show that neutrophils oxidize melatonin to 1-acetyl-5-formyl-5-methoxykynuramine (AFMK) in a reaction that is catalyzed by myeloperoxidase. Production of AFMK was highly dependent on superoxide but not hydrogen peroxide. It did not require hypochlorous acid, singlet oxygen, or hydroxyl radical. Purified myeloperoxidase and a superoxide-generating system oxidized melatonin to AFMK and a dimer. The dimer would result from coupling of melatonin radicals. Oxidation of melatonin was partially inhibited by catalase or superoxide dismutase. Formation of AFMK was almost completely eliminated by superoxide dismutase but weakly inhibited by catalase. In contrast, production of melatonin dimer was completely eliminated by superoxide dismutase but weakly inhibited by catalase. We propose that myeloperoxidase uses superoxide to oxidize melatonin by two distinct pathways. One pathway involves the classical peroxidase mechanism in which hydrogen peroxide is used to oxidize melatonin to radicals. Superoxide adds to these radicals to form an unstable peroxide that decays to AFMK. In the other pathway, myeloperoxidase uses superoxide to insert dioxygen into melatonin to form AFMK. This novel activity expands the types of oxidative reactions myeloperoxidase can catalyze. It should be relevant to the way neutrophils use superoxide to kill bacteria and how they metabolize xenobiotics.

Catalysis of hypochlorous acid production is the accepted physiological function of myeloperoxidase (MPO) (1, 2). This heme enzyme is the major protein in neutrophils and is also present in monocytes, macrophages, microglia (3), and neurons (4, 5). It reacts with hydrogen peroxide to form the redox intermediate compound I, which oxidizes chloride to hypochlorous acid with coincident regeneration of the native enzyme (Reactions 1 and 2). HOCI indicates hypochlorous acid.

\[
\text{MPO + H}_2\text{O}_2 \rightarrow \text{compound I + H}_2\text{O} \\
\text{REACTION 1}
\]

Myeloperoxidase also promotes the oxidation of numerous substrates (RH) to free radical intermediates via the classical peroxidase cycle involving compound I and compound II (Reactions 3 and 4).

\[
\text{compound I + RH} \rightarrow \text{compound II + R}^\cdot + \text{H}^+ \\
\text{REACTION 3}
\]

Compound III reacts sluggishly with potential substrates (11), but it is reduced by ascorbate (12) and acetaminophen (13). It is conceivable that compound III potentiates oxygen, like the analogous intermediates of related enzymes such as cytochromes P450 (14) and nitric-oxide synthase (15). Indeed, reactions of compound III have been invoked to explain the hydroxylation of phenol (16) and salicylate (17) by myeloperoxidase and superoxide.

Superoxide also reduces compound II of myeloperoxidase (Reaction 6). This reaction prevents accumulation of compound II and the associated inhibition of hypochlorous acid production (18, 19).

\[
\text{compound II + O}_2^- + 2\text{H}^+ \rightarrow \text{MPO + O}_2 + \text{H}_2\text{O} \\
\text{REACTION 6}
\]
We have found that neutrophils and macrophages oxidize melatonin to a chemiluminescent product, presumably $N^\circ$-acetyl-$N^\circ$-formyl-5-methoxykynuramidine (AFMK) (21–23). The reaction required superoxide and was blocked by azide, which is an inhibitor of heme enzymes. Catalase, which scavenges hydrogen peroxide, had little effect on formation of the chemiluminescent product. Collectively, these results suggested that myeloperoxidase uses superoxide to oxidize an organic substrate without the need for hydrogen peroxide. In this investigation our main objectives were to identify the chemiluminescent product and to establish whether it is formed by the classical peroxidase cycle (Reactions 1, 3, and 4) or by a novel mechanism that may be relevant to the physiological function of myeloperoxidase.

**MATERIALS AND METHODS**

Melatonin, 5-hydroxytryptamine hydrochloride (serotonin), superoxide dismutase, bovine liver catalase, xanthine oxidase, phorbol 12-myristate 13-acetate (PMA), DL-methionine, taurine, 3,3',5,5'-tetramethylbenzidine (TMB), acetaldehyde, dihydroxyeth compliments of melatonin as reported previously (27) and purified by HPLC. The identity of AFMK was confirmed by mass spectrometry ([M + 1] $^+$ $m/z = 265$) using a Quattro II electrospray ionization-mass spectrometer (Micromass, Manchester, UK) and by fluorescence spectrophotometry ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 460$ nm) using a Varian F-4500 spectrophotometer (21).

**Oxidation of Melatonin by Myeloperoxidase**—Melatonin was incubated in 50 mM phosphate buffer, pH 7.4, with 10 mM acetaldehyde and varying concentrations of myeloperoxidase and xanthine oxidase. Reactions were started by the addition of xanthine oxidase, and formation of AFMK was measured by HPLC as described above. Oxidation of melatonin was monitored by either following UV absorbance changes or increases in fluorescence ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 460$ nm). Absorbance changes were recorded on an Agilent 8453 diode array spectrophotometer, and fluorescence was measured using a Hitachi F-4500 spectrophotometer.

**Detection of Melatonin Oxidation Products by LC/MS**—The oxidation products of melatonin were separated isocratically on a Luna C18 reversed phase column (250 x 4.6 mm, 5 $\mu$m) using 1 mM KH$_2$PO$_4$, pH 4.0, acetonitrile (3:1) as the mobile phase with a flow rate of 1 ml/min. Absorbances were monitored at 254 nm, and fluorescence was recorded using excitation and emission wavelengths of 340 and 460 nm, respectively.

A standard curve was generated by adding reagent hypochlorous acid to PBS containing taurine. The absorbance measurements were made in Spectra Max 190 plate reader (Molecular Devices).

**Preparation of AFMK Standard**—AFMK was prepared by ozone oxidation of melatonin as reported previously (27) and purified by HPLC. The identity of AFMK was confirmed by mass spectrometry ([M + 1] $^+$ $m/z = 265$) using a Quattro II electrospray ionization-mass spectrometer (Micromass, Manchester, UK) and by fluorescence spectrophotometry ($\lambda_{ex} = 340$ nm; $\lambda_{em} = 460$ nm) using a Varian F-4500 spectrophotometer (21).

**Determination of AFMK Production by Activated Neutrophils**—Neutrophils (2 x 10$^6$ cells/ml) were preincubated for 10 min at 37 °C in Buffer A with 50 $\mu$m melatonin. Cells were stimulated with 50 ng/ml PMA, and subsequently reactions were stopped by adding 20 $\mu$g/ml catalase. Neutrophils were then pelleted by centrifugation and put on ice. Loss of melatonin and formation of AFMK were determined by HPLC after comparing peak areas with standard curves for the purified standards. The HPLC system used was a Shimatzu LC-10A coupled to SPD-10A UV-visible detector and RF535 fluorescence detector. Samples were separated isocratically on a Luna C18 reversed phase column (250 x 4.6 mm, 5 $\mu$m) using 1 mM KH$_2$PO$_4$, pH 4.0, acetonitrile (3:1) as the mobile phase with a flow rate of 1 ml/min. Absorbances were monitored at 254 nm, and fluorescence was recorded using excitation and emission wavelengths of 340 and 460 nm, respectively.

**Determination of Hypochlorous Acid Production by Neutrophils and Purified Myeloperoxidase**—Neutrophils (2 x 10$^6$ cells/ml) were preincubated at 37 °C in Buffer A with 5 mM taurine and varying concentrations of melatonin for 10 min. When used, superoxide dismutase was present at 20 $\mu$g/ml. Cells were stimulated by the addition of 100 ng/ml PMA. After 30 min the reactions were stopped by the addition of 20 $\mu$g/ml catalase. The neutrophils were then pelleted by centrifugation, and the supernatant put on ice. Formation of hypochlorous acid was measured by assaying accumulated taurine chloramine (see below). Purified myeloperoxidase (10 nM) was incubated in PBS with 5 mM taurine, and reactions were started by adding 50 mM H$_2$O$_2$. After 5 min, the reactions were stopped by adding catalase (20 $\mu$g/ml), and accumulated taurine chloramine was measured. When used, serotonin was present at 5 $\mu$M.

Taurine chloramine was assayed by adding 200 $\mu$l of neutrophil supernatant or the myeloperoxidase reaction system to 50 $\mu$l of a solution of containing 10 mM TMB and 100 $\mu$M potassium iodide in 50% dimethylformamide and 400 mM acetic acid. Under these conditions taurine chloramine oxidizes TMB to a blue product with an absorbance maximum at 655 nm. A standard curve was generated by adding reagent hypochlorous acid to PBS containing taurine. The absorbance measurements were made in Spectra Max 190 plate reader (Molecular Devices).
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**RESULTS**

**Oxidation of Melatonin by Stimulated Human Neutrophils**—Initially we sought to identify the chemiluminescent product formed when neutrophils oxidize melatonin. Neutrophils were incubated with melatonin and stimulated with the phorbol ester PMA to initiate production of superoxide and the release of myeloperoxidase. After 30 min, supernatants were assayed by liquid chromatography with an electrospray positive ionization mass detector to identify products of melatonin oxidation. Melatonin eluted at 17.7 min (m/z 233). Minor and major products eluted before melatonin (Fig. 1A). The minor product that eluted at 12.4 min had an m/z of 249 mass units (Fig. 1B), indicating a likely hydroxylated form of melatonin. The major product that eluted at 14.2 min had an m/z of 265 mass units (Fig. 1C), which corresponds to the mass of AFMK. This peak also co-eluted with authentic AFMK and had the same fluorescence and ultraviolet absorption properties as AFMK (data not shown).

Melatonin underwent progressive oxidation over the first 15 min of the reaction after which its rate of loss declined by ~75%. This slower loss was maintained for the subsequent 45 min of the incubation (Fig. 2). After 1 h ~40% of the melatonin was oxidized. The time course for AFMK formation mirrored the loss in melatonin. Over the 1-h incubation, 8 µM AFMK was formed, which accounted for about 45% of the oxidized melatonin. The nonstoichiometric yield of AFMK was not due to breakdown of the AFMK because the authentic compound was stable when added to stimulated cells (not shown). Thus, it is likely that melatonin was oxidized to additional products.

A range of inhibitors was added to neutrophils to help establish the mechanism by which the cells form AFMK (TABLE ONE). Minimal AFMK was produced when cells were not stimulated or when the NADPH oxidase was inhibited with diphenyliodonium. These results demonstrate that superoxide and/or hydrogen peroxide must be produced by neutrophils before they can oxidize melatonin. Superoxide dismutase was a strong inhibitor of AFMK formation. Catalase showed weak inhibition and blocked the reaction by only 50% at a high concentra-
hypochlorous acid, singlet oxygen, and ozone. Me₂SO and mannitol
Therefore, we added methionine to stimulated neutrophil to scavenge
trophils, including hypochlorous acid, singlet oxygen, hydroxyl radical,
inhibitor of myeloperoxidase, decreased production of AFMK by 70%.
Pathway was no more effective than superoxide dismutase alone. Azide, an
Results are means and ranges of duplicate experiments.

FIGURE 3. Effect of melatonin on hypochlorous acid production by myeloperoxidase. A, neutrophils (2 x 10⁶/ml) were stimulated with PMA in the presence of 5 mM taurine and variable concentrations of melatonin. After 30 min the amount of taurine chloramine that had accumulated was measured. Reactions were run in the absence (●) or presence (■) of 20 μg/ml superoxide dismutase (SOD). Results are typical of three experiments. B, hydrogen peroxide (50 μM) was added to 5 μM purified myeloperoxidase in PBS containing 5 mM taurine and variable concentrations of melatonin. Reactions were run in the absence (●) or presence (■) of 5 μM serotonin. They were stopped after 5 min by adding 20 μg/ml catalase, and accumulated taurine chloramine was measured. Results are means and ranges of duplicate experiments.

tration of 100 μg/ml. A combination of superoxide dismutase and catalase was no more effective than superoxide dismutase alone. Azide, an inhibitor of myeloperoxidase, decreased production of AFMK by 70%.

Various strong oxidants have been proposed to be produced by neutrophils, including hypochlorous acid, singlet oxygen, hydroxyl radical, and ozone (28). All of these oxidants are capable of oxidizing melatonin. Therefore, we added methionine to stimulated neutrophil to scavenge hypochlorous acid, singlet oxygen, and ozone. Me₂SO and mannitol were added to intercept the hydroxyl radical. None of these scavengers blocked formation of AFMK.

We also determined the effects of superoxide dismutase and catalase on the loss of melatonin. Superoxide dismutase (20 μg/ml) inhibited melatonin loss by 60% (7 ± 2 μM compared with 18 ± 2 μM). Catalase (20 μg/ml) was less effective, inhibiting by 39% (11 ± 2 μM compared with 18 ± 2 μM).

These results point to involvement of myeloperoxidase in the oxidation of melatonin to AFMK. However, the requirement for superoxide and the nonessential requirement for hydrogen peroxide suggests an unusual mechanism of oxidation by this heme enzyme. Diffusible oxidants are unlikely to be involved because their scavengers had no effect on formation of AFMK. It is more likely that melatonin reacted directly with myeloperoxidase.

Effect of Melatonin on Hypochlorous Acid Production by Neutrophils and Purified Myeloperoxidase—If myeloperoxidase was responsible for oxidizing melatonin, then production of hypochlorous acid by neutrophils is likely to have been affected. Therefore, we measured the effect of melatonin on extracellular production of hypochlorous acid by these cells. With increasing concentration of melatonin there was progressive inhibition of hypochlorous acid production (Fig. 3A). At 50 μM melatonin, little hypochlorous acid was formed. The concentration of melatonin that inhibited hypochlorous acid production by 50% (IC₅₀) was 18 μM. When superoxide dismutase was added to remove superoxide, the IC₅₀ for melatonin dropped to 4 μM.

To demonstrate that melatonin inhibits hypochlorous acid production by neutrophils through a direct reaction with myeloperoxidase, we determined the effect of melatonin on the chlorination activity of the isolated enzyme. Melatonin was a potent inhibitor of the initial rate of hypochlorous acid production by myeloperoxidase, which readily reduces compound II (29), substantially decreased the ability of melatonin to inhibit the chlorination activity of the enzyme.

From these results, we concluded that melatonin reacts with myeloperoxidase released by neutrophils and inhibits production of hypochlorous acid. It is likely to act by competing with chloride and reducing compound I to inactive compound II. This mechanism of inhibition has been shown to occur with tryptophan (30) and several phenolic and aromatic amines (31). Superoxide dismutase would enhance the ability of melatonin to inhibit hypochlorous acid production by neutrophils because it would prevent superoxide from recycling compound II (Reaction 6) so that the enzyme would be trapped in this inactive form.

In these assays hypochlorous acid was detected by trapping it as taurine chloramine. Therefore, it was possible that melatonin acted by scavenging hypochlorous acid, thereby preventing it from reacting with taurine in the buffer. However, at 100 μM melatonin was unable to prevent reagent hypochlorous acid from reacting with taurine (5 mM) (result not shown). Melatonin up to 100 μM had no effect on superoxide production by neutrophils (result not shown).
Oxidation of Melatonin by Purified Myeloperoxidase—To confirm that myeloperoxidase was responsible for the production of AFMK by neutrophils, we determined the ability of the purified enzyme to oxidize melatonin. Xanthine oxidase and acetaldehyde were used as a source of superoxide and hydrogen peroxide. Initially, we followed oxidation of melatonin by monitoring its difference spectrum (Fig. 4, top). With the complete reaction system there were progressive increases in absorbance peaks with maxima at 242 nm and between 320 and 360 nm. There was also a progressive decline in absorbance at 290 nm. There were minimal absorbance changes in the absence of myeloperoxidase, and no change in the absence of xanthine oxidase.

When superoxide dismutase was added to the reaction, oxidation of melatonin still occurred, but the absorbance changes were substantially different. There was a marked decrease in absorbance at 240 nm, smaller changes at 242 and 290 nm, a distinctive peak at 320 nm, and considerably less absorbance above 340 nm (Fig. 4, bottom). These results demonstrate that melatonin is oxidized by myeloperoxidase. Furthermore, the type of products formed in the presence of superoxide and their relative yields are different to those formed in the absence of superoxide.

Reaction mixtures were then assayed by liquid chromatography with mass spectrometry to identify oxidation products of melatonin. As with neutrophils, two UV-absorbing peaks eluted before melatonin. The first was a minor peak and had an m/z of 249 mass units. The major peak had an m/z of 265 mass units and the same fluorescent and UV properties as authentic AFMK (not shown). These products correspond to a hydroxylated form of melatonin and AFMK as found with stimulated neutrophils.

We also used selected ion monitoring to determine whether myeloperoxidase oxidizes melatonin to a dimer via the intermediate production of radicals species (Reactions 3 and 4) as it does with other organic substrates such as tyrosine (32). The expected dimer should have an m/z of 463 mass units when analyzed in the positive ion mode. Indeed, two products eluted with this m/z at about 17.5 and 18 min, indicating the formation of isomeric forms of melatonin dimer (Fig. 5A). The mass spectrum of the product eluting at 18 min gave fragments with m/z of 404.2 and 231 mass units (Fig. 5B), which are consistent with melatonin dimer (Fig. 5C).

To determine how myeloperoxidase oxidizes melatonin to AFMK, we undertook inhibitor studies similar to those with neutrophils. Super-
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TABLE TWO

Inhibitor profile for oxidation of melatonin to AFMK by purified myeloperoxidase

| Reaction system                  | AFMK % control |
|----------------------------------|-----------------|
| Complete system                  | 100             |
| −MPO                             | 15 ± 3          |
| −Xanthine                        | 0               |
| + SOD 20 μg/ml                   | 9 ± 2           |
| + Catalase 20 μg/ml              | 85 ± 3          |
| + Catalase 100 μg/ml             | 68 ± 2          |
| + SOD (20 μg/ml) + catalase (20 μg/ml) | 8 ± 1     |
| + Azide 100 μM                   | 33 ± 2          |
| + Methionine 1 mM               | 90 ± 3          |
| + Me2SO 1 mM                    | 92 ± 2          |

Oxidase dismutase blocked AFMK production by greater than 90%, whereas catalase was a poor inhibitor (TABLE TWO). A combination of superoxide dismutase and catalase was no more effective than superoxide dismutase alone. Azide inhibited by 70% but methionine and Me2SO had no effect. These results are very similar to the inhibition pattern observed with neutrophils (TABLE ONE). Thus, myeloperoxidase catalyzed the oxidation of melatonin to AFMK by a reaction that was reliant on superoxide. Although myeloperoxidase used hydrogen peroxide to promote formation of AFMK, the requirement for this substrate was not as great as that for superoxide. Hypochlorous acid, singlet oxygen, and hydroxyl radical could not have been responsible for the production of AFMK because their formation was blocked in the reaction system by excluding chloride and adding methionine or Me2SO.

We also undertook a separate set of experiments to determine the effect of superoxide on the formation of the oxidation products of melatonin. Superoxide could act either by being an essential reactant in the oxidation of melatonin, or it could react with myeloperoxidase to modulate the rates of formation of each of the products. To exclude its effect on the enzyme, we expressed product yields relative to the amount of melatonin oxidized (Fig. 6A). In accord with the data in TABLE TWO, AFMK production was highly reliant on superoxide. In contrast, there was less formation of dimer in the presence of superoxide. The minor hydroxylated product was unaffected by superoxide. These results suggested that superoxide was directly involved in the formation of AFMK. However, it limited formation of melatonin dimer presumably by reacting with melatonin radicals.

The inability of catalase to block AFMK production suggests that myeloperoxidase uses superoxide to oxidize melatonin in a reaction that is independent of hydrogen peroxide. However, it is possible that catalase may not be able to completely prevent hydrogen peroxide from reacting with myeloperoxidase. To assess this possibility, we contrasted the effect catalase has on formation of the different products of melatonin oxidation (Fig. 6B). We reasoned that if AFMK production is solely reliant on hydrogen peroxide, then catalase should inhibit its formation to the same extent as that of melatonin dimers. Conversely, if AFMK is formed independently of hydrogen peroxide, catalase should have a greater effect on the production of dimer. In this system, myeloperoxidase oxidized 50 ± 10 μM melatonin, and this was decreased to 13 ± 4 μM in the presence of catalase (74% inhibition). Products were not formed in the absence of myeloperoxidase (Fig. 6B). We found that catalase completely blocked formation of melatonin dimers, although it inhibited AFMK production by about 50% only. These results demonstrated that catalase prevented hydrogen peroxide from reacting with ferric myeloperoxidase to form compound I, and consequently the production of melatonin radicals. Hence, half the production of AFMK occurred via a route that did not require hydrogen peroxide.

We and others have measured the oxidation of melatonin by monitoring the formation of fluorescent products (21, 33, 34). Therefore, we determined the requirement for superoxide and hydrogen peroxide in formation of these products (Fig. 7). Both superoxide dismutase and catalase inhibited the rate of increase in fluorescence by ~60%. In combination they inhibited by greater than 90%. These effects are different from those observed when the enzymes were used to inhibit formation of AFMK (TABLE TWO). In the latter case, superoxide dismutase inhibited by ~90%, and it did not inhibit further when used in combination with catalase. From these results, we concluded that there are other fluorescent products formed besides AFMK when melatonin is oxidized by myeloperoxidase. Furthermore, oxidation occurs via two pathways: one that requires superoxide and the other that uses hydrogen peroxide.

The rate of formation of fluorescent products was dependent on the concentrations of xanthine oxidase, melatonin, and myeloperoxidase (Fig. 8). These results establish that at saturating concentrations of melatonin the rate of the reaction was dependent on the flux of superoxide. Conversely, at high fluxes of superoxide, the rate of the reaction was dependent on the concentration of melatonin. These results suggest...
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FIGURE 7. The effect of superoxide dismutase (SOD) and catalase (CAT) on the oxidation of melatonin by myeloperoxidase. Oxidation of melatonin was assayed by measuring the initial increase in fluorescence (λ<sub>ex</sub> = 340; λ<sub>em</sub> = 460) over the 1st min of the reaction. Reaction conditions were as described in Fig. 4 except the concentration of catalase was 70 μg/ml. Results are means and ranges of duplicate experiments.

FIGURE 8. The effect of superoxide, melatonin, and myeloperoxidase on the initial rate of melatonin oxidation. Oxidation of melatonin was assayed by measuring the initial increase in its fluorescence (λ<sub>ex</sub> = 340; λ<sub>em</sub> = 460) over the 1st min of the reaction. Either superoxide (A), melatonin (B), or myeloperoxidase (C) was varied, whereas the other two components were kept constant. The standard reaction was started by adding xanthine oxidase to a system containing xanthine oxidase, and CAT indicates catalase.

| Reaction system                  | ΔA<sub>456</sub>/ΔA<sub>456</sub> | % Compound II | % Compound III |
|----------------------------------|-----------------------------------|----------------|----------------|
| 1. MPO/H<sub>2</sub>O<sub>2</sub>/melatonin | 0.16                              | 100            | 0              |
| 2. MPO/XO<sup>a</sup>             | 0.16                              | 100            | 0              |
| 3. MPO/XO<sup>a</sup>/CAT         | 0.48                              | 10             | 90             |
| 4. MPO/XO<sup>a</sup>/melatonin   | 0.27                              | 70             | 30             |
| 5. MPO/XO<sup>a</sup>/melatonin/SOD| 0.16                              | 100            | 0              |
| 6. MPO/XO<sup>a</sup>/melatonin/CAT| 0.35                              | 50             | 50             |
| 7. MPO/XO<sup>a</sup>/melatonin/CAT| 0.48                              | 10             | 90             |

<sup>a</sup> The rate of superoxide production was 10 μM/min.

<sup>b</sup> The rate of superoxide production was 1 μM/min.

that the rate-determining step (or steps) in the product formation is (are) reliant on superoxide and melatonin.

Absorption Spectra of Myeloperoxidase during Oxidation of Melatonin—We monitored the absorption spectrum of myeloperoxidase during oxidation of melatonin to assist in elucidating the mechanisms by which melatonin is oxidized. Chloride was excluded to eliminate competition between it and melatonin for compound I. In all cases there was either a partial or complete shift of the Soret peak from 430 to 456 nm. This indicated conversion of the ferric enzyme to either compound II or compound III (35). There was no evidence of a peak at 472 nm, which is characteristic of the ferrous enzyme (36). Compound II and compound III are distinguished by the ratio of their absorbances at 625 and 456 nm, which are 0.17 and 0.52, respectively (35). As found previously, myeloperoxidase was present as 100% compound II when the enzyme was incubated with hydrogen peroxide and melatonin (34) (TABLE THREE, system 1). There was no indication of compound III formation as occurs with other substrates such as tryptophan and hydroquinone (30, 37). Thus, reduction of compound II by melatonin is the rate-determining step in the oxidation of melatonin by myeloperoxidase in the absence of superoxide.

We used the xanthine oxidase system to monitor turnover of myeloperoxidase in the presence of superoxide. As shown previously, this system converts myeloperoxidase to 100% compound II, pH 7.4 (9, 18) (TABLE THREE, system 2). Addition of catalase to scavenge hydrogen peroxide results in almost complete conversion of the ferric myeloperoxidase to compound III with a small percentage of compound II present (TABLE THREE, system 3).

In the presence of melatonin, the xanthine oxidase system converted ferric myeloperoxidase to a mixture of compound II and compound III (TABLE THREE, system 4). These results demonstrate that during oxidation of melatonin, superoxide reacts with ferric myeloperoxidase to form compound III. Furthermore, turnover of compound II and compound III is rate-determining in this system. Addition of superoxide dismutase resulted in complete conversion of the enzyme to compound II. Hence in the absence of superoxide, turnover of compound II is rate-determining as was the case when using reagent hydrogen peroxide. Addition of catalase raised the percentage of compound III present (TABLE THREE, system 5). With catalase at low fluxes of hydrogen peroxide and superoxide, there was only partial loss of the ferric enzyme, and it was converted mainly to compound III (TABLE THREE, system 6). The results with catalase indicate that under conditions where the nonclassical route for conversion of melatonin to AFMK predominates, turnover of compound III is rate-limiting.
Therefore, we investigated the direct reaction between compound III and melatonin. Compound III was formed by adding 2 mM hydrogen peroxide to myeloperoxidase and degrading excess hydrogen peroxide with catalase. Melatonin was then added to compound III, and spectral changes were monitored. Melatonin did not enhance the decay of compound III (results not shown). From these results we conclude that melatonin does not undergo an appreciable reaction with compound III.

**Oxidation of Melatonin by Compound III**—Myeloperoxidase could function like indoleamine 2,3-dioxygenase and use compound III to insert oxygen directly into its substrate. This enzyme shuttles between compound III and its ferrous form (38). Binding of molecular oxygen is then required for continued activity. If an analogous mechanism operated for myeloperoxidase, superoxide would be required only to initiate the first cycle of catalysis. Once the ferrous enzyme was formed, molecular oxygen would be sufficient to maintain production of AFMK. We conducted experiments in which myeloperoxidase was converted to 90% compound III by using xanthine oxidase, acetaldehyde, and catalase. Melatonin and superoxide dismutase were then added to the preformed compound III. Under these conditions no detectable AFMK was produced. Hence, myeloperoxidase requires a continuous source of superoxide for AFMK production. It does not act like indoleamine 2,3-dioxygenase.

**DISCUSSION**

In this investigation we have confirmed that neutrophils oxidize melatonin to AFMK in a reaction that is catalyzed by myeloperoxidase. The reaction mechanism utilized by myeloperoxidase is intriguing because the enzyme uses superoxide to oxidize melatonin. Although others have shown that myeloperoxidase uses hydrogen peroxide to oxidize melatonin to AFMK (34, 39), we found that production of AFMK was much greater in the presence of superoxide. Furthermore, hydrogen peroxide was not essential for conversion of melatonin to AFMK. Superoxide-dependent oxidation of melatonin was not a trivial side reaction because there was efficient conversion of melatonin to AFMK by both neutrophils and isolated myeloperoxidase. Under conditions where melatonin effectively prevented hypochlorous acid production by neutrophils, about half the oxidized melatonin was converted to AFMK. Our results demonstrate that in addition to halogenation and peroxidation, myeloperoxidase has an oxygenase activity that requires superoxide as a substrate.

We propose that myeloperoxidase oxidizes melatonin to AFMK via two independent pathways (Scheme 1). One pathway is the classical peroxidation mechanism (see Reactions 1, 3, and 4), and the second pathway involves superoxide and compound III. It has already been shown that myeloperoxidase uses hydrogen peroxide to oxidize melatonin (34). Reaction of melatonin with compound I is fast ($k = 6.1 \times 10^6$ M$^{-1}$ s$^{-1}$) but that with compound II is considerably slower ($k = 9.6 \times 10^5$ M$^{-1}$ s$^{-1}$) (34). Melatonin radicals are the expected products in both reactions. Once the radicals of melatonin are formed, they could either dimerize or react with superoxide to give an organic peroxide. This latter product would be expected to be unstable and rearrange to produce a dioxetane, and subsequently collapse to AFMK (Scheme 2).

This mechanism was originally proposed for oxidation of indoles by horseradish peroxidase (40). It would explain the absolute requirement for superoxide in the formation of AFMK. Addition of superoxide to the melatonin radical is supported by the analogous reaction of the N-centered radical of N-acetyltryptophan methyl ester (41). The rate for this reaction is close to diffusion-controlled ($k = 1.2 \times 10^9$ M$^{-1}$ s$^{-1}$) (41, 42), and the major products are the corresponding N-formylkynurenine and two organic peroxides (41). Dimers are also formed from coupling of the N-acetyltryptophan methyl ester radicals, which is in competition with their reaction with superoxide. Similar chemistry occurs when phenoxyl radicals react with superoxide (43). Also, neutrophils convert tyrosine to an organic peroxide via a comparable route (44). Superoxide would play an additional role in this pathway by reducing compound II (Reaction 6) and thereby enhancing the turnover of myeloperoxidase.

Reaction of superoxide with compound II also limits the effectiveness of melatonin as an inhibitor of hypochlorous acid production by neutrophils. Melatonin inhibits hypochlorous acid production by competing with chloride and reducing compound I to compound II. Superoxide is able to prevent accumulation of compound II by reducing it back to the native enzyme. Consequently, superoxide dismutase enhances the effect of melatonin by preventing superoxide from recycling compound II (45).

Our findings that catalase partially inhibited AFMK formation, loss of melatonin, and formation of fluorescent products support the involvement of the classical peroxidation mechanism. So too does the identification of melatonin dimers, which indicates that melatonin radicals were formed in the reaction. The extent of inhibition of melatonin oxidation and AFMK formation by catalase indicates that the classical peroxidation mechanism accounts for about 50% of the AFMK produced.

We propose that an oxygenase activity accounts for the remaining AFMK production by myeloperoxidase. The strong inhibition of AFMK production by superoxide dismutase indicates that superoxide is an essential substrate for this activity. It is independent of hydrogen peroxide because catalase only partially inhibited AFMK formation and yet completely blocked formation of melatonin dimers from the spectral...
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data it is apparent that compound III is formed during superoxide-de- 
dependent oxidation of melatonin and that its turnover is rate-limiting. 
Melatonin did not react directly with compound III. This reaction is 
expected to be much slower than the reaction of melatonin with 
compound II ($k = 9.6 \times 10^3 \text{M}^{-1} \text{s}^{-1}$ (34)), as is the case for other substrates 
of compound III, such as ascorbate (12, 46) and acetalaminophen (13). 
The substantial decrease in reactivity of substrates with compound III 
compared with compound II also holds with horseradish peroxidase 
(11). Turnover of compound III and formation of AFMK required a 
continuous source of superoxide. Hence, it is likely that superoxide and 
melatonin are involved in a concerted reaction with compound III to 
produce AFMK (Scheme 1). In this reaction compound III must be 
converted to ferric myeloperoxidase. It could not be reduced to com-
pound I or compound II. Otherwise radicals would be formed in the 
subsequent reactions of these intermediates with melatonin, and their 
production would not have been blocked by catalase.

Myeloperoxidase hydroxylates salicylate and phenol in reactions that 
require superoxide but not hydrogen peroxide (16, 17, 47). Also, com-
pound I or compound II also holds with horseradish peroxidase 
(16, 17, 47). Also, com-

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