Biochemical and Biophysical Research Communications **287,** 130–134 (2001)
doi:10.1006/bbrc.2001.5557, available online at http://www.idealibrary.com on IDEAL®

Oxidation of Melatonin and Tryptophan by an HRP Cycle Involving Compound III

Valdecir F. Ximenes,* Luiz H. Catalani,† and Ana Campa*,[1]
**Faculdade de Ciências Farmacêuticas and †Instituto de Química, Universidade de São Paulo, CEP 05508-900, São Paulo, Brazil*

Received July 16, 2001

We recently described that horseradish peroxidase (HRP) and myeloperoxidase (MPO) catalyze the oxidation of melatonin, forming the respective indole ring-opening product N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (*Biochem. Biophys. Res. Commun.* 279, 657–662, 2001). Although the classic peroxidatic enzyme cycle is expected to participate in the oxidation of melatonin, the requirement of a low HRP:H_2O_2 ratio suggested that other enzyme paths might also be operative. Here we followed the formation of AFMK under two experimental conditions: predominance of HRP compounds I and II or presence of compound III. Although the consumption of substrate is comparable under both conditions, AFMK is formed in significant amounts only when compound III predominates during the reaction. Using tryptophan as substrate, *N*-formyl-kynurenine is formed in the presence of compound III. Both, melatonin and tryptophan efficiently prevents the formation of p-670, the inactive form of HRP. Since superoxide dismutase (SOD) inhibits the production of AFMK, we proposed that compound III acts as a source of $O_2^{-\cdot}$ or participates directly in the reaction, as in the case of enzyme indoleamine 2,3-dioxygenase.

Key Words: **HRP; horseradish peroxidase; compound III; indolic compounds; indoleamine 2,3-dioxygenases, kynurenine, melatonin; oxidation; p-670; peroxidase; superoxide anion; tryptophan.**

We recently described that, when high concentrations of H_2O_2 are used, HRP and MPO catalyse the oxidation of indole compounds in a reaction that consumes oxygen, triggers chemiluminescence and forms indole ring opening products (1, 2).

Peroxidase uses H_2O_2 to form the active compound I that catalyses the dehydrogenation of several substrates in a peroxidatic cycle, classically involving compound I, compound II and native enzyme (3). Peroxidases also exhibit oxidase activity in the presence of NADH, in a enzyme cycle producing compound III and ferrous peroxidase (4, 5). In the latter case, hydroxylated products are formed, probably at expenses of hydroxyl radical produced from superoxide anion and H_2O_2 (6).

The increased interest in the peroxidase-catalyzed oxidation of biological indoles is noteworthy in the recent literature (1, 2, 7–9). The reactions of tryptophan or melatonin with compound I and II of MPO were recently reported. Tryptophan reacts rapidly with compound I (7) and melatonin reacts efficiently with both compound I and compound II (8). Although these data support a common peroxidatic cycle in the oxidation of these compounds, our initial observation that the oxidation of indolic compounds by HRP and MPO requires high concentrations of H_2O_2 is an indication that other enzyme paths might also be operative.

Here, we report the HRP-catalyzed production of indole ring-opening products of melatonin and tryptophan, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and *N*-formyl-kynurenine (NFK), respectively, under two different reaction conditions: where there is a predominance of HRP compounds I and II of the peroxidatic cycle or where compound III is present.

MATERIALS AND METHODS

Catalase (EC 1.11.1.6; from bovine liver), superoxide dismutase (SOD; EC 1.15.1.1; from bovine erythrocytes), horseradish peroxidase (HRP; EC 1.11.1.7; type VI), melatonin, L-tryptophan, DL-kynurenine, mannitol and NADH were from Sigma. Hydrogen peroxide (60%, from Interox) was diluted to the appropriate stock concentration and spectrophotometrically measured (10). The concentration of HRP was determined by absorption at 403 nm using a molar absorptivity of $1.02.10^5\ M^{-1}\ cm^{-1}$ (11).

UV-Vis spectra were recorded on a Shimadzu Multispec-1501 spectrophotometer. The reaction mixtures were analyzed by high performance liquid chromatography using a SHIMADZU LC-10A system coupled to SPD-10A UV-Vis and RF535 fluorescence detectors. The analyses were carried out on a Luna C-18 reversed phase column (25 × 4.6 mm, 5 μm) in isocratic mode using 1 mmol/L KH_2PO_4, pH4.0/acetonitrile 3:1 as mobile phase at a constant flow rate of 1

[1] To whom correspondence should be addressed. Fax: (55-11) 3813-2197. E-mail: anacampa@usp.br.

0006-291X/01 $35.00

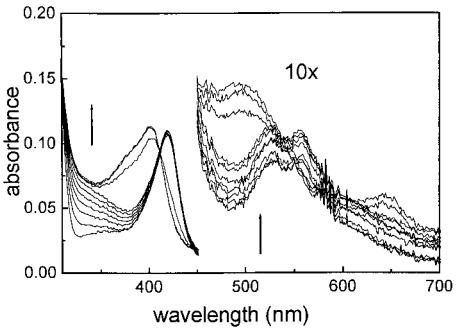

FIG. 1. Absorption spectral changes of HRP during melatonin oxidation under the standard reaction condition a. Compound II (Soret band at 420 nm and absorbances at 527 and 554 nm) predominates during the first 20 min, at which time it changes to the native form. Scans were recorded every 3 min.

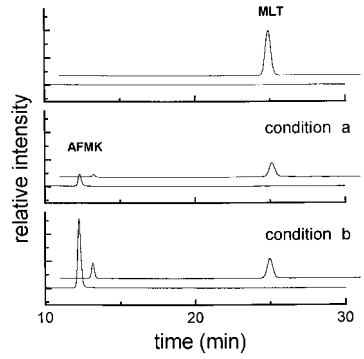

FIG. 3. HPLC profile of the melatonin/HRP/H_2O_2 standard reaction obtained after 30 min. From the top to the bottom the profiles are: melatonin (MLT), condition a and b. For each profile, the upper and lower lines correspond to absorbance and fluorescence detection, respectively.

mL/min. The mass spectra were obtained employing a Hewlett–Packard 5988 quadrupole mass spectrometer attached to a 5890 gas chromatograph using a HP1 (12 m × 0.25 mm × 0.25 μm) column. The formation of AFMK during the reaction was followed in a SPEX-FLUOROLOG 1681 fluorometer with a cooled photomultiplier.

Unless otherwise stated, the standard reaction mixture was: [HRP] = 1 μmol/L, [H_2O_2] = 10 μmol/L (condition a) or 2 mmol/L (condition b), melatonin and tryptophan = 50μmol/L in 0.05 mol/L phosphate buffer pH 7.4, at 37°C and final volumes of 3 mL. Typically, the reaction was initiated by addition of hydrogen peroxide.

RESULTS

The formation of indole ring-opening products and peroxidase spectral features were followed under two conditions: at low concentration of H_2O_2, where compounds I and II prevail (condition a), and at high H_2O_2 concentration, where there is a predominance of compound III (condition b).

Under condition a, the HRP:H_2O_2 ratio is 1:10 and the rate liming step is the conversion of compound II to the native form; if melatonin is present, the spectrum is dominated by compound II (Soret band at 420 nm and absorbances at 527 and 554 nm) (Fig. 1). After 20 min, the spectrum returns to that of the native enzyme.

Under condition b, the HRP:H_2O_2 ratio is 1:2000 and, as shown in Fig. 2, the characteristic compound III spectrum predominates over the entire 30 min of reaction (Soret band at 418 nm and absorbances at 544 and 577 nm). During the reaction an increased absorbance is clearly observed in the 340 nm region. This corresponds to the absorption of the indole ring-opening product from melatonin, AFMK. After 30 min, the HPLC profiles of the reactions performed under condition a and b (Fig. 3) show similar consumption of melatonin. However, a prominent signal corresponding to formation of AFMK (λ_{exc} = 340 nm; λ_{em} = 460 nm; MS (*m*/*z*): 264(7), 176(69), 160(100), 150(24), 117(13)) was observed only in condition b.

The differences in AFMK production under conditions a and b can be clearly seen in Fig. 4. The consumption of melatonin (initial 50 μM) in three experiments was found to be 30 ± 3 and 32 ± 7 μM under conditions a and b, respectively, while the formation of AFMK under condition a is only 13% of that observed for condition b.

The formation of p-670, an inactive form of HRP, is expected in the presence of high concentrations of H_2O_2

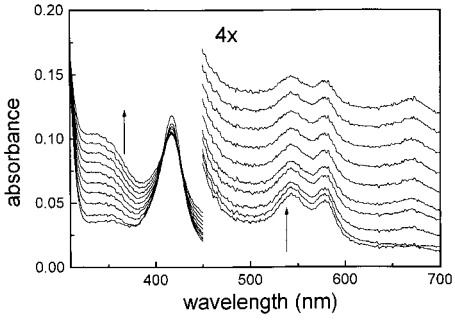

FIG. 2. Absorption spectral changes of HRP during melatonin oxidation under the standard reaction condition b. Compound III (Soret band at 418 nm and absorbances at 544 and 577 nm) predominates over the entire 30 min of reaction. Scans were recorded every 3 min.

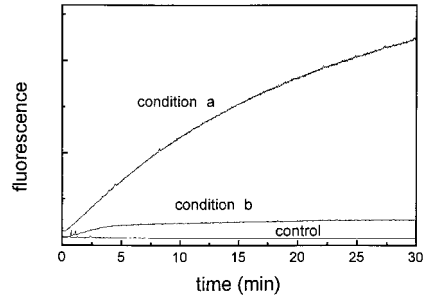

FIG. 4. Kinetics of AFMK production under condition a versus condition b measured by fluorescence at 460 nm (λ_{exc} = 340 nm). The melatonin/H_2O_2 control reaction is also shown.

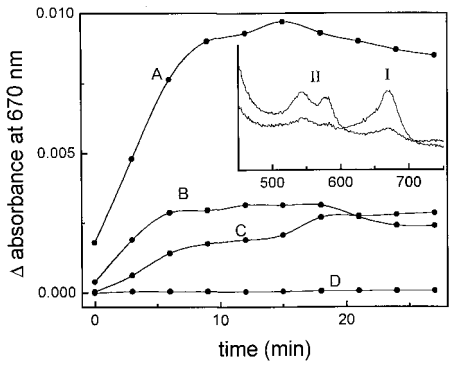

FIG. 5. Effect of melatonin (50 μmol/L and 0.5 mmol/L, B and D, respectively) and of tryptophan (0.5 mmol/L, C) in preventing p-670 formation during the reaction of HRP/H_2O_2 (A). The reactions were run under condition b. The inset corresponds to the HRP absorption spectra of reactions A and B taken after 15 min, showing the degradation of compound III and formation of p-670 (I) and conservation of compound III (II), respectively.

and occurs via the decomposition of compound III (11). Figure 5 shows the formation of p-670 when HRP is mixed with H_2O_2 at a 1:2000 ratio. The presence of melatonin clearly causes a protection of HRP, precluding the formation of p-670670. This protection depends on the melatonin concentration.

The effect of SOD (166 U/mL) and the hydroxyl radical scavenger mannitol (100 mM) on the oxidation of melatonin and AFMK production were examined. The addition of SOD inhibit the production of AFMK (Fig. 6), without affecting melatonin consumption (data not shown). Mannitol had no effect on either melatonin consumption or AFMK production.

The effect of pH on melatonin consumption and AFMK production was studied in the 5.0 to 9.0 range using phosphate buffer. Figure 7 shows that, at acidic pHs, the consumption of melatonin is much faster. However, no AFMK could be detected at pH 5.0 and only a small amount was observed at pH 6.0. The consumption of melatonin is similar at pH 7.4 and 9.0

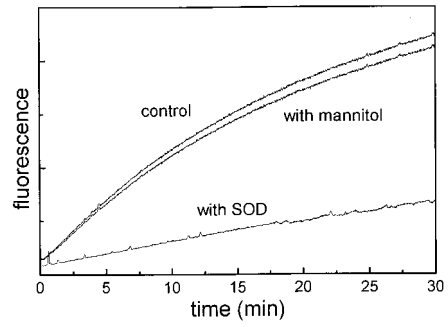

FIG. 6. Effect of SOD (166 units/mL) and mannitol (100 mmol/L) on the kinetics of AFMK production measured by fluorescence at 460 nm (λ_{exc} = 340 nm). The control is the reaction under condition b without any addition.

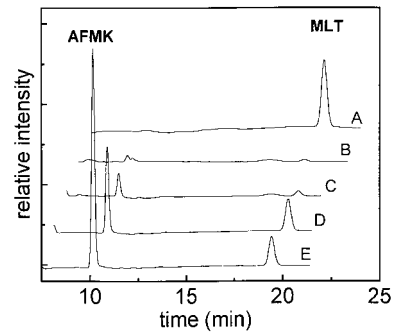

FIG. 7. HPLC profile of the melatonin/HRP/H_2O_2 system obtained after 30 min under condition b at different pHs: 5.0 (B); 6.0 (C); 7.4 (D); and 9.0 (E). Profile A corresponds to melatonin (50 μmol/L).

but, in the latter case, there is a pronounced formation of AFMK.

To verify whether a different compound III generating system was also able to catalyse the formation of AFMK, the reaction between NADH and HRP was tested. At pH 5.0, which is the classical condition for production of compound III (12), there is no melatonin consumption or AFMK formation (data not shown). Since neutral to basic pH seems to be required for AFMK production, the NADH/HRP system was tested at pH 7.4. In this condition, we found that continuous bubbling of O_2 resulted, after 8 min of reaction, in a spectrum dominated by compound III absorption (Fig. 8). Employing this condition, melatonin is consumed and AFMK is formed (Fig. 8, inset). The addition of catalase (150 U/mL) did not affect the compound III spectrum or AFMK production (data not shown).

In some selected experiments, tryptophan was employed to determine whether it could also be oxidized in a similar way as melatonin. Figure 9 shows the compound III spectrum in the presence of tryptophan at a

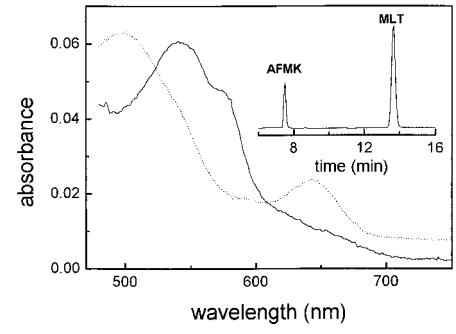

FIG. 8. Generation of compound III in the NADH system. A clear spectrum of compound III (—) appears at around 8 min under the reaction conditions: [HRP], 4 μmol/L; [NADH], 1.5 mmol/L; [MLT], 0.5 mmol/L in 50 mmol/L phosphate buffer, pH 7.4, at 37°C with continuous O_2 bubbling. The predominance of native enzyme can be observed after 30 min of reaction (· · ·). The inset shows the HPLC profile after 30 min of reaction.

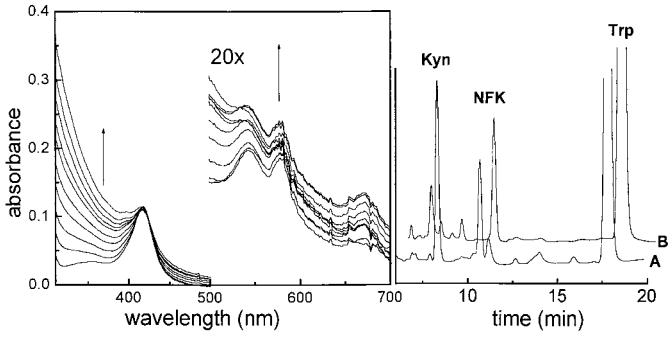

FIG. 9. Spectral change of HRP during tryptophan (0.5 mmol/L) oxidation under the reaction condition b; scans were recorded every 5 min (left) and HPLC profile of this reaction determined after 120 min (B) (right). The chromatograms also show the formation of NFK and its conversion to kynurenine (Kyn) after 1 h heating at 56°C (A).

high concentration of H_2O_2 (condition b) and the HPLC profile observed after 120 min of reaction, where NFK and kynurenine are seen. As in the case of melatonin, the presence of tryptophan also inhibits p-670 formation (see Fig. 5).

DISCUSSION

The oxidation of common substrates by peroxidases usually involves the native enzyme–compound I-compound II cycle (3). Compound I is formed from the native form by the addition of hydrogen peroxide or by the presence of contaminating peroxides and is the catalytically active form. Although generally less active, compound II also catalyzes the oxidation of a number of substrates. Recently, we reported that HRP catalyses the oxidative cleavage of several indolic compounds, via a reaction sequence in which the indolyl cation-radical is presumed to be the intermediate (2). We also showed that melatonin is oxidized by HRP, MPO and by activated neutrophils in a reaction from which AFMK was isolated (1). Although the classic native-compound I-compound II-native enzyme cycle is expected to participate in the oxidation of indole compounds, the requirement of a large amount of hydrogen peroxide indicated that HRP compound III was in same way involved. In this study, we specifically addressed the question of whether peroxidase compound III participates to the production of the kynurenine-like products formed in the oxidation of melatonin and tryptophan.

Recently, Allegra *et al.* (8) clearly showed that melatonin reacts with MPO compounds I and II. The condition utilized in the present work to produce compounds I and II (condition a) leads to melatonin consumption. However, the production of AFMK is incipient compared to that observed under condition b, where compound III prevails. It is also possible to form AFMK even in the absence of H_2O_2 if compound III formed by the HRP/NADH/catalase system is employed.

Native enzyme, compound I, compound II, compound III and ferrous enzyme are interconvertible forms, depending on the conditions. It is probable that, under condition b and in the HRP/NADH system, all these redox states of peroxidase coexist and that different enzyme cycles catalyze the formation of different oxidation products. Hence, we propose two routes for melatonin oxidation catalyzed by peroxidases. The first one involves the common HRP cycle and it is not the principal path responsible for AFMK formation, the second route involving compound III being required for AFMK formation. The inhibitory effect of SOD suggests that compound III might act as a source of $O_2^{-\bullet}$ involved in AFMK production. Superoxide anion would have to react with a melatonin radical since it does not react directly with melatonin (data not shown). In this case, the common peroxidatic cycle generating a melatonin radical would then take place simultaneously with the cycle forming compound III. In the experiments where compound III was obtained at the expense of NADH, melatonin cation radical was also proposed to be formed (9). Although $O_2^{-\bullet}$ is involved in AFMK formation, the participation of hydroxyl radical is excluded by the absence of a mannitol effect.

Since SOD also accelerates the decomposition of Compound III (13), another possibility is that compound III participates directly in AFMK formation. In this case, compound III would be acting similarly to the enzyme indoleamine 2,3-dioxygenase (14). In this context, Kettle and Winterbourn (15) have already mentioned the similarity between peroxidase compound III and the active form of indoleamine 2,3-dioxygenase. Since p-670 is produced from compound III (16), the strong protective effect of melatonin in inhibiting the formation of the inactive p-670 form supports the supposition of a direct reaction of compound III with melatonin. The pronounced pH dependence of AFMK formation may be related to the formation of protonated reactive substrates or intermediates. It is indeed curious to note that neutral to basic pH increases the affinity of the ferrous indoleamine 2,3-dioxygenase enzyme for its substrates (17).

Apart from the question as to the true operative mechanism(s), the protection promoted by melatonin and tryptophan against formation of p-670 suggests a role of these compounds in preventing the inhibition of peroxidases *in vivo*, e.g., in neutrophils and biological fluids where peroxidases or other hemeproteins are present. Furthermore, as can be inferred from the results of this and previous studies (1, 2), the formation of indole ring-opening products may also take place with other biological indoles and could be an alternative path for the production of kynurenine-like compounds, whose biological activity has been described (18, 19).

ACKNOWLEDGMENTS

The authors are indebted to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

1. Silva, S. O., Ximenes, V. F., Catalani, L. H., and Campa, A. (2000) Myeloperoxidase-catalyzed oxidation of melatonin by activated neutrophils. *Biochem. Biophys. Res. Commun.* **279,** 657–662.
2. Ximenes, V. F., Campa, A., and Catalani, L. H. (2001) The oxidation of indole derivatives catalyzed by horseradish peroxidase is highly chemiluminescent. *Arch. Biochem. Biophys.* **387,** 173–179.
3. Dunford, H. B. (1999) Heme Peroxidases, Wiley-VCH, New York.
4. Yokota, K., and Yamazaki, I. (1965) Reaction of peroxidase with reduced nicotinamide-adenine dinucleotide and reduced nicotinamide-adenine dinucleotide phosphate. *Biochim. Biophys. Acta* **105,** 301–312.
5. Odajima, T. (1971) Myeloperoxidase of leukocyte of normal blood. 2. Oxidation-reduction reaction mechanism of myeloperoxidase system. *Biochim. Biophys. Acta* **235,** 52–60.
6. Chen, S., and Schopfer, P. (1999) Hydroxyl-radical production in physiological reactions. A novel function of peroxidase. *Eur. J. Biochem.* **260,** 726–735.
7. Kettle, A. J., and Candaeis, L. P. (2000) Oxidation of tryptophan by redox intermediates of myeloperoxidase and inhibition of hypochlorous acid production. *Redox Rep.* **5,** 179–184.
8. Allegra, M., Furtmuller, P. G., Regelsberger, G., Turco-Liveri, M. L., Tesoriere, L., Peretti, M., Livrea, M. A., and Obinger, C. (2001) Mechanism of reaction of melatonin with human myeloperoxidase. *Biochem. Biophys. Res. Commun.* **282,** 380–386.
9. Olsen, L. F., Lunding, A., Lauritsen, F. R., and Allegra, M. (2001) Melatonin activates the peroxidase-oxidase reaction and promotes oscillations. *Biochem. Biophys Res. Commun.* **284,** 1071–1076.
10. Cotton, M. L., and Dunford, H. B. (1973) Studies on horseradish-peroxidase. 11. Nature of compounds I and II as determined from kinetics of oxidation of ferrocyanide. *Can. J. Chem.* **51,** 582–587.
11. Ohlsson, P. J., and Paul, K. G. (1976) Molar absorptivity of horseradish-peroxidase. *Acta Chem. Scand. B* **30,** 373–375.
12. Yokota. K., and Yamazaki, I. (1965) Reaction of peroxidase with reduced nicotinamide-adenine dinucleotide and reduced nicotinamide-adenine dinucleotide phosphate. *Biochim. Biophys. Acta* **105,** 301–312.
13. Metodiewa, D., and Dunford, H. B. (1989) The reactions of HRP, lactoperoxidase and MPO with enzymatically generated superoxide. *Arch. Biochem. Biophys.* **272,** 245–253.
14. Kobayashi, K., Hayashi, K., and Sono, M. (1989) Effects of tryptophan and pH on the kinetics of superoxide radical binding to indoleamine 2,3-dioxygenase studied by pulse radiolysis. *J. Biol. Chem.* **264,** 15280–15283.
15. Kettle, A. J., and Winterbourn, C. C. (1994) Superoxide-dependent hydroxylation by myeloperoxidase. *J. Biol. Chem.* **269,** 17146–17151.
16. Adediran, S. A. (1996) Kinetics of the formation of p 670 and of the decay of compound III of horseradish peroxidase. *Arch. Biochem. Biophys.* **327,** 279–284.
17. Sono, M. (1990) Spectroscopic and equilibrium studies of ligand and organic substrate binding to indolamine 2,3-dioxygenase. *Biochemistry* **29,** 1451–1460.
18. Leon, J., Vives, F., Crespo, E., Camacho, E., Espinosa, A., Gallo, M. A., Escames, G., and Acuna-Catroviejo, D. (1998) Modification of nitric oxide synthase activity and neural response in rat striatum by melatonin and kynurenine derivatives. *J. Neuroendocrinol.* **10,** 297–302.
19. Leon, J., Macias, M., Escames, G., Camacho, E., Khaldy, H., Martin, M., Espinosa, A., Gallo, M. A., and Acuna-Catroviejo, D. (2000) Structure-related inhibition of calmodulin-dependent neuronal nitric-oxide synthase activity by melatonin and synthetic kynurenines. *Mol. Pharmacol.* **58,** 967–975.