Research Article

Autoxidation of melatonin at excited state: mechanism proposal for formation of N^1-acetyl-N^2-formyl-5-methoxykynuramine

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ABSTRACT

N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) is one of the primary oxidation products of melatonin. There is growing evidence of its beneficial biological properties, including antioxidant features and modulators of cytokines and enzymes involved in the inflammatory process. Here, the autoxidation of melatonin mediated by UVC was studied regarding the formation of AFMK and the reaction mechanism. The parameters evaluated were irradiation, pH, dissolved oxygen, superoxide radical anion, and hydroxyl radical. We found that the AFMK yield is directly correlated with UVC irradiation. The AFMK concentration decreased 95% when a 280 nm cutoff filter blocked the irradiation. By removing the dissolved oxygen from the medium, the decrease was 90%. Superoxide dismutase, acting as a scavenger of superoxide radical anion, caused a 64% reduction. At pH 7.0, the AFMK yield was just 14% of those obtained at pH 10. These findings are consistent with a typical autoxidation reaction. In addition, the low yield of AFMK in the absence of UVC irradiation suggested that electronically excited melatonin is the species involved in the initial electron transfer. Density Functional Theory (DFT) calculations were performed to strengthen the proposal. Corroborant with the experimental results, the theoretical analyses revealed that electron transfer from melatonin to molecular oxygen is only energetically feasible in the excited state. In conclusion, the direct autoxidation of melatonin at excited state in alkaline pH is a straightforward approach to producing AFMK.

Key words: melatonin; AFMK; autoxidation; density functional theory.

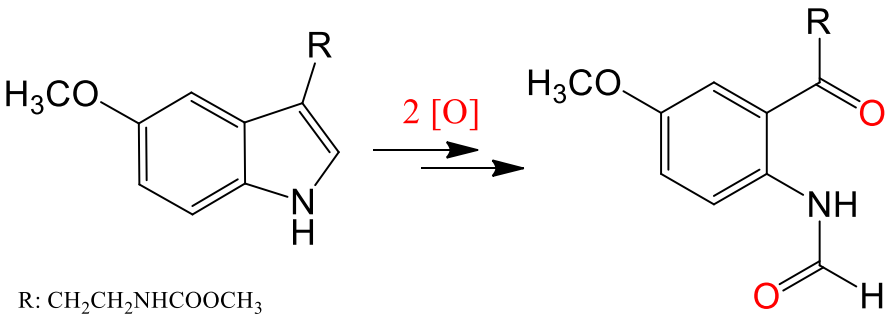
1. INTRODUCTION

Melatonin (MLT), an indoleamine derived from tryptophan, is ubiquitously presented in the animal kingdom and also produced in plants, unicellular organisms, algae, and bacteria (1–7). MLT is the primary secretory product of the pineal gland. Still, its biosynthesis has also been identified in the retina, gastrointestinal tract, airway epithelium, pancreas, adrenal gland, thyroid gland, thymus, urogenital tract, and placenta (8–11). MLT exhibits a variety of physiological functions. Among them, its action as an endogenous antioxidant is well

established (12). In this concern, the interaction of MLT with a multitude of reactive oxygen and nitrogen species (ROS and RNS) is well-documented (13, 14). Irrespective of the ROS or RNS employed in the oxidation, the major products of MLT oxidation are hydroxylated forms of MLT (HO-MLT), cyclic 3-hydroxymelatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), and its hydrolyzed derivative N^1-acetyl-5-methoxykynuramine (AMK) (15–18).

AFMK, the indole-ring cleavage product of MLT, is one of the major MLT oxidation products (15–21). Among the degradation products of MLT, AFMK is particularly relevant since many biological features of MLT have also been attributed to this metabolite. For instance, its action as a scavenger of ROS and RNS, inhibitor and modulator of cytokines, lipid mediators, and enzymes involved in the inflammatory process (22, 23). AFMK has been detected in many experimental models, including cell cultures, *in vivo* studies, and several enzymatic and non-enzymatic conditions (24–29).

From the molecular structure point of view, AFMK is just the product of MLT dioxygenation (Scheme 1). Accordingly, the similarity of AFMK with the tryptophan catabolism product, i.e., *N*-formylkynurenine, justifies the search for evidence of the role of indoleamine 2,3-dioxygenases (IDO) as the enzyme responsible for the generation of endogenous AFMK. However, although this pathway has been identified, it has been argued to be a minority (30). Actually, the dependence of hydrogen peroxide (H_2O_2) in the oxidation of MLT catalysed by IDO suggests that, even in this case, the classic enzymatic mechanism of IDO is not the main responsible for the generation of AFMK (30).



Scheme 1. **The chemical equation for the unspecific oxidation of melatonin leads to AFMK.**

Regarding the direct interaction of MLT with molecular oxygen, AFMK can be produced by the reaction with singlet oxygen via photosensitization with methylene blue (31). Another oxidative approach to producing AFMK is through photodegradation of MLT. These reactions are unspecific, and besides AFMK, the hydroxylated form of MLT (HO-MLT) is frequently detected (32–34). HO-MLT formation can be easily explained via hydroxyl radical (HO•) generated by photolysis and radiolysis. However, the generation of AFMK is mechanistically less obvious. In this concern, a seven-step pathway was proposed to explain the production of AFMK by MLT radiolysis and its dependence on molecular oxygen (O_2) (16). The proposed mechanism involves the formation of an intermediate HO-MLT peroxyl radical and its dimerization, leading to a tetroxide that decomposes, producing AFMK. It is essential to mention that besides the UV irradiation, the necessity of dissolved O_2 in the medium was identified as a requisite for AFMK production. Based on these literature data and the importance of AFMK, we studied the effect of the medium in the photo-induced oxidation of

MLT, aiming to understand the mechanism of formation of AFMK. Using experimental and theoretical approaches, we propose a simple autoxidation pathway to generate AFMK.

2. MATERIALS AND METHODS

2.1.1. Chemicals and solutions.

Melatonin (MLT), N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), dimethyl sulfoxide DMSO, superoxide dismutase (SOD) from bovine liver (ammonium sulfate suspension, 2,000-6,000 units/mg protein) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Stock solutions of MLT and AFMK (10 mmol L^{-1}) were prepared in ethyl alcohol. All reagents used to prepare solutions and buffers were of analytical grade. All solutions were prepared with water purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.1.2. Photo-induced oxidation.

The reactions were performed on a magnetically stirred solution (25 mL) containing MLT (100 μmol L^{-1}) in ultrapure Milli-Q® water. A 50 mL double-jacketed glass reactor was used, and the temperature was controlled using a water circulation bath (20 °C). The pH was adjusted with NaOH and HCl 10 mM solutions. When present, DMSO (1 mmol L^{-1}) and SOD (96 u/ml) were added before irradiation. The irradiations were performed using UVC lamps (model T5, potency 12 W, base G5, China) or LEDs (40 W, 353 mW/cm^2). The irradiation was carried out over the reactor. Aliquots of the solution (1.5 mL) were removed at 15 min intervals and submitted to high-performance liquid chromatography (HPLC) analysis (Jasco, Tokyo, Japan).

The concentrations of the studied compounds were evaluated based on MLT and AFMK standards and LC-MS analysis. The HPLC analyses were carried out under isocratic conditions on a Luna® C18 reversed-phase column (Phenomenex, 250 x 4.6 mm, 5 μm). The mobile phase consisted of solvent (A) 0.1% aqueous formic acid and solvent (B) 0.1% formic acid in acetonitrile. The mobile phase composition was 70 : 30% (A : B). The flow rate was 1 mL min^{-1}. The UV-Vis detector was set at 254 nm. LC-MS conditions: The reaction product (10 μL) was analyzed by the HPLC system Nexera XR Shimadzu (Kyoto, Japan) equipped with two LC 20AD pumps, SIL-20A autosampler, a DGU-20A degasser, a CTO-20A oven, and an interfaced CBM-20A system coupled to an AmaZon speed Ion Trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an Electrospray Ionization (ESI) interface. Using a split, a flow rate of 0.250 mL min^{-1} was sent to the ESI source in the following conditions: capillary voltage = 5000 V, end-plate voltage = 550 V, drying gas flow = 8 L min-1, drying temperature = 270 °C, and nebulizer pressure = 30 psi. The full scan mode was used, in a positive ionization mode, for a range of 100-400 m/z monitoring the ions $[M+H]^+$ 233 m/z (melatonin) and $[M+H]^+$ 249 m/z (hydroxylated melatonin) and $[M+H]^+$ 265 m/z (AFMK). The data were collected with the Bruker Daltonics Data Analysis software (version 4.3).

2.1.3. Theoretical studies.

The calculations were carried at Density Functional Theory (DFT) level of theory with Pople-type basis sets 6-311++G(2d,p) and 6-311++G(3df,2p) (35). The functionals used are the PBE0 functional, which mixes the Perdew-Burke-Ernzerhof (PBE) exchange energy and 25% Hartree-Fock exchange energy, along with the full PBE correlation energy. The symmetry of all molecules is C1. The ground state geometry was optimized at the PBE0/6-311++G(2d,p) level of theory (36). The first excited state geometry was optimized using time-dependent

density functional theory TD-PBE0, and the basis set 6-311++G(2d,p), selecting ten excited states. Grimme's D3 empirical dispersion correction was used with Becke-Johnson damping factors (GD3-BJ) (37). The solvent effect was evaluated using an implicit solvation model (Integral Equation Formalism Polarizable Continuum Model – IEFPCM) (38). The harmonic vibrational analyses were carried out for all optimized molecular geometries. All calculations were performed using the Gaussian09 suite of programs (39).

3. RESULTS AND DISCUSSION

MLT was submitted to oxidation under UVC. Figure 1 shows the time-dependent production of AFMK and consumption of MLT in different pHs. The medium's pH significantly impacted MLT consumption and AFMK formation, being more favorable in the alkaline range. The pH dependence was more critical to producing AFMK than MLT consumption. As can be seen, at pH 1.0, MLT was about 50% oxidized, and the formation was AFMK was undetected. On the other hand, at pH 10.0, the oxidation of MLT was just double, but the production of AFMK was about 40-fold higher. These results indicated that more than one product was generated during the reaction and that AFMK was the most affected by pH change. In this concern, the consumption of MLT and the formation of AFMK were monitored by HPLC (see Supporting Information, Figure S1), and LCMS identified the reaction products (Figure S2). Corroborant with reported studies, besides AFMK, the other major product was the hydroxylated form of MLT, i.e., HO-MLT.

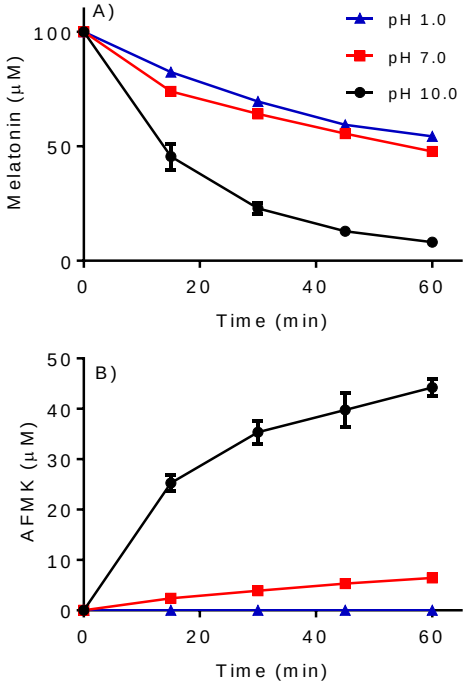


Fig. 1. pH effect on time-dependent photo-induced oxidation of melatonin and production of AFMK.

The results are the means and SD of three experiments.

The high AFMK yield at alkaline pH and its dependence on dissolved O_2 suggested that MLT's autoxidation can be the preferential pathway to generate AFMK. Indeed, deprotonation and involvement of O_2 are typical conditions for autoxidation reactions, i.e., catalysed or non-catalysed oxidation by O_2. Examples of the benefit of the alkaline medium for autoxidation are abundant in the literature, being particularly true for polyphenols (40–42). Corroborant with

our findings, Daescu *et al.* (43) reported the relevance of alkaline pH during the photodegradation of MLT, and Andrisano *et al.* described a similar effect on the photodegradation of melatonin and its determination in commercial formulations (33).

Another feature of typical autoxidation is the involvement of ROS. As has been reported, autoxidation reactions are characterized by the production of ROS via reduction of O_2 to superoxide radical anion ($O_2^{\bullet-}$) and, by dismutation, to hydrogen peroxide (H_2O_2) (44). Hence, aiming to advance in the comprehension of the factors involved in the increased efficiency of forming AFMK at alkaline pH, the following parameters were evaluated: irradiation dependence, presence of dissolved O_2 in the reaction medium, and the effect of scavengers of HO• and $O_2^{\bullet-}$.

The remotion of dissolved O_2 by bubbling N_2 was decisive in the comprehension of the phenomenon (Table 1). As can be seen, the AFMK yield decreased significantly by its remotion. On the other hand, the addition of DMSO, a scavenger of HO• (45), was less relevant. Finally, corroborant with the dependency of dissolved O_2, the yield of AFMK was strongly decreased by the addition of SOD, the enzyme responsible for the dismutation of $O_2^{\bullet-}$ (46), revealing the involvement of this reactive ROS, as should be expected for autoxidation (44). Our results agree entirely with the reported radio-induced ROS generated by gamma radiolysis of water. The authors described AFMK as the major product (84%) in aerated solutions, whereas HO-MLT was favored in the absence of oxygen (86%) (16).

Another central requirement for AFMK was electronic excitation. In this concern, two experimental approaches were used to study the dependence of irradiation. The first was to place a 280 nm cutoff filter between the reaction medium and the UVC lamp. The results depicted in Table 1 show that MLT consumption and formation of AFMK were almost totally blocked. In the second approach, LEDs (370 and 477 nm) replaced the UVC lamp. Again, a strong inhibition of MLT oxidation and AFMK production was obtained. As MLT has its maximum absorption of around 280 nm, these results strongly indicated the necessity of electronic excitation of the MLT for the reaction to occur.

Finally, it is worthy of note that the yield of AFMK might be underestimated. AFMK is susceptible to decomposition when submitted to UV light via carbon monoxide release and, thus, to deformylation leading to N^1-acetyl-5-methoxykynuramine (AMK) (47). In other words, the initial generation of AFMK can be closer to that of melatonin decomposition.

Table 1. Photo-induced oxidation of melatonin and the formation of AFMK: Effect of the reaction medium.

	MLT consumption (mM)	**AFMK production (mM)**
Control[#]	91 ± 3	44.3 ± 3
pH 7.0	53 ± 2	6.4 ± 3
Deaeration	17.5 ± 3	3.9 ± 3
+ DMSO	89 ± 3	38 ± 3
+ SOD	69.5 ± 3	16.2 ± 3
280 nm cutoff filter	9.0 ± 4	2.0 ± 3
LED (370 nm)	26.6 ± 3	11.5 ± 3
LED (467 nm)	5.7 ± 3	1.5 ± 3

[#]Control: Melatonin (100 mM), pH 10, UVC, 1 hour of irradiation. The results are the means and SD of the three experiments.

Based on these findings, we proposed that autoxidation is the mechanism involved in the increased yield of AFMK in the studied experimental condition. However, autoxidation of polyphenols does not need electronic excitation! Why could this be an issue with melatonin?

Addressing this question, we hypothesized that the electronic excitation could increase the reactivity of the deprotonated MLT, i.e., decreasing the energetic barrier of electron transfer from MLT to O_2. To pursue the hypothesis, DFT calculations were performed to simulate the reaction's thermodynamic profile and the influence of deprotonation and electronic excitation.

Figure 2 and Table S1 depict the reactants and products' total energy in the ground and excited states of protonated and deprotonated MLT. These results were obtained at the DFT level of theory. Corroborant with our experimental results, the electron transfer from MLT to O_2, the initial step in autoxidation, was a thermodynamically favorable process only when MLT was excited and deprotonated. Figure S3 and Tables S2-S11 show the frontier molecular orbitals and cartesian coordinates of the involved species.

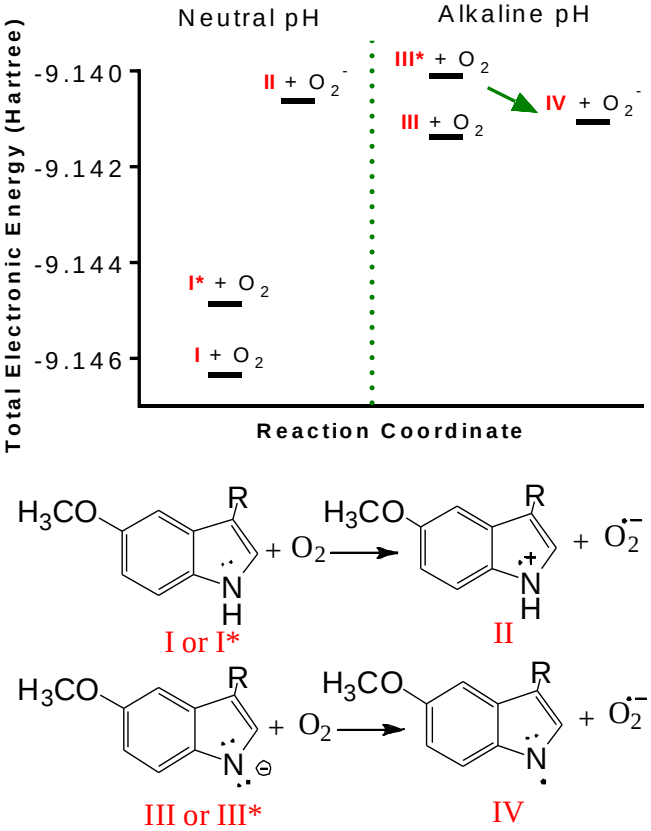


Fig. 2. pH and electronic excitation effects on electron transfer.

*DFT-based calculation of the total energy of reactants (melatonin protonated or deprotonated, and molecular oxygen) and products (melatonin radical or radical cation and superoxide anion radical. *Indicates excited state. The arrow indicates the energetically feasible electron transfer.*

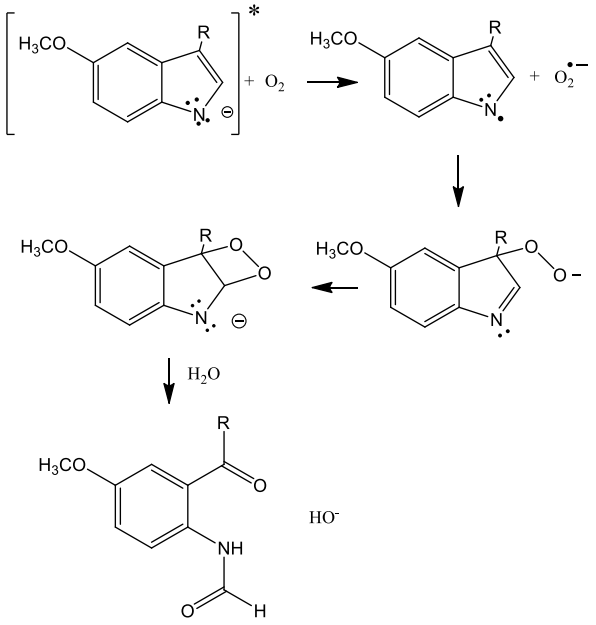
Based on these findings, the mechanism for autoxidation of MLT at excited state could be outlined as follows:

a) MLT deprotonation. Even though a weak acid (pKa = 12.7) (48), the *N*-hydrogen of indoles is more acidic than that of aromatic amines (pKa ~ 30), hence, at pH 10, even though a low concentration, the deprotonated form must be present in the reaction medium.

b) Electronic excitation promotes electron transfer from MLT to O_2.

c) Radical coupling between MLT radical and $O_2^{\bullet-}$ leads to the peroxyl intermediate.

d) Intramolecular nucleophilic attack leads to AFMK (Scheme 2).



Scheme 2. Mechanism proposal for the autoxidation of melatonin at excited state leading to AFMK.

4. CONCLUSION

Even though there are several chemical routes to produce AFMK, most of them are unclear about how the two oxygen atoms are incorporated into melatonin. It is not the case with autoxidation. The initial reduction of molecular oxygen leads to superoxide radical anion, and subsequent radical coupling leads directly to AFMK. A significant difference compared to typical autoxidations was the necessity of irradiation to increase the efficiency of the reaction. Our theoretical results showed that the electron transfer is thermodynamically favorable at the excited state, explaining the need for irradiation.

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AUTHORSHIP

Conceptualization, VFX; Methodology, VFX, MRP; Investigation, VFX, MRP, VRM, ARS, NHM; Data Curation, VFX, ARS, NHM; Writing – Original Draft Preparation, VFX; Writing – Review & Editing, VFX; Supervision, VFX, NHM; Project Administration, VFX; Funding Acquisition, VFX. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare. [

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