Scavenging of NO by Melatonin

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The hormone melatonin (*N*-acetyl-5-methoxytryptamine), mainly produced by the pineal gland during the hours of darkness, plays an important role as a transmitter of photoperiodic information and regulation of seasonal reproductive cycles.[1] Additionally, it mediates a variety of cellular, neuroendocrine, and physiological processes.[2] On the other hand, a large body of evidence suggests that melatonin is a potent endogenous free radical scavenger.[3] Recently, we have shown the thermodynamic feasibility of the reaction of melatonin with hydroxyl radicals.[4] Among the family of free radicals with biological relevance, nitric oxide (NO) deserves particular attention since it is involved in the regulation of a wide range of physiological functions as an intercellular and intracellular messenger.[5] Although it has been proposed that melatonin may scavenge NO,[6,7] the mechanism of this interaction is still an open issue. In this work we try to shed light on the following questions: Does melatonin directly scavenge NO? What are the main products of the reaction? What kind of mechanisms are operative in different environments?

The main product of the reaction of melatonin with NO in the presence of O_2 in aprotic and aqueous media, *N*-nitrosomelatonin, was isolated and characterized by ^{1}H NMR and X-ray spectroscopy[8] (Figure 1). The density functional theory (DFT)-optimized structure at the generalized gradient approximation (GGA) Becke−Perdew level[9] is in good agreement with experimental results. In solution, *N*-nitrosomelatonin is present in two isomeric forms, with O3−N3−N1−C9 dihedral angles of 0° and 180°. The isomerization barrier $\Delta G^{\#}_{330}$ was found to be 16 ± 1 kcal mol^{-1} by variable-temperature ^{1}H NMR.

Both ionic and free radical mechanisms have been postulated to explain nitrosation of nucleophiles by NO/O_2. The nitrosating species were suggested to be nitrosonium ion (NO^+) carriers, such as N_2O_3 and NOCl, or the free radicals NO_2 and NO.[10] The main reactions involving typical nitrosating agents are given in eqs 1−5.

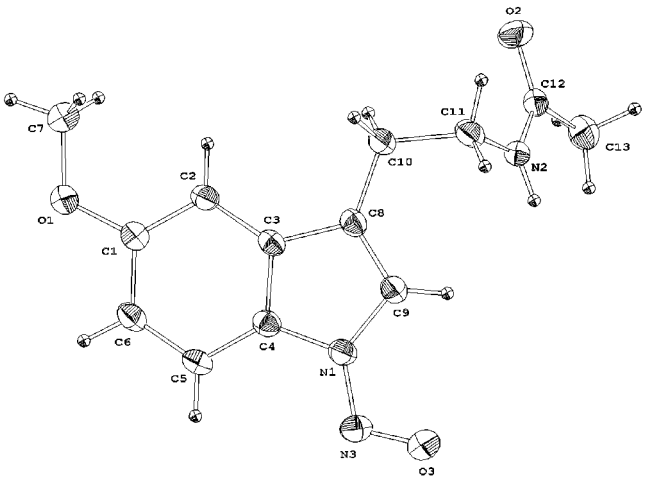


Figure 1. X-ray structure of *N*-nitrosomelatonin.

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$k_1 = 2.54 \times 10^6\ M^{-2}\ s^{-1}\ \text{(ref 11)}$$

$$NO + NO_2 \leftrightarrows N_2O_3 \quad (2)$$

$$k_2 = 1.1 \times 10^9\ M^{-1}\ s^{-1};\ k_{-2} = 8.1 \times 10^4\ s^{-1}\ \text{(ref 11)}$$

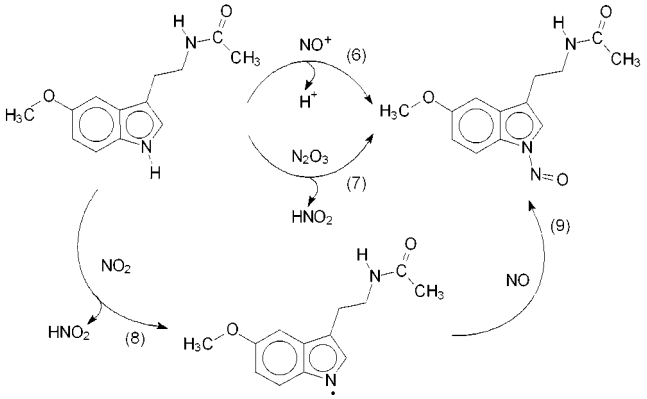
$$N_2O_3 + H_2O \leftrightarrows 2HNO_2 \leftrightarrows 2H^+ + 2NO_2^- \quad (3,4)$$

$$k_3 = 1.6 \times 10^3\ s^{-1};\ k_{-3} = 5.6\ M^{-1}\ s^{-1}\ \text{(ref 12)}$$

$$HNO_2 + H^+ \leftrightarrows NO^+ + H_2O \quad (5)$$

Rate constants for reactions 1−5 are given for aqueous media. Values for k_1 and k_2 in aprotic solvents were reported to be similar.[13] According to these reactions, melatonin could be nitrosated through three different pathways as outlined in Scheme 1:

Scheme 1



The concerted (7) and radical (8,9) pathways are consistent with the same global reaction:

$$4NO + O_2 + 2Mel \rightarrow 2Mel\text{-}NO + 2HNO_2 \quad (10)$$

A 0.2 M deareated solution of melatonin in anhydrous acetonitrile was saturated with NO gas. NO was previously passed through

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Table 1. DFT-Computed $\Delta E°$ in Vacuum and $\Delta G°$ in Acetonitrile and Aqueous Solution for the Proposed Reactions[a]

	GGA		
reaction	vacuum	acetonitrile	water
6	−21.3		−42.0
7	−10.3	−12.7	−14.3
8	14.2	11.2	8.3
9	−37.9	−38.0	−37.0
10	−95.4	−104.0	−107.8

[a] All values are in kcal/mol.

sodium hydroxide, to eliminate NO_2. Under these conditions, no reaction was observed. However, in the presence of O_2 and an excess of NO (O_2:melatonin = 1:2), complete conversion to *N*-nitrosomelatonin was achieved. The equilibria 3, 4, and 5 cannot be established in the absence of water. This implies that, in anhydrous acetonitrile, only the concerted and the free radical pathways are likely to occur. An alternative first step for the free radical pathway could involve abstraction of the indolic hydrogen by NO instead of NO_2. However, DFT calculations including solvent effects modeled by the polarizable continuum model (PCM)[14] in acetonitrile and aqueous solution predict a much larger $\Delta G°$ for the reaction with NO than with NO_2. The computed $\Delta G°$ values for this abstraction by NO and NO_2 in acetonitrile are 33.6 and 14.2 kcal/mol, respectively. This is consistent with earlier experimental evidence which suggests that NO is unable to directly nitrosate amines.[15]

A first-order rate constant was found for melatonin, $k_{obs} \approx 1 \times 10^{-2}$ s^{-1}, under pseudo-first-order conditions with respect to NO and O_2. The reaction was followed by an increase in the UV absorbance at 345 nm due to *N*-nitrosomelatonin ($\epsilon = 7070 \pm 50$ M^{-1}). Assuming the steady-state approximation for Mel$^{\bullet}$ and the preequilibrium for N_2O_3, and considering that reactions 1 and 2 are much faster than reactions 7 and 8, it can be shown that the observed constant is given by the following expression:

$$k_{obs} = 2[NO_2]\left(\frac{k_2 k_7}{k_{-2}}[NO] + k_8\right)$$

Under our experimental conditions in acetonitrile, [NO] and [NO_2] are about 8×10^{-4} and 2×10^{-3} M, respectively. Taking into account the value of the equilibrium constant for reaction 2, $K_2 = 1.36 \times 10^4$,[11] we can deduce that $(10k_7 + k_8) \approx 3$ M^{-1} s^{-1}. This implies that both pathways are slow compared with typical amine nitrosations.[11]

The limiting step for the radical pathway, reaction 8, is slightly endoergic (Table 1). This is consistent with the assumption that it may be an operative mechanism in acetonitrile. In the case of the concerted pathway (7), a favorable six-membered transition state could be proposed. In accord with the experimental results, DFT-PCM calculations yield a negative $\Delta G°$ value for the global reaction of formation of *N*-nitrosomelatonin (10) in acetonitrile. It is interesting to remark that, for the concerted and radical pathways, the computed solvent effects are small.

The hydrogen atom abstraction which produces a free radical in reaction 8 is feasible in this case due to stabilization of the nitrogen-centered free radical by the indolic ring. Moreover, the low nucleophilicity of the amine makes it less prone to attack by NO^+.

In aqueous media, two different situations were investigated: in plain water and in a phosphate buffer at pH 7.4. No reaction was observed at pH 7.4 in [HPO_4^{2-}] = 0.1 M. When the concentration of the buffer was diminished, low yields of product were obtained. The value of k_{obs} is $\sim 4 \times 10^{-3}$ s^{-1} under pseudo-first-order conditions with respect to NO and O_2. At pH 7.4, essentially all of the HNO_2 is completely dissociated to form NO_2^-, so reaction 5 is not feasible and only the radical or concerted pathways may be operative. Considering that [NO] = 2×10^{-3} M and [NO_2] = 2×10^{-3} M, and making the same approximations as described above, it can be shown that $(20k_7 + k_8) \approx 1$ M^{-1} s^{-1}. On the other hand, a value of $k_3 \approx 1 \times 10^5$ M^{-1} s^{-1} can be calculated at [HPO_4^{2-}] = 0.1 M by taking into account the fact that HPO_4^{2-} may catalyze N_2O_3 hydrolysis.[11,12] This implies that this process is much faster than the concerted and radical pathways of nitrosation. However, if the buffer concentration is decreased, both pathways may compete with reaction 3.

In the absence of buffer, reactions 1−4 take place, and the pH decreases to about 4 due to HNO_2 dissociation, making reactions 5 and 6 feasible. This ionic pathway in which NO^+ is the nitrosating agent has also been proposed for the nitrosation of the indolic amine tryptophane.[16] An additional confirmation that this pathway is operative at low values of pH is the fact that total conversion of melatonin to the nitroso derivative was obtained employing HNO_2 at pH 1. However, pathways 7 and 8,9 could also compete under these conditions due to the low nucleophilicity of melatonin.

DFT calculations predict the attack of melatonin by NO^+, reaction 6, to be spontaneous (Table 1). As expected for a reaction involving ions, solvent effects turned out to be significant.

Based on the experimental and DFT results, we proposed three different mechanisms in which NO in the presence of O_2 nitrosates melatonin to produce *N*-nitrosomelatonin, depending on the environment. If the reaction is performed in aprotic solvents, we propose a competition among a free radical and a concerted pathway, with NO_2 and N_2O_3 as the active species, respectively. In aqueous media, we expect the ionic reaction to be favored at acidic pH with NO^+ as the reactive species. At physiological pH, the concerted and free radical pathways may compete with N_2O_3 hydrolysis.

According to our findings, the formation of *N*-nitrosomelatonin under physiological conditions in which [NO] < 1 μM and [O_2] < 200 μM is not likely to occur by the mechanisms outlined previously, since other processes involving NO, for example binding to heme enzymes, are much faster. However, there may be an interplay of the proposed pathways with mechanisms involving other free radicals, such as hydroxyl or peroxynitrite. The demonstration that nitrosation of melatonin is feasible widens its rich physiological chemistry. In fact, NO and melatonin share many common intracellular sites. Since melatonin was shown to be protective against oxidative damage in situations where NO is known to account for molecular destruction,[17] cross-talk between the signaling systems associated with these molecules can be suggested. Furthermore, while melatonin may be involved in transnitrosation processes, *N*-nitrosomelatonin could act as a NO donor. Therefore, elucidation of the physiological relevance of the melatonin−NO interaction deserves particular attention. Further investigation of this issue is presently being carried out by our group.

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Supporting Information Available: Experimental and computational details, 200-MHz ^{1}H NMR of *N*-nitrosomelatonin, kinetic analysis, and Cartesian coordinates for the optimized species (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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