A Novel Metabolic Pathway of Melatonin: Oxidation by Cytochrome *c*†

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ABSTRACT: The indoleamine melatonin is ubiquitously distributed, and because of its small size and amphiphilic nature, it is able to reach easily all cellular compartments. The highest intracellular melatonin concentrations are found in the mitochondria, suggestive of local metabolism and/or direct participation in organelle function. In mitochondria cytochrome *c* (cyt *c*) could represent a melatonin target since it has the capability to oxidize organic molecules in the presence of H_2O_2, and mitochondria are the main site of H_2O_2 production in nonphagocytic cells. Therefore, we investigated oxidation of melatonin by cyt *c*/H_2O_2 couple as a potential pathway for its metabolism in the mitochondria. We found melatonin conversion into N^1-acetyl-N^2-formyl-5-methoxykynuramine via sequential steps that generate the intermediates 2-hydroxymelatonin and 2,3-dihydroxymelatonin. We experimentally excluded mediation by a Fenton/Haber-Weiss-type reaction and documented the dependence on oxoferryl heme for melatonin oxidation. Given the high mitochondrial concentrations of both melatonin and cyt *c* as well as the continuous generation of H_2O_2 during respiration, it is entirely possible that mitochondrial cyt *c*-mediated oxidation of melatonin may be a plausible pathway of its biotransformation in vivo.

Melatonin (*N*-acetyl-5-methoxytryptamine) has pleiotropic bioactivities that encompass numerous endocrinological and behavioral processes (*1*). Chemically, melatonin can function as an endogenous free radical scavenger and a broad-spectrum antioxidant, and it can easily reach all cellular compartments, because of its small size and amphiphilic nature (*2*−*4*). Indeed, melatonin is localized ubiquitously intracellularly in cytosolic, membrane, and nuclear compartments (*5*, *6*). By far, the highest melatonin concentrations are found in mitochondria (*7*), raising the possibility of functional significance for this targeting with involvement in situ in mitochondrial activities; for example, most apoptotic signals originate in the mitochondria, and melatonin has well-known antiapoptotic effects (*8*). Mitochondria are also the organelles with the highest production rate of reactive oxygen species (ROS), and melatonin is a powerful antioxidant (*9*). It has also been reported that melatonin can stimulate ATP synthesis (*10*).

The general metabolic fate of melatonin includes multiple intermediate products, suggestive of multienzyme involvement (Figure 1). In the liver melatonin undergoes cytochrome P-450 mediated O-demethylation and 6-hydroxylation to yield *N*-acetylserotonin (*N*-acetyl-5-hydroxytryptamine) and 6-hydroxymelatonin, respectively (*11*). The latter can be conjugated with either sulfate or glucuronide (*12*). Through accesory pathways, melatonin can be alternatively transformed into 5-methoxyindoleacetic acid or 5-methoxytryptophol (*13*). Cleavage of the melatonin pyrrole ring by indoleamine 2,3-dioxygenase yields N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK),[1] which is further degraded by arylamine formamidase to N^1-acetyl-5-methoxykynuramine (AMK) (*14*, *15*). Moreover, the hemoproteins myeloperoxidase and oxyferrylhemoglobin can also oxidize melatonin to AFMK (*16*, *17*), and under the action of catalase AFMK is deformylated to AMK, at least in vitro (*18*). Therefore, hemoproteins could share the property of melatonin oxidizing activity, particularly with mitochondrial cyt *c*. Cyt *c*, similar to most hemoproteins, can oxidize organic molecules in the presence of hydrogen peroxide through its pseudoperoxidase activity (*19*, *20*).

In nonphagocytic cells mitochondria are the main site of H_2O_2 production, although this is normally kept at relatively low local levels by the action of glutathione peroxidase. Under pathological conditions such as ischemia-reperfusion injury or inflammation, H_2O_2 production rises noticeably (*21*, *22*), increasing the likelihood for pseudoperoxidase oxidation of endogenous substrates, such as melatonin. In this study, we investigated melatonin oxidation by cyt *c*.

MATERIALS AND METHODS

Reagents. Horse heart cytochrome *c*, melatonin, diethylenetriaminepentaacetic acid (DTPA), bovine erythrocyte

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[1] Abbreviations: CDL, curved desolvation line; cyt *c*, cytochrome *c*; DTPA, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide; ETC, electron transport chain; ESI, electrospray interface; AFMK, N^1-acetyl-N^2-formyl-5-methoxykynuramine; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; AMK, N^1-acetyl-5-methoxykynuramine; ROS, reactive oxygen species; SIM, selected ion monitoring; SOD, superoxide dismutase; *t*-BuOOH, *tert*-butyl hydroperoxide.

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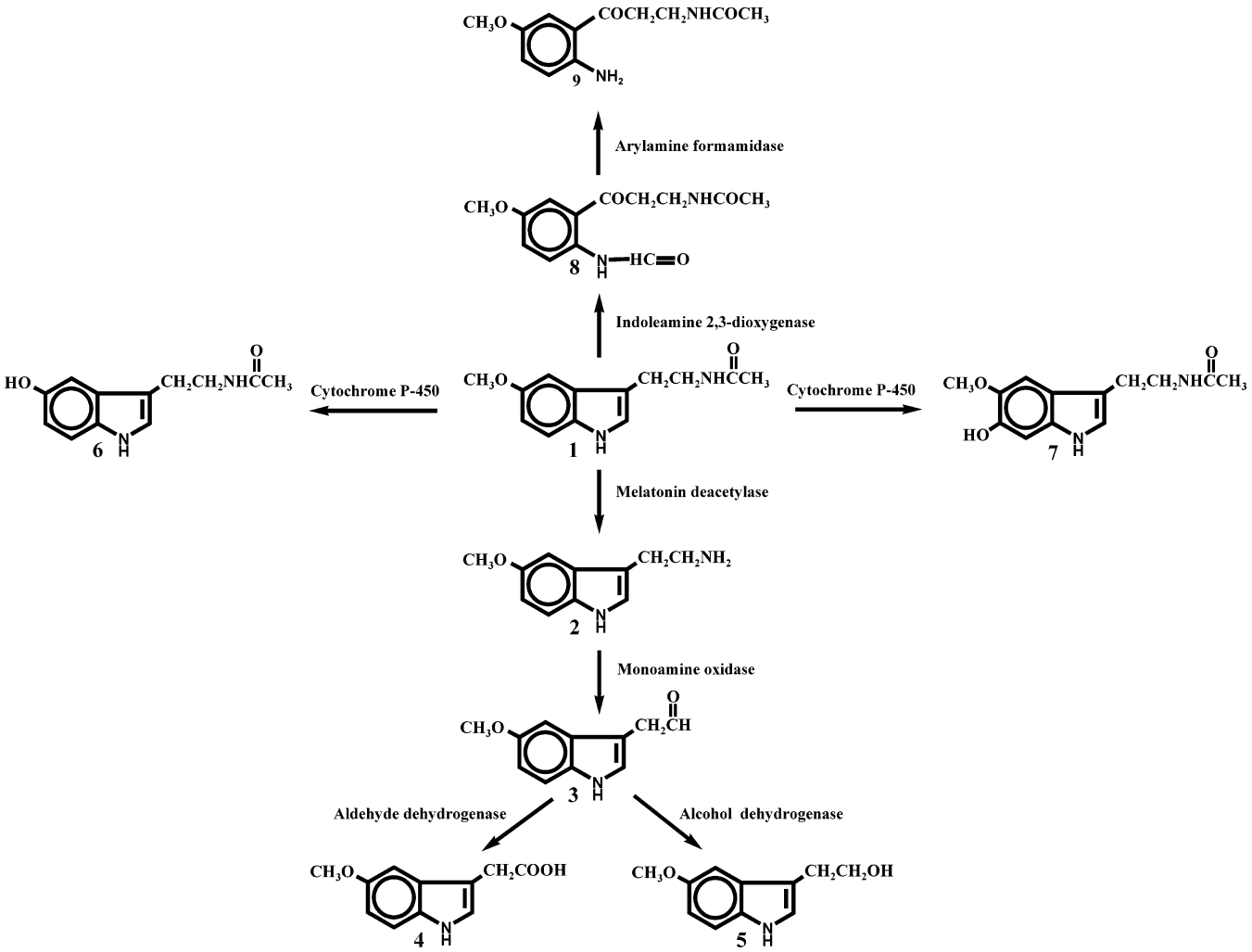

FIGURE 1: The biochemical pathways of melatonin metabolism: **1**, melatonin; **2**, 5-methoxytryptamine; **3**, 5-methoxyindoleacetaldehyde; **4**, 5-methoxyindoleacetic acid; **5**, 5-methoxytryptophol; **6**, *N*-acetylserotonin; **7**, 6-hydroxymelatonin; **8**, N^1-acetyl-N^2-formyl-5-methoxykynuramine; **9**, N^1-acetyl-5-methoxykynuramine.

superoxide dismutase (SOD), and *tert*-butyl hydroperoxide (*t*-BuOOH) were obtained from Sigma (St. Louis, MO). All reagents used in enzymatic assays and liquid chromatography−mass spectrometry (LC-MS) were of the highest purity.

Cytochrome *c* was fully oxidized by an excess of potassium ferricyanide and then desalted on a Sephadex G-25 column using 10 mM Tris-HCl (pH 7.4). The cyt c^{3+} concentration was determined spectrophotometrically using solid sodium dithionite as reducing agent and measuring absorbance at 550 nm ($\epsilon_M = 27700\ M^{-1}\ cm^{-1}$).

Hydrogen peroxide was prepared freshly immediately before use, and its concentration was determined spectrophotometrically using the extinction coefficient $\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$.

AFMK was synthesized and purified by HPLC (*23*) and its identity determined by spectrophotometry (bands at $\lambda_{max} = 231$, 262, and 342 nm) and verified by the LC-MS findings of molecular ion $[M + H]^+$ at *m*/*z* 265 and fragment ion at *m*/*z* 237 ($[(M - N\text{-acetyl}) + H]^+$), together with an additional fragment ion at *m*/*z* 178 ($[(M - (N\text{-acetyl} + \text{N-formyl})) + H]^+$) (*24*).

AMK was synthesized and purified by HPLC (*15*). The identity of the synthesized product was confirmed by LC-MS analysis, e.g., molecular ion $[M + H]^+$ at *m*/*z* 237, and by the UV absorbance maximum at $\lambda_{max} = 377$ nm (*15*).

Spectrophotometric Assay of Melatonin Oxidation. Measurements were performed in a Cary 50 spectrophotometer (Varian, Mulgrave, Australia) equipped with a thermostat-controlled cell holder set at 37 °C. The reaction mixture contained H_2O_2 (0.25 mM), cyt *c* (0.025 mM), and melatonin (0.1 mM) disolved in 10 mM Tris-HCl buffer (pH 7.4). In some experiments SOD, dimethyl sulfoxide (DMSO), DTPA, and imidazole were included at final concentrations of 166 units/mL, 10%, 1 mM, and 10 mM, respectively. The reaction was started by adding melatonin or H_2O_2 and recording the absorbance spectrum over the wavelength range of 220−700 nm during a 30 min period, at scanning intervals of 3 min.

Animals. Male Wistar rats (180−210 g) were used in the experiment. The animals were housed in a temperature-controlled room on a 12 h light/dark schedule with food and water available ad libitum. The animals were sacrificed between 10:00 and 12:00 h under ether anesthesia. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23), approved by the local veterinary service authority.

Isolation of Mitochondria. The mitochondrial fraction from rat heart was prepared by homogenizing the tissue in five volumes of ice-cold 0.25 M sucrose. The homogenate was

centrifuged at 1200*g* for 10 min at 4 °C, and the resulting supernatant was removed and centrifuged again at 10000*g* for 20 min at 4 °C. The pellet was resuspended in 0.25 M sucrose, and the centrifugation was repeated twice under the same conditions. The washed mitochondrial fraction was resuspended in 10 mM Tris-HCl buffer (pH 7.4) and used directly for the studies on melatonin metabolism.

Isolation of Mitoplasts. An appropriate volume of 2% digitonin solution was added to the mitochondrial suspension to a final concentration of 100 μg/mg of protein, and the mixture was shaken gently on ice for 5 min. Resultant mitoplasts were pelleted by centrifugation at 10000*g* for 10 min and washed two times with the isolation buffer [2 mM HEPES (pH 7.4), 70 mM sucrose, 220 mM D-mannitol, 2 mM EDTA], and the final pellet was suspended in 10 mM Tris-HCl (pH 7.4) and used for further experiments.

Melatonin Metabolism by Mitochondria (Mitoplasts). Melatonin was dissolved in 45% 2-hydroxypropyl-β-cyclodextrin immediately before its use. Isolated heart mitochondria (mitoplasts) were preincubated for 10 min at 37 °C with 75 μM *t*-BuOOH in 10 mM Tris-HCl buffer (pH 7.4). The reaction was started by adding melatonin (50 μM final concentration), and after incubation at 37 °C for 90 min, it was stopped by adding 1 mL of ice-cold methylene chloride. The reaction mixture was extracted twice with 1 mL methylene chloride, and the methylene chloride layers were combined and dried in an RVC 2-18 rotational vacuum concentrator (Christ, Osterode, Germany). The residues were dissolved in methanol and subjected to LC-MS analysis.

Determination of Endogenous Melatonin and Its Metabolites. Pineal glands or heart mitochondria pellet were homogenized in 0.1 M perchloric acid. The homogenate was centrifuged at 10000*g* for 20 min at 4 °C. The resulting supernatant was extracted twice with 5 volumes of methylene chloride, the methylene chloride layers were dried, and the residues were dissolved in methanol and subjected to LC-MS analysis.

LC-MS Analysis. Aliquots of the reaction mixture (20 μL) were separated on a QP8000a LC-MS (Shimadzu, City, Japan) equipped with diode array and single quadrupole mass spectrometry detectors. A Restec Allure C18 reverse-phase column (150 × 4.6 mm, 5 μm particle size, and 60 Å pore size) was used with a mobile phase consisting of 25% acetonitrile and 0.1% acetic acid. Elution was carried out isocratically at a flow rate of 0.75 mL/min and temperature of 40 °C. The eluent was routed to the mass spectrometric electrospray interface (ESI) set in the positive mode using nitrogen as the nebulizing gas. The mass spectrometry parameters were as follows: nebulizer gas flow rate, 4.5 L/min; electrospray voltage, 4.5 kV; curved desolvation line (CDL) heater temperature, 250 °C. Analyses were carried out in the scan mode from *m*/*z* 160 to *m*/*z* 550. The selected ion monitoring (SIM) mode was also used to detect ions with *m*/*z* = 249 (monohydroxymelatonin), *m*/*z* = 265 (dihydroxymelatonin and AFMK), *m*/*z* = 237 (AMK), *m*/*z* = 479 (dimer formed by residues of melatonin and hydroxymelatonin), and *m*/*z* = 233 (melatonin). System control and data acquisition were performed with the LC-MS workstation Class-8000 software (Shimadzu).

RESULTS AND DISCUSSION

Incubation of melatonin with cyt *c* in the presence of hydrogen peroxide resulted in a significant shift of the maximum absorbance in the melatonin spectrum (from 279 to 258 nm); there was also increased absorbance in the 310−350 nm region (Figure 2) indicating melatonin indole ring opening with formation of AFMK (*18*). On LC-MS analysis the predominant five products of melatonin oxidation had retention times (RT) of 2.45 min (product **1**), 3.43 min (product **2**), 3.72 min (product **3**), 4.28 min (product **4**), and 4.78 min (product **5**) (Figure 3). Under the same conditions neither cyt *c* nor H_2O_2 alone induced changes of melatonin spectrum, nor did they result in the appearance of melatonin products (Figure 3A,C).

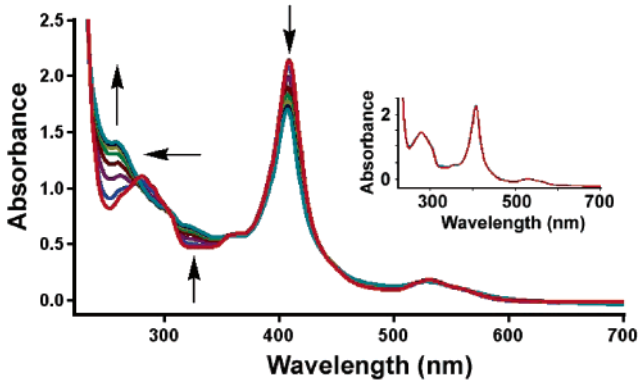

FIGURE 2: Spectrophotometric assay of melatonin oxidation by cyt *c*. Melatonin (0.1 mM) was incubated at 37 °C with cyt *c* (0.025 mM) and H_2O_2 (0.25 mM) in 10 mM Tris-HCl buffer (pH 7.4). The absorbance spectrum over the wavelength range 220−700 nm was recorded over 30 min after melatonin addition, at scanning intervals of 3 min (red trace, the initial scan). The direction of spectral changes is indicated by arrows. Downward and upward arrows indicate decrease or increase in absorbance in the trace of the spectrum. Insert: Effect of imidazole on melatonin oxidation by cyt *c*. Reaction conditions are as described above, except that cyt *c* was preincubated with 10 mM imidazole for 10 min before H_2O_2 and melatonin addition.

The mass spectrum of product **1** (RT 2.45 min) yielded a molecular ion $[M + H]^+$ at *m*/*z* = 265 with two more oxygen atoms than melatonin (Figure 3C,D). The absence of a fragment at *m*/*z* = 178 (characteristic of AFMK) indicates integrity of the pyrrole ring, while loss of water from the molecular ion at *m*/*z* = 265 resulted in a fragment at *m*/*z* = 247. These data suggest that product **1** corresponds to melatonin hydroxylated at two different positions, most likely positions 2 and 3, since disappearance of product **2** (identified as 2-hydroxymelatonin) was accompanied by simultaneous accumulation of product **1** and product **4** (identified as AFMK) (Figure 1B−E in Supporting Information). Since AFMK is formed by the opening of the pyrrole ring after melatonin has been hydroxylated at sites 2 and 3 (*24*), product **1** must be an intermediate between 2-hydroxymelatonin and AFMK.

Product **2** with RT 3.43 min had a molecular mass of 248 (molecular ion $[M + H]^+$ at *m*/*z* = 249), indicative of a monohydroxylated derivative of melatonin (Figure 3C,D). The mass spectra also contained an ion at *m*/*z* = 231 that originated from loss of water by the molecular ion ($[(M - H_2O) + H]^+$). Product **2** had a UV spectrum differing from 6-hydroxymelatonin (λ_{max} = 300.6 nm) and 4-hydroxymelatonin (λ_{max} = 308 and 238 nm) (*24*). Instead, the UV spectrum of product **2** (λ_{max} = 296 and 257 nm) was similar to that of the keto tautomer of 2-hydroxymelatonin (λ_{max} = 298 and 258 nm) (*24*). Together, LC-MS and UV

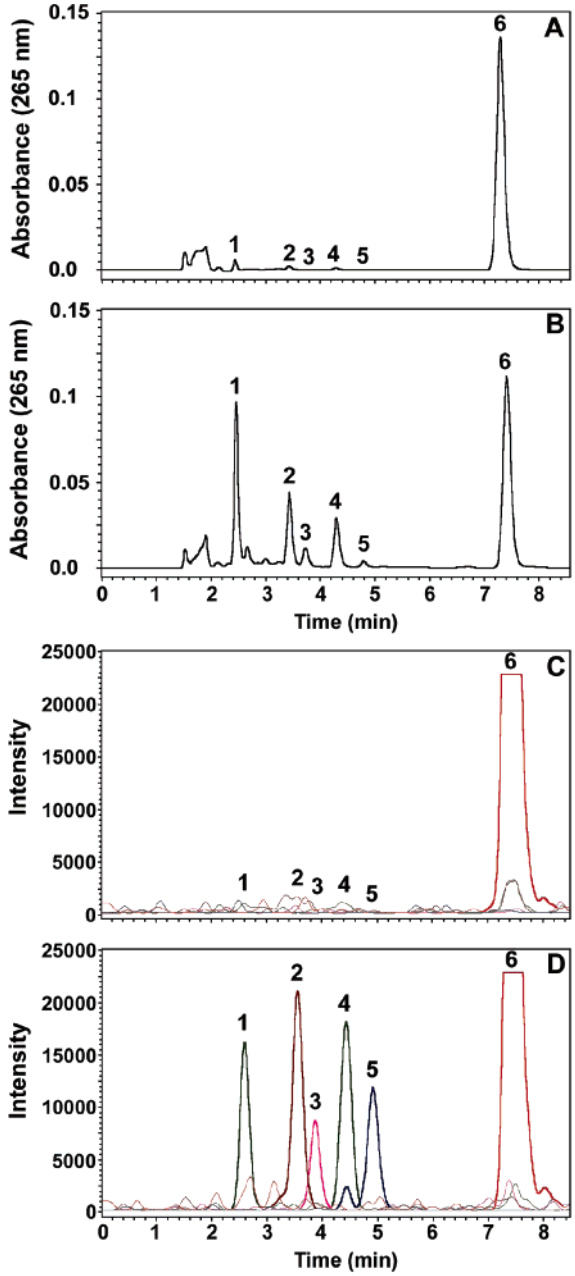

FIGURE 3: LC-MS analysis of products of melatonin oxidation by cyt *c*. HPLC elution profile (A) and mass chromatograms (C) of the reaction mixture with melatonin and H_2O_2 without cyt *c*. HPLC elution profile (B) and mass chromatograms (D) of reaction mixture containing melatonin, cyt *c*, and H_2O_2. See Materials and Methods section for experimental description. The HPLC elution profiles were monitored by absorbance at 265 nm. Mass spectrometric analysis was carried out in the scan mode from *m*/*z* 160 to *m*/*z* 550. Key: green, ion at *m*/*z* = 265; brown, ion at *m*/*z* = 249; purple, ion at *m*/*z* = 479; blue, ion at *m*/*z* = 237; red, ion at *m*/*z* = 233. The peaks designated as 1, 2, 3, 4, 5, and 6 correspond to product **1** (2,3-dihydroxymelatonin), product **2** (2-hydroxymelatonin), product **3** (dimer formed by residues of melatonin and hydroxymelatonin), product **4** (AFMK), product **5** (AMK), and melatonin, respectively.

spectral data indicate that product **2** corresponds to 2-hydroxymelatonin. Incubation of HPLC-purified product **2** with the cytochrome *c*/H_2O_2 pair yields product **1**, providing additional support in favor of 2-hydroxymelatonin and 2,3-dihydroxymelatonin as intermediates between melatonin and AFMK (Figure 2 in Supporting Information).

Product **3** with RT 3.72 min had a molecular mass of 478 (molecular ion $[M + H]^+$ at *m*/*z* = 479), corresponding to a dimer formed by residues of melatonin and hydroxymelatonin ([((melatonin (MW 232) + keto tautomer of 2-hydroxymelatonin (MW 248)) − 2H) + $H]^+$) (Figure 3C,D).

Product **4** with RT 4.28 min had a molecular ion $[M + H]^+$ at *m*/*z* = 265, indicating incorporation of two oxygen atoms into the melatonin molecule (Figure 3C,D). Product **4** was identical to the chemically synthesized AFMK standard by both LC-MS and spectrophotometry criteria.

Product **5** (RT 4.78 min) had a molecular ion $[M + H]^+$ at *m*/*z* = 237 indicative of AMK, a derivative of AFMK from which the formyl group has been cleaved (Figure 3C,D). Chromatography, mass spectrometry, and spectrophotometry characteristics of product **5** were identical to the chemically synthesized AMK standard.

The capability of the cyt *c*/H_2O_2 pair to oxidize organic molecules is well recognized, but the cyt *c* oxidant intermediates have so far not been identified. In this regard, it is known that in the presence of H_2O_2 ferricytochrome *c* can oxidize xenobiotics such as 2-keto-4-(thiomethyl)butyric acid, 4-aminoantipyrine, 2′,7′-dichlorofluorescein, 2,2′-azinobis-(ethylbenzothiazolinesulfonic acid), and luminol, as well as endogenous substrates such as GSH, NADH, and ascorbate (*19*, *20*). Ferricytochrome is, however, thought to only be a precursor for the catalytically active form of the hemoprotein that must contain iron in a higher oxidized state (*19*, *20*). Hence, the interaction between ferricytochrome *c* and hydrogen peroxide would initially form an oxoferryl derivative of hemoprotein (cyt $c^{+\bullet}$ Fe^{IV}═O):

$$\text{cyt } c\text{—}Fe^{III} + H_2O_2 \rightarrow \text{cyt } c^{+\bullet}\ Fe^{IV}{=}O + H_2O$$

Oxoferryl cyt *c*, similar to compound **I** of peroxidases, is assumed to contain two oxidizing equivalents, one in the form of oxoferryl heme (Fe^{IV}═O), and another as the porphyrin π radical (*19*). In the absence of a suitable substrate rapid electron transfer from the protein to the porphyrin moiety would ensue to generate the short-lived tyrosyl radical, which is subsequently oxidized to a long-lived tyrosine *o*-semiquinone radical (*25*, *26*). Since three tyrosine residues of cyt *c* are solvent-exposed (*25*), tyrosine radical formation would allow oxidation of the molecules to which the heme iron is inaccessible.

Spectrophotometric evidence on the formation of an oxoferryl form, which is available for other hemoproteins, has not been documented for cyt *c*. This may be related to the hexacoordinate configuration of heme iron in native cyt *c*, which is devoid of a coordinated water molecule that might be displaced by H_2O_2; this does not, however, preclude formation of a cyt *c* derivative similar to high-spin compound **I** of peroxidases by use of the sixth coordination place. The latter is occupied by an easily displaced sulfur ligand from methionine (Met-80) (*25*). Loss of the Met-80—heme iron bond may be detected as a reduction in the weak 695 nm absorption peak of native cyt *c* (*27*). Our experiments demonstrating that addition of H_2O_2 resulted in disappearance of the cyt *c* 695 nm absorbance and blue shift with discrete bleaching of the Soret band confirmed the expected replacement of the sixth heme ligand at Met-80 and change of cytochrome *c* low-spin species to high-spin species (Figure 4) (*28*).

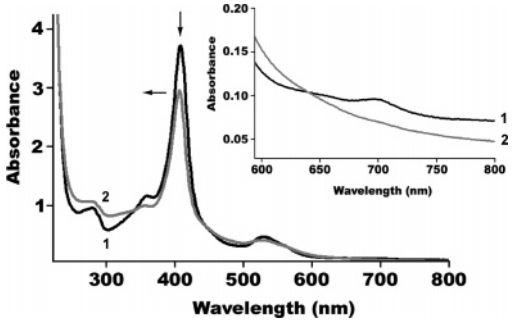

FIGURE 4: Absorption spectra of the cyt *c* reaction with H_2O_2. The reaction mixture contained cyt *c* (41 μM) in 10 mM Tris-HCl buffer (pH 7.4). Absorption spectra were recorded at 37 °C from 250 to 800 nm. After the initial scan of cyt *c* standard (trace 1), H_2O_2 at a final concentration of 357 μM (trace 2) was added, and spectral changes were monitored. Insert: zoom of absorption spectra area from 600 to 800 nm.

Theoretically, H_2O_2 could mediate melatonin oxidation via Fenton/Haber-Weiss-type reactions triggered by the release of iron from cyt *c*. In fact, AFMK and 2-hydroxymelatonin were indeed detected during incubation of melatonin with a Fenton-type $HO^{\bullet}$ generating system (24). Morever, addition of H_2O_2 to cyt *c* produced the bleaching of its Soret band at 408 nm; this indicates porphyrin ring opening directly proportional to the loss of iron (Figures 2 and 4) (*29*). Because up to 10% of cyt *c* was destroyed during the reaction complementary experiments were performed to test a presumptive role for free iron in melatonin oxidation.

Melatonin oxidation was nevertheless unaffected by addition of the chelating agent DTPA, conclusively excluding reaction mediation by free iron. Also, SOD and DMSO produced only slight to undetectable changes in reaction intensity, further excluding a role for $O_2^{-\bullet}$ and $^{\bullet}OH$ radicals in the oxidation of melatonin. Therefore, we can safely conclude that under the present experimental conditions melatonin oxidation is not a result of Fenton/Haber-Weiss-type reactions, in agreement with data showing that hydroxyl radicals are not generated during oxidation of organic substrates by the cyt c/H_2O_2 pair (*19*, *20*).

The candidate most likely responsible for melatonin oxidation is the family of oxoferryl heme species of cyt *c*. To test this possibility, we used imidazole, which prevents the formation of the oxoferryl derivative through its binding to heme iron, replacing the axial ligand Met-80 (*30*). Preincubation of ferricytochrome with imidazole did indeed completely inhibit the H_2O_2-induced melatonin oxidation and heme bleaching (Figure 2, insert).

Melatonin transformation to hydroxylated products is initiated by its interaction with strong oxidants and thought to proceed through a resonance-stabilized and relatively long-lived melatoninyl cation radical intermediate formed by the removal of one electron from the indole ring of melatonin (*14*). It has been proposed that indole derivatives are oxidized by horseradish peroxidase/H_2O_2 through addition of molecular oxygen to the indolyl radical producing the peroxide radical (*31*). The latter may cyclize to a dioxetane intermediate by an intramolecular nucleophilic ring closure. The decomposition of this dioxetane leads to a corresponding kynuric product. Melatonin is oxidized by horseradish peroxidase or myeloperoxidase, forming AFMK as a main product (*32*). We developed a tentative pathway presented in Figure 5 that explains the findings of 2-hydroxymelatonin and 2,3-dihydroxymelatonin, both being successive intermediates between melatonin and its final product, AFMK.

Melatonin transformation involves the removal of one electron from indoleamine by cyt $c^{+\bullet}$ Fe^{IV}=O, followed by addition of −OH to the melatoninyl cation radical, with oxidation of the adduct by a catalyst intermediate (cyt *c* Fe^{IV}=O). Cyt *c*−Fe^{III} is then formed, together with the keto tautomer of 2-hydroxymelatonin. The latter interacts with a new molecule of cyt $c^{+\bullet}$ Fe^{IV}=O forming an indolyl-type cation radical, which upon addition of −OH yields 2,3-dihydroxymelatonin. The latter is released into solution from the substrate-binding site of hemoprotein and undergoes oxidation by cyt $c^{+\bullet}$ Fe^{IV}=O or H_2O_2 accompanied by indole ring opening to form AFMK. In addition to the major pathway, the radical intermediates of melatonin and 2-hydroxymelatonin could also react with each other to yield a stable dimer as a byproduct.

Unlike melatonin nonenzymatic hydroxylations, the enzymatic additions of hydroxyl groups seem to be highly specific. In these reactions cytochrome P-450 metabolizes melatonin to 6-hydroxymelatonin, while cyt *c* oxidizes melatonin to 2-hydroxymelatonin and 2,3-dihydroxymelatonin. Likewise, melatonin incubation with a Fenton-type $HO^{\bullet}$ system generates a mixture of products hydroxylated at positions 2, 4, and 6 (*24*). Of these, the 2- and 6-hydroxylated metabolites of melatonin have been detected in vivo but not 4-hydroxymelatonin.

6-Hydroxymelatonin is firmly established as a major metabolite of melatonin at least in liver and kidney (*33*), whereas generation of 2-hydroxymelatonin is thought to be negligible (*12*). Nevertheless, the monooxygenated derivatives of melatonin identified as cyclic 2-hydroxymelatonin and cyclic 3-hydroxymelatonin have been detected in the urine of humans and rats; in the latter species, both oxidative stress (exposure to ionizing radiation) and the administration of melatonin itself increase the urinary cyclic 3-hydroxymelatonin excretion (*34*, *35*).

Of note, the monooxygenated derivative of melatonin originally referred to as cyclic 3-hydroxymelatonin, has recently been positively identified as the actual keto tautomer of 2-hydroxymelatonin (*36*). In fact, neither 3-hydroxymelatonin nor its cyclized forms have been identified as products of $^{\bullet}OH$-mediated oxidation of melatonin in a Fenton-type system (*24*). These observations should clarify apparent discrepancies on the structure of the melatonin monooxygenated product detected in the urine of humans and rats (*35*). Thus, if the monooxygenated product previously identified as cyclic 3-hydroxymelatonin is instead the keto tautomer of 2-hydroxymelatonin, then the cyt *c*-mediated 2-hydroxylation becomes a plausible pathway of melatonin biotransformation in vivo, at least under oxidative stress.

AFMK may also be formed from melatonin oxidation by neutrophil myeloperoxidase (*16*). In the brain where opening of the indole ring and formation of kinurenines are catalyzed by indoleamine 2,3-dioxygenase, both AFMK and its secondary product AMK are major metabolites of melatonin (*15*). Nevertheless, given the low basal level of activation of blood neutrophils and activity of indoleamine 2,3-dioxygenase (*37*), AFMK may be generated mostly as a product of cyt *c*-mediated transformation, a reaction enhanced under oxidative stress.

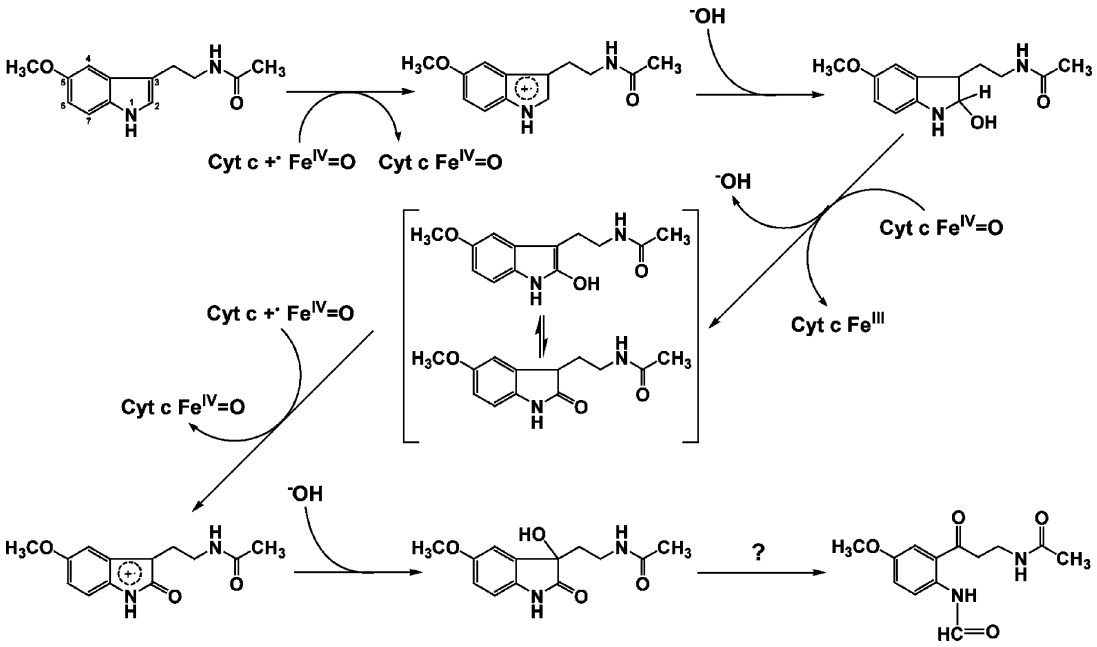

FIGURE 5: Proposed peroxidase-type pathway of melatonin metabolism to AFMK.

To confirm that melatonin is metabolized to AFMK in vivo, we measured endogenous melatonin and its metabolites in intact mitochondria. Endogenous melatonin and AFMK were found in intact heart mitochondria (Figure 3 in Supporting Information) as well as in mitochondria from liver and brainstem (data not shown). The LC-MS assay was not sensitive enough to measure endogenous hydroxylated derivatives of melatonin in intact mitochondria, but a peak corresponding to 2-hydroxymelatonin was easily detectable in heart mitochondria treated with *t*-BuOOH and exogenous melatonin (Figure 4 in Supporting Information). Moreover, we detected endogenous AFMK and 2-hydroxymelatonin in the pineal gland (Figure 5 in Supporting Information). *t*-BuOOH-induced formation of 2-hydroxymelatonin and AFMK was severalfold lower in cyt *c*-depleted mitoplasts than in heart mitochondria (Figure 4 in Supporting Information). Thus cyt *c* appears to be responsible, at least partly, for melatonin oxidation in mitochondria treated with *t*-BuOOH.

In most aerobic mammalian cells the leakage of electrons to molecular oxygen (O_2) occurring in mitochondria is the main source of ROS. The resulting superoxide is then rapidly dismutated to H_2O_2 either spontaneously or enzymatically (by mitochondrial superoxide dismutase) (*38*). H_2O_2 may in addition originate from local sources not linked to respiration, such as the oxidative deamination of biogenic amines by mitochondrial outer membrane monoamine oxidase (*39*). The mitochondrial H_2O_2 concentration is, however, kept at relatively low levels by the action of glutathione peroxidase, although some H_2O_2 molecules may interact directly with ferricytochrome *c* to form an oxyferryl derivative.

Also of interest with regard to melatonin oxidation by cyt *c* is the topology of ROS formation and that of its neutralizing enzymes. SOD, for example, is compartmentalized to sites complementary to the sources of superoxide. Thus, superoxide from complex **I** and complex **III** is released directly into the matrix where manganese−superoxide dismutase promotes conversion to H_2O_2; superoxide is also released by complex **III** into the intermembranous space, location of the intermembranous Cu,Zn-SOD in liver mitochondria (*40*). In the mitochondrial matrix glutathione peroxidase is thought to be the main H_2O_2 detoxification enzyme (*38*), whereas conventional H_2O_2 detoxification systems have not been described in the intermembranous space. Catalase has been detected in the matrix of cardiac mitochondria, but at extremely low activity levels, making it unlikely as a significant contributor to H_2O_2 removal (*41*). Therefore, melatonin oxidation by cyt *c* could provide a viable alternative for the elimination of H_2O_2 when the supply becomes excessive.

Cyt *c* exerts strong inhibition of H_2O_2 production in rat heart mitochondria via reverse electron transfer from succinate to NAD^+ (*42*). Mitochondrial H_2O_2 can also be scavenged by the cyt *c*-mediated "alternative electron-leakage pathway" (*43*). The latter mechanism that requires ferrocytochrome could remain active under physiological conditions, but in pathological situations when electron transport is disrupted, the melatonin-mediated pathway could become dominant. Thus if there is a block in electron transfer, ferricytochrome cannot be reduced, and cyt *c* would lose its capability to suppress H_2O_2 generation (*43*).

Melatonin's general availability and capability to regenerate ferricytochrome from oxoferryl cytochrome suggest that donation of its electron for H_2O_2 detoxification could be widespread. Moreover, unlike the progressive ineffectiveness of the alternative electron-leakage pathway, the melatonin-dependent mechanism would remain effective even if cyt *c* were to leave the respiratory chain. Thus, melatonin interactions with oxoferryl cyt *c* could play a significant role in the elimination of H_2O_2 in vivo, and melatonin metabolites themselves could further contribute to neutralization of ROS (*44*). In fact, AFMK with its low electrochemical potential would be particularly useful for that purpose, being capable of donating two electrons per molecule (*44*).

The pathophysiological role for oxoferryl cyt *c* remains to be defined, although several reports suggest its possible involvement in neurodegenerative disorders. Thus, in the presence of H_2O_2 cyt *c* is known to induce aggregation of

α-synuclein, a major component of Lewy bodies in Parkinson's disease (*45*). There is growing evidence that oxidative stress and inhibition of mitochondrial electron transport may underlie the pathogenesis of Alzheimer and Parkinson diseases and epilepsy (*46*). Accumulation of oxoferryl heme could impair the cyt *c*-mediated electron shuttle between complex **III** and complex **IV** occurring during mitochondrial respiration. It has been proposed that loss of the methionine ligand would result in failure of cyt *c* electron transport capability (*47*). Indeed, methionine oxidation, by HOCl, greatly reduces cyt *c*'s capability to accept electrons from ascorbate or from the cytochrome c_1 of complex **III** (*48*). Interaction of melatonin with oxoferryl hemoprotein could restore the normal redox cycle of cyt *c*, thereby allowing preservation of mitochondrial energy homeostasis whether in physiologic or pathological conditions. Indeed, melatonin administration would help to prevent development of neurodegenerative defects in experimental animals and, perhaps, in human subjects (*49*). In the rat exogenous melatonin does stimulate significantly the activity of complexes **I** and **IV**, leading to the suggestion that the indoleamine would donate electrons to these complexes, improving the function of the ETC (*10*).

To conclude, we report a dual reciprocal interaction between mitochondrial cyt *c* and the indoleamine melatonin. Thus, our findings provide direct evidence for a novel pathway in mitochondrial melatonin metabolism, namely, oxidation by cyt *c*. Thus, melatonin may have a new function added to its considerable repertoire of biological activities, a role as an intramitochondrial sensor of local redox status.

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SUPPORTING INFORMATION AVAILABLE

Figures 1−5 showing LC-MS analysis of (1) the reaction between melatonin, cyt *c*, and H_2O_2, (2) products of 2-hydroxymelatonin oxidation by cyt *c*, (3) endogenous melatonin and its metabolites in heart mitochondria, (4) the metabolism of exogenous melatonin in heart mitochondria and mitoplasts treated by *t*-BuOOH, and (5) endogenous melatonin and its metabolites in the pineal gland. This material is available free of charge via the Internet at http://pubs.acs.org.

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