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Oxidation of Melatonin by Oxoferryl Hemoglobin: A Mechanistic Study

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Reaction of melatonin with the hypervalent iron centre of oxoferryl hemoglobin, produced in aqueous solution from methemoglobin and H_2O_2, has been investigated at 37°C and pH 7.4, by absorption spectroscopy. The reaction results in reduction of the oxoferryl moiety with formation of a heme-ferric containing hemoprotein. Stopped-flow spectrophotometric measurements provide evidence that the reduction of oxoferryl-Hb by melatonin is first-order in oxoferryl-Hb and first-order in melatonin. The bimolecular reaction constant at pH 7.4 and 37°C is $112 \pm 1.0\,M^{-1}\,s^{-1}$.

Two major oxidation products from melatonin have been found by gas chromatography–mass spectroscopy: the cyclic compound 1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-b]indole (cyclic 3-hydroxy-melatonin), and *N*-acetyl-*N'*-formyl 5-methoxykynuramine (AFMK). The percentage yield of the two major products appears dependent on the ratio [oxoferryl-Hb]:[melatonin]—the higher the ratio the higher the yield of AFMK. The observed stoichiometry oxoferryl-$Hb_{reduced}$:$melatonin_{consumed}$ is 2, when the ratio [oxoferryl-Hb]:[melatonin] is 1:1, but appears >2 at higher molar ratios. The reduction of the hypervalent iron of the oxoferryl moiety may be consistent with an oxidation of melatonin by two one-electron steps.

Keywords: Gas chromatography–mass spectroscopy; Hemoglobin; Melatonin; Oxoferryl-Hb; Melatonin oxidation; ^{1}H-NMR; AFMK; Cyclic 3-hydroxymelatonin

Abbreviations: AFMK, *N*-acetyl-*N'*-formyl-5-methoxykynuramine; CI, chemical ionisation; EI, electronic ionisation; GC/MS, gas chromatography–mass spectrometry; Hb, hemoglobin; met-Hb, methemoglobin; PBS, phosphate-buffered saline; TLC, thin layer chromatography

INTRODUCTION

Similarly to indole[1–4] and tryptophan[5,6] derivatives, melatonin (*N*-acetyl-5-methoxytryptamine) has redox properties. Its reduction potential ($E^0 = 0.95 \pm 0.02$V),[7] allows the

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molecule to react with and neutralise a number of free radicals, including hydroxyl-,[8] alkoxyl-,[9] peroxyl-,[10] and to a lesser extent lipoperoxyl-[11,12] radicals. Literature data from experiments in pure chemical solution, provide evidence that nonenzymatic oxidation of melatonin can occur through more than one pathway.[8,13–16] Strong oxidants favour the release of one electron from melatonin, with formation of an indolyl-type cation radical.[7,14,16–19] Direct reaction of the cation radical with O_2 to produce *N*-acetyl *N'*-formyl-5-methoxykynuramine (AFMK) may account for the quenching of the melatoninyl radical to an inactive product.[17,19] However, the radical from melatonin can undergo other pathways, resulting in various metabolites.[14,16] Finally, it has been shown that $^{\bullet}OH$ radicals,[13] peroxynitrite,[14,16] and hypochlorous acid[20] can hydroxylate the indolamine.

The oxoferryl moieties from heme-proteins are strong oxidants[21,22] We have recently observed that melatonin can reduce the oxoferryl species formed after oxidation of methemoglobin with H_2O_2, which eventually prevents the oxidative denaturation of the protein and results in formation of methemoglobin.[23] Here, we report kinetic studies of the reaction of melatonin with the oxoferryl iron centre [Fe^{IV}=O] from hemoglobin, and identify the products from the indoleamine.

MATERIAL AND METHODS

Materials

Melatonin, 6-hydroxymelatonin, bovine heart methemoglobin, catalase, hydrogen peroxide, were from Sigma (St. Louis, MO). Alugram® Sil G/UV_{254} sheets for thin layer chromatography (TLC) were from Macherey–Nagel, Düren, Germany. All other chemicals were of research highest purity grade. Ion-free water and buffers used throughout this study were filtered through Chelex-100 (Sigma), and suitable plastic labware was used to avoid the effect of adventitious metals.

Optical Spectroscopy and Rapid Kinetic Measurements

Oxoferryl hemoglobin (oxoferryl-Hb) was prepared under spectral control using a Beckman DU 640 UV/vis spectrometer, equipped with a temperature controller. Bovine methemoglobin (met-Hb) in PBS was oxidised by a 10-fold excess H_2O_2 for 1 min, at 37°C, followed by addition of 250 IU catalase to remove excess H_2O_2.[24] Spectrophotometric scans (400–700 nm) were carried out, and concentration of met-Hb was calculated at 630 nm ($\varepsilon_{630} = 3.63\,mM\,cm^{-1}$ per heme group[25]), while the formation/disappearance of oxoferryl-Hb was monitored by recording the absorbance changes at 556 nm, the point at which the two forms differ most ($\Delta\varepsilon = 3.6\,mM\,cm^{-1}$).[26] Under all conditions used through the study, the concentration of oxoferryl-Hb measured 5 min after the addition of catalase, was one half of the starting met-Hb.

Kinetics of the oxoferryl reduction were evaluated by stopped-flow spectrophotometric measurements, with a temperature-controlled apparatus (High Tech Stopped Flow SF61, dead time 1 ms). Measurements were carried out by adding melatonin to the oxoferryl-Hb solution, 5 min after the addition of catalase. The reaction was monitored at 556 nm. To ensure first-order kinetics, the final concentrations of melatonin were 3–10 times that of oxoferryl-Hb. Determination of k_{obs}, the pseudo-first-order rate constant, were performed in triplicate for each substrate concentration. Second-order rate constants were calculated from the slope of the plot of the mean k_{obs} values versus concentration of substrate. Pseudo-first-order and the second-order rate constants for the reaction melatonin/oxoferryl-Hb were also determined by measuring melatonin consumption under pseudo-first-order conditions, and by plotting k_{obs} versus the oxoferryl-Hb concentrations.

Consumption of melatonin was determined by extracting 100 µl of the reaction mixture in a total water volume of 300 µl, with 2 ml of ethyl acetate.

After vortexing, the organic phase was gathered and 50 μl of the organic extract were analysed by a normal phase Supelco Nucleosil-100 HPLC column, eluted with ethyl acetate.[27] Fluorometric detection was with excitation at 285 nm and emission at 345 nm. Quantitation was by reference to a curve constructed with 0.5–5 nmol of melatonin.

GC-MS and ^{1}H-NMR Analysis

GC-MS analysis was carried out by a Varian 3400 analyser (column DB5-MS, 30 m; 0.25 mm ID; 0.25 μm), interfaced with a Saturn 3 (Varian) ion trap detector (electron beam energy 100 eV; emission current 20 mA; trap temperature 180°C). Thermal chromatographic program was as follows: initial temperature 120°C (1 min hold), first ramp 10°C min^{-1} to 210°C (30 min hold), second ramp 15°C min^{-1} to 280°C (5 min hold). Carrier gas (He) pressure was fixed at 20 psi on the head of column (injector temperature 290°C; transfer line temperature 180°C). Mass spectra of positive ions were obtained by both electronic ionisation (EI), and chemical ionisation (CI) using methane as reagent gas. Full scan spectra were acquired from m/z 40–350 at 1 scan s^{-1}.

Melatonin metabolites were separated by preparative TLC on silica gel with a fluorescent indicator. The mobile phase was ethyl acetate/methanol (90:10 v/v). Spectrophotometric analysis of the isolated compounds was carried out, and the ^{1}H-NMR spectra were recorded on a Bruker AC-250F (250 MHz) spectrometer in $CDCl_3$ solution, using tetramethylsilane as internal standard.

RESULTS

Reduction of Oxoferryl-Hb by Melatonin

The spectrum of met-Hb exhibits a characteristic absorption peak at 630 nm (Fig. 1A, dashed line). When 100 μM met-Hb is oxidised by a 10-fold excess H_2O_2, followed by addition of catalase, the marked spectral variations monitored in the 520–650 nm region after 5 min (Fig. 1A, trace a), may be ascribed to the formation of the oxoferryl

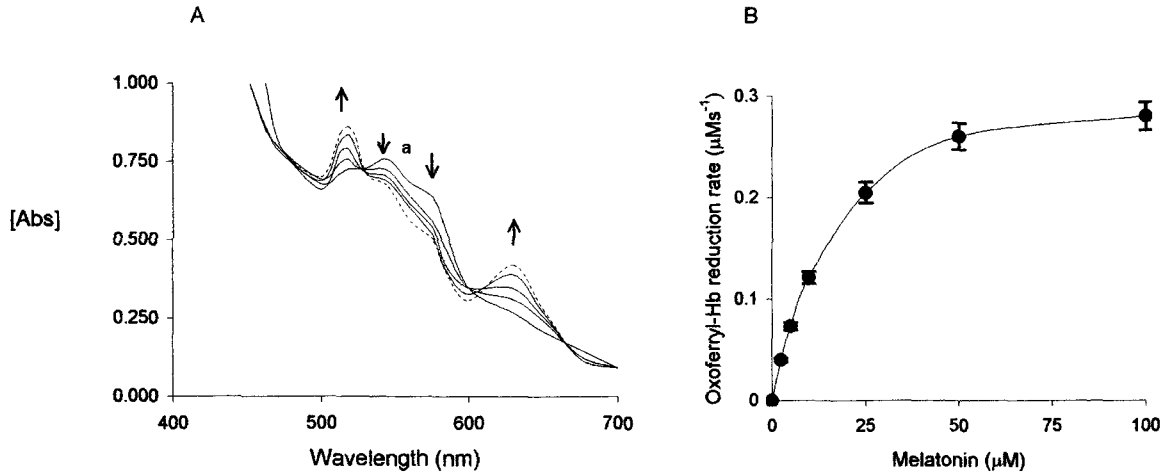

FIGURE 1 Reduction of oxoferryl-Hb by melatonin, at 37°C and pH 7.4. A. Met-Hb (100 μM, dashed line) was treated with a 10-fold excess of H_2O_2, followed by addition of catalase, and 50 μM oxoferryl-Hb (solid line, trace a) were generated after 5 min. Melatonin at 100 μM was added and repetitive scans were taken at 1 min intervals. The downward and upward arrows indicate decrease or increase in absorbance in the trace a spectrum. B. Dependence of the rate of oxoferryl-Hb reduction by melatonin concentrations. The rate of the oxoferryl reduction is calculated from the spectral variations monitored at 556 nm after 1 min from the addition of melatonin, and is corrected for the rate of spontaneous reduction. The points are the means ±SD of five separate experiments.

iron centre. The latter spontaneously reverts to its ferric state, according to a slow autoreduction process involving the oxidation of the protein moiety. Under our conditions the rate of the spontaneous reduction was $0.057\,\mu M\,s^{-1}$. Supplementation of an oxoferryl-Hb solution with melatonin results in a rapid reduction of the hypervalent hemoglobin to the ferric-heme hemoprotein (Fig. 1A), at a rate which increases with the melatonin concentrations 2.5–100 μM (Fig. 1B).

Stopped-flow spectrophotometric measurements at 556 nm were carried out to monitor the reduction of oxoferryl-Hb by melatonin. The kinetic traces displayed a single exponential character, and were fitted according to the single exponential equation ($Y = A_1 e^{-kt}$). A typical trace for the reduction of oxoferryl-Hb is shown in the inset of Fig. 2. The observed rate constants, k_{obs}, from the slopes of these traces increase linearly ($r^2 = 0.997$) with melatonin concentrations (Fig. 2), indicating that the reaction is also first-order in melatonin. The second-order constant calculated from the slope was $112 \pm 1.0\,M^{-1}s^{-1}$.

The time course of the melatonin consumption during the reaction with oxoferryl-Hb, under

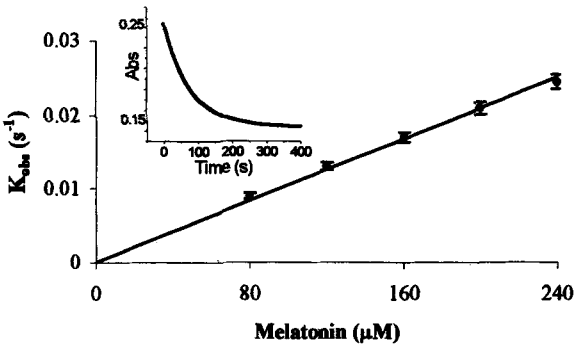

FIGURE 2 Secondary plot of the pseudo-first-order rate constants for the reduction of oxoferryl-Hb by melatonin (k_{obs}) versus the concentrations of melatonin. Each point is the mean ±SD of three separate experiments The inset shows a typical stopped-flow kinetic trace of the reduction of oxoferryl-Hb at 37°C, pH 7.4. The reaction was followed at 556 nm. After the mixing, but before the reaction, the mixture contained 25 μM oxoferryl-Hb and 160 μM melatonin.

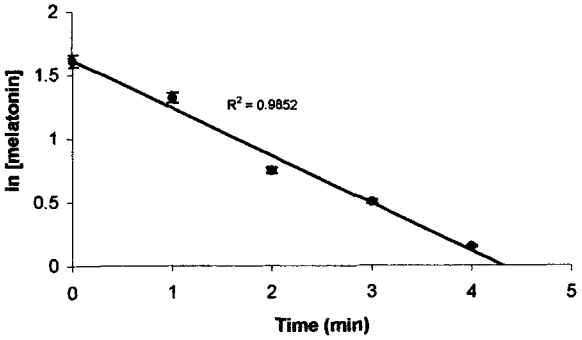

FIGURE 3 Semilogarithmic plot of consumption of 5 μM melatonin during the reaction with 50 μM oxoferryl-Hb, at 37°C, pH 7.4. Each point is the mean ±SD of three separate experiments.

pseudo-first-order conditions, confirmed that melatonin oxidation proceeds through first-order kinetics (Fig. 3). The observed rate constant k_{obs} from the slope of the curve ln[melatonin] versus time was $0.0058\,s^{-1}$. The second-order rate constant was calculated as $116 \pm 3.5\,M^{-1}s^{-1}$, in a good agreement with that calculated by the experiments described above.

Oxidation Products

The products from melatonin, after a 5 min incubation with a 25-fold molar excess of oxoferryl-Hb, were investigated. Under these conditions, a total consumption of melatonin was observed after organic extraction of the sample and HPLC analysis. Extraction of the sample and GC/MS analysis revealed the formation of two major products (metabolites 2 and 3, Fig. 4a).

The EI mass spectrum of the metabolite 2 (Fig. 4b) is quite comparable with that of an oxidation product previously identified as the cyclic 3-hydroxymelatonin (1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-b]indole, according to the IUPAC nomenclature).[13] Analysis by CI(CH_4) mass spectrometry produced a pattern with fragments at m/z 231 and 190, indicating loss of water and acetamide, respectively, from the protonated molecule, in addition to the $[M+H]^+$ ion at m/z 249. The

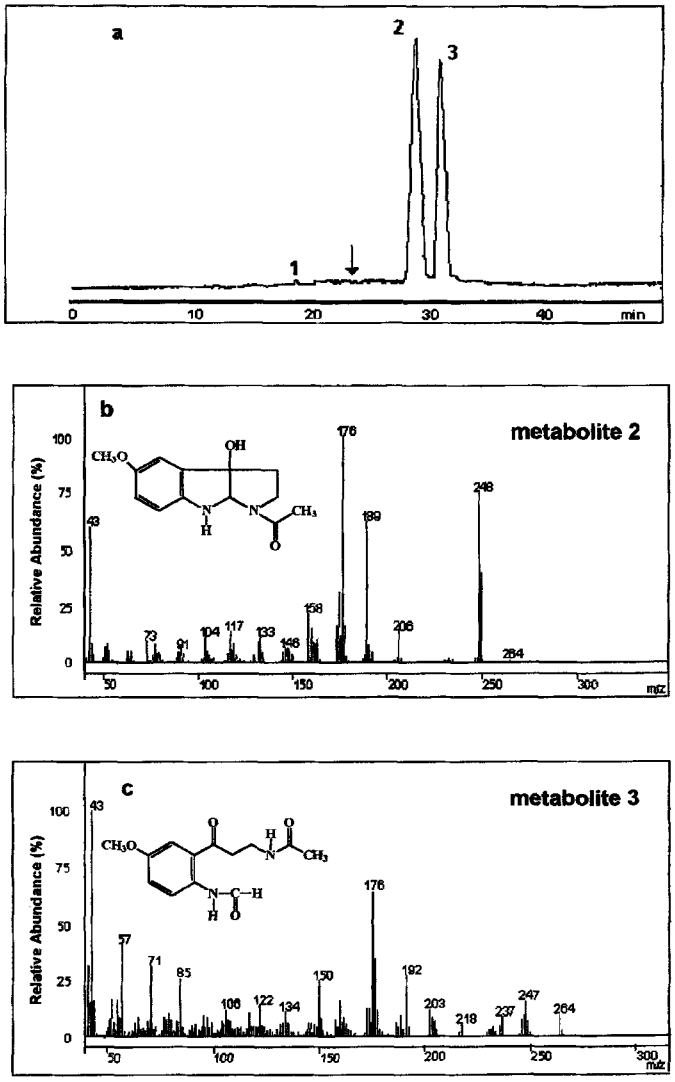

FIGURE 4 GC/MS chromatogram (a), and EI fragmentation patterns (b, c) of metabolites from melatonin after reaction with oxoferryl-Hb. The arrow indicates the elution time of melatonin. Incubation conditions, and GC/MS analysis were as reported in the Methods.

metabolite 3 (Fig. 4c) was identified as *N*-acetyl-*N'*-formyl-5-methoxykynuramine. The EI mass spectrum shows a molecular ion at m/z 264, 32 mass units in excess of the melatonin mass, which suggests the incorporation of two oxygen atoms. The low intensities of the high-mass ion fragments strongly indicate the opening of the indole ring. The fragments at m/z 192, 150, and 43 arise from a direct cleavage of the molecular ion, whereas the ion at m/z 176 possibly results from a rearrangement process of the m/z 247 $[M\text{-}OH]^+$ ion.

The structure of AFMK was also in accordance with the presence in the CI(CH_4) mass spectrum of ions at m/z 114 and m/z 178 (Fig. 5), which may result from protonation at different sites. Protonation of the phenyl ring may produce the m/z 114 ion, while protonation of the acetamide nitrogen, followed by acetamide loss (m/z 206) and ethylene removal gives the ion at m/z

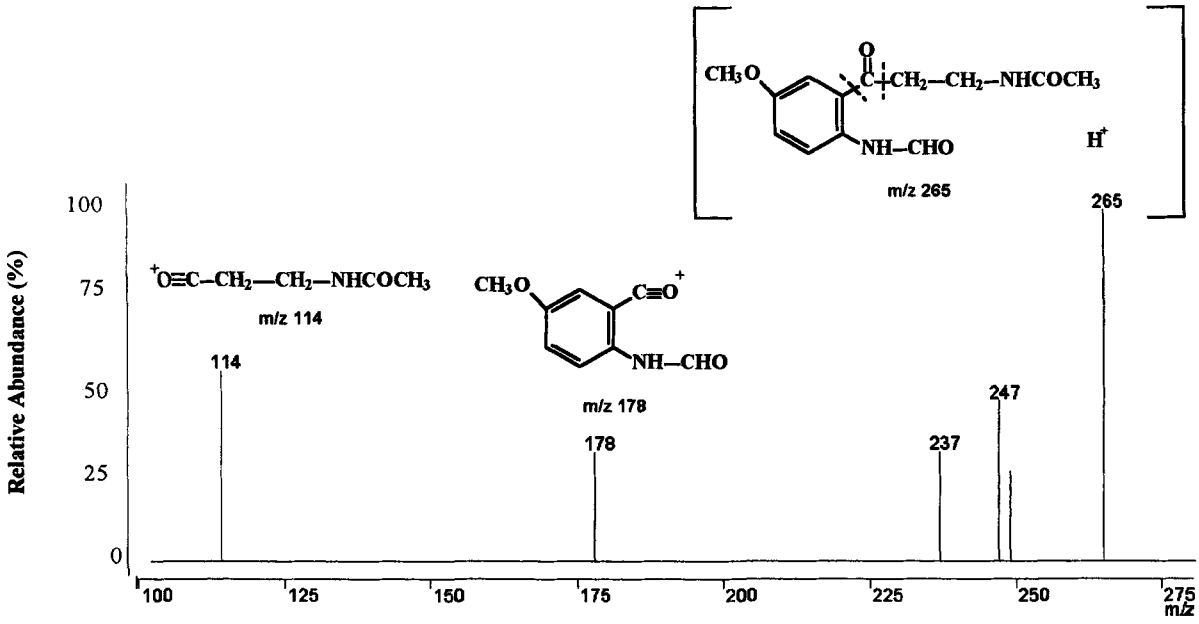

FIGURE 5 CI mass spectrum of AFMK.

178. Finally, the ^{1}H-NMR analysis agrees with the *N*-acetyl *N'*-formyl-5-methoxykynuramine structure.*

Traces of a third compound (metabolite 1, Fig. 4a) were also present in the GS/MS chromatogram. The EI mass spectra of the metabolite 1 show a molecular ion at m/z 248, which suggests the incorporation of an oxygen atom into the melatonin structure. Comparison with the retention time and fragmentation pattern of the commercially available compound, rules out that this metabolite is 6-hydroxymelatonin. Although the retention time relative to melatonin is comparable to that reported for 2-hydroxy melatonin,[14] our data do not allow to unequivocally identify this metabolite.

The absorption spectra of the two major melatonin metabolites are shown in Fig. 6. The absorption maxima are 305 nm, and 355 nm, for the cyclic 3-hydroxymelatonin and AFMK, respectively.

Stoichiometry of the Reaction and Yield of Products

Samples containing 50 μM oxoferryl-Hb and 1–50 μM melatonin were incubated for 2 min to monitor the consumption of melatonin and reduction of the oxoferryl heme. The experimental stoichiometric factor oxoferryl-$Hb_{reduced}$: $melatonin_{consumed}$ was about 2, in the range of the melatonin concentrations 5–50 μM. However, factors>2 were measured for concentrations of melatonin below 5 μM (Table I).

The melatonin consumption and percentage yield of the two major products vary with the oxoferryl-Hb:melatonin molar ratio. At a molar ratio of 1.0, the consumption of the indoleamine is 25–30% of the initial amount, and the cyclic 3-hydroxymelatonin represents the main product (Table I). The formation of a substantial amount of AFMK appears related to the availability of a large oxidant excess (Table I).

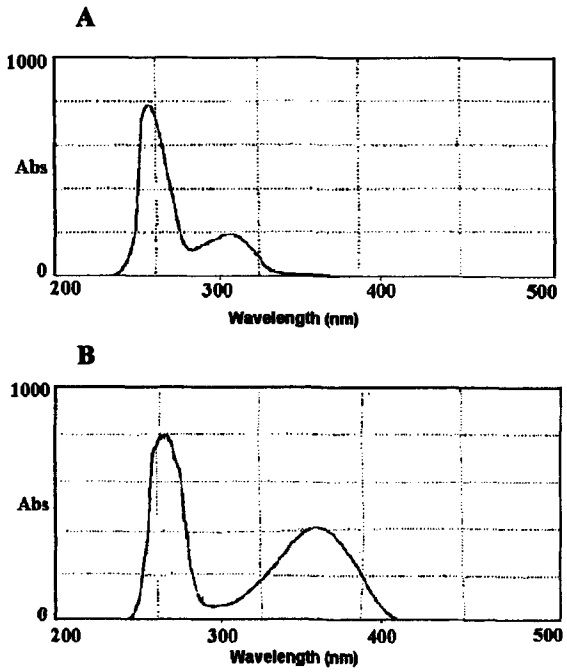

FIGURE 6 Absorption spectra in ethyl acetate of the two major metabolites after reaction with oxoferryl-Hb. (A) Cyclic 3-hydroxymelatonin (1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-b]indole). (B) *N*-acetyl *N'*-formyl 5-methoxykynuramine. The products were isolated by TLC chromatography and identified by GC/MS as reported in the Methods.

DISCUSSION

Since the discovery that melatonin could scavenge hydroxyl radicals,[8] numerous experimental models have been used to evaluate the interaction of the indoleamine with a variety of radical species or strong oxidants.[7,9–12,14,16,18] We have performed a kinetic analysis by stopped-flow absorption spectroscopy to study the reaction of melatonin with oxoferryl-Hb, a hypervalent iron species formed after reaction of met-Hb with H_2O_2. The reaction products from melatonin have also been investigated.

Reaction of methemoglobin with H_2O_2 leads to the so-called perferryl-Hb, two-equivalents oxidation above the ferric form, which comprises a transient radical localised on the globin, possibly an aromatic amino acid radical,[28,29] and a $Fe^{IV} = O$ oxoferryl-heme group[28,30,31] in which the oxygen maintains "its singlet state atomic character".[32,33] Once all peroxide is consumed, the radical spontaneously decays to the oxoferryl form, one equivalent oxidation above the native methemoglobin, with concomitant oxidation of the protein moiety. Melatonin is a two-electrons reducing molecule. Previous work from this laboratory has provided evidence that, under an experimental condition similar to that of the present study, i.e. 37°C and pH 7.4, melatonin can reduce both the perferryl radical from the H_2O_2-activated hemoglobin, and the oxoferryl moiety.[23] However, only the kinetic constant for the reduction of oxoferryl-Hb can be reliably measured. The measurement of the one-electron reduction kinetics of perferryl-Hb cannot be done because perferryl-Hb and oxoferryl-Hb are indistinguishable spectrophotometrically. On the other hand, the stopped-flow spectrophotometry

TABLE I Melatonin oxidation products from the reaction with oxoferryl-Hb, under different conditions

Oxoferryl-Hb:melatonin* (μM)	Melatonin consumed† (μM)	Stoichiometric ratio oxoferryl-$Hb_{reduced}$:$melatonin_{consumed}$	Products (percentage yields)‡	
			Cyclic 3-OH melatonin	AFMK
50:50	12.5 ± 1 (3)	2.1 ± 0.5 (3)	93 ± 1 (3)	6.8 ± 2 (3)
50:2	2 (3)	3.0 ± 0.6 (3)	58 ± 2.5 (3)	41 ± 2 (3)

* Oxoferryl-Hb was prepared from 100 μM met-Hb and H_2O_2 as reported in Methods, and the reaction melatonin/oxoferryl-Hb was carried out for 2 min at the indicated concentrations.
† Consumption of melatonin was evaluated by extraction of the reaction mixture and HPLC analysis; reduction of oxoferryl-Hb was evaluated spectrophotometrically.
‡ Suitable amounts of the reaction mixture were gathered, extracted and analyzed by GC/MS. The integrated peak area of each metabolite is expressed as percent of the peak areas.
The values are the mean ± SD of (n) seperate experiments.

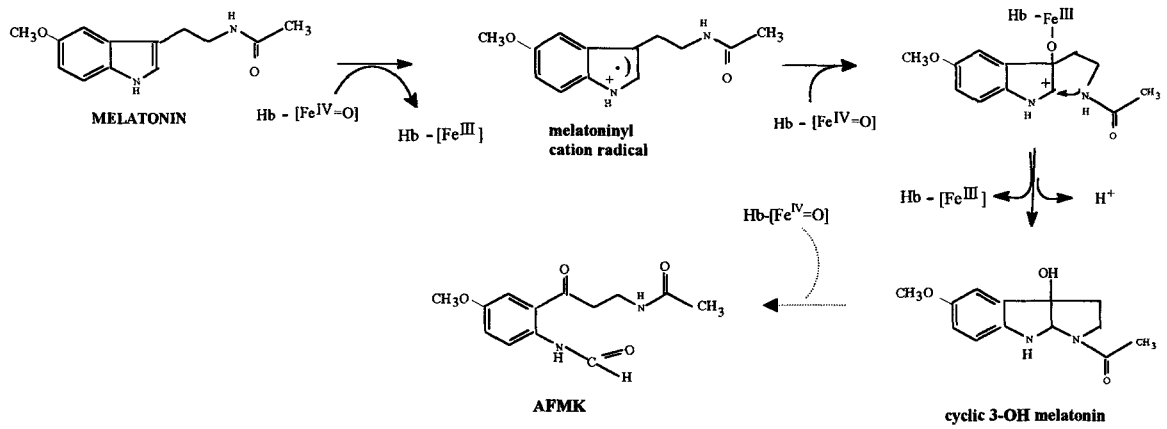

FIGURE 7 Proposed oxidation of melatonin by oxoferryl-Hb.

was not helpful to reveal the intermediate melatoninyl radical, the one-electron melatonin oxidation product, because of the strong interference, both in the UV and in the visible region, of the hemoglobin spectrum over that of the melatoninyl radical.[7,9] Experiments carried out by varying the standard conditions of the assay in order to minimise the interference were unsuccessful.

The bimolecular rate constant we have measured for the reduction of oxoferryl-Hb by melatonin is $112 \pm 1.0\,M^{-1}s^{-1}$. This value is similar to that reported for uric acid ($153\,M^{-1}s^{-1}$) and quite higher than that measured for ascorbic acid ($15\,M^{-1}s^{-1}$).[34] The experimental reduction potential E° for the Fe^{3+}/Fe^{4+}=O couple has been calculated 0.99 V.[35] Perferryl-Hb is a much stronger oxidant than the oxoferryl-Hb with an experimental redox potential of 1.4 V.[36] It is reasonable to hypothesise that electron transfer reactions may occur more easily between melatonin and perferryl-Hb.

On oxidation with oxoferryl-Hb, two major products from melatonin have been identified, the cyclic 3-hydroxymelatonin (1,2,3,3a,8,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-b]indole), and *N*-acetyl-*N'*-formyl 5-methoxykynuramine. In addition, traces of another monoxygenated metabolite, the structure of which has not been further investigated were found. Comparative analysis with the commercial compound rules out that it is 6-hydroxymelatonin.

Melatonin is metabolised to 6-hydroxymelatonin by a liver enzyme.[37] Other oxidation pathways, which lead to the cyclic 3-hydroxymelatonin, have been described. The cyclic 3-hydroxymelatonin has been found after oxidation by hydroxyl radical[13] or peroxynitrite.[16] Our results would further suggest that this compound is a major product of non-enzymatic oxidation of melatonin.

The formation of AFMK under the conditions of our assay appears peculiar. Production of this dioxygenated metabolite has been related to the reaction of superoxide anion with melatonin, either through an iron-porphyrin-mediated reaction during the catalytic mechanism of the brain indoleamine 2,3 dioxygenase,[38] or nonenzymatically, in a system generating this radical species by means of xanthine oxidase, in which hemin was an efficient catalyst.[39] AFMK can also be formed by treating melatonin with a very large excess of H_2O_2.[40] In contrast to the reported reactions, however, neither superoxide anions,[25,41] nor H_2O_2 are present in our system. In our hands the cyclic 3-hydroxymelatonin is the major metabolite, while very low amounts of AFMK are found when the oxidant oxoferryl-Hb is in a 1:1 molar ratio with melatonin. Large excess of oxoferryl-Hb generate the cyclic

3-hydroxymelatonin with substantial amounts of AFMK. These findings, and the observation that the stoichiometry oxoferryl-$Hb_{reduced}$: $melatonin_{consumed}$ raises up with the increasing of the oxoferryl-Hb:melatonin molar ratio, could imply that the cyclic product 3-hydroxymelatonin is oxidised by oxoferryl-Hb, thus forming AFMK. The reducing activity of the cyclic 3-hydroxymelatonin in this system requires further studies.

The formation of hydroxy derivatives of the indoleamine and of AFMK, after reaction of melatonin with strong oxidants, has been shown to proceed through a transient melatoninyl radical.[14,16,17] Such a species can be involved in the reaction of melatonin with oxoferryl-Hb. Taking into account the experimental evidence, a pathway for the oxidation of melatonin can be described. One-electron transfer reaction of melatonin with oxoferryl-Hb would generate met-Hb and a melatoninyl cation radical (Fig. 7). The formation of the cyclic 3-hydroxymelatonin can be consistent with a radical addition of the heme oxygen of oxoferryl-Hb to the C3 position of the melatoninyl cation radical with reduction of the oxoferryl moiety, followed by intramolecular nucleofilic attack by amidic nitrogen. This allows release of met-Hb and of the cyclic 3-hydroxymelatonin. Dehydration of the cyclic 3-hydroxymelatonin and attack by excess oxoferryl-Hb may start a sequence in which an indolyl-type cation radical reduces the oxoferryl moiety, releasing met-Hb. Rearrangement of the structure after nucleophilic attack by a water molecule would lead to AFMK.

The activity of heme-proteins such as myoglobin and hemoglobin versus hydrogen peroxide has been reported to as a pseudoperoxidase activity, in the mechanism of which the transient protein radical is considered as a compound I-like intermediate, and the oxoferryl form is comparable to the compound II intermediate, of human and animal peroxidases.[42] The presently observed reduction of oxoferryl hemoglobin may be considered to investigate the activity of melatonin with other oxoferryl moieties, such as those concerned with the activity of the catalytic heme-proteins involved in human pathology.[22] Investigation with myeloperoxidase is currently in progress.

Acknowledgements

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References

[1] Jovanovic, S.V. and Steenken, S. (1992) "Substituent effect on the spectral, acid base and redox properties of indolyl radicals: a pulse radiolysis study", *Journal of Physical Chemistry* **96**, 6674–6679.

[2] Al-Kazwini, A.T., O'Neill, P., Adams, G.E. and Cundall, R.B. (1990) "One-electron oxidation of methoxylated and hydroxylated indoles by azide 1. Characterization of primary indolic radicals", *Journal of Physical Chemistry* **94**, 6666–6670.

[3] Solar, S., Getoff, N., Surdhar, P.S. and Armstrong, D. (1991) "Oxidation of tryptophan and *N*-methylindole by N_3, Br_2^-, $(SNC)_2^-$ radicals in light and heavy water solutions. A pulse radiolysis study", *Journal of Physical Chemistry* **95**, 3639–3643.

[4] Shen, X., Lind, J. and Merenyi, G. (1987) "One-electron oxidation of indoles and acid base properties of the indolyl radicals", *Journal of Phyical. Chemistry* **91**, 4403–4406.

[5] Land, E.J. and Prütz, W.A. (1969) "Fast one-electron oxidation of tryptophan by azide radicals", *International Journal of Radiation Biology* **32**, 203–207.

[6] Redpath, J.L., Santus, R., Ovadia, J. and Grossweiner, L.I. (1975) "The oxidation of tryptophan by radical anions", *International Journal of Radiation Biology* **27**, 201–204.

[7] Mahal, H.S., Sharma, H.S. and Mukherjee, T. (1999) "Antioxidant properties of melatonin: a pulse radiolysis study", *Free Radical Biology and Medicine* **26**, 557–565.

[8] Tan, D.-X., Chen, L.D., Poeggeler, B., Manchester, L.D. and Reiter, R.J. (1993) "Melatonin: a potent, endogenous hydroxyl radical scavenger", *Endocrine Journal* **1**, 57–60.

[9] Scaiano, J.C. (1995) "Exploratory laser flash photolysis study of free radical reactions and magnetic field effects in melatonin chemistry", *Journal of Pineal Research* **19**, 189–195.

[10] Pieri, C., Marra, M., Moroni, F., Recchioni, R. and Marcheselli, F. (1994) "Melatonin: a peroxyl radical scavenger more effective than vitamin E", *Life Sciences* **55**, 271–276.

[11] Livrea, M.A., Tesoriere, L., D'Arpa, D. and Morreale, M. (1997) "Reaction of melatonin with lipoperoxyl radicals

in phospholipid bilayers", *Free Radical Biology and Medicine* **23**, 706–711.
[12] Marshall, K.-A., Reiter, R.J., Poeggeler, B., Aruoma, O.I. and Halliwell, B. (1996) "Evaluation of the antioxidant activity of melatonin *in vitro*", *Free Radical Biology and Medicine* **21**, 307–315.
[13] Tan, D.-X., Manchester, L.C., Reiter, R.J., Plummer, B.F., Hardies, L.J., Weintraub, S.T., Vijayalaxmi, S.T. and Shepherd, A.M.M. (1998) "A novel melatonin metabolite, cyclic 3-hydroxymelatonin: a biomarker of *in vivo* hydroxyl radical generation", *Biochemical and Biophysical Research Communications* **253**, 614–620.
[14] Zhang, H., Squadrito, G.L. and Pryor, W.A. (1998) "The reaction of melatonin with peroxinitrite: formation of melatonin radical cation and absence of stable nitrated products", *Biochemical and Biophysical Research Communications* **251**, 83–87.
[15] Roberts, J.E., Hu, J.E. and Wishart, J.F. (1998) "Pulse radiolysis study of melatonin and chloromelatonin", *Journal of Photochemistry and Photobiology B—Biology* **42**, 125–132.
[16] Zhang, H., Squadrito, G.L., Uppu, R. and Pryor, W.A. (1999) "Reaction of peroxyxynitrite with melatonin: a mechanistic study", *Chemical Research in Toxicology* **12**, 526–534.
[17] Poeggeler, B., Saarela, S., Reiter, R.J., Tan, D.-X., Chen, L.-D., Manchester, L.C. and Barlow-Walden, L.R. (1994) "Melatonin. A highly potent endogenous scavenger and electron donor: new aspects of the oxidation chemistry of this indole accessed in vitro", *Annals of the New York Academy of Sciences* **738**, 419–420.
[18] Stasica, P.P., Ulanski, P. and Rosiak, J.M. (1998) "Reactions of melatonin with radicals in deoxygenated aqueous solution", *Journal of Radioanalytical and Nuclear Chemistry* **232**, 107–113.
[19] Hardeland, R., Reiter, R.J., Poeggeler, B. and Tan, D.-X. (1993) "The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances", *Neuroscience and Biobehavioral Reviews* **17**, 347–357.
[20] Dellegar, S.M., Murphy, S.A., Bourne, A.E., Di Cesare, J.C. and Purser, G.H. (1999) "Identification of the factors affecting the rate of deactivation of hypochlorous acid by melatonin", *Biochemical and Biophysical Research Communications* **257**, 431–439.
[21] Everse, J. and Hsia, N. (1997) "The toxicities of native and modified hemoglobins", *Free Radical Biology and Medicine* **22**, 1075–1099.
[22] Halliwell, B. and Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicine, 3 (Clarendon Press, Oxford).
[23] Tesoriere, L., Allegra, M., D'Arpa, D., Butera, D. and Livrea, M.A. (2001) "Reaction of melatonin with hemoglobin-derived oxoferryl radicals and inhibition of the hydroperoxide-induced hemoglobin denaturation in red blood cells", *Journal of Pineal Research*.
[24] Patel, R.P., Svistunenko, D.A., Darley-Usmar, V.M., Symons, M.C.R. and Wilson, M.T. (1996) "Redox cycling of human methaemoglobin by H_2O_2 yields persistent ferryl iron and protein based radicals", *Free Radical Research Communications* **25**, 117–123.
[25] Winterbourn, C.C. (1990) "Oxidative reactions of hemoglobin", In: Packer, L. and Glazer, A.N., eds, Methods in Enzymology (Academic Press, San Diego, CA) **186**, pp. 265–272.
[26] Gringberg, L.N., Rachmilewitz, E.A. and Newmark, H. (1994) "Protective effects of rutin against hemoglobin oxidation", *Biochemical Pharmacology* **48**, 643–649.
[27] Vitale, A., Ferrari, C., Aldana, H. and Affanni, J. (1996) "Highly sensitive method for the determination of melatonin by normal-phase high performance liquid chromatography with fluorimetric detection", *Journal of Chromatography B* **681**, 381–384.
[28] McArthur, K.M. and Davies, M.J. (1993) "Detection and reactions of the globin radical in haemoglobin", *Biochimica et Biophysica Acta* **1202**, 173–181.
[29] Giulivi, C. and Cadenas, E. (1998) "Heme protein radicals: formation fate, and biological consequences", *Free Radical Biology and Medicine* **24**, 269–279.
[30] Davies, M.J. (1988) "Detection of peroxyl and alkoxyl radicals produced by reaction of hydroperoxides with heme-proteins by electron spin resonance spectroscopy", *Biochimica et Biophysica Acta* **964**, 28–35.
[31] Davies, M.J. (1991) "Identification of a globin free radical in equine myoglobin treated with peroxides", *Biochimica et Biophysica Acta* **1077**, 86–90.
[32] Everse, J. (1998) "The structure of heme-proteins compounds I and II: some misconceptions", *Free Radical Biology and Medicine* **24**, 1338–1346.
[33] Sugimoto, H., Tung, H.-C. and Sawyer, D.T. (1988) "Formation, characterization and reactivity of the oxene adduct of [tetrakis(2,6-dichloro-phenyl)porphinato]iron(III) perchlorate in acetonitrile. Model for the reactive intermediate of cytochrome P-450", *Journal of the American Chemical Society* **110**, 2465–2470.
[34] Giulivi, C. and Davies, K.J.A. (1990) "A novel antioxidant role for hemoglobin", *Journal of Biological Chemistry* **265**, 19453–19460.
[35] Koppenol, W.H. and Liebman, J.F. (1983) "The oxidizing nature of the hydroxyl radical: a comparison with the ferryl ion (FeO^{2+})", *Journal of Physical Chemistry* **88**, 99–101.
[36] Shiga, T. and Imaizumi, K. (1975) "Electron spin resonance study on peroxidase- and oxidase-reactions of horseradish peroxidase and methemoglobin", *Archives of Biochemistry and Biophysics* **167**, 469–479.
[37] Kopin, I.J., Pare, C.M.B., Axelrod, J. and Werissbach, H. (1961) "The fate of melatonin in animals", *Journal of Biological Chemistry* **236**, 3072–3075.
[38] Hirata, F., Hayaishi, O., Tokuyama, T. and Senoh, S. (1974) "*In vitro* and *in vivo* formation of two new metabolites of melatonin", *Journal of Biological Chemistry* **249**, 1311–1313.
[39] Hardeland, R., Fuhrberg, B., Behrmann, G. and Balzer, I. (1993) "Sleep-latency reducing pineal hormone melatonin as a scavenger of free radicals: hemin-catalysed formation of N_1-acetyl-N_2-formyl-5-methoxykynuramine", *Sleep Research* **22**, 621–628.
[40] Tan, D.-X., Manchester, L.C., Reiter, R.J., Plummer, B.F., Limson, J., Weintraub, S.T. and Qi, W. (2000) "Melatonin directly scavenges hydrogen peroxide: a potentially new metabolic pathway of melatonin biotransformation", *Free Radical Biology and Medicine* **29**, 1177–1185.
[41] Nagababu, E. and Rifkind, J.M. (2000) "Reaction of hydroperoxide with ferryl hemoglobin: superoxide production and heme degradation", *Biochemistry* **39**, 12503–12511.
[42] Dunford, H.B. (1999) Heme peroxidases (Wiley and Sons, New York).