MICROSOMAL METHOXYMELANIN FORMATION FROM 6-HYDROXYMELATONIN

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1. Introduction

In [1] we demonstrated that the superoxide anion (O_2^-)-dependent formation of microsomal protein-bound metabolite(s) of 5-HT or 5-HTP was accompanied by the consumption of NADPH and molecular oxygen. In addition to 5-hydroxyindoles, 6-HT was found to be as active as 5-HT in stimulating the microsomal NADPH oxidase activity and oxygen consumption [1]. In [2], we reported the O_2^--dependent microsomal melanogenesis from 5-HT and related 5-hydroxyindoles by demonstrating a free radical signal derived from a protein-bound metabolite(s) of 5-hydroxyindoles.

The formation of 5-methoxy-6-hydroxyindole from 5,6-DHI mainly by the action of HIOMT may not be important in melanogenesis whereas 5-hydroxy-6-methoxyindoles derived from 5,6-DHI by the action of COMT can be converted spontaneously to methylated melanins [3]. Here, we present data showing that 6-HM is a good substrate for the formation of methylated melanin, when incubated with microsomes in the presence of an NADPH-generating system and molecular oxygen. Thus, the position of a free hydroxyl-group at either 5 or 6 on the benzene ring of the indole nucleus is not of major importance, at least, in the O_2^--dependent microsomal melanogenesis.

Abbreviations: 6-HM, 6-hydroxymelatonin; 6-HT, 6-hydroxytryptamine; 6-HTP, 6-hydroxytryptophan; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; 5-MT, 5-methoxytryptamine; 5,6-DHI, 5,6-dihydroxyindole; DPPH, α,α-diphenyl-β-picrylhydrazyl; HIOMT, hydroxyindole-*O*-methyltransferase; COMT, catechol-*O*-methyltransferase; SOD, superoxide dismutase; f_{p_2}, NADPH-cytochrome *c* (cytochrome P450) reductase; EPR, electron paramagnetic resonance.

2. Materials and methods

Microsomal NADPH-cytochrome *c* reductase was purified from either trypsin-digested microsomes [4] or detergent-solubilized microsomes [5] of the livers of male Wistar rats (250–300 g) fasted for 12 h before sacrifice, except that Renex-690, a non-ionic detergent, was replaced by Triton X-100 [6] and that an affinity chromatography on 2′,5′-ADP–Sepharose 4B [7] was used. The A_{277}/A_{455} ratios for the trypsin-solubilized enzyme and the detergent-solubilized enzyme were 6.9 and 9.9, respectively, and the specific activities of the former and the latter enzymes in 0.1 M Tris–HCl buffer (pH 7.8) were 35 and 28 μmol cytochrome *c* reduced . min^{-1} . mg $protein^{-1}$ at 28°C, respectively. Superoxide dismutase was purified as in [8]. The microsomal protein-bound pigments from 6-HM, 6-HT and 6-HTP were prepared as in [2] except that 0.1 M K-phosphate buffer (pH 6.8) was substituted for 0.1 M Tris–HCl buffer (pH 7.8) when 6-HT and 6-HTP were used as substrates since 6-HT has been reported to be relatively unstable at high pH values [9].

Oxygen consumption and NADPH oxidation were measured as in [1]. EPR spectra were measured as in [2]. Protein was determined according to [10].

6-HM and 6-HT were purchased from Sigma. 6-HTP was kindly supplied to us by Professor V. Erspamer, Institute of Medical Pharmacology, University of Rome. 2′,5′-ADP–Sepharose 4B was obtained from Pharmacia.

3. Results

3.1. *Effect of 6-HM on the NADPH oxidase activity and oxygen consumption catalyzed by trypsin-solubilized NADPH-cytochrome* c *reductase*

As shown in fig.1, the very slow oxidation of

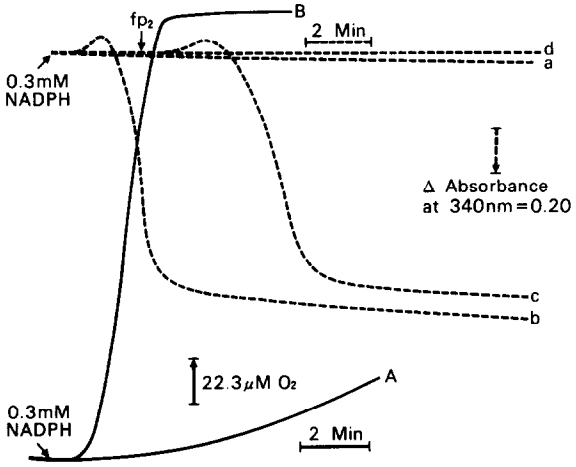

Fig.1. 6-HM-dependent NADPH oxidation and O_2 consumption by trypsin-solubilized NADPH-cytochrome *c* reductase. For the measurement of both NADPH oxidation and O_2 consumption, the reaction mixture, in 3.0 ml final vol. contained 51.3 μg flavoprotein, 0.1 M Tris–HCl buffer (pH 7.8), 1.5 mM KCN, 0.4 mM 6-HM (dissolved in ethanol and 20 μl of the solution added), and 0.3 mM NADPH added at the arrows at 35°C: (· · ·) NADPH oxidation; (——) O_2 uptake of which increase was shown upward in the vertical axis; (a,A) in the absence of 6-HM; (b,B) in the presence of 6-HM; (c) initially 6-HM and NADPH, then 25.6 μg flavoprotein was added during the steady state; (d) in the absence of flavoprotein.

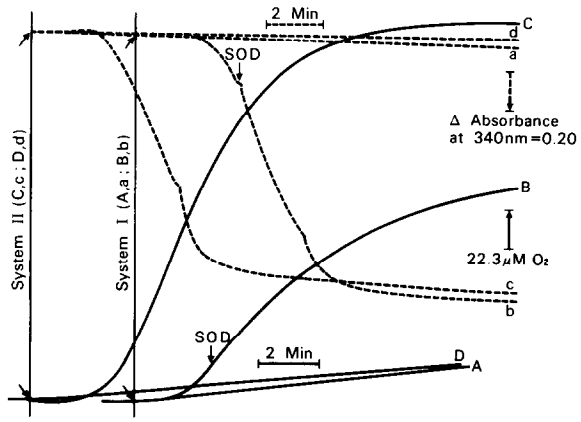

Fig.2. Effect of superoxide dismutase on 6-HM-dependent NADPH oxidation and O_2 consumption catalyzed by detergent-solubilized NADPH-cytochrome *c* reductase. The experimental conditions were those fig.1, except that 30 μg detergent-solubilized flavoprotein and 0.2 mM 6-HM (added in 10 μl ethanol) were used and that KCN was omitted: (· · ·) NADPH oxidation; (——) O_2 uptake of which increase was shown upward in the vertical axis; system I (a,A) pre-existence of 0.1 mg SOD; (b,B) addition of 0.3 mg SOD where indicated; system II (c,C), in the absence of SOD; (d,D) in the absence of 6-HM and SOD. The reaction was initiated by the addition of 0.3 mM NADPH at the arrows.

NADPH in the presence of the flavoprotein (a) was strongly accelerated by the presence of 6-HM (b). This accelerated oxidation of NADPH was actually accompanied by an O_2 uptake since the slow O_2 consumption catalyzed by the flavoprotein (A) was greatly stimulated by the presence of 6-HM (B). The reaction mixture composed of 6-HM and NADPH alone showed no decrease in A_{340} (d). On addition of the flavoprotein during the steady state, NADPH oxidation was induced after a small lag period (c). These results indicate that 6-HM is metabolized by this flavoprotein in the presence of NADPH.

The observation of the small increase in A_{340} after the initiation of the reaction was reproducible. We are currently examining the nature of this absorbing species.

3.2. *Involvement of superoxide anion radical (O_2^-) in 6-HM-dependent NADPH oxidation and O_2 consumption catalyzed by detergent-solubilized NADPH-cytochrome c reductase*

The effect of SOD on the NADPH oxidase activity and oxygen consumption was investigated since:

(i) This flavoprotein is known to be a good O_2^- generator [11];

(ii) O_2^- may participate in microsomal 5-HT-dependent NADPH oxidation, in O_2 consumption and in the formation of the protein-bound metabolite(s) from 5-HT [1].

Fig.2 shows the NADPH oxidase activity and oxygen consumption both in the presence (system I) and absence (system II) of SOD. The very slow oxidation of NADPH (d) and O_2 uptake (D) in the presence of detergent-solubilized flavoprotein were strongly stimulated in the presence of 6-HM (c,C) as in the case of trypsin-solubilized flavoprotein. The amounts of NADPH and O_2 consumed in the reaction mixture calculated at the final states of (c,C) after 15 min were 21 and 20 μmol/mg protein, respectively, giving a NADPH/O_2 ratio of ~1/1, which confirms the microsomal system in [1]. The pre-existence of SOD in the reaction mixture inhibited almost completely both 6-HM-stimulated NADPH oxidation and O_2 uptake (a,A). The addition of SOD during the steady state, however, inhibited only the 6-HM-stimulated O_2 uptake by ~50% (B) and had no effect on 6-HM-dependent NADPH oxidation (b) as in the case of 5-HT [1] or of microsomal oxidation of epinephrine [12].

3.3. *EPR spectra of the protein-bound metabolite(s) from 6-HM, 6-HT and 6-HTP*

As shown in fig.3, when 6-HM, 6-HT or 6-HTP was incubated with rat liver microsomes in the presence of NADPH generating system, a free radical signal with $g \sim 2.00$ was observed (1,4,7). The linewidth of 3 signals derived from 6-hydroxyindoles was ~10 G which agrees well with that of other melanins [13,14]. The spin density calculated by double integration with DPPH as standard, gave $\sim 9.6 \times 10^{12}$, 1.5×10^{13} and $4.1. \times 10^{12}$ spins/mg wet wt moist powder, prepared from the reaction mixture of 6-HM, 6-HT and 6-HTP, respectively. Without NADPH-generating system, no signal appeared (3,6,9). Moreover, the incubation of microsomes with NADPH generating system alone gave almost no signal (10). Therefore it can be said that the observed signals are derived from the metabolites of 6-HM, 6-HT and 6-HTP formed by the microsomal oxidation system. The spin densities calculated from the radical species obtained in [2], where 5-HT and 5-HTP were used as substrates, are of the same order as these.

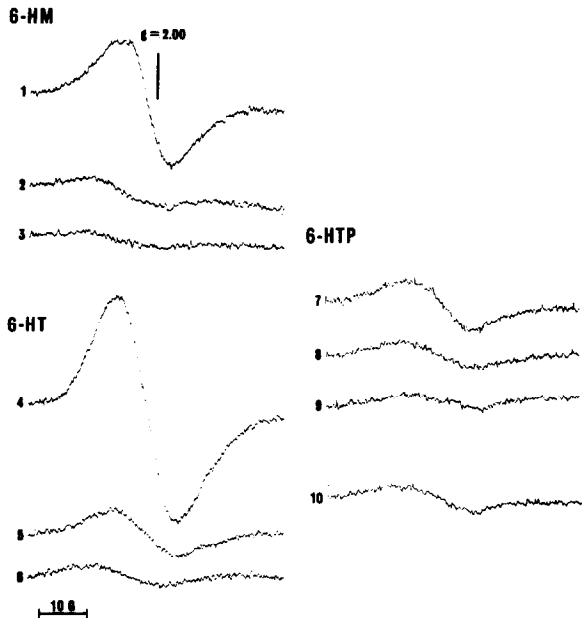

Fig.3. EPR spectra of melanin prepared by incubating 6-HM, 6-HT or 6-HTP with rat liver microsomes in the presence or absence of NADPH-generating system and the effect of SOD on the formation of free radical signals. The reaction mixture, in 10 ml final vol. contained 15 mg microsomal protein, 0.1 M Tris–HCl buffer (pH 7.8) for 0.48 mM 6-HT or 0.1 M K-phosphate buffer (pH 6.8) for 0.5 mM 6-HT or 6-HTP, 1.5 mM KCN, and NADPH-generating system composed of 0.6 mM NADP, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 35 U glucose 6-phosphate dehydrogenase, and was incubated at 35°C for 30 min with constant shaking in air. 0.5 mg SOD was added if necessary: (1,4,7) in the presence of NADPH-generating system (samples wet wt, (1) 131 mg, (4) 141 mg, (7) 144 mg); (2,5,8) in the presence of NADPH-generating system and SOD; (3,6,9) in the absence of NADPH-generating system; (10) microsome + NADPH-generating system alone.

The inclusion of SOD in the reaction mixture produced very weak signals (2,5,8). This means the participation of O_2^- in this type of melanogenesis from 6-hydroxyindoles.

4. Discussion

From our data concerning the O_2 consumptions, NADPH oxidations, and the spin densities, there was no preference between 5- and 6-hydroxyindoles as the substrate for the microsomal system. Although the melanins from these substrates might be different in their fine structures, EPR experiments were incapable of detecting such differences. The fact that no preference between 5- and 6-hydroxyindoles existed for melanin formation here may be in contrast to the concept in [3], where *O*-methylation of 5,6-DHI at position 6 has a preference to that at position 5 in melanogenesis.

Concerning the degradation pathway of indole nucleus of indole compounds, there are generally two pathways:

(1) The hydroxylation of the benzene moiety of the indole nucleus at position 6 in liver microsomes [9,15,16];
(2) The cleavage reaction of pyrrole ring moiety by indoleamine dioxygenase, especially in brain [17].

Tryptamine and melatonin follow both types of degradation [9,15,17,18]. As for tryptophan, the hydroxylation at position 6 does not occur [9,15]. Nevertheless, the endogenous formation of 6-HTP from *N*-acetyltryptophan may be possible since the presence of the latter in human urine has been reported [19] and it is known to be hydroxylated at position 6 by liver microsomes [9] and the removal of the acetyl group from *N*-acetyl-tryptophan is highly active in kidney [20].

Thus, the 6-hydroxy compounds formed are subsequently conjugated by either glucuronide or sulphate [18,21]. Besides the conjugation pathways, however, there is another O_2^--dependent degradation pathway to that presented here. The reaction product may be

a quinoid of an unknown structure or its semiquinone form which binds to the microsomal protein covalently resulting in the formation of a melanin-like pigment as evidenced by the free radical signals (fig.3). This seems to be another synthetic pathway of methoxymelanin which is different from that in [3]. It is of interest to see whether another possible endogenous 6-hydroxyindole compound, e.g., 5-methoxy-6-hydroxytryptamine probably formed from 5-MT by 6-hydroxylation, can be transformed to methoxymelanin by a microsomal system.

Acknowledgements

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References

[1] Uemura, T., Matsushita, H., Ozawa, M., Fiori, A. and Chiesara, E. (1979) FEBS Lett. 101, 59–62.
[2] Uemura, T., Shimazu, T., Miura, R. and Yamano, T. (1980) Biochem. Biophys. Res. Commun. 93, 1074–1081.
[3] Axelrod, J. and Lerner, A. B. (1963) Biochim. Biophys. Acta 71, 650–655.
[4] Omura, T. and Takesue, S. (1970) J. Biochem. (Tokyo) 67, 249–257.
[5] Van der Hoeven, T. A. and Coon, M. J. (1974) J. Biol. Chem. 249, 6302–6310.
[6] Miki, N., Sugiyama, T. and Yamano, T. (1980) J. Biochem. (Tokyo) 88, 307–316.
[7] Yasukochi, Y. and Masters, B. S. S. (1976) J. Biol. Chem. 251, 5337–5344.
[8] McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049–6055.
[9] Jepson, J. B., Zaltzman, P. and Udenfriend, S. (1962) Biochim. Biophys. Acta 62, 91–102.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Aust, S. D., Roering, D. L. and Pederson, T. C. (1972) Biochem. Biophys. Res. Commun. 47, 1133–1137.
[12] Schenkman, J. B., Jansson, I., Powis, G. and Kappus, H. (1979) Mol. Pharmacol. 15, 428–438.
[13] Van Woert, M. H., Prasad, K. N. and Borg, D. C. (1967) J. Neurochem. 14, 707–716.
[14] Blois, M. S., Zahlan, A. B. and Maling, J. E. (1964) Biophys. J. 4, 471–490.
[15] Jepson, J. B., Udenfriend, S. and Zaltzman, P. (1959) Fed. Proc. FASEB 18, 254.
[16] Otani, T., Akagi, K. and Sakamoto, Y. (1962) J. Biochem. (Tokyo) 52, 428–432.
[17] Hirata, F., Hayaishi, O., Tokuyama, T. and Senoh, S. (1974) J. Biol. Chem. 249, 1311–1313.
[18] Kopin, I. J., Pare, C. M. B., Axelrod, J. and Weissbach, H. (1960) Biochim. Biophys. Acta 40, 377–378.
[19] Armstrong, M. D., Show, K. N. F., Gortakowski, M. J. and Singer, H. (1958) J. Biol. Chem. 234, 17–30.
[20] Erspamer, V., Glässer, A. and Nobili, M. B. (1961) Arch. Biochem. Biophys. 93, 673–674.
[21] Szara, S. (1961) Experientia 17, 76.