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# OXIDATION OF NADH BY MELANIN AND MELANOPROTEINS

E. V. GAN, H. F. HABERMAN AND I. A. MENON

*Clinical Sciences Division, Medical Sciences Building, University of Toronto, Toronto, M5S 1A8 (Canada)*

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## SUMMARY

The oxidation of NADH by melanin was employed as a means for studying the properties of melanin isolated from melanoma. The NADH-oxidizing activity of melanin isolated from B16 melanoma (tumour-melanin) was compared with that of the melanin synthesized by the action of monophenol monooxygenase (tyrosinase, EC 1.14.18.1) on 3,4-dihydroxyphenylalanine (dopa-melanin). Unlike dopa-melanin, the tumour-melanin did not oxidize NADH. When the proteins associated with the tumour-melanin were hydrolyzed by acid treatment, the resulting product readily oxidized NADH. Conversely, addition of bovine serum albumin to the melanin-synthesizing system or to the melanin already synthesized decreased the oxidation of NADH by the melanin. Similarly, polyarginine and polylysine decreased the NADH oxidizing activity while polyglutamate and polyaspartate did not have any effect. The results indicate that basic components of the proteins bound to melanin may be blocking the active sites of melanin involved in the oxidation of NADH. It is postulated that the electron transfer properties of melanin may be important in its protective role against radiation and toxic free radicals and that the inactive melanoprotein, as it occurs in the cell, may be converted to active melanin upon dissociation or degradation of the protein by agents such as irradiation.

## INTRODUCTION

Melanosomes are subcellular organelles located in the cytoplasm of melanocytes of various sources. Melanosomes are the sites for the synthesis and storage of melanin. In these cells melanin is synthesized by the oxidation of tyrosine to 3,4-dihydroxyphenylalanine and subsequently to phenylalanine-3,4-quinone, followed by polymerization of the indole derivatives formed from phenylalanine-3,4-quinone. Among the series of steps involved in melanogenesis, the oxidations of tyrosine and 3,4-dihydroxyphenylalanine are the only reactions catalyzed by enzymes. One proposal holds that in mammals both reactions are catalyzed by one enzyme termed monophenol monooxygenase ("tyrosinase", EC 1.14.18.1) [1–3], and another holds that 3,4-dihydroxyphenylalanine oxidase only mediates the oxidation of 3,4-dihydroxyphenylalanine formed by the action of peroxidase [4–7].

The ability of 3,4-dihydroxyphenylalanine oxidase to use molecular oxygen

has led to speculation that it may contribute to the respiration of melanomas [8–10]. The 3,4-dihydroxyphenylalanine oxidase activity of melanosomes has been found to be decreased as melanization progressed within the melanosomes, and the activity of soluble 3,4-dihydroxyphenylalanine oxidase from melanoma is decreased after this enzyme was employed for melanin synthesis [11, 12]. Therefore, information regarding the structure of melanosomes is important not only for knowledge about the control of pigmentation but also for the estimation of the respiratory activity of normal and malignant melanocytes. Very little information is available concerning the composition of melanosomes. It has been found that besides these enzymes, melanosomes contain several other proteins and some of these proteins are firmly bound to melanin [13, 14]. Duchon et al. [15] have reported that melanosomes from various sources have different proportions of melanin and protein. No detailed information is available regarding the types of proteins contained in the melanosomes and the nature of the binding of proteins to melanin.

The present studies were undertaken in order to investigate properties of melanin granules as isolated from melanocytes. For this purpose the non-enzymatic oxidation of NADH in the presence of melanin has been employed. Van Woert [16,17] who first reported this reaction attributed this property of melanin to the fact that it is a stable free radical and could act as a one-dimensional semiconductor and electron acceptor as suggested by Pullman and Pullman [18]. The physiological role of this reaction, if any, is not known. However, we thought that this property of melanin could be used as a tool to study the function of melanin within the melanosomes.

## MATERIALS AND METHODS

### *Materials*

Mushroom monophenol monooxygenase Grade III, $\delta$-chymotrypsin (Type I, 40–50 units/mg), trypsin (Type IX, 10 000–13 000 BAEE units/mg), pepsin (2500–3200 units/mg), protease (Type III, 0.3–0.5 unit/mg), bovine serum albumin, L-3,4-dihydroxyphenylalanine, reduced nicotinamide adenine dinucleotide ($\beta$-NADH), poly-L-arginine hydrochloride (Type II-B), poly-L-lysine hydrobromide (Type I-B), and the sodium salts of poly-L-glutamic acid and poly-L-aspartic acid were obtained from Sigma Chemical Co. Ninhydrin, hydrindantin, and 2-methoxyethanol were purchased from British Drug Houses Ltd.

### *Preparation of melanin*

Melanin granules were isolated from 15 g of B16 melanoma tumour maintained in C57BL/6J mice as described by Haberman and Menon [19]. The melanin granules were resuspended in 5.0 ml of distilled water and dialyzed against water for 18 h. Approx. 26 mg (dry weight) of melanin was obtained. This preparation is referred to as "tumour-melanin". Melanin was prepared in vitro (a) by the autooxidation of 3,4-dihydroxyphenylalanine at pH 8.0 according to Swan [20], and (b) enzymically from 100 mg of 3,4-dihydroxyphenylalanine and 10 mg of purified mushroom monophenol monooxygenase in 25 ml of 0.1 M phosphate buffer, pH 6.8, as described by Van Woert [16, 17]. The insoluble melanin was separated by centrifugation at 100 000 $\times$ $g$ for 30 min and washed twice with water. The melanin

from (b) was resuspended in 5.0 ml of water. Approx. 20 mg (dry weight) of melanin was obtained. This preparation is referred to as "dopa-melanin".

*Enzymic hydrolysis*

Enzymic hydrolysis of melanin with chymotrypsin, trypsin, or protease was carried out at 37 °C for 22 h in 0.1 M phosphate buffer, pH 7.0, and at an enzyme concentration of 100 $\mu$g/ml. The digestion with pepsin was performed in 0.02 M HCl. The incubation mixture consisted of 2.0 ml of a suitable dilution of the melanin suspension and 0.2 ml of the enzyme solution in a total volume of 4.0 ml. After incubation, the melanin was separated by centrifugation at 100 000 $\times$ $g$ for 30 min, washed twice with water, resuspended in 4.0 ml of water, and assayed for its reactivity towards NADH.

*Acid hydrolysis*

Acid hydrolysis of melanin was carried out in 2 M HCl in sealed tubes at 100 °C for various periods of time. The hydrolyzed samples were centrifuged, washed and assayed as described above. The course of the hydrolysis was also followed by a direct ninhydrin analysis [21] on the supernatant fractions. The results are expressed in terms of leucine equivalents.

*Effect of albumin and polyamino acids*

Dopa-melanin was prepared from 10 mg of 3,4-dihydroxyphenylalanine and 1 mg of mushroom monophenol monooxygenase in 2.5 ml of 0.1 M phosphate buffer, pH 6.8 at 37 °C for 18 h in the presence of varying amounts of bovine serum albumin. In a separate experiment, varying amounts of albumin were added after melanin synthesis and incubation was carried out for another 18 h at 37 °C. After incubation, the insoluble melanin was separated by centrifugation at 100 000 $\times$ $g$ for 30 min, washed twice with water, and resuspended in 1.0 ml water. Oxidation of NADH was carried out with the melanin suspension diluted 20 times.

Parallel experiments were performed using varying amounts of poly-L-glutamate, poly-L-aspartate, poly-L-lysine, and poly-L-arginine, except that the oxidation of NADH was carried out with 1:10 dilution of the melanin suspension.

*Oxidation of NADH*

The oxidation of NADH was monitored spectrophotometrically at 340 nm for 1 h at room temperature. The standard reaction mixture contained 0.9 ml of the melanin suspension, 0.3 ml of 1.28 mM NADH, and 1.8 ml of 0.1 M phosphate buffer, pH 7.2. A blank containing the same concentration of melanin but without NADH was run simultaneously with the standard reaction.

*Amino acid analysis*

Hydrolysis of 2 mg of melanin was carried out in 1.0 ml of constant boiling 6 M HCl at 110 °C for 24 h in evacuated sealed tubes. After hydrolysis, the contents were dried in a dessicator over NaOH, and the residue was dissolved in 1.0 ml of Spinco pH 2.2 buffer. The hydrolyzate was analyzed with a Beckman amino acid analyzer, Model 121 using $\alpha$-aminoguanido propionic acid and norleucine as internal standards.

RESULTS

*Oxidation of NADH by melanin*

The oxidation of NADH by the dopa-melanin and the melanosomes from melanoma is shown in Fig. 1. The absorbance in the cuvette containing NADH and dopa-melanin decreased linearly during the 60-min period. On the other hand, the absorbance in the cuvette containing NADH and tumour-melanin was not decreased during the same period. There was no change in the absorbance in the control

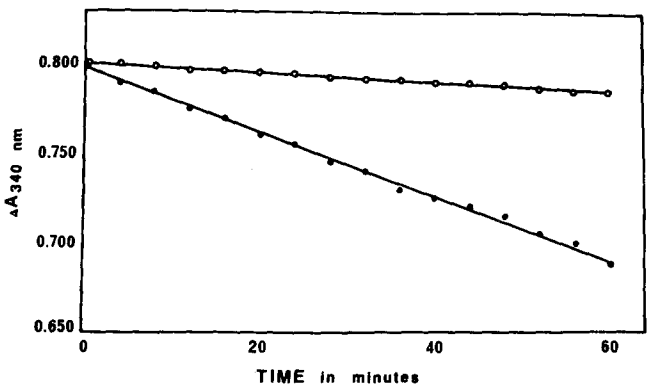


Fig. 1. Oxidation of NADH by dopa-melanin and tumour-melanin. ●—●, dopa-melanin; ○—○, tumour-melanin.

cuvettes where either melanin or NADH was omitted. The above results were corroborated using another parameter for following the reaction. The oxygen uptake by the system was determined by using an oxygraph. Oxygen uptake was observed only with the dopa-melanin but not with the tumour-melanin. Melanin obtained by the non-enzymatic oxidation of 3,4-dihydroxyphenylalanine as described by Swan [20] was found to oxidize NADH.

*Effect of hydrolysis of protein on the oxidation of NADH*

The possibility that the inability of the tumour-melanin to oxidize NADH may be because the active sites are blocked by proteins bound to the melanin was investigated. The question was explored by the hydrolysis of protein by acid and then determining the oxidation of NADH by the melanin obtained after the treatment. As seen from the results shown in Fig. 2, the amount of protein hydrolyzed increased as the period of hydrolysis was increased. After the hydrolysis procedure the tumour-melanin was found to oxidize NADH. In fact, the ability to oxidize NADH gradually increased as the hydrolysis was prolonged, and followed a pattern similar to the amounts of protein hydrolyzed. There was considerably smaller amounts of protein hydrolyzed from dopa-melanin. Similarly, the oxidation of NADH also was enhanced to a relatively lesser extent. Attempts to hydrolyze the proteins bound to melanin by means of several proteolytic enzymes were not successful as seen by the fact that the resulting melanin did not have NADH-oxidizing activity.

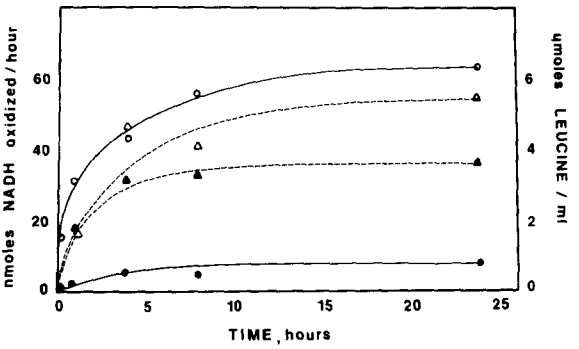


Fig. 2. Oxidation of NADH by melanin and hydrolysis of melanin-bound protein by treatment with acid. ○—○, NADH oxidized by dopa-melanin; △---△, NADH oxidized by tumour-melanin; ●—●, amino acids (leucine equivalents) from dopa-melanin; ▲---▲, amino acids from tumour-melanin.

*Effect of addition of albumin upon the oxidation of NADH by dopa-melanin*

The above results indicated that the hydrolysis of protein bound to melanin increased the ability of the melanin to oxidize NADH. The question whether addition of protein to melanin would decrease this activity was next examined. The results are given in Fig. 3. When varying amounts of albumin were added to the melanin-synthesizing system the oxidation of NADH was decreased. Similarly, experiments where albumin was added to melanin which had already been synthesized showed that addition of increasing amounts of albumin decreased the oxidation of NADH to greater extent.

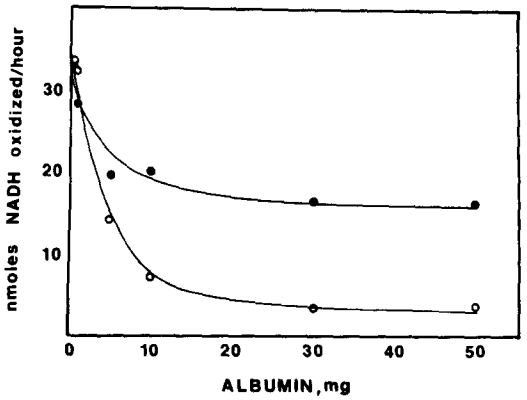


Fig. 3. Effect of albumin on the oxidation of NADH by dopa-melanin. ○—○, albumin added during the synthesis of melanin. ●—●, albumin added after melanin was synthesized and isolated.

*Effects of polyamino acids on the oxidation of NADH by melanin*

The effects of acidic and basic polyamino acids upon the oxidation of NADH by melanin were next investigated. In the first series of experiments the polyamino acids were added to the monophenol monooxygenase system during the melanin synthesis. As seen from Fig. 4 polyglutamate and polyaspartate did not have any

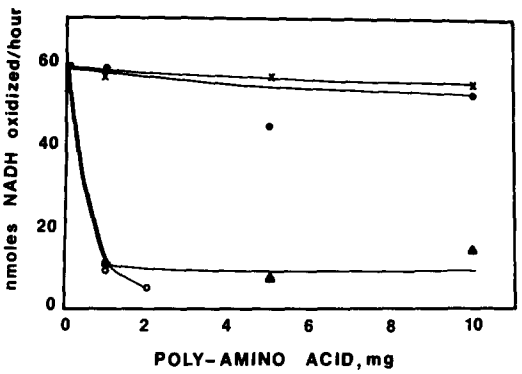


Fig. 4. Effects of addition of polyamino acids during the synthesis of melanin on the oxidation of NADH by melanin. ×—×, polyaspartate; ●—●, polyglutamate; ▲—▲, polylysine; ○—○, polyarginine.

significant effect on the NADH oxidation. On the other hand, polylysine and polyarginine produced a considerable decrease in the oxidation of NADH. The addition of polyglutamate and polyaspartate to melanin already prepared did not have any significant effect on the oxidation of NADH. Addition of polylysine or polyarginine to the melanin suspension resulted in aggregation of the melanin and rapid sedimentation of the particles. Probably these polypeptides form cross-linkages between the melanin granules causing the aggregation. Therefore, the effects of these substances under these conditions could not be studied.

*Composition of amino acids from dopa-melanin and tumour-melanin*

The protein contents and amino acid compositions of dopa-melanin and tumour-melanin were compared. These results are given in Table I. No detectable amounts of unusual amino acids e.g., dopa as reported by Duchon et al. [2, 15] were present in either sample. As expected, the composition of amino acids from dopa-melanin was closely similar to that of the amino acids from mushroom monophenol monooxygenase employed for the preparation of the melanin. From the total weight of all amino acids and the dry weight of melanin taken for hydrolysis, 63.0% of tumour-melanin was protein compared to 16.7% for dopa-melanin.

## DISCUSSION

The results presented above show a striking difference in the oxidation of NADH by the melanin isolated from melanoma and that synthesized by the action of monophenol monooxygenase on 3,4-dihydroxyphenylalanine. In conformation with the observation of Van Woert [16, 17], the dopa-melanin readily oxidized NADH. However, the melanin from the tumour did not oxidize NADH. When the tumour-melanin was subjected to acid hydrolysis under conditions routinely employed for the hydrolysis of proteins, the resulting product had considerable NADH-oxidizing activity. When bovine serum albumin was added to the melanin-synthesizing system or to melanin already synthesized, the NADH-oxidizing activity was decreased. These results show that when proteins are bound to melanin the resulting melanoproteins have lower NADH-oxidizing activity. It may be con-

TABLE 1

COMPOSITION OF AMINO ACIDS OBTAINED AFTER THE HYDROLYSIS OF DOPA-MELANIN AND TUMOUR-MELANIN

| Amino acid | Weight percent of amino acid* | |
|---|---|---|
| | Dopa-melanin | Tumour-melanin |
| Lysine | 5.97 | 6.87 |
| Histidine | 4.92 | 3.16 |
| Arginine | 5.94 | 7.30 |
| Aspartic acid | 12.48 | 9.74 |
| Threonine | 6.58 | 5.27 |
| Serine | 5.60 | 5.42 |
| Glutamic acid | 13.26 | 12.45 |
| Proline | 4.68 | 5.39 |
| Glycine | 5.34 | 4.91 |
| Alanine | 6.87 | 5.57 |
| Half-cystine | 0.09 | 2.44 |
| Valine | 5.98 | 5.76 |
| Methionine | 1.45 | 2.74 |
| Isoleucine | 5.29 | 4.43 |
| Leucine | 7.39 | 9.01 |
| Tyrosine | 3.11 | 4.28 |
| Phenylalanine | 5.03 | 5.24 |
| Protein** (%) | 16.66 | 62.94 |

* The amino acid composition is expressed in weight percent of each amino acid from the total weight of all amino acids recovered from the analysis.

** Percent of protein is calculated from the total weight of all amino acids and the dry weight of melanin taken for hydrolysis.

cluded from these results that melanin as isolated from the melanoma is present as melanoprotein. The proteins are presumably bound to melanin in such a manner that the free radical sites on the melanin are blocked.

Studies on the effects of the synthetic polypeptides of known composition showed that polyglutamate and polyaspartate had no effect on the NADH while polylysine and polyarginine produced a marked inhibition. These results show that basic polypeptides are more effective in decreasing the NADH-oxidizing activity and may also form complexes with melanin more effectively. Analysis of the amino acid composition of the proteins in the melanosomes showed that there was no obvious abundance of either the basic or acidic groups of amino acids. It is possible that specific regions of proteins having concentrations of basic amino acids may be responsible for the binding to melanin.

It is not possible to make definite conclusions regarding the biological significance of the present results. However, we would like to postulate the following probable role for melanoproteins. It has been proposed that the free radical structure of melanin may be important in its protective role against radiation and toxic free radicals [22, 23]. It has also been postulated that melanin may act as a biological electron exchange polymer and may protect cells and tissues against oxidizing and reducing conditions which would have harmful effects on cellular structure and metabolism [24]. Our observations on the presence of melanin in the melanoma as

melanoproteins not capable of electron transfer may have relevance in these functions of melanin. This may represent the state in which melanin may be stored within the cell. Our results showing that removal of proteins bound to melanin could produce melanin active in electron transfer could lead to the postulate that similar mechanisms for "activation" of melanin may be prevalent in cells. For example it may be proposed that high energy photons may dissociate the melanoproteins and thereby produce free melanin active as a free radical. The storage of melanin as relatively inactive melanoproteins would eliminate the possible side effects which may be caused by the presence of active melanin within the cells all the time. Thus by this means it may be possible to preserve a potentially active defense mechanism to be utilized only when the conditions demand its function.

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