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Isolation and Identification of the Protein Component of Hair Melanin

KATHLEEN HALL, B.Sc. and LESZEK J. WOLFRAM, Ph. D.*

Synopsis—ELECTRON MICROSCOPIC STUDIES have previously revealed that, in the MELANOCYTE, granules appear to exist in varying stages of development. They represent preliminary synthesis of NONMELANIZED GRANULES and are composed of coiled protein filaments or membranes. This protein matrix serves as a substrate for subsequent deposition of the melanin polymer and thus becomes totally encapsulated by the pigment. Upon reaching complete melanization, the granules appear uniformly dense, and no internal structure can be resolved by conventional techniques.

Previous attempts to identify the MELANO-PROTEIN have been hindered by the chemical inertness of the melanin capsule. A technique for solubilizing the melanin was found and provides a facile method of isolating the protein moiety present in the pigment granule. The chemical composition of this protein has also been determined.

I. Introduction

The site of melanin formation in vertebrates is the melanocyte. This is a specialized, distinctive cell characterized by two or more dendritic processes and usually located in a region adjacent to the developing cells endowed with the function of pigment transfer. In the case of hair follicles, the melanocytes are normally found dispersed among the rapidly dividing cortical cells of the hair bulb at the apex of a small cavity known as the dermal papilla. Pigment granules formed in these melanocytes are transferred to the cortical cells of the forming hair, and during the process of extrusion and keratinization the melanin pigment becomes aligned to the axis of the fiber.

The fine structure of both melanocytes and melanosomes has attracted much attention. The pioneering work of Birbeck (1), Drochmans (2), and Moyer (3) has demonstrated the developmental changes in the elaboration of black pigment granules. Two main successive steps in the formation of a

*Gillette Company, Personal Care Division, Boston, Mass. 02106.

melanosome can be represented as follows: first, the formation of a protein matrix composed of coiled filaments or membranes; second, the deposition of melanin polymer onto the protein matrix. As the melanin deposition continues, the membranous support becomes so obliterated that the granules appear uniformly electron dense and devoid of any structural detail. From an investigative point of view, complete melanization results in somewhat of a paradox. The known resistance of the melanin polymer to chemical reagents allows for easy separation of the pigment and its encapsulated protein from the fibrous keratin, but at the same time effectively prevents the isolation of the proteinaceous matrix from its melanin environment. Several attempts have been made to extract the protein component from the matrix granules. Thus Serra (4) claims to have isolated a melano protein complex free from fiber contamination. However, his high values for protein content of the pigment (over 50%) appear incompatible with the available elemental analysis of the composite materials. Laxer (5), who had isolated the pigment from hair by treatment of the latter in phenol hydrate-thioglycollic acid mixtures, refluxed the melanin granules with 6*N* HCl for extended periods of time (>48 hours) and analyzed the hydrolysate by paper chromatography. Most of the amino-acids present in the keratin were found in the hydrolysate, but no quantitative determinations were made.

Recent investigations by Gjesdal (6) have confirmed the presence of protein in pigment granules isolated by hydrolyzing variously colored fibers with dilute alkali. Again, a quantitative analysis was not attempted, and Gjesdal noted that the protein present in the pigment is strongly bound within the granule.

In the course of our work on hair bleaching we have developed a very mild, yet effective procedure for solubilization of the melanin pigment (7). It appeared to us that this method offers a unique way for a very facile separation of the protein-bound pigment. The utility of such an approach is the subject of this paper.

II. Materials and methods

A. *Poodle Hair*

Black poodle hair was obtained from random samples of hair clippings. The hair was purified by Soxhlet extraction with methylene chloride followed by absolute methanol for four hours each. The hair was then rinsed well with deionized water and allowed to dry.

B. *Oriental Hair*

Samples of Oriental hair, approximately 5 in. in length, were obtained from an individual known to have untreated hair. The distal ends of the fibers were trimmed and discarded; the remaining hair was purified by the extraction procedure described above.

C. *Hair Melanin*

The melanin was isolated by careful dissection of the ink sacks, previously hair by the nonhydrolytic method of Laxer (5). The hair sample was refluxed for 24 hours with a phenol hydrate-thioglycollic acid mixture (PHT). The pigment was separated by centrifuging the solution in cellulose nitrate tubes at 1800 rpm for 45 min. The melanin was then washed with two successive portions of PHT, rinsed several times with 40% aqueous ethanol, then dried *in vacuo* at 60°C.

D. *Squid Melanin*

The melanin was isolated by careful dissection of the ink sacs, previously removed from approximately 1 lb of squid. The pigment was rinsed several times with water, then soaked in 6*N* HCl for 24 hours at ambient temperature. Removal of the acid was accomplished by thorough water rinsing, and centrifugation of pigment followed by air drying.

E. *Chemical Reagents*

Commercially available, reagent grade solvents were utilized in this study without further purification.

F. *Amino-acid analysis*

Amino-acid analysis was performed on the Phoenix M7800 automatic amino-acid analyzer.

III. Results and Discussion

We have recently shown (7) that the bleaching of melanin by aqueous solutions of H_2O_2 involves two successive processes: solubilization of the pigment granules followed by decolorization of the solubilize melanin. The reaction can be restricted to the solubilization stage alone if dilute solutions of H_2O_2 are used. Under such conditions, the polymeric character of the pigment is retained, and the extent of oxidative degradation of the protein component is marginal. The molecular weight of the solubilized pigment was calculated to be 11,400 and 15,000 via osmotic pressure measurements and thin-layer gel filtration, respectively (7).

The solubilized melanin was prepared as follows: 100 mg of the pigment was suspended with stirring in 20 ml of 1% hydrogen preoxide at pH 10 (ammonia was used for pH adjustment) and 25°C. Complete dissolution of the pigment took place within 60 min. At this point the excess peroxide was destroyed by platinum black, and the product was lyophilized yielding a black, highly lustrous water-soluble material. It is of interest to note that the solubility characteristics of the oxidized melanin could be changed by esterifica-

tion of its carboxylic side chains by treatment in methanol/HCl. This methylated melanin was now water insoluble under acidic and neutral conditions but dissolved readily at pH 9 and above as the groups of the hydroxyindole residues began to ionize.

The solubility characteristic of the solubilized melanin thus appeared to be controlled by the ionization of the carboxylic side chains present in this melano-protein moiety. Aqueous solutions of the latter were found to form copious precipitates upon acidification below pH 4. Although both the melanin and the protein were present in the precipitate, only the protein was found in the supernatant. The melanin-free protein present in the supernatant accounted for 23% of the total protein associated originally with the pigment. Complete separation of the protein component was attained by acid hydrolysis of the combined fractions, i.e., the precipitate and supernate in 6*N* HCl for 16 hours at 105°C. Table I lists the amino-acid content of the protein component of the melanins obtained from human hair as well as from dog hair. For comparison, the amino-acid composition of the corresponding hair keratin is also given.

It is clearly evident that the chemical composition of the melano-protein differs greatly from that of the keratin. Particularly striking is the high content of basic amino-acids present in the former. Although the extent of amidation of the glutamic acid is unknown, it is tempting to suggest that the protein, in its native state, is positively charged and thus offers an ideal site for the firm embedding of negatively charged melanin precursors and their polymers. This way, the cohesion of alternate protein-melanin layers is greatly enhanced, and thus the electrostatic interaction may be the instrumenal factor in the initial stages of formation of high-density pigment granules.

The melano-protein contains two acids which have not been found as the components of keratins. These are taurine and β-alanine. Although these compounds might be by-products of the solubilization process, the possibility that they are associated with the biosynthesis of the melanin should not be overlooked.

The protein content of the dog hair melanin, calculated from the amino-acid data, accounts to 7.8%, based on the weight of the solubilized pigment. A similar value of 9.4% was obtained for the melano-protein content of melanin which was isolated from Oriental hair. These appear to be reasonable values. The solubilizing treatment, although effective in bringing about physical destruction of the pigment granules, is not likely to cause an extensive oxidative degradation of the encased protein. The chemical attack in this case appears to be confined to the residues of sulfur-containing amino-acids. Evidence for the latter is supported by chemical analysis of extensively bleached hair; in this case the reaction between keratin and hydrogen peroxide is mainly confined to the cystine cross-links giving rise to the formation of cysteic acid residues. Thus the cysteic acid found in large quantities in the melano-

Table I

Amino-Acid Composition of the Protein Isolated from the Melanin of Oriental Hair and Poodle Hair

Type of Side Chain and Amino Acid	Melano-Protein μM/g	Oriental Hair μM/g	Melano-Protein μM/g	Poodle Hair μM/g
Aliphatic	2656	1963	2755	1962
Glycine	1005	455	1143	572
Alanine	459	365	504	392
Valine	478	448	372	386
Isoleucine	210	207	162	184
Leucine	466	488	500	428
β-Alanine	38	...	74	...
Aliphatic hydroxyl	967	1425	747	1396
Threonine	417	533	342	552
Serine	550	892	405	844
Aromatic	276	288	346	299
Tyrosine	101	159	129	166
Phenylanine	175	129	217	133
Acidic	1619	1407	1579	1256
Aspartic acid	682	427	559	428
Glutamic acid	937	980	1020	828
Basic	1037	771	1507	518
Lysine	311	211	700	152
Histidine	156	71	230	48
Arginine	570	489	577	318
Sulfur containing	787	1391	521	1469
Cysteic acid	463	41	337	42
Taurine	36	...	14	...
Methionine sulfoxide	22	...	...	...
Methionine sulfone	184	...	101	...
Half-cystine	45	1312	42	1370
Methionine	37	38	27	56
Heterocyclic	453	600	358	601
Proline	453	600	358	601

protein is partially accounted for as a result of the oxidation of cystine occurring during the solubilization process. However, the bulk of the cysteic acid is not likely to arise from the mild oxidative treatment employed. Some support for this is obtained by the presence of cysteic acid in the hydrolyzates of intact melanin. The possibility of cysteic acid formation via disproportionation of products effected by the redox system of melanin's quinonoid structure is also rejected. A known quantity of cystine was added to solubilized melanin samples prior to hydrolysis; no increase in the cysteic acid content was detected.

Whether the disulfide bond of cystine is also the covalent link between the melanin polymer and the protein component of the granule is not known at this stage. We would like to point out, however, that the solubilization treatment does release some of the protein from its melano-protein moiety. As much as one-third of the total protein content can be separated from the melanin polymer by mere acidification of the solubilized pigment. The recently introduced technique of enzymatic hydrolysis (8) may prove to be an invaluable tool in determining both the frequency and the nature of melanin-protein covalent bonding.

A third melanin, a sepia melanin, was isolated from the squid ink sac and solubilized in the same manner previously described. This melanin exists as electron dense spheres measuring 0.1 to 0.3 μm across (9); the melanins isolated from the hair keratin were in the form of discrete granules, approximately 0.35 by 1.0 μ. Despite the physical difference and origin of the squid melanin, a similarity in the melano-protein compositions was observed for these melanins.

Calculation of the protein content of the squid melanin based on the data in Table II gave the value of 4.3%, a somewhat lower value than the one obtained in the case of the hair melanins examined. The basicity of the squid melano-protein was considerably less than in the corresponding proteins isolated from poodle hair melanin and Oriental hair melanin. This may be due to the need for more acidic functions within this particular structure in order to aid in the dispersion of the melanin ink in aqueous media. A comparison of the analytical data given in Tables I and II reveals that in many cases, either the concentrations or proportions of the respective amino acids are in close proximity. This is in spite of the diverse sources of these three proteins and possibly connotes that a chemically specific protein matrix is a prerequisite for the formation of melanin granules, irrespective of their animal origin. Such a hypothesis of protein specificity is new, and in view of the scarce experimental evidence available (namely, the chemical composition of 3 melano-proteins), it may be classified as speculation. Yet it might be the key to the activity of the melanosomes and the rate at which the melanin polymer is formed within these cells.

Table II

Amino-Acid Composition of the Protein Isolated from Squid Melanin

Type of Side Chain and Amino Acid	Melano-Protein μM/g
Aliphatic	2669
Glycine	975
Alanine	461
Valine	352
Isoleucine	327
Leucine	526
β-Alanine	28
Aliphatic Hydroxyl	462
Threonine	228
Serine	234
Aromatic	623
Tyrosine	237
Phenylalanine	386
Acidic	2002
Aspartic acid	1158
Glutamic acid	844
Basic	887
Lysine	240
Histidine	144
Arginine	503
Sulfur Containing	663
Cysteic acid	287
Taurine	163
Methionine sulfoxide	...
Methionine sulfone	139
Half-cystine	nil
Methionine	74
Heterocyclic	400
Proline	400

IV. Conclusion

The technique of melanin solubilization was successfully used in this investigation to elucidate the nature of proteins associated with pigments. There is no reason why this technique could not be extended to examine melanins of various origins to assert or reject our postulate of protein-matrix specificity.

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V. References

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