Mammalian Melanosomal Proteins: Characterization by Polyacrylamide Gel Electrophoresis

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INTRODUCTION

For many years the major problem impeding the elucidation of the structure of melanin and the melanosome has been the insolvibility of the melanin polymer. To date, alkaline and acid hydrolysis seem to be the only methods available which can completely solubilize the melanosome (1); unfortunately, both methods degrade the constituents of the organelle other than the melanin polymer. The recent emergence of refined techniques for the solubilization of the more insoluble cellular components, such as ribosomes and membranes, justified a reinvestigation of melanosome solubility. This paper reports the successful solubilization of the melanosome, and a partial characterization of its proteins.

MATERIALS AND METHODS

Melanosome Isolation

Black mice (C57BL/6N) (30 days old) were sacrificed by CO₂ asphyxiation, their eyes were immediately dissected into 0.25 M sucrose (with 1 mM EDTA, 10 mM Tris–HCl, pH 7.2) at 4°C and homogenized for 30 sec in a Virtis Polytron Sonicator. The homogenate was then centrifuged at 500 g for 10 min to pellet unbroken cells and debris; the resulting supernatant fraction was spun at 10,000 g for 30 min to pellet the melanosomes. These were then resuspended in 0.25 M sucrose and centrifuged at 500 g for 10 min and 10,000 g for 30 min as before; the pellet was again resuspended and subsequently layered on 30% sucrose and pelleted at 20,000 g for 30 min. The melanosomes were washed twice more through 30% sucrose, then lyophilized and stored frozen. For solubility studies, portions of the lyophilized pellet were weighed out, then stirred for 24 hr at 20°C or 70°C in the solvents as described in the tables. The digests were then spun at 100,000 g.

1 Abbreviations used: DOC, deoxycholate, sodium; SDS, dodecyl sulfate, sodium; NaDS, decyl sulfate, sodium; ami-diol, 2-amino-2-methyl-1,3-propanediol.
g for 30 min, and protein concentration determinations were run on the supernatant fractions. Protein concentrations were determined by UV absorption and the Lowry method (2).

**Electron Microscopy**

Specimens were fixed in 6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 2 hr at 4°C, washed overnight in buffer with 0.1 M sucrose, then postfixed in 1% OsO₄ for 2 hr. After dehydration in a graded series of ethanol, tissues were embedded in Epon, sectioned on an LKB Ultratome II, stained with uranyl acetate and lead citrate, then viewed in a Siemens IA electron microscope.

**Polyacrylamide Gel Electrophoresis**

The electrophoresis system used was the Tris–glycine system described by Jovin et al. (3), system 400; when the NaDS¹–gel system was run, 0.02 M NaDS was added to the upper buffer (11); when the 8 M urea gel system was run, 8 M urea was added to all phases of the system. The ami-diol gel system and the imidazole gel system used were numbers 2964 and 1241, respectively (3). Bromphenol blue was used as a tracking dye, but relative mobilities were calculated against the migration of the true front (4). Gels were stained with either fast green FCF or Coomassie blue, then scanned at 700 nm with a Gilford spectrophotometer, Model 2000, equipped with a linear transport scanner.

**IR–VIS–UV Spectroscopy**

Samples to be scanned spectrophotometrically were dialyzed against water exhaustively, then scanned with a Beckman DK-2A Ratio Recording Spectrophotometer.

**RESULTS AND DISCUSSION**

**Melanosome Isolation**

Since previously published preparative procedures for melanosome fractions showed a considerable amount of contamination as assayed by electron microscopy (5, 6), it was considered critical to purify further the melanosomal preparation, and centrifugation through 30% sucrose (taking advantage of the extremely high density of the granules) yielded very pure fractions. Figure 1 is a light micrograph of the melanosomal fraction prepared as described; Fig. 2 is an electron micrograph of a representative portion of the same pellet. Little evidence of contaminating membranes or other particles is visible.

**Melanosome Solubility**

In Table 1, the results of initial solubilization attempts on the melanosomal preparation are presented. As can readily be seen, most of the solvents used had little or no effect on melanosomal protein solubilization. All three detergents tested (DOC, SDS, and NaDS), however, solubilized a small amount of the melanosomes, and a representative detergent (NaDS), as well as 8 M urea, were studied more closely. In addition, since melanin has previously been solubilized by NaOH rather readily, two additional solvents at high pH (imidazole and ami-diol) were also used. In Table 2, the results of melanosomal solubilization at an elevated tempera-
Fig. 1. Light micrograph of melanosome fraction, prepared as described in Methods. Line = 100 μm.

Fig. 2. Electron micrograph of melanosome fraction. Little contamination of any type is visible. Line = 1 μm.
TABLE 1
INITIAL SOLUBILIZATION STUDIES ON MELANOSOMES

| Solvent                          | Protein concn (mg/ml) 100,000 g supernatant fraction |
|----------------------------------|-----------------------------------------------------|
| Acetic acid (0.1 M)              | 0.00                                                |
| Ami-diol (0.1 M)                 | 0.00                                                |
| Ammonium hydroxide (0.02 M)      | 0.00                                                |
| Butyl alcohol                    | 0.00                                                |
| Decyl sulfate (0.1 M)            | 0.11                                                |
| Deoxycholate (0.1 %)             | 0.10                                                |
| Dodecyl sulfate (0.1 M)          | 0.10                                                |
| Guanidine–HCl (6 M)              | 0.00                                                |
| Guanidine-thiocyanate (1 M)       | 0.00                                                |
| Imidazole (1 M)                  | 0.00                                                |
| Phenol–acetic acid (40:60)       | 0.05                                                |
| Triethanolamine                  | 0.00                                                |
| Urea (8 M)                       | 0.15                                                |

* Initial concentrations were 1 mg/ml for all solvents; left stirring for 24 hr at 70°C.

TABLE 2
FURTHER SOLUBILIZATION STUDIES ON MELANOSOMES

| Solvent             | Protein concn (mg/ml) 100,000 g supernatant fraction |
|---------------------|-----------------------------------------------------|
| Ami-diol (1 M)      | 0.08                                                |
| Imidazole (1 M)     | 0.11                                                |
| Decyl sulfate (0.1 M) | 0.75                                      |
| Urea (8 M)          | 0.50                                                |

* Initial concentrations were 1 mg/ml for all solvents; left stirring for 24 hr at 70°C.

ture (70°C) are shown. Ami-diol and imidazole solubilized a relatively small amount of the preparation, while urea and NaDS solubilized the melanosomes almost completely.

Disc Gel Electrophoresis

All four of the solubilized fractions reported in Table 2 were subjected to acrylamide gel electrophoresis and Fig. 3 shows the resulting banding patterns. Both the imidazole and amido-diol gel system show a single band only, a very small molecule (less than 1000 in MW) which migrates with the front. In the case of the imidazole gel system, this band is darkly pigmented, suggesting that it is either melanin or a melanoprotein. The latter possibility is most likely since the darkly pigmented band can also be stained with several protein stains. In the amido-diol gel system, a low molecular-weight band only is seen, with no pigment visible. Urea and NaDS gels display seven bands each. Figure 4 is a scanning profile of a NaDS gel as seen in Fig. 3. When the melanosome fraction is initially extracted with NaDS for several hours only at 70°C, then centrifuged at 100,000 g for 30 min and the pellet reextracted with NaDS, electrophoresis of the resulting supernatant fraction shows only one protein band—2, as well as the pigment migrating
Fig. 3. Polyacrylamide gels of electrophoresed melanosomal proteins. Gels were run as described in methods, from left to right: 0.02 M NaDS, 8 M urea, 0.1 M imidazole, and 0.1 M amido-diol.

Fig. 4. Scanning profile of melanosome proteins separated on 6% gel, 0.02 M NaDS. Arrow marks location of bromphenol blue. Repeated extraction demonstrates only peak 2.

with the front. We have interpreted this to indicate that the six other proteins are probably located externally on the granule, quite possibly being the constituents of the limiting membrane, while the remaining protein (band 2) is located internally with the melanin and might represent the structural fibrillar protein of the melanosome. The seven bands depicted in Fig. 4 were subjected to analysis by electrophoresis in conjunction with the data-processing programs as described by Rodbard and Chrambach (4), and Table 3 shows several of the physical param-
TABLE 3

PHYSICAL CHARACTERISTICS OF MELANOSOME PROTEINS

| Protein # | Rel. Mob. | Kr  | MW (±5%) | Radius (Å) |
|-----------|-----------|-----|----------|------------|
| 1         | .508      | .0371 | 6,400    | 12         |
| 2         | .398      | .0551 | 74,300   | 28         |
| 3         | .264      | .1444 | 410,000  | 49         |
| 4         | .209      | .1596 | 470,000  | 52         |
| 5         | .098      | .1122 | 290,000  | 44         |
| 6         | .081      | .2147 | 680,000  | 58         |
| 7         | .065      | .2315 | 740,000  | 60         |

Gel electrophoresis system used was #400, with 0.02 M NaDS, as described in Methods. Relative mobilities reported were at 6% T, 3% C. For further explanation of parameters arrived at, or equations used for their calculation, cf. reference 4.

Parameters of these proteins as obtained through this data-processing output. There are several major points of interest in this table; first, the reported molecular weight of protein band 2 (74,000) is very close to the accepted molecular weight of the mammalian tyrosinase monomer (T1 66,000) (7), in fact, it is the only protein band which even approaches this molecular weight (see Table 3). Second, it is interesting to note that the approximate mean radius of protein band 2 is 28 Å. This latter value is based on several tentative assumptions (4), but 28 Å is still definitely in the range of the 30 to 35-Å figure already reported to be the thickness of the melanosomal fibril (8, 9), and again it is the only protein band which is in such a range. Both these pieces of data support the view that protein band 2 is actually the fibrillar protein, and that this protein, in turn, might also represent the tyrosinase located in the melanosome.

Spectroscopy

When the urea-solubilized melanosome supernatant fraction is electrophoresed and the darkly pigmented band which runs with the front is collected, dialyzed exhaustively against water, then scanned spectrophotometrically, a profile such as that presented in Fig. 5 is seen. The uniform absorbance displayed here has been reported to be characteristic for melanin (10).

![Spectrophotometric absorbance scan](image_url)

**Fig. 5.** Spectrophotometric absorbance scan of melanin solubilized by urea. Uniform absorbance in this range is characteristic for melanin.
SUMMARY

1. Two solvents, 0.1 M NaDS and 8 M urea, are capable of completely solubilizing a melanosome preparation, without degrading the proteinaceous components.

2. This solubilization can be achieved in approximately 24 hr at 70°C at a concentration of 1 mg dry weight melanosome per ml solvent.

3. Analysis of solubilized melanosome preparations by gel electrophoresis in the presence of 8 M urea or 0.1 M NaDS reveals the presence of seven protein bands, as well as a small molecular-weight, darkly pigmented band which runs with the front.

4. This darkly pigmented band represents melanin or a melanoprotein as evidenced by its spectrophotometric absorbance pattern.

5. The possibility that band 2 in the NaDS gel system may represent the structural fibrillar protein and/or the site of tyrosinase activity must be considered.

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