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The structure of neuromelanin and its iron binding site studied by infrared spectroscopy

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Abstract The binding of neuromelanin (NM) to iron is of interest due to its role in brain aging and Parkinson's disease. In the present work, infrared spectra of both NM isolated from human brain and of synthetic NM analogues are reported with the aim of identifying the main functional groups and their chelating ability for iron. It is observed that a peptide and an aliphatic chain are present in the NM structure. The coordination of iron in NM occurs through –OH phenolic units. In synthetic melanin samples, the preferred sites for iron binding are –OH phenolic and >NH indolic groups. Amino acid analysis confirmed the presence of a peptide component in NM and synthetic melanin incubated in putamen homogenate. In addition, the elemental analysis demonstrated the presence of an aliphatic component specific of NM.

Key words: Neuromelanin; Infrared spectroscopy; Melanin; Parkinson's disease; Iron

1. Introduction

Neuromelanin (NM) is an insoluble black-brown granular pigment that accumulates in neurons of the substantia nigra (SN) and locus coeruleus. Its neuronal content in man increases during life up to a maximum at around 60 years and then decreases afterwards [1]. This pigment is considered as a melanin in all respects [2,3], sharing several characteristics with the pigment of eyes and hair, such as the presence of a stable free radical, low solubility, the affinity for transition metals and others [4,5]. The precursors of NM synthesis are probably sulfated derivatives of dopamine and it is still questioned whether its synthesis goes through autoxidation or enzyme involvement. Several papers in the last years have been devoted to the study of the complex iron-NM [4,6–10] but the role of the pigment in modulating the neuronal dynamics of metal ions is not yet well understood. The most recent studies propose a 2-fold role for NM: (1) cytoprotective in the binding of toxic metal ions, (2) cytotoxic in the generation of reactive radical species and subsequent peroxidation processes [11]. In Parkinson's disease brains, the neuronal loss is accompanied by an increase of nigral iron [12,13] and considering the known ability of NM in chelating iron, it appears that this pigment may play a role in the degenerative processes of SN cells [4,6]. In fact, Fenton chemistry predicts that metal ions, such as iron and copper, may significantly contribute to the formation of toxic species ($OH^{\bullet}$, $O_2^{\bullet -}$, H_2O_2 or singlet dioxygen). Research into the involvement of NM in the neurodegenerative processes cannot disregard the elucidation of the structure of this biopolymer directed at the identification of the number and the kind of the binding sites for the metals. Infrared (IR) spectroscopy is an important non-destructive method providing information on functional groups present in small size samples. In early studies [14], IR spectroscopy was used to investigate the structure of dopamine melanin as a model of human brain pigment and identified the following groups as preferential binding sites for iron: –OH carboxylic and phenolic, >NH indolic. More recently [15], a FTIR spectrum of NM isolated from SN showing the presence of aliphatic chains has been reported, using samples of NM isolated from individual subjects, thus demonstrating the homogeneity of the NM structure in different subjects.

The IR study of NM in the present work has been performed by a 2-fold strategy: (1) to compare the native NM sample, therefore rich in iron, with the same sample after treatment with a strong metal chelator (disodium ethylenediaminetetracetate (EDTA)), (2) to compare IR spectra of NM with those of different synthetic melanins prepared with different procedures and iron content.

2. Materials and methods

2.1. NM

Samples of human SN were obtained during the autopsy of patients (age between 50–80 years) who died of non-neurological and non-degenerative diseases. Autopsies were carried out within 48 h after death. The tissue samples were frozen in dry ice and kept at −80°C until the isolation procedure. SN tissues were homogenized in water and shaken thoroughly. After centrifugation, the obtained pellet was washed twice with 50 mM phosphate buffer (pH 7.4) and then incubated for 3 h with a solution containing Tris buffer (75 mM, pH 7.4) sodium dodecylsulfate (35 mg/ml). The suspension was centrifuged and the precipitate incubated for 3 h, again with the same medium as above also containing proteinase K (0.5 mg/ml). The NM was washed twice with NaCl solution (9 mg/ml) and water. Finally, it was washed with methanol, hexane and evaporated to dryness under a nitrogen flow at 40°C. The sample was divided into two parts, one was washed twice with water and freeze-dried and the other one was suspended in 0.15 M EDTA-Na_2 and incubated at room temperature for 8 h. After centrifugation at 10 000 rpm for 10 min, the pellet was resuspended in fresh EDTA-Na_2 solution and incubated for an additional 14 h, then centrifuged and the resulting pellet was freeze-dried.

2.2. Synthetic melanins

These samples were prepared by autoxidation and enzymatically.

Sample A: this polymer was obtained by autoxidation of dopamine and cysteine. A 300 mg amount of dopamine and 39.5 mg cysteine were dissolved in a 200 ml volume of 0.050 M phosphate buffer (pH 7.4) solution. The solution was allowed to autoxidize in air at 37°C for 3 days (protected from light), centrifuged at 5000 rpm for 30 min and then washed twice, first in 1% acetic acid and in 9% NaCl. The pellet was finally treated with EDTA as described for the natural

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Abbreviations: EDTA, disodium ethylenediaminetetracetate; NM, neuromelanin; SN, substantia nigra; IR, infrared

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sample, then washed twice in bidistilled water and dried under a N_2 flow.

Sample A-Fe: part of the sample A was exposed to iron. It was rehydrated (0.5 mg/ml) in 50 mM citrate buffer (pH 7.2) for 10 h, then, it was incubated with a 1 mM solution of $Fe_2(SO_4)_3$ in the same citrate buffer for 2 days in air. The pellet was washed one time with citrate buffer, two times with bidistilled water and then dried under a N_2 flow.

Sample E: this synthetic NM was obtained by enzymatic oxidation (tyrosinase) of dopamine and cysteine. Into one flask containing 200 ml of 0.05 M sodium phosphate buffer (pH 7.4) were dissolved, 60 mg dopamine, 7.4 mg cysteine and 7 mg tyrosinase. The solution was allowed to react at 37°C for 2 days. The reaction was stopped by adding CH_3COOH ($3 < pH < 4$). The solution was sonicated for 10 min and centrifuged at 5000 rpm for 30 min. The precipitate was suspended in 1% acetic acid, centrifuged and resuspended in 0.5% SDS+50 mM Tris (pH 7.4). The solution was incubated for 2 h at 37°C and then washed with NaCl 0.9%. The pellet was finally treated with EDTA as described for the natural sample, then washed twice in bidistilled water and dried under a N_2 flow.

Sample E-Fe: part of sample E was exposed to iron according to the procedure described for sample A-Fe.

Sample AP: sample A was suspended in water, sonicated in an ultrasound bath to obtain a fine suspension. The latter was placed into a tube containing freshly dissected putamen tissue and the mixture was thoroughly homogenized, then incubated at 37°C for 48 h under a flowing gas of O_2/CO_2 (90/100). At the end, melanin was isolated as described above for the isolation of NM from SN.

2.3. Methods

IR absorption spectra were performed in a dispersive spectrophotometer Jasco DS-702G, operating in the wavelength range 4000–200 cm^{-1}. The samples were prepared as pellets (diameter 13 mm and thickness ~0.20 mm) of melanin (~1 mg) in KBr (70 mg), the weight ratio being chosen to avoid saturation effects in the absorption bands. Before performing IR absorption measurements, the specimens were stored in a dry box in the presence of P_2O_5 for 1 week to assure the same hydration degree in all of them.

Elemental analysis (CHNS %) was carried out as previously reported by the combustion technique [15]. The content of amino acids in NM and sample AP was determined by using the hydrolysis with 6 M HCl containing 0.5% mercaptoethanol and 0.01% phenol under nitrogen into a sealed glass tube, which was heated at 110°C for 10 h. Then, the amino acids were analyzed by HPLC [16].

3. Results

3.1. NM

Fig. 1 shows the IR spectra of the NM isolated from SN as extracted (NM) and the same sample treated with EDTA (NM+EDTA), according to the described procedure.

Detailed analysis of the NM spectrum provides information on the main chemical groups responsible for the vibration at characteristic wavenumbers: OH stretching (3440 cm^{-1}), >NH stretching (3160 cm^{-1}), $-CH_3$ asymmetrical stretching (2970 cm^{-1}), $>CH_2$ stretching (2946 cm^{-1}), $-CH_3$ symmetrical stretching (2860 cm^{-1}), C=O stretching (1694 cm^{-1}), >N-H bending (1528 cm^{-1}), $-CH_2-CH_3$ bending (1425 cm^{-1}), C–N stretching (1368 cm^{-1}), phenolic C–OH stretching (1245 cm^{-1}), C–O stretching (1150 cm^{-1}), C–O–C asymmetrical stretching (1060 cm^{-1}).

The strong C=O band (1694 cm^{-1}) together with the absorption at 1150 cm^{-1} is a typical feature of ketones, as the sharp carbonyl band at 1694 cm^{-1} is indicative of the conjugation of the C=O with a benzene ring, which corresponds to the typical quinone structure of melanins. The two features at 1528 and at 1368 cm^{-1} are strongly suggestive of a pyrrole or indole >NH group. It is worthwhile noting that the three bands around 2950 cm^{-1} and the feature centered at 1060 cm^{-1} appear as distinctive characters in the spectrum of the NM from SN, as is evident by comparing this with the spectra of other natural melanins previously investigated [14,17] and those of the synthetic ones as shown afterwards (Fig. 2). In addition to the typical character of the aromatic structure of the sample, the CH triplet between 2930 and 2860 cm^{-1} and the peak at 1425 cm^{-1} demonstrate the presence of a substantial amount of aliphatic groups in the NM structure. The shoulder at 1060 cm^{-1} appears to be more difficult to attribute, as the interpretation is limited by the complexity of a system like NM. The involved wavenumber domain suggests an association with C–O–C groups stretching vibrations, but the comparison with the spectra of the synthetic samples would suggest an alternative explanation as reported in Section 4.

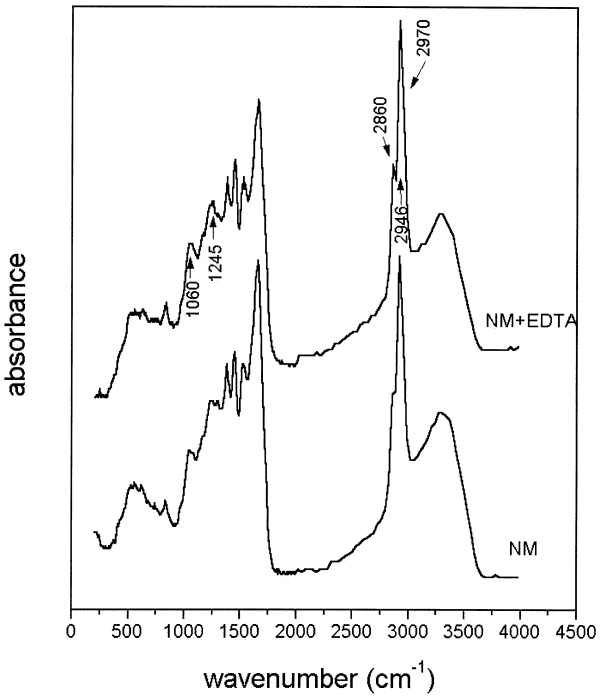

Fig. 1. IR spectra of isolated NM and the same sample after treatment with EDTA.

The IR spectrum of NM treated with EDTA (Fig. 1) appears to be very similar in all the main features to that of NM but the band at 1245 cm^{-1} is enhanced with respect to the corresponding feature in the spectrum of the untreated sample. Since –C–OH phenolic groups exhibit absorption in this spectral region and it is known that the position of the C–O stretching band is strongly influenced by the substitution of –H, here, the replacement with a heavy metal such as an iron atom could drastically modify the absorption frequency of the group by shifting the band to a lower wavenumber region. Then, the observed increase of amplitude in the –C–OH absorption band after EDTA treatment further demonstrates that this group is involved in binding to iron. According to our previous determinations (L. Zecca, personal communication), the starting sample of NM contains 10.9μg/mg iron and after the mentioned EDTA treatment, the content is 6.4 μg/mg. The rate of decrease in the iron content is proportional to the rate of increase in the amplitude of the 1245 cm^{-1} band.

3.2. Synthetic melanins

In Fig. 2, the IR spectra of all the synthetic melanins ob-

tained by the various procedures are shown. The main bands are given here and the corresponding functional groups: C═O asymmetrical stretching (1620 cm^{-1}), C═C stretching (aromatic system) (1600 cm^{-1}), N–H bending (indole ring) (1513 cm^{-1}), C═O symmetrical stretching (1450 cm^{-1}), C–N stretching (indole ring) (1365 cm^{-1}), –C–OH stretching (phenolic) (1280 cm^{-1}). By comparing the spectrum of melanin obtained by autoxidation of cys-dopamine (sample A) with the spectrum of melanin polymerized by tyrosinase from the same precursors (sample E), it appears that the two polymerization pathways drive to very similar complexes. In fact, the IR spectra show a large number of common features. The amplitude of the absorption bands in the spectrum of sample E appear to be generally weaker in respect to sample A and in particular in the region ~1600 cm^{-1} and in the 1200–1400 cm^{-1} spectral domain. The reduced amplitude and the enlargement of the band at 1600 cm^{-1} and the appearance of a shoulder at 1620 cm^{-1} in the sample E spectrum are likely due to the increase of the C═O number with respect to the amount of the aromatic C═C groups as a consequence of the oxidizing activity of tyrosinase on simple mono- and *o*-diphenols. This is consistent with results from chemical degradation studies [18]. No evidence has been recorded of enzyme incorporation in the sample polymerized in the presence of tyrosinase, since the typical amide bands are absent. The main changes in the spectra of the two samples after iron

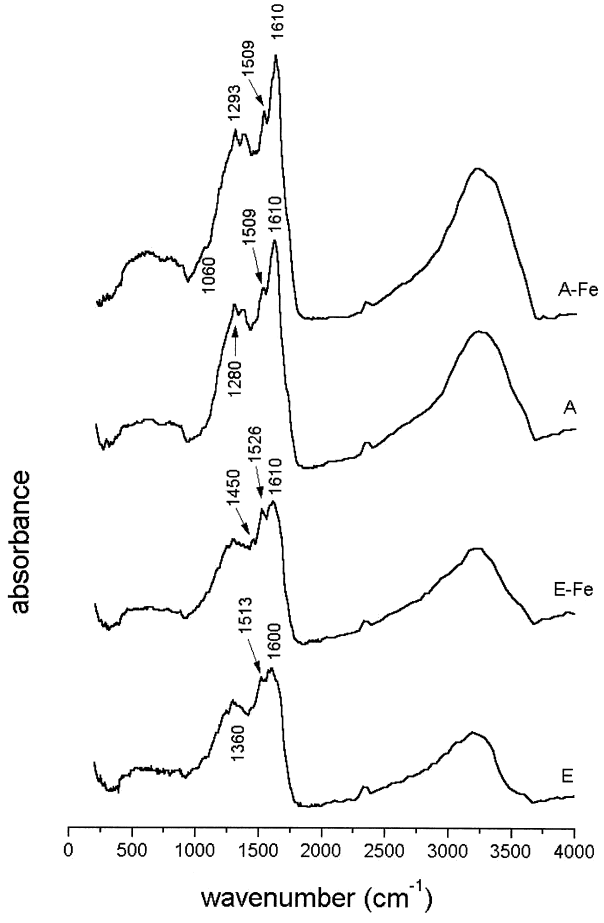

Fig. 2. IR spectra of synthetic melanins prepared by autoxidation (A) and enzymatically (E) and the same samples after treatment with iron (A-Fe, E-Fe).

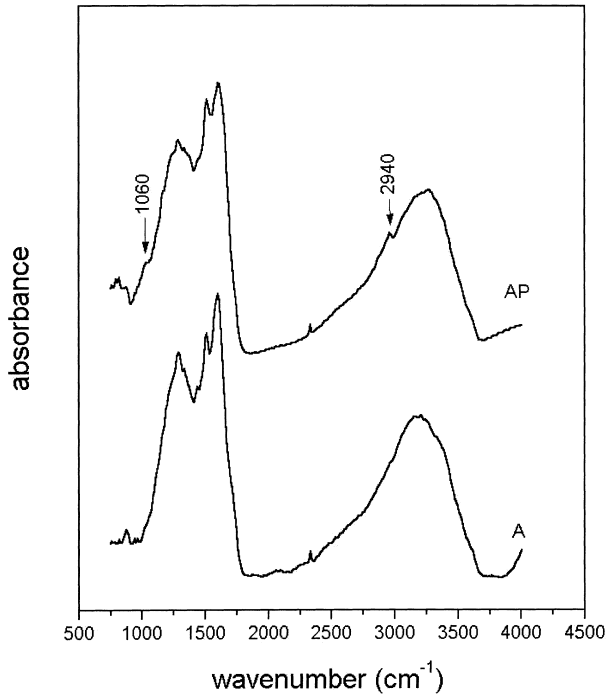

Fig. 3. IR spectra of synthetic melanin prepared by autoxidation (A) and the same sample after incubation in putamen homogenate and isolation (AP).

binding can be summarized as follows. In the sample E-Fe spectrum, the band at 1513 cm^{-1} shifts at 1526 cm^{-1} and a little feature appears at 1450 cm^{-1}, the absorption region around 1360 cm^{-1} is flattened. In the sample A-Fe spectrum, the band at 1280 cm^{-1} is shifted towards a high wavenumber region like 1293 cm^{-1} and a shoulder appears at 1060 cm^{-1}. Due to the group attribution in the spectral regions affected by the iron addition, it is easy to recognize that in the enzymatically prepared sample, probably both phenolic OH and indolic NH groups appear to be involved while in the sample polymerized by autoxidation, only phenolic groups are bound to iron.

However, in the spectra of samples E and E-Fe, the C–OH stretching bands do not appear clearly in the region 1200–1300 cm^{-1} due to their overlapping with many peaks. Then, the shift and intensity variations caused by the iron binding are hidden.

3.3. Synthetic melanin incubated in brain tissue homogenate

The analysis of the sample AP spectrum deserves particular noting. As shown in Fig. 3, the spectrum of the synthetic polymer incubated in putamen (AP) displays two additional absorptions if compared with sample A. In the narrow wavenumbers range around 2940 cm^{-1}, a little but net feature appears and at 1060 cm^{-1}, a shoulder is seen which resembles a characteristic absorption of NM spectrum. The band at 2940 cm^{-1} can be assigned to the alkyl groups of amino acids as shown by the determination of amino acids (Table 1) in this sample. During this incubation, the melanin A chemically binds peptides from brain tissue. The enlargement of the band at 1660 cm^{-1} is consistent with the overlapping of the C═O stretching band characteristic of the pigment spectrum with the amide I band typical of the amino acidic component derived from the incubation matrix. Another important change

Table 1
Elemental and amino acid contents (mean ± S.E.M., $n = 2$) of different melanins are reported

	% C	% H	% N	% S	C/H (molar ratio)	Amino acid content (nmol/mg)
Synthetic melanin A	50.66 ± 0.16	3.65 ± 0.05	8.41 ± 0.05	3.17 ± 0.08	1.14 ± 0.02	–
Synthetic melanin incubated with putamen AP	51.10 ± 0.75	5.12 ± 0.13*	7.22 ± 0.11*	3.07 ± 0.10	0.83 ± 0.01*	618.40 ± 70.20
NM from SN	60.00 ± 1.23#	7.66 ± 0.50#	6.09 ± 0.42	2.50 ± 0.01#	0.65 ± 0.03#	741.90 ± 72.80

Calculated C/H molar ratios are also shown for each sample.
*t-Test: $P < 0.05$, synthetic melanin A versus synthetic melanin incubated with putamen AP.
#t-Test: $P < 0.05$, synthetic melanin incubated with putamen AP versus NM from SN.

that occurred after incubation in putamen is the decrease of the intensity of the –C–OH band because of the documented uptake of the iron contained in brain tissue [8]. A shift of the absorption to a lower wavenumber is also observed again due to the binding of iron.

3.4. Elemental and amino acid content

The synthetic melanin (A) was prepared and incubated in putamen homogenate in order to mimic the behavior of NM. In Table 1, the elemental analysis shows that the percentage content of carbon and sulfur does not change after incubation of melanin in putamen homogenate. On the contrary, the hydrogen percentage increases and that of nitrogen decreases significantly because of the incorporation of peptides as shown by the total amino acid concentrations. The compositions of carbon, hydrogen and sulfur in NM from SN are all significantly different from those in synthetic melanin (AP) after incubation in putamen, while the amino acid content was similar in both types of melanins. The molar ratio C/H was calculated as a marker of the unsaturation degree of these samples. In fact, such a ratio was significantly higher in the synthetic melanin (A) compared to the same melanin after incubation in putamen (AP) since a peptide component was bound during this treatment. The melanin after incubation (AP) had a C/H molar ratio significantly higher than NM, even if AP and NM samples had the same content of amino acids. Then, these data show a lower degree of unsaturation in NM, thus further supporting the presence of aliphatic groups in NM.

4. Discussion

In this work, the main bands present in spectra of NM and synthetic melanins have been associated with characteristic functional groups in agreement with the attributions described by several authors [15,19,20].

In comparison to the model melanins, the NM spectrum appears to be dominated in the high wavenumber region by a strong and differentiated absorption at about 2900 cm^{-1} due to alifatic chains as shown also by elemental analysis and previous mass spectrometry measurements [15]. The origin of these aliphatic components at the present is quite unknown. This is typical of NM as synthetic melanin incubated in putamen homogenate shows only a weak band in this region due to the peptide frame. The second important feature of the NM spectrum is at 1060 and at 1660 cm^{-1} and relates to the peptide component which was also evidenced by amino acid analysis. FTIR spectra performed on some phytomelanins [21] highlighted the existence of a similar absorption at 1060 cm^{-1} in the spectra of these compounds, attributed by the authors to the incorporation of protein moieties in the pigment structure. This hypothesis is in agreement with the analysis of the sample AP spectrum which demonstrates that synthetic sample A, after incubation in a brain area like putamen (sample AP), exhibits both the described absorption bands. The third important feature of the NM spectrum is the absorption at 1245 cm^{-1}, corresponding to a –C–OH phenol group which participates in the chelation of iron. As shown by XAFS studies [10], iron is bound to oxygen-derived phenolic groups in an octahedral configuration. However, IR spectroscopy shows quite different wavenumbers for the expected C–OH stretching in NM, autoxidation melanin and enzymatic melanin. This change in wavenumbers could result from the presence of different substituting groups in the aromatic ring of NM and synthetic melanins.

Synthetic analogues of NM obtained by autoxidation or by enzymatic polymerization exhibit –OH phenolic and indolic $>$NH residues at sites mainly involved in the binding to the metal. The NM and synthetic melanins exhibited several structural similarities and the chemical groups involved in the coordination of iron seem to be the same, with the exception of the E-Fe sample where indole groups seem to be involved in iron chelation. In addition to the main peaks here discussed, there are several weak signals in the reported spectra but we have no hypothesis for an attribution of these.

The lack of good structural models for NM and other natural melanins places limits on the interpretation of some spectroscopic results but it can be suggested that autoxidative compounds seem to resemble NM more from the structural point of view. The results of this work on the type of coordination of iron agree quite well with previous EPR, Mössbauer and XAFS studies [8,10,22]. However, several open questions remain on the organic structure of NM.

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