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Iron-binding characteristics of neuromelanin of the human substantia nigra

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Abstract

The vulnerability of the dopaminergic neurons of the substantia nigra (SN) in Parkinson's disease has been related to the presence of the pigment neuromelanin (NM) in these neurons. It is hypothesised that NM may act as an endogenous storage molecule for iron, an interaction suggested to influence free radical production. The current study quantified and characterised the interaction between NM and iron. Iron-binding studies demonstrated that both NM and synthetically-produced dopamine melanin contain equivalent numbers of high and low-affinity binding sites for iron but that the affinity of NM for iron is higher than that of synthetic melanin. Quantification of the total iron content in iron-loaded NM and synthetic melanin demonstrated that the iron-binding capacity of NM is 10-fold greater than that of the model melanin. This data was in agreement with the larger iron cluster size demonstrated by Mössbauer spectroscopy in the native pigment compared with the synthetic melanin. These findings are consistent with the hypothesis that NM may act as an endogenous iron-binding molecule in dopaminergic neurons of the SN in the human brain. The interaction between NM and iron has implications for disorders such as Parkinson's disease where an increase in iron in the SN is associated with increased indices of oxidative stress.

Keywords: Neuromelanin; Iron binding; Mössbauer spectroscopy; Parkinson's disease; Ferritin

1. Introduction

In recent years the oxidative stress hypothesis of neurodegenerative diseases has been intensively examined. Oxidative stress is suggested to be important in PD, either as a primary causal factor or alternatively as a secondary contributory factor. Pivotal to this hypothesis is the finding that iron homeostasis is changed in PD.

Neurochemical, physical, histochemical and imaging techniques have demonstrated increased iron levels in PD patients and in the parkinsonian SN post-mortem [4,6,11,15,32,36]. The reason for this localised increase in iron is unknown but it is hypothesised that iron may increase oxidative load because of its ability to stimulate free radical production [5,15,23,37]. SN neurons in the

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Abbreviations: cysDAM, synthetic dopamine melanin prepared with cysteine; DAM, synthetic dopamine melanin; Isol. DAM, synthetic dopamine melanin subjected to neuromelanin isolation procedure; NM, neuromelanin; PD, Parkinson's disease; Protein-NM, neuromelanin isolated with proteinase inhibitor; SN, substantia nigra.

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human are characterised by the presence of a dark polymer pigment NM, the presence of which has been directly related to their fate in PD [19,22,24]. Melanins in other body tissues actively protect against oxidative damage [25,29,33,34], possibly as a consequence of their ability to bind reactive molecules, such as transition metals. The formation of an apparently similar pigment in the SN, a tissue which suffers a high intrinsic oxidative load [17], suggests a parallel function in the brain. In support of this hypothesis NM and DAM attenuate oxidative membrane damage *in vitro* [5,12]. In the presence of high concentrations of iron, however, DAM acts as an effective pro-oxidant, rather than as an antioxidant [5,31,38]. This effect is attributed to the binding of iron to melanin and the subsequent reduction of bound ferric iron to a more weakly bound ferrous state, stimulating hydroxyl radical production [31]. Further, incubation of human NM or DAM with iron *in vitro* stimulates, rather than decreases, oxidative tissue damage [5,13,28,30]. NM has been suggested to act as an iron storage molecule *in vivo* but this function might also increase the potential for oxidative damage within the vulnerable dopaminergic neurons if tissue iron levels are increased, for example in PD [5,20,23,27]. We have previously shown that DAM binds iron in a quantifiable manner *in vitro* [5] but we have also shown that DAM differs structurally to NM [14]; thus it is unclear if this synthetic molecule is an adequate model of the native pigment. NM-bound iron has been quantified in the post-mortem brain [20,23] but the iron-binding characteristics of NM cannot be determined in post-mortem tissue. The aim of the current study was to characterise for the first time the binding of iron to human NM.

2. Experimental procedures

2.1. Preparation of melanins

NM was isolated from the SN of neurologically normal adult individuals as previously described [14]. The SN were dissected from the brain within 40 hr of death at −10° and pooled in a glass–Teflon homogeniser. The samples were homogenised in 20 mL water and centrifuged at 12,000 *g* for 10 min. The resulting pellets were washed twice with 50 mM phosphate buffer (pH 7.4), then incubated in 50 mM Tris buffer (pH 7.4) containing 0.5 mg/mL SDS at 37° for 3 hr, followed by a further 3-hr incubation with addition of 0.2 mg/mL proteinase K in the same buffer which depletes the amino acid content of NM by 65% [14]. The pellets were pooled and consecutively washed with saline, water, methanol and hexane. Finally, the resulting dark pellet was incubated for three periods of 8 hr each in 150 mM EDTA (pH 7.4) before being washed twice with water and dried under vacuum. Additional samples of SN tissue were prepared in the same manner in the absence of proteinase K but with the addition of a proteinase inhibitor cocktail (Sigma Chemical Co.) to preserve the proteineaceous component of NM (Protein-NM, [14]). NM was also isolated using SN tissue previously fixed in 5% phosphate-buffered formaldehyde for a minimum of 18 months. DAM was prepared as previously described [14]. Two millimolar dopamine (DA; Sigma) was incubated in 50 mM Tris buffer (pH 8) containing 0.1 mM $CuSO_4$ for 24 hr at 26°. Oxidation was stopped by the addition of concentrated HCl to pH 2, and the resulting liquid was centrifuged at 9000 *g* for 15 min. The pellet was resuspended three times in 0.01% KCl and centrifuged at 27,000 *g* for 20 min; the final pellet was resuspended by homogenisation in 0.01% KCl and dialysed in double distilled water at 4° for 48 hr. The resulting melanin was finally lyophilised. As a control for the isolation procedures, one sample of prepared DAM was subsequently submitted to the same procedure used for the isolation of NM from the human brain (Isol. DAM). A further sample of cysDAM at a molar ratio of 6:1 dopamine:cysteine as previously described to more accurately reflect the chemical composition of native NM [16,41]. All melanin samples were lyophilised and stored protected from light at 4°.

2.2. In vitro melanin binding

Binding experiments using melanin suspensions were performed according to the modified method of Ben-Shachar *et al.* [5]. Directly prior to use, all melanin samples were resuspended in double distilled water and sonicated for 3 min, a drop of the solution was examined microscopically to ensure the presence of a fine, homogenous melanin suspension. The specific binding of 19 concentrations of $^{59}FeCl_3$ (1–400 nM, 28.22 mCi/mg) to 3 μg of human NM or DAMs was assayed in the presence of 4 or 1 mM $FeCl_3$ in a final volume of 250 μL in 5 mM Tris buffer (pH 7.4 at 37°). The incubation period of 2 hr at 37° was terminated by the addition of 2 mL cold buffer and filtration through GF/B filters. The filters were washed three times each with 2 mL ice-cold buffer and radioactivity quantified by liquid scintillation spectrometry. Non-specific binding at 10 μM $^{59}FeCl_3$ represented 24% of total binding, while binding of 10 μM $^{59}FeCl_3$ to filters incubated with 10 or 100 nM $FeCl_3$ in the absence of melanin represented 16 and 8% of binding in the presence of 3 μg melanin, respectively. In further experiments to define the low-affinity binding site of NM 12 μg NM or DAM were incubated in the presence of 150 nM $FeCl_3$ and 15 concentrations of $^{59}FeCl_3$ ranging from 1 μM to 5 mM and binding determined in 5 mM Tris buffer (250 μL final volume, pH 7.4) following a 2 hr incubation at 37° as described.

2.3. Mössbauer spectroscopy

NM was isolated from normal adult human brain as described above. Following lyophilisation, NM and synthetic melanin were enriched with ^{57}Fe as this isotope has a

typical Mössbauer effect. 10.08 mg ^{57}Fe was dissolved in 200 mL 37% HCl and 100 mL of 70% HNO_3 in a glass vial and reduced to approximately 0.1 mL at 90°. A 300-mL aliquot of 37% HCl was added and the solution re-concentrated to approximately 0.1 mL by heating at 90°. This $^{57}FeCl_3$ solution was added to 3.7 mL 1 M citrate buffer (pH 7.3) and stored at −70°. Thermogravimetric analysis of isolated NM indicate that the molecule is stabile under these conditions [40].

To prepare ^{57}Fe NM or synthetic melanin, samples were suspended in 1 M pH 7.3 citrate buffer (0.5 mg/mL) and reduced to a fine suspension by shaking for 48 hr. Samples (2 mL) of NM or synthetic melanin suspension were shaken at 4° with 0.5 mL of ^{57}Fe citrate solution for 72 hr. The resultant suspension was centrifuged, the pellet washed with 1 mL of citrate buffer then twice with 2 mL of water and finally lyophilised. The iron content of the prepared native and synthetic melanin (0.8–1 mg) were measured using electrothermal atomic absorption spectroscopy and quantified by the use of external standards.

Mössbauer spectra from samples of approximately 2.5 mg iron-loaded NM or DAM were measured in a cryostat in the horizontal transmission geometry using a constant-acceleration spectrometer in conjunction with a 512 channel analyser in the time-scale mode. Spectra of NM were measured at 4.2, 120 and 300 K and of DAM at 4.2, 77 and 300 K. The source consisted of 1.85 GBq of ^{57}Co diffused in Rh foil. Zero velocity was taken as the centroid of the absorption spectrum of α-Fe at room temperature. The calibration spectrum exhibited a typical line width of 0.24 mm/s.

2.4. Data analysis

Data from the iron-binding experiments consisted of three separate experiments performed in triplicate and were analysed using non-linear computer curve fitting using the program Prism (Graphpad Software). One-way ANOVA followed by Bonferroni's *t*-test was used to compare binding characteristics defined for each melanin type. Results are expressed as means ± SEM. The Mössbauer spectra were analysed with a least-squares computer program which fitted Lorentzian lines to the experimental spectra.

3. Results

Both native NM- and DAM-bound iron in a saturable manner. Nonlinear binding curve analysis revealed two binding sites representing low- and high-affinity binding populations in NM and DAM described in Table 1. The iron-binding characteristics of DAM described in the present study are comparable with our previously published results (Table 1). Iron-binding characteristics of the low-affinity binding sites are comparable in NM and DAM, in contrast, the dissociation constant (K_d) of the high-affinity binding sites on NM was significantly lower than that of DAM, but not of that for cysDAM ($P = 0.32$). The binding characteristics of NM isolated in the presence of proteinase inhibitors (Protein-NM) were similar to that of NM. The iron-binding characteristics of cysDAM and DAM submitted to the isolation procedure described for NM (Isol. DAM) did not differ significantly from that of DAM (Table 1), suggesting that the differences were not due to the isolation procedure. No iron-binding characteristics could be determined from NM isolated from formalin-fixed brain tissue suggesting that the fixation process disturbs the iron-binding sites.

Mössbauer spectra obtained from ^{57}Fe-loaded human NM exhibited a doublet at 300 K and a sextet (92%) at 4.2 K characteristic of trivalent iron (Fig. 1a and c). The spectrum at 120 K exhibited superparamagnetic relaxation, indicative of ferritin-like iron clusters (Fig. 1b). In support of our previous work using iron-depleted NM [18], no spectra characteristic of divalent iron were measured (within an error margin of approximately 5% of overall iron content; data not shown).

Mössbauer parameters describing the electronic structure of the iron complexes in NM and ^{57}Fe-cysDAM are given in Table 2. For purposes of comparison, our previously published data obtained from NM incubated with the iron chelator EDTA [18] and from human SN tissue measured *ex vivo* [39] are also presented. The broad line widths Γ of ^{57}Fe-loaded NM and DAM measured at 300 and 4.2 K represent a distribution of structurally inequivalent iron sites, so that the distribution of the ΔE_Q values of the sextet at 4.2 K could not be determined. In comparison, iron sites in NM with a depleted iron content and

Table 1
Iron-binding characteristics of NM and DAMs

Melanin	High-affinity site		Low-affinity site	
	B_{max} (nmol/mg melanin)	K_d (nM)	B_{max} (nmol/mg melanin)	K_d (nM)
NM	3.27 ± 0.32	7.18 ± 1.08*	16.62 ± 1.21	94.31 ± 6.55
Protein-NM	4.11 ± 0.33	8.80 ± 0.56	17.84 ± 1.52	82.31 ± 8.82
DAM	3.11 ± 0.35	11.39 ± 1.31	18.23 ± 2.65	86.36 ± 8.45
DAM[a]	1.13 ± 0.15	13 ± 1.61	17.40 ± 1.39	200 ± 46
Isol. DAM	3.55 ± 0.24	10.98 ± 1.87	15.58 ± 1.46	101.46 ± 22.32
cysDAM	3.17 ± 0.20	9.03 ± 1.07	16.89 ± 1.41	99.33 ± 8.94

[a] Results published by Ben-Shachar *et al.* [5].
* $P = 0.04$, compared with DAM.

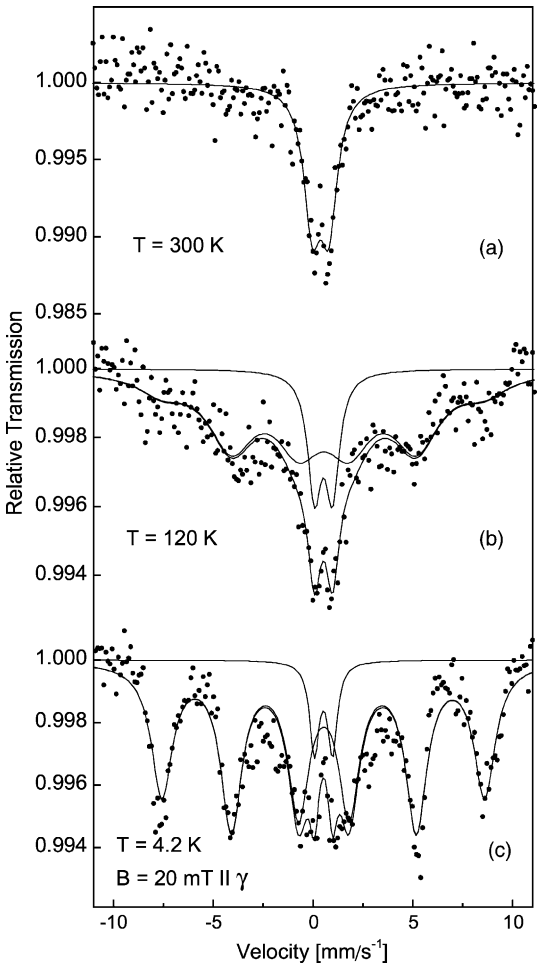

Fig. 1. Mössbauer spectra obtained from ^{57}Fe-loaded human NM exhibited a doublet at 300 K (a) and a sextet (92%) at 4.2 K (c) characteristic of trivalent iron. The spectrum at 120 K exhibited superparamagnetic relaxation, indicative of ferritin-like iron clusters (b). The spectrum at 4.2 K was measured in an externally applied field of 20 mT, applied parallel to the γ-beam. The parameters obtained from a least-square fit using Lorentzian lines are summarised in Table 2.

those in human SN tissue measured *ex vivo* were structurally more homogenous, as indicated by the smaller line widths and by the observed ΔE_Q value at 4.2 K in these two cases (Table 2). In addition, blocking temperature and hence also the ferritin-like iron oxyhydroxy-cluster size increased in the sequence: SN tissue < DAM < NM. The iron content of the synthetic and human melanin samples used for the Mössbauer measurements were 451 ± 38 and 5204 ± 432 ng/mg melanin, respectively (mean ± SD).

4. Discussion

We have previously reported the binding of iron to DAM [5] but the iron-binding characteristics of NM have not been previously investigated. These data extend previous reports of iron bound to NM granules measured in the post-mortem brain [3,20,23] by demonstrating that NM binds iron in a saturable manner. Two binding sites for iron were identified in the endogenous molecule and an important and novel finding of the current work is the significantly higher affinity of NM for iron at the high-affinity site. This is consistent with structural differences demonstrated between the native and synthetic molecules [14] and previous studies suggesting the involvement of different functional groups in the iron complex in the native and synthetic pigments [8,26]. Nevertheless, cysDAM appears to mimic the structure of NM more accurately than the simpler DAM polymer [14,16] and this greater similarity is reflected in the current study by the similar iron-binding characteristics of these two molecules. In contrast to DAM, NM contains an unidentified proteineaceous element (approximately 5% mass, [14]). The comparable iron binding demonstrated for NM prepared in the presence of proteinase K and that prepared in the presence of proteinase inhibitors supports previous data suggesting

Table 2
Mössbauer parameters of ^{57}Fe-loaded NM, ^{57}Fe-loaded DAM, Fe-depleted NM and SN tissue measured *ex vivo*

Component	Temperature (K)	ISO (mm/s)	ΔE_Q (mm/s)	Γ (mm/s)	H_{eff} (kG)	Proportion (%)	Reference
NM	300	0.36	0.82	1.02	–	100	This study
	120	0.51	0.90	0.83	–	20	
		Broad six-line pattern (superparamagnetic relaxation)				80	
	4.2	0.51	0.90	0.56	–	8	
		0.51	0	1.28	495	92	
DAM	300	0.40	0.85	0.80	–	100	This study
	77	0.50	0.90	0.60	–	50	
		Broad six-line pattern (superparamagnetic relaxation)				50	
	4.2	0.50	0	1.30	495	100	
Fe-depleted NM	300	0.37	0.59	0.40	–	100	[18]
	4.2	0.50	−0.26	0.65	495	100	
SN tissue *ex vivo*	77	0.48	0.70	0.50	–	100	[39]
	4.2	0.50	−0.26	0.60	495	100	

ISO, isomer shift; ΔE_Q, quadrupole splitting; Γ, line width; H_{eff}, magnetic hyperfine field. Standard error of ISO, ΔE_Q and Γ of the two-line spectra at 300 and 77 K is ±0.05 mm/s. Due to the very broad line widths of the six-line spectra at 4.2 K, refinement of ΔE_Q was not attempted, i.e. ΔE_Q was kept at zero. Fe-depleted NM was isolated in the presence of 150 mM EDTA to chelate weakly bound iron [18]. The parameters of SN tissue represent mean parameters from three normal adult SN [39].

that iron binds to a melaninaceous, rather than a proteineaceous component, of the molecule [26].

The broad line width observed in the Mössbauer spectra of NM and DAM (quadrupole doublet at 300 K and sextet at 4.2 K) represent structurally inequivalent iron sites and are consistent with the presence of two binding sites in both molecules. Despite comparable numbers of high- and low-affinity binding sites, the total iron content of the iron-loaded NM sample used for the Mössbauer spectroscopy was 10-fold greater than that of the synthetic pigment. The Mössbauer blocking temperature of iron-loaded NM (over 120 K) is higher than that of both iron-loaded DAM (77 K, Table 2) and iron-depleted NM (77 K, [18]). These data are indicative of a larger iron cluster size in NM compared with DAM and suggest that more iron may be bound at each site in NM. In comparison, frozen nigral tissue measured *ex vivo* exhibits a lower blocking temperature (below 77 K, [39]) and thus a smaller cluster size than both NM and DAM. The difference in cluster size in hydrated brain tissue and dried samples of iron-loaded ^{57}Fe-NM is likely to result from the increased uptake of iron by NM during the isolation and iron-loading procedures [35,39]. The ability of NM to bind additional iron during the isolation process is consistent with the hypothesis that the iron-binding capacity of NM *in vivo* is unsaturated. In the normal brain, NM is suggested to be only 50% loaded with iron [35] suggesting that the polymer retains a significant chelating potential, should intraneuronal iron concentrations increase. The Mössbauer data in Table 2 support previous reports that NM, like ferritin, binds iron in oxyhydroxy clusters in the ferric form [1,18,23,32,35,42], although several groups have suggested that the iron oxyhydroxy polymer in NM is simpler [7], smaller and less regular [1] than the iron core of ferritin.

A larger cluster size in iron-loaded NM compared with iron-depleted NM and intact nigral tissue samples suggest that additional iron is added to existing iron clusters in NM, analogous to the formation and growth of the ferritin iron core [21]. Iron storage in ferritin represents a mechanism by which iron can be stored in a chemically inert form to minimise iron-mediated toxicity [10]. Iron bound to high-affinity binding sites on NM may also be redox inactive. In an environment of low iron concentrations, NM may thus act as a cytoprotectant within the neuron. At high iron concentrations, however, the high-affinity binding sites may become saturated; iron subsequently bound to low-affinity binding sites may remain redox active with possible neurotoxic consequences for the cell. Indeed, both increased concentrations and redoxactive iron are reported to be associated with NM granules in the parkinsonian brain post-mortem [9,20,23]. High local iron concentrations reported in the parkinsonian SN might thus favour a toxic, rather than a protective, role for NM. Recent studies of the NM iron-binding domain in parkinsonian brains suggest that the polymer may differ structurally in this tissue, with the consequence that iron-binding capacity is reduced [2,27]. This is supported by magnetic studies of parkinsonian NM which indicate a reduction in both the size and number of iron clusters [7]. Changes in NM in PD have not yet been well described but any reduction in NM's capacity to bind iron may lead to an increase in redox active iron within the SN and a subsequent increase in cell damage. A comparison of the iron-binding characteristics of NM obtained from the parkinsonian brain with those of NM from the normal brain described in the current work would clarify whether the iron-binding capacity of NM differs in PD.

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