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Optical absorption and fluorescence spectroscopy studies of ground state melanin–cationic porphyrins complexes

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Abstract

Optical absorption and fluorescence spectroscopies were employed in the study of the interaction between synthetic L-dopa (dihydroxyphenylalanine) melanin and the cationic porphyrins tetrakis(4-*N*-methylpyridyl) porphyrin (TMPyP), tetrakis(4-*N*-benzylpyridyl)porphyrin (TBzPyP), zinc tetrakis(4-*N*-methylpyridyl)porphyrin (ZnTMPyP) and zinc tetrakis (4-*N*-benzylpyridyl)porphyrin (ZnTBzPyP). Optical absorption and fluorescence properties of the porphyrins were dependent on the symmetry of the central ring. No evidence was found for dimerization of the porphyrins in phosphate buffer, pH 7, in the concentration range between 4×10^{-8} to 5×10^{-5} *M*. Addition of L-dopa melanin red shifted the optical absorption spectra of porphyrins, concomitant to broadening and reduction in intensity of the bands. L-Dopa melanin also strongly quenched the fluorescence of the porphyrins. Time resolution of the fluorescence decay of porphyrins showed at least two lifetimes that were only slightly modified in the presence of melanin. The interaction between melanin and porphyrin resulted in the formation of non-fluorescent ground state complexes. It was found that there are two different classes of binding sites in melanin for complexation with cationic porphyrins and the values of dissociation constants are of the order of 10^{-8} *M*. These values and the number of binding sites are dependent on the nature of the porphyrins. It was shown that the binding has electrostatic origin, but it is also affected by metal coordination and hydrophobic interaction.

Keywords: Melanin pigment; Cationic porphyrin; Ground state non-fluorescent complexes; Fluorescence lifetime; Optical spectroscopy

1. Introduction

Melanins are widespread polymers originating from redox conversion of precursors including tyrosine, dihydroxyphenylalanine and dopamine. Present in illuminated (skin, hair, eyes) and non-illuminated organs (brain, inner ear), the physiological role of the polymer should be dependent on intrinsic properties of the polymer (e.g. the stabilization of free radicals), as well as on the interaction with other molecules. It is believed that the main biological function of the melanins

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is the cell photoprotection but even in the absence of light melanin takes part in processes of electron transfer [1–3] and it was verified that in the brain, melanin binds to the neurotoxic metabolite MPP^+ (methylphenylpyridine), with relevant consequences as the Parkinsonism [4]. It is also important the interaction of melanin with O_2, leading to superoxide [5] formation and, under illumination, originating hydrogen peroxide and hydroxyl radicals [6]. In melanin photochemistry the interaction between melanin and dyes like Rose Bengal [7,8] leads to significant photosensitization effects such as increase in the number of free radicals induced by light and enhancement of oxygen consumption.

The binding of melanin to dyes can modify their photophysical properties, the extent of the changes being dependent on the nature of the binding. It was suggested that the formation of complexes between melanins and flavins occurs through hydrophobic interaction and that the singlet excited state of the flavins is not quenched by melanin [9]. On the other hand, it was shown that synthetic cationic porphyrins may bind to melanins through ionic type interaction, and that the excited states of tetra(4-*N*,*N*,*N*,*N*-trimethylanilinium)porphyrin are quenched after binding to melanin [10].

Melanin interaction with sensitizers can be important in phototherapy. Natural porphyrins have been widely studied and used as sensitizers in photodynamic therapy, particularly protoporphyrin IX [11] and the haematoporphyrin derivative HPD [12] that actually is a complex where the active fraction is a haematoporphyrin dimer called protofrin II [13]. Less attention is given to the utilization of synthetic porphyrin derivatives. The studies with synthetic derivatives may be useful due to the fact that one can handle with structural diversity, related to parameters such as hydrophobicity, global charge and incorporation of coordinated metal.

In this work we studied the binding of synthetic melanin, obtained from autooxidation of L-3,4-dihydroxyphenylalanine (L-dopa), to synthetic cationic porphyrins, and how the binding was affected by electronic charge, metal coordination and hydrophobicity. The porphyrins studied were the novel synthesized tetrakis(4-*N*-benzylpyridyl)porphyrin (TBzPyP), the tetrakis(4-*N*-methylpyridyl)porphyrin (TMPyP), and their zinc complexes, zinc tetrakis(4-*N*-benzylpyridyl) porphyrin (ZnTBzPyP) and zinc tetrakis(4-*N*-methylpyridyl)porphyrin (ZnTMPyP). Chemical structures of the porphyrins are shown in Fig. 1. Optical absorption, steady-state fluorescence and time-resolved fluorescence spectroscopies were employed in the study of the nature and the character of the interaction between melanin and porphyrins. The changes induced by melanin on the electronic structure of the porphyrins were investigated, as well as the action of the pigment as quencher of the porphyrin fluorescence. It was evidenced the high efficiency of melanin as a promoter of non-radiative pathways for the decay of electronic excited states of porphyrins.

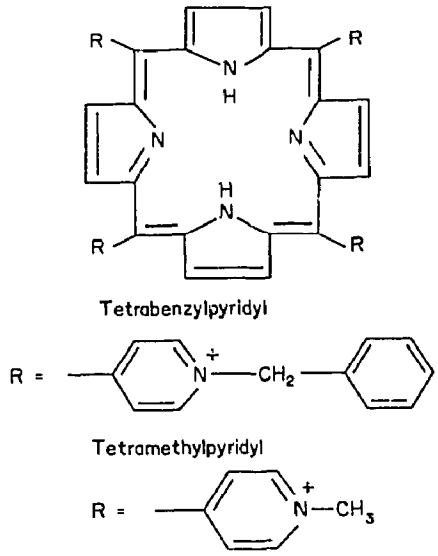

Fig. 1. Chemical structures of cationic porphyrins.

2. Material and methods

2.1 Materials

Melanin has been synthesized by autooxidation of L-3,4-dihydroxyphenylalanine (hereafter called L-dopa). Fifty milligram of L-dopa were

added to 100 ml of bidistilled water and the pH of the solution was set at 9.0 by addition of NaOH. After 5 days under oxygen flux the resulting black solution was dialysed to eliminate molecules with molecular weight lower than 5000. The final solution was evaporated at 40°C, resulting in a black powder soluble in water at pH higher than 6.0.

Tetrakis(4-*N*-methylpyridyl)porphyrin (TMPyP) and zinc tetrakis(4-*N*-methylpyridyl) porphyrin (ZnTMPyP) were prepared according to the method of Fleischer [14]. Tetrakis(4-*N*-benzylpyridyl)porphyrin (TBzPyP) was prepared by alkylation of tetra pyridyl porphyrin (TPyP, obtained from condensation of 4 pyridine carboxyaldehyde and pyrrole in propionic acid) with benzyl chloride in dimethylformamide or chloroform : ethanol mixtures. Zinc tetrakis(4-*N*-benzylpyridyl)porphyrin (ZnTBzPyP) was obtained from alkylation of ZnTPyP with benzyl chloride in a chloroform : ethanol mixture. The anionic porphyrin tetra sulfonatepyridylporphyrin (TSPP) was purchased from Aldrich (Milwaukee) and sodium dodecyl sulfate (SDS) from Sigma Chem. Co. (St. Louis, MO).

The experiments were carried out in solutions at pH 7.0, in phosphate buffer 0.01 *M* or 0.1 *M*.

2.2 Experimental apparatus

Optical absorption measurements were performed in Varian DMS 200 or Beckman DU 70 spectrometers. To observe the effect of melanin addition on the porphyrin spectra, melanin was used as blank in the same concentration as added to the porphyrins.

Steady-state fluorescence was measured with a SLM Aminco 8000 fluorimeter. When necessary, corrections were made due to light absorption by melanin.

Time-resolved fluorescence experiments were performed using an apparatus based on the single photon counting method, described in detail elsewhere [15]. Basically, the excitation source was a cavity dumped dye laser synchronously pumped by a mode-locked argon laser (Spectra Physics) operating at 8.25 kHz and giving a pulse width of 15 ps. Emission was detected on a Hamamatsu 1564U-01 microchannel plate photomultiplier. Deconvolution of the experimental curve and adjustment to a multi-exponential decay process was done by non-linear least-square method. Adequacy of the exponential decay fitting was judged by inspection of the plots of weighted residuals and by the statistical parameters reduced chi-square and serial variance ratio.

3. Results

3.1 Porphyrins optical absorption

Optical absorption spectra of TMPyP and its zinc complex ZnTMPyP in phosphate buffer 0.01 *M*, pH 7.0, were similar to previous reports [16], showing the Soret band at 420 and 430 nm, respectively. A major difference between the free base and the zinc porphyrin appeared in the *Q*-bands, reflecting the symmetry of the central ring: the zinc porphyrin has D_{4h} symmetry and presented two bands, $Q(0,0)$ at 607 nm and $Q(0,1)$ at 565 nm, while the free base porphyrin has the symmetry lowered to D_{2h}, showing the splitting of *Q*-bands to $Q_x(0,0)$, $Q_x(0,1)$, $Q_y(0,0)$ and $Q_y(0,1)$ that appeared at 643, 586, 557 and 519 nm, respectively. The tetrabenzyl pyridylporphyrins presented the same general behaviour, as seen in Table 1, that gives the spectral position of maxima of absorption bands and their relative intensities for all the porphyrins studied.

3.2 Porphyrins fluorescence

3.2.1 Steady state fluorescence

The porphyrin derivatives studied showed fluorescence in phosphate buffer pH 7.0. As the porhyrins studied were free-base or closed shell metal porphyrin complexes, the fluorescent emission is attributed to the radiative deactivation of the electronic excited state associated with the porphyrin macrocycle. The emission of free-base porphyrins, like the optical absorption spectra, differ from metallic porphyrin emission due to the differences in their symmetries, the former being significantly red shifted. In Figs. 2(a) and 2(b) fluorescence emission spectra are shown,

Table 1

Absorption properties of porphyrins measured in phosphate buffer 0.01 *M*, pH 7.0. Numbers in parentheses are relative intensities

Porphyrin	Peak positions (nm)				
	B(0,0)	Q_y(0,1)	Q_y(0,0)	Q_x(0,1)	Q_x(0,0)
TMPyP	422(16)	519(1)	557(0.37)	586(0.43)	643(0.10)
TBzPyP	427(14)	521(1)	558(0.46)	587(0.46)	643(0.19)
Porphyrin	*B*(0,0)	*Q*(0,1)	*Q*(0,0)		
ZnTMPyP	437(12)	565(1)	607(0.32)		
ZnTBzPyP	440(11)	566(1)	609(0.40)		
Porphyrin	Extinction coefficients at *B*(0,0) (10^5 *M* cm^{-1})				
TMPyP	2.4				
TBzPyP	1.9				
ZnTMPyP	2.1				
ZnTBzPyP	1.7				

corrected for photomultiplier response as well as for the dependence of lamp emission on wavelength. The lineshape of emission bands for all the porphyrins investigated remained practically constant over the entire range of porphyrin concentrations studied, which covered the interval between 4×10^{-8} to 5×10^{-5} *M*. Excitation was performed at wavelengths in which the optical density (OD) of the porphyrins were lower than 0.1 for strong inner filter effects were observed when the OD in excitation wavelength was higher than 0.15. The values of quantum yields of porphyrins fluorescence, estimated using the quantum yield for TPP as reference, were low, as can be seen in Table 2.

3.2.2 *Time-resolved fluorescence*

Time-resolved fluorescence measurements were performed using excitation at 586 nm for the free-base porphyrins and 580 nm for the zinc porphyrins. Porphyrin concentrations were chosen so that the OD in these wavelengths were approximately 0.1, case in which the concentration values were near to 1×10^{-5} *M*. The decay parameters for each derivative are listed in Table 2 and resulted from global analysis of time-resolved fluorescence data obtained at several emission wavelengths in the range between 610 and 730 nm. In the case of TMPyP and TBzPyP the fluorescence decay was best fit by three components at shorter wavelengths and the very short component was not present at longer wave-

Table 2

Fluorescence properties of porphyrins (concentration 10^{-5} *M*) measured in phosphate buffer 10 m*M*, pH 7.0. Time-resolved results from global analysis over a set of emission wavelengths from 610 to 730 nm

Porphyrin	Quantum yield	Lifetime (ns)			Fract. contr. (%)			Emission wavelength (nm)
		T_1	T_2	T_3	F_1	F_2	F_3	
TMPyP	0.024	5.06	1.56	0.026	94.7	4.6	0.7	640
					97.6	2.4	–	700
TBzPyP	0.040	4.32	1.94	0.039	90.4	8.6	1.0	640
					90.5	11.6	–	700
ZnTMPyP	0.022	1.29	0.33	–	98.5	1.5	–	640
ZnTBzPyP	0.031	1.17	0.026		99.0	1.0	–	640

lengths. The Zn derivatives had only two exponential decay components. In all cases, the long decay time component had more than 90% fractional contribution to the total fluorescence. As can be seen in Table 2, the decay times for the zinc porphyrins were significantly shorter than the corresponding values for the parent porphyrin compounds. The ratio of the values of the long decay time component for TMPyP and ZnTMPyP was 3.85, and that for TBzPyP and ZnTBzPyP was 3.46. These values are not in agreement with the quantum yield ratios, indicating that the radiative lifetimes of the zinc derivatives are different from the corresponding free-base porphyrins.

3.3 Porphyrin aggregation

The shape of the fluorescence spectra of TMPyP and TBzPyP did not change over the concentration range between 4×10^{-8} to 2×10^{-5} M. The fluorescence intensity varied linearly with concentration over the entire range studied. Figure 3 shows the data for TMPyP measured at pH 7.0 in phosphate buffer 0.01 M, with excitation at 420 nm. At concentrations higher than those presented in Fig. 3 the linearity is maintained, but the measurements had to be done with excitation at 580 or 520 nm (Q-bands)

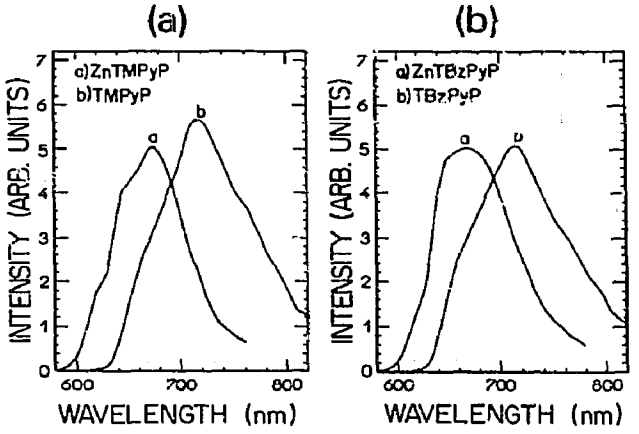

Fig. 2. Corrected fluorescence spectra of porphyrins. Concentration 6×10^{-6} M. Excitation wavelength 520 nm for TMPyP and TBzPyP and 565 nm for zinc derivatives.

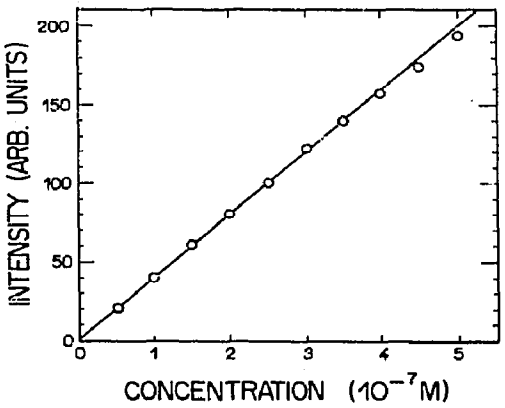

Fig. 3. Dependence of fluorescence intensity on TMPyP concentration. Excitation at 420 nm, in phosphate buffer 0.01 M, pH 7.0.

in order to avoid inner filter effects that were present when the OD exceeded 0.15 in the Soret band region. These results clearly indicate that aggregation of the porphyrin derivatives studied did not occur under the conditions of the experiment. This is not in agreement with previous reports, wich suggested the occurrence of dimerization of TMPyP at concentrations as low as 10^{-6} M [17,18].

3.4 Effect of melanin optical absorption

The optical absorption spectrum of autooxidized L-dopa melanin showed the usual large and structureless feature, extending through the visible region and with increasing absorption intensity towards the UV region. Extinction coefficient values for L-dopa melanin, expressed in (cm μg/ml)$^{-1}$ are, typically, 0.017 at 430 nm and 0.0072 at 580 nm. These values remained constant in the melanin concentration range used (lower than 10 μg/ml), leading to a linear dependence between the optical density and the concentration of melanin in solution.

The interaction between melanin and porphyrins changed the optical absorption spectra of the porphyrins. Addition of melanin caused red shift in the absorption spectrum of TBzPyP, reduction in absorption intensity and broadening of the bands (Fig. 4). The behavior observed in the Soret band is similar to that verified in the Q-

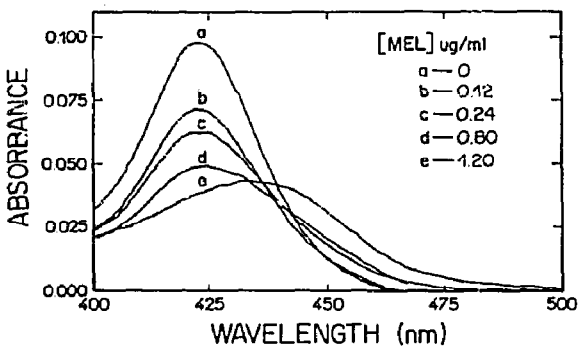

Fig. 4. Optical absorption spectra of TBzPyP in the presence of melanin. Porphyrin concentration 0.6×10^{-6} M, phosphate buffer 0.01 M, pH 7.0.

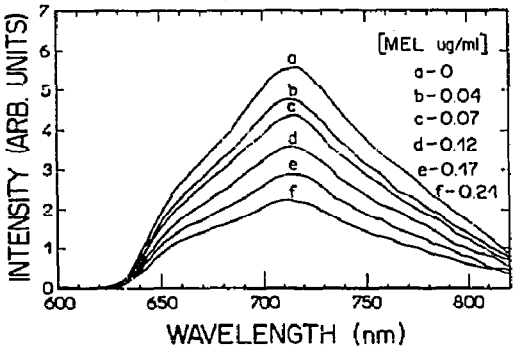

Fig. 5. Fluorescence spectra of TBzPyP in the presence of melanin. Porphyrin concentration 0.6×10^{-6} M, phosphate buffer 0.01 M, pH 7.0. Excitation wavelength 425 nm.

bands. A rise in the base line also was observed, which might be attributed to an increase in scattering due to the formation of complexes between melanin and porphyrin. The same effect was observed when melanin was added to TMPyP and to the zinc porphyrins. No changes in optical absorption spectra were verified when melanin was added to tetrasulfonatophenylporphyrin (TPPS), an anionic porphyrin.

3.5 Effect of melanin fluorescence

Fluorescence due to melanin was negligible. The addition of melanin resulted in strong quenching of porphyrin fluorescence. This behavior is illustrated in Fig. 5, that shows the corrected fluorescence emission spectra of TBzPyP at several concentrations of melanin. Similar results were obtained for the other porphyrins, but the extent of quenching by a given amount of melanin is not the same (Fig. 6). In Fig. 5 the relative decrease in the intensity of fluorescence, calculated as the area under the emission spectrum, was plotted as a function of the concentration of melanin added to the solution. The values were corrected for melanin absorption in the few cases when this was relevant and it can be observed that the quenching effect is more pronounced for TBzPyP.

The time-resolved fluorescence of porphyrins in the presence of melanin also was investigated. For each porphyrin, a set of time-resolved fluorescence data obtained at several melanin concentrations was submitted to global analysis. The results are presented in Table 3 and the quality of the adjustments was based on the analysis of the plots of weighted residuals and of the statistical parameters reduced chi-square and serial variance ratio. From this analysis it may be said that the lifetimes for decay of fluorescence of porphyrins remained constant, independent of the concentration of melanin. Comparison between Tables 2 and 3 shows that the decay times of porphyrin fluorescence were not significantly

Table 3

Time-resolved fluorescence parameters of porphyrins (10^{-5} M) after addition of melanin. Results from global analysis over a set of melanin concentrations between 0 to the maximum [M] indicated in the Table

Complex	$[M]_{max}$ (μg/ml)	Emission wavelength	Lifetime (ns)			Fract. contr. (%)		
			T_1	T_2	T_3	F_1	F_2	F_3
TMPyP	6.0	675	5.00	1.33	0.008	90.1	9.3	0.6
TBzPyP	7.2	675	4.10	1.44	0.005	90.2	9.4	0.4
ZnTMPyP	7.2	640	1.30	0.62	0.008	94.9	4.1	1.0
ZnTBzPyP	6.0	640	1.20	0.72	0.003	92.3	6.1	1.6

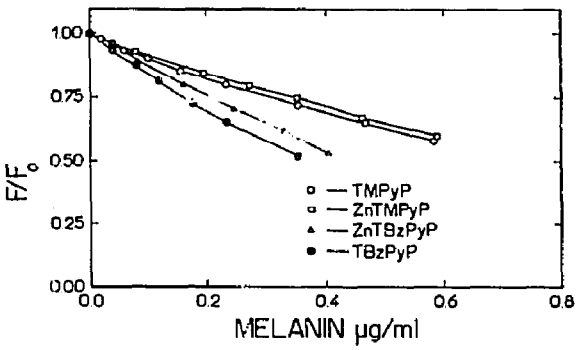

Fig. 6. Quenching of porphyrin fluorescence by melanin. Porphyrin concentrations 6×10^{-6} *M*, phosphate buffer 0.1 *M*, pH 7.0. Excitation wavelength 520 nm for free base porphyrins and 565 nm for zinc derivatives.

changed after addition of melanin, even at melanin concentrations in which the intensity of emission was strongly diminished. Some small changes in the relative population, with increase of the short lifetime contribution may be attributed to an increase in the scattering, associated to the formation of a less soluble compound. There was some evidence of melanin–porphyrin sedimentation after a period of time.

4. Discussion

The occurrence of interaction between the porphyrins and melanin synthesized from L-dopa autooxidation was demonstrated by the changes in the optical absorption spectra of porphyrins after addition of melanin. Independent of the meso group attached to the central ring or of the presence of metal, addition of melanin caused red shift of the spectrum, broadened the absorption bands and decreased their intensities. These effects were verified in Soret as well as in *Q*-bands. Such spectral alterations are similar to the observed in the formation of complexes between TMPyP and aromatic compounds like PFL^+ (proflavin 3,6-dimethylacridinium cation) and AQS^- (9,10-anthraquinone-2-sulfonate) [19]. The spectral modifications observed, according to the Gouterman's model [20], can be attributed to a greater delocalization of π-orbitals and to a relative intensification of vibrational components of *Q*-bands, indicating a larger coupling between S_1 and S_2 states. A raise of the base line upon addition of melanin is also verified, due to a larger scattering in the solution. The change in the baseline due to scattering introduced a difficulty in the separation of the contributions from free porphyrins and complexed porphyrins to the total spectrum, specially in the region of *Q*-bands. We were then directed to fluorescence results to investigate the interaction between melanin and porphyrin.

Steady state fluorescent emission of the cationic porphyrins were strongly quenched by melanin. On the other hand we verified that the emission from the anionic porphyrin tetrasulfonatophenylporphyrin (TPPS) was only slightly affected by melanin. Compared to the quantity of melanin added to the cationic porphyrin TMPyP, an amount ten times higher of melanin must be added to the anionic TPPS, in order to achieve the same reduction in fluorescence emission. The suggestion of the involvement of charges in the interaction is further supported by the decrease of quenching by melanin in the presence of SDS micelles. When SDS was present at concentrations above its critical micellar concentration there was only very small effect of quenching of porphyrin fluorescence due to melanin. The amount of melanin that reduced the TMPyP fluorescence to 50% of its initial value was 40 times higher in the presence of SDS compared to the situation without the micelles. The value for the quenching of ZnTBzPyP fluorescence was 96. The origin of these effects are electrostatic, for the SDS micelle acts as a negatively charged cage around the porphyrins, as discussed by Kano et al. [18], decreasing the extent of interaction between porphyrin and melanin.

Additional evidence for the involvement of charges in the interaction came from experiments with different ionic strength. Fluorescence quenching experiments were performed at pH 7.0 with phosphate buffer 0.1 *M* and 0.01 *M* and it was seen that the extent of quenching of TMPyP by melanin was greatly enhanced when the buffer was at 0.01 *M* concentration (Fig. 7). Similar results were obtained with the other cationic porphyrins. Thus it may be said that the interaction

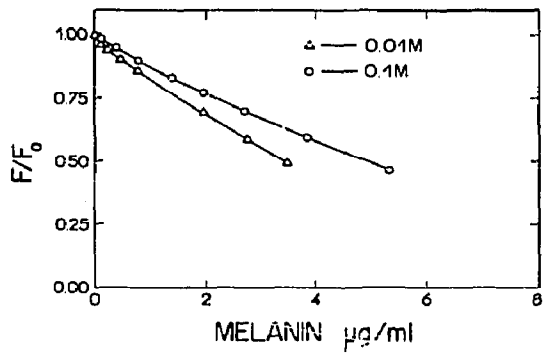

Fig. 7. TMPyP fluorescence quenching by melanin. Effect of ionic strength. Phosphate buffer pH 7.0. Porphyrin concentration 0.6×10^{-6} *M*, excitation wavelength 420 nm.

between melanin and the porphyrins studied is predominantly of ionic nature, as observed in the binding of tetra(4-*N*-trimethylanilinium)porphyrin to melanin [10]. However, the extent of the interaction with melanin is not the same for the different cationic porphyrins we studied. In order to analyse the differences between the porphyrins when interacting with melanin, we determined the dissociation constants for the binding equilibrium.

4.1 Dissociation constants of melanin porphyrin complexes

As pointed before, optical absorption spectra of porphyrins were drastically changed in the presence of melanin, simultaneous to a quenching in the fluorescence of the dye. On the other hand, time-resolved fluorescence showed that the lifetimes of the emitting species remained practically constant in the presence or absence of melanin. The results may be interpreted as a consequence of the formation of a non-fluorescent complex in a ground state reaction. This reaction in the ground state produces a melanin–porphyrin complex with electronic structure distinct from either porphyrin or melanin. As this complex is non-fluorescent, the emission is due only to free porphyrin in solution, resulting in fluorescent spectrum with decreased intensity and without modification both in the shape of the spectrum as in the lifetimes of the fluorescence decay. The process may be represented by the relation

$$M + P \rightarrow MP \quad (1)$$

where M is melanin, P is porphyrin and MP is the complex melanin–porphyrin.

Melanin is an heterogeneous polymer, in which negative charges may be stabilized, either as a result of phenol or carboxylic groups present in the final molecule [21] or, alternatively, due to the existence of trapping levels for electrons in ligand states as shown by molecular orbital calculations [22]. It is possible then to make the assumption that more than one binding site is present in the melanin for complexation with porphyrin. Furthermore, there is a strong positive deviation of the Stern–Volmer plot for static quenching, suggesting that the complex formation occurs at a ratio higher than 1:1 porphyrin to melanin. In this way, the complex formation should obey the relation

$$M + nP \rightarrow MP_n \quad (2)$$

Within this model, assuming n independent binding sites, it is possible to determine a dissociation constant for the process, by using a Scatchard plot. In this case we have

$$r/[C_f] = n/K_d - r/K_d \quad (3)$$

where $[C_f]$ is the free melanin concentration, K_d is the dissociation constant, n the number of binding sites and the parameter r is the ratio between the concentration of porphyrin bound and the total melanin concentration. Steady state fluorescence was employed for the determination of these values, as follows.

Known aliquots of porphyrin were added to a given quantity of melanin (between 0 to 1.0 µg/ml in phosphate buffer 0.1 M) present in excess in relation to the porphyrin concentrations. Assuming, as discussed above, that the complex does not fluoresce, all emission from free porphyrin. The fluorescence intensity and lifetimes obtained for the emission of free porphyrin concentrations

way we could determine the concentration of free porphyrin present in the solution containing melanin and the amount of bound porphyrin complexed with melanin.

The Scatchard plot for the binding of melanin to ZnTMPyP is non-linear, suggesting the existence of more than one class of independent binding sites in melanin for interaction with the porphyrins (Fig. 8). Numerical simulation to a model of two classes of independent sites gave good fit to experimental data (continuous line in Fig. 8). Similar results were obtained for the other porphyrins studied and the values of the dissociation constants obtained from the numerical simulations are shown in Table 4, with the corresponding values of the parameter n_i. This parameter means the concentration of porphyrin bound to a concentration of melanin equal to 1 μg/ml at the ith class of binding sites. In the studies of interaction between melanin and flavins the existence of two different classes of binding sites for the complex formation also was observed. As the interaction of porphyrins with melanin is primarily ionic, the values for dissociation constants are in the range of 10^{-8} M, substantially lower than those obtained for melanin–flavin complexation that are of hydrophobic nature [9].

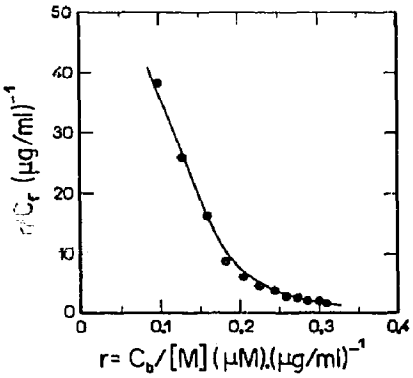

Fig. 8. Scatchard plot for melanin–ZnTMPyP complexation. Parameter r stands for concentration of bound porphyrin(10^{-6} M) per unit concentration of melanin (μg/ml). C_f is the concentration of free porphyrin (μM). Data from fluorescence spectra of ZnTMPyP in phosphate buffer 0.1 M pH 7.0, excitation wavelength 420 nm. Melanin concentration 1.0 μg/ml, range of porphyrin concentration, 0.1 to 0.6 μM. Solid line results from numerical simulation for two classes of independent binding sites model.

Table 4

Dissociation constants (K_{di}) and concentration (n_i) of bound porphyrin per unit concentration (μg/ml) of melanin

Complexes	K_{d1} (10^{-8} M)	K_{d2} (10^{-8} M)	n_1 (μM)	n_2 (μM)
TMPyP	0.34±0.01	3.5±0.1	0.17±0.01	0.24±0.1
TBzPyP	0.68±0.03	4.6±0.2	0.24±0.01	0.44±0.2
ZnTMPyP	0.19±0.01	2.4±0.2	0.13±0.01	0.21±0.1
ZnTBzPyP	0.24±0.02	2.7±0.2	0.22±0.01	0.30±0.1

The values in Table 4 allow the comparison between different porphyrins. It can be observed that the affinity of porphyrins to melanin is higher for the zinc porphyrins. It is known that melanin binds different di- and trivalent metal ions such as Mg^{2+}, Zn^{2+}, Al^{3+}, with the formation of chelates through carboxyl or semiquinone residues [21]. Although zinc atoms are already coordinated to the porphyrin ring through a tetradentated stable linkage, it is still possible to obtain pentacoordination with basic groups in melanin acting as axial ligands perpendicular to the porphyrin plane. This structural arrangement leads to lower dissociation constants for zinc porphyrins compared to free base ones, but imposes some limitations on the number of sites in melanin accessible to interaction via zinc coordination. A consequence would be that the parameter n_i is lower for the zinc porphyrins compared to the free base porphyrins.

On the other hand, the results of Table 4 also show a dependence on the substituent present in the porphyrin. Lower affinities and higher values for n_i were obtained when benzyl groups were the substituents, both for free base porphyrins as for zinc porphyrins. Even if the melanin polymer presents many ionized groups responsible for the strong electrostatic interaction with positively charged porphyrins, their distribution within the polymer is heterogeneous. Indeed, in addition to many experimental evidences for this heterogeneity, molecular orbital calculations [22] suggested that the negative charges on melanin should be located preferentially at the extremes of the polymer. Then we may consider that the distribution

of the charged groups within melanin can originate regions with hydrophobic character in the polymer, making available a higher number of binding sites for interaction with the more hydrophobic porphyrins. Therefore, higher values of n_i are obtained for benzylporphyrins, either free base or zinc porphyrin. At the same time, higher values for dissociation constants, due to the weaker hydrophobic interaction, are obtained for these porphyrins.

5. Concluding remarks

It was shown that the interaction between melanin and cationic porphyrin has an strong electrostatic character, leading to dissociation constants of the order of 10^{-8} M. Some other aspects related to the properties of the melanin polymer are relevant: the possibility of coordination with metal results in lower dissociation constants for complexation with the zinc porphyrins, and hydrophobic interaction allows for a higher number of binding sites available within the polymer for the benzylporphyrins.

The formation of complexes with melanin significantly modifies the deexcitation processes of electronically excited porphyrins. There is strong quenching of porphyrin fluorescence and radiation absorbed by the complexed porphyrin turns out to be dissipated through non-radiative pathways, a typical property of the melanin polymer. These pathways could include the direct coupling between absorbed photons and molecular vibrations extended over the whole polymer–porphyrin complex, as observed recently by photoacoustic spectroscopy on pure melanin [23]. Another possibility could be the production of photo induced free radical products as verified in melanin photosensitization induced by molecules such as Rose Bengal. In this case, deexcitation processes may include electron transfer or charge transfer and studies are currently being performed to test these possibilities. In any case it may be said that the photoprotective action of melanin progresses through the quenching of the excited state of the dye. On the other hand, radical species produced through the mediation of melanin could become physiologically important. Due to the interaction with the porphyrins, this indirect photoinduction of free radicals in melanins may be occurring at wavelengths where the light absorption due to melanin itself is low.

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