Biophysical Chemistry, 38 (1990) 39–48
Elsevier

BIOCHE 01458

Interactions of flavins with melanin

Studies on equilibrium binding of riboflavin to DOPA-melanin and some spectroscopic characteristics of flavin-melanin complex

A. Kozik [a], W. Korytowski [b], T. Sarna [b] and A.S. Bloom [c]

[a] *Department of Animal Biochemistry and* [b] *Department of Biophysics, Institute of Molecular Biology, Jagiellonian University, Krakow, Poland and* [c] *Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, WI 53226, U.S.A.*

Received 9 October 1989
Revised manuscript received 26 February 1990
Accepted 5 March 1990

Melanin pigment; Flavin; Binding interaction; Equilibrium dialysis; Fluorescence lifetime; Absorption spectroscopy

Natural melanins are photoprotective pigments that in mammals are principally found in the skin, hair, and eyes. Although the molecular mechanism of photoprotection of pigmented cells has not yet been established, several hypotheses have been proposed with melanin acting as a light filter, free radical scavenger, and quencher of electronically excited states of reactive intermediates. It can be expected that the detoxicating efficiency of melanin should be enhanced if the melanin and potentially cytotoxic species are brought close together. Such a situation may occur for a number of photosensitizing dyes that have the ability to bind to melanin. The interaction of melanin with flavins has been studied under strictly controlled experimental conditions. The equilibrium dialysis method has been employed to determine dissociation constants and the number of binding sites in melanin at pH 5–9. The data reveal that synthetic DOPA-melanin has two different classes of binding sites with dissociation constants of 10^{-6} and 10^{-5} M, respectively. The overall binding capacity of melanin, at pH 7, is 250 nmol RF/mg melanin. The amount of bound-to-melanin RF increases with pH. The absorption spectra of melanin complexes with RF and lumiflavin indicate that hydrophobic interaction may be involved in the binding of these flavins by melanin. No changes in flavin fluorescence have been detected after binding of flavin to melanin. It appears that, contrary to cationic photosensitizing dyes, the singlet excited state of flavin molecules is not quenched by melanin.

1. Introduction

Melanins are high-molecular-weight polymers of catechol(amine) origin that in animals are present in the skin, hair, eyes, and sometimes, in internal organs – the liver, brain, and inner ear [1]. It is believed that the most important biological function of this ubiquitous pigment is photoprotection of pigmented cells [2].

Melanin is a unique biopolymer in that its synthesis in vivo is only partially controlled by an enzyme — the tyrosinase [3]. It appears that redox conversions of the melanin precursors (tyrosine, dopa, dopamine, etc), after the formation of dopaquinone (or related quinones), are mostly spontaneous with the involvement of molecular oxygen and transition metal ions [4]. The end-product of such a polymerization is a complex macromolecule consisting of different monomeric units linked together via a variety of bonds. This polymer exhibits an array of unusual physico-

Correspondence address: T. Sarna, Biophysics Section, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A.

Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; HPLC, high-pressure liquid chromatography; FMN, riboflavin 5′-phosphate (flavin mononucleotide); RF, riboflavin.

chemical properties. It contains persistent radicals [5], is capable of undergoing redox reactions [6] and can bind a number of different molecules [7]. Binding of multivalent metal ions and organic cations can be explained in terms of ion-exchange ability of this substance. It has been documented that melanin contains carboxylic, amine, phenolic hydroxyl, *o*-semiquinone, and *o*-semiquinonimine groups that deprotonize at specific pH values and bind other cations [1,8].

An interesting observation has recently been reported: If a cationic dye molecule such as tetra(4-*N,N,N,N*-trimethylanilinium)porphyrin binds to melanin, a very efficient quenching of the excited states of the dye occurs [9]. Melanin-bound dye is no longer able to participate in photosensitized reactions, induce electron transfer or generate singlet oxygen. Although the mechanism of these quenching phenomena induced by melanin is not known, it is of interest to note that the photosensitizing ability of certain dyes was in part retained, if these dyes were covalently attached to melanin [10]. No study has been reported concerning whether or not other types of binding of dyes to melanin — hydrophobic, hydrogen, or Van der Waals — could significantly modify their photosensitizing properties.

It has been found that chlorpromazine and chloroquine used therapeutically could cause phototoxic side-effects [11]. Since these drugs, on prolonged use, tend to accumulate in pigmented tissue, this phototoxicity has been attributed to their binding with cellular melanin [12]. It was speculated that non-ionic binding of these drugs to melanin may be responsible for their sensitizing activity within the pigmented cells.

One of the potent photosensitizing dyes that naturally occurs in the cells is flavin. Flavins in vitro are capable of inducing photosensitized oxidation of a number of important cellular constituents [13]. Both type I (free radical) and type II (singlet oxygen) mechanisms of flavin-induced photosensitized oxidation have been found. On the other hand, flavins by themselves are quite photolabile: in the presence of an appropriate reductant they can undergo photoreduction and, if oxygen is present, they could generate superoxide anion and hydrogen peroxide [14,15]. It is also known that photoreduction of riboflavin does not require an extrinsic reductant; the dye photodegradation could result from intramolecular electron transfer with the ribitol side chain serving as an intrinsic electron donor [16].

Riboflavin and fluorescent pteridines are commonly found in the colored integuments and eyes of lower vertebrates [17]. It has been reported that the flavin content in cat choroid was related to the presence of melanin [18]. It has been postulated that flavin accumulation in pigmented tissues may arise from flavin-melanin binding that occurs in vivo [19].

In our work we studied the effect of binding of flavin molecules by synthetic melanin on the spectroscopic properties of the flavins. We wanted to determine if such binding would modify the photosensitizing ability and photolability of the flavins. This is the first part of our study in which we report on melanin-flavin interaction under strictly controlled experimental conditions. In the follow-up study, we will describe the kinetics of flavin photodegradation in the presence and absence of melanin and the photosensitizing efficiency of free and melanin-bound flavin.

2. Materials and methods

2.1. Chemicals and standard solutions

Lumiflavin and riboflavin were obtained from Sigma (St. Louis, MO) and FMN from Serva (F.R.G.). Stock water solutions of these flavins were standardized spectrophotometrically using published values of the molar extinction coefficients: 12 800 $M^{-1}\,cm^{-1}$ at 441 nm for lumiflavin [20], and 12 500 $M^{-1}\,cm^{-1}$ at 445 nm for riboflavin and FMN [21]. 3-Carboxymethyllumiflavin and 3-ethyl-7,8-dimethyl-10-tetraacetylribitylisoalloxazine were kindly provided by Dr Z. Zak. Concentrations of stock solutions of these riboflavin analogs were determined by fluorometric titration with a standardized solution of riboflavin-binding protein. All operations with flavin preparations and solutions were performed in the dark.

Riboflavin-binding protein was isolated from egg white [22] and kept frozen as a concentrated

solution in water (15–20 mg/ml). The flavin-binding capacity of protein solutions was determined from fluorometric titration of a riboflavin sample of known concentration.

DOPA-melanin was synthesized at pH 8 by the autooxidation method as described elsewhere [23].

All standard chemicals were of analytical grade from Merck, Serva (F.R.G.), Fluka (Switzerland) or POCh (Poland). For use in HPLC flavin separations, methanol and water were redistilled twice from all-glass apparatus.

2.2. Fluorometric determination of flavins

Fluorescence of riboflavin or other isoalloxazine derivatives was measured using a Spekol 11 fluorometer (Carl-Zeiss-Jena, G.D.R.), with the excitation wavelength set at 365 nm. At emission detection a suitable filter was used to cut off light of wavelength shorter than 500 nm. A method of fluorometric titration based on complete quenching of flavin fluorescence in the complex with riboflavin-binding apoprotein [24] was used for determination of riboflavin in diluted solutions (10^{-7}–10^{-6} M). To 1.5-ml samples (in 0.05 M phosphate buffer, pH 7) 10-μl portions of a standardized apoprotein solution (1.5–2 mg/ml) were added and the fluorescence changes were determined. A similar method was applied for standardization of 3-ethyl-7,8-dimethyl-10-tetraacetylribitylisoalloxazine stock solutions. Fluorometric determinations of lumiflavin, FMN, 3-carboxymethyl-lumiflavin and 3-ethyl-7,8-dimethyl-10-tetraacetylribitylisoalloxazine were performed by direct reading of the fluorescence intensity of the sample [25] and recording of the fluorescence increase on addition of flavin standard. Finally, flavin fluorescence was quenched by addition of excess (approx. 3-fold) riboflavin-binding apoprotein to determine a residual 'non-flavin' signal.

2.3. Quantitation of flavin binding to DOPA-melanin

Binding isotherms for flavin-melanin systems were determined by the equilibrium dialysis method in a minidialyser described elsewhere [26]. In a unit dialysis cell, 0.7 ml of DOPA-melanin solution (0.5 mg/ml) was put into a 1 ml-chamber at one side of the dialysis membrane, and 0.7 ml of flavin solution was placed into the chamber at the other side. In a series of dialysis cells, the concentrations of flavin solutions were varied from 5×10^{-6} to 1×10^{-4} M. These initial flavin concentrations (C_0) were accurately determined by one method. The dialysis was performed at 5° C, in the dark; under these conditions no degradation of flavins was observed. After 5 days, necessary to reach equilibrium, solutions from the chambers on the melanin-free membrane side were removed in order to determine equilibrium flavin concentrations (C_{eq}). These were analysed fluorometrically for most samples (see above). When binding of riboflavin was under study, the highest flavin concentrations were determined spectrophotometrically after subtracting the absorbance of samples from control cells, in which melanin was dialyzed against flavin-free buffer. Concentrations of melanin-bound flavin were calculated from $C_b = C_0 - 2C_{eq}$.

Some minor modifications of the standard procedure for equilibrium dialysis are described in the figure legends.

2.4. Recovering of flavins from complexes with DOPA-melanin

Samples (0.7 ml) from several mixtures of riboflavin (1×10^{-4} M) and melanin (0.5 mg/ml) in 0.05 M phosphate buffer (pH 7), equilibrated and kept in darkness, were placed in chambers of the minidialyser and dialyzed against solutions (0.7 ml) of riboflavin-binding apoprotein (at least 2 mg/ml) in the phosphate buffer. Other conditions of equilibrium are described above. Because of the high affinity of the apoprotein to many flavins, all flavin reversibly bound to melanin was expected to be quantitatively transferred into protein complex. After dialysis, protein samples were taken out, adjusted to pH 3.5 with acetic acid, and applied onto small columns (1.5 ml) of CM-32 cellulose, equilibrated and eluted with 0.1 M sodium acetate buffer (pH 3.5). Under these conditions, the protein was retained by the column but flavins were eluted [22]. The first 4–5 ml of eluate were collected, concentrated on a rotary evaporator to the original volume (about 0.5 ml)

and used for HPLC analyses of flavin content and composition.

2.5. Absorption spectra of flavins complexed with melanin

In a typical experiment, a solution of DOPA-melanin was equilibrated with the given flavin in phosphate (pH 7) or borate (pH 9) buffer. Absorption spectra were recorded using an HP8452 diode array spectrophotometer and a 1 mm optical cell.

2.6. Fluorescence spectra and fluorescence lifetimes of flavins complexed with melanin

The fluorescence lifetimes for free and bound flavins were determined using an SLM 8000C lifetime spectrofluorometer (SLM Instruments, Urbana, IL). The methods and instrumentation have previously been described [27]. An excitation wavelength of 451 nm was used and modulation was at 30 and 18 MHz. A KV-501 filter was placed in the emission pathway. Apparent absolute phase lifetimes (τ_ϕ) were calculated against a glycogen standard using a computer program provided by SLM.

3. Results and discussion

Since natural melanins are complex supermolecular systems consisting of proteins, lipids, and the melanin polymer [1] that are insoluble at neutral pH, we have decided to use a synthetic polymer to study the interactions of flavins with melanin. Such synthetic melanins, obtained by autooxidation of DOPA, have proven to be a reasonably good model for studying the physicochemical properties of eumelanin [5–10].

Flavin binding to melanin could readily be detected in aqueous, unbuffered solutions of DM (0.5 mg/ml) and riboflavin. As shown in fig. 1 (lower curve), the plot of bound riboflavin vs free riboflavin concentration forms a typical saturation-type curve. It can be concluded that such binding is a reversible process. Firstly, when riboflavin had been added to melanin solution and

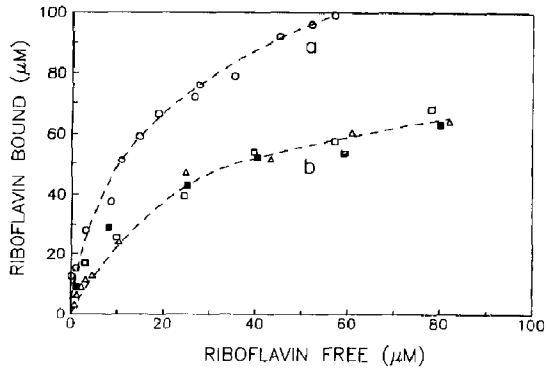

Fig. 1. Isotherms of riboflavin binding to DOPA-melanin in solutions of various pH values. When the binding in unbuffered solution (pH 5) was studied (b), the equilibrium dialysis was performed at room temperature (△) or in the cold (5 °C). In the latter case, the binding isotherms were determined twice: first, by dialysis of the riboflavin-melanin mixture against water (□); second, using the standard procedure, i.e., riboflavin dialyzed to melanin from the opposite membrane side (■). The outer isotherm (○) was determined in 0.5 M sodium bicarbonate buffer (pH 9) at 5 °C (a). Concentration of DOPA-melanin was 0.5 mg/ml for all experiments; other details are described in section 2.

then the sample was dialyzed against water, the binding isotherm obtained was identical to that determined by the standard procedure (riboflavin dialyzed to melanin from the opposite membrane side). Secondly, as pre-incubated riboflavin-melanin mixtures were dialyzed vs a specific riboflavin-binding apoprotein, at least 95% of the added flavin could be recovered. No conversion of riboflavin to other compounds could be detected by HPLC methods. Thus, the flavin-DM interaction can be regarded as a chemical equilibrium.

A solution of DOPA-melanin in distilled water has a pH value of about 5. The binding of riboflavin to melanin was greater in buffered solutions of higher pH (fig. 1). The amount of bound riboflavin increased markedly where the pH was raised from 5 to 9.

At pH 5 (unbuffered solution), the binding isotherm was also determined at room temperature. Although no pronounced temperature effects on the amount of melanin-bound flavin could be seen, changes in the shape of the binding isotherm were apparent at different temperatures.

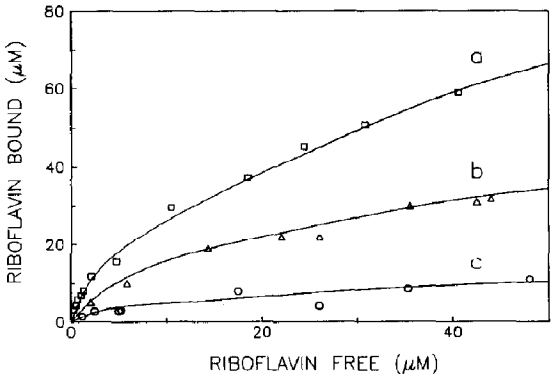

Fig. 2. Dependence of riboflavin binding to DOPA-melanin at pH 7 on polymer concentration. Isotherms from representative experiments are presented. Equilibrium dialysis was run in 0.05 M sodium phosphate buffer at 5°C. [Melanin]: 0.5 mg/ml (a), 0.25 mg/ml (b), 0.05 mg/ml (c). Theoretical isotherms drawn through the experimental points were calculated using the following binding parameters C_{bmax}, $k_d^{(1)}$, C_{bmax}, $k_d^{(2)}$ (each in μM): (□) 12.4, 1.44, 146, 84, respectively; (△) 6.0, 1.7, 54, 44, respectively; (○) 2.8, 0.86, 16.2, 56, respectively.

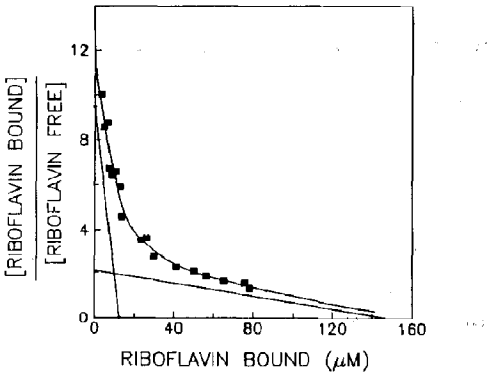

Fig. 3. Scatchard plot for riboflavin binding to DOPA-melanin at pH 7 vs polymer concentration. Isotherms from representative experiments are presented. Equilibrium dialysis was run in 0.05 M sodium phosphate buffer; [melanin]: 0.5 mg/ml. The fitting curve was obtained by non-linear regression computer analysis [28]. A model of two classes of independent identical binding sites was found to fit optimally the data. Linear components of the theoretical curve are also drawn.

The dependence of riboflavin binding on DOPA-melanin concentration is shown in fig. 2. At a constant free riboflavin concentration, the amount of riboflavin bound seemed roughly proportional to the polymer concentration.

Fig. 3 depicts a Scatchard plot taken from a representative experiment where binding of riboflavin to DOPA-melanin was measured at pH 7. Some curvilinearity of the Scatchard plot was observed under all experimental conditions studied. This may be interpreted as an indication of some heterogeneity of the binding sites of the polymer. The analysis is based on the assumption that several classes of melanin-binding sites can be distinguished; these binding sites within one class are identical and non-interacting with each other. Fitting of theoretical models to the experimental points and solving for binding parameters were performed using the program LIGAND of Munson and Rodbard [28]. This computerized non-linear weighted regression procedure works in the most appropriate coordinate system: total ligand added vs ligand bound. Moreover, any improvement of the fit by introducing a more complicated model (i.e., more parameters to be fitted) is tested

TABLE 1

Parameters for riboflavin binding to DOPA-melanin

Conditions of equilibrium dialysis (buffer, temperature)	Binding capacities (nmol/mg melanin)			Dissociation constants (μM)	
	Class 1	Class 2	Total	Class 1	Class 2
Water (pH 5, 20°C)	6	162	168	1.0	26
Water (pH 5, 5°C)	48	165	213	2.4	79
0.05 M phosphate [a] (pH 7, 5°C)	25–35	260–295	285–330	1.0–2.0	70–90
0.05 M bicarbonate [b] (pH 9, 5°C)	40–48	260–308	300–356	0.8–3.1	31–35

[a] Range from 6 independent runs.
[b] Range from 3 independent runs.

(F-test) for statistical significance. For most experiments on riboflavin binding to DOPA-melanin, the two-class model appeared statistically justified, and thus:

$$b = \frac{b_{max}^{(1)} - C_{eq}}{k_d^{(1)} + C_{eq}} + \frac{b_{max}^{(2)} - C_{eq}}{k_d^{(2)} + C_{eq}} \quad (1)$$

where b denotes the amount of bound riboflavin and k_d the intrinsic dissociation constant. The ranges of binding parameters found are collected in table 1, and a representative sample is shown graphically in fig. 3. Intrinsic dissociation constants for higher-affinity sites were in the micromolar range, and the binding to sites of the second class was 10 times weaker. At pH 7, the overall binding capacity was about 300 nmol riboflavin per mg melanin. Typically, 10–20% of that belonged to the higher-affinity sites. The relative proportions of the two classes, however, seemed slightly variable for different batches of synthetic polymer. The pronounced effect of pH on riboflavin binding to DOPA-melanin is quantitatively described by a decrease in the dissociation constants of both classes of binding sites, and an increase in total binding capacity mainly because of increase in the amount of lower-affinity sites.

Some riboflavin analogs were studied for their ability to bind to DOPA-melanin in neutral pH buffer (0.05 M phosphate, pH 7). The binding isotherms for lumiflavin and FMN are shown in fig. 4, and those for 3-carboxymethyllumiflavin and 3-ethyl-7,8-dimethyl-10-tetraacetylribitylisoalloxazine in fig. 5. Flavins with negatively charged substituents were poorly bound by melanin; this may reflect electrostatic repulsion due to negative groups of the polymer. Lumiflavin was bound to DOPA-melanin stronger than riboflavin (2–3-times lower dissociation constants for both classes of binding sites). This finding suggests that the ribitol chain does not contribute to the overall binding energy. This rather bulky substituent may even exert some steric hindrance so that the interaction with melanin is weakened. Similar steric effects may be responsible for relatively weak binding (in terms of binding capacity rather than dissociation constants) of the tetraacetyl derivative to melanin. On the other hand, results with

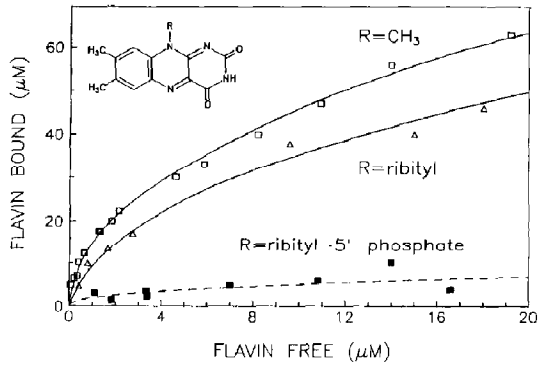

Fig. 4. Isotherms for binding of lumiflavin (□) and FMN (■) to DOPA-melanin. Equilibrium dialysis was performed in 0.05 M phosphate buffer at 5 °C; [melanin]: 0.5 mg/ml. Experimental points from a control run with riboflavin (△) are also presented. Theoretical curves (solid lines) were found by a graphical analysis of the Scatchard plot using the limiting-slopes method [29]. For lumiflavin it was assumed that the total binding capacity is similar to that for riboflavin. The following binding parameters C_{bmax}, $k_d^{(1)}$, C_{bmax}, $k_d^{(2)}$ (each in μM) were used to draw lines: for lumiflavin, 14.7, 0.39, 105, 22.7, respectively; for control plot, 21, 1.9, 109, 51.3, respectively.

riboflavin analogs may suggest that the flavin-melanin interaction is of hydrophobic nature.

It has been recognized long ago that melanin can bind many drugs of polycyclic aromatic structure [30–32]. Reported dissociation constants were

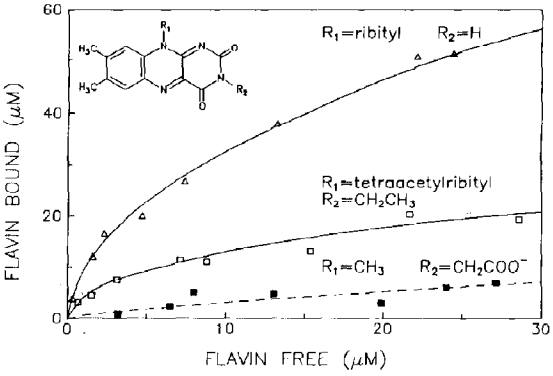

Fig. 5. Isotherms for binding of 3-carboxymethyllumiflavin (■), and 3-ethyl-7,8-dimethyl-10-tetraacetylribitylisoalloxazine (□) to DOPA-melanin at 5 °C and pH 7. A control isotherm for riboflavin (△) is also shown. Lines were drawn using the following parameters C_{bmax}, $k_d^{(1)}$, C_{bmax}, $k_d^{(2)}$ (each in μM): (□) 5.8, 0.98, 29, 27.8, respectively; (△) 17.8, 1.5, 112, 56, respectively. The parameters were estimated graphically [29].

at best in the low micromolar range. Thus, riboflavin and lumiflavin should be classified into a group of compounds of highest affinity to DOPA-melanin.

There is extensive literature reporting that basic spectral properties of flavins such as absorption spectra and fluorescence lifetimes change markedly on binding to biopolymers. Those studies, however, were almost exclusively limited to proteins [20,24,33]. Because of the strong absorptivity of melanin throughout the ultraviolet/visible range, the melanin-flavin system is not very convenient for analogous characterization. Nevertheless, our attempts to measure the absorption spectra of equilibrated riboflavin-melanin mixtures were partially successful even though the absorbance of such samples was rather high. Under conditions favorable for binding (pH 9.0, riboflavin/melanin ratio not excessive), subtle spectral changes of such riboflavin were observed (fig. 6). Some hypochromism was typically detected on both 370 nm and 450 nm bands, and was much more pronounced in the latter. Thus, the distinguishable

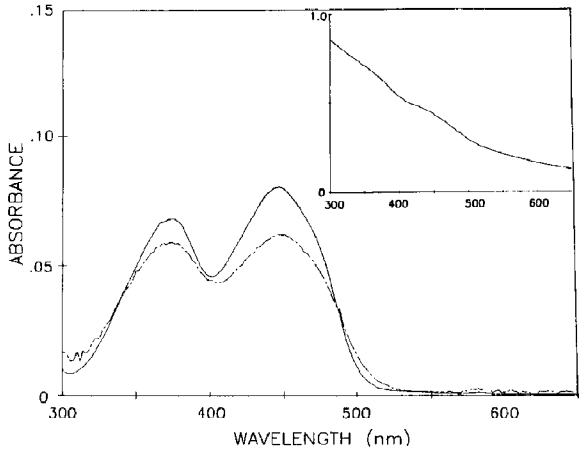

Fig. 6. Effect of binding to DOPA-melanin on the electronic absorption spectrum of riboflavin at pH 9.0. Riboflavin (50 μM) was equilibrated with DOPA-melanin (0.25 mg/ml) in 0.05 M borate buffer, and then the absorption spectrum in a 1 mm cuvette was recorded in the near-ultraviolet/visible range (inset). After subtracting a melanin blank (0.250 mg/ml), a spectrum was obtained that can be regarded as occurring from melanin-bound (plus some free) riboflavin (dashed line). Absorption of free riboflavin (50 μM) in the buffer is also presented (solid line). For more details see section 2.

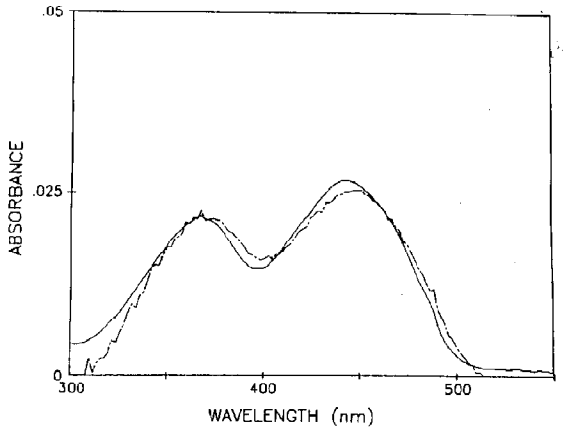

Fig. 7. Effect of binding to DOPA-melanin on the absorption spectrum of lumiflavin. The buffer used was 0.05 M borate (pH 9.0); [lumiflavin] = 20 μM; [melanin]: 0.25 mg/ml. Spectra were recorded in a 1 mm cuvette, as described in section 2 and fig. 6.

effect of melanin binding on the absorption spectra of riboflavin seemed best described as the lowering of the ratio of intensities at 450 to 370 nm bands.

As lumiflavin binds to DOPA-melanin even more tightly than riboflavin (see above), changes in the absorption spectra of this flavin are likely to be detectable on binding. As shown in fig. 7, some spectral effect does occur and is manifested by a hypochromism in both bands and a small (some 2–3 nm) but reproducible batochromic shift in the 450 nm band. These changes are again small and visible only at pH 9.0 and low lumiflavin/melanin ratios. In contrast, the absorption spectra of FMN remain unchanged in melanin solution (results not shown). This is consistent with the observed very weak affinity of riboflavin phosphate to melanin.

The observed spectral changes can be interpreted, by comparison, in terms of the effect of organic solvents and protein binding on the absorption spectra of flavin. Transfer of flavins from water to solvents of lower dielectric constant is generally believed to result in a hypochromic shift with hypochromism in the near ultraviolet band and a resolution of vibronic structure in the visible band [25,34]. Flavin-binding sites in typical flavoproteins are hydrophobic, but the solvent ef-

fect itself could never suffice fully to explain the spectral changes observed on binding. This is because other forces such as hydrogen bonding and stacking to aromatic amino acid residues [35] are involved in the binding mechanism. Thus, as an example, spectra of flavins in complexes with riboflavin-binding apoprotein are characterized by a hypochromism with no shift in the near-ultraviolet band and a hypochromism, a batochromic shift and some resolution in the visible region [30,36].

The subtle effect of melanin binding on the absorption spectra of interacting flavins (riboflavin and lumiflavin) can hardly support the hypothesis of a hydrophobic character of the flavin-melanin interaction as is suggested from studies of flavin analogs (see above). The hypochromism of the near-ultraviolet band seems to indicate some involvement of hydrophobic forces. Obviously the mechanism of flavin binding to melanin is much more complex. A number of hydrogen bonds to isoalloxazine (but not to ribitol hydroxyls!) might also form, resulting in the observed temperature effect on the binding parameters.

The apparent phase lifetime (τ_ϕ), detected in time-resolved experiments with melanin-bound riboflavin at pH 9, was 3.68 ± 0.06 ns when a modulation frequency of 30 or 18 MHz was used, respectively. Heterogeneity analysis indicated that the fluorescence decay observed could best be described by a single-exponential decay. This can be compared with the values of 3.70 ± 0.02 and 3.97 ± 0.03 ns determined for free riboflavin under similar instrumental settings. Thus, it is evident that the phase lifetime of riboflavin is not markedly altered by its binding to melanin. On the other hand, this parameter was readily modified by changing the pH; the phase lifetime of free riboflavin at pH 7 was found to be 4.7 ns. Since this latter value corresponds quite well to the fluorescence lifetime of free riboflavin at pH 7 reported previously (4.64 ± 0.05 ns [37]), we can reasonably assume that the phase lifetimes determined in our measurements are true lifetimes of the lowest singlet excited state of the riboflavin. No meaningful quenching of the emission intensity of riboflavin excited at 451 nm was observed in samples containing riboflavin and melanin, even

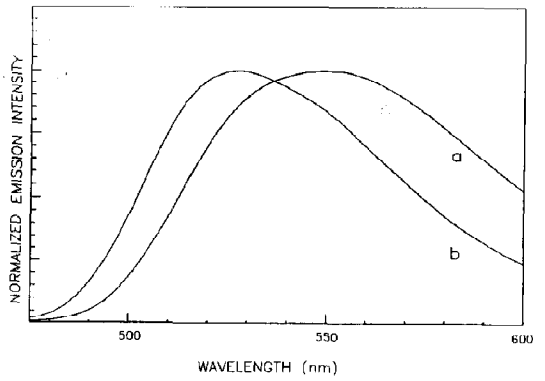

Fig. 8. CW-fluorescence spectra of riboflavin equilibrated with DOPA-melanin at pH 7 (a) and of riboflavin alone (b); [riboflavin] = 50 μM; [melanin] = 50 μg/ml.

though the experimental conditions (pH, RF/melanin ratio) were favorable for the binding of a significant percentage of the riboflavin by melanin. In such samples an apparent red-shift of the maximum of fluorescence emission of riboflavin was evident (fig. 8). The magnitude of this shift depended on the ratio of bound to free riboflavin. It was also related to the concentration of melanin which determines the inner-filter effect in the samples studied. Since the absorbance of a solution of 250 μg/ml melanin at 528 nm (maximum of free RF) and 550 nm (apparent maximum of the sample with 50% of bound RF) was 2.3 and 2.0, respectively, this effect could be explained by wavelength-dependent attenuation of the RF emission brought about by melanin.

Thus, it can be concluded that complexation of melanin with riboflavin does not change its fluorescence properties, i.e., lifetime, emission intensity and spectral dependence. This is rather an unusual result in view of the strong quenching of flavin fluorescence that is induced by molecules that form complexes with this fluorophore [38]. For instance, it has been shown that binding of RF by apoprotein of hen egg white (RBP) caused both static and dynamic quenching of flavin fluorescence [37]. It was argued that the mechanism of static quenching, which was the dominant type of fluorescence quenching induced by RBP, involved charge-transfer complexes (CT). CT interaction between the singlet excited state of flavins and

electron donors such as indoles and phenols has been directly observed using picosecond laser flash photolysis [39]. This latter study revealed that while interaction between the ground-state flavin and indole molecules was rather weak (dissociation constant $\sim 10^{-1}$ M), very efficient quenching of the flavin fluorescence was due to electron transfer from the quencher to the excited flavin. Although melanin contains a number of indolic and phenolic groups [1], it appears that binding of flavins to melanin, studied in this work, is determined by another type of interaction. It is not clear why these abundant functional groups of melanin cannot form a similar type of CT complex with flavins. Perhaps steric hindrance, imposed by the polymer structure, prevents intimate contact between the flavin molecule and the functional groups of melanin that is necessary for the formation of CT complexes. Also, it is possible that the redox properties of the melanin moieties, compared to free monomer units, are strongly modified by intramolecular interactions. It remains to be shown if flavin bound to melanin exhibits photochemical properties similar to those of free flavin, whether melanin-bound and free flavins are equally susceptible to photodegradation and whether the photosensitizing ability of flavin is modified by binding to melanin. We are presently studying these aspects of melanin-flavin interaction.

Acknowledgements

We wish to thank Dr Peter J. Munson and David Rodbard for making the program LIGAND-PC (v. 3.0) available. This work was supported in part by a grant CPBR3.13.4.4.3 from the Polish Academy of Sciences, a grant RPII.13. 2.11 from the Polish Ministry of Sciences and grants GM27665-09 from NIH and DA03725 from NIDA.

References

1 R.A. Nicolaus, in: Melanins, ed. E. Lederer (Hermann, Paris, 1968).
2 M.A. Pathak and T.B. Fitzpatrick, in: Sunlight and man, eds T.B. Fitzpatrick, M.A. Pathak, L.C. Harber, M. Seiji and A. Kukita (University of Tokyo Press, Tokyo, 1974) p. 725.
3 G.A. Swan, in: Fortschritte der Chemie organischer Naturschaffe, eds W. Herz, H. Griesback and W. Kirby (Springer, New York, 1974) vol. 31, p. 522.
4 G. Prota, Med. Res. Rev. 8 (1988) 525.
5 R.C. Sealy, C.C. Felix, J.S. Hyde and H.M. Swartz, in: Free radicals in biology, ed. W.A. Pryor (Academic Press, New York, 1980) vol. 4, 209.
6 T. Sarna, W. Korytowski and R.C. Sealy, Arch. Biochem. Biophys. 200 (1985) 140.
7 B. Larsson, Ph.D. Thesis, Uppsala University, Uppsala (1979).
8 T. Sarna, W. Froncisz and J.S. Hyde, Arch. Biochem. Biophys. 202 (1980) 304.
9 J. Bielec, B. Pilas, T. Sarna and T.G. Truscott, J. Chem. Soc. Faraday Trans. 82 (1986) 1469.
10 J. Bielec, B. Pilas, T. Sarna, C. Knox and T.G. Truscott, J. Chem. Soc. Faraday Trans. 84 (1988) 149.
11 T.B. Fitzpatrick, M.A. Pathak, I.A. Magnus and W.L. Curuen, Annu. Rev. Med. 14 (1963) 195.
12 R. Baweja, T.D. Sokolski and P.H. Patil, J. Pharm. Sci. 66 (1977) 1547.
13 R.C. Straight and J.D. Spikes, in: Singlet O_2, ed. A.A. Frimer (CRC Press, Boca Raton, 1985) vol. IV, p. 91.
14 P.F. Heelis, B.J. Parsons, G.D. Phillips and J.F. McKellar, Photochem. Photobiol. 30 (1978) 343.
15 A.M. Michelson, in: Superoxide and superoxide dismutases, eds A.M. Michelson, J.M. McCord and I. Fridovich (Academic Press, London, 1977) p. 87.
16 W.M. Moore and R.C. Ireton, Photochem. Photobiol. 25 (1977) 347.
17 J.H. Elliot and S. Futterman, Arch. Ophthalmol. 70 (1963) 137.
18 K. Matsui and K. Yamagiya, J. Nutr. Sci. Vitaminol. 19 (1973) 193.
19 M. Obika, Comp. Biochem. Physiol. 53B (1976) 521.
20 J. Becvar, Ph.D. Dissertation, University of Michigan, Michigan (1973).
21 L.G. Whitby, Biochem. J. 54 (1953) 437.
22 M.B. Rhodes, N. Bennett and R.E. Feeney, J. Biol. Chem. 234 (1959) 2054.
23 C.C. Felix, J.S. Hyde, T. Sarna and R.C. Sealy, J. Am. Chem. Soc. 100 (1978) 3922.
24 M. Nishikimi and Y. Kyogoku, J. Biochem. 73 (1973) 1233.
25 J. Koziol, Methods Enzymol. 18B (1971) 253.
26 Z. Wasylewski and A. Kozik, Eur. J. Biochem. 95 (1979) 121.
27 J.R. Lakowicz, Principles of fluorescence spectroscopy (Plenum, New York, 1983).
28 P.J. Munson and D. Rodbard, Anal. Biochem. 107 (1980) 220.
29 I.M. Klotz and D.L. Hunston, Biochemistry 10 (1971) 3065.
30 K. Shimada, R. Baweja, T. Sokolowski and P.N. Patil 65 (1976) 1057.

31 B. Larsson and H. Tjalve, Biochem. Pharmacol. 28 (1979) 1181.
32 K.B. Stepien and T. Wilczok, Biochem. Pharmacol. 31 (1982) 3359.
33 J.-D. Choi and D.B. McCormick, Arch. Biochem. Biophys. 204 (1980) 41.
34 H.A. Harbury, K.F. Lanoue, P.A. Loach and R.M. Amick, Proc. Natl. Acad. Sci. U.S.A. 45 (1959) 1708.
35 D.B. McCormick, Photochem. Photobiol. 26 (1977) 169.
36 M. Nishikimi and K. Yagi, J. Biochem. 66 (1969) 427.
37 K. Bystra-Mieloszyk, A. Balter and R. Drabent, Photochem. Photobiol. 41 (1985) 141.
38 D.B. McCormick, in: Molecular associations in biology, ed. B. Bullman (Academic Press, New York, 1968) p. 377.
39 A. Karen, N. Ikeda, N. Mataga and F. Tanaka, Photochem. Photobiol. 37 (1983) 495.