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Quenching of the Excited States of 8-Methoxypsoralen by Synthetic Eumelanin

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The interaction of 8-methoxypsoralen (8-MOP) with synthetic eumelanin was investigated using static and time-resolved fluorescence and pulsed photoacoustic calorimetry. Spectroscopic data indicate the absence of interaction in the ground state, whereas the singlet excited state of 8-MOP is quenched by the pigment; the average fluorescence lifetimes are independent of the melanin concentration, thus indicating a static mechanism. Photoacoustic data show that the quenching process involves an increased intersystem crossing probability, which is almost unaffected by the presence of oxygen, as expected for a molecule essentially acting as a type I photosensitizing agent.

KEY WORDS: 8-Methoxypsoralen; melanin; photoacoustics.

INTRODUCTION

In recent years many spectroscopic investigations have dealt with the photophysics and the photochemistry of the interaction between 8-methoxypsoralen (8-MOP) and various subcellular components such as DNA, proteins, and membranes (1,2). Due to the relevance of this psoralen in the photochemotherapy of skin disorders, it seems interesting to characterize the binding of 8-MOP to melanins since this can affect both the bioavailability and the potential toxicity of the drug (3), usually elicited via photochemical reactions involving the triplet state of the psoralen. The two chromophores under investigation show some optical features, such as a strong overlap of absorption bands, that render the use of spectroscopical techniques quite delicate; nevertheless, a careful examination of experimental data and the comparative use of fluorescence and pulsed laser time-resolved photoacoustics should allow a good characterization of the interaction. The latter technique monitors the energy content and the time profile of nonradiative relaxations of optically excited states, including internal conversion and energy transfer to molecular oxygen. This comparative approach allows us to draw a more complete picture of the fate of the energy absorbed by melanin 8-MOP complexes. This paper reports the first results of a study on the photophysics of 8-MOP in the presence of synthetic eumelanin.

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MATERIALS AND METHODS

All measurements were performed in 10 m*M* phosphate buffer, pH 7, at room temperature.

Eumelanin was prepared by autooxidation of 0.5 g L-dopa in 150 ml water; the pH was raised to 9 and the solution stirred for 48 h; after acidification the solution was centrifuged and the pellet dried up at 40°C; the powder thus obtained was soluble in phosphate buffer at pH 7. The concentration of melanin is expressed as micrograms per milliliter since the molecular weight of the compound is known only approximately. 8-MOP was obtained by Sigma and used as received.

Both fluorescence and photoacoustic measurements

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were performed at optical densities below 0.2 to minimize inner filter effects.

Deoxygenation of the samples in photoacoustic measurements was achieved by directly bubbling nitrogen inside the photoacoustic cell.

Sample absorbance was measured with a Jasco 7850 spectrometer, while fluorescence was carried out with a Perkin–Elmer LS-50 luminescence spectrometer. Time-resolved fluorescence (single-photon counting) was performed on a home built apparatus.

Photoacoustic measurements were performed with a home-built apparatus that has been described previously [4]. The light source was a XeCl excimer laser (EMG 50 Lambda Physik; 5-ns pulse width, 308 nm), pumping a dye laser (FL 3001 Lambda Physik) operating in the UV (336- to 350-nm) and visible (470- to 510-nm) regions; the photoacoustic signal was detected by a Panametrics V-103 PZT-based pressure transducer and amplified (60 db) by an ultrasonic preamplifier; an RJ-7620 (Laser Precision Corp.) energy meter was used to monitor the laser pulse energy. The signal was recorded by a LeCroy 9450A digital oscilloscope operated at 2.5 ns/channel; the data were collected and analyzed with an IBM ps/2 50 computer.

Typically 500 laser shots are averaged to give a single photoacoustic waveform, which is normalized for the incident laser pulse energy and subtracted from the experimental baseline. The instrumental transfer function is determined by recording the photoacoustic waveform of a compound that releases the absorbed energy as heat with unit efficiency within a few nanoseconds.

Numerical deconvolution of photoacoustic waveforms is performed by means of an iterative nonlinear least-squares algorithm, which provides the fractional amplitudes and the lifetimes of the heat transients. When the lifetime of the transient is shorter than a few nanoseconds, the evolved heat is integrated by the transducer and the amplitude of the corresponding decay is usually called prompt heat α [5]; the ratio of the amplitudes of the sample and reference waveforms gives the same value, provided that the lifetime of the sample is not in the range 50 ns–5 μs. The heat deposited in times longer than several microseconds is not sensed by the transducer and may be regarded as "lost" energy.

RESULTS

As stated above, investigating the spectroscopical properties of the 8-MOP/melanin system involves some experimental difficulties, mainly pertaining to the strong overlap of their absorption bands. The presence of competitive absorption by the two cromophores requires careful evaluation of the energy effectively absorbed and successively released under various forms, both radiatively and nonradiatively. The optical filter effect exerted by each chromophore on the other may be taken into account by considering a generalized expression derived from the Beer–Lambert law. To describe the prompt heat released by a two-absorber system, we use the following expression [4]:

$$\alpha_{tot} = \left[\alpha_{1A} \frac{A_A}{A_{tot}} + \alpha_{1B} \frac{A_B}{A_{tot}} \right] \quad (1)$$

where α_{1A} and α_{1B} are the fractions of prompt heat released by the two chromophores, A_A and A_B are the individual absorbances, $A_{tot} = A_A + A_B$, and α_{tot} is the fraction of prompt heat as measured for the system. The individual fractions of prompt heat for the two chromophores by photoacoustic data were obtained by means of Eq. (1); a similar correction was performed on the fluorescence data.

The absorbance spectrum of 8-MOP is not affected by the presence of melanin, whereas the static fluorescence of the drug is quenched by the pigment (see Fig. 1); fitting the experimental fluorescence emission am-

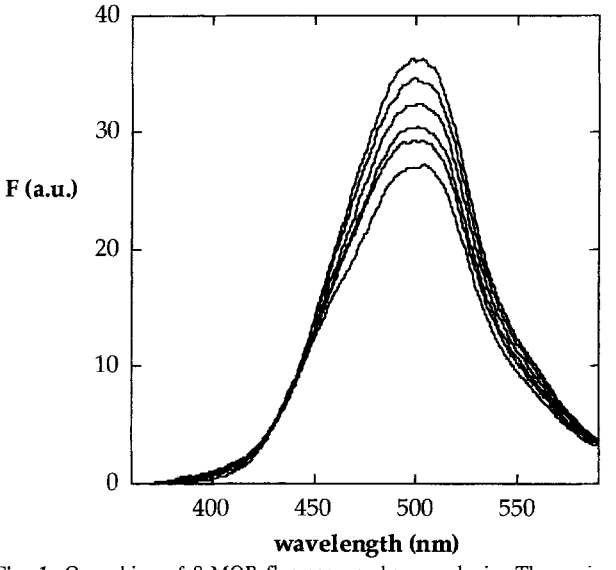

Fig. 1. Quenching of 8-MOP fluorescence by eumelanin. The excitation wavelength was 308 nm; the concentration of 8-MOP was kept constant at 4.8 μM, whereas the melanin concentration was varied between 0 and 4.5 μg/ml. Emission spectra were corrected for absorbance and spectral sensitivity of the detection system.

plitudes assuming Stern–Volmer kinetics [6] gives the value 0.02 $(\mu g/ml)^{-1}$ for the quenching constant K and is shown in Fig. 2 as a solid line.

Eumelanin alone showed a very low fluorescence between 360 and 560 nm, as reported previously [7], that was subtracted from the emission spectra of the system; performing measurements at different temperatures, ranging between 291 and 318 K, does not affect the results, thus indicating a static mechanism of quenching [6]. This hypothesis is confirmed by fluorescence lifetime measurements (see Table I). In the absence of eumelanin, the time profile of the fluorescence emission of 8-MOP can be best fitted by a double exponential; both components are essentially unaffected by binding to eumelanin.

8-MOP is characterized by a very low fluorescence quantum yield [8]; the fraction of prompt heat for 8-MOP is close to unity, being equal to 0.97 in aqueous solution, in accordance with the scarcely efficient intersystem crossing of the psoralen. The triplet lifetime of 8-MOP has been reported by Craw *et al.* [9] to be of the order of 3 μs and falls on the long-lifetime side of our temporal resolution; in accordance with this result, deconvolution analysis of the photoacoustic signals did not give any evidence of heat release occurring within our temporal resolution, apart from release of prompt heat. The energy stored by the triplet state of 8-MOP is then "lost" to the photoacoustic instrument. Melanin itself shows a prompt heat very close to unity, $\alpha = 0.98$. The addition of increasing quantities of melanin causes a lowering of the total prompt heat, α_{tot}, as well as of the prompt heat of 8-MOP, as shown in Figs. 3 and 4. It is evident from Fig. 3 that binding to melanin results in a lowering of both the total prompt heat and the prompt heat emitted by the psoralen; the extent of the effect is, nevertheless, different in the two cases.

This suggests that an increased rate of intersystem crossing is induced by melanin, which quenches the fluorescence of the psoralen, giving rise to transient species decaying on a time scale that is outside the instrumental sensitivity and therefore not sensed by the pressure transducer. We have proposed previously [4] a kinetic

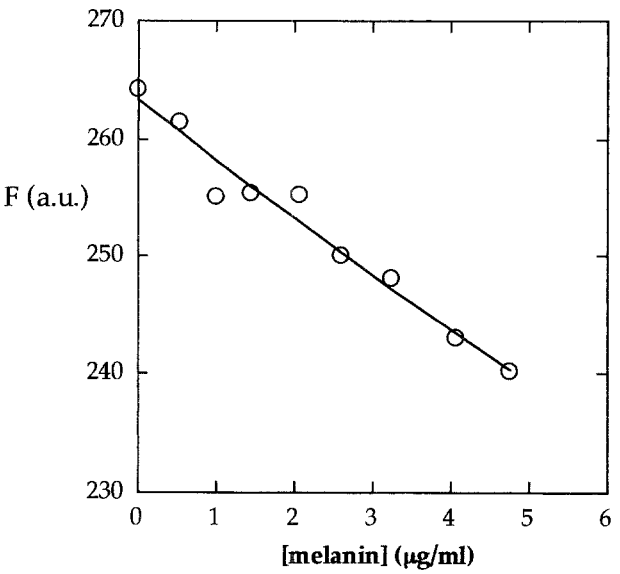

Fig. 2. Quenching of 8-MOP fluorescence by eumelanin; conditions were the same as for Fig. 1. The solid line is the best fit to the data with the usual Stern–Volmer equation, yielding the value $K = 0.02$ $(\mu g/ml)^{-1}$.

Table I. Fluorescence Lifetimes of 8-MOP Alone and in the Presence of Eumelanin[a]

	τ_1 (ns)	P_1 (%)	τ_2 (ns)	P_2 (%)
8-MOP	1.22	76	0.7	23.3
8-MOP + melanin	1.2	73.5	0.76	26.5

[a]Concentrations were as follows: 8-MOP, 10 μM; eumelanin, 8 μg/ml.

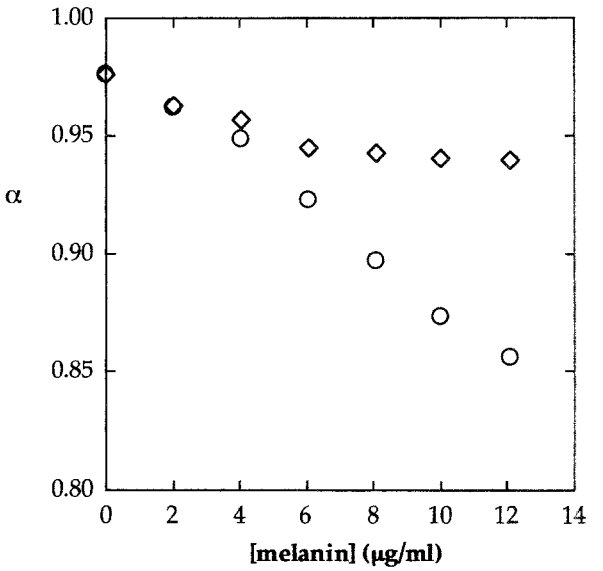

Fig. 3. Total prompt heat for 8-MOP-melanin (◇) and prompt heat of 8-MOP (○) versus melanin concentration. The 8-MOP concentration was 9.7-μ*M*; the melanin concentration ranged between 0 and 12.10 μg/ml.

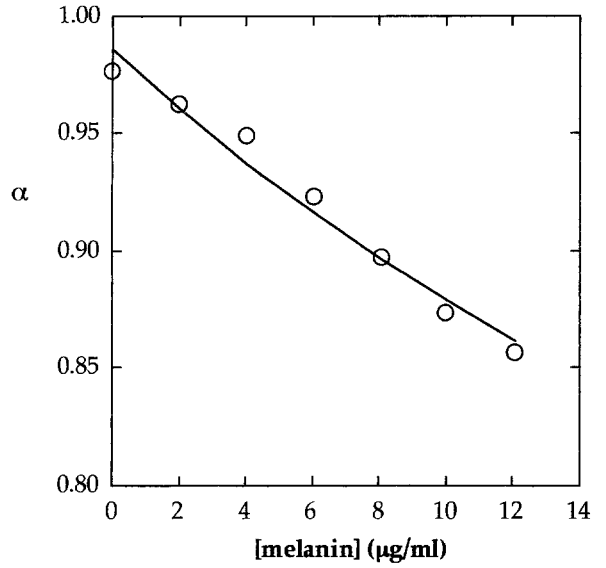

Fig. 4. Prompt heat released by 8-MOP versus melanin concentration. The 8-MOP concentration was 9.7-μ*M*; the melanin concentration ranged between 0 and 12.10 μg/ml. The solid line is the best fit to experimental data of Eq. (2). We use for E_T the value reported previously, corresponding to 535 nm (9).

model to describe this quenching process and the expression for the fraction of prompt heat released by 8-MOP is reported below:

$$\alpha_1 = 1 - \frac{\nu_{em}}{\nu_{ex}} \frac{\Phi_F}{1 + K[M]} - \frac{\Phi_{ISC} + (1 + K[M])}{(1 + K[M])} \frac{E_T}{E} \quad (2)$$

where Φ_F and Φ_{ISC} represent the fluorescence and intersystem crossing quantum yields, υ_{ex} and υ_{em} are the incident and average emission light frequencies; E is the molar energy content of laser pulses, and E_T is the triplet-state energy.

Fitting the experimental points with Eq. (2) gives the value 0.023 $(\mu g/ml)^{-1}$ for K, which agrees with that obtained by fluorescence measurements, indicating that the model is consistent. The value of the intersystem crossing quantum yield for 8-MOP obtained from our fitting is 0.023, which is comparable to that reported previously [10].

Upon deoxygenation of the samples, no significative changes in the photoacoustic parameters were observed, neither for free 8-MOP nor for the melanin-containing samples. This evidence supports the idea that the role of oxygen in the photobiological activity of 8-MOP is not a major one [11]. This behavior can be contrasted with that of a typical photodynamic agent such as zinc-tetrabenzylpyridylporphyrin, for which the interaction with eumelanin is dramatically affected by the presence of oxygen [4].

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