Comparison of the Ultrafast Absorption Dynamics of Eumelanin and Pheomelanin

Tong Ye and John D. Simon*

Departments of Chemistry and Biochemistry, Duke University, Durham, North Carolina 27708

Received: May 9, 2003; In Final Form: August 13, 2003

An enhanced photoreactivity of the red melanin, pheomelanin compared to the black melanin, eumelanin is commonly invoked to explain why fair-skinned individuals are more susceptible to skin cancers. Ultrafast pump−probe spectroscopy is used to examine the primary photodynamics of eumelanin from *Sepia officinalis* and synthetic pheomelanin following excitation at 303 nm. Both melanins reveal a transient absorption throughout the visible spectrum that rises within the instrument response. On the picosecond time scale, the transient absorption signal decays to a constant nonzero value, and the dynamics to nonexponential. Thus, both melanins do not exhibit complete ground-state recovery, suggestive of the formation of long-lived intermediates. Comparison to the initial transient absorption indicates the yield of such species is identical for the two pigments. The shape of the transient spectrum for the two melanins differ; however, global fits to the 13 different probe wavelengths used to construct each spectrum reveal both pheomelanin and eumelanin exhibit similar dynamics. The decay associated spectrum for each time constant is examined. Comparison of the transient absorption dynamics to data obtained from time-resolved emission experiments of melanins suggest that different molecular species are responsible for the data recorded in the two experiments.

Introduction

Melanins are commonly divided into two types: the black eumelanins and the reddish-brown pheomelanins. Eumelanin and pheomelanin are distinguished by differences in their molecular precursors. Eumelanins are composed of indolic units derived from the oxidation of tyrosine.[1,2] Pheomelanins are composed of benzothiazines derived from the oxidation of cysteinyldopa units.[3] It is generally believed that melanin is photoprotective, shielding against ultraviolet (UV) and visible (VIS) radiation.[4−10] Such photoprotection is believed to be responsible for a lower incidence of UV-induced skin damage in individuals with darker skin and/or dark hair.

A higher rate of incidence of UV-induced skin damage among red-haired and fair-skinned individuals is hypothesized to result from, in part, photosensitization effects of pheomelanin.[11−15] In support of this hypothesis, Prota and co-workers reported a correlation between the eumelanin/pheomelanin ratio and the minimal erythemal dose, suggesting that UV sensitivity is associated with high pheomelanin and low eumelanin levels and that the eumelanin/pheomelanin ratio may be a chemical parameter for predicting individuals at high risk for skin cancer and melanoma.[12] UV-A-induced DNA single-strand breaks in human melanocytes differing only in the amount of pigment produced showed photosensitization by intrinsic chromophores, most likely pheomelanin and/or melanin intermediates.[13−15]

Several studies focus on comparing the reactivity of eumelanin and pheomelanin. UV-A/B irradiation of pheomelanin causes the reduction of nitroblue tetrazolium by superoxide.[11] Under similar conditions, eumelanin does not. The cumulative studies to date suggest that there are not substantial differences between the photoreactivity of eumelanin and pheomelanin.[16−23] The photochemical uptake of oxygen by eumelanin and pheomelanin are comparable.[18,24] Both melanins also inhibit UV-induced liposomal lipid peroxidation.[25] However, it is commonly hypothesized that photoreactivity differences between the two pigments underlie observed epidemiological trends in the skin cancer rates between different skin types. Nonphotochemical functions are also ascribed to melanins, including chelation of metal ions[26] and mitigation of oxidative stress by scavenging ROS such as superoxide anion and hydrogen peroxide.[27] A quantitative comparison of the relative effectiveness of eumelanin and pheomelanin in performing these functions has not been reported.

Previously, we examined ultrafast dynamics of pheomelanin following UV-A excitation.[28,29] A transient absorption spectrum from 490 to 1000 nm was presented. Throughout the probe wavelength range there is an instantaneous rise in sample absorbance upon photolysis, and the transient absorption then decays nonexponentially. In the case of eumelanin, we had previously presented degenerate pump−probe data in the UV-B and UV-A regions.[30] Upon photolysis, immediate bleach was observed. The signal then recovered nonexponentially. No studies have been presented discussing the possible visibly absorbing transient for this pigment.

In this paper, the transient absorption spectra in the visible region and their decay dynamics following excitation of synthetic pheomelanin and *Sepia* eumelanin at 303 nm are compared. It would be desireable to compare biologically relevant samples of these pigments. However, our understanding of the isolation and characterization of natural pheomelanins is not as advanced as in the case of eumelanins. At this point, the best-characterized pheomelanin is synthetic, and we have chosen to compare this pigment to the best characterized natural eumelanin, that isolated from the ink sacs of *Sepia officinalis*.

Experimental Section

Sample Preparation. Pheomelanin was synthesized according to published procedures[31] and dissolved in sodium phosphate buffer (pH of 7.2, KH_2PO_4; 0.2008 M, NaOH 0.01201 M).

* To whom correspondence should be addressed. E-mail: jsimon@duke.edu.

10.1021/jp0352837 CCC: $25.00
Published on Web 09/16/2003

Eumelanin was isolated from the ink sac of *Sepia officinalis* as previously described.[32]

Ultrafast Spectroscopy. Experiments were conducted using a commercial regeneratively amplified, Ti/Sapphire laser system (Spitfire, Spectra Physics, 120 fs (fwhm), 0.9 mJ/pulse centered at 800 nm,1 kHz repetition rate). Excitation light pulses at 303 nm were generated using optical parametric amplifier (OPA800, Spectra Physics). Probe pulses in the visible region were generated using interference filters to select the desired wavelength from a white light continuum, which was produced by focusing 800 nm pulses on a 2 mm sapphire plate. The pump−probe transient absorption measurement setup was described previously in detail.[29] The beam diameters of both the pump and probe at the region of spatial overlap within the sample were determined, enabling quantitative analyses on observed signal intensities. In the experiments, the beam diameter of the pump was kept around 200 μm, while that of probe was smaller, around 100 μm. The excitation power was attenuated by the variable neutral density filter and kept at 100 nj/pulse. The optical densities of the samples in a 1-mm quartz cuvette were 0.1 OD at the excitation wavelength. The use of the same optical densities ensures that the same number of photons are absorbed by the two pigments. Unfortunately, molecular structure and therefore molecular weight information is not available for melanins, and so the spectra cannot be normalized to concentration of absorbing species. Normalization by matched optical density at the excitation wavelength enables comparison of the oscillator strengths of the transient absorptions of the two pigment. All transient optical experiments were performed using magic angle between the polarization of the pump and probe pulses in a 1 mm path length flowing cuvette. During photolysis, a peristaltic pump circulated the solution.

Data Analysis. Nonlinear least-squares analysis was used to simultaneously fit the entire set of transient absorption decays (13 different probe wavelengths) to

$$y(\lambda_i, t) = y_{0i} + A_{1i}e^{-t/\tau_1} + A_{2i}e^{-t/\tau_2} + A_{3i}e^{-t/\tau_3}$$

where, λ_i is the *i*th probe wavelength, *t* is the time delay between the pump and probe pulses, y_{0i} is the offset, and A_{1i}, A_{2i}, and A_{3i} are the preexponential factors for the time components,τ_1, τ_2 and τ_3, at the *i*th probe wavelength, respectively. The preexponential factors obtained by such a global fitting procedure can be used to construct a spectrum for the each time component, or decay associated spectrum (DAS). The transient signals do not decay to zero on the time scale of the experiment, but level off at a small transient absorption value, which is denoted by y_{0i}.

Results and Discussion

Transient Spectrum of Eumelanin and Pheomelanin. Figure 1 shows the transient absorption spectrum at 1 ps following photoexcitation of *Sepia* eumelanin and synthetic pheomelanin at 303 nm. The data shown in the graph are normalized to excitation power density and sample absorption at 303 nm in a 1 mm cell. The plot shows that the intensity of the transient signal for *Sepia* eumelanin is about a factor of 2 greater than that of synthetic pheomelanin. Both spectra rise within the instrument response and then decay with increased delay time. The spectra differ in the relative intensities of the three absorption maxima revealed but have an overall similarity in shape. The ratio of the intensity of the bands at ~650 nm and ~575 nm are similar for the two pigments. The major spectral difference between pheomelanin and eumelanin is the wavelength of maximum absorbance, 750 nm for pheomelanin and ~800 nm for eumelanin. For the pheomelanin spectrum, the five peaks appear at 750 nm (13 333 cm^{-1}), 700 nm (14 285 cm^{-1}), ~640 nm (15 625 cm^{-1}), 585 nm (17 094 cm^{-1}), and 520 nm (19 231). The energy differences of these peaks from the 750 nm band are 952, 2292, 3761, and 5898 cm^{-1}, respectively, which one can reasonably associate with vibronic structure. It appears that the intensities of vibronic peaks are enhanced with increasing wavelength. The spectra alone cannot enable determination of the number of molecular chromophores that contribute. If different molecules correspond to the different transient features, one could expect differences in the dynamics of these spectral features.

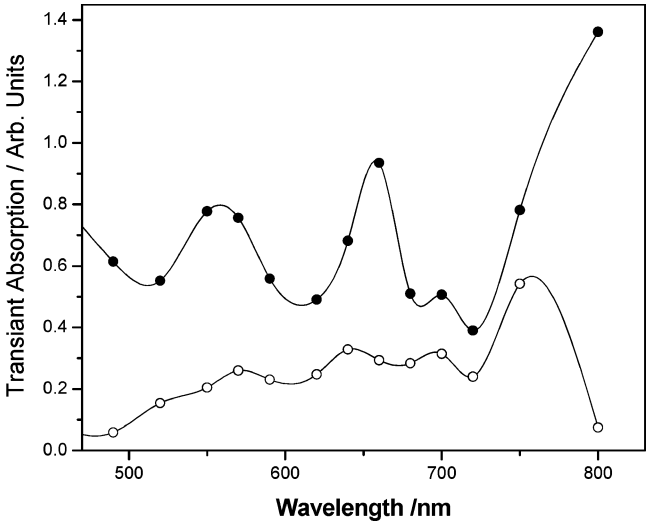

Figure 1. Transient spectra recorded 1 ps following the excitation of eumelanin (●) and pheomelanin (○) by 303 nm. The spectra are recorded for identical optical density solutions and under identical spectrometer conditions. The signal intensities observed therefore indicate that the transient spectrum associated with eumelanin has a larger oscillator strength than that for pheomelanin. The spectra show similar structural features.

Transient Absorption Decay Dynamics and Decay Associated Spectra. Figure 2 shows the time-dependent dynamics for the probe wavelength of 520 nm for both eumelanin (A) and pheomelanin (B). In fitting, the time constants are determined by a global fit to the entire data set (13 wavelengths), and amplitudes vary to obtain the best fit (as measured by r^2). The time constants obtained from the global fit are given in Table 1. The solid lines in Figure 2 are the calculated fits to these transient data. The data reveal that the times associated with the deactivation of the electronically excited melanins are essentially identical.

Figures 3 shows the DAS for pheomelanin and eumelanin, respectively. For pheomelanin, the DAS for the three time constants are similar. The results for eumelanin are not as straightforward. The DAS corresponding to the three time constants all show three pronounced maxima, but the relative amplitudes of these features differ for the three time constants. In addition, small spectral shifts are observed in the band ~570 nm.

The 800-nm component to the DAS dominates for all three time constants. Relative to the 800-nm component, the 650-nm and 570-nm components increase in importance as the time constant becomes longer. The ratio of the 650-nm and 570-nm components for τ_1, τ_2, and τ_3 are 1.6:1, 1.2:1, and 0.9:1, respectively, revealing that these absorption bands play dominant roles in different time constants. The analysis does not cleanly assign a specific absorption feature in the transient spectrum to

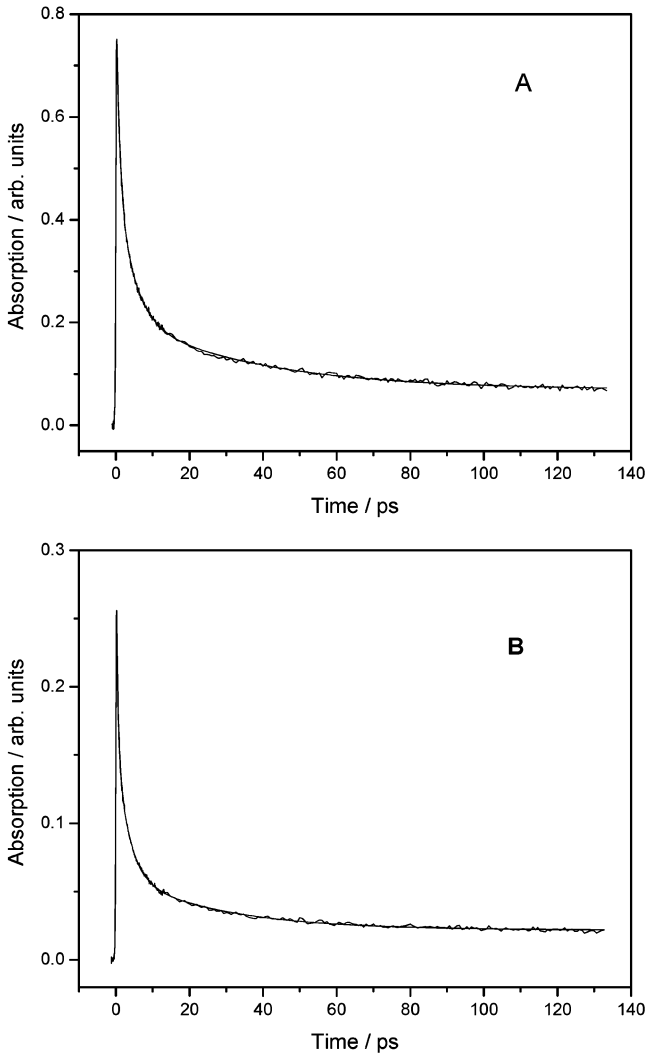

Figure 2. Transient decay at 520 nm for eumelanin (A) and pheomelanin (B) following excitation at 303 nm. The solid lines through the data are a result of the global fit of a multiexponential decay function to the entire set of probe wavelengths. The time constants obtained from the fits are given in Table 1.

TABLE 1: Global Fitting Parameters Obtained from the Transient Absorption Data for Melanins Following Excitation at 303 nm

sample	τ_1/ps	τ_2/ps	τ_3/ps	r^2
eumelanin	0.56 ± 0.8	3.2 ± 0.5	31 ± 5	0.9985
pheomelanin	0.46 ± 0.7	2.9 ± 0.5	27 ± 5	0.9993

a given time constant. The resolution afforded by the global data analysis suggests that all spectral features present in the transient spectrum contribute to the multiexponential decay. It is likely that these spectra are derived from a small set of structurally similar chromophores within the eumelanin pigment. This would explain the nonexponentiality of the decays, the similar vibronic structure observed in the transient spectrum, and the differences in the relative intensities of the vibronic bands. Given that the connectivity of the monomer units in the structure of eumelanin is not known, it is currently not possible to speculate on the molecular details that give rise to these spectral differences.

The total delay time examined in these experiments corresponds to five times the longest relaxation time (ca. 30 ps) obtained from the analytical analysis of the data set. Thus, the values for $y_0(\lambda)$ obtained from the global fitting procedure

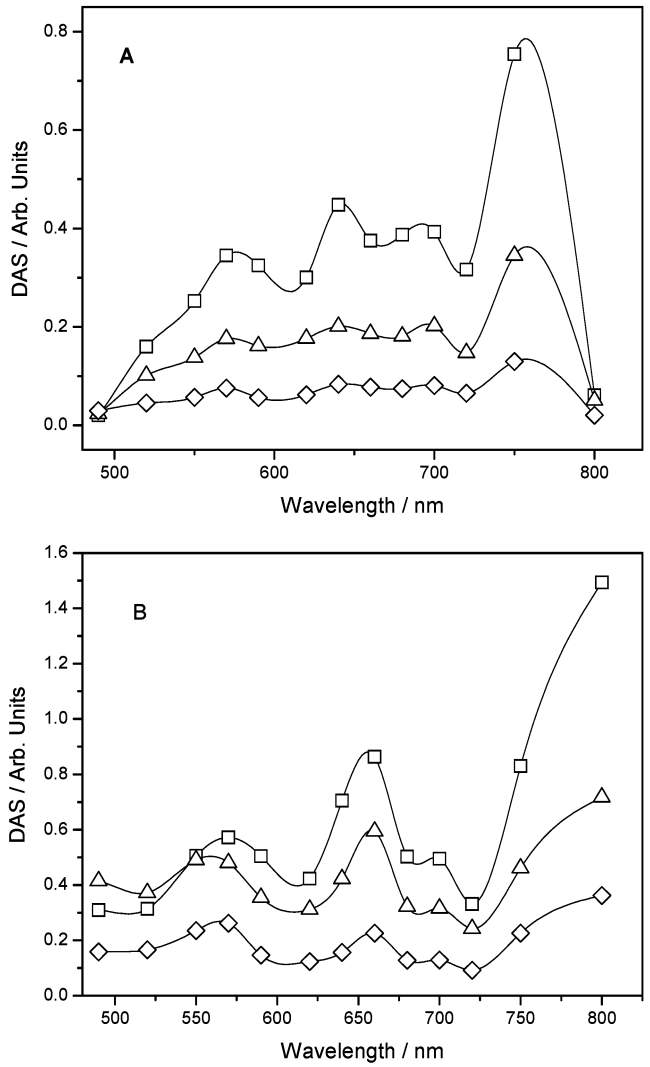

Figure 3. The DAS for the transient data following the photoexictation of pheomelanin (A) and eumelanin (B) at 303 nm. The symbols (□), (△), and (◇) correspond to the three time constants τ_1, τ_2, and τ_3 tabulated in Table 1, respectively.

represents the transient spectrum of the long-lived ($t > 150$ ps) species formed following the ultrafast recovery dynamics observed. The lifetime of the intermediate(s) is not known, but given that no dynamics are reflected in the decay for the time scale probed, the shortest lifetime will be on the order of a few nanoseconds. Optical transients of melanin have not been observed in nanosecond laser flash-photolysis studies, and our attempts to record such data have also not bee successful. The resolution of transient absorption signals in the nanosecond experiments are on the order of 10^{-2} OD units. The femtosecond experiments reveal that the $y_0(\lambda)$ signals corresponds to an absolute signal of 5×10^{-6} OD units. Thus, in this case, signals resolved on the femtosecond time scale are not observable on the nanosecond time scale.

It is interesting to compare the transient spectrum obtained immediately following photolysis (0.2 ps) with the DAS corresponding to the values of $y_0(\lambda)$. The comparisons of these spectra are shown for both melanins in Figure 4. For each melanin, the two spectra have been normalized at the wavelength of maximum absorption. This comparison shows that the shape of the transient spectrum following the nonexponential ultrafast decay is different than that observed immediately following photolysis. On a relative scale, the majority of the spectral decay on the picosecond time scale takes place in the region between

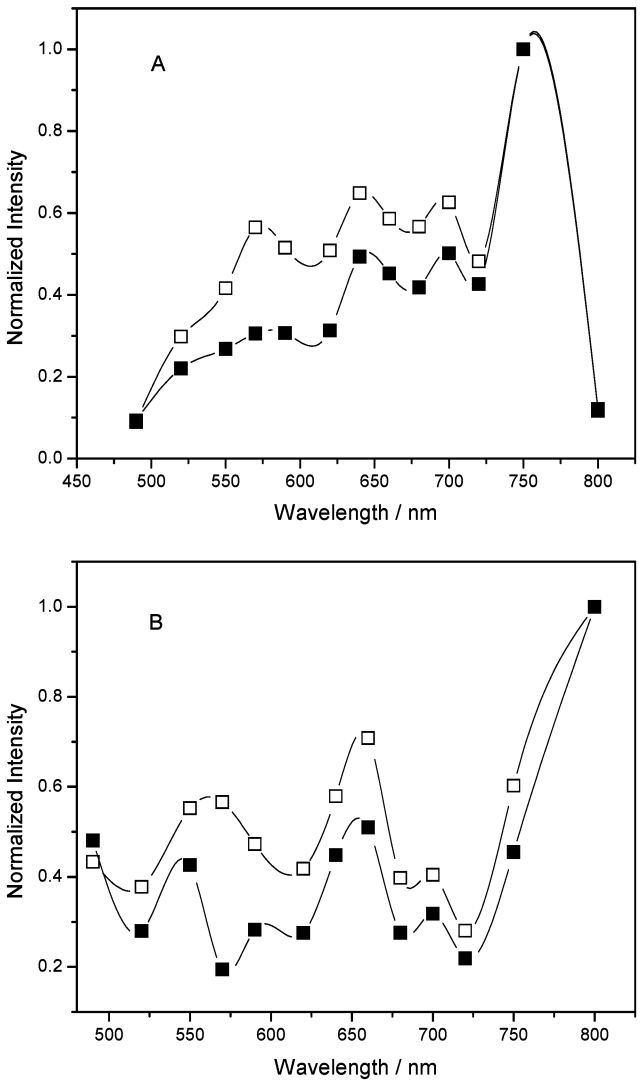

Figure 4. Compared of the transient absorption spectrum 0.2 ps following photolysis at 303 nm (□) with the DAS corresponding to the *y*0 value (■) for pheomelanin (A) and eumelanin (B). This DAS spectrum is identical to that observed experimentally for time delays between 100 and 150 ps and therefore reflects the absorption of the transient that does not decay on ultrashort time scales.

500 and 700 nm. These data further suggest that the transient dynamics arise from the excitation of a set of chromophores within the pigment.

The similarity in the time constants (Table 1) for eumelanin and pheomelanin certainly support the thought that similar chemical structures control the observed dynamics. Eumelanin and pheomelanin are both polyphenolic species, and it is possible that such a chromophore (e.g., catechol) is responsible for the dynamics reported herein. Further work in the elucidation of the molecular structure of melanin constituents is needed before such a definitive assignment can be made.

Comparison of the Transient Absorption and Emission Decay Dynamics. It is interesting to compare these dynamics with those obtained from fluorescence lifetime measurements. The emission dynamics of melanin are nonexponential and require a sum of exponentials to generate functional forms that provide fits to experimental data.[33−36] In a previous report on *Sepia* eumelanin, for excitation at 335 nm, the four lifetimes and amplitudes (given in parentheses) for emission collected at 520 nm, near the maximum of the spectrum, are 58 ps (0.54); 0.51 ns (0.22), 2.9 ns (0.16) and 7.0 ns (0.08).[33] Likewise, the fluorescence from pheomelanin is nonexponential. A function form that is the sum of three exponentials provides a fit to experimental data; the lifetimes and amplitudes (given in parentheses) are 46 ps (0.66), 1.2 ns (0.16), and 6.2 ns (0.18).[37]

The origins of the nonexponential decays (both in absorption and emission) are not known. However, the fastest lifetime component of the emission is in rough agreement with the slowest time constant revealed by the ultrafast absorption measurements, and thus it is possible that both experimental techniques are reflecting the decay of the same molecular species. Because attempts to perform nanosecond flash photolysis experiments on the melanins were unsuccessful, we are unable to address whether there are decay components on nanosecond time scale in the transient absorption signal, similar to what is observed in the emission data. Similarly, the time-resolution of single-photon counting detection renders it nearly impossible to resolve dynamics components faster than 10 ps, and so it is not clear whether the ultrafast components observed in the transient absorption measurement are present in the emission decays and simply not resolved because of the inherent resolution of the instrument. The quantum efficiency of the emission (ca. 10^{-3}) is too low to enable detection of the emission dynamics of upconversion techniques. Thus, technical limitations make comparison of the dynamic data difficult, and one cannot determine from these data alone whether the emission and absorption signals arise from the same chromophore(s) within the pigments.

Insight into this issue can be obtained by comparing the emission excitation spectrum and the action spectrum for the generation of the ultrafast transient absorption. The emission excitation spectrum provides the absorption profile of the molecule(s) that generate emission at the wavelength detected. Using tunable-excitation wavelengths and ultrafast transient absorption detection, if we assume the molecular chromophore responsible for the observed transient absorption spectrum does not exhibit wavelength-dependent photochemistry, and then the shape of the action spectrum is a measure of the absorption profile of the molecule(s) that generate(s) the observed transient. The emission excitation and action spectrum for the ultrafast transient for the pheomelanin sample studied herein peak at 400 and 360 nm, respectively. Because these spectra provide direct information on the absorption properties of the constituent(s) responsible for the emission and transient absorption, respectively, the fact that these spectra differ reveals that time-resolved emission and transient absorption experiments probe *different species* in the pigment. These results clearly show that one must exercise caution in interpreting the transient spectroscopic results of melanins in terms of the photobiological properties of the pigments. While interesting dynamics are revealed, it remains to be established whether either transient absorption or emission experiments probe the molecules responsible for the photoaerobic reactivity of melanins. To address such issues, it will be important to measure the transient optical properties and the action spectra for photoaroebic processes (oxygen photoconsumption, superoxide formation) on the same set of samples. Comparison between existing published literatures is potentially problematic, resulting from the range of methods used to isolate and prepare melanins and the effects these procedures have on the integrity and molecular structure of the pigment.

Acknowledgment. This work is supported by the National Institute of General Medical Sciences and Unilever Research US.

References and Notes

(1) Ito, S. *Biochim. Biophys. Acta* **1986**, *883*, 155−161.
(2) Ozeki, H.; Wakamatsu, K.; Ito, S.; Ishiguro, I. *Anal. Biochem.* **1997**, *248*, 149−157.
(3) Ito, S. *J. Invest. Dermatol.* **1993**, *100*, S166−S171.
(4) Zeise, L.; Chedekel, M. R.; Fitzpatrick, T. B., Eds. *Melanin: Its role in human photoprotection*; Valdnmar Press: Kansas, 1995.
(5) Giacomoni, P. U. *J. Photochem. Photobiol., B* **1995**, *29*, 87−89.
(6) Kollias, N.; Sayre, R. M.; Zeise, L.; Chedekel, M. R. *J. Photochem. Photobiol., B* **1991**, *9*, 135−160.
(7) Kollias, N.; Baqer, A. H. *J. Soc. Cosmet. Chem.* **1988**, *39*, 347−354.
(8) Hill, H. Z.; Hill, G. J. *Pigm. Cell. Res.* **2000**, *13*, 140−144.
(9) Hill, H. Z. *Bioessays* **1992**, *14*, 49−56.
(10) Hill, H. Z. *J. Invest. Dermatol.* **1994**, *102*, 265−265.
(11) Chedekel, M. R.; Smith, S. K.; Post, P. W.; Pokora, A.; Vessel, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5395−5399.
(12) Vincensi, M. R.; d'Ischia, M.; Napolitano, A.; Procaccini, E. M.; Riccio, G.; Monfrecola, G.; Santoianni, P.; Prota, G. *Melanoma Res.* **1998**, *8*, 53−58.
(13) Wenczl, E.; van der Schans, G. P.; Roza, L.; Kolb, R.; Smit, N.; Schothorst, A. A. *J. Invest. Dermatol.* **1998**, *110*, 693−693.
(14) Wenczl, E.; Van der Schans, G. P.; Roza, L.; Kolb, R. M.; Timmerman, A. J.; Smit, N. P. M.; Pavel, S.; Schothorst, A. A. *J. Invest. Dermatol.* **1998**, *111*, 678−682.
(15) Wenezl, E.; Timmerman, A. J.; vanderSchans, G. P.; Roza, L.; Smit, N.; Kolb, R.; Schothorst, A. A. *J. Invest. Dermatol.* **1997**, *109*, 433−433.
(16) Ezzahir, A. *J. Photochem. Photobiol. B* **1989**, *3*, 341−349.
(17) Schmitz, S.; Thomas, P. D.; Allen, T. M.; Poznansky, M. J.; Jimbow, K. *Photochem. Photobiol.* **1995**, *61*, 650−655.
(18) Sarna, T.; Menon, I. A.; Sealy, R. C. *Photochem. Photobiol.* **1985**, *42*, 529−532.
(19) Sarna, T.; Sealy, R. C. *Photochem. Photobiol.* **1984**, *39*, 69−74.
(20) Sarna, T.; Sealy, R. C. *Arch. Biochem. Biophys.* **1984**, *232*, 574−578.
(21) Sarna, T.; Sealy, R. C.; Menon, I. A. *Yale J. Biol. Med.* **1984**, *57*, 416−416.
(22) Rozanowska, M.; Sarna, T.; Land, E. J.; Truscott, T. G. *Free Radic. Biol. Med.* **1999**, *26*, 518−525.
(23) Chedekel, M. R.; Agin, P. P.; Sayre, R. M. *Photochem. Photobiol.* **1980**, *31*, 553−555.
(24) Sarna, T.; Menon, I. A.; Sealy, R. C. *Photochem. Photobiol.* **1984**, *39*, 805−809.
(25) Krol, E. S.; Liebler, D. C. *Chem. Res. Toxicol.* **1998**, *11*, 1434−1440.
(26) Sarna, T. *J. Photochem. Photobiol., B* **1992**, *12*, 215−258.
(27) Bustamante, J.; Bredeston, L.; Malanga, G.; Mordoh, J. *Pigm. Cell. Res.* **1993**, *6*, 348−353.
(28) Ye, T.; Simon, J. D. *J. Phys. Chem. B* **2002**, *106*, 6133−6135.
(29) Ye, T.; Simon, J. D. *Photochem. Photobiol.* **2003**, *77*, 41−45.
(30) Nofsinger, J. B.; Ye, T.; Simon, J. D. *J. Phys. Chem. B* **2001**, *105*, 2864−2866.
(31) Ito, S. *Pigm. Cell. Res.* **1989**, *2*, 53−56.
(32) Liu, Y.; Simon, J. D. *Pigm. Cell. Res.* **2003**, *16*, 72−80.
(33) Forest, S. E.; Lam, W. C.; Millar, D. P.; Nofsinger, J. B.; Simon, J. D. *J. Phys. Chem. B* **2000**, *104*, 811−814.
(34) Nofsinger, J. B.; Simon, J. D. *Photochem. Photobiol.* **2001**, *74*, 31−37.
(35) Teuchner, K.; Ehlert, J.; Freyer, W.; Leupold, D.; Altmeyer, P.; Stucker, M.; Hoffmann, K. *J. Fluoresc.* **2000**, *10*, 275−281.
(36) Teuchner, K.; Freyer, W.; Leupold, D.; Volkmer, A.; Birch, D. J. S.; Altmeyer, P.; Stucker, M.; Hoffmann, K. *Photochem. Photobiol.* **1999**, *70*, 146−151.
(37) Ye, T.; Simon, J. D. *in preparation* **2003**.