Explanation for the Disparity among Absorption and Action Spectra of Eumelanin

J. Brian Nofsinger,[†] Susan E. Forest,[†,‡] and John D. Simon*[,†,§]

Department of Chemistry, Duke University, Durham, North Carolina 27708, and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received: July 28, 1999; In Final Form: October 11, 1999

The optical properties of eumelanin (from *Sepia officinalis*) are found to vary with particle size. The absorption spectrum for small eumelanin particles agrees quantitatively with the reported action spectra for photoinduced oxygen consumption and free radical generation by eumelanin. These small particles, unlike the large ones, generate long-lived reactive intermediates upon absorption of UV light. The data presented suggest that the small eumelanin particles may be involved in UV-A-induced photochemical processes believed to lead to DNA damage in skin cells, whereas the large particles efficiently dispose of UV-A energy through rapid nonradiative decay processes. These results provide new insight into the dichotomy that eumelanin is both photoprotective and photosensitizing. This size-dependent photoreactivity may be one of the contributing factors to the observed variations in skin cancer rates among different skin types.

Introduction

Half of all new cancers today are skin cancers.[1] The incidences of skin cancers (both melanoma and nonmelanoma) are increasing in most parts of the world.[2] In the United States, there has been about a 600% increase in the rate of incidence of this disease since the 1930s.[3] The lifetime risk of developing malignant melanoma for a person born in 1950 is 1 in 600, while the current estimate for someone born in 1987 is 1 in 135. An even higher rate of incidence is predicted for those born at the turn of the century.[3] The American Cancer Society estimates that in the United States about 44 200 new melanomas will be diagnosed[1] and about 7300 people will die from this disease in 1999.[4] Among skin diseases, melanoma is the most frequent cause of death.

It is clear that one of the dominant causes of skin damage and skin cancer is exposure to ultraviolet (UV) radiation. While significant attention has been paid to UV-B (280−320 nm) exposure[5,6] (the region of direct DNA absorption), reports clearly indicate that UV-A (320−400 nm) radiation also plays an important role in skin diseases.[5,7−9] In general, the pathways by which UV-A induced physiological responses are thought to occur are by mechanisms that involve the photochemical generation of singlet oxygen and/or reactive species such as superoxide, peroxide, and the hydroxyl radical by the absorbing molecule.[7−9] One of the UV-A photoreceptors in melanocytes, the cells that give rise to melanoma, is melanin. Excitation of melanin by UV light has been shown to produce the damaging oxygen species mentioned above.[10,11] Therefore, elucidating the characteristics behind the photogeneration of reactive species by melanin is important for understanding the possible roles of this pigment in causing melanoma cancers. In addition, because melanin is also present near the nuclei of keratinocytes, the UV- (and visible-) induced photochemistry of this pigment can also be involved in the initial photoreactions that ultimately lead to nonmelanoma cancers.

Photoreactions of melanins have been studied, and in vitro action spectra for particular chemical processes have been reported.[12,13] In general, an action spectrum follows the absorption spectrum of the primary photoreceptor involved in the photochemical process.[14] However, in the case of melanin, this relationship has not been observed. For example, for eumelanin (the brown-black pigment) the action spectra for the photoconsumption of oxygen and the photoproduction of radicals are essentially identical, but these spectra differ dramatically from the absorption spectrum of the pigment.[12,13] Determining why these action spectra reveal a wavelength dependence different from that of the absorption spectrum is central to developing an understanding of the photochemical properties of this pigment and its relationship to DNA damage and skin cancer. In this paper we propose an explanation for the observed difference between the absorption and action spectra.

An unresolved and germane topic to this issue is the relative contributions of scatter and absorption to the optical spectrum of eumelanin. It is possible that the optical spectrum as measured in an absorption spectrometer is dominated by scattering and that the published action spectra represent the true shape of eumelanin's absorption spectrum. For example, previous work suggests that the scattering cross section is about 15% of that of the absorption cross section at 580 and 633 nm.[15]

It is also possible that the difference between the optical and action spectra reflects an underlying molecular property of the pigment. In this regard, the heterogeneity of eumelanin (both in composition and in size) could be important. Previous work by Zeise and co-workers clearly reveals a large distribution of particle sizes comprising eumelanin.[16] Specifically, their studies reveal that eumelanin particles from *Sepia officinalis* are spherical in shape and range in size from 70 to 460 nm in diameter. The peak of the particle size distribution is approximately 160 nm. The distribution is not symmetric about the peak value. These particles can aggregate to form units that can range in size to tens of micrometers.[16] In addition to these observations, we have found that there is additional substructure consisting of spherical particles less than 60 nm in diameter.[17] In this paper we examine optical and photoacoustic properties

[†] Duke University.
[‡] Current address: Procter & Gamble, Miami Valley Labs, 11810 East Miami River Road, Ross, Ohio 45061.
[§] Duke University Medical Center.

10.1021/jp992640y CCC: $18.00
Published on Web 11/30/1999

of eumelanin as a function of particle size. These data provide both insight into the contribution of scattering to the optical spectrum and a molecular explanation for the above-mentioned action spectra for light-induced oxygen consumption and free radical production.

Experimental Section

Eumelanin extracted from the ink sacs of *Sepia officinalis* was obtained from Sigma. An amount of 1 L of HPLC grade water was added to 1 g of eumelanin. The solution was then sonicated (Branson Ultrasonics Corporation) for 30 min at a temperature between 18° and 23 °C and then centrifuged (RC-5 Superspeed Refrigerated Centrifuge, DuPont Co., Sorvall Centrifuge Products) at 3000 rpm for 30 min. The eumelanin solution was separated by molecular weight, MW, using an Amicon stirred cell equipped with ultrafiltration disk membranes (Millipore-Amicon). Ultrafiltration disk membranes are rated in terms of molecular weight through the retention of globular proteins. By use of a series of membranes, the following solutions were collected and examined: (a) MW > 10 000, (b) 10 000 > MW > 3000, (c) 3000 > MW > 1000, (d) MW < 1000. All filtrations were performed at 5 °C. The samples for photoacoustic measurements were made in a pH 7.2 potassium phosphate buffer.

Scanning electron microscopy (SEM) was used to determine the particle sizes for the various fractions. Melanin particles in the stock and MW > 10 000 solutions were mainly between 2 and 25 μm in diameter, while the 10 000 > MW > 3000, 3000 > MW > 1000, and MW < 1000 solutions contained particles smaller than 60 nm. Our SEM results on the various particle sizes are discussed in detail elsewhere.[17]

For comparison purposes, eumelanin was also synthesized by an autoxidation procedure.[18] An amount of 1 g of 3,4-dihydroxyphenylalanine (Sigma) was added to 1 L of a pH 7.5 potassium phosphate buffer. The solution was kept in the dark for approximately 2 weeks with occasional monitoring of pH. After 2 weeks, the pH was adjusted to 2.0 using 2 M HCl. The solution was then centrifuged for 15 min at 2000 rpm. The solution was washed with deionized water until the pH reached 5.0. The eumelanin paste was stored under 5−10 mL of deionized water in a dark refrigerator. The product was characterized using optical absorption. Molar absorptivities were in agreement with literature values.[19]

Absorption experiments were carried out using a diode array spectrophotometer (Hewlett-Packard 8452). The solutions studied had an optical density of 0.114 ± 0.004 at 266 nm in a 1 cm cuvette.

The setup for photoacoustic experiments is similar to that described by Hanson et al.[20] Samples of eumelanin contained in a quartz cuvette were excited by 0.8−8.0 μJ, 10 ps pulses at 351 nm. The 10 ps, 351 nm pulses were created by third harmonic generation of an amplified, diode-pumped Nd:YLF (1054 nm) laser. The photoacoustic signal was detected perpendicular to the excitation source by a 1 MHz piezoelectric transducer (Panametrics A103.S) attached to the sample cell with a thin layer of silicon grease. The signal was preamplified (Panametrics 5660b) and then recorded by a digital oscilloscope (LeCroy 9450AM). Data were collected at a 125 Hz repetition rate and were transferred to a computer for analysis. The laser beam was not focused into the sample, but rather a pinhole was used to define a beam diameter of approximately 2 mm. Each photoacoustic waveform was generated from an average of 1000 laser shots. Photoacoustic signals of eumelanin were compared to that of a reference molecule, bromocresol purple,[20,21] under identical conditions. Data were collected for both degassed and oxygen-saturated samples. A stir bar was used to circulate the sample in the cuvette during data accumulation. The photoacoustic signal varies linearly with the optical density of the sample and the excitation energy,[21,22] as expected. The data collected were normalized both to the excitation energy using a photodiode that detected part of the excitation pulse and to the optical density as measured by an absorption spectrometer. The optical density of the solutions studied was 0.100 ± 0.004 at 351 nm in a 1 cm cuvette.

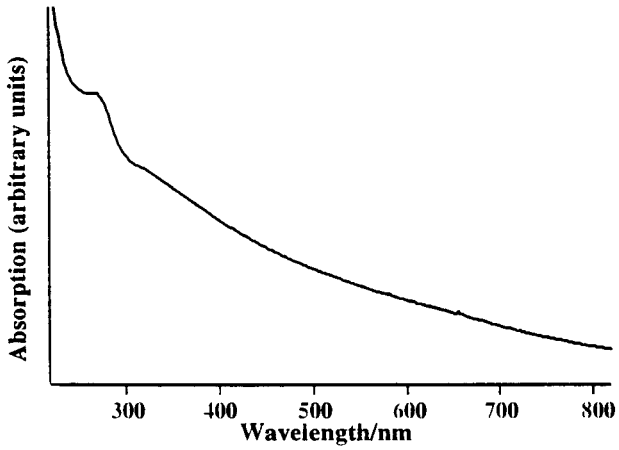

Figure 1. Optical spectrum of an aqueous solution of eumelanin from *Sepia officinalis* at room temperature.

From measurements of the acoustic wave generated by the heat released during radiationless transitions from a photoexcited molecule, both energetic and kinetic data can be obtained for short-lived species that cannot be easily monitored by traditional time-resolved techniques. Energetic changes reflected in the amplitude of the photoacoustic signal and kinetics are manifested by the time dependence of the signal. For the particular transducer used in these experiments, kinetics that occur on a time scale of nanoseconds to a few hundred nanoseconds can be resolved. Faster events appear to occur instantaneously, and slower events are not detected by the device. Further details of the theory and data analysis of photoacoustic calorimetry can be found in the literature.[21−24]

A distribution of results was obtained for different aliquots of each melanin sample studied. For example, for different measurements on the 3000 > MW > 1000 solution of eumelanin, the percentage of the photon energy released as heat ranged from 71% to 101%. On the other hand, results for the MW < 1000 aliquot showed a much smaller and more normal distribution ranging from 36% to 47%. The large range in signals recorded for the different size fractions is most likely due to the heterogeneity of the samples.[17] Photoacoustic calorimetry is generally both precise and accurate, having an experimental error of around 2%.[22] This is consistent with the observed variations in signal for our reference compound bromocresol purple. A statistical analysis of the data sets recorded for the melanin samples gives an error of 11% and 3% for the 3000 > MW > 1000 and MW < 1000 solutions, respectively.

Results and Discussion

The optical spectrum of a pH 7.2 solution of eumelanin from *Sepia officinalis* is shown in Figure 1. The corresponding spectra for four size-selected eumelanin samples derived from this bulk solution are shown in Figure 2. These data have been arbitrarily normalized at 270 nm in order to illustrate two spectral features worth noting. First, consider the peak at 270 nm. Eumelanin from *Sepia officinalis* has a protein coat associated with it that

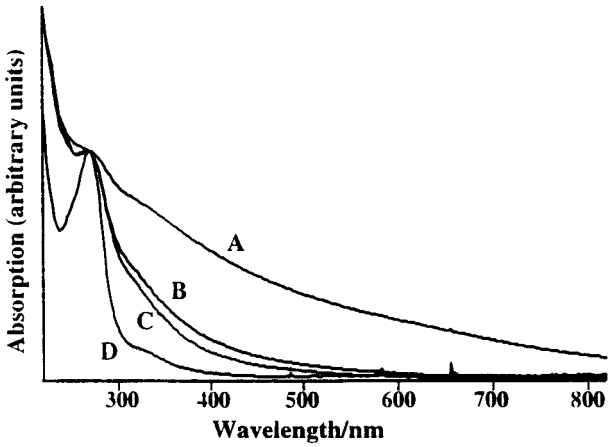

Figure 2. Optical spectra for aqueous solutions of size-dependent fractions of eumelanin from *Sepia officinalis* at room temperature: (A) MW > 10 000; (B) 10 000 > MW > 3000; (C) 3000 > MW > 1000; (D) MW < 1000. The spectra are normalized at 270 nm. The spectral feature around 270 nm arises from the absorption by the protein coat associated with the natural eumelanin.

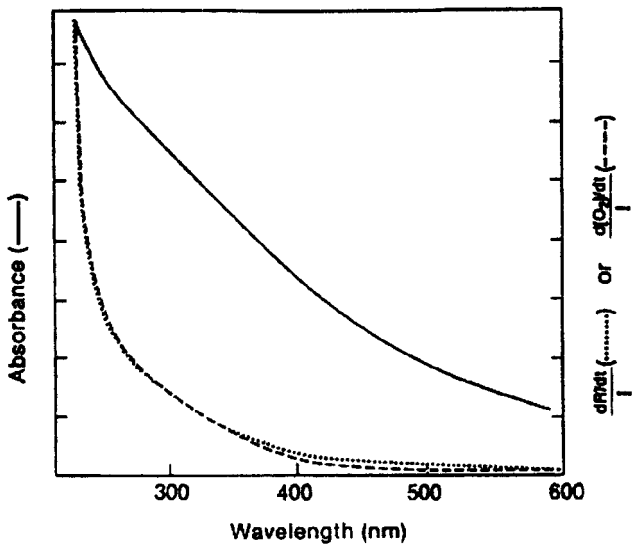

Figure 3. Absorption spectrum of synthetic eumelanin compared to the action spectra for photoinitiated free radical production and oxygen consumption. The figure is reproduced from ref 12 with permission.

can constitute up to 50% of a sample's weight.[25] This coat cannot be fully removed in the isolation and purification process of the pigment. About 8% of the weight of the samples used in this study is protein.[26] The protein associated with these samples has been shown to contain tyrosine and phenylalanine;[27] these amino acids have an absorption peak around 270 nm. Synthetic eumelanin that is generated by the autoxidation of 3,4-dihydroxyphenylalanine lacks a protein coat and has an optical spectrum that matches the natural pigment *except* for the lack of the 270 nm component (see data in Figure 3). Thus, the spectral feature at 270 nm observed in the natural samples can be assigned to absorption of light by the protein coat. The data in Figure 2 show that the relative contribution of the protein fragments to the optical spectrum varies with particle size.

The second feature worth noting is that the optical density changes in the UV-A and visible regions of the spectrum as a function of particle size. Specifically, the spectrum extends to longer wavelengths with increasing particle size. Because the structure of eumelanin is not known, it is not possible to determine the concentration of each sample. Therefore, the absolute absorption cross section at various wavelengths cannot

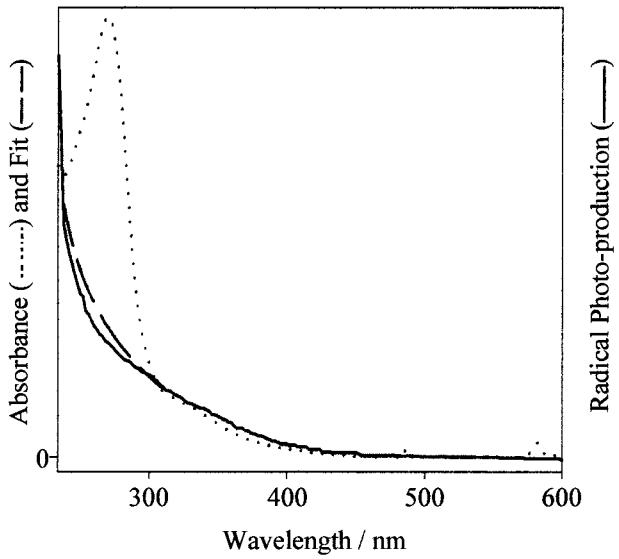

Figure 4. Absorption spectrum of the eumelanin size fraction MW < 1000 (dotted line) compared to the action spectrum for photoinitiated free radical production (solid line). The estimated eumelanin absorbance in the absence of protein (dashed line) is also plotted (see text for details).

be determined. However, it is clear from the data that the large particles (MW > 10 000) dominate the optical spectrum of the bulk solution. From a fit of the optical spectrum of the bulk solution to a linear combination of the size-selected optical spectra, we estimate that particles with molecular weights less than 10 000 comprise no more than 1% of the optical density of bulk melanin in the UV-A.

Figure 3 reproduces the results of Sarna and Sealy, in which they compared the absorption spectrum of synthetic eumelanin to the action spectra for the photoinduced consumption of oxygen and free radical production.[12] They found that the action spectra are quantitatively similar for synthetic eumelanin and bovine eye melanin with and without the protein coat. Because of this similarity, the spectrum reported in ref 12 and reproduced in Figure 3 is the average of the measured action spectra for these eumelanins. The differences between the absorption and action spectra have not been explained. We now know that different sized particles have different absorption properties, so it is of interest to compare these action spectra to the data shown in Figure 2. As shown in Figure 4, we find that the absorption spectrum of small eumelanin particles (MW < 1000) is the only spectrum in Figure 2 that has the same shape as the action spectra for wavelengths longer than 310 nm. This comparison suggests that small eumelanin particles are responsible for the photochemical generation of reactive free radicals and consumption of oxygen. The wavelength dependence of the photoinduced consumption of oxygen and free radical production does not depend on the presence of protein; the action spectra for the bovine eye melanin is the same with and without the protein coat.[12] Therefore, we should compare the action and absorption spectra without regard to protein absorption. This is difficult because the protein component cannot be isolated independently so that its absorption properties can be determined and then subtracted from the spectra shown in Figures 2 and 4. The following was done to estimate the absorption spectrum of eumelanin in this region. The absorption spectrum for the wavelength regions well separated from the protein component ($220 \text{ nm} < \lambda < 236$ and $310 \text{ nm} < \lambda < 328$ nm) was fit to a biexponential function. The resulting parameters were then used to interpolate the absorption spectrum from 236 to 310 nm. This

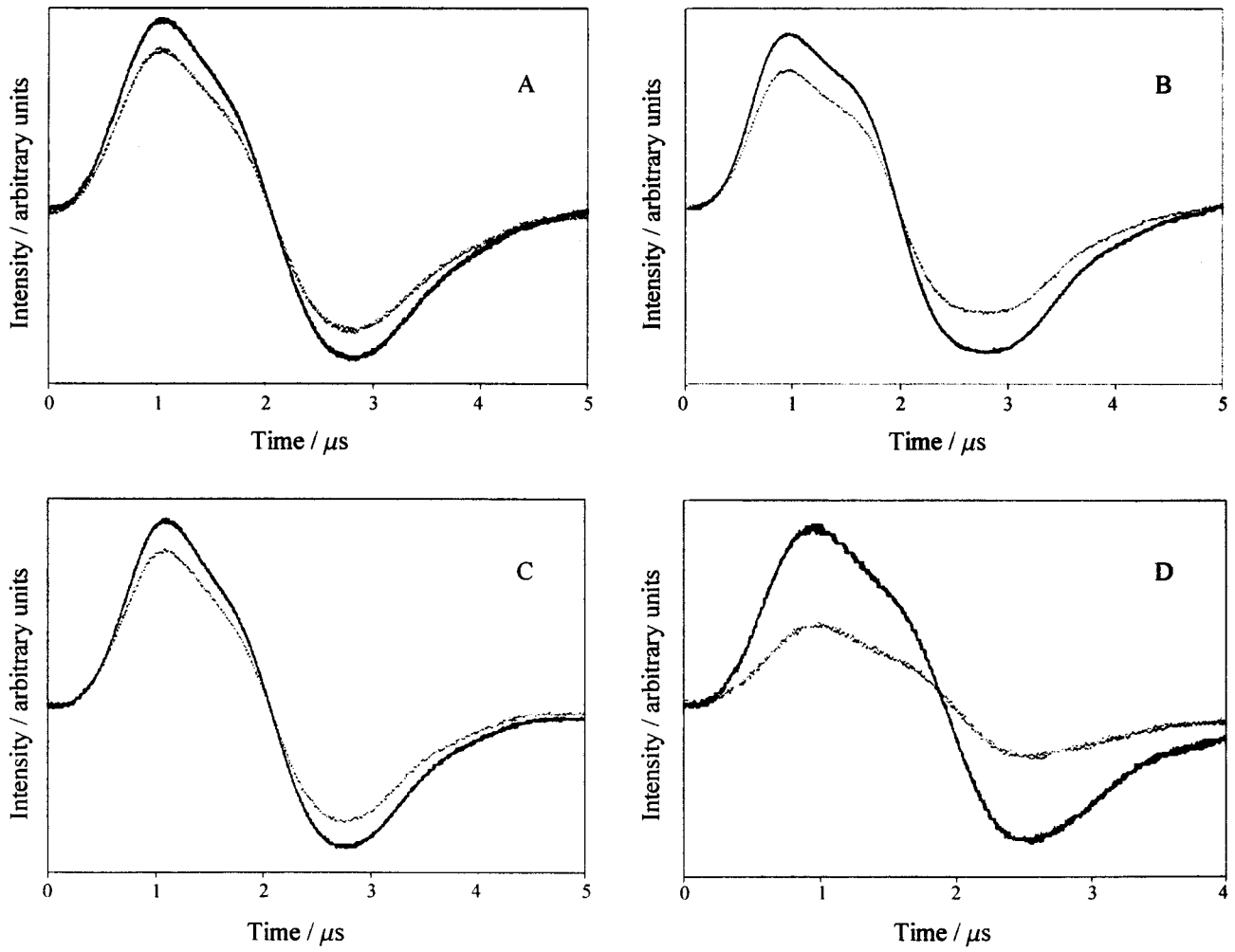

Figure 5. The 351 nm induced photoacoustic waveforms observed for size-dependent fractions of eumelanin from *Sepia officinalis* (dotted line) and the reference compound bromocresol purple (solid line) at room temperature: (A) MW > 10 000; (B) 10 000 > MW > 3000; (C) 3000 > MW > 1000; (D) MW < 1000.

gives the dashed curve in Figure 4. Within this approximation, the absorption spectrum of the small particles matches the action spectra throughout the entire ultraviolet region.

The role of small melanin particles in the photogeneration of radical species can be further substantiated by the photoacoustic data for the different size fractions using an excitation wavelength of 351 nm. These data, shown in Figure 5, reveal that the percent of absorbed energy that is released as heat by the MW > 10 000, 10 000 > MW > 3000, 3000 > MW > 1000, and MW < 1000 eumelanin samples is 89 ± 7%, 91 ± 9%, 83 ± 11%, and 44 ± 3%, respectively. For the MW > 1000 samples, most of the absorbed energy is released as heat. Compared to the bromocresol reference, there are no shifts in the photoacoustics waveforms for these melanin samples, and so this release of energy occurs on a nanosecond or faster time scale. The incomplete conversion of photon energy into heat observed for these samples could result from the scattering of excitation light and/or the photogeneration of a chemical intermediate that persists at least on the hundreds of nanosecond time scale. Light scattering affects the photoacoustic signals in the following manner. If we assume that all light energy absorbed by the melanin samples is converted into heat, then for samples of matched optical density the photoacoustic waveforms for melanin should be the same as that of bromocresol purple. However, this requires that the optical density results from the absorption of light. If the sample scatters light, then the measured optical density is a combination of absorption and scattering, and only the former component contributes to the photoacoustic signal. The absolute contribution of scattering to the optical spectrum of melanin is controversial. Regardless of its role, the decreased photoacoustic signal observed upon comparing the MW > 1000 and MW < 1000 samples cannot be attributed to scattering because scattering cross sections decrease with decreasing particle size.[28] Therefore, these data must reflect the formation of a long-lived energetic intermediate by the MW < 1000 small particles. This long-lived transient could be a primary intermediate in the mechanism by which the photoproduction of radicals and photoconsumption of oxygen occur. Only the MW < 1000 particles exhibit efficient formation of a reactive intermediate, which is consistent with the above agreement between the MW < 1000 absorption spectrum and the action spectrum for these chemical processes. One ESR study supports this conclusion by suggesting that an early intermediate in the photochemical process is a reactive triplet state.[29] If we assume that the intermediate revealed by the photoacoustic measurements is a triplet state, then the minimum energy of the triplet state of MW < 1000 eumelanin particles is ~190 kJ mol^{-1} above the ground state.

Because the action spectra for photoinitiated radical production and oxygen consumption are identical,[12] they must involve a common mechanism. We can gain insight into the order of events (radical production, reaction with oxygen) by comparing photoacoustic data for the small particle fraction taken in the absence of O_2 with that recorded for O_2 saturated solutions. In

our experiments, identical photoacoustic signals (amplitude and time profile) are observed for argon-saturated and O_2-saturated solutions of eumelanin. Kinetic events that occur on the nanosecond to a few hundreds of nanosecond time scale result in changes in the temporal profile of the photoacoustic waveform. Thus, quenching by or reaction with O_2 would lead to an increase in signal amplitude. Because neither temporal shifts in the photoacoustic waveform or changes in signal amplitude are observed upon comparison between the argon-saturated and O_2-saturated solutions of eumelanin, we can conclude that reaction with oxygen occurs on a time scale longer than several hundred nanoseconds. The photoacoustic data unambiguously establish that the reaction with O_2 occurs after the formation of a long-lived reactive species.

Interestingly, the work of Sarna and Sealy shows that synthetic eumelanin has lower efficiencies for radical photoproduction and oxygen photoconsumption compared to natural samples from bovine eyes.[12] However, the synthetic eumelanin studied was filtered, removing most of the MW < 50 000 particles. On the basis of the work presented herein, the differences in size distributions for both the natural and synthetic eumelanins could account for the observed differences in rates of radical production and oxygen consumption for the synthetic and natural samples. This size-dependent reactivity combined with the variations in eumelanin particle size in skin may also then be one of the contributing reasons for the variations observed in cancer rates among different skin types.

Acknowledgment. This work is supported by a grant from the National Institute of General Medical Sciences and the North Carolina Biotechnology Center through a faculty recruitment grant. We thank Dr. Jeffery M. Tosk for discussions about the autoxidation method for eumelanin synthesis and Dr. Shannon Studer-Martinez for her assistance in sample preparation.

References and Notes

(1) Ries, L. A. G.; Kosary, C. L.; Hankey, B. F.; Miller, B. A.; Harras, A.; Edwards, B. K *SEER Cancer Statistic Review, 1973−1995*; National Cancer Institute: Bethesda, MD, 1998.

(2) Gasparro, F. P.; Mitchnick, M.; Nash, J. F. *Photochem. Photobiol*. **1998**, *68*, 243−256.

(3) Rigel, D. S.; Kopf, A. W.; Friedman, R. J. *J. Am. Acad. Dermatol*. **1987**, *17*, 1050−1053.

(4) National Center for Health Statistics. *Vital Statistics of the United States 1994 Vol. II, Mortality, Part A*; Public Health Service: Washington, DC, 1998.

(5) Kvam, E.; Tyrrell, R. M. *Carcinogenesis* **1997**, *18*, 2379−2383.

(6) Madronich, S.; DeGruijl, F. R. *Photochem. Photobiol*. **1994**, *59*, 541−546.

(7) Peak, M. J.; Peak, J. G.; Churchill, M. E. In *Biological Responses to Ultraviolet A Radiation*; Urbach, F., Ed.; Valdenmar Publishing: Overland Park, KS, 1992; pp 39−45.

(8) Ranadive, N. S.; Menon, I. A. *Pathol. Immunopathol. Res*. **1986**, *5*, 118−139.

(9) Williams, R. J. P. *The Biology and Chemistry of Active Oxygen*; Bannister, J. V., Bannister, W. H., Eds.; Elsevier: New York, 1984; pp 1−15.

(10) Felix, C. C.; Hyde, J. S.; Sarna, T.; Sealy, R. C. *Biochem. Biophys. Res. Commun*. **1978**, *84*, 335−341.

(11) Korytowski, W.; Pilas, B.; Sarna, T.; Kalyanaraman, B. *Photochem. Photobiol*. **1987**, *45*, 185−190.

(12) Free radical action spectra were obtained from ESR measurements of the second-order radical decay kinetics following excitation of melanins at various wavelengths. The rate of radical production is proportional to the square of the radical concentration. The action spectra for oxygen consumption were compiled by measuring changes in the superhyperfine structure of the $M_I = +1$ transition for a nitroxide spin probe. Both radical production and oxygen consumption measurements were corrected by photon fluence rates; see the following. Sarna, T.; Sealy, R. C. *Arch. Biochem. Biophys*. **1984**, *232*, 574−578.

(13) Sarna, T.; Sealy, R. C. *Photochem. Photobiol*. **1984**, *39*, 69−74.

(14) Kochevar, I. E. *Photoimmunology*; Krutmann, J., Elmets, C. A., Eds.; Blackwell Science: Oxford, 1995; pp 19−33.

(15) Vitkin, I. A.; Woolsey, J.; Wilson, B. C.; Anderson, R. R. *Photochem. Photobiol*. **1994**, *59*, 455−462.

(16) Zeise, L.; Murr, B. L.; Chedekel, M. R. *Pigm. Cell Res*. **1992**, *5*, 132−142.

(17) Nofsinger, J. B.; Forest, S. E.; Eibest, L. M.; Moss, J. A.; Gold, K. A.; Fitzgerald, M. C.; Simon, J. D. *Pigm. Cell Res.*, submitted.

(18) Enochs, W. S.; Hyslop, W. B.; Bennett, H. F.; Brown, R. D., 3rd; Koenig, S. H.; Swartz, H. M. *Invest. Radiol*. **1989**, *24*, 794−804.

(19) Sarna, T. *J. Photochem. Photobiol., B* **1992**, *12*, 215−258.

(20) Hanson, K. M.; Li, B. L.; Simon, J. D. *J. Am. Chem. Soc*. **1997**, *119*, 2715−2721.

(21) Laarhoven, L. J. J.; Mulder, P.; Wayner, D. D. M. *Acc. Chem. Res.* **1999**, *32*, 342−349.

(22) Braslavsky, S. E.; Heibel, G. E. *Chem. Rev*. **1992**, *92*, 1381−1410.

(23) Peters, K. S. *Angew. Chem., Int. Ed. Engl*. **1994**, *33*, 294−302.

(24) Rudzki, J. E.; Goodman, J. L.; Peters, K. S. *J. Am. Chem. Soc*. **1985**, *107*, 7849−7854.

(25) Sealy, R. C.; Felix, C. C.; Hyde, J. S.; Swartz, H. M. *Free Radicals in Biology*; Pryor, W. A., Ed.; Academic Press: New York, 1980; Vol. IV, pp 209−259.

(26) Chedekel, M. R.; Ahene, A. G.; Zeise, L. *Pigm. Cell Res*. **1992**, *5*, 240−246.

(27) Zeise, L.; Addison, R. B.; Chedekel, M. R. *Pigm. Cell Res., Suppl*. **1992**, *2*, 48−53.

(28) Kerker, M. *The Scattering of Light and Other Electromagnetic Radiation*; Academic Press: New York, 1969; pp 414−486.

(29) Felix, C. C.; Hyde, J. S.; Sealy, R. C. *Biochem. Biophys. Res. Commun*. **1979**, *88*, 456−461.