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Investigation of Photoexcited States in Porcine Eumelanin through their Transient Radical Products

Alice Wang^{†,¶}, Anthony R. Marino[†], Elzbieta M. Gasyna[†], Tadeusz Sarna[‡], and James R Norris Jr.^{*,†}

[†]Department of Chemistry, University of Chicago, Chicago, Illinois [‡]Department of Biophysics, Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University, Krakow, Poland

Abstract

Time-resolved electron paramagnetic resonance (TREPR) was used to monitor the photochemistry of radical pairs from melanin in porcine retinal pigment epithelial (RPE) cells on the submicrosecond timescale. Two distinct signals were found: one of enhanced absorption/emission at early times and one mostly emissive at later times. The emissive character of the longer-lived feature suggests participation of an excited triplet precursor, something not generally thought to exist in melanins. The radicals in the early-time signal were separated by about 21 Å and those in the later-time signal were separated by about 22–24 Å.

Introduction

The biopolymer eumelanin protects tissue from photodamage by quickly converting light energy into heat and preempting harmful photochemical reactions. This process is generally believed so fast as to preclude intersystem crossing into excited triplet states.¹ Nonetheless, the residual photochemistry of melanin is responsible for the photochemical generation of semiquinone-like melanin radicals and, in the presence of oxygen, potentially cytotoxic superoxide anion and hydrogen peroxide.^{1,2} Recent studies have shown that, in addition to melanin's photoprotective behavior, photoexcited melanin has potential to cause DNA damage³ and even melanoma.⁴

Because triplet states are long-lasting as deactivation to the ground state is spin-forbidden, they have opportunity to induce photochemical side reactions, yet their role in the production of so-called "reactive oxygen species" in the aerobic photochemistry of melanin has been all but dismissed.^{1,2,5} The photophysical properties of melanin depend on its disordered aggregation, preventing traditional x-ray structural analysis, and its insolubility and light scattering complicate traditional optical techniques.¹ Ultrafast optical and time-resolved photoacoustic studies have shown that melanin quickly and efficiently converts the majority of light energy into heat.^{6,7} Previously and contrary to the common view, time-resolved electron paramagnetic resonance (TREPR) studies, sensitive only to the free radical population, have suggested that triplet states might indeed be formed in low concentrations in both synthetic melanin⁸ and that from human retinal pigment epithelial (RPE) cells.⁹ These previous TREPR studies were limited in time resolution and sensitivity and have been questioned regarding the role of the excited triplet state.¹ Here we've used the increase in time and field resolution and the better sensitivity of our spectrometer to determine inter-radical distances for two distinct radical pair

Email: jrnorris@uchicago.edu.

[¶]Current address: Duke University School of Medicine, Durham, North Carolina.

signals from eumelanin in porcine RPE cells, one coming from an excited singlet precursor and the other seemingly born from an excited triplet—the former never seen before—and so probe the mechanisms of energy dissipation in melanin.

Experimental Methods

Porcine eyes (Grant Park Packing, Chicago, IL) were opened by circumferential incision above the ora serrata near the limbus. The lens, neural retina, and remaining vitreous were removed. RPE cells were gently scraped from the choroid, washed with PBS without Ca^{2+} and Mg^{2+} (Hyclone HyQ PBS [1X] 0.0067 M), and then centrifuged at 212 g. The supernatant was removed and the pellet was suspended in 5 mL PBS.

Samples were flowed through a 0.25 mm Suprasil quartz EPR flat cell (Wilmad) to minimize sample degradation caused by the laser. Oxygen was purged by bubbling the sample with argon gas for 30 minutes prior to sample irradiation and continually during it. The gas first passed through a buffer solution to saturate it with water and prevent water loss in the sample. To minimize oxygen diffusion through the 1/64" PTFE tubing connecting the sample reservoir, flat cell, and peristaltic pump (Pharmacia Biotech P1), the tubing was sheathed in 1/4" polyethylene tubing through which a blanket of nitrogen flowed.

The EPR signal was measured at room temperature in a rectangular TE_{102} resonator (Bruker 4102ST). The microwave bridge (Bruker ER041MR) was equipped with a GaAs FET amplifier (Miteq AMF-4F-090100-15-10P-L) to provide enhanced time resolution. TREPR signals were collected through direct detection—no field modulation was used. The phase of the TREPR signal could be determined by checking the phase of the steady-state EPR signal.

Photoexcitation was provided through a hole in the front plate of the resonator by the third harmonic (355nm) of an Nd:YAG laser (SpectraPhysics DR4), pulse width 15 ns, that was used at a repetition rate of 10 Hz and an energy of 10 mJ/pulse. Time traces were collected on a digital oscilloscope (LeCroy Waverunner 2) for 200 laser pulses at each field point, and traces of all 128 field points were compiled into a dataset containing time, field, and signal amplitude information. Instrumentation was controlled from a PC using a homemade MATLAB script.

Laser pickup was removed by a linear baseline correction between the low- and high-field edges of the spectrum. The TREPR signals were weak and required smoothing to see them, and this was accomplished using unweighted moving average—first along the time axis, then along the field axis. Smoothing in Figure 1 was over a window of 550 ns and 0.17 mT. Traces and fits in Figure 2 and field spectra in Figure 3 were smoothed over a window of 350 ns and 0.07 mT.

Results and Discussion

Two distinct signals emerge from the time-resolved experiment, one rising and falling within the first microsecond and the second emerging thereafter, both with line widths indicative of radical pairs. The early-time signal is one of low-field absorption and high-field emission (A/E), and the later-time signal is mostly emissive (E) (Figure 1). The centers of both spectra align with that of the melanin steady-state EPR spectrum ($g = 2.004$). These two transient spectra appear to be markers of two different processes rather than parts of the same. Exponential fits of the time traces indicate that the decay of the first signal is faster than the emergence of the second at all field points across the spectrum (Figure 2). Additionally, trials with different samples gave different intensity ratios of the two signals.

The radical pair spectra were simulated by solving the Liouville equation for a four-level system,

$$\frac{d\rho}{dt} = [H, \rho]. \quad (1)$$

The upper and lower levels are essentially the T_+ and T_- levels of a triplet molecule and are separated by the sum of the two radicals' Zeeman and hyperfine energies, $2\hbar\omega_0$. The middle two levels are separated by the exchange (J) and dipolar (Drp) energies of the radical pair so that the Hamiltonian in the singlet—triplet basis is¹⁰⁻¹²

$$H = \begin{matrix} & \begin{matrix} |T_+\rangle & |S\rangle & |T_0\rangle & |T_-\rangle \end{matrix} \\ \begin{matrix} \langle T_+| \\ \langle S| \\ \langle T_0| \\ \langle T_-| \end{matrix} & \begin{bmatrix} \hbar\omega_0 - J + d & 0 & 0 & 0 \\ 0 & +J & \hbar\Delta\omega & 0 \\ 0 & \hbar\Delta\omega & -J - 2d & 0 \\ 0 & 0 & 0 & -\hbar\omega_0 - J + d \end{bmatrix} \end{matrix} \quad (2)$$

where $d = \frac{D_{rp}}{3} (1 - 3\cos^2\theta)$, θ is the angle between the radical pair axis and the external magnetic field, and $\hbar\Delta\omega$ is the difference in Zeeman and hyperfine energies of the two radicals and modulates the ST_0 coherence. Microwave perturbations are omitted here under the assumption of low microwave power. The hyperfine energies are assumed to follow a Gaussian distribution whose standard deviation can be related to a first derivative steady-state EPR spectrum peak-to-peak line width as $2\sigma = lw_{pp}$.

To solve the integrated version of Eq. 1 we need to define our initial populations, $\rho(0)$. The most straightforward approach is to propose that all the population begins in the singlet level, as with a singlet precursor. This assumption follows from the ultrafast optical data,^{5,6} which appears to exclude intersystem crossing to triplet states and well describes the spectral features found at early time (Figure 3a). As absorption to the T_+ state and emission to the T_- state are equally probable,^{13,14} the spectrum should have an equal intensity absorption and emission. The melanin granules are treated as a powder so, to treat the dipolar interaction, the calculation was averaged over all orientations of the radical pair with respect to the external magnetic field direction. The separation of the absorptive and emissive components indicates a radical pair Drp value of about -0.3 mT, and hence an radical pair separation of about 21 Å (point dipole approximation), but the spectral features are dominated by the width of the hyperfine (0.5 mT lw_{pp} —the width of the steady-state EPR spectrum), so that the error of Drp is about ± 0.1 mT. A precise value for J , the difference in g factors of the radicals (Δg), and g anisotropies are inaccessible for the same reason, but would likely be recoverable using higher frequency TREPR.

The later-time component to the spectrum, however, cannot be simulated assuming a population coming exclusively from the singlet level. The net polarization found in it is usually associated with the triplet mechanism (TM), where intersystem crossing from an excited singlet state to an excited triplet state will occur more rapidly along molecular axes with orbital angular momentum which can couple to the electron spin angular momentum.^{13,15} The radical pair populations depend on the difference in intersystem crossing into the T_X , T_Y , and T_Z sublevels of the excited triplet and its zero-field splitting parameters, D_T and E_T .

If we forget the widely-held belief that triplets are not formed in melanin, we can simulate the later-time spectrum (Figure 3b). Excited triplet polarization transfer was performed as had been done previously^{16,17} and extended to a radical pair situation as recently reported by Kobori et

al.¹⁸ For simplicity we assume random orientation between the triplet molecular axis and the radical pair axis. The net emission requires either a $D_T < 0$ with population primarily in the T_Z and T_Y sublevels or a $D_T > 0$ with population coming primarily through T_X and T_Y . D_{rp} values for the spectrum are -0.25 mT ($22 \text{ \AA}$) for $D < 0$ and -0.20 mT ($24 \text{ \AA}$) for $D > 0$. As with the early-time signal, the smallness of D_{rp} , J , and Δg with respect to the width of the hyperfine distribution limits their precise determination. The radical hyperfine distribution needed to simulate the transient spectrum is large at 0.74 mT (first derivative lw_{pp}) as compared to that of the steady-state EPR spectrum. A plausible explanation for the extra width in the spectral wings is that a distribution of inter-radical distances might be involved, the numbers reported above better describing the center of the spectrum (radicals with smaller D_{rp} , at greater separation), a possibility we are currently investigating.

All spectra were simulated with $J < 0$, the normal case, though the spectrum in Figure 3a could have been simulated as coming from a thermalized triplet precursor with $J > 0$, though this interpretation would be much more difficult to justify.

We cannot see how net polarization in Figure 3b could arise from a singlet precursor in any common fashion. One could interpret the emissive signal as no signal at all but instead the transient masking of free radicals already present in melanin. For instance, the generation of a radical pair from a singlet precursor, EPR silent at distances where J is large, could broaden a preexisting free radical signal and so seem to make it disappear. The transient emissive signal is, however, wider than the steady-state signal, and the explanation doesn't account for the signal's shape, either. Level crossing phenomena could explain net polarization created from a singlet precursor, but net emission would require the unlikely ST_+ mechanism in a radical pair system with $J > 0$. ST_+ and ST_- also generally require large hyperfine anisotropies to couple the crossing levels,^{19,20} and these do not seem to exist in melanin. The presence of preexisting radicals in melanin suggest the possibility of radical—excited state interactions, such as in the radical—triplet polarization mechanism (RTPM),^{21,22} some of which might be able to explain the net emissive signal without requiring a triplet precursor, and we are currently investigating these possibilities. Regardless of the interpretation one chooses, it is clear that the mechanisms of energy dissipation in melanin are not satisfactorily understood.

Conclusion

Two distinct radical pair species were seen in the melanin from porcine RPE cells, their approximate inter-radical distances between 19 and $24 \text{ \AA}$. Despite the general consensus that excited triplet states do not exist in melanin, we present evidence suggesting the contrary. The TREPR signals in porcine RPE cells are small and in no way challenge the view that the majority of light energy absorbed by melanin is quickly turned into heat. This minority process that we observe, however, has the potential to drive melanin photochemistry, and so the assumption of purely singlet chemistry in melanin's phototoxicity is perhaps premature.

Acknowledgments

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Figure 1.
The TREPR spectrum of porcine RPE cells shows absorption/emission at early time and mostly emission at later time.

Figure 2.

Time traces averaged over early-time absorptive (345.00–345.25 mT, in red) and early-time emissive (345.50–345.75 mT, in blue) features. Exponential fits include rate constants for the rise and fall of both early- and later-time features. k_{fall} of the early-time feature in both traces is 400 ns, while k_{rise} for the later time feature is 2.5 μ s (red trace) and 1.0 μ s (blue trace). The inset gives a closer look at the area in the dashed box.

Figure 3.

TREPR spectra at a) early and b) late time, time-averaged over the window given. Black dashed lines are simulations. The equal-intensity A/E of a) suggests a singlet precursor while the emissive nature of b) seems to preclude it. Excited triplet parameters in b) are $D_T = -0.15 \text{ cm}^{-1}$, $D_T = +0.023 \text{ cm}^{-1}$, and inter-system crossing rates $k_{isc,X}:k_{isc,Y}:k_{isc,Z} = 0:0.45:0.55$.