Photochemistry of Pheomelanin Building Blocks and Model Chromophores: Excited-State Intra- and Intermolecular Proton Transfer

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Supporting Information

ABSTRACT: Pheomelanins, the epidermal pigments of red-haired people responsible for their enhanced UV susceptibility, contain 1,4-benzothiazines and 1,3-benzothiazole as main structural components. Despite the major role played in pheomelanin phototoxicity, the photoreactivity of these species has so far remained unexplored. Static and time-resolved fluorescence spectroscopy was used to identify excited-state reactions of the two main pheomelanin benzothiazole building blocks, namely, the 6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole (BT) and the 2-carboxy derivative (BTCA) together with model chromophores lacking some of the ionizable functions. The results show that in aqueous buffer solution the OH at 4-position and the benzothiazole nitrogen atom control the photochemistry of both BT and BTCA via excited-state proton transfer to solvent (ESPT) and excited-state intramolecular proton transfer (ESIPT), while the amino acidic groups of the alanyl chain have a minor influence on the photochemistry. The ESPT and ESIPT produce several different excited-state ionic species with lifetimes ranging from ~100 ps to ~3 ns.

SECTION: Spectroscopy, Photochemistry, and Excited States

The characteristic phenotype of red-haired individuals of Celtic origin, with pale skin, blue-green eyes, and freckles, is due to the production of pheomelanin pigments. The positive correlation between the red hair phenotype and the UV-susceptibility trait, the high tendency to sunburn and the increased risk for skin tumors and melanoma of these subjects, has been attributed to the poor antioxidant and photoprotective properties of pheomelanins compared with the dark eumelanins. Despite the major role played in pheomelanin phototoxicity, the photoreactivity of these species has so far remained unexplored. Static and time-resolved fluorescence spectroscopy was used to identify excited-state reactions of the two main pheomelanin benzothiazole building blocks, namely, the 6-(2-amino-2-carboxyethyl)-4-hydroxy-1,3-benzothiazole (BT) and the 2-carboxy derivative (BTCA) together with model chromophores lacking some of the ionizable functions. The results show that in aqueous buffer solution the OH at 4-position and the benzothiazole nitrogen atom control the photochemistry of both BT and BTCA via excited-state proton transfer to solvent (ESPT) and excited-state intramolecular proton transfer (ESIPT), while the amino acidic groups of the alanyl chain have a minor influence on the photochemistry. The ESPT and ESIPT produce several different excited-state ionic species with lifetimes ranging from ~100 ps to ~3 ns.

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1.84 of the heterocyclic nitrogen (Table 1), at this pH, the BTCA is in its trianionic form BTCA\(^{3-}\) protonation states when it was not possible to distinguish the spectra of MB measured at pH 1 (in HCl solution), 2.5, 7, and according to their protonation states.)

Therefore, we assign the 410 nm band centered at 410 nm (Figure 1b). On the basis of the pK\(_a\) of MB (88%, NH\(^+\)).

The absorption spectrum measured at pH 9.5 shows a shift of ~4 nm for all of its spectral features compared with that at pH 2.5 and exhibits an extra band centered at ~335 nm. On the basis of the pK\(_a\) values listed in Table 1, at this pH the majority of the BT molecules are in the deprotonated (O\(^-\)) form. The additional absorption band can therefore be assigned to this species. A similar red-shifted absorption band of hydroxyphenylbenzothiazole in aqueous ethanol solution, increasing in intensity with increasing pH, was attributed to the deprotonated (O\(^-\)) species, although in that structure a more extended delocalization of the anion compared with BT is expected.

The absorption spectra of BT in sodium phosphate buffer at different pH values as well as in MeOH are shown in Figure 2a. The spectra in buffer at pH 2.5 and in MeOH are very similar. The absorption spectrum measured at pH 9.5 shows a shift of ~4 nm for all of its spectral features compared with that at pH 2.5 and exhibits an extra band centered at ~335 nm. On the basis of the pK\(_a\) values listed in Table 1, at this pH the majority of the BT molecules are in the deprotonated (O\(^-\)) form. The additional absorption band can therefore be assigned to this species. A similar red-shifted absorption band of hydroxyphenylbenzothiazole in aqueous ethanol solution, increasing in intensity with increasing pH, was attributed to the deprotonated (O\(^-\)) species, although in that structure a more extended delocalization of the anion compared with BT is expected.

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At pH 12, dianionic BT\(^{2-}\) is the dominating ground-state species with only a small (<1%) contribution of monoanionic BT\(^{-}\) (Table 1). This sample composition gives rise to the fluorescence spectrum (Figure 2b) peaking at \(\sim 480\) nm with a
shoulder at ~430 nm (Figure S3 in the Supporting Information), which we consequently assign to BT$^{−2}$ (~480 nm) and BT$^−$ (~430 nm) (more later). At pH 9.5, BT$^−$ and BT$^{−2}$ are the dominating species at about the same concentrations with a static fluorescence spectrum composed of the ~430 nm BT$^-$ and ~480 nm BT$^{−2}$ emission bands. (There is a minor (~17%) fraction of species with the 4-OH group protonated: OH; N; NH$_3$; COO$^−$ and OH; N; NH$_2$; COO$^{−}$.)

At pH 2.5, the ground-state population is a mixture of species with heterocyclic N deprotonated (BT$^+$) at 82% of the total benzothiazole concentration and 18% protonated also at the heterocyclic nitrogen (BTH$_{2}^{2+}$), based on pK$_a$ in Table 1. Nevertheless, the steady-state emission spectrum is not that of the BT$^+$ but red-shifted by ~200 nm from the peak of the absorption band to $\lambda_{\text{max}}$ ~490 nm (Figure 2b, Figure S3 in the Supporting Information). By analogy to what was observed for MB and as will be clear from the time-resolved fluorescence results (see later), this emission originates mainly from BTH$_{2}^{2+}$ or BT$^{−2+}$ (At this point, we cannot distinguish between the fluorescence spectra of these two species. It appears that the protonation of the heterocyclic N is the determining factor for red-shifting the fluorescence to ~490 nm.) formed through excited-state proton transfer to the heterocyclic N. At pH 7, ground-state BT is in its neutral form and the static fluorescence spectrum exhibits a double-peak structure with maxima at ~430 and ~490 nm, that is, those of BT$^−$ and BTH$_{2}^{2+}$/BT$^{−2+}$. The ground state concentrations of BT$^−$ and BTH$_{2}^{2+}$ are negligible at pH 7, suggesting that these fluorescence bands must be a result of deprotonation of the 4-OH group due to ESPT/ESIPT and protonation of the heterocyclic N through ESPT from the solvent or ESIPT from the 4-OH, respectively.

The picture of excited-state proton transfer in BT hinted by the static fluorescence spectra may be substantiated with the help of time-resolved fluorescence results. In the pH range 2.5–9.5, the time-resolved fluorescence spectrum exhibits a fast <10 ps time-scale red shift from ~420 to ~500 nm. On a slower, several hundred picosecond time scale, the spectrum shifts back to ~450 nm and then decays without further change on the nanosecond scale (Figure 2c). This time evolution (Figure S4 in the Supporting Information) can be described by three pH-dependent DAS components having wavelength maxima approximately consistent with the static fluorescence bands in Figure 2b and characterized by exponential lifetimes: ~420 nm (<10 ps) (the <10 ps component is limited by the streak camera instrumental response, implying that the early time spectrum most likely has a maximum at somewhat shorter wavelength, ~400 nm). ~450 ± 20 nm (1 ns) (the very low amplitude of this component makes the determination of its wavelength maximum uncertain); and ~490 nm (230 ps) (Figure 2d). At pH 12, where BT$^{−2}$ is the dominating ground-state species (~100%), the time-resolved fluorescence spectrum is dominated by a ~480 nm DAS component with a ~140 ps lifetime and only a very low amplitude of the ~450 nm DAS (1 ns). The time-resolved DASs can thus now be correlated to the various benzothiazole protonation states in the following manner: <10 ps (~400 nm) DAS~BT$^{−}$; 140 ps (~480 nm) DAS~dianionic BT$^{−2}$; 230 ps (~490 nm) DAS~BTH$_{2}^{2+}$/ BT$^{−2+}$; and 1 ns (~450 nm) DAS~monoanionic BT$^{−}$.

These correlations between BT protonation states, their fluorescence spectra, and associated lifetimes now allow us to suggest the following excited-state reaction scheme. Upon photoexcitation of BT, the excited state decays in <10 ps (~400 nm fluorescence) to form the excited-state BTH$_{2}^{2+}$/BT$^{−2+}$ and BT$^−$, which then decay with lifetimes of ~230 ps (490 nm fluorescence) and 1 ns (~450 nm fluorescence), respectively. The processes responsible for this are ESPT from the 4-OH group to the solvent for formation of the anion and 4-OH to heterocyclic-N ESIPT to form BT$^{−2+}$ or solvent to heterocyclic-N ESPT for BTH$_{2}^{2+}$ formation. Similarly to MB, the latter process could significantly contribute at low pH. These processes control the fluorescence dynamics of benzothiazole at pH values where the 4-OH group is protonated (<9). At higher pH with this group deprotonated (4-O$^−$), no ESIPT and thus no significant protonation of the heterocyclic-N occurs, as is demonstrated by the much lower amplitude of 490 nm (230 ps) fluorescence at pH 9.5. (As a matter of fact, part of the ~200 ps amplitude here must be due to BT$^{−2+}$ (see previous).) At this pH, the proton concentration in solution is also low, making ESPT from the solvent negligible. At even higher pH 12, another red-shifted (~480 nm) fluorescence band appears, with almost the same maximum as that of BTH$_{2}^{2+}$/BT$^{−2+}$, due to the ground-state concentration of BT$^{−2+}$ present at this pH. The fluorescence lifetime of this species is ~140 ps.

Forster calculations using the fluorescence spectra for BT$^+$ ($\lambda_{\text{max}}$ ~390 nm) and BTH$_{2}^{2+}$ ($\lambda_{\text{max}}$ ~490 nm) forms suggest that proton transfer from the solvent to the heterocyclic nitrogen is thermodynamically strongly favored, as in the case of MB. Using the fluorescence spectra in Figure 2b of BT$^+$ and anionic BT$^−$ forms, with maxima at ~400 and ~450 nm, respectively, the pK$_a$ change in the excited state, $\Delta$pK$_{a}^*$, can be estimated to ~6.3 pH units. Because pK$_a$ of the 4-OH group is ~8.8, this suggests that ESPT occurs if the solution pH is >2.5. This is consistent with the lower spectral amplitude of BT$^−$ fluorescence at pH 2.5 in both static and time-resolved spectra, as compared with pH 7 and 9.5 (Figures 2b,d). The excited-state proton transfers previously discussed are schematically summarized in Scheme 1.

The role of the amino acid side chain in the excited-state properties of BT was examined with the help of M-BT, lacking

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Scheme 1. Summarizing the Reaction Model$^a$

$^a$Shown are the proton transfers occurring on the excited species at pH >3.
the the alanyl side chain. The time-resolved fluorescence spectra and kinetics at both pH 2.5 (Figure S5 in the Supporting Information) and 9.5 (not shown) exhibit the same main pattern as that of BT: two spectral components characterized by maxima at 420–440 nm (10–20 ps) and ∼480 nm (100–150 ps). A component corresponding to the very low amplitude superposition of BTCA and BTCA−2 from the time-resolved measurements, the 450 nm band is a component which is associated with a switching from eumelanin to pheomelanin backbone or its formation by benzothiazine ring contraction may change the excited-state dynamics is a question that future work is addressing. The different fluorescence characteristics of eumelanin and pheomelanin building blocks can now be summarized in a few points. (i) No photochemistry occurs in methanol, as demonstrated by a small fluorescence Stokes shift and relatively long fluorescence lifetime (250~300 ps for BT), representing the sum of radiative and nonphotochemical radiationless processes of the benzothiazole core. (ii) In aqueous buffer solution, the OH at 4-position and the benzothiazole nitrogen atom control the photochemistry of both BT and BTCA via ESPT and ESIP processes. (iii) The amino acidic groups of the alanyl chain have a minor influence on the photochemistry (but protonation state of the amino group determines the excited-state lifetime of anionic species). (iv) The ESPT and ESIP produce several different excited-state ionic species with lifetimes ranging from ~100 ps to ~3 ns.

The findings here for BT and BTCA pheomelanin building blocks can now be compared with those of 5,6-dihydroxyindole (DHI) and 2-carboxy-5,6-dihydroxyindole (DHICA), the building blocks of eumelanin. On the monomer level there does not seem to be a dramatic difference between the various building blocks; in buffer solution they all exhibit ESPT or ESIP processes (to a varying extent), and the resulting excited states decay on the hundreds of picoseconds to nanoseconds time scale. In alcohol solution (methanol), these processes are much slower or completely stop. Dimers and larger units of DHICA have remarkably different excited-state dynamics compared with larger units of DHI: subpicosecond decay of the excited states through excited-state proton transfers. This unique feature was correlated to the photoprotective functions of eumelanin. How incorporation of benzothiazole in pheomelanin backbone or its formation by benzothiazine ring contraction may change the excited-state dynamics is a question that future work is addressing.

The different fluorescence characteristics of eumelanin and pheomelanin is an interest of the possible diagnostic tool for the detection of malignant transformation in melanocytes, which is associated with a switching from eumelanin to

Figure 3. (a) Absorption and (b) steady-state fluorescence (upon 266 nm excitation) spectra of BTCA.
pseudomelanin production. The steady-state fluorescence of model synthetic pheomelanins has been previously investigated, but up to now no interpretation of the species responsible for the phenomena has been provided because of the lack of information on the behavior of the putative building blocks inside the pigment. In this regard, the present investigation provide the first description of the steady-state and excited-state dynamics of the two main benzothiazole units of pheomelanin under conditions of relevance to the physiological process and provide a new groundwork for the interpretation of the photoreactivity of pheomelanin and the associated damages of the epidermal tissues.

ASSOCIATED CONTENT

Supporting Information

Experimental details and static and time-resolved fluorescence data at several more pH values and wavelengths are given as associated content. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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