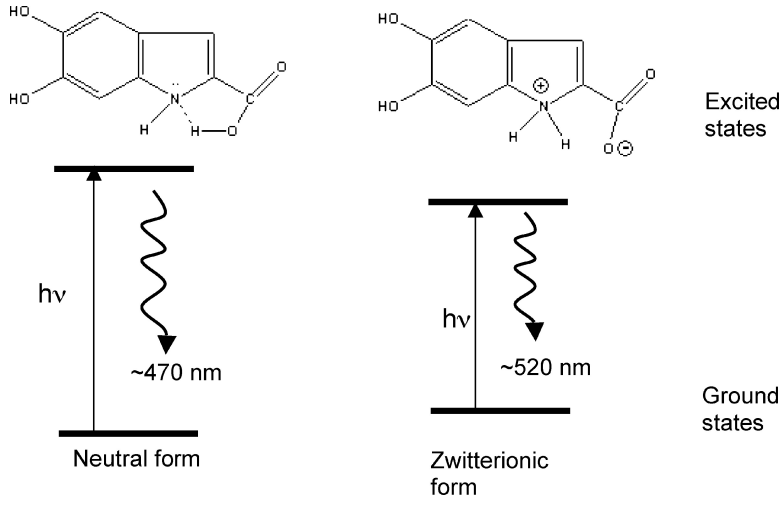
Article

Role of Solvent, pH, and Molecular Size in Excited-State Deactivation of Key Eumelanin Building Blocks: Implications for Melanin Pigment Photostability

M. Gauden, A. Pezzella, L. Panzella, M. T. Neves-Petersen, E. Skovsen, S. B. Petersen, K. M. Mullen, A. Napolitano, M. d'Ischia, and V. Sundstro#m

J. Am. Chem. Soc., **2008**, 130 (50), 17038-17043• DOI: 10.1021/ja806345q • Publication Date (Web): 13 November 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES

Published on Web 11/13/2008

Role of Solvent, pH, and Molecular Size in Excited-State Deactivation of Key Eumelanin Building Blocks: Implications for Melanin Pigment Photostability

M. Gauden,*,† A. Pezzella,‡ L. Panzella,‡ M. T. Neves-Petersen,§ E. Skovsen,§ S. B. Petersen,§ K. M. Mullen,|| A. Napolitano,‡ M. d'Ischia,‡ and V. Sundström†

Department of Chemical Physics, Lund University, Box 124, S 22100 Lund, Sweden, Department of Organic Chemistry and Biochemistry, University of Naples Federico II, Naples, Italy, Department of Physics and Nanotechnology, Aalborg University, Denmark, and Biophysics Group, Department of Physics and Astronomy, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam

Received August 11, 2008; E-mail: Magdalena.Gauden@chemphys.lu.se

Abstract: Ultrafast time-resolved fluorescence spectroscopy has been used to investigate the excited-state dynamics of the basic eumelanin building block 5,6-dihydroxyindole-2-carboxylic acid (DHICA), its acetylated, methylated, and carboxylic ester derivatives, and two oligomers, a dimer and a trimer in the O-acetylated forms. The results show that (1) excited-state decays are faster for the trimer relative to the monomer; (2) for parent DHICA, excited-state lifetimes are much shorter in aqueous acidic medium (380 ps) as compared to organic solvent (acetonitrile, 2.6 ns); and (3) variation of fluorescence spectra and excited-state dynamics can be understood as a result of excited-state intramolecular proton transfer (ESIPT). The dependence on the DHICA oligomer size of the excited-state deactivation and its ESIPT mechanism provides important insight into the photostability and the photoprotective function of eumelanin. Mechanistic analogies with the corresponding processes in DNA and other biomolecules are recognized.

Introduction

Melanins are a peculiar group of pigments responsible for the broad range of colorations of mammalian skin, hair, and eyes. Production of melanins takes place within epidermal melanocytes by the tyrosinase-catalyzed oxidation of tyrosine. In the process of melanogenesis, two types of melanin are formed: eumelanin (black-brown) and pheomelanin (yellow to reddish brown). The dark eumelanin is thought to be involved in skin and eye protection against UV damage, mainly because of its peculiar broad-band monotonic absorption in the UV−visible range (∼300−800 nm), a photoinducible free radical character,[1] and strong nonradiative relaxation of photoexcited electronic states. Nevertheless, solid experimental evidence supporting the photoprotective concept is still lacking. Eumelanin is composed of two main monomeric units, 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) at various levels of oxidation and linked in a random manner.[2] The role of DHICA as a basic eumelanin building block is well established[3−5] and is illustrated by the rapid conversion of this indole to a dark, eumelanin-like pigment on exposure to oxidizing agents or to UV radiation or even on standing at neutral physiologic pH. It has been proposed mainly based on X-ray studies that indole monomers are joined into oligomeric units that interact noncovalently, perhaps by $\pi-\pi$ stacking.[6−9] As a result, larger aggregates would be formed, leading to eumelanin particles.[10,11] Atomic force microscopy investigations of the eumelanin isolated from the ink sac of *sepia officinalis* suggested that particle formation is a hierarchical process in which small units are assembled into hundred-nanometer structures, which then aggregate to form the morphology of the macroscopic pigment.[12−14] However, despite considerable efforts, the structures and photophysics involved in eumelanin photoprotection remain poorly understood. Studies of the optical properties of eumelanin[15] revealed a marked particle size-dependence: small particles generate long-lived

† Lund University.
‡ University of Naples Federico II.
§ Aalborg University.
|| Vrije Universiteit.

(1) Seagle, B.-L. L.; Rezai, K. A.; Gasyna, E. M.; Kobori, Y.; Rezaei, K. A.; Norris, J. R. *J. Am. Chem. Soc.* **2005**, *127*, 11220–11221.
(2) Tran, M. L.; Powell, B. J.; Meredith, P. *Biophys. J.* **2006**, *90*, 743–752.
(3) Ito, S. *Biochim. Biophys. Acta* **1986**, *883*, 155–161.
(4) Pezzella, A.; d'Ischia, M.; Napolitano, A.; Palumbo, A.; Prota, G. *Tetrahedron* **1997**, *53*, 8281–8286.
(5) Tsukamoto, K.; Palumbo, A.; Dischia, M.; Hearing, V. J.; Prota, G. *Biochem. J.* **1992**, *286*, 491–495.
(6) Zajac, G. W.; Gallas, J. M.; Cheng, J.; Eisner, M.; Moss, S. C.; Alvarado-Swaisgood, A. E. *Biochim. Biophys. Acta, Gen. Subj.* **1994**, *1199*, 271–278.
(7) Cheng, J.; Moss, S. C.; Eisner, M. *Pigm. Cell Res.* **1994**, *7*, 263–273.
(8) Cheng, J.; Moss, S. C.; Eisner, M.; Zschack, P. *Pigm. Cell Res.* **1994**, *7*, 255–262.
(9) Gallas, J. M.; Littrell, K. C.; Seifert, S.; Zajac, G. W.; Thiyagarajan, P. *Biophys. J.* **1999**, *77*, 1135–1142.
(10) Nofsinger, J. B.; Forest, S. E.; Eibest, L. M.; Gold, K. A.; Simon, J. D. *Pigm. Cell Res.* **2000**, *13*, 179–184.
(11) Zeise, L.; Murr, B. L.; Chedekel, M. R. *Pigm. Cell Res.* **1992**, *5*, 132–142.
(12) Clancy, C. M. R.; Simon, J. D. *Biochemistry* **2001**, *40*, 13353–13360.
(13) Liu, Y.; Simon, J. D. *Pigm. Cell Res.* **2003**, *16*, 72–80.
(14) Liu, Y.; Simon, J. D. *Pigm. Cell Res.* **2003**, *16*, 606–618.
(15) Nofsinger, B.; Simon, J. D. *Photochem. Photobiol.* **2001**, *74*, 31–37.

 10.1021/ja806345q CCC: $40.75

reactive intermediates upon absorption of UV light, whereby they may be involved in UV-A-induced photochemical processes believed to lead to DNA damage, whereas the large particles efficiently dispose of UV-A energy through rapid nonradiative decay processes. This size-dependent photoreactivity has been suggested to be one of the contributing factors to the observed variations in skin cancer rates among different skin types.

Steady-state fluorescence experiments have shown that the emission spectra and fluorescence quantum yield depend on the excitation wavelengths. Time resolved fluorescence and transient absorption studies have been performed to elucidate excited-state dynamics and photoproduct formation.[15−17] Transient absorption spectra of eumelanins are difficult to interpret, presumably reflecting quite complex dynamics where several processes occur simultaneously.[17] Time resolved fluorescence experiments revealed multiexponential decay of fluorescence,[18−20] while time-resolved polarization experiments on Sepia eumelanin resulted in an ∼80 ps depolarization time constant that was attributed to energy transfer between chromophores.[19] Overall, these observations suggest the existence of several chromophores within the pigment.[21]

Recently, evidence has been presented for mirror-image rule violation in spectra of DHICA, and it has been proposed that this phenomenon is due to convergent adiabatic and nonadiabatic excited-state intramolecular proton transfer (ESIPT) processes.[22] Specifically, excitation into the S_1 or S_2 states of a catecholate anion of DHICA, represented by dual bands in the absorption spectrum, leads to emission from the S_1 state of its proton-transfer conjugate. It was proposed that intramonomer ESIPT may function as an energy dissipation mechanism in the pigment. Recent calculations have predicted that hydrogen shifts may be involved in the photophysics of 5,6-dihydroxyindole (DHI).[23]

Since eumelanin occurs *in vivo* at a high degree of aggregation, it is very difficult to draw detailed conclusions regarding the reaction mechanism. Already over 30 years ago there were proposed several mechanisms in order to explain how the photoprotection was achieved by the eumelanin pigment.[24] It has been speculated that energy dissipation occurs through efficient radiationless processes in large aggregates;[15] however, no direct evidence has been available. In fact, investigation of the photophysical and photochemical properties of 5,6-dihydroxyindole building blocks remains to-date the most useful approach toward an understanding of photoprotective mechanisms. Time-resolved fluorescence study of excited-state dynamics under different conditions might shed new light on the mechanisms of eumelanin photoprotection. Added to this is the interest of 5,6-dihydroxyindole photochemistry in relation to the possible involvement of the circulating metabolites in immediate tanning[25] and in the erythemogenic response of skin to UV radiation. In humans, the serum and urine concentrations of 5,6-dihydroxyindoles increase dramatically following exposure to sunlight, UVA (315−400 nm) or UVB (290−315 nm), as well as during PUVA therapy, suggesting that these indoles may play important biological roles in the response of skin to actinic damage.

Herein, we provide the first experimental evidence that DHICA oligomers have shorter excited-state lifetimes than their monomeric building blocks, a result of direct relevance for the photoprotective function of eumelanin. The present work, in addition, determines the fluorescence spectra and decay times of DHICA at neutral and acidic pH, as well as in acetonitrile solvent. We show that the variation of spectra and excited-state dynamics can be understood as a result of excited-state intramolecular proton transfer, and we propose this as the mechanism to achieve the photostability of eumelanin, in analogy with what has been proposed for DNA and other biomolecules.[26−28]

Results

Ionization States. Fluorescence measurements were performed on the following substrates (Figure 1): DHICA, its *O*-acetyl (DAICA), methyl ester (DHICA-Me), and *O,O*-dimethyl methyl ester (DMICA-Me) derivatives, and the symmetric 4,4′-dimer (DHICA-dimer) and trimer (DHICA-trimer), both as the *O*-acetyl derivatives. Among the molecules studied, only the parent DHICA is water-soluble. It possesses three potentially ionizable functional groups, a carboxylic acid at C-2 and two hydroxyl groups at C-5 and C-6. The pK_a's for these three groups are 4.25, 9.76, and 13.2, respectively, as determined by potentiometric and spectrophotometric titrations.[29] Thus, in the pH range accessible to experiment, several ionization states and tautomers can be populated. At pH 3, the fully protonated state dominates; at pH ∼ 6, the dominant state has only the carboxylic group in its deprotonated form (anion); at pH > 10, the dominant state has both the carboxylate and the C-6 hydroxyl deprotonated (dianion) (see Figure 1B). At pH values of physiological relevance, i.e. 7.4, DHICA exists as a mixture of the anion and dianion, in an approximately 1000:1 ratio, and the anion/protonated form ratio is ∼200:1.

DHICA derivatives, DAICA, and oligomers (DAICA-dimer and -trimer) were specifically designed to determine the effects of functional group and molecular size modification on the fluorescence properties and excited-state dynamics of DHICA. Functional group modifications reflected by these compounds include the following: *O*-acetylation, which substantially blunts the electron releasing properties of the OH groups; *O*-methylation, which also does so in alkaline medium, though to a much lesser extent, preventing pH-dependent ionization; and carboxyl group conversion to methyl esters, which increases the electron withdrawing effects on the indole ring and precludes ionization in aqueous medium and dimerization equilibria in organic

(16) Nofsinger, J. B.; Ye, T.; Simon, J. D. *J. Phys. Chem. B* **2001**, *105*, 2864–2866.
(17) Ye, T.; Simon, J. D. *J. Phys. Chem. B* **2003**, *107*, 11240–11244.
(18) Docchio, F.; Boulton, M.; Cubeddu, R.; Ramponi, R.; Barker, P. D. *Photochem. Photobiol.* **1991**, *54*, 247–253.
(19) Forest, S. E.; Lam, W. C.; Millar, D. P.; Nofsinger, J. B.; Simon, J. D. *J. Phys. Chem. B* **2000**, *104*, 811–814.
(20) Teuchner, K.; Freyer, W.; Leupold, D.; Volkmer, A.; Birch, D. J. S.; Altmeyer, P.; Stucker, M.; Hoffmann, K. *Photochem. Photobiol.* **1999**, *70*, 146–151.
(21) Meredith, P.; Powell, B. J.; Riesz, J.; Nighswander-Rempel, S. P.; Pederson, M. R.; Moore, E. G. *Soft Matter* **2006**, *2*, 37–44.
(22) Olsen, S.; Riesz, J.; Mahadevan, I.; Coutts, A.; Bothma, J. P.; Powell, B. J.; McKenzie, R. H.; Smith, S. C.; Meredith, P. *J. Am. Chem. Soc.* **2007**, *129*, 6672–6673.
(23) Sobolewski, A. L.; Domcke, W. *ChemPhysChem* **2007**, *8*, 756–762.
(24) Pathak, M. A.; Fitzpatrick, T. B. *The role of natural photoprotective agents in human skin, Sunlight and Man: Normal and Abnormal Photobiologic Responses*; University of Tokyo Press: Toyko, 1974.
(25) Maeda, K.; Hatao, M. *J. Invest. Dermatol.* **2004**, *122*, 503–509.
(26) Sobolewski, A. L.; Domcke, W.; Dedonder-Lardeux, C.; Jouvet, C. *Phys. Chem. Chem. Phys.* **2002**, *4*, 1093–1100.
(27) Abo-Riziq, A.; Grace, L.; Nir, E.; Kabelac, M.; Hobza, P.; de Vries, M. S. *Proc. Natl. Acad. Sci.* **2005**, *102*, 20–23.
(28) Sobolewski, A. L.; Domcke, W.; Hattig, C. *Proc. Natl. Acad. Sci.* **2005**, *102*, 17903–17906.
(29) Charkoudian, L. K.; Franz, K. J. *Inorg. Chem.* **2006**, *45*, 3657–3664.

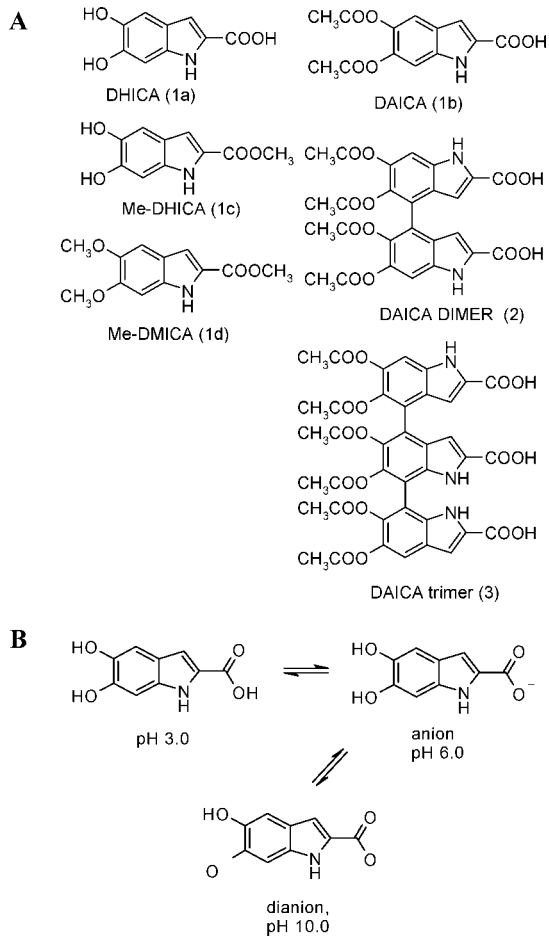


Figure 1. (A) Structures of the studied molecules. (B) Protonation equilibria of DHICA and dominant forms at various pH values.

solvents. Comparison of data from DAICA and the DHICA-dimer and -trimer was aimed at gaining insights into the effects of molecular size and oligomerization on the fluorescence of DHICA, an issue of special relevance to the photophysical properties of eumelanin pigments.

Absorption Steady-State Spectra. The eumelanin optical absorption spectrum is a broad, structureless curve that is monotonically decreasing as a function of wavelength. DHICA and its derivatives and higher oligomers all exhibit the same qualitative spectral features: two main absorption bands, one at ~225 nm and another at ~315 nm (Figure 2). The high-energy band is narrower than the low-energy band, and both bands exhibit a more or less pronounced substructure. The relative intensities and peak positions of the bands vary somewhat between the different derivatives and oligomers.

Time-Resolved Fluorescence. With time-resolved fluorescence, we measure the time evolution of the spontaneous emission following excitation of the molecule. Although the fluorescence signal is often weak due to low quantum yield, it has a great ability to exclusively monitor the excited-state dynamics and thus provide a less complex and more easily interpreted picture of the photodynamics than transient absorption, which monitors all species that happen to absorb the probe light, i.e. both excited states and ground states. We have performed time-resolved fluorescence streak camera experiments in order to examine the excited-state dynamics of DHICA in its acetylated, methylated, and carboxylic ester derivatives as well as two oligomers: a dimer and a trimer in the *O*-acetylated

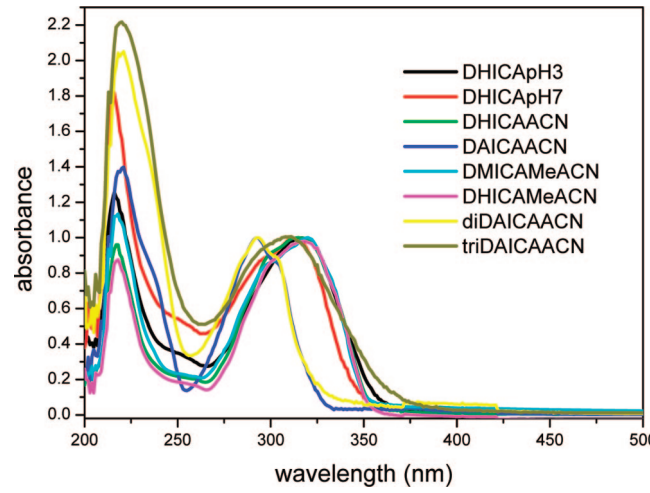


Figure 2. Absorption spectra of DHICA and its derivatives (DAICA-**d**) and oligomers (DHICA-dimer and DHICA-trimer) normalized at the lower energy band.

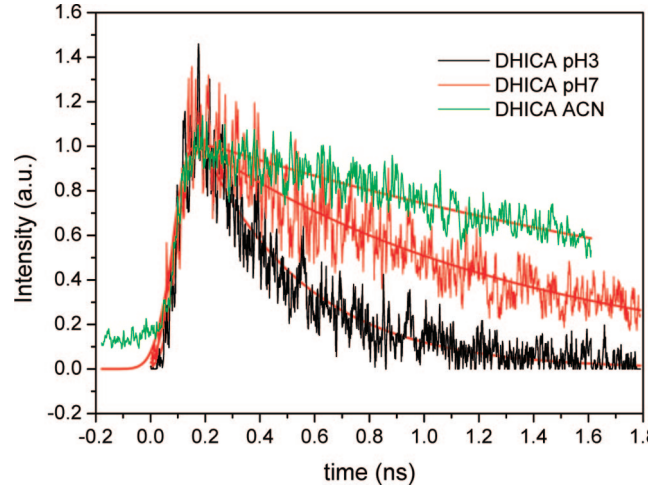


Figure 3. Fluorescence kinetic traces of DHICA at pH 3, at pH 7, and in acetonitrile.

forms. Additionally, we have examined the pH dependence of the excited-state lifetime of DHICA. We used 280 nm as an excitation wavelength, and we monitored the fluorescence in the 350−510-nm wavelength range. Application of a global analysis procedure yielded a single decay component for all molecules. In acetonitrile, DHICA has an excited-state lifetime of 2.6 ns (3 ns after degassing the sample with nitrogen; thus, further samples were not routinely degassed prior to measurement). Strikingly, the excited-state lifetime in aqueous buffer solution is much shorter than that in acetonitrile and is pH-dependent: at pH 3, DHICA has an excited-state lifetime of 380 ps, which increases to 1.2 ns at pH 7. The pH dependence of the excited-state lifetime is illustrated in Figure 3. As we will discuss in more detail below, the 3- to 4-fold shorter excited-state lifetime for DHICA at pH 3 as compared to pH 7 we assign to excited-state intramolecular proton transfer.

A comparison of the kinetic traces at the maximum emission for DHICA, DHICA-dimer, and DHICA-trimer demonstrates a shortening of the excited-state lifetime for higher oligomers (Figure 4). A possible explanation and implications of this result are discussed below. Fluorescence lifetimes for all the studied molecules are summarized in Table 1.

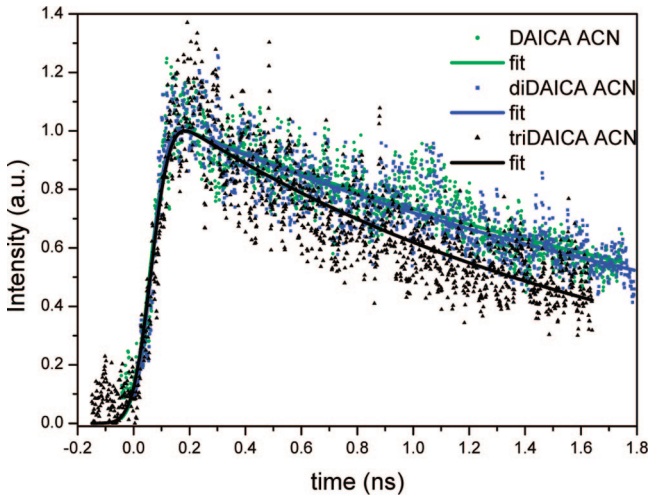


Figure 4. Fluorescence kinetic traces of DAICA, DAICA-dimer, and DAICA-trimer in acetonitrile.

Table 1. Summary of Measured Fluorescence Lifetimes

	pH 3 (ns)	pH 7 (ns)	acetonitrile (ns)
DHICA	0.4	1.2	2.6
DAICA			2.6
DHICA-Me			2.5
DMICA-Me			2.6
DHICA-dimer			2.5
DHICA-trimer			1.7

Examining the decay-associated spectra (DAS) of the studied molecules (Figure 5) reveals that they depend on the substitution pattern as well as on solvent and pH. In 0.1 M phosphate buffer at pH 3, the DAS of DHICA has a maximum at ~440 nm. At pH 7, the maximum is blue-shifted by ~30 nm, and in acetonitrile, there is a slight further blue-shift to ~408 nm. Derivatives of DHICA show characteristic spectral shifts in acetonitrile but cannot be studied in aqueous solution. The decay-associated spectrum of DHICA-Me presents a maximum at ~425 nm, while for DAICA the maximum is at ~390 nm; DMICA-Me shows an emission maximum at ~410 nm. DAS of the dimer and trimer of DHICA show further spectral changes: the DHICA-dimer presents a maximum at ~405 nm, whereas the DHICA-trimer presents a maximum at ~450 nm. The carboxyl and hydroxyl groups of the molecules offer rich opportunities for intra- and intermolecular hydrogen bonding and consequently proton transfer in the excited state. We will show below that much of the spectral variation described above can be understood as a result of hydrogen bonding and excited-state proton transfer equilibria involving the COOH and NH groups (and possibly also the 5,6-OH groups).

Discussion

We start the discussion by assigning the DAS fluorescence spectra obtained from the global analysis (Figure 5). Since all fluorescence decays are single exponential, these spectra are identical to conventional steady-state fluorescence spectra. The variation of fluorescence spectra with pH, solvent, and substitution pattern offers the first clue to an understanding of the fluorescence properties. Excited-state intramolecular proton transfer (ESIPT) is known to lead to effects such as dual fluorescence and fluorescence quenching. The effect was first discovered by Weller[30] and has since then been extensively studied.[31] The excited-state proton transfer process is often facilitated if an intramolecular hydrogen bond exists in the ground state. The COOH and NH groups of the molecules with protonated carboxylic groups can be envisaged to be involved in an intramolecular hydrogen bond. In fact, it was shown[32] for molecules related to DHICA, indole-2-carboxylic acid, and indole-5-carboxylic acid that there is an excited-state equilibrium between the neutral hydrogen bonded form and the zwitterionic form, where the carboxylic hydrogen has been transferred to the nitrogen. In most solvents, a very broad and sometimes dual band fluorescence was observed, with a red-shifted band due to the zwitterionic form and a blue-shifted indole-like fluorescence due to the neutral form. Acidic conditions with a protonated COOH group and a developed HN----HOOC hydrogen bond were seen to give rise to red-shifted zwitterionic fluorescence, whereas basic conditions favoring the anionic form (and thus no possibility of hydrogen bonding and proton transfer) gave weak indole-like blue-shifted fluorescence. These results are directly relevant and applicable to the molecules studied here. The molecules with a protonated COOH group all exhibit very broad fluorescence spectra with more or less pronounced multiple bands or wings (Figure 5). A change of pH from 3 to 7 in aqueous solution results in a ~30 nm blue-shift of the fluorescence spectrum of DHICA, similar to what was observed for indole-2-carboxylic acid and indole-5-carboxylic acid.[32] A spectral band analysis of the fluorescence spectra of the above-mentioned molecules (Figure 5 inset) shows that the spectra can be described by two Gaussian bands centered at ~390−410 and ~440 nm. The slight variation of the position of the component spectra for the different DHICA derivatives can be understood as a substituent effect.

In analogy with the assignments made for indole-2-carboxylic acid and indole-5-carboxylic acid,[32] we propose the presence of an excited-state equilibrium between hydrogen bonded and zwitterionic forms to explain the fluorescence properties of the compounds studied here (except for DHICA-Me and DMICA-Me) (Figure 6). The zwitterionic form is characterized by red-shifted fluorescence at ~440 nm, and the neutral hydrogen bonded form has fluorescence at 390−410 nm. Excited-state intramolecular proton transfer is known to be very fast (sub-picosecond to picosecond time scale),[31] implying that the excited-state equilibrium is established very quickly, much faster than the decay of the two excited species and also much faster than the time resolution of our experiment. This leads to the observed single exponential fluorescence decays. With this model, we can now rationalize the observed fluorescence behavior under the various experimental conditions used. The red-shifted short-lived (380 ps) fluorescence in acidic aqueous solution is due to the zwitterionic form, which is stabilized in the polar protic environment. At neutral pH, the DHICA is in its anionic form, having blue-shifted fluorescence (390−410 nm; 1.2 ns lifetime), and in analogy with the picture obtained for indole-2-carboxylic acid and indole-5-carboxylic acid,[32] this fluorescence can be assigned to an indole-like fluorescence. However, the situation is more complex for DHICA possessing the two 5,6-OH groups, which also can be involved in H-bonding and proton transfer. Thus, according to the calcula-

(30) Weller, A. *Z. Elektrochem.* **1956**, *60*, 1144–1147.
(31) Kosower, E. M.; Huppert, D. *Annu. Rev. Phys. Chem.* **1986**, *37*, 127–156.
(32) Bangal, P. R.; Chakravorti, S. *J. Phys. Chem. A* **1999**, *103*, 8585–8594.

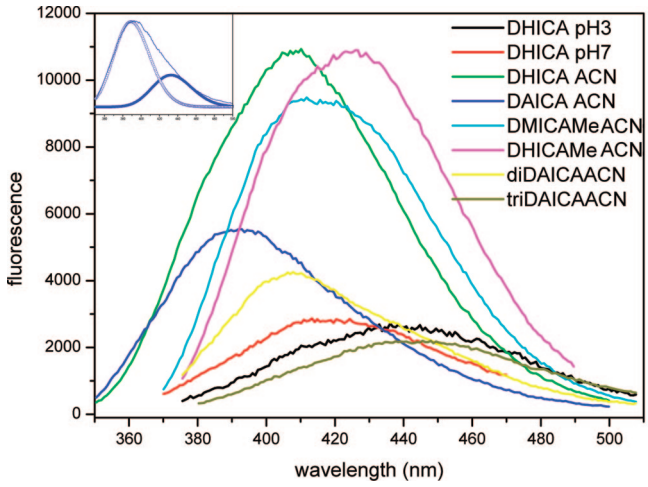


Figure 5. Decay-associated spectra (DAS) of the fluorescence decay that resulted from a global analysis of streak camera experiments on DHICA, DAICA, and the oligomers DHICA-dimer and DHICA-trimer. The inset shows band-shape analysis of the fluorescence spectra of DAICA in acetonitrile.

tions of Olsen et al.,[22] for the anion there is a ground-state equilibrium between the form with a deprotonated carboxyl group and the catecholate anion with a deprotonated hydroxyl attached to the C-6 atom, with the catecholate having lower energy. Calculations including a dielectric environment[22] suggested that carboxylate and cathecolate forms are of similar ground-state energies. The result of these considerations is that, upon photoexcitation of DHICA in aqueous solution at neutral pH, the excited state could be deactivated through a similar ESIPT process, as discussed above for the protonated species, or through the proton transfer suggested in ref 22 involving the two hydroxyl groups.

For DHICA and its derivatives in the acetylated forms, the observed fluorescence spectrum in acetonitrile is a superposition of fluorescence from the neutral and zwitterionic forms, as revealed by the broad spectra with underlying band structure and wings. The varying relative amplitudes of blue and red bands suggest different excited-state equilibrium constants for the different molecules. The two methyl esters (DHICA-Me and DMICA-Me) lacking a carboxylic proton do not have the HN----HOOC hydrogen bond and proton transfer, but it is well-known that carboxylic oxygen can be involved in hydrogen bonding and ESIPT reactions with alcoholic[33] or amine protons.[34] Thus, also the methyl esters could be involved in ESIPT reactions and dual fluorescence. An example of this kind of ESIPT is that proposed for salicylic acid.[35] In this case, the reaction is driven in the opposite direction, from phenol to the carboxylic group, resulting in formation of a zwitterionic form. Nevertheless, there are a few known examples where the nitrogen can act as a donor and carbon can be an acceptor.[32] In order to further establish the nature of the ESIPT in DHICAs, quantum chemical calculations need to be performed. Future experimental work on variously substituted DHICAs and DHIs (blocking various proton transfer possibilities) will hopefully also allow us to better distinguish the role of the different proton transfer processes. In conclusion of this part, we propose that DHICA molecules and their derivatives studied here undergo excited-state proton transfer processes, which leads to dual fluorescence and, in aqueous solution, short excited-state lifetime. In analogy with previously observed ESIPT processes for similar molecules (indole-2-carboxylic acid and indole-5-carboxylic acid[32]), we propose that for the parent DHICA the carboxylic proton and indole nitrogen are important mediators of the process, but for the molecules with *O*-acetylation, *O*-methylation, and carboxyl group conversion to methyl esters, other proton transfer reactions could also be active.

Are the results discussed above of relevance to the photoprotective function of melanin? In acetonitrile, DHICA has a relatively long excited-state lifetime of 2.6 ns, but in aqueous solution, it is drastically shortened to 1.2 ns at pH 7 and only 380 ps at pH 3. The excited-state lifetime is also decreased in going from DHICA to the trimer, indicating that the nonradiative decay is faster for the higher oligomers than for monomers. This is the first direct experimental evidence that oligomers of DHICA have shorter excited-state lifetimes than the corresponding monomeric building blocks, and it is consistent with the view that, in order for the melanin pigment to work as a photoprotecting agent, the nonradiative decay rate of oligomers has to be very fast. The oligomers could unfortunately only be studied in acetonitrile, owing to their low solubility in aqueous solution. Therefore, we do not know what is the excited-state lifetime of an oligomer in an aqueous environment at physiological conditions (pH 7). However, it is not unreasonable to assume that if the shortening of the excited-state lifetime observed for dimers and trimers in acetonitrile (Figure 4) also occurs in an aqueous environment, the expected trimer lifetime would be subnanosecond, and for larger oligomers, it can be hypothesized to be in the tens to hundreds of picosecond range. Such short lifetimes would represent a very efficient conversion of UV-light energy into heat.

The difference in the excited-state lifetime between monomers and higher oligomers suggests that a relaxation pathway depends on the molecular size and structure. The molecular structure must have been a factor that played a role in evolution in the selection of a specific molecule for photostability purposes. DNA is a prominent example of a molecule with photoprotective mechanisms that prevent UV radiation damage. Although the biological functions of DNA and melanin are different, they both need to be photostable. Efficient excited-state deactivation is critical to DNA photostability, since a long excited-state lifetime may promote mutagenic reactions. The very short excited-state lifetimes of DNA bases have been suggested to have their origins in conical intersections between various excited-state potential surfaces and the ground state,[26,36,37] leading to ultrafast internal conversion processes. Very efficient excited-state deactivation, specific to the Watson−Crick (WC) structure of a GC (guanine-cytosine) base pair,[27] was explained by Sobolewski et al.[28] as a barrierless proton transfer process that connects the spectroscopic $^1\pi\pi^*$ excited state with the electronic ground state via two conical intersections. Similarly, femtosecond mass spectrometry showed that a model base pair of 2-aminopyridine of WC geometry has a considerably shorter excited-state lifetime than the monomer or dimers of other structure. The results were explained as a result of conical

(33) Van Benthem, M. H.; Gillispie, G. D. *J. Phys. Chem.* **1984**, *88*, 2954–2960.
(34) Fahrni, C. J.; Henary, M. M.; VanDerveer, D. G. *J. Phys. Chem. A* **2002**, *106*, 7655–7663.
(35) Lahmani, F.; ZehnackerRentien, A. *J. Phys. Chem. A* **1997**, *101*, 6141–6147.
(36) Ismail, N.; Blancafort, L.; Olivucci, M.; Kohler, B.; Robb, M. A. *J. Am. Chem. Soc.* **2002**, *124*, 6818–6819.
(37) Merchan, M.; Serrano-Andres, L. *J. Am. Chem. Soc.* **2003**, *125*, 8108–8109.

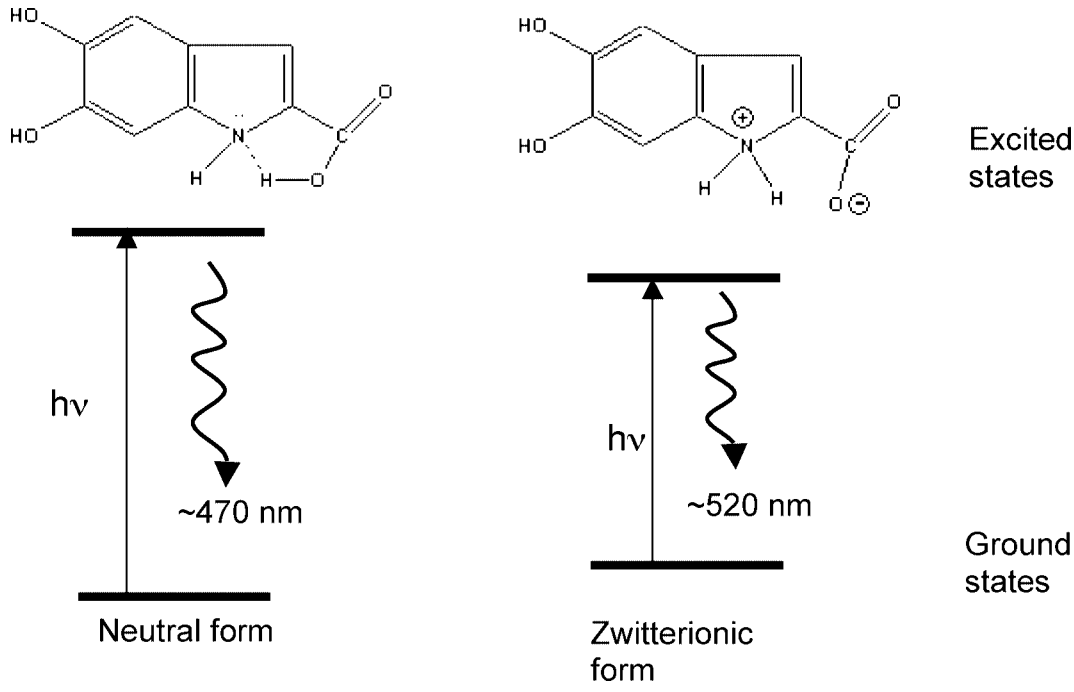


Figure 6. Proposed energy level diagram for an excited-state proton transfer in DHICA. Proposed energy level diagram for an excited-state proton transfer in DHICA.

intersections connecting the locally excited $^1\pi\pi^*$ state and the electronic ground state with a charge transfer state.[38] More long-lived so-called excimer states have also been suggested as intermediates in the excited-state relaxation pathways of oligonucleotides, with possible roles in photoprotection.[39]

From the discussion above, we conclude that, in both base pairs of DNA and oligomers of DHICA, which are building blocks of melanin, excited-state proton transfer reactions (most likely giving rise to conical intersections) are essential features for achieving efficient excited-state deactivation. Efficient excited-state relaxation through similar processes has also been proposed for amino acids[26] and other organic molecules.[40,41] Thus, it appears that Nature is using a general and common theme to achieve photostability and photoprotection of molecules with key functions.

Materials and Methods

Sample Preparation. DHICA,[42] DHICA-Me,[43] DHICA-dimer,[44] and DHICA-trimer[44] were prepared according to literature procedures. DAICA was prepared by acetylation of DHICA with acetic anhydride/pyridine. DMICA-Me was prepared by treating DHICA with an excess of diazomethane in ethyl ether.

Fluorescence Streak Camera Measurements. The near-infrared output pulses of a femtosecond laser (Spectra-Physics, Tsunami) were sent through a pulse selector (Spectra-Physics) to reduce the repetition rate from 80 to 8 MHz. The beam of infrared pulses was then used to pump a harmonic generator (Spectra-Physics, GWU) to generate the third harmonic of the fundamental wavelength. The resulting UV output pulses were approximately 200 fs and centered at 280 nm. The average power of the beam of UV pulses was ~1.8 mW at a repetition rate of 8 MHz, corresponding to a pulse energy of 0.2 nJ. These UV pulses were used to excite the sample solution kept in a rotating cuvette, and the fluorescence emission was collected using a one inch diameter 50 mm focal length quartz lens and then focused onto the input slit (width 100 μm) of a spectrograph (Oriel, MS257). The grating used in the spectrograph was blazed at 300 nm and had 300 lines/mm. The output from the spectrograph was sent onto the streak camera (Optronis, GmbH), and data were collected with a sweep speed of 0.01 mm/ps, which corresponds to a time resolution of 250 ps at the used entrance slit of 100 μm. The streak-camera fluorescence data were analyzed globally[45] in terms of a sum of exponential decays, using a parallel model yielding species associated spectra (DAS) and corresponding lifetimes.

Acknowledgment. This work was supported by funding from the Swedish Energy Agency (STEM), Wennergrenska Stiftelsen, the Crafoord Foundation, and the Swedish Research Council (VR) through an excellence grant to the Lund Laser Centre and LaserLab Europe Contract No. RII3-CT-2003-506350. L.P. thanks "L'ORÉAL Italia Per le Donne e la Scienza" for funding.

Note Added after ASAP Publication. Figure 5, originally published November 13, 2008, was replaced by the author. Wavelength values relating to the figure were changed in the first and third paragraphs of the Time-Resolved Fluorescence section and the first and second paragraphs of the Discussion section. The corrected version was published November 18, 2008.

JA806345Q

(38) Schultz, T.; Samoylova, E.; Radloff, W.; Hertel, I. V.; Sobolewski, A. L.; Domcke, W. *Science* **2004**, *306*, 1765–1768.
(39) Crespo-Hernandez, C. E.; Cohen, B.; Kohler, B. *Nature* **2005**, *436*, 1141–1144.
(40) Vallet, V.; Lan, Z. G.; Mahapatra, S.; Sobolewski, A. L.; Domcke, W. *J. Chem. Phys.* **2005**, *123*, 144307–144322.
(41) Lan, Z.; Domcke, W.; Vallet, V.; Sobolewski, A. L.; Mahapatra, S. *J. Chem. Phys.* **2005**, *122*, 224315–224328.
(42) Edge, R.; d'Ischia, M.; Land, E. J.; Napolitano, A.; Navaratnam, S.; Panzella, L.; Pezzella, A.; Ramsden, C. A.; Riley, P. A. *Pigm. Cell Res.* **2006**, *19*, 443–450.
(43) Palumbo, P.; Dischia, M.; Crescenzi, O.; Prota, G. *Tetrahedron Lett.* **1987**, *28*, 467–470.
(44) Pezzella, A.; Vogna, D.; Prota, G. *Tetrahedron* **2002**, *58*, 3681–3687.
(45) van Stokkum, I. H. M.; Larsen, D. S.; van Grondelle, R. *Biochim. Biophys. Acta, Bioenerg.* **2004**, *1657*, 82–104.