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Temperature dependence of photochemical fluorescence fading in Skh-1 hairless mouse collagen

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Summary

Background—Type I mammalian collagens have several photolabile fluorescent moieties that absorb UV rays capable of reaching the dermis. We studied the temperature dependence of fluorescence fading as a marker of photochemical damage.

Methods—Collagen solutions were exposed to radiation from 0 to 240 min from either a UVG-11 hand lamp, total dose = 1.173×10^3 J/m²; a UVL-21 hand lamp total dose = 2.030×10^3 J/m²; or the fluorometer, at 325 ± 5 nm, total dose = 0.156×10^3 J/m². We recorded intensities at excitation/emission wavelengths 270/300, 270/330, 270/360, 270/400, 325/400, and 370/450 nm at $T = 9.0$ – 59.3 °C.

Results—Results indicated simultaneous forward and reverse reactions. However, the 270/360 nm fluorophore could be analyzed as a second-order reaction. The Arrhenius curve showed two straight lines intersecting near the denaturation temperature, with helix activation energy $E_a \sim 0$ and coil $E_a = 7.6 \pm 0.6$ kcal/mol (31.7 ± 2.5 kJ/mol).

Discussion—Collagen-bound fluorophores are not just passive markers of oxidative stress and age-related damage. Their photolability to wavelengths reaching the dermis may result in pathological conditions, particularly at elevated body temperatures.

Keywords

collagen; fluorescence; photochemical fluorescence fading

Type I mammalian collagens may have one or more fluorescent moieties that absorb ultraviolet (UV) rays capable of reaching the dermis. Exposure to solar wavelengths leads to irreversible photochemical changes in collagen structure and supramolecular scaffolding, as evidenced by fluorescence fading. Newly synthesized collagen contains two to three tyrosine residues per 1000 amino acids (1, 2). Tyrosine has excitation and emission maxima at

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270/300 nm. Posttranslational modifications and/or cross links include 3,4-dihydroxyphenylalanine (DOPA; 280/330 nm) (3) two adjacent interacting 'excimer-like' residues in close proximity (280/360 nm) (4), dityrosine, formed by covalent photochemical linkage between two proximal tyrosine residues (325/400 nm) (5), advanced glycation endproducts (AGE) (6), and/or DOPA oxidation products (7), both types 370/450 nm.

It has long been known that the result of the interaction of UVA and UVB wavelengths with dermal collagen can lead to photoaging and/or other skin pathologies (8). This may occur as a result of changes in collagen solubility (9, 10), increased collagenase induction (11–13), increased susceptibility to collagenase (14), and decreased procollagen production (15, 16).

In recent years, several of these collagen fluorophores have been used as markers of aging and/or oxidative stress (17–20). As it has become evident that these fluorophores are photolabile to solar wavelengths (4), more recent studies have focused on their photochemical (4) and photosensitization (21) properties as they affect the biopolymers to which they are bound and their surroundings.

We have previously (22) studied the temperature dependence of acid-soluble of Skh-1 hairless mouse collagen fluorescence emission. Arrhenius plots for Skh-1 collagen were non-linear and indicated a temperature-independent component above the denaturation temperature.

This communication is concerned with the temperature dependence of photochemical fluorescence fading. We expect *a priori* to observe complex kinetic behavior in most instances because of the presence of multiple simultaneous forward and backward reactions whose course is dependent on the temperature *per se* as well as on the excitation wavelength and on the conformation of the collagen (i.e. helix vs. coil). However, the photochemical disappearance of the 270/360 nm fluorophore can be analyzed as a simple second-order reaction under UVC excitation (4). Again, the calculated Arrhenius curve is non-linear and can be separated into two straight lines that intersect near the denaturation temperature. This time, however, we found that the low-temperature component (helix) had a negligible activation energy ($E_a \sim 0$), while that from the coil was $E_a = 7.6 \pm 0.6$ kcal/mol (31.7 ± 2.5 kJ/mol).

Methods

Materials and apparatus

Acid soluble type I collagen was extracted from 6- to 10-week-old Skh-1 hairless mice as described previously (4). Fluorescence measurements were carried out 0.5 M HOAc in a Perkin-Elmer 650-40 Fluorescence Spectrometer (Perkin-Elmer, Norwalk, CT, USA) with both excitation and emission slits at 10 nm. Total fluorescence and excitation spectra (4) were measured at ambient temperature. For the temperature-dependent studies, 1.0 ml of 0.05% collagen solutions were irradiated in a thermostatted cuvette (Hellma Cells, Plainview, NY, USA) and readings were taken at excitation/emission wavelengths of 270/300, 270/330, 270/360, 270/400, 325/400, and 370/450 nm after equilibration at room temperature. Irradiations at times from 0 to 240 min were carried out with one of three

sources: (A) a 4 W UVG-11 hand lamp, (B) a 4 W UVL-21 hand lamp (both from Ultra Violet Products, San Gabriel, CA, USA) in combination with a 1 mm Schott WG-345 filter (D 58640, Iserlohn, Germany), or (C) with 325 nm excitation (10 nm bandwidth) from the spectrofluorometer itself. Source (A) emits predominantly UVC at 254 nm (irradiance = 0.0815 W/m^2 ; total dose = $1.173 \times 10^3 \text{ J/m}^2$). Source (B) emits broad-band UVA radiation peaking at 366 nm, irradiance = 0.141 W/m^2 , total dose = $2.030 \times 10^3 \text{ J/m}^2$, with no appreciable emission below 325 nm. The irradiance emitted by the spectrometer source (C) is 0.0108 W/m^2 , total dose = $0.156 \times 10^3 \text{ J/m}^2$ at $325 \pm 5 \text{ nm}$. All fluence rates were measured with an Epply thermopile (Epply, Newport, RI, USA). Source spectral distribution was measured with a calibrated OL-754 Spectroradiometer (Optronic Laboratories Inc., Orlando, FL, USA).

Data analysis

At each temperature and for each fluorophore, the resulting $I_f(t)$ vs. irradiation time t plots were analyzed kinetically. In case of simple first-order kinetics, there is a logarithmic relationship between $I_f(t)$ and time t , proportional to the rate of photochemical fading. With second-order kinetics between two like fluorophores, valid for the 276/360 nm pair under 254 nm irradiation (4), one sees a $1/I_f$ vs. t relationship. In this case, we have

$$1/I_n(t) = k(T)t + 1 \quad (1)$$

where $1/I_n(t)$ is the normalized reciprocal fluorescence intensity at time t . Slope $k(T)$, first order parameter (in units of s^{-1}), is an empirical parameter proportional to the bimolecular rate constant for the photochemical disappearance of the 270/360 nm fluorescence at (absolute) temperature $T(\text{K})$. We determined the proportionality constants $k(T)$ from the resulting slopes and plotted $\log k(T)$ as a function of $1/T(\text{K}^{-1})$ to obtain Arrhenius plots (23). Under normal conditions, Arrhenius plots yield a straight line, with slope = $E_a/2.3R$, where E_a is the activation energy (cal/mol or J/mol), R = the gas constant, 1.987 cal/K/mol ($R = 8.314 \text{ J/K/mol}$). The slope of the Arrhenius plot is independent of the units used for $k(T)$.

Mixed kinetics, where neither first- nor second-order plots are linear, imply the presence of simultaneous competing photochemical reactions. Deviation of Arrhenius plots from linearity suggests a change of phase (in this case helix-coil transition) and/or the simultaneous presence of more than one reaction.

Results

Figure 1 shows the time course results for UVC (top) and UVA (bottom) at 37°C . Collagen fluorescence fades non-uniformly across the spectrum. Except for the fluorescence at 270/360 nm, the kinetic curves did not fit either a simple first- or second-order plot. All of the other curves were consistent with the presence of simultaneous temperature- and wavelength-dependent forward and backward reactions. Results with 325 nm irradiation were similar to those for UVA excitation (not shown). In Fig. 1, only the results at 37°C are shown for brevity, but the curves are qualitatively similar at all the temperatures studied.

The kinetics of disappearance of 270/360 nm fluorescence are second order, and fit Equation (1). Figure 2 shows the family of linear regression lines with slope = $k(T)$ for $9.1 < T < 59.3$ °C (282.2 < $T(K) < 332.5$ K). Below 300 K (27 °C), $k(T)$ is constant at $(12.4 \pm 1.0) \times 10^{-3}$ /s. Above this temperature, $k(T)$ increases with increasing temperature to a value of 22.3×10^{-3} (intensity units)/s at 332.5 K (59.3 °C). An Arrhenius plot (Fig. 3) of $1/T(K^{-1})$ vs. $\log k(T)$ is distinctly non-linear, but the resulting curve can be resolved into two straight lines intersecting at ca. 303–306 K (30–33 °C). At $T < 300$ K (30 °C) $E_a \approx 0$, while at $T > 306$ K (33 °C) $E_a = 7.6 \pm 0.6$ kcal/mol (31.7 ± 2.5 kJ/mol).

Discussion

Except for the 270/360 band, fluorescence fading of Skh-1 collagen fluorophores indicates the presence of several opposing forward and reverse reactions that are temperature and wavelength dependent (Fig. 1). UVC wavelengths tend to create species having long-wave absorption and fluorescence characteristics, e.g. dityrosine and AGE/DOPA oxidation products; UVA wavelengths tend to cause their disappearance. Our data support earlier findings that UVC wavelengths dimerize two molecules of interacting tyrosine to dityrosine (4, 5) but that the latter species is photolabile to UVA (4).

The photochemical fading of the 270/360 nm species, is consistent with the disappearance of an excimer-like dimer of interacting tyrosine residues in close proximity (4). Under UVC radiation, we found an empirical straight-line relationship between reciprocal normalized fluorescence intensity, $1/I_f(t)$, and irradiation time t (Fig. 2). Evidently, the reaction responsible for dimer fading is relatively fast in comparison with other competing reactions under these conditions.

The Arrhenius plot (Fig. 3) is non-linear, but it can be approximated by two straight lines intersecting at ca. 30–33 °C; i.e. roughly at the denaturation temperature T_m . This point is uncertain because significant amounts of native and denatured collagen co-exist in this region. This curve indicates the presence of at least two photochemical components, one of which is temperature independent ($E_a \sim 0$), and takes place in the triple helix. The temperature-dependent component(s) has an activation energy of 7.6 ± 0.6 kcal/mol (31.7 ± 2.5 kJ/mol), and take(s) place only at higher temperatures where the amount of random coil is significant. This type of behavior is consistent with the existence of an additional photochemical reaction not likely in the helix (25) (e.g. reaction with molecular O_2) that may require thermal energy for activation (25). Alternatively, photodimerization could also occur at higher temperatures, but the more flexible coil conformation requires energy to place the tyrosine molecules in a favorable orientation for reaction. These two possibilities are not mutually exclusive.

These results, as compared with previous fluorescence temperature-dependence studies (22), strongly suggest that the fluorescence emission (22) and photochemical fluorescence fading behavior of these fluorophores may complement each other as markers of collagen conformation, chemical behavior, excited state properties, and supramolecular structure. The Arrhenius plots differ significantly from each other both qualitatively and quantitatively, and

the activation energies obtained for each process are consistent with those found in other systems (25, 26).

Finally, this study further emphasizes that collagen-bound fluorophores are not just passive markers of oxidative stress and age-related damage. Their photolability to wavelengths that can reach the dermis may produce alterations that can themselves result in pathological conditions such as photoaging, photosensitivity, or wound-healing disorders. Our temperature data indicate that elevated body temperatures may render dermal collagen significantly more susceptible to photodamage, and the deleterious sequelae outlined above.

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References

1. Gallop, PM., Seifter, S. Preparation and properties of soluble collagens. In: Colowick, SP., Kaplan, NO., editors. *Methods in enzymology*. Vol. 6. New York: Academic Press; 1963. p. 635-641.
2. Rubin AL, Pfahl D, Speakman PT, Davison PF, Schmitt FO. Tropocollagen: significance of protease-induced alterations. *Science*. 1963; 139:37-39. [PubMed: 13982985]
3. Kato Y, Nishikawa T, Kawakishi S. Formation of protein-bound 3, 4-dihydroxyphenylalanine in collagen types I and IV exposed to ultraviolet light. *Photochem Photobiol*. 1995; 61:367-372. [PubMed: 7740080]
4. Menter JM, Williamson GD, Carlyle K, Moore CL, Willis I. Photochemistry of type I acid-soluble calf skin collagen: dependence on excitation wavelength. *Photochem Photobiol*. 1995; 62:402-408. [PubMed: 8570699]
5. Lehrer SS, Fasman GD. Ultraviolet irradiation effects in poly-L-tyrosine and model compounds. Identification of bityrosine as a photoproduct. *Biochemistry*. 1967; 6:757-767. [PubMed: 6025562]
6. Milšik I, Deyl Z. Change in the amount of e-hexosyllysine, UV absorbance and fluorescence of collagen with age in different animal species. *J Gerontol*. 1991; 46:B111-B116. [PubMed: 1903139]
7. Menter JM, Abukhalaf IK, Patta AM, Silvestrov NA, Willis I. Fluorescence of putative chromophores in Skh-1 and citrate- soluble calf skin collagens. *Photodermatol Photoimmunol Photomed*. 2007; 23:222-228. [PubMed: 17986057]
8. Kligman LH. The hairless mouse model for photoaging. *Clinics Dermatol*. 1996; 14:183-195.
9. Kligman LH, Gebre M. Biochemical changes in hairless mouse skin collagen after chronic exposure to ultraviolet A radiation. *Photochem Photobiol*. 1991; 54:233-237. [PubMed: 1780360]
10. Trautinger F, Mazzucco K, Knobler RM, Trenz A, Kokoschka EM. UVA- and UVB-induced changes in hairless mouse skin. *Arch Dermatol Res*. 1994; 286:490-494. [PubMed: 7864664]
11. Herrmann G, Wlaschek M, Lange TS, Prenzel K, Goerz G, Scharffetter-Kochanek K. UVA radiation stimulates the synthesis of various matrix metalloproteinases (MMPs) in cultured human fibroblasts. *Exp Dermatol*. 1993; 2:92-97. [PubMed: 8156175]
12. Fisher GJ, Datta SC, Harvinder S, et al. Molecular basis of sun-induced premature skin aging and retinoid antagonism. *Nature*. 1996; 379:335-339. [PubMed: 8552187]
13. Petersen M, Hamilton T, Li H-L. Regulation and inhibition of collagenase expression by long-wavelength ultraviolet radiation in cultured human skin fibroblasts. *Photochem Photobiol*. 1995; 62:444-448. [PubMed: 8570703]
14. Menter JM, Cornelison LM, Cannick L, et al. Effect of UV on the susceptibility of acid-soluble Skh-1 hairless mouse collagen to collagenase. *Photochem Photoimmunol Photomed*. 2003; 19:28-34.

15. Quan T, Tianyuan H, Kang S, Voorhees JJ, Fisher GJ. Solar ultraviolet irradiation reduces collagen in photoaged skin by blocking transforming growth factor- β type II receptor/Smad signaling. *Am J Pathol.* 2004; 165:741–751. [PubMed: 15331399]
16. Schwartz E, Cruickshank FA, Christensen CC, Perlish JS, Lebwohl M. Collagen alterations in chronically sun-damaged skin. *Photochem Photobiol.* 1993; 62:841–844.
17. McCarthy AD, Etcheverry SB, Brussone L, Lettieri G, Barrio DA, Cortizo AM. Non-enzymatic glycosylation of a type I collagen matrix: effects on osteoblastic development and oxidative stress. *BMC Cell Biol.* 2001; 2:1471–2121/2/16.
18. Versul N, DeGroot J, Lodehinkel E, et al. Age-related accumulation of Maillard reaction products in human articular cartilage collagen. *Biochem J.* 2000; 350:381–387. [PubMed: 10947951]
19. Balasubramanian B, Kanwar R. Molecular pathology of dityrosine crosslinks in proteins: Structural and functional analysis of four proteins. *Mol Cell Biochem.* 2002; 234/235:27–38.
20. DiMarco T, Guilivi C. Current analytical methods for detection of dityrosine, a biomarker of oxidative stress, in biological systems. *Mass Spectrom Rev.* 2007; 26:108–120. [PubMed: 17019703]
21. Ortwerth BJ, Linetsky M, Olesen PR. Ascorbic acid glycation of lens proteins produces UVA sensitizers similar to those in human lens. *Photochem Photobiol.* 1995; 62:452–462.
22. Menter JM. Temperature dependence of collagen fluorescence. *Photochem Photobiol Sci.* 2006; 5:403–410. [PubMed: 16583021]
23. Moore, W. *Physical chemistry*. 3rd. Englewood Cliffs, NJ: Prentice-Hall; 1962. p. 272-273.
24. Helseth DL, Lechner JH, Veis A. Role of the amino-terminal extrahelical region of type I collagen in directing the 4D overlap in fibrillogenesis. *Biopolymers.* 1979; 18:3005–3014.
25. Turro, NJ. *Modern molecular photochemistry*. Menlo Park, CA: The Benjamin/Cummings Publishing Co. Inc; 1978. p. 187-188.
26. Bowen EJ, Sahu J. The effect of temperature on fluorescence of solutions. *J Phys Chem.* 1959; 63:4–7.

Fig. 1.

(A) Time course of collagen fluorophore fading under irradiation with a 4 W UVG-11 ultraviolet C (UVC) fluorescent hand lamp, irradiance = 0.0815 W/m^2 ; total dose = $1.173 \times 10^3 \text{ J/m}^2$. These curves reflect the complexity of the reactions involving these fluorophores. The time course of tyrosine fluorescence (270/300 nm) shows the presence of reverse reactions at $t > 30 \text{ min}$, probably as a result of non-covalent excimer-like tyrosine(s) and covalent dityrosine decomposition. Covalent dityrosine reflects a quasi-stationary state; it is formed from photolysis of tyrosine, and the dityrosine product decomposes on UVC radiation. Similar phenomena take place with the 370/450 nm species (most likely DOPA oxidation products). Under UVC, the decomposition of the 270/360 nm (interacting tyrosine) species predominates, and it disappears according to simple second-order analysis (see Fig. 2). The other fading curves are not amenable to simple first- or second-order kinetic analysis. (B) Time course of collagen fluorophore fading under irradiation with a 4 W UVL-21 UVA fluorescent hand lamp in conjunction with a 1 mm Schott WG 345 filter, irradiance = 0.141 W/m^2 ; total dose = $2.030 \times 10^3 \text{ J/m}^2$. All curves exhibit complicated kinetic behavior owing to the presence of simultaneous forward and back reactions. These curves confirm that the fluorescent products generated by UVC wavelengths are decomposed by longer UVA radiation. None of these curves are amenable to simple first- or second-order kinetic analysis.

Fig. 2.

Second-order plots of normalized reciprocal fluorescence intensity, $1/I_n$, vs. irradiation time t for Skh-1 collagen 270/360 nm fluorescence under UVG-11 UVC irradiation, irradiance = 0.141 W/m^2 ; total dose = $2.030 \times 10^3 \text{ J/m}^2$. The observation of second-order kinetics is consistent with a reaction between two similar tyrosine molecules in close proximity that results in a non-fluorescent product. The slope of each curve determines a second-order rate parameter, $k(T)$, at each temperature. Some plots at intermediate temperatures are omitted solely for clarity.

Fig. 3.

Arrhenius Plot of $1/T(\text{K})$ vs. $\log k(T)$ as evaluated from the data depicted in Fig. 2. In simple cases, such a plot yields a straight line whose slope is proportional to activation energy E_a for fluorescence fading. In the present case, we obtain a non-linear plot, which can be expressed as two straight lines, intersecting at $T \sim 303\text{--}306\text{ K}$ ($30\text{--}33\text{ }^\circ\text{C}$), i.e. near the denaturation temperature. In the low-temperature (helical) domain, fluorescence fading is independent of temperature ($E_a \approx 0$), while at $T > 306\text{ K}$ ($33\text{ }^\circ\text{C}$), where collagen is in the disordered coil, $E_a = 7.6 \pm 0.6\text{ kcal/mol}$ ($31.7 \pm 2.5\text{ kJ/mol}$).