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Fluorescent-Labeled Cross-Links in Collagen: Pyrenesulfonylhydrazine†

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ABSTRACT: Aldol condensation products of two lysyl-derived aldehyde (allysine) residues are involved in cross-linking of collagen. However, the distribution of these cross-links and their age-related changes remain largely unanswered. We have found that the unsaturated aldehydes of aldol condensation cross-links can be fluorescent labeled. When labeled with pyrenesulfonylhydrazine, pyrene dimers and excimers fluoresce at 383 and 485, nm, respectively. (The pyrene dimer is stable in benzene, whereas in polar solvents it exhibts an exponential decay to monomer fluorescing at 378 nm.) Dimers bound to collagen also decay to monomers, but at a more complicated, nonexponential rate. This dissociation in collagen is also associated with gradual decrease in the excimer fluorescence. While dissociated monomers appear to be reassociated by redialysis, the excimer is not regenerated. During fibril formation in vitro of the labeled collagen, two fluorescence changes take place: a very rapid decrease of the excimer fluorescence and a gradual increase of the monomer fluorescence. These changes indicate very early and early conformational changes at the nonhelical terminal telopeptides. The excimer fluorescence also decreases upon thermal and guanidine denaturation. Two different environments for excimer formation are suggested by the latter. It is concluded that pyrenesulfonylhydrazine offers a unique and sensitive probe for the proximity of aldehyde groups as well as for the mobility and conformation changes of the telopeptides in collagen.

In a previous study on the fluorescent labeling of acid-soluble type I collagen with pyrenebutyrylhydrazine (PBH),[1] we have shown the formation of excimer fluorescence, indicating the presence of two adjacent pyrene labels bound to the same collagen molecule (Shambaugh & Fujimori, 1981). Most pyrene molecules were at the nonhelical terminal telopeptides. Unsaturated aldehydes of aldol condensation cross-links in the β chain of collagen (Bornstein & Traub, 1979) were labeled with the pyrene groups. Thus, excimer formation indicated the proximity of two unsaturated aldehydes in collagen.

In the present study, another pyrene derivative, PSH,[1] has been found to have unique characteristics distinct from those of PBH. The solubility of PSH in aqueous solution is higher than that of PBH. The pyrene aggregate fluorescence observed in the PBH–collagen complex is not prominently formed in the PSH–collagen complex. Instead, PSH forms pyrene ground-state dimers which are gradually dissociated to monomers in aqueous solution. In order to obtain more information concerning the proximity of unsaturated aldehydes and the structural changes in the telopeptide, further research has been conducted to investigate PSH excimer formation and related phenomena in collagen. This paper describes the pronounced formation of excimer fluorescence by labeling of collagen with PSH dimers as well as changes in excimer fluorescence. In addition, the dissociation and association of the PSH dimer bound to collagen will be reported in connection with their relation to subtle structural changes at the telopeptides.

Materials and Methods

The preparation of acid-soluble type I collagen, from rat tail of young (4–6 weeks) and old (about 2 years) rats, and $NaBH_4$-reduced collagen, as well as pepsin treatment, was performed as previously described (Crabtree & Fujimori, 1980; Shambaugh & Fujimori, 1981). A small amount of PSH crystals (Molecular Probes, Inc.) was added to 0.2% collagen in 0.005 M acetic acid and stirred at 4 °C overnight. Subsequently, the mixtures were centrifuged twice at 55000*g* for 45 min and dialyzed extensively against 0.005 M acetic acid.

Fluorescence and excitation spectra were measured on a Perkin-Elmer fluorescence spectrophotometer, Model 650-10S, equipped with a temperature-controlled cell holder. A Cary 15 spectrophotometer was used to measure absorption spectra. Fibril formation and gel-filtration experiments were also carried out as previously described (Shambaugh & Fujimori, 1981).

Results

Figure 1 shows the fluorescence and excitation spectra of the freshly prepared PSH–young collagen complex, measured immediately after extensive dialysis against 0.005 M acetic acid. When excited at 330 nm, structural fluorescence bands were at 383, 402, and 425 nm, and an excimer fluorescence appeared at about 485 nm (Figure 1, curve A). Excitation at 360 nm gave rise to increased fluorescence bands (Figure 1, curve B) compared to those excited at 330 nm. The excitation spectrum for the 383-nm fluorescence exhibited maxima at 356 and 363 nm with a shoulder at 340 nm (Figure 1, curve C). These fluorescence and excitation maxima were entirely different from those of a pyrene monomer (see below), indicating that the emitting species is not a monomeric pyrene. Another maximum at about 380 nm was present in the excitation spectrum for the second fluorescence band at 402 nm (not shown). The excitation spectrum for the excimer fluorescence at 485 nm showed two peaks at 360 and 384 nm

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[1] Abbreviations used: PBH, pyrenebutyrylhydrazine; PSH, pyrenesulfonylhydrazine.

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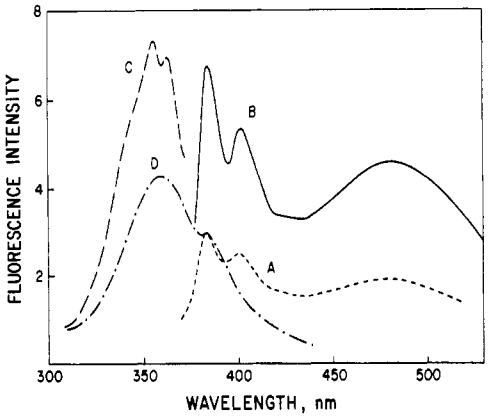

FIGURE 1: Fluorescence (A, B) and excitation (C, D) spectra of the fresh PSH–young collagen (0.1%) complex. (A) Excited at 330 nm; (B) excited at 360 nm; (C) for 383-nm fluorescence; (D) for 485-nm fluorescence.

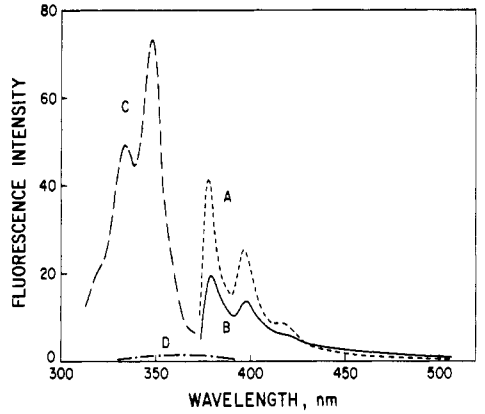

FIGURE 2: Fluorescence (A, B) and excitation (C, D) spectra of the aged PSH–young collagen (0.1%) complex. (A) Excited at 330 nm; (B) excited at 360 nm; (C) for 378-nm fluorescence; (D) for 485-nm fluorescnce.

(Figure 1, curve D). It should be noticed that spectrum D is similar in the wavelength range to spectrum C, but with less clear structure.

These fluorescence bands were found to change upon standing for 1 week at room temperature, or more slowly at 4 °C. The fluorescence and excitation spectra of the aged PSH–collagen complex, measured after 2 weeks at room temperature, are shown in Figure 2. The following striking changes took place: (1) The structural fluorescence bands blue-shifted to 378, 397, and 420 nm and increased their intensity (Figure 2, curves A and B). The excitation spectrum for the new enhanced fluorescence at 378 nm had maxima at 334 and 348 nm with a shoulder at 320 nm (Figure 2, curve C). These fluorescence and excitation maxima correspond to those of a monomeric pyrene. (2) The excimer fluorescence almost disappeared. The excitation spectrum for this 485-nm fluorescence exhibited only a residual band at 360 nm (Figure 2, curve D). The increase of the 378-nm fluorescence and the decrease of the 485-nm fluorescence are depicted as a function of time in Figure 3, curves A and B, respectively. Since the tail of the increased monomer spectrum covers the excimer spectral region, curve B does not show a large change. These changes suggest the dissociation of PSH aggregates to monomers upon aging of the PSH–collagen complex.

The same fluorescent characteristics and changes occurred in a fresh aqueous solution containing 10^{-5} M PSH, except that the excimer fluorescence was absent. The decrease of the 383-nm fluorescence and the increase of the 378-nm fluorescence are shown in Figure 3, curves C and D, respec-

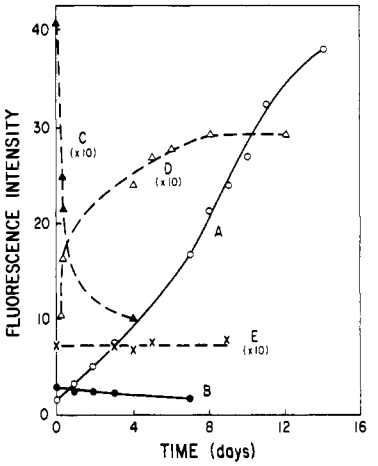

FIGURE 3: Time-dependent fluorescence changes of the PSH–young collagen (0.1%) complex (A, B) and PSH (10^{-5} M) in aqueous (C, D) and benzene (E) solution at room temperature. (A and D) 378-nm fluorescence (excited at 330 nm); (B) 485-nm fluorescence (excited at 360 nm); (C and E) 383-nm fluorescence (excited at 330 nm). ×10 indicates 10-fold intensity in fluorescence scale.

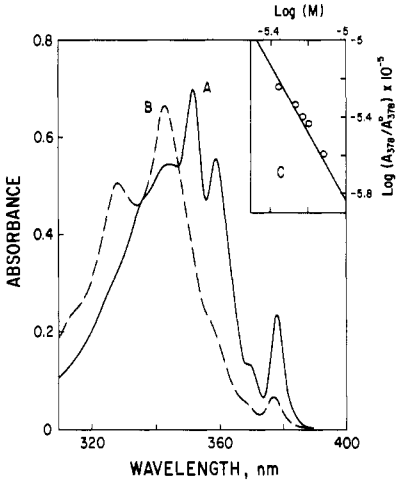

FIGURE 4: Absorption spectra of PSH (2.5×10^{-5} M) in benzene (A) and methanol (B). (C) is a plot of log (M) against log $(A_{378}/A^0_{378}) \times 10^{-5}$ (see text).

tively. These changes were exponential with respect to time, indicating a monomolecular reaction in agreement with the dissociation of PSH aggregates. In contrast to the first-order, faster rise of the 378-nm fluorescence in PSH aqueous solution, the increase in the PHS–collagen complex was much slower and sigmoidal instead of exponential (Figure 3, curve A). On the other hand, PSH (10^{-5} M) in benzene solution showed a 383-nm fluorescence which was stable (Figure 3, line E).

The absorption spectrum of PSH (2.5×10^{-5} M) in benzene, shown in Figure 4, curve A, was also stable and exhibited peaks at 378, 370, 359, and 352 nm. PSH in benzene fluoresced at 383, 402, and 422 nm as in the fresh PSH–collagen complex. The fluorescence of a fresh PSH–methanol solution was also at 383 nm but shifted to 378 nm upon aging as in aqueous solution. The aged PSH–methanol solution showed main absorption maxima at 343 and 328 nm with a minor band at 377 nm of the remaining PSH aggregates (Figure 4, curve B). When excited at 330 nm, this aged methanol solution emitted pyrene monomer fluorescence at 378, 397, and 418 nm as in the aged PSH–collagen complex. By changing the volume ratio of benzene to methanol (100, 80, 60, 50, 40, and 0%), one could measure the 378-nm absorbance (A_{378}) in these mixtures containing 10^{-5} M PSH. These solutions were allowed to stand for more than a week at room temperature to

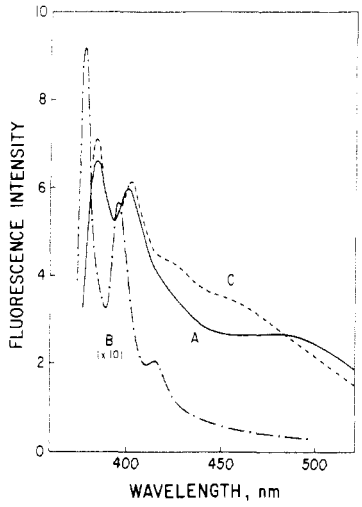

FIGURE 5: Fluorescence spectral changes of the PSH–old collagen (0.1%) complex. (A) Fresh; (B) aged for 3 weeks at room temperature; (C) dialyzed for 1 week at 4 °C. Excited at 330 nm.

assure a monomer–aggregate equilibrium. In this equilibrium, $K = (M)^n/(Mn)$ and $n \log (M) = \log (K/n) + \log (A_{378}/A^0_{378}) \times 10^{-5}$, where n is the degree of aggregation, K is an equilibrium constant, A^0_{378} is A_{378} in 100% benzene, and (M) and (Mn) are the monomer and aggregate concentrations, respectively. As shown in Figure 4, line C, a plot of log $(A_{378}/A^0_{378}) \times 10^{-5}$ against log (M) [$(M) = 10^{-5} - (A_{378}/A^0_{378}) \times 10^{-5}$] gave a straight line with a slope (n) of about 1.8, indicating that the PSH aggregate is actually a PSH dimer.

When the aged PSH–collagen complex was again dialyzed against 0.005 M acetic acid at 4 °C, dissociated PSH apparently reassociated to dimers. This peculiar change is shown in Figure 5 for old collagen. In contrast to young collagen, the fresh PHS–old collagen complex exhibited weaker excimer fluorescence compared to dimer fluorescence, but a relatively stronger fluorescence at 440–450 nm (Figure 5, curve A). When aged for 3 weeks at room temperature, PSH dimers were dissociated into monomers, and the excimer fluorescence was lost (Figure 5, curve B). The apparent reassociation of PSH monomers to its dimers by dialysis for 1 week did not regenerate the excimer, but the 440–450-nm fluorescence became more visible (Figure 5, curve C). Young collagen behaved similarly except that the latter was not as prominent as in old collagen. This fluorescence could be due to PSH higher aggregates which are similar to PBH aggregates in our previous study. When collagen solutions were mixed with PSH crystals for 1–2 h, centrifuged, and dialyzed, excimer formation was already visible in young collagen, but not in old collagen with the 440–450-nm fluorescence.

During fibril formation of the fresh PSH–young collagen complex, in which turbidity change was slightly slowed down compared to that of native collagen, the following fluorescence changes took place. (1) The excimer fluorescence decreased very rapidly within 1 min (Figure 6, curve A). This decrease was accompanied by a similar quick increase of the 378-nm monomer fluorescence (Figure 6, curve B). (2) During the turbidity lag period (100 min), the 378-nm fluorescence gradually increased (Figure 6, curve B). This fluorescence increase was followed by increased light scattering which is also manifested by the Raman scattering of water at 370 nm (Figure 6, curve C). On the contrary, for the aged PSH–collagen complex, whose fibril formation was further delayed compared to that of the fresh complex, no change in the 378-nm monomer fluorescence was observed during the turbidity lag period.

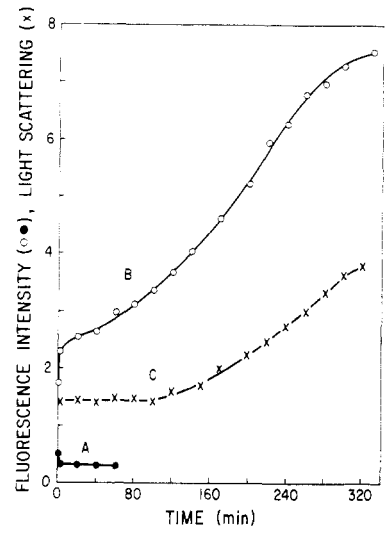

FIGURE 6: Fluorescence and light-scattering changes during fibril formation of the PSH–young collagen (0.0125%) complex at 25 °C. (A) Fluorescence at 480 nm; (B) 378-nm fluorescence; (C) Raman scattering of water at 370 nm. Excited at 330 nm.

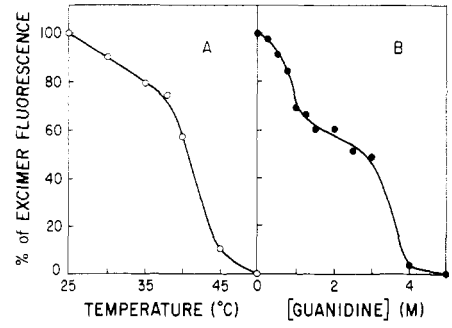

FIGURE 7: Excimer fluorescence changes of the fresh PSH–young collagen (0.1%) complex as a function of temperature (A) and guanidine-HCl concentration (B). Excited at 360 nm.

The excimer fluorescence was also affected by temperature increase and guanidine treatment. When the temperature was increased, the excimer intensity gradually decreased, exhibiting a sharp drop at the denaturation temperature (Figure 7, curve A). Heating also accelerated the conversion of the dimer to the monomer fluorescence. In contrast, guanidine treatment demonstrated a two-step decrease at 1 and 4 M guanidine-HCl (Figure 7, curve B), suggesting the possible formation of excimers in two different environments. During denaturation by guanidine, the increase of the pyrene monomer fluorescence was not observed, but the dimer fluorescence slightly increased and red-shifted from 383 to 388 nm in the presence of 5 M guanidine. The fluorescence peak shifted back to 380 nm but only with a slight increase upon standing overnight at room temperature. As was shown in Figure 3, curve A, the nontreated PSH–collagen complex underwent more efficient dissociation to monomers. This indicates that guanidine inhibits the dissociation of dimers but destroys the excimer configuration of pyrene dimers.

With pepsin treatment, the excimer was also diminished, and PSH dimers dissociated. After dialysis and KCl precipitation twice, the pyrene fluorescence was totally eliminated from the collagen, indicating that all pyrenes are at the nonhelical terminal telopeptides. With the non-pepsin-treated complex, KCl precipitation allowed partial release of noncovalently bound PSH from the protein. Finally, it should be noted that reduced young collagen, when treated with PSH, did not form excimer but only a weak monomer fluorescence (about 10% compared to that of native collagen), verifying

that PSH reacts specifically with aldehyde groups.

Discussion

The present study reveals that PSH is bound to collagen as a dimer which remains stable during dialysis. Since reduced collagen, whose aldehyde groups are reduced by $NaBH_4$, does not form excimer, excimer arises from PSH dimers bound to unsaturated aldhyde groups at the telopeptides of β chains. (Gel-filtration studies on PSH–collagen complexes showed the same result as found in PBH–collagen complexes. Pyrene groups were retained with β and γ chains, but not with α chains.) It appears that one PSH in the dimer reacts with the aldehyde group, while the other PSH remains unreacted and is noncovalently bound in a hydrophobic environment. Pyrene molecules involved in excimer emission have an excitation spectrum which is similar to but not identical with that of pyrene dimer fluorescence. This nonidentical excitation spectrum indicates that pyrene dimers emitting the excimer fluorescence are in an environment different from those for the dimer fluorescence. Furthermore, the presence of two different environments for excimer formation is indicated by the effect of guanidine. Detailed information concerning the location of excimer formation awaits further investigation.

Ground-state hydrocarbon dimers, differing from dye molecules, are usually unstable in solution at room temperature. Only under suitable conditions at low temperature are stable pyrene pairs formed (Ferguson, 1965). Sandwich dimers of other hydrocarbons can also be produced from supercooled solutions in vitreously solidifying solvents by controlled heating and recooling (Ferguson, 1966). The absorption and fluorescence spectra of these dimers differ considerably from those of monomers but are similar to those of crystals. Excimer flurescence was also observed from pyrene crystals, in which the adjacent molecules occur in parallel pairs (Ferguson, 1958).

PSH dimers, probably stabilized by their polar resonance structure, are the first pyrene dimers to be reported which are stable at room temperature even in a low concentration (10^{-5} M) in the nonpolar solvent benzene. In polar solvents such as water, methanol, or dimethyl sulfoxide, however, PSH dimers are gradually dissociated in dilute solution. [It should be noted here that pyrene monomer fluorescence also undergoes significant changes on going from nonpolar to polar solvents (Kalyanasundaram & Thomas, 1977). The enhancement of fluorescene in polar solvents has been attributed to complex formation with the solvent (Lianos & Georghiou, 1979).] PSH dimers bound to collagen also dissociate, but much more slowly with different rates in different environments. Some delayed dissociation, coupled with this heterogeneous process, would be responsible for the nonexponential dissociation of PSH dimers in collagen. PSH dimers involved in excimer formation could be different in their rate of dissociation from those of nonexcimer dimers. Differently prepared PSH–collagen complexes also demonstrate different rates of dissociation. For example, the PSH–collagen complex, prepared by mixing equal volumes of PSH-saturated aqueous solution with collagen solution, showed somewhat exponential dissociation.

Excimer fluorescence occurs more favorably with two dimers in proximity than in dissociated monomer states. When PSH dimers dissociate, the necessary excimer conformation is lost. When two pyrene molecules are connected by a chain, as in 1,1′-dipyrenyl methyl ether (Georegescauld et al., 1980), conformation-dependent intramolecular excimer is formed. Excimer formation in this case is a unimolecular process, as opposed to a diffusion-controlled bimolecular process in pyrene solution. Two adjacent PSH dimers attached to collagen could also form excimer as in the case of intramolecular excimer formation.

Apparent reassociation of PSH monomers to dimers by redialysis implies that dissociation does not separate the two dissociated PSH monomers far apart. Upon reassociation, however, required excimer configuration is not restored. The nonhelical telopeptides are unstructured and mobile in acid solution (Chandrakasan et al., 1976). This would allow dimers to dissociate as in free PSH in solution. The mobile peptides may be changed in favor of dimer formation under dialysis conditions. This possibility for the regeneration of PSH dimers is under further investigation.

In the process of fibril formation in vitro, the very rapid loss of excimer emission was followed by the gradual dissociation of PSH dimers during the turbidity lag phase. These changes took place much more quickly than those while standing in acid solution and are similar to a combination of our two previous findings. These were found separately in the rapid decrease of the PBH excimer fluorescence (Shambaugh & Fujimori, 1981) and in the gradual increase of the dansyl fluorescence (Crabtree & Fujimori, 1980). The present result further verifies those conformational changes occurring at the telopeptides in both the very early and early events (Gelman et al., 1979a,b; Silver et al., 1979; Helseth et al., 1979) during fibril formation.

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