BLUE FLUORESCENCE AND CROSSLINKING OF PHOTOOXIDIZED PROTEINS

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1. Introduction

Upon aging, ocular lens proteins increase crosslinking and visible fluorescence [1]. The direct or sensitized photooxidation of tryptophan residues in proteins produces the primary photoproduct *N*-formylkynurenine (FK) [2,3], whose blue fluorescence excited at 350 nm emits at 400 and 435 nm [2,4]. In the UV (300 nm)-induced photooxidation of calf-lens α-crystallin, we have discovered the formation of new blue fluorescence bands at 440 and 460 nm, which can be excited at 400–420 nm [4–6]. When UV (300 nm)-irradiated α-crystallin is further irradiated with UV (365 nm) light which is absorbed by FK, the 400/435 nm fluorescence is converted to the 440/460 nm fluorescence [5]. Furthermore, photooxidized α-crystallin is crosslinked, while subsequent UV (365 nm)-irradiation increases crosslinking [6]. To compare with calf-lens α-crystallin, other tryptophan-containing proteins have been investigated. A parallelism between crosslinking and 440/460 nm fluorescence has been found in other photooxidized proteins. In addition, 440/460 nm fluorescence and crosslinking were also found to be affected by hydroxylamine or hydrazine.

2. Materials and methods

Porcine pepsin and bovine serum albumin (BSA) were obtained from Sigma and porcine pepsinogen, bovine trypsin and hen egg white lysozyme from Worthington. Myosin (1.5% in 0.5 M KCl) was a gift from Dr Lu of the Dept. of Muscle Research in our Institute. α-, β_H-, β_L- and γ-Crystallins were isolated from calf lenses by gel-filtration on Sephadex G-200, as in [5]. The 0.2% or 0.5% protein solution (1 ml in 0.1 M or 0.5 M borate buffer, pH 8.2) in a rectangular quartz cell (1 cm × 1 cm) was irradiated for 2 h with UV (300 nm) light from a Xe lamp (150 W), connected to a Bausch and Lomb high intensity monochromator (band width ±10 nm), at 10 cm from the exit slit. For subsequent UV (365 nm) irradiation for 1 h, a Hg lamp with a filter for the 365 nm Hg line was used at 20 cm from the filter (intensity 2.8–3 mW/cm^2). Before and after UV-irradiation, fluorescence (excited at 350 and 400 nm) and excitation (for 450 nm fluorescence) spectra were measured using a Perkin-Elmer fluorescence spectrophotometer model 650-10 S. Gel-electrophoresis experiments (6% acrylamide gel for myosin and BSA, 13% gel for other proteins) were also performed as in [6].

3. Results

Fig.1 and 2 show excitation spectral changes in the blue fluorescence at 450 nm of BSA, β_L-crystallin and pepsinogen. As seen in fig.1A(2), UV (300 nm)-irradiated BSA efficiently produced 450 nm fluorescence excited at 400–420 nm (composite 440/460 nm fluorescence) in addition to the fluoresence from FK excited around 350 nm. Since these 2 fluorescence bands overlap at 450 nm, the excitation spectrum exhibited 2 bands: a structural band with 3 peaks at 400, 415 and 422 nm for the former fluorescence and a single broad band around 350 nm for the latter. This excitation spectrum of UV (300 nm)-irradiated BSA is the same as that reported for α-crystallin [5,6]. Another excitation peak at 295 nm is due to energy transfer from tryptophan residues. The decrease of tryptophan peak was accompanied by the concomitant increase of the above-mentioned 2 excitation bands (fig.1A(1 → 2)). Subsequent UV (365 nm) irradiation increased 400–420 nm excitable fluorescence at the expense of 330–370 nm excitable fluorescence

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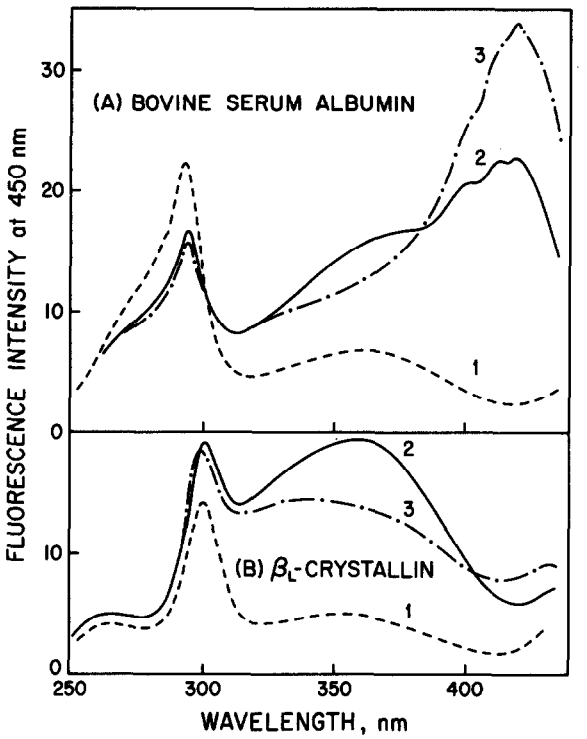

Fig.1. UV-induced excitation spectral changes in the 450 nm fluorescence of 0.2% BSA (A) and β_L-crystallin (B) in 0.1 M borate buffer (pH 8.2): (1) before irradiation; (2) after UV (300 nm) irradiation for 2 h; (3) after further UV (365 nm) irradiation for 1 h.

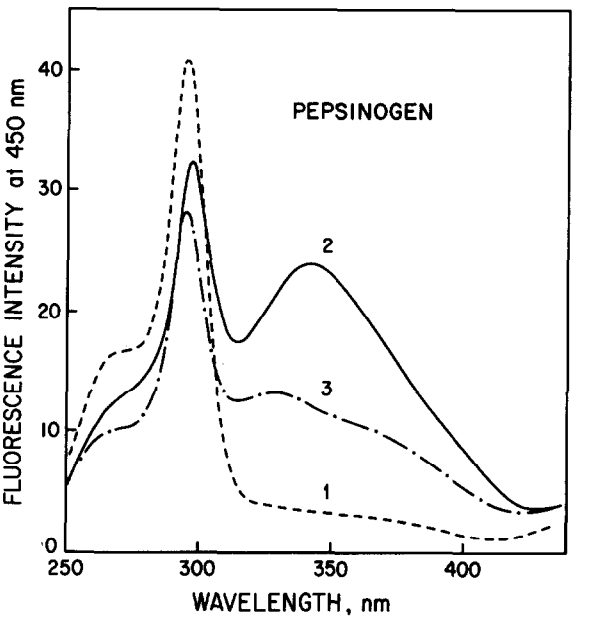

Fig.2. UV-induced excitation spectral changes in the 450 nm fluorescence of 0.2% pepsinogen in 0.1 M borate buffer (pH 8.2): (1–3) as in fig.1.

(fig.1A(2 → 3)). In contrast to BSA, β_L-crystallin exhibited only a small increase of the 450 nm fluorescence excited at 420 nm (fig.1B(2 → 3)), whereas pepsinogen did not produce it (fig.2(2 → 3)). In both cases, FK fluorescence excited at 330–370 nm was produced by UV (300 nm) irradiation (fig.1B,2(1 → 2)), but was converted by UV (365 nm) light either partially (fig.1B(2 → 3)) or entirely (fig.2(2 → 3) to non-450 nm fluorescent forms.

The results including other proteins are summarized in table 1 for both 440/460 nm fluorescence (excited at 400–420 nm) and crosslinking. Intermolecular or intersubunit crosslinking by UV-irradiation was investigated with SDS–polyacrylamide gel electrophoresis. No intermolecular crosslinking was found in the case of trypsin, pepsin, pepsinogen and BSA. Trypsin and pepsin, like pepsinogen, did not produce 440/460 nm fluorescence. BSA was exceptional in efficiently producing 440/460 nm fluorescence without any intermolecular crosslinking and will be discussed below. With myosin, photo-crosslinking produced dimers (400 000 M_r) and higher polymers of myosin heavy chains with some formation of 440/460 nm fluorescence. Under irradiation conditions used, lysozyme and γ-crystallin produced dimers (28 000 and 40 000 M_r, respectively) together with moderate 440/460 nm fluorescence, whereas β_L- and β_H-crystallins formed only a small amount of dimer-like cross-

Table 1
Crosslinking and 440/460 nm fluorescence of photooxidized proteins

Protein	M_r	440/460 nm Fluorescence	Crosslinking (intermolecular or intersubunit)
Trypsin	23 300	–	–
Pepsin	35 000	–	–
Pepsinogen	39 000	–	–
BSA	67 000	+++	–
Lysozyme	14 600	+	+
Myosin	500 000	+	++
γ-Crystallin	20 000	++	+
β_L-Crystallin	50 000	+	+
β_H-Crystallin	200 000	+	+
α-Crystallin	800 000	+++	++

linked subunits (53 500 and 57 000 M_r, respectively) with a small increase in 440/460 nm fluorescence. The photo-polymerization of *S*-carboxymethyl-lysozyme to its various multimers was shown after irradiation with light over 300 nm for 72 h [7].

When α-crystallin or BSA was irradiated with UV (300 nm) light in the presence of 0.05 M hydroxylamine or hydrazine, 440/460 nm fluorescence was not produced. These reagents, added to UV (300 nm)-irradiated proteins, were found to reduce 440/460 nm fluorescence (excited at 400–420 nm). The changes in the case of photooxidized BSA with hydroxylamine are shown in fig.3A by excitation spectra. Little change occurred in the fluorescence excited at 330–370 nm. Fig.3B shows the decrease of 450 nm fluorescence (excited at 420 nm) for both photooxidized α-crystallin and BSA in the presence of hydroxylamine or hydrazine. The decrease was slower in BSA than in α-crystallin. Although the change appeared to be faster with hydroxylamine than with hydrazine, there may be some instantaneous quenching of the conformation-sensitive fluorescence [6] by hydroxylamine, followed by a gradual decrease. Gel-electrophoresis experiments further revealed that crosslinking is inhibited, and crosslinked proteins are partially dissociated, by these reagents (0.1 M). This was studied with α- and γ-crystallins and lysozyme. In all cases, hydrazine was more effective than hydroxylamine. The effect of hydroxylamine was stronger with γ-crystallin and lysozyme than with α-crystallin.

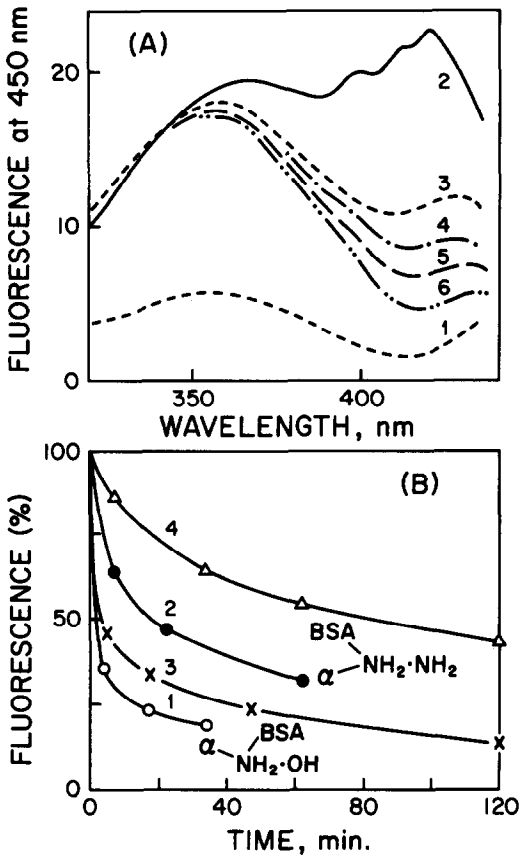

Fig.3. (A) Excitation spectral changes in the 450 nm fluorescence of photooxidized BSA (0.2%) with hydroxylamine (0.1 M) in 0.5 M borate buffer (pH 8.2): (1) before irradiation; (2) after UV (300 nm) irradiation for 2 h; (3) 3–4 min; (4) 16–17 min; (5) 46–47 min; (6) 120–121 min; after the addition of 0.1 M hydroxylamine. (B) Changes in the 450 nm fluorescence (excited at 420 nm) of UV (300 nm)-irradiated (2 h) 0.2% α-crystallin (1,2) and BSA (3,4) in the presence of 0.1 M hydroxylamine (1,3) or hydrazine (2,4) in 0.5 M borate buffer (pH 8.2).

4. Discussion

A parallel relationship between crosslinking and 440/460 nm fluorescence supports the possibility that these blue-fluorescent products participate in photo-crosslinking. BSA is unique and contains only 2 tryptophan residues at positions 134 and 212 and 59 lysine residues in a single polypeptide chain of 581 amino acids. In the regions around tryptophan residues, there is a large accumulation of lysine residues [8]. If the carbonyl group of FK reacts with a lysyl amino group to form a crosslink, intramolecular crosslinking could be expected in this protein. This would explain the absence of intermolecular crosslinking and the efficient formation of 440/460 nm fluorescence due to intramolecular crosslink. On the other hand, trypsin, pepsin and pepsinogen form neither crosslink nor 440/460 nm fluorescence. In trypsin, all the amino groups of lysine residues occupy positions on the surface, whereas tryptophan residues are more or less in the interior of the protein molecule [9]. A proximity between tryptophan residues to be converted to FK and lysine amino groups would determine the formation of crosslinks. In the case of pepsin [10] and pepsinogen [11], tryptophan residues could also be remote intra- and inter-molecularly from lysine residues. In contrast, one or more tryptophan residues in lysozyme exist sufficiently exposed on the surface [12]. Lysozyme, as well as myosin (most tryptophan residues, ~7–8, present in the head subfragment-1 of heavy meromyosin in the heavy

chain [13]), calf-lens α-, β- and γ-crystallins, demonstrated both crosslinking and 440/460 nm fluorescence in a different degree.

The effect of hydroxylamine and hydrazine upon 440/460 nm fluorescence and crosslink is consistent with the possible involvement of adjacent amino groups in their interaction with FK. It is known that FK reacts with hydrazine to form hydrazone (prior to the cyclization to a pyridazone derivative) [14–16]. While the formyl group is eliminated at some unidentified stage, the carbonyl group at the *ortho*-position reacts with hydrazine. Hydroxylamine is expected to react with the same carbonyl group. The increased reactivity of carbonyl groups at the excited state of FK could lead to the formation of fluorescent crosslinks in certain protein environments. A specific interaction (possibly charge or electron transfer) between the FK triplet state and an amino group has been demonstrated [17]. The reaction between carbonyl and amino groups is also supported by a red-shift of the absorption maximum from 320 nm for FK to ~420 nm for 440/460 nm fluorescent chromophores and by the blue-light (420 nm)-induced conversion of 440/460 nm fluorescent chromophores back to FK. The latter photoconvertible reactions were previously reported with calf-lens α-crystallin [5], but BSA also exhibited the same photo-reversible changes.

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