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Photooxidation of Specific Residues in α-Crystallin Polypeptides†

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ABSTRACT: Singlet oxygen is a biologically important, photochemically generated species that preferentially oxidizes His, Trp, and Met residues of protein molecules. Calf α-crystallin was photooxidized with use of *meso*-tetra(*p*-sulfonatophenyl)porphyrin (TPPS) and uroporphyrin (UP) as singlet oxygen generators. The effects of photooxidation were monitored by analyzing the changes in α-crystallin peptide maps obtained by reversed-phase HPLC using a photodiode array absorbance detector. The reaction led to the loss of six specific peptides, five of which contained photooxidizable residues. Peptides containing His-97 and His-154 from the A chain and Met-68 from the B chain are preferentially photooxidized, suggesting that those residues have access to singlet oxygen. Trp residues in the N-terminal region are converted to NFK, whereas Trp-60 in the B chain is not photooxidized strongly suggesting that the former are close to the surface of α-crystallin while the latter Trp residue is buried. Only one peptide that is lost from the peptide maps does not contain a photooxidizable group; however, this peptide does contain an apparently undigested Lys residue. It is suggested that it forms a cross-link with a photooxidized His residue.

Wavelengths of light greater than 293 nm are transmitted through the cornea, exposing the vertebrate eye lens to considerable, potentially deleterious far-UV irradiation (Taylor et al., 1988; Urbach, 1989; Lee, 1989; Frederick et al., 1989). It is well documented that such exposure initiates significant structural alterations to the lens proteins, including subtle conformational changes (Andley et al., 1984; Mandal et al., 1988; Andley, 1988) and photooxidation of reactive amino acid residues (Garcia-Castineiras et al., 1978; Inoue et al., 1982; Fujimori, 1982; Dillon et al., 1987; Tallmadge & Borkman,

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1989; Dillon & Atherton, 1990) as well as disulfide and non-disulfide covalent cross-linking (Fujimori, 1982; Gossey et al., 1980; Dillon et al., 1985; Roy et al., 1984; Saito & Matsuura, 1985, Schiavon & Veronese, 1986; Borkman et al., 1986). As a result of various therapies and diseases, metabolites such as porphyrins, which are efficient photosensitizers, can reach the eye (Kessel et al., 1987; Fraunfelder, 1982). In vitro and in vivo studies with two members of this family of compounds, uroporphyrin (UP)[1] and *meso*-tetra(*p*-sulfonatophenyl)porphyrin (TPPS) have indicated that their presence greatly accelerates the photooxidation of lens crystallins (Roberts & Dillon, 1987, 1989; Dillon et al., 1988; Roberts et al., 1991). These studies have also shown that TPPS, but not UP, binds to lens proteins (Roberts et al., 1990), resulting in the production of longer lived triplet excited states of the porphyrin capable of mediating the modification of specific residues within the protein (Dubbelman et al., 1978; Girotti, 1979; Verweij & van Steveninck, 1982). Binding can be expected to be important in vivo since it increases the residence time of the scavenger and, therefore, the extent of photooxidative damage (Roberts et al., 1990).

α-Crystallin is isolated from lens fiber cells as an 800 000-Da aggregate (Bloemendal, 1981; Maisel, 1985) composed of the constitutive 20 000-Da αA and αB subunits and their phosphorylated forms, αAp and αBp, respectively. It is generally accepted that similar aggregates are present in vivo (Benedek, 1971; Tardieu et al., 1986) in the lens. Several models have been proposed for the tertiary and quaternary structure of α-crystallin (Siezen, 1981; Augusteyn & Koretz, 1987; Thomson & Augusteyn, 1988, 1989); however, there is no consensus regarding the tertiary structure or the precise arrangement of the α-crystallin subunits within the aggregate. The photochemistry of α-crystallin has been of interest to us because knowing the relative photolytic rates of reactive residues will provide data concerning the tertiary, and perhaps quaternary, structure of the protein. The porphyrins TPPS and UP, which greatly accelerate photooxidative reactions through the production of singlet oxygen (Dubbelman et al., 1978; Gitotti, 1979; Verweij, 1982), can be used as tools to study the photochemistry and structure of lens crystallins. This is due to the fact that singlet oxygen reacts preferentially with only three amino acids, histidine, tryptophan, and methionine, and since the lifetime of singlet oxygen in water is short (microseconds) (Foote, 1976), externally generated singlet oxygen would be expected to react with external residues.

We report here the kinetics of the photosensitized oxidation of α-crystallin in the presence of these porphyrins. The specific sites of photooxidation within the primary structure of α-crystallin are determined by comparison of the HPLC tryptic peptide maps of the photooxidized material with nonirradiated control samples. The loss of material from specific peaks, accompanying the concomitant appearance of peaks unique to the photolyzed α-crystallin, was observed. The identities of these peptides were determined by analysis of their absorption spectra, amino acid compositions, and, in some instances, sequence analyses. It is apparent from these data that the photooxidation of α-crystallin initially involves only a very limited number of residues, most of which are present in the αA chain. This finding may be a reflection of the tertiary and quaternary structure of α-crystallin.

[1] Abbreviations: NFK, *N*-formylkynurenine; UP, uroporphyrin; TPPS, *meso*-tetra(*p*-sulfonatophenyl)porphyrin; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-pressure liquid chromatography.

Experimental Procedures

Isolation of α-Crystallin. Lenses were dissected from fresh calf eyes (Marx Brothers Veal, Red Bank, NJ). After removal of the capsule, the outer cortex, corresponding to approximately 20% of the lens wet weight, was obtained by stirring the lens tissue in 50 mM Tris/0.2 M NaCl/1 mM EDTA/10 mM 2-mercaptoethanol, pH 7.4, at 4 °C, using 4 mL of buffer/g of tissue wet weight. The outer cortex tissue suspension was homogenized and centrifuged at 105000*g* for 45 min at 4 °C. The resultant protein concentration of the resultant supernatant was adjusted to 50 mg of protein/mL. A total of 30 mL of supernatant was loaded onto a 5 cm × 100 cm Sepharose-CL6B (Phamacia-LKB) gel filtration column (Van den Oetelaar et al., 1985), eluted at a flow rate of 1 mL/min, and monitored by absorbance at 280 nm. α-Crystallin eluting as a single symmetrical peak at 1200 mL corresponding to an apparent molecular mass of 800 000 Da was collected, extensively dialyzed against water, lyophilized, and stored at −20 °C. The purity of these cortical α-crystallin preparations was routinely determined by SDS–PAGE and IEF analysis.

Photochemical Modification of α-Crystallin. The 2 mg/mL α-crystallin solutions in 10 mM sodium phosphate, pH 7.4, were irradiated for various periods of time (0–30 min) in the presence or absence of either 1.0 mM *meso*-tetra(*p*-sulfonatophenyl)porphyrin (TPPS) or uroporphyrin (UP) using a 450-W medium-pressure mercury lamp (Conrad-Hanovia) in a "merry-go-round" apparatus (Dillon et al., 1987). The light from the lamp was filtered through a 5% solution of $CuSO_4$, which effectively absorbs wavelengths >297 nm. The irradiated protein was dialyzed extensively against water in the dark, lyophilized, and stored at −20 °C.

Proteolysis of α-Crystallin. Both control and irradiated samples of α-crystallin were digested with trypsin as previously described (Dillon et al., 1987). Briefly, the digestion mixtures containing 10 mg/mL α-crystallin, 0.2 mg/mL TPCK-treated trypsin (Sigma Chemical Co), and 0.1 N *N*-ethylmorpholine adjusted to pH 8.6 with acetic acid were incubated at 37 °C for 24 h. The reaction was stopped by the addition of glacial acetic acid to a final concentration of 8.5 N. The digests were lyophilized and stored at −20 °C.

HPLC Analysis of Tryptic Digests. The lyophilized tryptic digests were solubilized in 0.1% trifluoroacetic acid (TFA) to a final concentration of 2 mg of protein/0.8 mL, sonicated, and centrifuged at 12000*g* for 4 min. The 200-mL samples of the resultant supernatant, containing >95% of the total protein, were injected onto a C_{18} reversed-phase HPLC (RP-HPLC) column (Beckman ultrasphere, 5-mm beads, 4.6 mm × 150 mm), and the peptides were resolved with use of a Waters 840 HPLC system with a complex gradient using 0% CH_3CN containing 0.1% trifluoroacetic acid (TFA) as buffer A and 50% CH_3CN containing 0.1% TFA as buffer B. The gradient contained the following steps: 0% buffer B, 0–5 min; 0–20% B, 5–45 min; 20–40% B, 45–75 min; 40–100% B, 75–125 min. The elution of peptides was monitored either by measuring absorbance simultaneously at 214 and 280 nm, (Waters Detector Model 441), or by using a photodiode array detector (Waters Model 990). Peptides were collected, lyophilized, redissolved in buffer A, and purified in the same RP-HPLC system but with a different gradient: 0% B, 0–5 min; 0–20% B, 5–55 min; 20–40% B, 55–120 min; 40–60% B, 120–150 min; 60–100% B, 150–160 min. In this way, nine peptides of interest were purified to apparent homogeneity.

Characterization of Tryptic Peptides. The peptides of interest were hydrolyzed with 6 N HCl at 110 °C in vacuo for

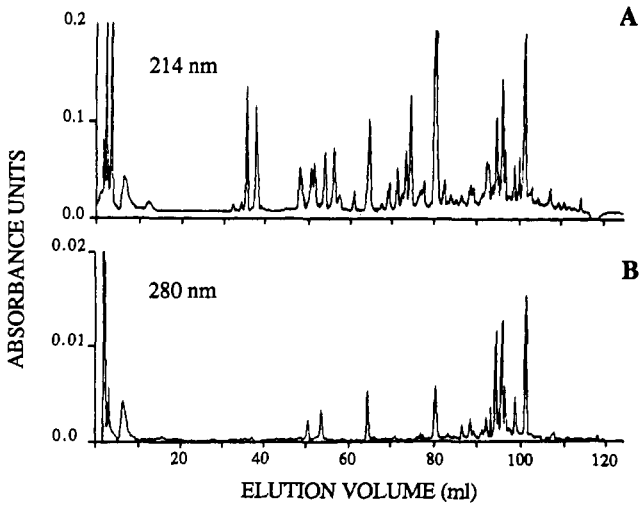

FIGURE 1: HPLC chromatograms of tryptic digests of α-crystallin monitored at 214 and 280 nm. α-Crystallin (2 mg/mL) was dissolved in 10 mM phosphate, pH 7.4, in the presence or absence of 1 mM either UP or TPPS4 without irradiation. The samples were immediately dialyzed extensively against water, lyophilized, and digested with trypsin and analyzed by HPLC as described in the text. Both the 214- and 280-nm profiles of all three samples were essentially identical.

24 h, and their amino acid compositions were determined with use of a Beckman 6300 amino acid analyzer. Several peptides were also subjected to automated Edman degradation in a gas-phase amino acid sequencer (Applied Biosystems Model 470A). The data from these analyses was used to conclusively determine the identity of various α-crystallin tryptic fragments and their location in the primary structure of the A and B polypeptide chains. In addition, complete absorption spectra (190–500 nm) of the resolved peptides were obtained with use of the photodiode array detector and the appropriate analysis software (Waters Model 990).

RESULTS

Photochemical Alteration of α-Crystallin. Tryptic digests of both control and irradiated samples of α-crystallin were analyzed by HPLC using a reversed-phase C_{18} column. The absorbance of the eluting peptides was monitored at 190–500 nm using a photodiode array detector (Waters Model 990) and also at 214 and 280 nm (Waters 441 monitor, 840 HPLC system) as described under Experimental Procedures. Approximately 30–35 peptides are consistently resolved at 214 nm from the digests of control samples, with additional material eluting near the void volume apparently containing very hydrophilic peptides or single amino acid residues (Figure 1). This is in close agreement with the theoretical number of possible peptides predicted from the tryptic digestion of total α-crystallin (van der Ouderaa, 1973, 1974).

Approximately eight major peptides with absorbance at 280 nm are present in the HPLC profiles of the α-crystallin digests (Figure 1). Several minor peptides are also present. Tryptic digestion of α-crystallin is expected to yield eight peptides with strong 280-nm absorbance: three containing tryptophan (T1 of αA; T1 and T6 of αB) and five containing tyrosine (T3, T4, T13, and T16 of αA; T4 and T17 of αB; note that T16 of αA and T17 of αB are identical peptides) (Figure 2). Tryptophan-, tyrosine-, and phenylalanine-containing peptides can be identified by the differences in their absorption spectra. Tryptophan has a broad peak of absorbance from 270 to 290 nm, while the absorption peak of tyrosine is narrower, ranging from 270 to 280 nm. Tyrosine has negligible absorbance at 290 nm. Phenylalanine displays only weak absorbance from 255 to 280 nm, with a small peak at 258 nm. Displaying the absorption profiles of the tryptic peptides simultaneously at 250, 270, and 290 nm (Figure 3) enables us to distinguish between tryptophan- and tyrosine-containing peptides. The five major tryptic peptides eluting at 3, 51, 55, 66, and 81 mL have no absorbance at 290 nm and, therefore, contain tyrosine, while those eluting relatively later, at 95–101 mL, absorb strongly at all three wavelengths and, therefore, contain tryptophan (Figure 3A). Figure 3B, which shows the spectrum analysis of both a tyrosine-containing (66 mL) and tryptophan-containing (101 mL) peptide, illustrates clearly their differences in absorption spectra.

The irradiation of α-crystallin in the presence of 1 mM UP or TPPS results in the apparent modification of six major specific peptides (labeled A–F in Figure 4). Their modification is reflected by their greatly reduced absorbance at 214 nm during the time course or irradiation. Three of these peptides (A–C), eluting at 37, 55, and 72 mL correspond to peptides displaying no absorbance at 280 nm and, therefore, contain neither tyrosine nor tryptophan. However, the other three peptides (D–F) correspond to tryptophan-containing peptides (Figure 3). A striking feature of the photochemistry of α-crystallin is the apparent stability of the tyrosine residues in the presence of the porphyrin sensitizers.

There was a greater than 50% loss in both 214- and 280-nm absorbance in the tryptophan peptides (D–F) upon irradiation of α-crystallin in the presence of TPPS (data not shown) and UP (Figure 4) after 5 and 10 min, respectively. Although the same major peptides become photooxidized in the presence of both TPPS and UP, the overall rate of photolysis is greater with TPPS, which has been shown to bind to α-crystallin (Roberts et al., 1990).

Although the photooxidation of α-crystallin clearly results in the modifications of several specific tryptic peptides, the appearance of new, modified peptides is limited. After only 10 min of irradiation in the presence of UP, there is a 3–4-fold increase in both the 214- and 280-nm absorbance of peptides eluting at 87 mL (P1) and 89 mL (P2) (Figures 4 and 5). In samples exposed to TPPS for 10 min, the increase in the amount of P1 and P2 is about 50% of that observed in the samples containing UP. Interestingly, spectrum analysis of these photoproducts indicates that tryptophan is present (data not shown), suggesting strongly that they contain intact tryptophan and other, unidentified modified residues.

Photooxidation of tryptophan has been reported to produce *N*-formylkynurenine, a compound with strong absorbance at 320 nm (Walrant & Santus, 1974). Figure 6A shows that several peaks absorbing at 320 nm are resolved from samples irradiated in the presence of either TPPS or UP. Spectrum analysis of the largest of these eluting at 55 min shows that this peptide has a broad absorbance peak from 315 to 330 nm (Figure 6B). Although the relative amount of material photolyzed is greatly in excess of the amount of material contained in identifiable new peptides, other photooxidized, hydrophilic peptides may be unresolved and present in the void volume of the HPLC profiles.

The six major peptides modified by irradiation (A–F), the two photoproduct peaks (P1 and P2), and one tyrosine peptide unaffected by irradiation (G) were collected, lyophilized, and repurified to apparent homogeneity by HPLC as described under Experimental Procedures with use of a modified CH_3CN gradient. The amino acid compositions of each peptide were determined (Table I), and their sequences were deduced from the known primary structure of the A and B chains of α-crystallin (van der Ouderaa, 1973, 1974; Figure 2). As a further control, the tryptophan peptide (D) and the tyrosine

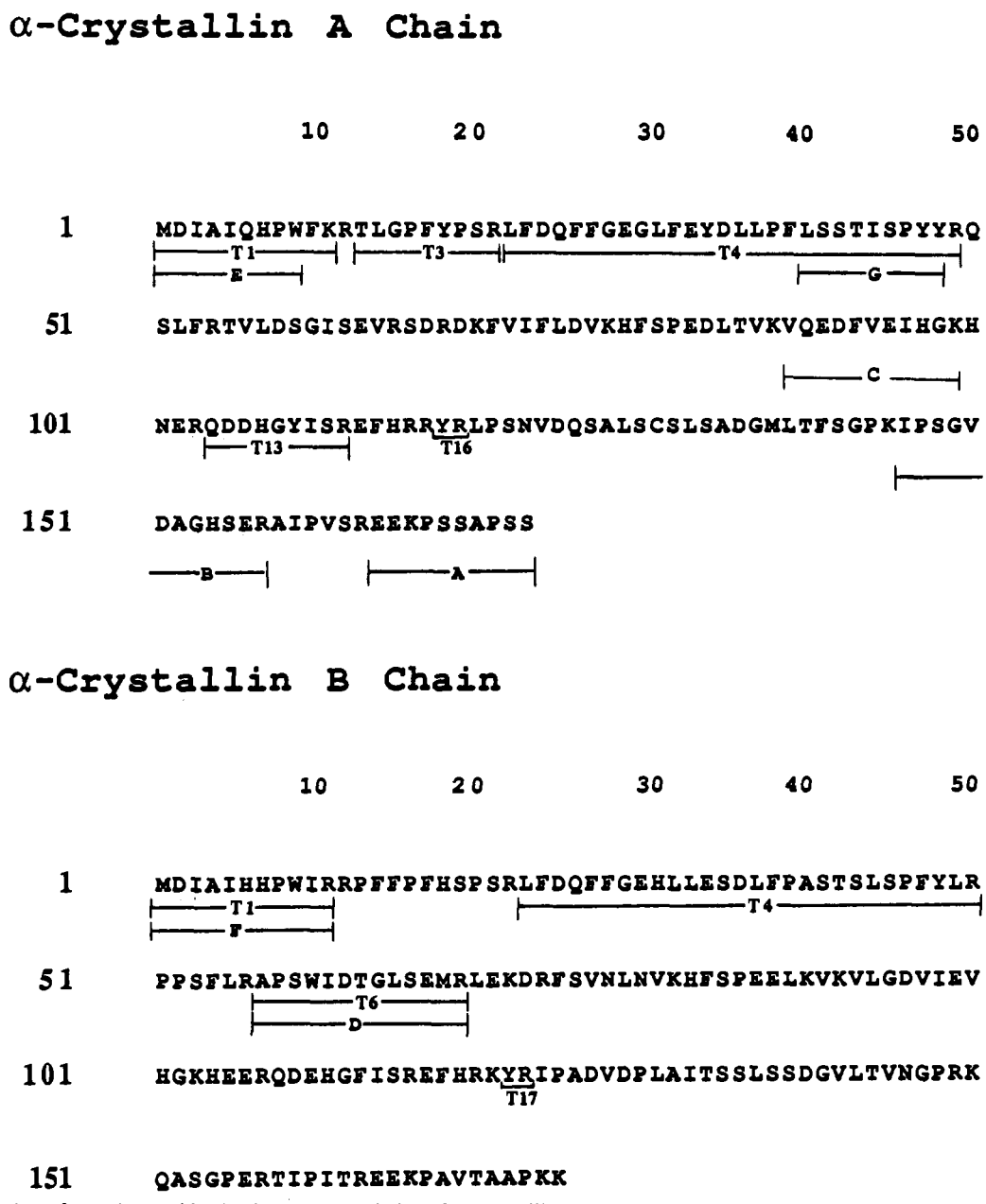

FIGURE 2: Location of tryptic peptides in the A and B chains of α-crystallin containing tryptophan (αA, T1; αB, T1 and T6) and tyrosine (αA, T3, T4, T13, and T16; αB, T4 and T17). The location of peptides A–F and G are shown for comparison.

peptide (G) were subjected to NH_2-terminal sequence analysis to confirm the sequences deduced from their amino acid compositions. The peptides P1 and P2 were also subjected to sequence analysis.

Peptides A, B, C, D, and F correspond to authentic tryptic peptides from either the A or B chain of α-crystallin; peptide E results from a chymotryptic cleavage of the NH_2 terminus of the αA chain (Figure 7). Interestingly, four of the six peptides modified are from the αA chain. The deduced sequences of peptides A, B, and C, which exhibit no absorbance at 280 nm, confirm the absence of tryptophan and tyrosine; however, the highly photoreactive residue, histidine, is present in peptides B (His-154) and C (His-97) (Figure 7). The spectrum analysis of peptides D, E, and F (Figure 3) indicates, and the deduced sequences confirm, that tryptophan is present in all three of these peptides (Figure 7). The locations of the six major peptides modified by photooxidation within the primary structure of α-crystallin are indicated in Figure 2. Peptide E is the NH_2 terminus of the αA chain resulting from a chymotryptic cleavage at Trp-9. Peptides D and F are the only peptides from the αB chain modified during irradiation, both of which contain tryptophan. Peptide F corresponds to the NH_2 terminus of the αB chain (T1), containing Trp-9, and peptide D is the tryptic peptide (T6) containing the final tryptophan residue, Trp-60.

Peaks P1 and P2 resolved from tryptic digests of α-crystallin photolyzed in the presence of UP, were collected, lyophylized, and rerun on the C_{18} column with use of a modified CH_3CN gradient. A major peptide component was purified to apparent homogeneity from each peak. The amino acid composition (Table I and II) and the sequence analysis of P1 confirm that it is apparently identical with peptide D. This indicates not only that Trp-60 is intact in P1 but that other modifications induced by singlet oxygen must be responsible for its altered elution volume (from 95 mL to 87 mL; Figure 5). This may be the conversion of Met to methionine sulfoxide. The absorption spectrum of P2 might also indicate that tryptophan is present; however, sequence analysis of P2 shows that it corresponds to a chymotryptic peptide comprised of residues 49–56 of the B chain, LRPPSFLR, which does not contain

Table I: Amino Acid Composition of α-Crystallin Peptides Purified by HPLC[a]

	A mol %	B mol %	C mol %	D mol %	E mol %	F mol %	P1 mol %	P2 mol %	G mol %
Asp	0.6	8.7 (1)	9.6 (1)	9.3 (1)	11.7 (1)	10.6 (1)	9.2 (1)	8.7 (1+)	2.5
Glu	23.1 (2)[b]	9.4 (1)	28.5 (3)	10.4 (1)	12.8	4.2	10.3 (1)	7.5 (1)	4.6
Ser	32.8 (4)	13.5 (2)	1.7	16.3 (2)	4.6	5.1	13.6 (2)	5.6 (1)	30.1 (3)
Gly	3.7	15.2 (2)	10.7 (1)	12.0 (1)	6.6	5.7	9.0 (1)	1.9	4.7
His	0.2	7.6 (1)	7.3 (1)	0.9	7.8 (1)	13.3 (2)	0.73	5.3 (1)	1.8
Arg	2.1	8.2 (1)	0.5	7.7 (1)	2.5	8.5 (1)	7.4 (1)	9.4 (2)	1.8
Thr	0.2	2.5	0.4	7.4 (1)	1.0	1.5	6.6 (1)	0.8	11.1 (1)
Ala	10.4 (1)	7.7 (1)	0.4	7.7 (1)	10.5 (1)	9.2 (1)	6.9 (1)	7.0 (1)	0.7
Pro	18.9 (2)	8.7 (1)	0.4	7.6 (1)	10.6 (1)	8.0 (1)	8.2 (1)	16.3 (3)	9.0 (1)
Tyr	0.1	0.5	0.1	0.3	0.6	0.5	0.8	0.6	8.7 (1)
Val	0.4	7.5 (1)	17.4 (2)	0.8	1.1	1.2	2.3	1.8	0.3
Met	0.1	0.1		4.4 (1)	5.9 (1)	5.3 (1)	5.0 (1)	5.1 (1)	0.1
Ile		9.0 (1)	7.3 (1)	5.5 (1)	16.5 (2)	19.2 (3)	7.9 (1)	12.8 (3)	10.4 (1)
Leu	0.3	0.7	1.0	7.9 (1)	4.1	4.2	8.5 (1)	10.9 (2)	11.1 (1)
Phe	0.1	0.2	7.7 (1)	1.1	2.6	2.3	2.3	5.2 (1)	1.8
Lys	7.0 (1)	0.7	6.4 (1)	0.6	0.8	0.6	1.1	0.7	0.3
	8.0[c]	**7.5**	**7.6**	**8.0**	**8.0**	**8.4**	**8.0**	**5.0**	**10.0**

[a] As described in the text, A–F are the peptides modified during UV irradition. G is a tyrosine peptide unmodified during UV irradiation. P1 and P2 are peptides whose appearance can be correlated with a decrease in the 214- and 280-nm absorbance of peptides D, E, and F. [b] Values in parentheses represent the number of residues per mole of peptide (rounded to nearest integer). [c] Mole percent arbitrarily designated as equivalent to 1 residue/mol.

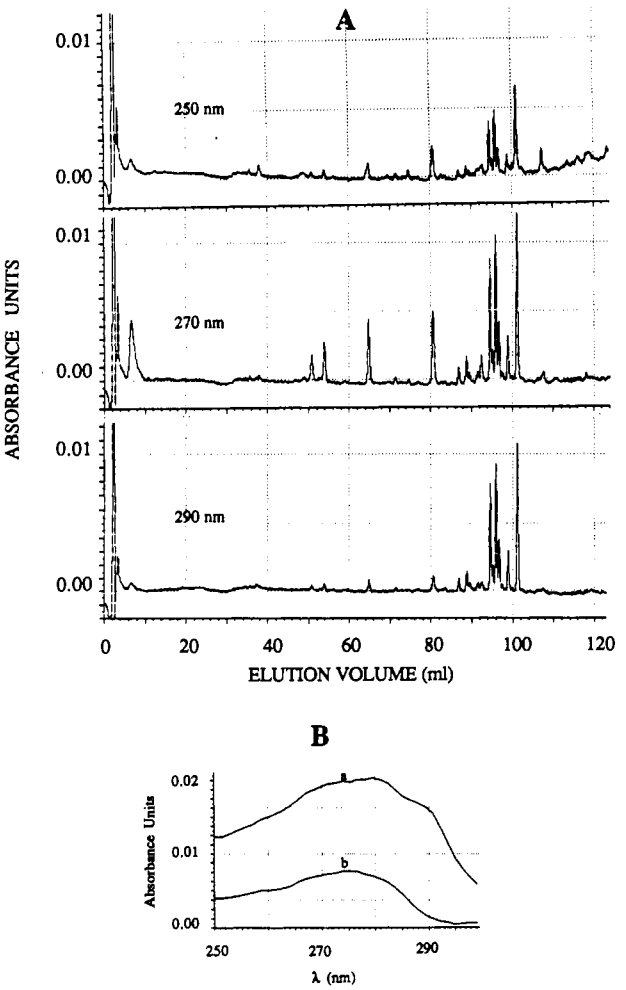

FIGURE 3: (A) HPLC chromatograms of tryptic digests of α-crystallin (+TPPS, $t = 0$) monitored at 250, 270, and 290 nm. (B) Absorption spectra of the tryptophan-containing peptide eluting at 101 mL (a) and the tyrosine containing peptide eluting at 66 mL (b).

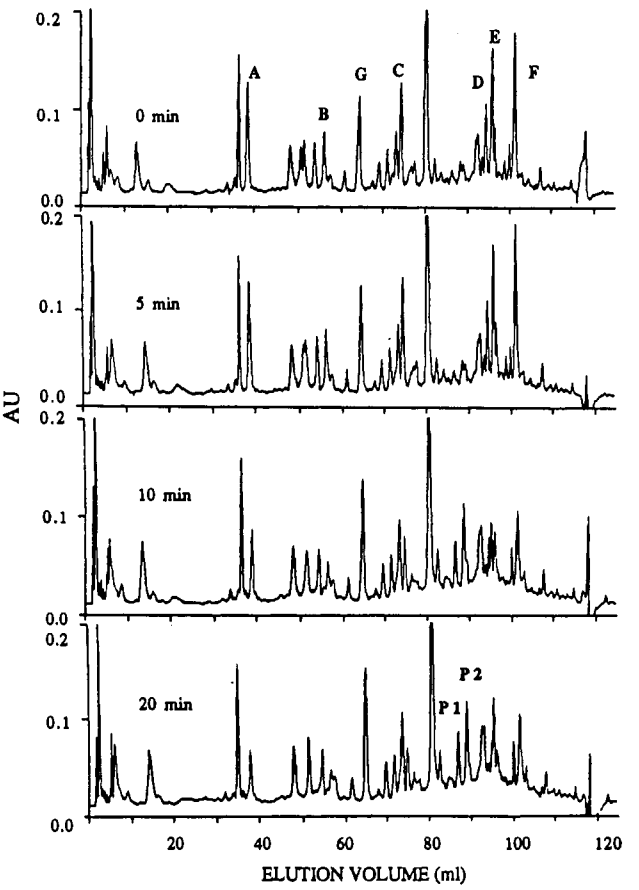

FIGURE 4: HPLC chromatograms of tryptic digests of α-crystallin exposed to UV irradiation in the presence of 1 mM UP for 0, 5, 10, and 20 min. Chromatograms were monitored at 214 nm. Peptides specifically modified by UV irradiation are labeled A–F. Peptide G is the tyrosine-containing peptide subjected to amino acid and sequence analysis. Note that the decrease in absorbance of peptides D, E, and F can be correlated with an increase in absorbance of peptides P1 and P2.

tryptophan. Furthermore, the amino acid composition of P2 (Table II) indicates that significant amounts of other amino acid residues not detected by sequence analysis are also present. These include 1 residue/mol each of Asp, Glu, His, Pro, Met, and Phe and 2 residues/mol of Ile.

Discussion

Tryptic digestion of α-crystallin results in the generation of 12 peptides that contain the photooxidizable residues, His, Met or Trp. In the present investigation we report that only 5 of the 12 tryptic peptides containing His, Met, or Trp are

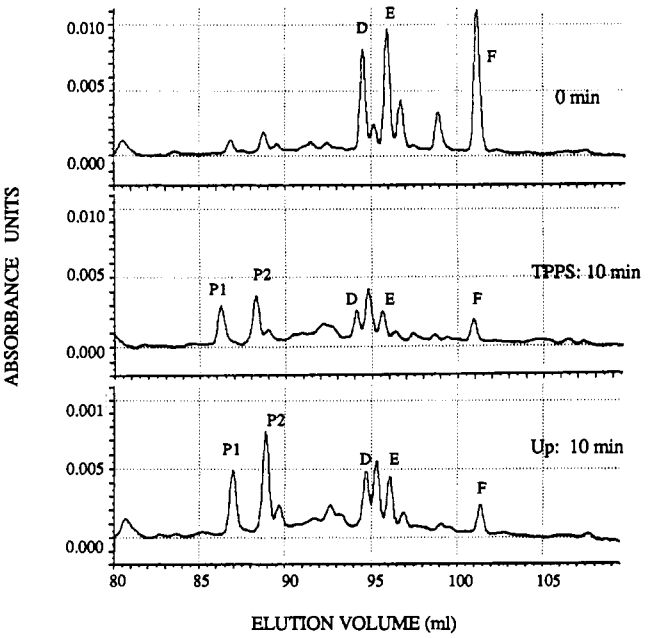

FIGURE 5: HPLC chromatograms (80–110 mL) of tryptic digests of α-crystallin subjected to UV irradiation in the presence of 1 mM TPPS or 1 mM UP for 0 and 10 min. The peptides were monitored at 280 nm. Peptides D, E, and F are the tryptophan-containing peptides of α-crystallin. The decrease in the 280-nm absorbance of peptides D, E, and F can be correlated with an increase in the 280-nm absorbance of peptides P1 and P2.

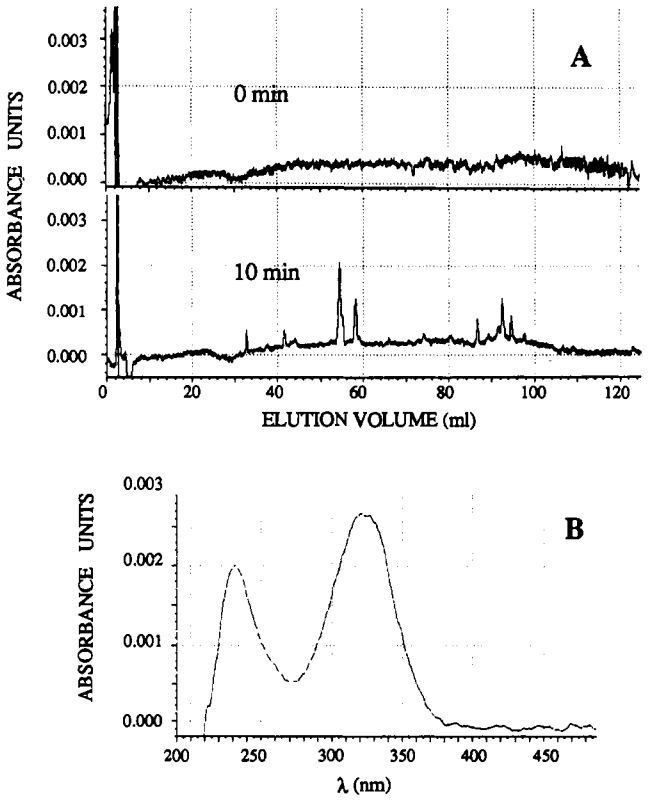

FIGURE 6: (A) HPLC chromatograms of tryptic digests of α-crystallin subjected to UV irradiation in the presence of 1 mM TPPS for 0 and 10 min. The peptides were monitored at 320 nm. (B) Absorbance spectrum of the peptide eluting at 54 mL (A). Similar results were obtained with use of samples subjected to photolysis in the presence of 1 mM UP.

apparently photooxidized. In addition, a sixth peptide, which contains none of these residues, corresponding to the carboxyl terminus of the αA chain, is also apparently photooxidized. UP or TPPS catalyzed the rapid photooxidation of all of the

Table II: Amino Acid Composition (Expressed as Residues per Mole) of Peptides P1 and P2 Deduced from Table I Compared with the Theoretical Amino Acid Composition of (I) Residues 57–69 of the αB Chain, APSWIDTGLSEMR, (II) Residues 49–56 of the αB Chain, LRPPSFLR, (III) Residues 1–8 of the αA Chain, AcMDIAQHPW, and (IV) Residues 1–8 of the αA Chain and Residues 49–56 of the αB Chain

	P1	I	P2	II	III	IV
Asp	1	1	1+		1	1
Glu	1	1	1		1	1
Ser	2	2	1	1		1
Gly	1	1				
His			1		1	1
Arg	1	1	2	2		2
Thr	1	1				
Ala	1	1	1		1	1
Pro	1	1	3	2	1	3
Tyr						
Val						
Met	1	1	1		1	1
Ile	1	1	3		2	3
Leu	1	1	2	2		2
Phe			1	1		1
Lys						

tryptophan-containing peptides in α-crystallin (Trp-9 of the A chain; Trp-9 and Trp-60 of the B chain). The modification of the tryptophan residues is indicated by reduced 214- and 280-nm absorbance of peptides D, E, and F (Figures 4 and 5). The rate of photolysis of the tryptophan-containing peptides is significantly greater in the presence of TPPS than in the presence of UP (Figure 4), probably because TPPS binds to α-crystallin, resulting in the localized production of singlet oxygen (Roberts et al., 1990; Dubbelman et al., 1978; Girotti, 1979; Verweij & van Steveninck, 1982). Besides the tryptophan-containing peptides, only three other peptides (A–C), all from the A chain of α-crystallin, were affected by singlet oxygen (Figure 4). The presence of histidine in peptides B and C (Figure 7) may explain their susceptibility to photooxidation; however, at least 10 other tryptic peptides containing histidine (four in the A chain and six in the B chain; van der Ouderaa et al., 1973, 1974) were not affected. The observation that only two specific histidine-containing peptides in the entire α-crystallin molecule become modified during photolysis suggests that these histidine residues are on the surface (hydrophilic region) of the protein. The lack of photooxidation of other peptides containing His or Met may be due to the ionization state of the individual His or to the fact that they are buried in the hydrophobic region of the protein. A concern is that photooxidation may lead to denaturation exposing "buried" residues to photooxidation. This is not supported by the limited number of residues that reacted.

The rapid loss of 214-nm absorbance of peptide A suggests the photooxidation of a susceptible residue. However, this peptide corresponding to the carboxyl terminal of the α-chain does not contain His, Met, or Trp. The loss of the peptide A may be due to altered digestion of the photooxidized material; however, this would result in the symmetrical loss of two peptides from the chromatogram and not one as is observed. A more intriguing explanation is that the undigestible Lys-164 in peptide A (Figure 7) has reacted with a photooxidized residue resulting in an inter- or intramolecular cross-link. This is consistent with model experiments (Verweij & van Steveninck, 1982) where it was found that the most probable cross-link is formed in a dark reaction between Lys and photooxidized His.

Despite the apparently limited number of regions in the α-crystallin molecule affected by singlet oxygen, characterization of the modifications themselves is complicated by the

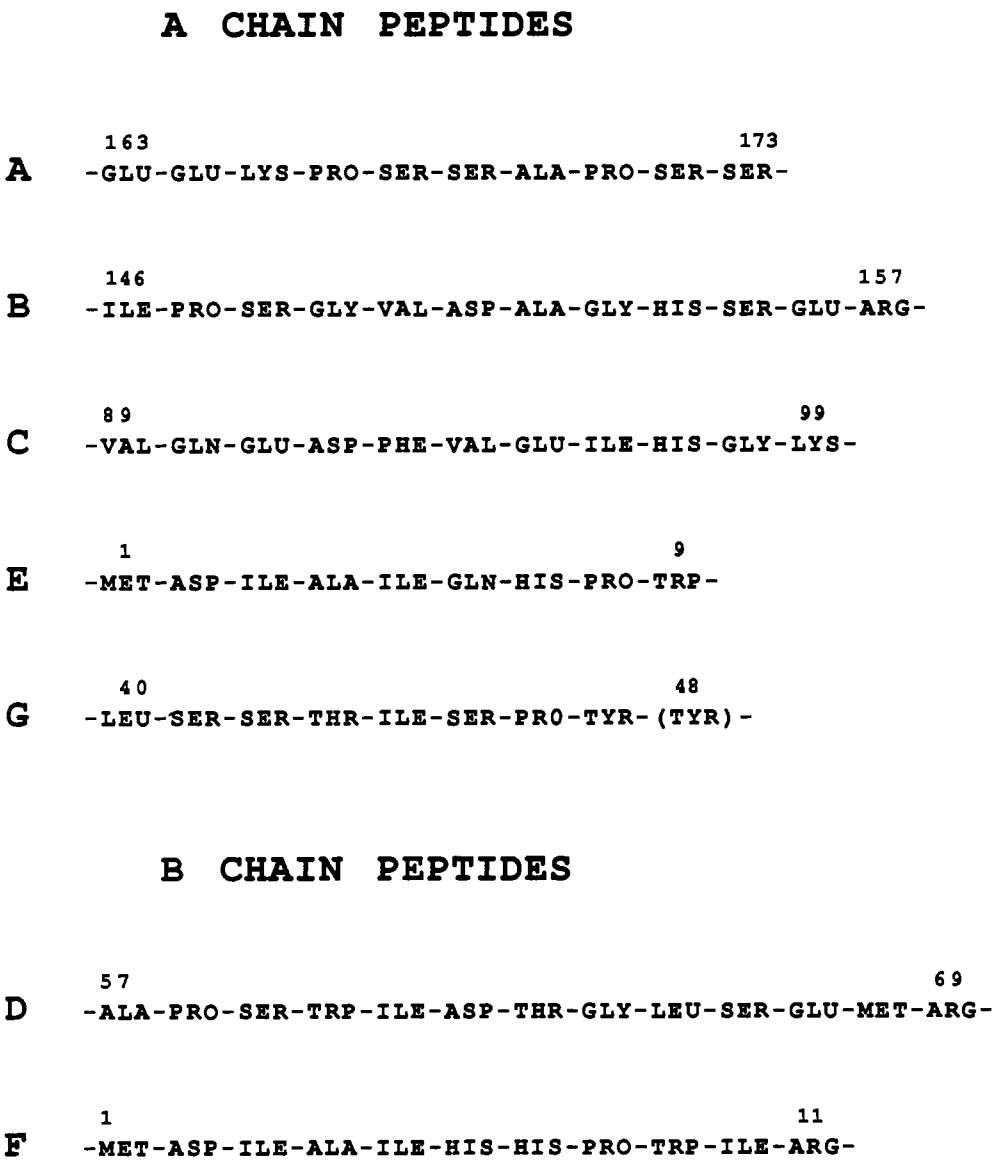

FIGURE 7: Sequences of α-crystallin peptides deduced from the amino acid compositions of purified peptides isolated by HPLC. Peptides A–F are the major peptides modified by UV irradiation in the presence of either UP or TPPS. Peptide G is a tyrosine peptide analyzed as a control. The amino acid sequences of peptides D and G were also determined by automated Edman degradation as described in the text.

number of different possible product peptides generated when more than one photooxidizable residue is present in a peptide. It can be predicted that a number of peptides, each containing different modifications, will be generated by the T-1 peptides in α-crystallin since they contain His, Met, and Trp. This possibly explains the complex mixture of product peptides that contain NFK. However, the modification of the tryptophan residues, indicated by reduced 214- and 280-nm absorbance of peptides D, E, and F, can also be correlated with a concomitant increase in the appearance of peptides P1 and P2, both of which also contain intact tryptophan (Figure 5). The maximum amount of material detected at both 214 and 280 nm in P1 and P2 is significantly greater (2–3 times) in the samples photolyzed in the presence of UP than in those samples photolyzed with TPPS. This indicates that a greater proportion of the indole ring of the tryptophan residues remains intact during irradiation in the presence of UP than in the presence of TPPS.

The amino acid composition and sequence analysis of both P1 (elution volume 87 mL) isolated from samples containing UP and peptide D (elution volume 95 mL) match the tryptic peptide, T6 (Figure 2) of the B chain. Therefore, although Trp-9 in this peptide is apparently unaltered by photooxidation, the significant change in the elution volume (8 mL) of P1 strongly suggests that another residue or residues are modified and that these modifications are responsible for the altered behavior of P1 on the C_{18} RP column (Figure 5). The photooxidation of Met-68 to methionine sulfoxide (Met-SO) would be expected to make T6 more hydrophilic and may contribute to the altered elution volume of P1.

Sequence analysis of P2 showed the presence of a single peptide with an unblocked NH_2 terminus corresponding to a chymotryptic peptide comprised of residues 49–59 of the B chain, LRPPSFLR, which does not contain tryptophan. However, the absorption spectrum of P2 indicates that tryptophan is present in the purified material, and its amino acid composition further indicates that, besides tryptophan, significant amounts of other residues not detected by Edman degradation are also present (Table II). These data strongly suggest that another peptide component, presumably with a blocked NH_2 terminus, is present in P2. Inspection of the amino acid composition (Table II) of P2 leads to the conclusion that, in addition to the sequenced peptide, 1 residue/mol each of Asp, Glu, His, Pro, Met, and Phe and 2 residues/mol of Ile are present. Since tryptophan is also present, this amino acid composition closely matches the NH_2-terminal chymo-

tryptic peptide of the A chain: AcMDIAIQHPW (Table II, column III; Figure 8), i.e., peptide E. Since P2 behaves on the HPLC column as an apparently homogeneous peptide, it seems reasonable to speculate that it may contain a cross-linked component, residues 49–56 of the B chain, LRPPSFLR, and residues 1–9 of the A chain, AcMDIAIQHPW. Further experiments are currently underway to further characterize P2 with the use of mass spectroscopy.

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Registry No. NFK, 1022-31-7; UP, 26316-36-9; TPPS, 35218-75-8; peptide A, 135105-91-8; peptide B, 135105-92-9; peptide C, 135105-93-0; peptide D, 135105-94-1; peptide E, 135105-95-2; peptide F, 135105-96-3; oxygen, 7782-44-7.

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