Hydroxyl Radical Mediated Damage to Proteins, with Special Reference to the Crystallins

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ABSTRACT: Oxidative modification of the eye lens proteins, the crystallins, is known to cause protein cross-links and aggregates which lead to lens opacification or cataracts. We focus attention here on oxidative damage occurring in crystallins and some "control" proteins upon reaction with the hydroxyl radical (•OH) which, in the lens, is generated by photosensitization or by the Fenton reaction. In the present study, we have synthesized and used the bishydroperoxide I as a "photo-Fenton" reagent, in order to photolytically generate pure •OH, free of other oxyradicals. Our findings are the following: (i) Trp residues are oxidized by •OH to *N*-formylkynurenine and related compounds, but this in itself does not lead to covalent aggregation of the protein. (ii) Tyr residues react with •OH, but apparently do not produce dihydroxyphenylalanine or bityrosine. Nor do protein cross-links occur as a result. (iii) Oxidation of His residues appears to be obligatory for protein cross-linking. Histidine-free proteins do not form high molecular weight products upon reaction with •OH. Protection of His residues by adduct formation in other proteins inhibits cross-linking. (iv) Lys residues seem to participate in the cross-linking reaction. Protection of the Lys residues by maleylation of the protein inhibits cross-linking. (v) The oxidized protein is more acidic in nature than the parent, and it might have altered conformational features.

The eye lens is a tissue made up largely (35% by weight) of three closely related proteins called the crystallins, with the remainder being essentially water. Metabolism occurs mostly in the thin outer layer epithelial cells, which differentiate to produce the lens fiber cells that constitute the bulk of the lens. The fiber cells themselves are enucleated and free of organelles. This makes the eye lens very sluggish in metabolic and turnover activities. The crystallins that constitute the lens are thus very long lived, with half-lives measured in decades. Since the generalized strategy of increased turnover and replacement of damaged proteins (Stadtman, 1986; Wolff et al., 1986; Davies, 1987) is not available to the lens, changes in the crystallins arising from environmental and metabolic assult, even at low levels, tend to accumulate with time and can adversely affect the transparency of the lens. Development of color and protein covalent cross-linking are two events that occur in the lens with age, as well as during cataractogenesis (Bloemendal, 1981; Duncan, 1981).

Oxidative modification is one significant factor in crystallin cross-linking that leads to high molecular weight aggregates, loss of solubility, and lens opacity (van Haard et al., 1978; Garland et al., 1988). The lens has a number of endogenous defense mechanisms against oxidative damage, involving ascorbate, glutathione, and the antioxidant enzymes (Srivastava, 1980). When the levels or efficiencies of these antioxidants are compromised, or when the oxidant stress becomes abnormal, oxidative damage to the lens constituents can occur, leading to lens dysfunction. Active oxygen species, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the superoxide ($O_2^{\cdot-}$) and hydroxyl radicals (•OH), can be generated in the eye through photochemical means (Zigman, 1981; Zigler & Goosey, 1981; Pitts et al., 1986) or "in the dark", e.g., through Fenton-type redox reactions (Zigler et al., 1985; Garland et al., 1986) mediated by metal ions like Fe^{2+} which might accumulate over a period of time in a slow-turnover tissue such as the lens.

Of the various active oxygen species, singlet oxygen usually arises via the type II photosensitization pathway (Foote, 1969) and needs molecular oxygen, whose concentration in the lens is significantly low. Singlet oxygen-mediated reactions would thus be somewhat less important in the lens, except in hyperbaric conditions. The other oxyradicals can be generated both by photochemical means and in the dark. The hydroxyl radical and the superoxide anion are of particular relevance with respect to both their availability and reactivity in cells and tissues. Since oxidation of the various crystallins is one factor in the cataract process, it becomes important to evaluate, compare, and contrast the reaction of each of these active oxygen species with proteins. We have earlier studied the reaction of pure $^1O_2$ with proteins (Balasubramanian et al., 1990), while others (McNamara & Augusteyn, 1984; Huang & Hu, 1990; Thomas et al., 1990; Reddan et al., 1990; Patterson & Walsh, 1990) have investigated the oxidation of crystallins by H_2O_2. We present here some results of our investigations on the oxidative modifications caused by hydroxyl radicals (•OH), using a compound devised by us earlier (Saito et al., 1989, 1990; Matsugo et al., 1991) for the generation of pure •OH, free of any other oxyradicals. This compound (see Figure 1 for its structure, denoted as I) is termed a photo-Fenton reagent, since it generates •OH upon photolysis at 366 nm. We have used this reagent to induce oxidative damage in DNA (Saito et al., 1990) and in microsomes (Konishi et al., 1991) earlier. We describe here the use of this photo-Fenton reagent with proteins, which has enabled us to evaluate the nature and extent of damage occurring in proteins in general, and in the lens crystallins in particular, upon reaction with hydroxyl radicals. This is of relevance to the process of lens opacification, which occurs due to the effects

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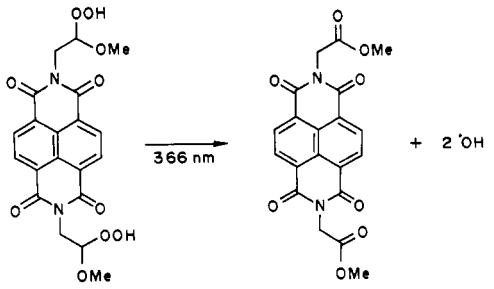

(I) (II)

FIGURE 1: The chemical structure of the hydroperoxide (compound I) and its photolytic product the methyl ester compound II.

of oxidative modification of the constituent proteins.

Experimental Procedures

The Photo-Fenton Reagent. We have synthesized a class of aromatic hydroperoxides which, upon irradiation at appropriate wavelengths, decompose and generate pure •OH in high yields, through a process of γ-hydrogen abstraction that leads to the cleavage of the O–O bond (Saito et al., 1989, 1990). Bis(hydroperoxy)naphthaldiimide (termed here compound I), which has been designed to act as a photo-Fenton reagent, was prepared as follows. To a solution of *N,N'*-bis-(2,2-dimethoxyethyl)-1,4,5,8-naphthaldiimide (1.11 g, 2.5 mmol), which was prepared from commercially available naphthalene-1,4,5,8-tetracarboxylic anhydride and 2,2-dimethoxyethylamine, in dry methylene chloride (50 mL) was added excess ethereal hydrogen peroxide obtained by extracting 30% aqueous hydrogen peroxide with ether (Saito et al., 1989). To the solution was added triflic acid (0.24 mL, 2.5 mmol) at 0 °C by a syringe. The solution was stirred at 0 °C for 1 h. After the solution was poured into ice water, the methylene chloride layer was separated and washed with cold water. Evaporation of the extracts at 0 °C under reduced pressure gave a yellow solid which was washed twice with cold ether to give compound I (745 mg, 67%): mp 142–143 °C (decomp); 200-MHz ^{1}H NMR ($CDCl_3$) δ 3.59 (s, 6 H), 4.40 (d, d, 2 H, J = 2.8, 14.0 Hz), 4.67 (d, d, 2 H, J = 8.1, 14.0 Hz), 4.91 (d, d, 2 H, J = 2.8, 8.2 Hz), 8.85 (s, 4 H), 9.56 (s, 2 H, OOH); ^{13}C NMR ($CDCl_3$) δ 40.73, 56.22, 104.05, 126.95, 127.28, 132.30, 164.27; MS *m/e* (rel intensity) 446 (M^+, 0.18), 410 (M^+-36, 8.6), 378 (M^+-68, 6.4), 351 (M^+-95, 29); IR 1715, 1670, 1360, 1245 cm^{-1}; UV max (acetonitrile) 377 nm (log ε 4.44), 357 (4.38), 340 (4.20).

Bishydroperoxide I was completely insoluble in aqueous solution but slightly soluble in aqueous organic solvents, e.g., up to 3 mM in acetonitrile:water (8:92). No decomposition was observed in an acetonitrile solution without irradiation at ambient temperature for at least 24 h. Photolysis of compound I in acetonitrile at 366-nm light from a transilluminator resulted in its rapid decomposition to give the methyl ester compound II quantitatively with a quantum yield of 0.18 with the generation of hydroxyl radicals. The generation of hydroxyl radicals from compound I was confirmed by the chemical trapping reactions and ESR spin trapping technique using 5,5-dimethylpyrroline *N*-oxide, as described previously (Saito et al., 1990).

The following procedure has been found to be the most desirable for efficient generation of •OH, in a manner that would allow it to react with an aqueous protein solution. The procedure also allows us to remove unreacted compound I and the product compound II from the reaction mixture. A total of 4 g of Kieselgel-60 beads (E Merck, Darmstadt, Germany), of particle size 0.040–0.063 mm (230–400 mesh), was added to a solution of 4 mg of compound I in 40 mL of DMSO:EtOH (3:5 volume ratio). The suspension was subjected to rotary evaporation under reduced pressure at 55 °C and then lyophilized overnight. The dry beads, coated uniformly with compound I which was adsorbed on the surface, were found to be excellent for the photolytic generation of hydroxyl radicals. In control experiments, no dissociation of compound I from the silica gel beads was observed in a suspension in water, as determined by UV spectroscopy of the aqueous phase.

Proteins. α-, β-, and γ-crystallins were purified and isolated from bovine eye lens by gel filtration on Sephadex G-200 in a pH 7.6 buffer containing 50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA,[1] and 3 mM NaN_3. δ-Crystallin was purified and isolated from 3-day-old chick lenses by Sephacryl S-200 chromatography in a pH 9.1 buffer containing 50 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, and 3 mM NaN_3. The isolated, purified crystallins were later dialyzed, lyophilized, and stored in a freezer prior to use. Ribonuclease A (RNase A) was obtained from Boehringer Mannheim, Mannheim. Bovine pancreatic trypsin inhibitor (BPTI), melittin, superoxide dismutase (SOD), and catalase were obtained from Sigma Chemical Co., St. Louis, MO.

Maleylation of δ-crystallin was done by modification of the procedure of Butler et al. (1969). A total of 4 mg of δ-crystallin was dissolved in 2 mL of carbonate buffer (0.1 M, pH 9), to which 100 μL of 1 M maleic anhydride in dioxane was added in five additions at 3-min intervals. The reaction was conducted at 2 °C, with the pH maintained at 9 by the addition of NaOH as necessary. The product was run down a Sephadex G-25 desalting minicolumn, and the void volume contained the maleylated protein. Maleylation was checked by absorption at 250 nm, as well as by the product's inability to be stained by alkaline ammoniacal silver nitrate on polyacrylamide gels, while being stained as usual by the dye Coomassie blue R 350. Silver staining in alkaline medium occurs largely by the reaction of silver with free amino groups in the protein (Wray et al., 1981).

Protection of the histidyl groups of the protein was done using the reagent diethyl pyrocarbonate (DEPC), using the method outlined by Miles (1977). To 200 μL of 10 mM DEPC in ethanol was added 1 mL of a 1.5 mg/mL solution of δ-crystallin in water. After 10 min, the reaction mixture was run through a Sephadex G-25 desalting minicolumn preequilibrated with 10 mM Tris buffer, pH 7, and the DEPC-protected protein collected in the void volume.

Reaction of Proteins with •OH. In a typical reaction, 100 mg of Kieselgel beads coated with compound I was added to 1 mL of the protein solution (of concentration ca. 1 mg/mL) in a quartz cuvette of 1 cm path length. The cuvette was placed in the cell compartment of a Hitachi F-4000 spectrofluorometer, and the solution was constantly stirred so as to keep the Kieselgel beads from sedimenting, and in the light path. The excitation monochromator was set at 366 nm, the entrance slit was opened to a bandwidth of 20 nm, and the solution was irradiated using the 150-W Xe arc lamp source of the instrument. The light intensity falling on the sample is estimated to have been about 0.5 mW/cm^2 (10^{14}–10^{15} photons incident/s).

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DEPC, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; IEF, isoelectric focusing; NFK, *N*-formylkynurenine; PAGE, polyacrylamide gel electrophoresis; RNase A, ribonuclease A; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; Tris, tris(hydroxymethyl)aminomethane.

Distilled deionized water was used to make the protein solutions used in the above reactions since buffers tend to affect •OH-mediated reactions (Davies, 1987). All reactions for a given protein were performed on solutions of identical protein concentration with relevant exogenous components. In every case, a portion of the solution which was not reacted as above served as the control. It may be noted that the range of concentrations of crystallin solutions is the same as that used in earlier studies of oxidative damage to crystallins (Zigler & Goosey, 1981; Garland et al., 1986, 1988; Balasubramanian et al., 1990). Intermolecular cross-linking was monitored by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS). The samples for these were boiled in the presence of SDS and β-mercaptoethanol.

The time course of the reaction of proteins with •OH under these conditions was conveniently monitored by following the time-dependent loss of the fluorescence intensity of the protein in the 300–350-nm region (due to the Tyr and Trp residues). For δ-crystallin, the drop in fluorescence intensity was found to be exponential-like, tailing down to a value of about 60% of the initial intensity in 90 min, under the experimental conditions described above. Electrophoretic assay of the protein at this time also revealed the presence of cross-linked high molecular weight covalent aggregates. In this connection, it is known with crystallins that the Trp, Tyr, His, and Met contents decrease, and nondisulfide cross-links increase with age and in cataracts (Bloemendal, 1981). Keeping this in view, we decided to use a standard period of 90 min for all the reactions described here, since longer reaction times could damage the less reactive groups as well.

After irradiation, the solution was centrifuged and the beads were discarded. Since both compound I and its photolytic product compound II after •OH generation remained adsorbed on the Kieselgel beads, the supernatant had only the protein solution. This point was independently confirmed spectroscopically, and it provided the basis for the immobilization of compound I and the choice of Kieselgel as the adsorbent. It was thus confirmed that neither compound I nor its photoproduct compound II is involved in any covalent or noncovalent association with the proteins.

Fluorescence Spectra. A Hitachi F-4000 steady-state spectrofluorometer was routinely used, with excitation and emission band-passes of 3–5 nm and cuvettes of path lengths 1 cm and 0.3 cm. Tyrosine emission was monitored near 305 nm, and tryptophan emission was monitored near 335–345 nm (excitation around 280 nm in both cases), while other emissions (e.g., due to bityrosine or *N*-formylkynurenine) were monitored in the 400–440-nm region (excitation around 320 nm).

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was done using a Pharmacia Phastsystem assembly, with precast gels of acrylamide percentage appropriate to the molecular weights of the protein studied. Isoelectric focusing (IEF) electrophoresis runs were made on δ-crystallin, in the pH range 3–9 on the same system.

DEPC modification (and maleylation) of the protein had the effect of weakening the intensity of the protein stain in the gel, in comparison to that of the control unmodified protein band, even when equal amounts of the protein solutions were loaded (e.g., lanes 1, 3, 9, and 10 in Figure 4). Hence, quantitation of the bands in the gels was done using a molecular dynamics computing densitometer. It was ensured that the intensities of the bands in the control lanes and the sum of those of the bands in the test lanes were about the same, so as to enable appropriate comparisons.

Amino Acid Analysis. Fresh and oxidized samples of δ-crystallin were hydrolyzed with 6 N HCl for 24 h (110 °C) prior to analysis. The amino acid analysis was done using a LKB 4151 Alpha Plus analyzer, calibrated with a standard mixture of amino acids, and in duplicate runs. The detection limit was about 0.2 nM for each amino acid.

Results and Discussion

Though all amino acid residues in a protein chain are susceptible to modification by the hydroxyl radical, the rate constants of the reaction suggest that tryptophan (Trp), histidine (His), cysteine (Cys), and methionine (Met) are the most vulnerable, while tyrosine (Tyr) residues are also thought to be sensitive, and perhaps phenylalanine (Phe) might also add to •OH to produce tyrosine (Dorfman & Adams, 1973). In the case of crystallins, Garland et al. (1986) have seen the Trp, His, Met, and Cys residues to be readily modified in the Fenton oxidation reaction. Davies et al. (1987) have reacted bovine serum albumin (BSA) with •OH and compared the amino acid composition before and after the reaction and found that in addition to the above-mentioned residues, Asx [aspartate (Asp) and asparagine (Asn)], Glx [glutamate (Gln) plus glutamine (Gln)], and lysine (Lys) were also modified. As a result of such a reaction with •OH, significant aggregation of proteins to higher molecular weight forms (dimers, trimers, and even tetramers) was seen (Davies, 1987), of which over 90% arose due to nondisulfide covalent cross-links and less than 10% was assignable to noncovalent interactions or to disulfide bonds. No peptide bond degradation, leading to lower molecular weight forms, was seen upon reaction with •OH alone, though it did occur when the proteins were exposed to a mixture of hydroxyl and superoxide anion radicals, i.e. •OH + $O_2^{\bullet-}$. A more recent paper (Vissers & Winterbourn, 1991) reports that pure •OH oxidizes the Trp residues in fibronectin, but not its Cys residues, and also that it promotes only cross-linking and not degradation of the peptide chain. Thus, the question of particular interest is what amino acid residue needs to be modified in order to produce eventual covalent (nondisulfide) crosslinking?

Choice of Test Proteins. In an effort to delineate which amino acid residues play the primary role in the protein cross-linking, we have studied the reaction of •OH with melittin, BPTI, RNase A, and the crystallins for the same duration under identical conditions and monitored the extent of protein cross-linking by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol (so as to detect only nondisulfide covalent cross-links). Melittin was chosen as a control protein that contains no Cys, Tyr, His, or Met in its sequence, but it does have one Trp, one Ser, two Gln, and three Lys residues. Reaction of •OH with melittin and monitoring of the formation of high molecular weight protein cross-links would thus be informative. Likewise, BPTI has in its sequence the vulnerable residues Cys, Met, Phe, and Tyr but no Trp or His, while RNase A, which like BPTI has Tyr but no Trp, serves as a "histidine control" among these three proteins, since it has four His residues. The four crystallins α-, β-, γ-, and δ-crystallin were used in order to demonstrate that reaction with •OH leads to covalent cross-linking of crystallins. All of them were oxidized in a similar fashion, but to varying extents, as shown later. α- and γ-crystallin solutions tended to become hazy and turbid after the reaction, while β- and δ-crystallins stayed in solution. We exemplify the results obtained with δ-crystallin here.

Modification of Trp Residues. As mentioned above, since Trp reacts rapidly with •OH, we have used the changes in the

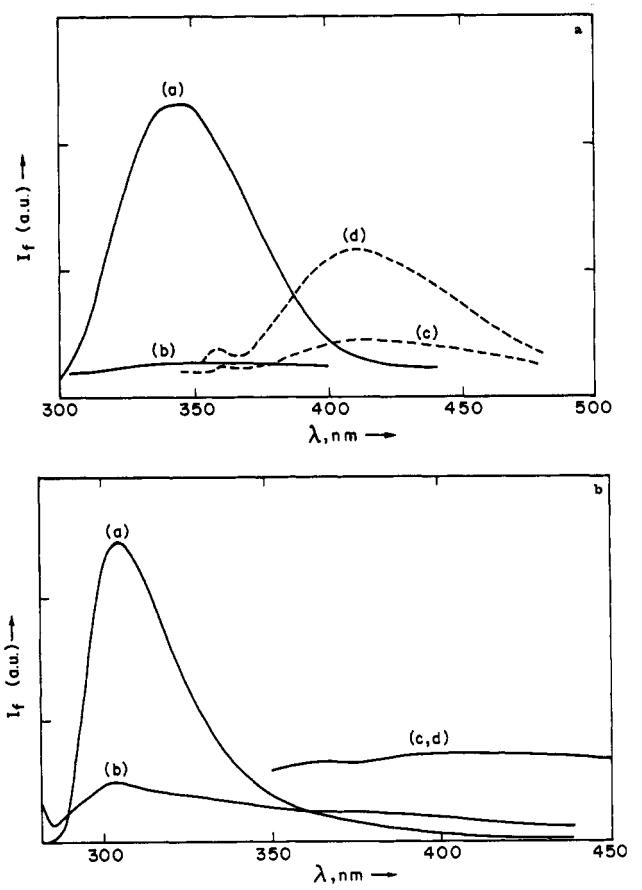

FIGURE 2: Panel a: The fluorescence spectrum of melittin before (curves a and c) and after reaction for 90 min with •OH generated by compound I (curves b and d). Curves a and b were obtained upon excitation at 290 nm (Trp), while curves c and d were obtained upon excitation with 320-nm light. The excitation and emission band-passes were 5 nm each, and the sensitivities for curves c and d were 5-fold higher than for a and b. Quartz microcells of path length 5 mm and volume 0.2 mL were used. Panel b: The fluorescence spectra of RNase A before (curves a and c) and after reaction with •OH for 90 min (curves b and d). The excitation wavelength for curves a and b was 275 nm, while that for curves c and d was 325 nm. No other emission bands were seen, even with a variety of other excitation wavelengths.

fluorescence spectrum of the Trp residue as a convenient assay for its modification. Figure 2 shows that the Trp residue in melittin is completely lost after reaction with •OH generated by compound I. That the loss is indeed due to reaction of the protein with hydroxyl radicals was confirmed in several ways: (i) irradiation of the protein alone, in the absence of compound I, with 366-nm light does not produce any changes in the emission spectrum; (ii) addition of the •OH scavenger mannitol (1 mM) to the solution completely inhibited the loss of the Trp fluorescence; and (iii) the presence of superoxide dismutase and catalase in the reaction mixture during irradiation with 366-nm light did not inhibit the reaction. Figure 2 shows that concomitant with the Trp emission loss, a new emission band at 435 nm (excitation 320 nm) is generated. On the basis of its emission position and intensity profile, as well as on the basis of comparison with the spectrum of an authentic sample, this band is assignable to the *N*-formylkynurenine (NFK) residue and other closely related products of the oxidation of Trp by singlet oxygen (Balasubramanian et al., 1990) and by photochemical means (van Heyningen, 1973; Walrant & Santus, 1974; Zingman, 1981). It is worth reiterating here that melittin has just a lone Trp residue and that it has no Tyr or Phe in its sequence.

Modification of Tyr Residues. The reaction of the two other control proteins with •OH is interesting in this connection. Both BPTI and RNase A have no Trp in them, but they have Phe and Tyr. Figure 2 shows that upon reaction with •OH the emission band of RNase A near 305 nm, largely due to Tyr, was lost, but no new fluorescence band was detected elsewhere. Identical results were obtained with BPTI as well. That the Tyr band is essentially lost after exposure to •OH suggests that the hydroxylation of Phe residues by •OH in a manner that would generate Tyr does not occur to any significant extent; alternately, it is also possible that the Tyr produced by the hydroxylation of the Phe residues was further oxidized by the steady-state concentration of the hydroxyl radicals produced in this experiment. The absence of any emission spectrum in the 410-nm region (or an excitation band near 320 nm) suggests that no detectable amount of bityrosine is generated either.

Davies (1987) had used the presence of an emission band in the 410-nm region (excitation 325 nm) as the sole method for the detection of bityrosine, in the reaction of •OH with 12 different proteins. Vissers and Winterbourn (1991) have also used the appearance of a fluorescence band around 400 nm as indicative of the formation of bityrosine. However, a note of caution is in order here, with respect to such interpretation.

It is to be noted that all the proteins studied by the above authors contain Trp residues, which would also yield emission (and excitation) bands in the same region upon oxidation (to NFK and related species) by the hydroxyl radical, as noted above. Thus the presence of a fluorescence band near 410 nm, per se, need not indicate the formation of bityrosine, unless it occurs when a tryptophan-free protein is oxidized and unless additional independent evidence for its formation is obtained using methods such as amino acid analysis or chromatography. Verweij et al. (1982) used all the three assays to detect bityrosine cross-links in a few proteins that were oxidized using ozone. Out of a handful of reports on bityrosine formation in Trp-free proteins, the ones on calmodulin, troponin C, and parvalbumin (Malencik & Anderson, 1987; Anderson & Malencik, 1989) are notable since they underscore the importance of the proximity of the two Tyr residues in order to form the bityrosine link; it is formed only in the calcium-bound form of calmodulin where the two Tyr are brought together, but not in the calcium-free form, in thrombin digests of calmodulin, or in troponin C or parvalbumin, where they are distally placed. It is of course formed in poly(L-tyrosine) (Lehrer & Fasman, 1967). It would thus appear that neither in BPTI nor in RNase A do two Tyr residues come close together, intramolecularly or intermolecularly under the present experimental conditions, to be able to form bityrosine upon •OH reaction. It would also appear that the extensive cross-linking seen in fibronectin upon reaction with •OH (Vissers & Winterbourn, 1991) need not necessarily arise through bityrosine links.

It is further worth noting that the Folin–Lowry reagent used for protein estimation (Lowry et al., 1951) did not develop color with RNase A that was modified upon reaction with hydroxyl radical, though it did with the unmodified protein. This method involves reaction of the reagent with Tyr and Trp in the protein; bityrosine would be expected to react and generate color.

Though Tyr residues are modified by •OH, as evidenced by fluorescence loss at 305 nm, no new absorption or emission spectral band was detected concomitantly. Thus, while the structure of the modified product needs to be elucidated, it does not appear to be dihydroxyphenylalanine (dopa), bityrosine, or dopachrome (pink color, with absorption bands at 305 and 415 nm). This behavior of BPTI and RNase A

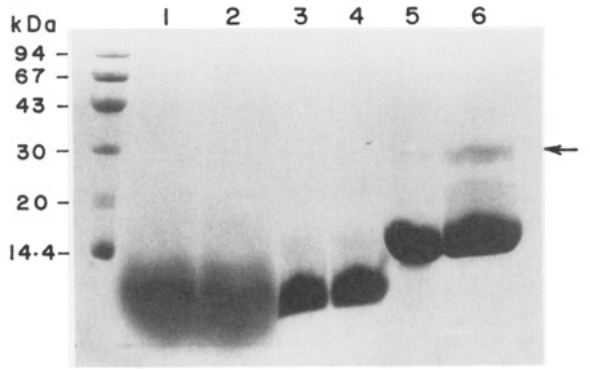

FIGURE 3: Effects of •OH reaction as seen by polyacrylamide gel electrophoresis (PAGE). Samples were reacted with •OH radicals for 90 min and loaded on a 20% gel after briefly being boiled with sodium dodecyl sulfate (SDS) in the presence of β-mercaptoethanol. Lanes 1 and 2: melittin control and reacted, respectively. Lanes 3 and 4: BPTI control and reacted. Lanes 5 and 6: RNase A control and reacted. Staining of the bands was done using Coomassie blue.

is similar to that of troponin C in that the 305-nm band is lost upon oxidative modification but that no new emission band appears (Anderson & Malencik, 1989). Joshi et al. (1987) have found that photosensitized oxidation of L-tyrosine (and L-dopa) produced dopachrome and subsequently melanin-type pigments and that the active oxygen species involved in such oxidations are $^1O_2$ and $O_2^{\bullet-}$ and not •OH, since •OH quenchers did not inhibit the reaction. It is also worth noting here that Stadtman and Berlett (1991) have recently pointed out that the oxidation pathway of aromatic amino acids such as Tyr depends on the mode of oxidation; radiolysis appears to attack the ring and generate hydroxyphenylalanines, while Fenton oxidation does not.

His Residue Oxidation Leads to Protein Cross-Linking. The SDS–PAGE patterns shown in Figure 3 reveal that while the reaction of •OH with melittin and with BPTI leads to Trp or Tyr modification, it does not lead to the production of covalent high molecular weight aggregates involving nondisulfide cross-linking. In other words, the Trp, Ser, Gln, and Lys residues of melittin do not seem to be involved in covalent oxidative cross-linking; neither are the Met, Phe, and Tyr of BPTI. RNase A, on the other hand, contains the additionally vulnerable residue His, which is absent in melittin and BPTI and, as Figure 3 shows, does produce nondisulfide cross-links or high molecular weight products. These results are remarkably similar to those of our earlier studies on the oxidation of these proteins with singlet oxygen (Balasubramanian et al., 1990), where we had shown that histidine residues are necessary for protein cross-linking brought about upon oxidation with $^1O_2$. Analysis of the histidine content of RNase A after such modification and cross-linking, using the diethyl pyrocarbonate (DEPC) adduct assay (Miles, 1977), revealed progressive loss of His residues (from the original value of 4 per protein molecule) with the time of exposure to the oxidant.

Table I: Changes in the Amino Acid Composition of δ-Crystallin upon Reaction with •OH Generated from Compound I[a]

amino acid residue	nmol of control	change after reaction	amino acid	nmol of control	change after reaction
Ala	32.5	–	Leu	54.8	+0.5
Arg	19.1	−1.5	Lys	34.3	–
Asx	32.5	+0.2	Met	5.2	−5.2
Cys	0	–	Phe	10.9	−0.6
Gly	24.0	+2.0	Ser	40.6	+0.6
Glx	60.4	+3.5			
His	8.0	−2.5	Thr	35.4	–
Ile	32.3	–	Tyr	5.0	−5.0
			Val	30.5	+0.5

[a] All values are in nanomoles, accurate to 0.2 nmol. Asx refers to Asp and Asn, and Glx refers to Glu and Gln.

Turning to the crystallins, Figure 4 shows that δ-crystallin, chosen as a representative example, also gets covalently cross-linked and produces high molecular weight oligomers upon reaction with •OH. Chicken δ_1-crystallin contains five histidines and no cysteine in its sequence of 446 residues (Yasuda et al., 1984). The cross-links here would thus be necessarily nondisulfide in nature.

We next analyzed the amino acid composition of δ-crystallin after reacting it with hydroxyl radicals. Table I reveals that all the Tyr residues are oxidized. So are the Met residues, presumably to the corresponding sulfoxide or sulfone. The 30% loss in His would mean that between one and two of the five His residues are oxidized in the 90 min of the reaction. In addition to Tyr and His, the side chains of Phe and Arg also appear affected. That the Trp residues are essentially lost was shown by fluorescence spectral assay. Oxidation seems to increase the composition of Glx Gly, Ser, Leu, Val, and Asx, while the other residues are not affected. Oxidation of His side chains is known to produce Asp and Asn derivatives (Uchida & Kawakishi, 1988, 1989, 1990; Ranganathan et al., 1991) and Gln derivatives under certain conditions (Ranganathan et al., 1987). Attack of •OH at the β-carbon center (rather than the aromatic ring) of Tyr has been suggested to lead to the generation of Gly (Ranganathan, 1991).

We then argued that if His residues are involved in protein oxidative cross-linking, it should be possible to inhibit the

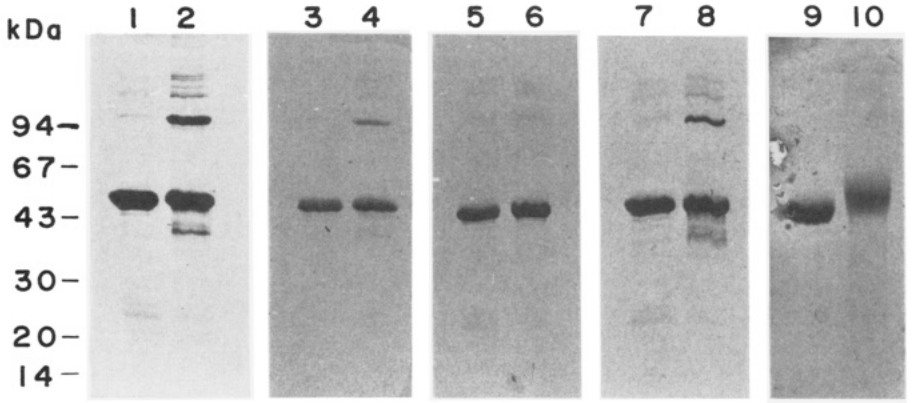

FIGURE 4: SDS–polyacrylamide gel (12.5%) of δ-crystallin samples under various conditions: lanes 1 and 2, protein (control and reacted with •OH, respectively); lanes 3 and 4, protein pretreated with DEPC (control and reacted); lanes 5 and 6, protein in the presence of 100 mM L-histidine (control and reacted); lanes 7 and 8, protein in the presence of 100 mM L-lysine (control and reacted); lanes 9 and 10, protein pretreated with maleic anhydride (control and reacted, respectively). Bands were visualized by silver staining.

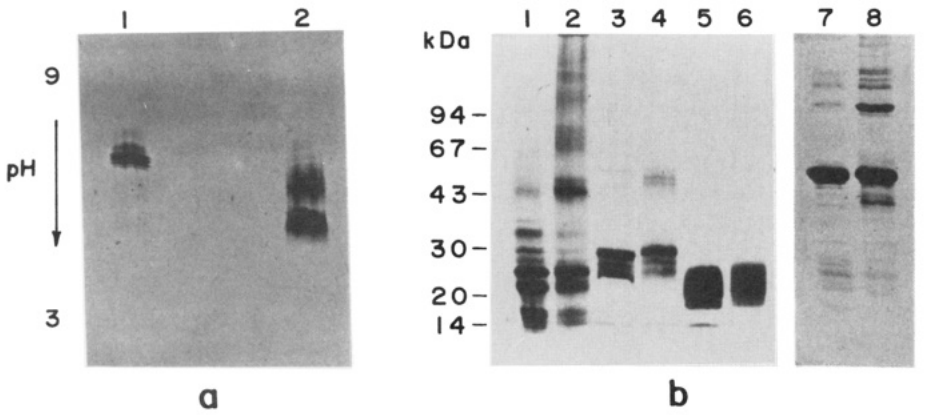

FIGURE 5: (a) Isoelectric focussing gel (pH 3–9) of δ-crystallin unreacted (lane 1) and reacted (lane 2) with •OH. (b) 12.5% SDS–polyacrylamide gels of crystallins: lanes 1 and 2 correspond to fresh and •OH-reacted α-crystallin; lanes 3 and 4 are likewise for β-crystallin, lanes 5 and 6 are likewise for γ-crystallin, and lanes 7 and 8 are likewise for δ-crystallin, respectively.

extent of cross-linking by blocking the His residues in a protein by, say, adduct formation with DEPC itself (Balasubramanian et al., 1990; Miles, 1977). Accordingly, we incubated δ-crystallin with a 4 M excess of DEPC for 5 min and eluted the "protected" protein rapidly using a Sephadex G-25 column preequilibrated with saline, so as to remove unreacted DEPC (Wolf et al., 1970). Figure 4 (lanes 3 and 4) shows that such protection of δ-crystallin by DEPC prior to exposure to •OH inhibits the formation of high molecular weight products significantly. Gel scanning quantitation revealed that compared to the 52% cross-linking seen in the control (relative intensity of the high molecular weight bands in lane 2 with respect to that of the 50-kDa parent band) DEPC protection led to a reduced value of 31% cross-linking. Lanes 5 and 6 of Figure 4 show that the addition of 100 mM L-histidine to the solution prior to reaction with •OH leads to the prevention of cross-linking. The externally added His can compete with the His side chains in the protein and thus arrest protein damage, as well as act as a scavenger of hydroxyl radicals.

Lanes 7 and 8 of Figure 4 show that the prior addition of L-lysine to the protein solution causes some inhibition of cross-linking (about 33% cross-linking, based on the intensity ratio of the high molecular weight bands to parent band in lane 8). Several other amino acids, both in the free form as well as in the α-amino group blocked form, were also tried but were found not to inhibit cross-linking. Likewise, when the free amino groups of the lysyl side chains of the protein were blocked by maleylation (lanes 9 and 10), hydroxyl radical mediated cross-linking was inhibited. These results are in accord with the earlier ones of Verweij et al. (1981) and Dubbelman et al. (1978), who had studied the photodynamic cross-linking of the proteins and implicated Lys as important to the process. The nature of the nondisulfide cross-link is yet to be worked out, but an amide (or peptide) bond involving oxidized His residues and free amino groups has been suggested (Dubbelman et al., 1978). Current efforts in our laboratory are directed toward the synthesis and study of peptides with appropriately placed His and Lys residues in the sequence as test peptides in order to investigate this suggestion.

Likewise, it is not clear what the exact products of the modification of His are, but aspartic acid and its derivatives (and Glx derivatives) are seen as final products (Tomita et al., 1969; Wasserman, 1970; Uchida & Kawakishi, 1989; Ranganathan et al., 1987, 1991). The isoelectric focusing (IEF) electrophoretic patterns of native δ-crystallin shown in Figure 5a reveal a change in the net electric charge on the molecule. The modified crystallin is seen to be more acidic. Table I indicates a loss of Arg and His residues and an increase in the levels of Glx and Asx upon the reaction of •OH with δ-crystallin. These findings are in accord with those of Davies (1987), who has found the changes in the charge patterns of several proteins after exposure to hydroxyl radicals. Garland et al. (1986, 1988) have noted that crystallins become more acidic upon oxidative damage, e.g., via the Fenton reaction, and it is known that the crystallins in a cataractous lens often have a lower isoelectric point in comparison to those in normal lenses (Augusteyn, 1981).

It is instructive to compare these results with those obtained on the oxidation of proteins using the copper(II)/ascorbate and the Fe–EDTA/H_2O_2/ascorbate systems as the oxidizing agents (Garland et al., 1986; Vissers & Winterbourn, 1991; Uchida & Kawakishi, 1988, 1989, 1990; Stadtman & Berlett, 1991). The former system selectively oxidizes the Trp and His residues of bovine serum albumin, leaving the Tyr and Phe residues intact. Likewise, one of the two His residues of angiotensin I is oxidized by this oxidant, but not its Tyr and Phe residues. In both cases, however, the active oxidizing species is not exclusively •OH, since the hydroxyl radical scavengers mannitol, urea, or dimethyl sulfoxide do not inhibit the reaction. Since catalase (and partly superoxide dismutase) does inhibit the oxidation, the oxyradicals of major importance here are derived from the copper-catalyzed Fenton reaction. Likewise, it has been reported (Vissers & Winterbourn, 1991) that only •OH generated by γ-radiolysis was able to oxidize Trp residues in fibronectin. H_2O_2 and the iron Fenton reagents were not able to do so. None of the oxidants affected the Cys content of fibronectin. The point of interest, in any event, is that His and Trp are the most vulnerable residues, just as we have seen in the present study using photogenerated •OH.

Oxidative damage to proteins is reported to be accompanied by increased carbonyl derivatives within proteins (Stadtman, 1986; Levine, 1983; Oliver et al., 1987; Lenz et al., 1989), which are usually detected by reaction with phenylhydrazines or borohydride, though the unreactive 2-imidazolone has also been detected (Uchida & Kawakishi, 1990). We are currently analyzing crystallins from aged and cataractous eye lenses in an effort to identify such carbonyl compounds that may accumulate either with time or upon oxidative damage.

Comparison of the Crystallins. Crystallins are very long lived proteins, since the eye lens in which they exist has extremely slow metabolic and turnover rates. Even minor covalent modifications of the proteins tend to accumulate over time and lead to polymerization and precipitation of the proteins and lens opacification. In the analysis of cataracts, it is of importance to study opacification of the various subregions of the lens tissue, such as the nucleus, cortex, or the

posterior subcapsular region. And since the nucleus is enriched in γ-crystallin while the midregion and cortex have a greater abundance of β- and α-crystallins, it is of interest to compare the vulnerability of the various crystallins to oxidative damage and reaction with •OH. Figure 5b shows the SDS–PAGE profiles of α-, β-, γ-, and δ-crystallins reacted with •OH under identical experimental conditions. α-Crystallin is seen to be the most extensively modified, in terms of both the number of high molecular weight bands and their amounts, as monitored by the intensity of staining. Gel scanning densitometry of lanes 1 and 2 of Figure 5b revealed an increase in the proportion of the high molecular mass (43 kDa and above) bands, from 7% in the control lane to about 53% in the oxidized protein in lane 2. δ-Crystallin of the avian lens nucleus appears to be about equally reactive (50% cross-linking as seen by densitometry of its bands), followed by β-crystallin (about 21%, as seen in lane 4), and γ-crystallin seems to be modified the least under the conditions. It is not clear whether this reactivity order reflects the order of accessibility of reactive residues in the proteins, since the vulnerability of the proteins to modification by singlet oxygen follows the order γ- > β- > α-crystallins (Balasubramanian et al., 1990), but the sensitivity to direct photooxidation at 300 nm varies in the order α > γ > β (Andley et al., 1984). We note parenthetically that among the crystallins α-crystallins are known to self-aggregate extensively akin to micellar association (Augusteyn & Koretz, 1987) and to bind to liposomes (Ifeanyi & Takemoto, 1991). This amphiphilic nature of α-crystallin might enable it to adsorb on the Kieselgel surface and interact better with compound I that is immobilized there. δ-Crystallin is a tetrameric aggregate in solution, and β-crystallin is also oligomeric, while γ-crystallin is the lone member of the family which exists as monomers in solution. Such hydropathy differences among the crystallins might have influenced their interaction with the surface-bound compound I. We note, however, that no covalent or noncovalent association of the compounds I or II with crystallins occurred, as monitored by spectroscopic analysis.

Circular dichroism spectra that we ran on the oxidized samples suggest that the conformational characteristics of the •OH-treated protein might be somewhat different from those of the unreacted protein, in a fashion reminiscent of what has been seen earlier with photodynamically damaged α-crystallin (Mandal et al., 1986; Chakrabarti et al., 1986).

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Registry No. •OH, 3352-57-6; TI, 9035-81-8; RNaseA, 9001-99-4; Ala, 56-41-7; Arg, 74-79-3; Asp, 56-84-8; Cys, 52-90-4; Gly, 56-40-6; Asn, 70-47-3; His, 71-00-1; Ile, 73-32-5; Leu, 61-90-5; Lys, 56-87-1; Met, 63-68-3; Phe, 63-91-2; Ser, 56-45-1; Thr, 72-19-5; Tyr, 60-18-4; Val, 72-18-4; Glu, 56-86-0; Gln, 56-85-9; melittin, 20449-79-0.

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A Possible Initial Folding Intermediate: The C-Terminal Proteolytic Domain of Tryptophan Synthase β Chains Folds in Less Than 4 Milliseconds into a Condensed State with Non-native-like Secondary Structure†

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ABSTRACT: The isolated F2-V8 peptide corresponding to the 101 C-terminal residues of *Escherichia coli* tryptophan synthase β chains folds into a heat-stable, yet fluctuating, condensed state that contains a lot of secondary structure. However, this state has non-native-like secondary and supersecondary structures [Chaffotte, A., Guillou, Y., Delepierre, M., Hinz, H.-J., & Goldberg, M. E. (1991) *Biochemistry 30*, 8067–8074]. To characterize the rate of appearance of this state, stopped-flow studies on the far-ultraviolet circular dichroism (CD) and on the binding of 1-anilino-8-naphthalenesulfonate (ANS) have been conducted during the folding of guanidine-unfolded F2-V8. It was shown that both the CD signal at 222 nm and the ANS binding properties of folded isolated F2-V8 were regained, at 20 °C, within the dead time of the stopped-flow apparatus, which was 4 ms. At 12 °C, the binding of ANS was also completed within this dead time, but the ellipticity showed some minor later changes. After a rapid overshoot of the CD signal that occurred during the 4-ms dead time, a small readjustment of the ellipticity to the final value occurred more slowly and was completed after about 25 ms. Thus, even at 12 °C, the hydrophobic core and most of the secondary structure of folded F2-V8 were formed in less than 4 ms. These observations strongly suggest that the previously described condensed non-native-like state of F2-V8 results from a very rapid, nonspecific, hydrophobic collapse. It is proposed that such a state may be a general early intermediate in protein folding.

The mechanisms by which a polypeptide chain folds in vitro into its native conformation are currently the subject of active investigations. According to the general picture which has emerged in recent years (Kim & Baldwin, 1982, 1990; Jaenicke, 1991; Baldwin, 1991), the folding of a small monomeric protein made of a single domain would proceed as follows. First, the polypeptide chain would undergo local folding to give stretches of nativelike secondary structure (α helices or β strands) that build the "framework" (Kim & Baldwin, 1982) for forming the hydrophobic core of the native structure. Then, these elements of secondary structure would "adhere" (Kim

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