Exposure of β_L -crystallin to oxidizing free radicals enhances its susceptibility to transglutaminase activity

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1. The effects of free radicals on the capacity of β_L -crystallin to act as a substrate for the enzyme transglutaminase were investigated. 2. β_L -Crystallin was exposed to defined radical species that were generated radiolytically, and transglutaminase activity, using the modified protein as substrate, was subsequently measured by monitoring the incorporation of [14 C]putrescine. 3. Exposure of β_L -crystallin to hydroxyl radicals, thymine peroxy radicals and acetone peroxy radicals at concentrations of up to 135 μ M increased the capacity of the protein to incorporate putrescine. With higher concentrations of these radicals this capacity of β_L -crystallin to act as a transglutaminase substrate declined to control levels or lower. 4. Superoxide radicals were inactive in this regard; hydroperoxy radicals were active only at high concentrations. 5. It has previously been suggested that changes in the crystallins that occur during aging and with cataract may be due to oxidative reactions and to transglutaminase activity. This study suggests that these phenomena may be considered together rather than separately.

INTRODUCTION

The crystallins, the major structural proteins of the lens, have proved to be very useful in studying post-translational alterations of protein structures in relation to the phenomena of cell aging. The vertebrate lens contains some of the body's oldest proteins, some of them having been synthesized before birth. Therefore, in a long-lived vertebrate such as man, the lens proteins have a very long time to accumulate age-related modifications. There are several well-documented changes which occur in the human lens: conversion of the protein from a soluble into an insoluble form, increase in disulphide and a decrease in thiol groups, development of pigmentation, specific limited cleavages of the polypeptide chains and decreased antioxidant protection, to name but a few (Zigler & Goosey, 1981; Taylor & Davies, 1987). The changes to the proteins of the lens are complex and not fully characterized, and they probably involve several mechanisms acting in concert (see Harding, 1981). These changes result in a high level of covalently cross-linked proteins that bring about a loss of transparency and a characteristic opacity of the lens, resulting in reduction of visual function and culminating in senile cataract (Kramps *et al.*, 1976). The ocular lens is exposed *in vivo* to a high level of oxidative stress, both from oxidants present in the fluids surrounding the lens and from oxidants generated within the lens itself, particularly by photochemical reactions (Spector, 1985). Thus the thiol groups of the human lens proteins may undergo oxidation to form disulphide bonds that cross-link protein molecules (Harding, 1972; Truscott & Augustein, 1977a,b; Anderson & Spector, 1978). Aged and cataractous lens contains also polypeptides that are resistant to disaggregation by urea and SDS (Kramps *et al.*, 1978). This suggests that these aggregates have formed from lens proteins by a process of cross-linking involving covalent bonds other than disulphide bonds. The isolation of γ -glutamyl- ϵ -lysine isopeptides from polymers characteristically present only in specimens of human cataract, and the finding of transglutaminase (TGase) activity in the lens suggest a possible role of this enzyme in the terminal differ-

entiation of lens fibre cells and also in the process of cataractogenesis (Lorand *et al.*, 1981). TGase activity is dependent on a relatively high Ca^{2+} concentration, but, in the lens, the Ca^{2+} concentration varies between 0.5 and 2 mM (Clark *et al.*, 1980, and references cited therein), this being sufficient to activate the enzyme to an appreciable extent. Furthermore, Ca^{2+} levels increase in aged and cataractous lenses (Krause, 1934; Duncan & Bushell, 1975), and it has been demonstrated that Ca^{2+} is capable of inducing aggregates of normal α -crystallin in the human lens *in vitro* (Jedziniak *et al.*, 1972). A second type of control can occur by an increased substrate susceptibility to TGase possibly dependent on conformational changes induced by post-translational modifications. Such conformational changes might be caused by oxidative stress; reactive free radicals may increase the accessibility of amine acceptor sites, making crystallins more susceptible to TGase activity. Preliminary studies indicate that oxidant injury is able to increase the susceptibility of β_L -crystallin to TGase (Brossa *et al.*, 1990). We have used γ -irradiation to generate specific oxygen free radicals and investigated the effect of these radicals on the capacity of crystallin to act as a substrate for exogenous TGase activity. Our results indicate that oxidative stress may contribute to the cross-linking of lens crystallins not only directly, but also indirectly, by increasing the susceptibility of the protein to TGase activity.

MATERIALS AND METHODS

Bovine lens β_L -crystallin and guinea-pig liver transglutaminase were purchased from Sigma. [14 C]Putrescine was purchased from Amersham International. Protosol solubilizer and Econofluor scintillation fluid were obtained from New England Nuclear.

Exposure of crystallin to free radicals

β_L -Crystallin, in double-distilled and deionized water, at a concentration of 1 mg/ml, was exposed to various concentrations of oxygen-centred radicals using the Brunel University ^{60}Co

Abbreviations used: TGase, transglutaminase; e^-_{aq} , solvated electron; $\text{OH}^\cdot$, hydroxyl radical; $\text{H}^\cdot$, hydrogen atom; O_2^- , superoxide radical; $\text{HO}_2^\cdot$, hydroperoxyl radical; Nbs_{ss}, 5,5'-dithiobis-(2-nitrobenzoic acid).

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source at a dose rate of 7.8 Gy/min as measured by Fricke & Hart (1966) dosimetry, up to a total dose of 1000 Gy. By using techniques defined previously (Willson, 1978), specific radical formation was achieved by careful design of the irradiation system. Briefly, in the radiolysis of water, the main primary species formed are $\text{OH}^\bullet$, e_{aq}^- and $\text{H}^\bullet$. Radiolytic yields are expressed as G values (μM -radicals/10 Gy): $G_{\text{OH}^\bullet} = 2.7$; $G_{\text{e}_{\text{aq}}^-} = 2.7$, whereas $G_{\text{H}^\bullet} = 0.55$. If the system is saturated with N_2O , the solvated electron reacts quantitatively such that 90% of the free radicals produced are $\text{OH}^\bullet$:

If O_2 is present in the system ($\text{N}_2\text{O}/\text{O}_2$, 4:1, v/v), it can react with carbon-centred substrate radicals generated by $\text{OH}^\bullet$ radicals, converting them into the corresponding substrate-peroxyl radicals.

Superoxide radicals ($\text{O}_2^{\bullet-}$) can be generated in this system if sodium formate (0.01 M) is included. Adjustment of this system to pH 4 enables the conversion of most of the $\text{O}_2^{\bullet-}$ to hydroperoxyl radical ($\text{HO}_2^\bullet$). In order to generate examples of organic peroxyl radicals, acetone or thymine is added to the solution (saturated with $\text{N}_2\text{O}/\text{O}_2$, 4:1, v/v), where $\text{OH}^\bullet$ radicals react with them by hydrogen abstraction. Thus, for acetone:

At the acetone concentration used (1 mM) the system consists primarily of acetylonyl peroxyl radicals ($G = 5.2$; Zegota *et al.*, 1986).

In a solution containing thymine (1 mM) the $\text{OH}^\bullet$ radicals will react with the 5,6 double bond, giving the hydroxyl-free-radical adduct of thymine [$\text{T}(\text{OH}^\bullet)$] that, in the presence of O_2 , will be transformed to the corresponding peroxyl radical:

TGase-directed putrescine incorporation

The substrate capacity of β_L -crystallin for TGase was tested after irradiation by determining the TGase-directed Ca^{2+} -dependent incorporation of [^{14}C]putrescine into trichloroacetic acid-insoluble material. The assay reaction mixture (0.2 ml) contained 0.1 mg of β_L -crystallin, 5 mM- CaCl_2 , 0.01 unit of TGase and 50 mM-Tris/HCl, pH 7.5. [^{14}C]Putrescine was added to a final concentration of 1 mM (0.5 μCi). After 30 min of incubation at 37 °C the reaction was stopped with 10% trichloroacetic acid (1 ml). The precipitates, collected on Sartorius 25 S glass-fibre filters, were washed with 5% trichloroacetic acid, dried, and transferred into scintillation vials. The precipitates were dissolved in 1 ml of Protosol solubilizer and scintillation solution was added (7 ml). Radioactivity was determined with a liquid-scintillation counter.

Protein fragmentation

SDS/PAGE was performed with the discontinuous buffer system of Laemmli (1970) with a Protean slab-gel apparatus (Bio-Rad). A stacking gel of 2.5% (w/v) acrylamide, a resolving gel of 15% acrylamide and a running buffer of 25 mM-Tris/HCl/0.19 M-glycine/0.1% SDS were employed, and 2.5 μg of protein sample was applied per lane. Gels were stained with Coomassie Brilliant Blue and were calibrated with Boehringer molecular-mass standards of trypsin inhibitor (20.1 kDa), lactate dehydrogenase (36.5 kDa), glutamate dehydrogenase (55.4 kDa) and phosphorylase b (97.4 kDa).

Tryptophan oxidation and thiol determination

Tryptophan oxidation was measured as loss of β_L -crystallin fluorescence peaks at 290 nm excitation and 350 nm emission after irradiation compared with unirradiated controls.

The measurement of thiol content was performed with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid), Nbs_2] by the method of Di Simplicio (1983). The samples containing irradiated crystallin were assayed at final concentration of 0.1 mg/ml in 0.1 M-phosphate buffer, pH 7.5 (1.1 ml). The reaction was started by the addition of 50 μl of 20 mM- Nbs_2 , and the absorbance at 412 nm was measured 20 min later.

RESULTS

Effect of the hydroxyl radical

The effect of the hydroxyl radical was determined by using irradiation of solutions saturated with either N_2O or with $\text{N}_2\text{O}/\text{O}_2$ (4:1, v/v). Under all these conditions a biphasic response was seen: an increase in putrescine-incorporation capacity that was concentration-dependent up to 135 μM free radicals, followed by a decrease in the value of this parameter at higher radical concentrations (Fig. 1). The maximum increase in putrescine incorporation was seen between 54 and 135 μM - $\text{OH}^\bullet$ under these conditions. The maximum increase above control activity was around 57%, regardless of the presence or absence of O_2 . The presence of O_2 seemed to play a more marked role at higher $\text{OH}^\bullet$ concentrations: at 540 μM - $\text{OH}^\bullet$ the amount of putrescine incorporation returned to control levels in samples irradiated in the absence of O_2 , but declined to 40% below control levels in the presence of O_2 .

Effect of superoxide and hydroperoxyl radicals

In contrast with the effect of $\text{OH}^\bullet$, it was found that the superoxide radical, $\text{O}_2^{\bullet-}$, was completely ineffective in changing the substrate capacity of β -crystallin at similar radical concentrations (Fig. 2). We also examined the effect of the more reactive protonated form of this radical, $\text{HO}_2^\bullet$ (Fig. 2). Again, this radical was largely ineffective until high concentrations were generated, but at 540 μM - $\text{HO}_2^\bullet$ the increase (45%) in putrescine incorporation capacity was marked.

Effect of organic peroxyl radicals

Two organic peroxyl radicals species were tested for their effects on β_L -crystallin: the thymine peroxyl radical and the acetone peroxyl radical (Fig. 3). With both of these radical species we found a pattern of effect similar to that found with $\text{OH}^\bullet$. A biphasic response was again seen, with maximum increases in putrescine incorporation reaching similar levels to those induced by $\text{OH}^\bullet$ attack and occurring at similar radical concentrations. However, the decline in putrescine incorporation at higher radical concentrations was not so marked as with $\text{OH}^\bullet$. Thus, at peroxyl radical concentrations of about 300 μM , incorporation was still 30–40% above control levels whereas, with similar concentrations of $\text{OH}^\bullet$, putrescine incorporation was no longer above control levels.

Protein fragmentation

Analysis by SDS/PAGE of β_L -crystallin after exposure to the various free radicals under study enabled us to compare the effects of the radicals on putrescine incorporation with their effects on β_L -crystallin fragmentation. The $\text{OH}^\bullet$ radical was found to be the most damaging, with extensive loss of the β_L -crystallin subunit occurring at radical concentrations above 270 μM . The thymine and peroxyl free radicals also caused major

Fig. 1. Effect of $\text{OH}^\bullet$ radicals on putrescine-incorporation capacity of β -crystallin

Solutions of crystallin (1 mg/ml) were irradiated under conditions generating hydroxyl radicals in the presence ($\square$) or the absence ($\blacksquare$) of O_2 . After irradiation, aliquots of crystallin were incubated with transglutaminase and [^{14}C]putrescine and the extent of putrescine incorporation was measured. Full details are given in the Materials and methods section.

Fig. 2. Effect of $\text{O}_2^{\bullet-}$ ($\blacksquare$) and $\text{HO}_2^\bullet$ ($\square$) radicals on the putrescine-incorporation capacity of β -crystallin

The procedure was as indicated in the legend to Fig. 1, except for the specific irradiation conditions (see the Materials and methods section).

fragmentation of the β_L -crystallin at radical concentrations above 260 μM . In contrast, the $\text{O}_2^{\bullet-}$ and $\text{HO}_2^\bullet$ radicals produced no detectable fragmentation, even at the highest concentrations. These data are summarized in Fig. 4, where, for reasons of space, only the results using 0, 135 and 540 μM concentrations of the radicals $\text{OH}^\bullet$, $\text{O}_2^{\bullet-}$, $\text{HO}_2^\bullet$ and thymine peroxy are shown. None of the fragments were sufficiently large to be retained by the gel, and no radical-induced polymerization products were evident.

Fig. 3. Effect of thymine peroxy radicals ($\blacksquare$) and acetone peroxy radicals ($\square$) on the putrescine-incorporation capacity of β -crystallin

The procedure as indicated in legend to Fig. 1, except for the specific irradiation conditions.

Fig. 4. SDS/PAGE of β_L -crystallin before and after exposure to free radicals

The protein was exposed to $\text{OH}^\bullet$ (in the presence and absence of O_2), $\text{O}_2^{\bullet-}$ or thymine peroxy radical ($\text{ThyO}_2^\bullet$) at a range of radical concentrations. For reasons of space only the results of exposure to 0 (lane a), 135 (lane b) and 270 (lane c) μM -radical are shown. Abbreviation: Std, standards of indicated molecular mass.

Protein thiol content and tryptophan fluorescence

The effects of the various radical species on the thiol concentration of β_L -crystallin and on its intrinsic tryptophan-dependent fluorescence were also monitored. In summary, $\text{OH}^\bullet$ and the thymine and acetone peroxy free radicals were very active in terms of oxidizing the thiol groups in a concentration-dependent manner, whereas the $\text{O}_2^{\bullet-}$ and $\text{HO}_2^\bullet$ radicals were without effect, except at the highest concentrations. Similarly, tryptophan fluorescence was strongly decreased by the $\text{OH}^\bullet$ and organic peroxy radicals in a concentration-dependent manner, whereas the $\text{O}_2^{\bullet-}$ and $\text{HO}_2^\bullet$ were again ineffective at all concentrations. These data are summarized in Tables 1 and 2.

Table 1. Effect of various free radicals on crystallin thiol concentration

Crystallin was exposed to free radicals and its thiol content measured as described in the text. Results are means $\pm$ S.E.M. from three independent experiments. Abbreviations: ThyO₂[•], thymine peroxy radical; AceO₂[•], acetone peroxy radical. Statistical significance (Student's *t* test; relative to unirradiated control): ^a*P* < 0.001; ^b*P* < 0.01; ^c*P* < 0.05; no symbol, not significantly different.

[Radical] (μ M)	Free radical...	Crystallin [SH] (nmol/mg of protein)					
		OH [•]	OH [•] /O ₂	O ₂ ^{•-}	HO ₂ [•]	ThyO ₂ [•]	AceO ₂ [•]
0		68.3 $\pm$ 2.3	77.7 $\pm$ 7.3	65.2 $\pm$ 2.0	59.7 $\pm$ 5.9	61.7 $\pm$ 2.3	59.7 $\pm$ 3.4
27		61.3 $\pm$ 2.4	58.7 $\pm$ 0.7	62.7 $\pm$ 6.0	55.3 $\pm$ 6.4	48.7 $\pm$ 4.5	61.7 $\pm$ 14.7
54		54.2 $\pm$ 4.5 ^c	60.2 $\pm$ 1.3	61.5 $\pm$ 6.5	51.3 $\pm$ 11.3	48.0 $\pm$ 5.0	52.0 $\pm$ 1.0
135		52.0 $\pm$ 4.0 ^c	45.6 $\pm$ 2.7 ^c	62.0 $\pm$ 7.0	52.3 $\pm$ 6.2	33.5 $\pm$ 4.5 ^b	33.1 $\pm$ 1.1 ^b
270		46.7 $\pm$ 7.1 ^c	39.0 $\pm$ 8.1 ^c	43.0 $\pm$ 3.0 ^b	47.7 $\pm$ 7.4	20.3 $\pm$ 5.4 ^b	39.0 $\pm$ 7.0
540		11.0	27.5 $\pm$ 5.5 ^b	43.0 $\pm$ 3.8 ^b	52.0 $\pm$ 1.5	14.3 $\pm$ 3.8 ^a	21.6 $\pm$ 2.6 ^a

Table 2. Effect of various free radicals on crystallin tryptophan fluorescence

Crystallin was exposed to free radicals and tryptophan fluorescence was determined as described in the text. Data are means $\pm$ S.E.M. from three independent experiments. Abbreviations and statistical significances are as in Table 1.

[Radical] (μ M)	Free radical...	Tryptophan fluorescence (arbitrary units)					
		OH [•]	OH [•] /O ₂	O ₂ ^{•-}	HO ₂ [•]	ThyO ₂ [•]	AceO ₂ [•]
0		165 $\pm$ 10	161 $\pm$ 3	161 $\pm$ 2	142 $\pm$ 3	127 $\pm$ 6	121 $\pm$ 2
27		142 $\pm$ 7	139 $\pm$ 2 ^a	164 $\pm$ 5	157 $\pm$ 9	109 $\pm$ 6	102 $\pm$ 5 ^b
54		145 $\pm$ 9	139 $\pm$ 1 ^a	152 $\pm$ 4	147 $\pm$ 5	99 $\pm$ 6 ^b	103 $\pm$ 9
135		123 $\pm$ 6 ^b	108 $\pm$ 1 ^a	159 $\pm$ 1	137 $\pm$ 11	87 $\pm$ 5 ^a	74 $\pm$ 4 ^a
270		93 $\pm$ 7 ^a	84 $\pm$ 3 ^a	162 $\pm$ 4	149 $\pm$ 4	59 $\pm$ 1 ^a	58 $\pm$ 3 ^a
540		57 $\pm$ 7 ^a	29 $\pm$ 4 ^a	159 $\pm$ 2	145 $\pm$ 1	39 $\pm$ 2 ^a	42 $\pm$ 5 ^a

DISCUSSION

The lens proteins afford unique advantages for the study of age-related changes, owing to their extremely slow turnover. The changes in lens crystallins that have been most intensively studied are those produced by oxidation due to the production of reactive oxygen species (see Taylor & Davies, 1987) and those produced by transglutaminase activity (Lorand *et al.*, 1985; Velasco & Lorand, 1987; Zigler *et al.*, 1989). Previously these have been considered as independent phenomena, but we propose that they are related, in that exposure of crystallins to oxidants may predispose them to enhanced transglutaminase activity, leading to the formation of cross-links. Our preliminary data in support of this hypothesis came from studies in which β_L -crystallin was exposed to a glucose/glucose oxidase mixture that generates H₂O₂ and may indirectly yield oxygen free radicals (Brossa *et al.*, 1990). β_L -Crystallin susceptibility to TGase activity was increased by over 100%, but it was not clear from those experiments which oxidizing species was responsible for this effect. The results presented here confirm this original observation that oxidative reactions enhance the susceptibility of β_L -crystallin to TGase activity. It is clear, however, that O₂^{•-} is incapable of modifying β_L -crystallin so as to increase its susceptibility to TGase, and even its more reactive protonated form (HO₂[•]) only shows such activity towards β_L -crystallin at the highest concentration studied here. Although O₂^{•-} is probably the most commonly produced oxygen free radical in animal cells, it is generally held that it is not a particularly damaging species in itself, owing to its relatively low chemical reactivity. The lack of effect on crystallin thiol groups, tryptophan fluorescence and

polypeptide fragmentation, used here as general markers of protein damage for comparison, support this. The significance of O₂^{•-} production in cells lies more in its potential as a precursor for OH[•] production, the latter being a far more reactive oxidizing species. Here we demonstrate that the OH[•] radical is by itself capable of modifying β_L -crystallin so as to enhance its capacity as a TGase substrate. Simultaneously, at all concentrations studied, OH[•] damages β_L -crystallin in terms of thiol oxidation and loss of tryptophan fluorescence. Fragmentation of the polypeptide chain was difficult to quantify, and the degree of fragmentation at low OH[•] concentrations cannot be estimated. The biphasic response curve for putrescine incorporation suggests that a limited amount of protein modification enhances TGase substrate capacity, but that higher concentrations of OH[•] fragment the protein so much as to begin to destroy its capacity to act as a substrate. The same argument also applies to the organic peroxy free radicals studied. Organic peroxy radicals are less reactive in general than OH[•], but may be more damaging by being more selective in their targets, and they are increasingly implicated as important damaging species (Willson, 1985). In the present study, thymine peroxy and acetone peroxy radicals showed an activity profile very similar to that of OH[•], with the exception that, at the higher concentrations, the peroxy radicals still enhance putrescine-incorporation capacity, possibly indicating that they are less destructive. By way of comparison, Wolff & Dean (1986) found thymine peroxy radicals incapable of fragmenting albumin, whereas Gee *et al.* (1985) found this radical capable of inactivating alcohol dehydrogenase. These are important observations, since it is more likely that organic peroxy radicals, with their longer half-lives, will be the ultimate

damaging species in the cell rather than $\text{OH}^\bullet$, which is so reactive as to be limited to reacting with whatever biomolecule is present at its site of production (Slater, 1976).

The effect of the presence of O_2 seems to be to promote the damaging effects of $\text{OH}^\bullet$, tipping the balance from enhanced TGase susceptibility to excessive destruction of the polypeptide chain, probably as a consequence of the formation of peroxy radicals on the protein itself. In general terms, these data are in agreement with recent reports by other workers (Dean *et al.*, 1986; Wolff & Dean, 1986; Wolff *et al.*, 1986; Davies, 1987) who, in studies of radical attack on other proteins, conclude that $\text{OH}^\bullet$ is readily capable of fragmenting polypeptide chains, whereas $\text{O}_2^{\bullet -}$ is not.

In conclusion, the present study has confirmed our hypothesis that the action of reactive oxygen species upon β_L -crystallin increases the susceptibility of this protein to TGase activity. Further, it identifies which free radicals are likely to be responsible for this effect and which are not. Further work will be directed towards fully understanding the mechanism of this effect, which probably depends on changes in the conformation of the protein so as to expose more glutamine residues. This study not only reinforces the theory that oxidizing free radicals are important in pathological changes to crystallins, but also enables us to consider TGase-dependent changes as a related phenomenon rather than a separate one.

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