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Structural modifications of human β_2 microglobulin treated with oxygen-derived radicals

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Treatment of human β_2 microglobulin (β_2m) with defined oxygen-derived species generated by treatment with γ-radiation was studied. As assessed by SDS/PAGE, the hydroxyl radicals ($^{\bullet}OH$) caused the disappearance of the protein band at 12 kDa that represents β_2m, and cross-linked the protein into protein bands stable to both SDS and reducing conditions. However, when $^{\bullet}OH$ was generated under oxygen in equimolar combination with the superoxide anion radical ($O_2^{\bullet-}$), the high-molecular-mass protein products were less represented, and fragmented derivatives were not obviously detectable. Exposure to $^{\bullet}OH$ alone, or to $^{\bullet}OH + O_2^{\bullet-}$ in the presence of O_2, induced the formation of β_2m protein derivatives with a more acidic net electrical charge than the parent molecule. In contrast, $O_2^{\bullet-}$ alone had virtually no effect on molecular mass or pI. Changes in u.v. fluorescence during $^{\bullet}OH$ attack indicated changes in conformation, as confirmed by c.d. spectrometry. A high concentration of radicals caused the disappearance of the β-pleated sheet structure and the formation of a random coil structure. Loss of tryptophan and significant production of dityrosine (2,2′-biphenol type) were noted, exhibiting a clear dose-dependence with $^{\bullet}OH$ alone or with $^{\bullet}OH + O_2^{\bullet-}$. The combination of $^{\bullet}OH + O_2^{\bullet-}$ induced a pattern of changes similar to that with $^{\bullet}OH$ alone, but more extensive for c.d. and tryptophan oxidation (2 Trp/β_2m molecule), and more limited for dityrosine formation. Lower levels of these oxidative agents caused the reproducible formation of species at 18 and 25 kDa which were recognized by antibodies against native β_2m. These findings provide a model for the protein pattern observed in β_2m amyloidosis described in the literature.

INTRODUCTION

Human β_2 microglobulin (β_2m) is a low-molecular-mass protein of 99 amino acids, with one intrachain disulphide bridge between cysteine residues 25 and 80 [1,2]. Its primary structure is highly conserved among species and relatively invariant within a species [3]. Study of the three-dimensional structure of bovine β_2m shows that the β_2m molecule contains two antiparallel β-pleated sheets [4], as previously postulated from the study of its physicochemical properties [5]. β_2m is found on the cell surface of all nucleated cells and has been identified as the light chain of the class I HL-A antigens [6]. It is non-covalently linked to the heavy chain of the major histocompatibility complex and is essential to its serological specificity [7].

β_2m is normally present as a free monomer at low concentrations in various body fluids [1,8]. The major site of β_2m catabolism is localized in the kidney [9]. Therefore in conditions characterized by a decreased glomerular filtration rate, such as in uraemic and long-term haemodialysed patients, serum levels of β_2m increase [8,10,11]. Furthermore, in some haemodialysed patients β_2m exists in an insoluble form as tissue deposits of fibrillar protein, designated as amyloidosis [12–14]. Deposits are mainly located in carpal tunnel structures [12,15], subchondrial bone and bone marrow [13,16] and joint capsule synovial membranes [17,18]. It remains uncertain how amyloid fibril is formed from β_2m. Amyloidosis was reported to originate from an intact precursor or its metabolites in the serum [19], and may depend on the tissue origin. Intact β_2m molecules of molecular mass 12 and 24 kDa were found mainly in carpal tunnel structures and subchondrial bone [12,13]. Derivatives of β_2m with molecular masses of 24, 17, 12 and 8 kDa were described in synovial amyloid from dialysed patients [17,18], and fragments of 8 kDa were found in amyloid kidney stones of uraemic patients [20]. Furthermore, in the serum of patients on long-term haemodialysis, a novel β_2m was characterized, in addition to normal β_2m [21], with a lower molecular mass and a lower isoelectric point (pI 5.2) than normal β_2m (pI 5.7) [1,5].

It is known that oxygen-derived free radicals can readily fragment proteins in solution and inactive proteins by modification of amino acid residues [22–28]. Cross-linking reactions also occur [27–31]. The high reactivity of these oxygen-derived species has led to their implication in pathophysiology. Several studies have suggested that oxygen-derived radicals may be involved in the degradation of connective tissues that occurs under the conditions that accompany chronic inflammation [32–35].

Phagocyte activation was shown to occur during dialysis on conventional membranes [36–38]. Phagocytic cells are capable of producing large amounts of reactive oxygen metabolites at their surfaces during the oxidative burst. The wide distribution of β_2m makes it a plausible target for the action of oxygen-derived radicals. In the present paper, we study the ability of qualitatively and quantitatively defined oxygen-derived radicals generated by γ-radiation lysis to degrade β_2m solution. We described in detail some structural modifications that occur as a result of treatment with hydroxyl radicals ($^{\bullet}OH$) alone, or with an equimolar mixture of hydroxyl radicals and superoxide radicals (50% $^{\bullet}OH$ + 50% $O_2^{\bullet-}$) in the presence of oxygen. Results showed that, in the presence of low amounts of $^{\bullet}OH$, β_2m is cross-linked in a highly regular manner to produce molecularly defined components.

MATERIALS AND METHODS

Protein

Human β_2m, purified from urine according to the method in [1], was obtained from Sigma (M4890, batch nos. 127F-07991 and 29F-06161). β_2m concentrations were determined at 280 nm

Abbreviation used: β_2m, β_2 microglobulin.
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from u.v. absorption spectra recorded on a Cary 2200 spectrophotometer, using a molar absorption coefficient of 19.6 $mM^{-1} \cdot cm^{-1}$ [1].

Oxygen radical generation

Radical species were homogeneously generated in protein solutions by γ-radiolysis with a ^{60}Co source (performed in the Laboratoire de Chimie Physique directed by Professor C. Ferradini, Université R. Descartes, Paris, France) [39]. A 1 ml solution of 5 μM-β_2m in 10 mM-sodium/potassium phosphate buffer, pH 7.4, at room temperature was saturated for 30 min with the appropriate gas before irradiation in closed vessels. The selective bubbling of N_2O, O_2 and O_2 in presence of 10 mM-sodium formate allowed the generation of 100% hydroxyl radicals ($^{\cdot}OH$), an equimolar mixture of hydroxyl radicals and anion superoxide radicals (50% $^{\cdot}OH + 50\%$ $O_2^{\cdot -}$) and 100% anion superoxide radicals ($O_2^{\cdot -}$) respectively, according to the well-known yields of formation of the various radicals [40]. The doses were delivered at a rate of 2.48 ± 0.11 Gy/min, as measured by Fricke & Hart dosimetry [41]. Doses varied between 30 and 900 Gy, thus enabling us to study ratios of oxygen radical/protein (nmol of radicals/nmol of protein) between 5 and 100.

SDS/PAGE and blotting

SDS/PAGE was performed at room temperature in vertical slab gels containing 0.1% (w/v) SDS, 4 M-urea and a 10–20% gradient of total acrylamide, using the Laemmli discontinuous buffer system [42]. Samples and molecular mass standards in 60 mM-Tris/HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol and 5% glycerol were heated for 5 min at 95 °C and layered on to a 4% (w/v) stacking gel (0.75 mm thick). The relative molecular masses of the proteins were calibrated using the molecular mass markers (Pharmacia) as standards. To optimize detection of any fragment formed during exposure of β_2m to oxygen radicals, proteins were stained with a sensitive silver stain under reducing conditions [43] or subjected to immunoblotting (see below). New protein bands were detected in the 58–66 kDa molecular mass range due to an excess of 2-mercaptoethanol in electrophoresis sample buffer, as discussed in [44,45].

Electrophoretic transfer from SDS/polyacrylamide gels to a nitrocellulose membrane (0.2 μm) was performed as described by Towbin *et al.* [46]. After Ponceau Red staining, the blots were probed using a polyclonal rabbit antiserum against human β_2m (Calbiochem Corp., San Diego, CA, U.S.A.). Immunoreactions were detected using alkaline phosphatase-coupled anti-rabbit IgG antiserum (Biosys, Compiegne, France).

Isoelectric point measurements

Isoelectric focusing was performed on a flat-bed apparatus (Multiphor II 2117; Pharmacia) in ultrathin polyacrylamide layers (LKB Ampholine PAGplates: 1804-102; Pharmacia), chosen to give an effective pH range of 6.5–4.0, according to the instruction manual. The pH values of the gels were calibrated using pI markers (Pharmacia) as standards. Gels were silver-stained under reducing conditions [43].

C.d. measurements

C.d. spectra were recorded with a mark V Dichrograph (Jobin et Yvon, Palaiseau, France) at 20 °C. Data in the range 250–185 nm were collected at 0.2 nm intervals and expressed as mean residue ellipticity, $[\theta]$, in degrees $\cdot cm^2 \cdot dmol^{-1}$. The c.d. spectropolarimeter was interfaced to an on-line data acquisition system using an Apple II computer fitted with the appropriate program. The spectra reported are the averages of three independent scans from which a buffer blank was subtracted. β_2m was used at a concentration of 5 μM in cells of 0.1 cm path length.

Fluorescence measurements

Fluorescence spectra were recorded on a Perkin–Elmer LS-5 spectrofluorimeter with automatic balance of the excitation spectrum performed with Rhodamine B. Protein fluorescence emission was recorded from 300 to 460 nm after excitation at 280 nm or 295 nm. Absorbance did not exceed 0.01 at 280 nm. All experiments were performed in 10 mM-phosphate buffer, pH 7.4, at 20 °C.

Tryptophan residue quantification was determined by the fluorimetric method described in [47]. Briefly, the typical fluorescence emission of tryptophan from protein denatured in 6 M-guanidine hydrochloride in the presence of 30 mM-2-mercaptoethanol was measured at its maximum near 350 nm after excitation at 295 nm (wavelength selected to obtain only tryptophan fluorescence) and compared by inner calibration with the fluorescence emission of L-tryptophan (Serva, Heidelberg, Germany) in phosphate buffer (ϵ_{280} 5.71 $mM^{-1} \cdot cm^{-1}$ at pH 7.0). For the calculations, the fluorescence intensity of a tryptophan residue in denatured protein samples was approximated to be equal to that of free tryptophan in the same guanidine medium. Measurements made twice on the same sample were reproducible within 5–7%, provided that the guanidine hydrochloride incubation was maintained at room temperature for 20 min before recording.

Dityrosine residue quantification was performed using a fluorimetric method based on a principle similar to that for tryptophan quantification. The fluorescence emission of dityrosine [48] generated in a protein sample denatured in 6 M-urea was recorded and measured at its maximum near 410 nm after excitation at 325 nm, and compared by inner calibration with the fluorescence of 2,2'-biphenol (Aldrich-Chemie, Steinheim, Germany; ϵ_{316} 5.79 and ϵ_{280} 4.51 $mM^{-1} \cdot cm^{-1}$ in basic and acidic media respectively [49]), a product chemically analogous to the predominant form obtained by oxidation of free tyrosine [30,50]. Such a calibration assumes a similar quantum yield for the free biphenol and for the dityrosine generated in the protein sample. Assays on duplicate samples generally agreed within 5%.

RESULTS

Changes in β_2m conformation induced by oxygen radicals

C.d. spectroscopy was used to monitor changes in the secondary structure of β_2m during the course of structural transitions induced by oxygen radicals. Fig. 1(*a*) shows the far-u.v. c.d. spectrum of native β_2m which was found in this study, similar to previously reported spectra [5,7]. The spectrum was characterized by a negative band centred at 218 nm, which crosses over to positive values at 214 nm, reaching a maximum near 195 nm. The feature at 218 nm was attributed to peptide bonds in a β-pleated sheet structure [51]. The β structure was inferred from crystallographic data of bovine β_2m showing the involvement of 35% of the amino acids in the β conformation [4]. The minor positive band observed at 232 nm was probably due to the disulphide bond maintained in an asymmetrical environment, as discussed in [52] for immunoglobulin structure.

Upon exposure to $^{\cdot}OH$ alone or to $^{\cdot}OH + O_2^{\cdot -}$ in the presence of 100% O_2, the spectral features characteristic of the native protein were lost (Fig. 1*b*). The minimum at 218 nm was replaced by an intense negative ellipticity centred near 198 nm. A band in this position was typically seen in randomly coiled polypeptides and proteins, and had features similar to those reported for unfolded protein in 6 M-guanidine hydrochloride [51,52]. The magnitude of the trough observed on exposure to $^{\cdot}OH + O_2^{\cdot -}$ was greater than that observed on exposure to $^{\cdot}OH$ alone. In contrast,

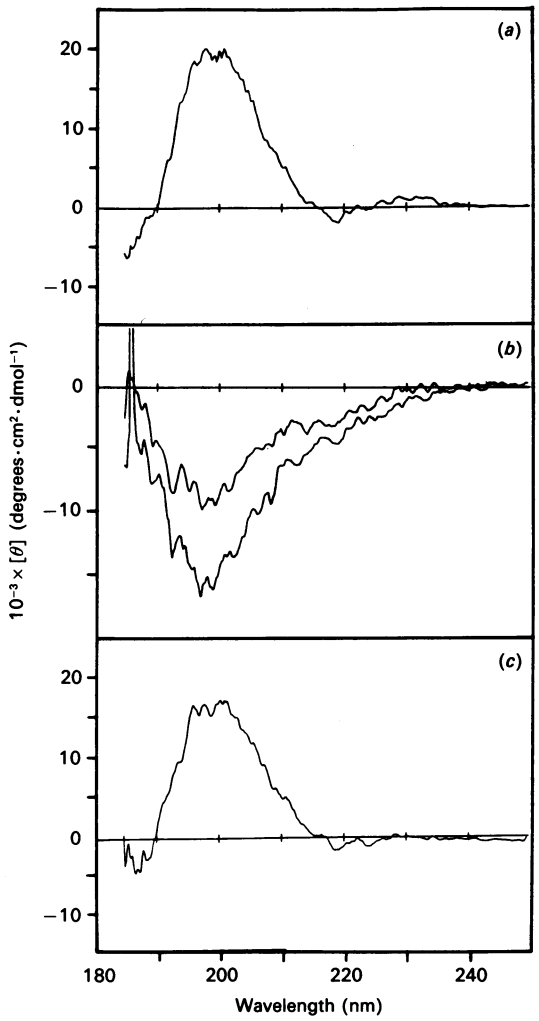

Fig. 1. Effect of long-term exposure to γ-radiation on the far-u.v. c.d. spectrum of β_2m

The ellipticity per mean residue, $[\theta]_\lambda$, given in degrees·cm²·dmol⁻¹, was recorded as a function of wavelength (nm). (*a*) Native β_2m in 10 mM-phosphate buffer, pH 7.4. (*b*) and (*c*) Irradiated samples: 1 ml of a 5 μM solution of β_2m was exposed in (*b*) to 500 nmol of ·OH generated under N_2O flow (upper trace), or to 250 nmol of ·OH plus 250 nmol of $O_2^{\cdot-}$, generated under oxygen flow (lower trace), i.e. at a ratio of 100 nmol of oxygen radicals/nmol of β_2m; or in (*c*) to 500 nmol of $O_2^{\cdot-}$ generated in the presence of 10 mM-sodium formate under oxygen flow.

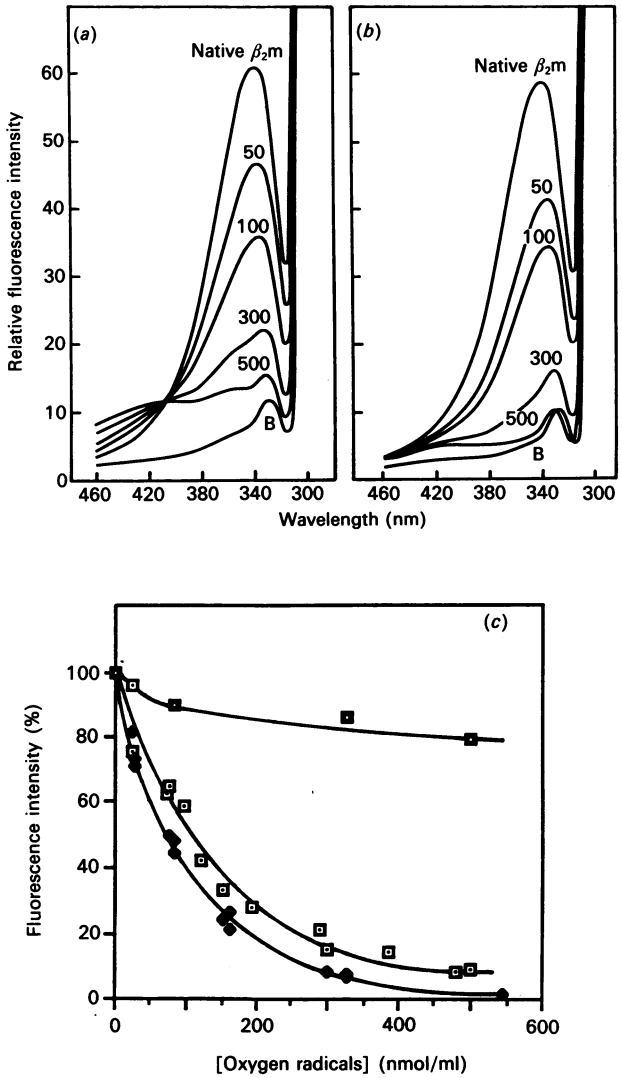

Fig. 2. Dose-dependent effect of γ-radiolysis on β_2m structure, as indicated by fluorescence emission spectra in phosphate buffer

(*a*) and (*b*) Typical fluorescence emission spectra from β_2m samples (1.25 μM) were recorded after excitation at 295 nm. From top to bottom: native β_2m in 10 mM-phosphate buffer, pH 7.4, and β_2m samples (5 μM) treated at the indicated doses, expressed in nmol/ml, of ·OH (*a*) or an equimolar mixture of ·OH + $O_2^{\cdot-}$ (*b*) in the presence of N_2O or O_2 respectively. B, buffer alone. (*c*) Dependence of the fluorescence emission monitored at maximum peak emission on the concentrations of oxygen radicals generated during γ-radiolysis. ⊡, ·OH alone; ■, $O_2^{\cdot-}$ alone; ◆, equimolar mixture of ·OH + $O_2^{\cdot-}$. Excitation wavelength, 295 nm; emission wavelength, 340 nm. Each data point represents the mean value of at least two determinations in a single experiment. Data correspond to three independent series of experiments performed with a mean concentration of 5 μM-β_2m.

exposure to $O_2^{\cdot-}$ alone caused no change in the β-pleated sheet structure detected by the minimum at 218 nm; only the disappearance of the positive band near 230 nm was observed (Fig. 1*c*).

The alteration in protein conformation was confirmed by fluorescence studies. The major fluorescent species in β_2m were two tryptophan residues per molecule [53]. Tryptophan fluorescence measured in buffer was greatly decreased by treatment with ·OH + $O_2^{\cdot-}$, whereas ·OH produced a smaller decrease (Fig. 2). Both decreases were dose-dependent. No detectable change was observed upon exposure to $O_2^{\cdot-}$ alone. The quenching of fluorescence correlated with a blue shift of the maximum emission peak from 340 to 330 nm. Furthermore, upon exposure to large amounts of ·OH, an additional fluorescence emission with a maximum at 410–420 nm was detected (Fig. 2*a*). Typical dityrosine emission was characterized upon excitation at 325 nm (Fig. 3) (see below).

Perturbations in the tryptophan fluorescence spectrum may be due either to tryptophan oxidation or to a change in the ionization of a neighbouring group, or may be caused by a conformational change. However, the induction of a newly organized protein structure, characterized by the shielding of the tryptophan residues and the blue shift of the emission maximum, is not consistent with the c.d. results showing the absence of a defined structure in the β_2m molecule upon exposure to large amounts of ·OH or ·OH + $O_2^{\cdot-}$ radicals. Analysis of the net charge of the molecule and titration of tryptophan residues will shed light on these interpretations (see below).

Following treatment with oxygen radicals, changes in the overall electric charge of the β_2m protein were revealed by

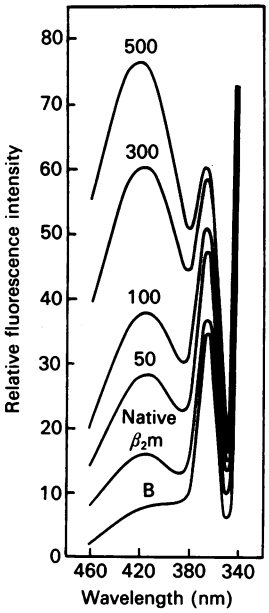

Fig. 3. Dose-dependent effect of γ-radiolysis on dityrosine formation in β_2m, monitored by fluorescence emission spectra in phosphate buffer

Typical fluorescence emission spectra of dityrosine from β_2m samples (1.25 μM) treated in the presence of various concentrations of ·OH as indicated were recorded after excitation at 325 nm, with the *y*-scale increased by a factor of 8 compared to Figs. 2(*a*) and 2(*b*). B, buffer alone.

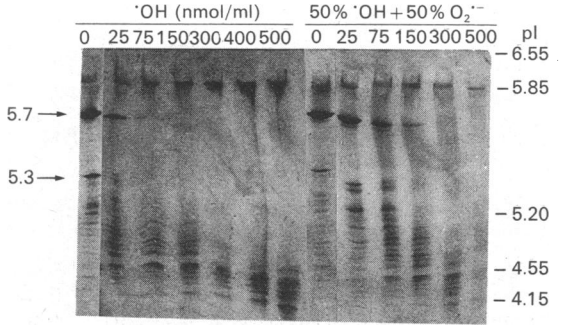

Fig. 4. Effect of exposure to oxygen radicals on the net charges of β_2m samples

Solutions of 5 μM-β_2m (1 ml) were treated with increasing doses (nmol/ml) of oxygen radicals; either ·OH or an equimolar mixture of ·OH + $O_2^{\cdot-}$ as indicated and analysed for net charge determination by isoelectric focusing gel electrophoresis (pH 6.5–4.5) using 1.2 μg of protein per lane. Protein bands were visualized by silver staining. Calibration of the pH values in the gel was obtained using the following pI markers: from top to bottom, human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase B (pI 5.85), β lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55) and glucose oxidase (pI 4.15). Arrows indicate the positions of the two main components of native β_2m.

analysis of isoelectric focusing gels, which showed that the β_2m molecule became significantly more acidic. As shown in Fig. 4, the pI of the native protein was estimated to be 5.7 for the major form, with a slight contribution at 5.3, as detected by silver staining. Differently charged forms of β_2m have been reported in normal urine [54] and sera [55–57]. Upon exposure to oxygen radicals, the native form at pI 5.7 disappeared at molar ratios (nmol of radicals/nmol of protein) of 15 for ·OH and 60 for

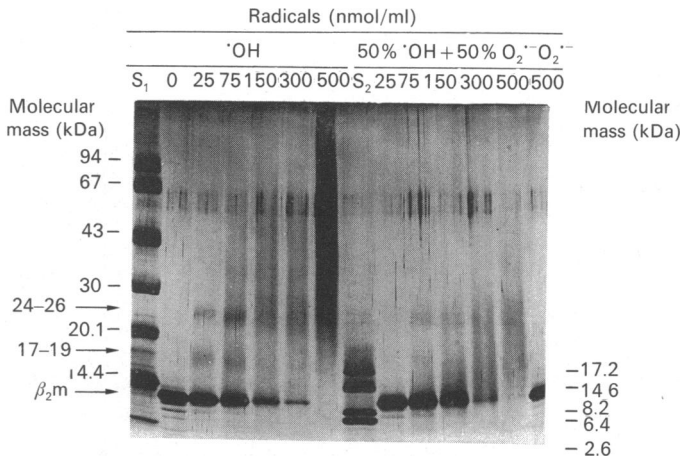

Fig. 5. Degradation of β_2m by oxygen radicals

β_2m solutions (5 μM) were exposed to oxygen radicals at various doses (expressed in nmol/ml) of ·OH, an equimolar mixture of ·OH + $O_2^{\cdot-}$ or $O_2^{\cdot-}$, as indicated. After treatment, β_2m samples were subjected to SDS/PAGE under denaturing and reducing conditions in a 10–20% polyacrylamide gradient gel at 1.4 μg of protein/lane. Gels were silver-stained. Lane S_1 contains standard proteins of known molecular mass (in kDa): phosphorylase *b* (94), BSA (67), egg albumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1) and α-lactalbumin (14.4). Lane S_2 contains low-molecular-mass markers as follows: intact form of myoglobin (17.2) and myoglobin derivatives I + II (14.6), I (8.2), II (6.4), III (2.6) and 1–14 (1.7). Arrows indicate the positions of the main protein bands generated upon oxygen radical treatment. The gel is representative of several experiments.

·OH + $O_2^{\cdot-}$. Long exposures generated a series of new polypeptide species with lower pI values extending down to pI 4.5. Such charge changes were indicative of modifications of amino acid residues. Both ·OH and ·OH + $O_2^{\cdot-}$ induced similar charge modifications. The general pattern of new band formation was different for the two exposure conditions, and the bands which were most acidic were obtained by exposure to ·OH alone.

Effect of oxygen radicals on β_2m molecular mass

After exposure to oxygen radicals, β_2m samples were subjected to polyacrylamide slab gel electrophoresis in the presence of SDS and urea under reducing conditions to determine their molecular mass (Fig. 5). Non-treated β_2m samples showed a dominant polypeptide band at 12 kDa, together with a fragment at 8 kDa that was constitutively present in the starting material and represented less than 7% of the total protein. Both bands at 12 kDa and 8 kDa were detected by anti-β_2m antibodies (results not shown) and disappeared upon radiation at low doses. Incubation with ·OH along or with the mixture ·OH + $O_2^{\cdot-}$ caused a loss of protein silver staining bands in a dose-dependent manner. In contrast, exposure to $O_2^{\cdot-}$ caused no change in the molecular mass of the protein, even up to a theoretical radical/protein molar ratio of 100.

A high molar ratio of ·OH/protein led to a diffused smear, corresponding to higher-molecular-mass covalent aggregates which were stable to reduction, and which expanded over 30–100 kDa, presumably due to cross-linking. The ·OH + $O_2^{\cdot-}$ mixture caused no such aggregation, but gave a faintly visible smear in the range 17–26 kDa. Moreover, at a low molar ratio of 5 with ·OH alone, two additional polypeptides of molecular mass 17–19 and 24–26 kDa were generated. In contrast, the incubation with the ·OH + $O_2^{\cdot-}$ mixture (molar ratio 15) produced at most one slightly stained species at 24–26 kDa. These new species at

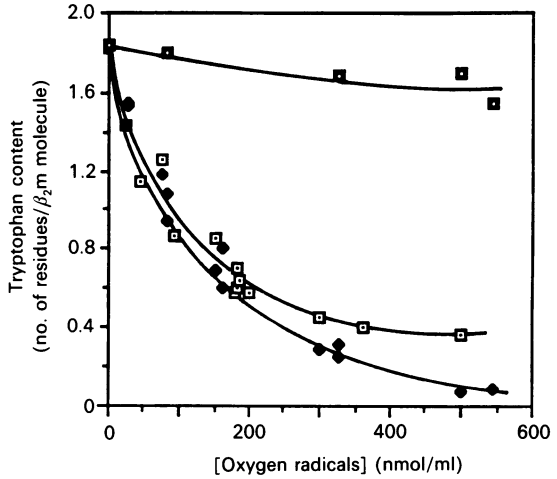

Fig. 6. Destruction of tryptophan residues in β_2m on exposure to oxygen radicals

Solutions of 5 μM-β_2m (1 ml) were treated with increasing doses of oxygen radicals, expressed in nmol/ml (⊡, $^{\bullet}OH$ alone; ■, $O_2^{\bullet-}$ alone; ◆, equimolar mixture of $^{\bullet}OH+O_2^{\bullet-}$). Aliquots were denatured in 6 M-guanidine hydrochloride and assessed for tryptophan content in the irradiated sample by recording fluorescence emission spectra after excitation at 295 nm, as described in the Materials and methods section. From sequence data, each β_2m molecule has two tryptophan residues [3]. Each data point represents the mean value of at least two determinations in a single experiment. Data correspond to three independent series of experiments performed at a mean concentration of 5 μM-β_2m.

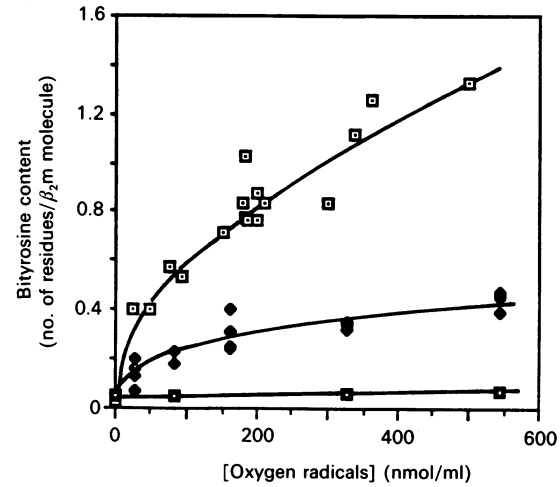

Fig. 7. Formation of dityrosine residues in β_2m as a function of oxygen radical exposure

β_2m samples (5 μM, 1 ml) were treated with increasing doses of oxygen radicals (⊡, $^{\bullet}OH$ alone; ■, $O_2^{\bullet-}$ alone; ◆, equimolar mixture of $^{\bullet}OH+O_2^{\bullet-}$). Aliquots were denatured in 6 M-urea and assessed for dityrosine content by comparing fluorescence emission at 415 nm after excitation at 325 nm, with that of 2,2′-biphenol taken as reference, as described in the Materials and methods section. Each data point represents the mean value of at least two determinations in a single experiment. Data correspond to three independent series of experiments.

17–19 and 24–26 kDa were detected in immunoblots by anti-β_2m antibodies (results not shown).

Interestingly, prolonged exposure to oxygen radicals caused extensive destruction of the whole molecule into smaller fragments that were no longer detectable by our silver staining technique. The difference in the sizes of derivatives observed on exposure to the protein to $^{\bullet}OH$ in the absence or presence of $O_2^{\bullet-}$ implied a difference in mechanism and/or kinetics of transformation.

Changes in β_2m tryptophan content on exposure to oxygen radicals

Titrations of tryptophan residues in irradiated β_2m samples were performed by monitoring tryptophan fluorescence after complete denaturation of protein samples in 6 M-guanidine hydrochloride and 30 mM-2-mercaptoethanol, as described in [47]. After excitation at 295 nm, the fluorescence emission of tryptophan residues was recorded between 300 and 450 nm. Spectra were characterized by a maximum peak emission at 352 nm, indicating that most chromophores (i.e. tryptophan residues) were totally accessible to the solvent upon unfolding the protein. Analysis of the decrease in intensity of the maximum emission peak illustrated in Fig. 6 indicated a decrease in the number of tryptophan residues per β_2m molecule upon exposure to radicals, in a dose-dependent manner. In the initial portion of the curves, one tryptophan residue per β_2m molecule was destroyed by 100 nmol of $^{\bullet}OH$ or 50 nmol of $^{\bullet}OH$ in association with 50 nmol of $O_2^{\bullet-}$. In both cases the profiles followed a biphasic exponential time course. The shape of the tryptophan destruction curves indicated that modified tryptophan residues compete with native tryptophan residues for the available oxygen radicals. In contrast, exposure to $O_2^{\bullet-}$ alone produced no significant tryptophan loss. These results were consistent with the concept that $^{\bullet}OH$ initiated tryptophan oxidation. However, the addition of $O_2^{\bullet-}$ and the presence of oxygen appeared to exacerbate many of the effects of $^{\bullet}OH$, since large amounts of the mixture $^{\bullet}OH+O_2^{\bullet-}$ led to the complete destruction of two tryptophan residues per β_2m molecule, but $^{\bullet}OH$ along did not have this effect.

Generation of dityrosine residues on exposure of β_2m to oxygen radicals

Upon exposure to $^{\bullet}OH$ attack, tyrosine residues generated as major product 2,2′-biphenol or dityrosine, characterized by a maximum emission peak at 415 nm upon excitation at 325 nm [30,50] (Fig. 3). Titrations of dityrosine production in irradiated β_2m samples were assessed in denatured samples by comparing their fluorescence emission with that of a known concentration of free 2,2′-biphenol in the same urea medium, assuming equivalent quantum yield. Results presented in Fig. 7 showed a quasi-linear production of dityrosine residues. At the higher doses tested, 1.3 molecules of dityrosine per β_2m molecule were produced by 500 nmol of $^{\bullet}OH$. In contrast, exposure to $^{\bullet}OH+O_2^{\bullet-}$ induced a more limited response, which reached a plateau at 0.4 molecule of dityrosine per β_2m molecule with 200 nmol of $^{\bullet}OH+O_2^{\bullet-}$. Little or no dityrosine was produced by exposure to $O_2^{\bullet-}$ alone.

DISCUSSION

The results presented here on the modifications of the structure of a β-pleated sheet serum protein, i.e. β_2m, by hydroxyl and superoxide radicals generated upon γ-radiolysis provide evidence for an initial $^{\bullet}OH$ attack on the polypeptide chain and for the modulation of the initiated reaction by the presence of oxygen. For a given dose of γ-irradiation, a smaller degree of cross-linked β_2m is observed in the presence of oxygen radicals, i.e. $^{\bullet}OH+O_2^{\bullet-}$, than in their absence. In contrast, superoxide radical alone had little effect on β_2m and appeared only to reduce the essential disulphide bridge which holds the native molecule in a fairly rigid

conformation. However, when such cross-linkage was cleaved, c.d. data indicated that the β-sheet and β-turn structure did not collapse, and that the polypeptide chain did not adopt the unfolded state, as was the case after reactions with $^{\bullet}OH$ or $^{\bullet}OH + O_2^{\bullet-}$.

The mechanism of $^{\bullet}OH$ attack is well established as involving the direct reaction of $^{\bullet}OH$, via hydrogen abstraction, at a carbon atom in the α-position of a carbonyl group located either in the peptide bond or in the amino acid side chain [22]. The radical intermediate might decay harmlessly or could react randomly in forming covalent cross-links, by either inter- or intra-molecular reaction with an amino acid radical generated close by, as documented from reports using BSA as a model [24,27,31,58–61]. The probability of a reaction will depend not only on the distance from the radical species, but also on the intramolecular environment and flexibility of the molecule.

Interestingly, we found that dityrosine species were formed predominantly in irradiated β_2m samples containing aggregated proteins, i.e. at higher amounts of $^{\bullet}OH$ alone than in the presence of both $^{\bullet}OH + O_2^{\bullet-}$. This suggests the intermolecular rather than the intramolecular preference of transient tyrosyl-radical–tyrosine-residue reaction. Indeed, the reaction depended on the availability of a pair of tyrosine residues. Native β_2m contained about four (out of a total of six) tyrosyl residues which were accessible for reaction [62] and which were localized in β-strands belonging to separate sheets. Thus formation of dityrosine on conversion of aromatic rings to their radical form was highly probable as a mechanism for protein cross-linking (or protein aggregation). However, it was highly unlikely that dityrosine was the only covalent bond, although ring-structured radicals were known to be the most reactive [29,30]. However, any amino acid radical formed within a peptide chain could cross-link with an amino acid radical (or residue) in another protein chain [60,61].

In oxygenated β_2m solutions, we showed that the degree of both dimerization of tyrosine residues and cross-linkage of the β_2m molecule was not as efficient as in N_2O-bubbled solutions. Indeed, the aerated solution contained, as reactive agents, both $^{\bullet}OH$ and $O_2^{\bullet-}$. The latter might react with transient tyrosyl radicals [50], resulting in generation of tyrosine, thus preventing their transformation of dityrosine and the formation of chain cross-links. Moreover, in the presence of oxygen, the carbon radical was converted into a peroxyl radical known to be highly reactive and to cause extensive destruction of the molecule into fragments. Indeed, as a consequence of peroxyl radical decomposition, adjacent peptide bond cleavage and side-chain scission might occur [22,27,31]. In the present study, evidence for degradation was provided, first by the gradual decrease in the intensity of the starting native protein band and, secondly, by the generation of new protein species characterized by more acidic pI values than the starting sample, the latter becoming undetectable at high irradiation doses in the presence of oxygen. However, the lack of detection of smaller-sized fragments suggests that β_2m was not degraded in a regular manner to produce defined fragments which could be accumulated significantly. Further investigation using more resolutive methods (Tricine–SDS/PAGE [63] or avidin–biotin reaction [64]) produced no evidence for chain breaks (results not shown). Indeed, any amino acid might be susceptible to modification by $^{\bullet}OH$ radical action [60,61] and probably would cause multiple cleavage sites, although the protein structure could greatly influence reactivity with oxygen radicals.

In the present study, evidence for extensive oxidative protein modification was provided by the presence of an excess of negative charges on the newly generated protein species upon irradiation. Moreover, the destruction of tryptophan residues was observed and occurred at different rates for the two exposure conditions. Cross-linked aggregates would naturally become more resistant than unfolded fragments to further radical attack. The protective conformation of aggregates would shield a previously exposed tryptophan side-chain from $^{\bullet}OH$ attack and consequently would account for a partial protection of tryptophan against oxidation upon exposure to $^{\bullet}OH$ alone. In contrast, if the presence of oxygen caused protein fragmentation rather than protein aggregation, then the two tryptophan residues per β_2m molecule would be accessible and thus destroyed by oxidation.

Another major point of interest concerned the transient accumulation of two discrete protein species, with molecular mass values averaging 18 and 25 kDa, upon limited exposure to radicals. Concomitantly, a decrease in band intensity of the native β_2m polypeptide chain at 12 kDa and the β_2m derivative at 8 kDa was observed, together with the titration of 0.5 bityrosine residues/β_2m molecule upon exposure to 75 nmol of $^{\bullet}OH$ radicals. Therefore our overall data suggest that one major chain linkage occurred, which reflected the location of an intermolecular dityrosine bridge established between either two identical subunits (i.e. 2×12 kDa or 2×8 kDa) or the two initial reactants ($8 + 12$ kDa). Random interactions between the reactants were very likely to depend on their relative concentrations, and the least probable association would be 2×8 kDa. However, in the presence of oxygen, where protein fragmentation was much more pronounced, the species at 25 kDa was predominant. This observation was consistent with the disappearance of the two initial species at 12 and 8 kDa by cleavage at the same rate. Some cross-linkage might also occur, but would preferentially involve the more representative species, i.e. that remaining at 12 kDa.

It is interesting to note that our results on the co-existence of native β_2m at 12 kDa and its fragment at 8 kDa with other species at 18 and 25 kDa are compatible with the polypeptide composition comprising 8.5, 12, 17 and 24 kDa β_2m derivatives reported in amyloid deposits [15–18]. This indicates that the proposed mechanism of radical attack of β_2m could be of functional significance. This assumption is supported by data on the amino acid composition of the amyloid fibril protein purified from four patients with carpal tunnel syndrome [15]. Careful analysis of the relative deviation of content of each amino acid residue compared with native β_2m shows significant variation in glutamic acid, lysine, glycine, alanine, proline and tyrosine content (Table 1). These differences can be explained in terms of the three major characteristics reported in the present study for the structural modifications of β_2m induced by oxygen radicals. The generation of protein species with a more acidic pI could be due to the decrease in lysine residues and the slight increase in glutamic acid residues. A fragmentation mechanism, implying lateral chain scission, could be involved in the accumulation of an excess of glycine and alanine residues. Moreover, peptide bond cleavage at proline residues, as proposed in [27] for BSA, could be predicted from the disappearance of proline residues. Finally, cross-linkage involving the dityrosine bridge would be consistent with a significant decrease in tyrosine residues.

In conclusion, the present data support our initial hypothesis on the involvement of oxygen radicals in the formation of modified β_2m derivatives. These derivatives probably differ from the native β_2m by their molecular interactions with other proteins, thus leading to their precipitation as amyloid deposits. Whether a radical attack may occur *in vivo* and contribute to these modifications is conceivable, particularly as activated neutrophils, concurrently releasing oxidants and granule proteinases, were found together with amyloidosis in synovia. Therefore the possibility remains that synergy between radical-mediated polypeptide damage and proteolytic attack might be

Table 1. Amino acid composition of serum β_2m and amyloid β_2m protein

The amino acid composition of serum β_2m was established from sequence data published in [2]. The mean value for amyloid β_2m protein was calculated from the data published by Gejyo *et al.* [15], from four different patients.

Amino acid	No. of residues β_2m	No. of residues Amyloid β_2m protein	Relative change (%)
Asp	12	10.8	−10
Thr	5	4.1	−17
Ser	9	8.3	−8
Glu	11	11.9	+8
Pro	5	3.8	−23
Gly	3	4.5	+50
Ala	2	4.3	+116
Cys	2	2.1	−3
Val	7	6.2	−11
Met	1	0.6	−43
Ile	5	3.9	−20
Leu	7	6.6	−5
Tyr	6	3.3	−44
Phe	5	4.4	−12
Lys	8	6.1	−23
His	4	3.8	−3
Arg	5	4.3	−13
Trp	2	–	–

involved. This possibility is in agreement with reports from several laboratories showing that oxygen radicals cause susceptibility to proteolysis [26,65–68].

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