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Fragmentation of proteins by free radicals and its effect on their susceptibility to enzymic hydrolysis

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1. Defined radical species generated radiolytically were allowed to attack proteins in solution. 2. The hydroxyl radical ($OH^{\cdot}$) in the presence of O_2 degraded bovine serum albumin (BSA) to specific fragments detectable by SDS/polyacrylamide-gel electrophoresis; fragmentation was not obvious when the products were analysed by h.p.l.c. In the absence of O_2 the $OH^{\cdot}$ cross-linked the protein with bonds stable to SDS and reducing conditions. 3. The superoxide ($O_2^{-\cdot}$) and hydroperoxyl ($HO_2^{\cdot}$) radicals were virtually inactive in these respects, as were several other peroxyl radicals. 4. Fragmentation and cross-linking could also be observed when a mixture of biosynthetically labelled cellular proteins was used as substrate. 5. Carbonyl and amino groups were generated during the reaction of $OH^{\cdot}$ with BSA in the presence of O_2. 6. Changes in fluorescence during $OH^{\cdot}$ attack in the absence of O_2 revealed both loss of tryptophan and changes in conformation during $OH^{\cdot}$ attack in the presence of O_2. Increased susceptibility to enzymic proteolysis was observed when BSA was attacked by most radical systems, with the sole exception of $O_2^{-\cdot}$. 7. The transition-metal cations Cu^{2+} and Fe^{3+}, in the presence of H_2O_2, could also fragment BSA. The reactions were inhibited by EDTA, or by desferal and diethylenetriaminepenta-acetic acid ('DETAPAC') respectively. 8. The increased susceptibility to enzymic hydrolysis of radical-damaged proteins may have biological significance.

INTRODUCTION

Oxygen radicals are believed to be generated by a number of processes *in vivo*, including the 'respiratory burst' of phagocytic cells, metal-catalysed substrate autoxidations, mitochondrial electron transfer and the reduction of hydroperoxides by redox-active transition metals such as iron and copper [reviewed by Halliwell & Gutteridge (1984)]. The high reactivity of these species (by virtue of their unpaired electrons) has led to their implication in pathophysiology. One such instance is rheumatoid arthritis, in which radicals may degrade macromolecules in cartilage (Monboisse *et al.*, 1983; Dean *et al.*, 1984) and in synovial fluid (McCord, 1974). We have previously demonstrated that radicals generated within whole cartilage discs can degrade proteoglycan, apparently by selective fragmentation of the core protein (Dean *et al.*, 1984). This may simply reflect the higher reactivity of $OH^{\cdot}$ with proteins than with polysaccharides (Ross & Ross, 1977).

Considerable data indicate that radicals can inactivate proteins by modification of amino acid residues [reviewed, for instance, by Willson (1983)]. We have now studied the ability of a variety of quantitatively and qualitatively defined free radicals to fragment protein in solution, to extend previous work of Schuessler & Schilling (1984). We also consider changes in the susceptibility of proteins to enzymic hydrolysis after radical modification and fragmentation. We have also examined the ability of H_2O_2 to fragment protein in the presence of several transition-metal chelates. H_2O_2 has previously been shown to modify mucus glycoproteins (Creeth *et al.*, 1983*a*,*b*) [and damage many simple proteins (Gutteridge & Wilkins, 1983)] in reactions dependent upon the presence of adventitious metal complexes (Halliwell & Gutteridge, 1984).

MATERIALS AND METHODS

Proteins

Bovine serum albumin (BSA) was obtained from Boehringer Mannheim and dialysed for 24 h against deionized water before use. BSA (defatted) from Sigma was also used in several experiments, with similar results. ^{3}H-labelled proteins were prepared by labelling Chinese-hamster ovary (CHO) cells with a tritiated amino acid mixture (Amersham) for 24 h as described previously (Cockle & Dean, 1984). Cells were harvested by scraping; washed and repelleted in phosphate-buffered saline (Dulbecco A) three times and passed ten times through a 19-gauge syringe needle. Finally, cells were sonicated and membranes sedimented by centrifugation at 30000 *g* for 3 h. The resultant supernatant was dialysed for 24 h against deionized water and consisted of equal amounts of 5%-(w/v)-trichloroacetic acid-soluble and -insoluble protein at equilibrium (CHO-cell extracts contain high proteolytic activity). Picrylsulphonic acid and dinitrophenylhydrazine were obtained from Sigma. The BCA protein assay reagent was obtained from Pierce and

Abbreviations used: $OH^{\cdot}$, hydroxyl radical; BSA, bovine serum albumin; CHO, Chinese-hamster ovary; DETAPAC, diethylenetriaminepenta-acetic acid; $O_2^{-\cdot}$, superoxide radical; $HO_2^{\cdot}$, hydroperoxyl radical.

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Warriner (Chester, U.K.) and sodium cyanoborohydride ($NaCNBH_3$) from Aldrich (Gillingham, Dorset, UK). All other reagents were of the highest purity available.

Radical generation

Radical species were generated (homogeneously in solution) by using a 2000 Ci cobalt source (Willson, 1978; Dean *et al.*, 1984) at a dose rate of 4.39 krad/min. For 1 ml of a 1 mg/ml solution of BSA (150 μM), a theoretical radical/protein molar ratio of 10 corresponds to 28.3 krad or 150 nmol of radicals. Irradiation of proteins was performed in 10 mM-potassium phosphate buffer, pH 7.4, at room temperature, a total volume of 1 ml being used. Solutions were saturated with the appropriate gas by blowing gas over the surface for 15 min before irradiation and continuously during it. All reagents were made up in deionized water. In some experiments H_2O_2 (2.5 mM) was present with various combinations of Fe^{3+}, Cu^{2+} (100 μM) and the chelating agents DETAPAC, EDTA and desferrioxamine mesylate (110 μM) in 10 mM-potassium phosphate buffer, pH 7.2, at 37 °C.

Analysis of products of radical attack

SDS/polyacrylamide-gel electrophoresis on 10% (w/v) gels was run with dithiothreitol as reducing agent. Loss of 68 kDa starting material was measured from gel scans after Coomassie Blue staining. The extent of staining was proportional to the amount of protein in the gel over the range studied. Fragmentation of the starting material was studied by using a sensitive silver stain (Oakley *et al.*, 1980). All the observations described here were, however, made with both staining techniques. Damaged protein was also studied under non-denaturing conditions by h.p.l.c. on a TSK 3000SW column (Pierce and Warriner). The generation of material soluble in 5% trichloroacetic acid was assessed by using the Pierce BCA assay. Catalase (0.1 μM) was used to remove remaining H_2O_2 (which interferes with the Lowry assay) in relevant experiments. For the ^3H-labelled proteins, distribution of counts between 5%-trichloroacetic acid-soluble and -insoluble fractions (formic acid-solubilized) was measured. Carbonyl groups were estimated by the dinitrophenylhydrazine method (Friedemann & Haugen, 1943), and free amino groups by using picrylsulphonic acid in the presence of 1% SDS (Habeeb, 1966).

Protein-modification studies

Susceptibility to tryptic digestion (Rupley, 1967) and tryptophan-fluorescence alterations (Shaklai *et al.*, 1984) were studied. In the former case, 5 mg of modified protein was incubated with 2 or 6 μg of trypsin at 37 °C in 10 mM-potassium phosphate, pH 7.2. At different time intervals the incubation mixture was made 5% with respect to trichloroacetic acid and precipitated in a bench centrifuge. Trichloroacetic acid-soluble peptides were measured. Tryptophan fluorescence was measured between 320 and 420 nm during excitation at 275 nm of BSA at 10 μg/ml in 10 mM-potassium phosphate buffer, pH 7.2, or 4 M-guanidinium chloride (precleaned with activated charcoal).

Data points on graphs represent the means of at least three determinations (unless otherwise stated). S.D. values are omitted for clarity, but in all cases were less than 3% of the mean.

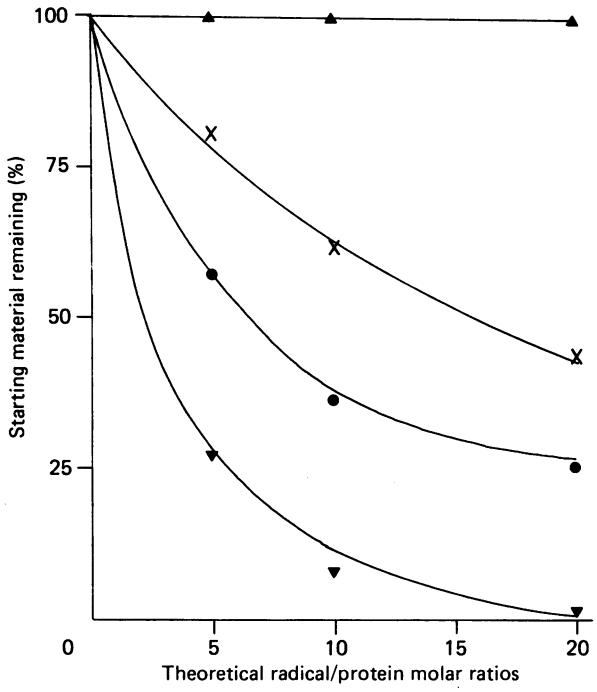

Fig. 1. Destruction of BSA monomer by radical species

The percentage loss of starting BSA monomer (15 μM in 10 mM-potassium phosphate buffer, pH 7.2) was estimated by scanning SDS/10% polyacrylamide gels after staining. Peaks were cut out and weighed. $OH^{\cdot}$ converters were added to 10 mM; the Cu^{2+}/BSA molar ratio was 3. Symbols: ▲, $O_2^{-\cdot}$, $HO_2^{\cdot}$, $OH^{\cdot}/O_2$/aspirin, $OH^{\cdot}/O_2$/thymine, $OH^{\cdot}/O_2$/glucose; ×, $OH^{\cdot}$; ●, $OH^{\cdot}/O_2$; ▼, $OH^{\cdot}/O_2^{-\cdot}/O_2$.

RESULTS

Protein M_r changes induced by radical species: defined fragments are produced

Fig. 1 shows the destruction of BSA monomer by various radical species. The $OH^{\cdot}$ radical itself is essential for destruction of the 68 kDa monomer, and this is sensitized by the presence of molecular O_2 and surprisingly, by $O_2^{-\cdot}$. As judged by SDS/polyacrylamide-gel electrophoresis, $OH^{\cdot}$ alone (without O_2) causes production of high-M_r covalent aggregates stable to reduction, whereas $OH^{\cdot}/O_2$ causes no such aggregation but, rather, extensive fragmentation. In contrast with $OH^{\cdot}$, $O_2^{-\cdot}$ or $HO_2^{\cdot}$ as well as the reactive peroxy radicals derived from thymine and aspirin (Willson, 1978), glucose and glyceraldehyde cause no change in the M_r of the protein, even up to a theoretical radical/protein molar ratio of 50. Alanine and leucine were added (at 10 mM) in some experiments, and gave 20 and 80% protection respectively, at a theoretical radical/protein molar ratio of 25. Cu^{2+}, added at a 3-fold molar excess to the protein, did not sensitize the protein to $O_2^{-\cdot}$-mediated damage (at least with respect to M_r changes) (Fig. 1).

SDS/polyacrylamide-gel electrophoresis of the attacked protein (not shown) reveals the generation of specific fragments by $OH^{\cdot}$ attack in the presence of O_2. Formation of the fragments (about ten in the M_r range 68000–25000) occurs up to about 25 radicals per monomer, after which there is a generalized smearing of the BSA monomer towards the lower-M_r position. The

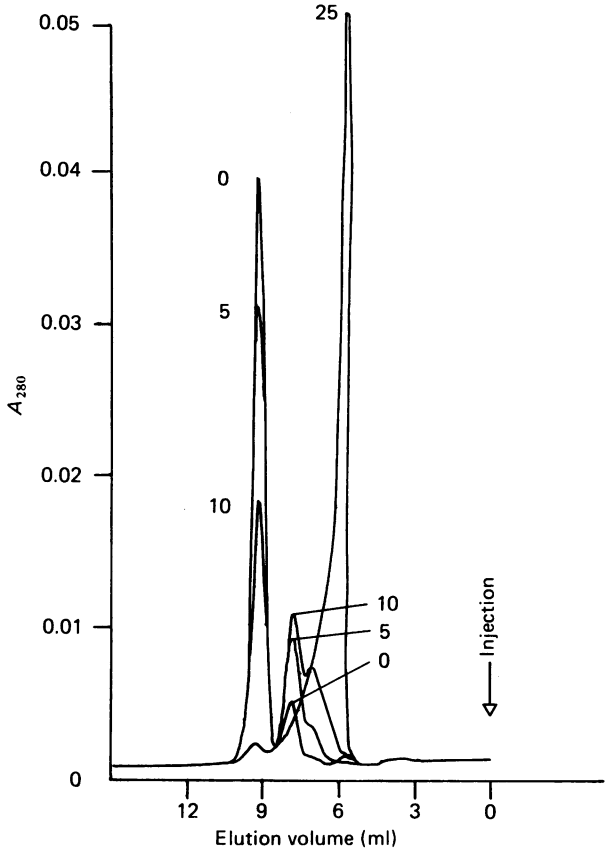

Fig. 2. H.p.l.c. elution profiles of BSA at increasing radical/protein molar ratios (OH· system)

A Pierce TSK 3000SW gel-filtration column was used. A 20 μl portion of BSA (1 mg/ml) was injected in each case and eluted in 10 mM-potassium phosphate, pH 7.2, containing 100 mM-KCl; the flow rate was 1.5 ml/min.

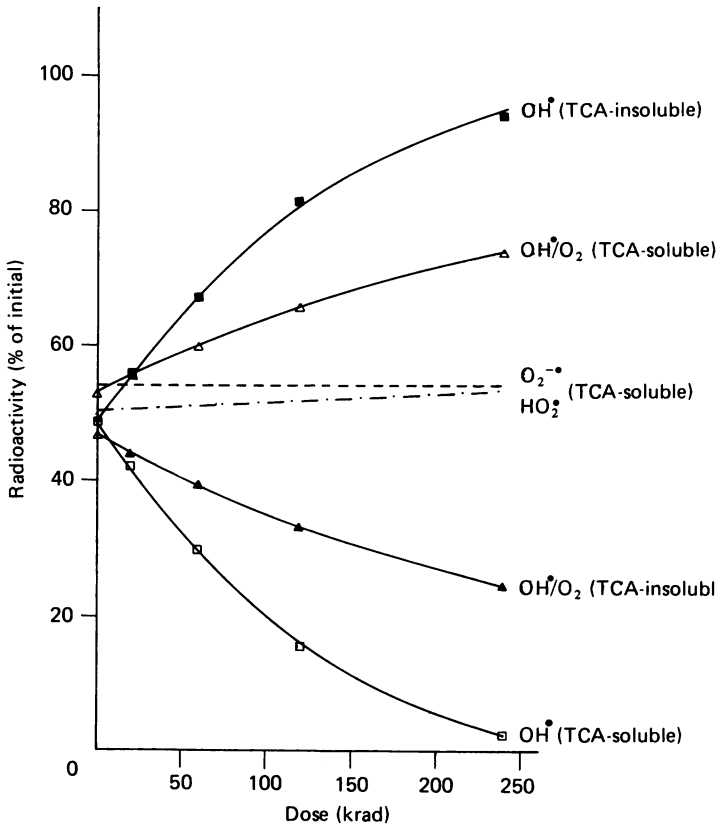

Fig. 3. Fragmentation and cross-linking by OH·

CHO-cell soluble proteins (see the Materials and methods section) labelled with tritiated amino acids (10 mg/ml) together with BSA (1 mg/ml) were exposed to radical attack. Doses of up to 240 krad were used, after which trichloroacetic acid (TCA)-soluble and -insoluble radioactivity was measured. No radioactivity was lost from the system. Similar results were also obtained in parallel experiments using human monocytes labelled with [1-^{14}C]leucine.

data for OH· attack were very similar to those shown by Schuessler & Schilling (1984). The gels confirm that the O_2-dependent fragmentation process is enhanced by $O_2^{-\cdot}$ (as in Fig. 1).

H.p.l.c. of the damaged protein under non-denaturing conditions, by contrast, shows only slight evidence of fragmentation by OH· in the presence of O_2 (not shown), although polymerization by OH· alone (Fig. 2) is evident. This suggests that fragments produced by O_2 are only dissociated by the aggressive conditions needed for SDS/polyacrylamide-gel electrophoresis.

The generation of carbonyl and amino groups during radical attack was examined: amino groups increased initially and then decreased with increasing radical attack. Carbonyl groups increased progressively (not shown). In each case positive results were only obtained with OH·/O_2 [as shown previously for carbonyl groups (Garrison, 1968; Dean *et al.*, 1985)].

Fig. 3 represents the distribution of radioactivity in trichloroacetic acid-soluble and -insoluble fractions after radical damage to CHO proteins and peptides in the presence of BSA (1 mg/ml). OH· in the absence of O_2 causes the relocation of trichloroacetic acid-soluble radioactivity into the trichloroacetic acid-insoluble fraction and thus causes the cross-linking of protein. Other results are consonant with those for BSA alone, and in both cases fragmentation by OH· is dependent on the presence of O_2. $O_2^{-\cdot}$ and $HO_2^{\cdot}$ produce no M_r changes; this result shows also that intrinsic proteolysis is not a significant factor over the time periods involved. OH· interconverts the trichloroacetic acid-soluble and -insoluble fractions more rapidly in the absence of O_2 than in its presence. The converse is seen for destruction of the BSA monomer (Fig. 1). However, the former case concerns exchange between species of M_r above or below a value of about 5000, and not all fragmenting attacks will produce species with M_r values of less than 5000. In contrast, all fragmenting attacks will cause loss of the starting 68000-M_r monomer.

Radical-induced conformational change

BSA attacked by OH·/O_2 became decreasingly resistant to tryptic hydrolysis as the number of radical events per monomer increased (Fig. 4). Enhancement of proteolysis was, however, not dependent upon direct fragmentation reactions (Fig. 5). OH· (in the absence of O_2), which cross-links the protein, and the thymine-derived peroxy radical (which induces no M_r changes) both render the protein less resistant to enzymic proteolysis. $O_2^{-\cdot}$ was the

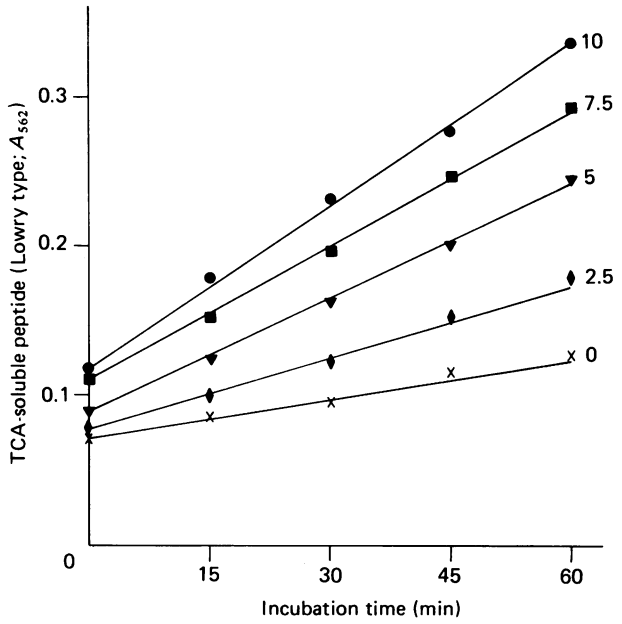

Fig. 4. Enhanced tryptic susceptibility of BSA after limited radical (OH/O_2) attack

BSA at 10 mg/ml was exposed to 0, 2.5, 5, 7.5 or 10 radicals per molecule (as indicated by the respective lines on the Figure). After the appropriate irradiation, 0.5 ml of BSA was removed, 0.04 ml of trypsin (50 μg/ml) added, and the mixture incubated at 37 °C for the specified times. Subsequently, material soluble in 5% trichloroacetic acid (TCA) was prepared, and the Pierce BCA assay used to measure peptides therein.

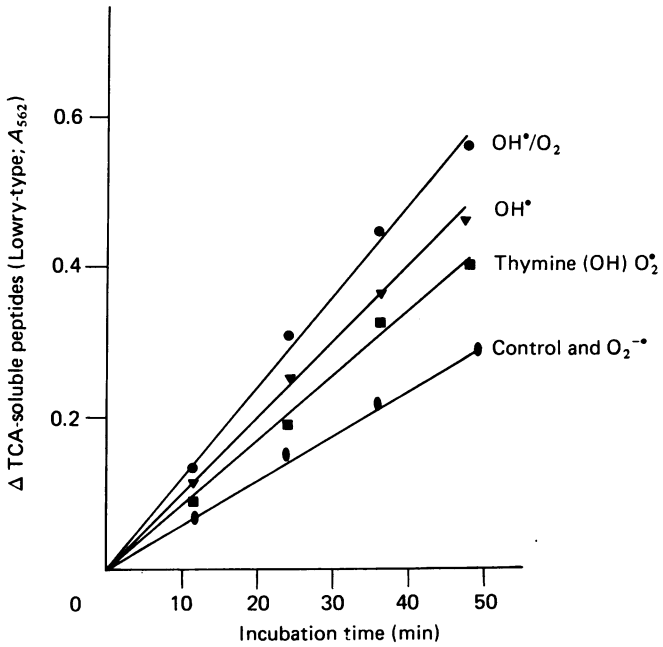

Fig. 5. Tryptic proteolysis of BSA after attack by various radical species

In each case BSA was exposed to 5 radicals/molecule before enzymic hydrolysis. TCA (trichloroacetic acid)-soluble products after hydrolysis were measured as for Fig. 6, but the trichloroacetic acid-soluble reactivity present at zero time in each system has been substracted from all corresponding values.

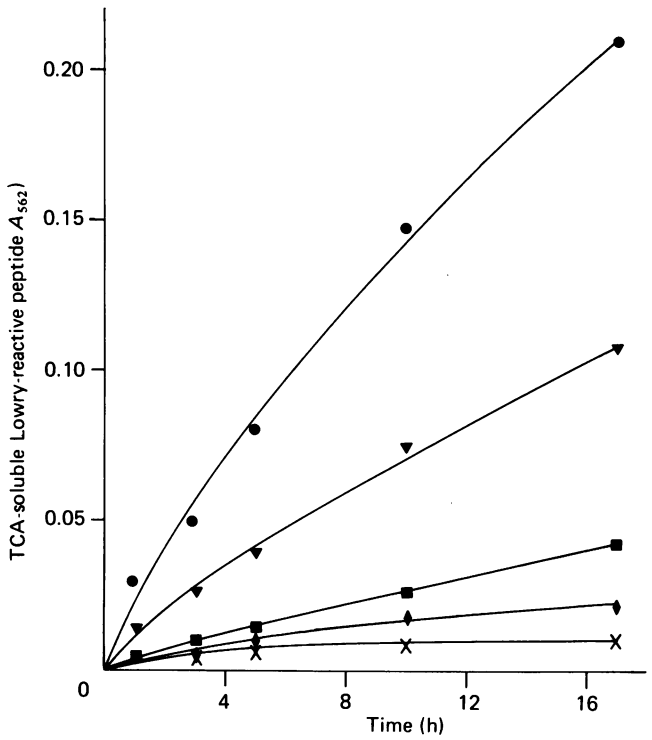

Fig. 6. Release of trichloroacetic acid (TCA)-soluble peptides from BSA by H_2O_2 in the presence of transition metals

BSA (1 mg/ml) was incubated with 2.5 mM-H_2O_2 at pH 7.2 (10 mM-potassium phosphate buffer) at 37 °C. Metals were added at 100 μM and chelators at 110 μM (except phosphate, which was 10 mM in all cases). After the specified times, aliquots were incubated (15 min at room temperature) with catalase (100 nM) to remove H_2O_2 before precipitation of protein in 5% trichloroacetic acid (added as 0.08 ml of a 37.5% solution). Trichloroacetic-acid-soluble fragments in 0.1 ml of the resulting supernatant were then measured by using the Pierce BCA modification of the Lowry assay (with which H_2O_2 interferes). SDS/polyacrylamide gels showed that, after 17 h of degradation by Cu^{2+} and H_2O_2, no intact BSA monomer remained, and a smear of smaller products was obtained. Essentially similar results were obtained when H_2O_2 was used at 25 mM. Symbols: ●, Cu^{2+} (100 μM); ▼, Fe^{3+}/EDTA; ■, Cu^{2+}/EDTA; ◆, Cu^{2+} (10 μM); ×, Fe^{3+}/DETAPAC, Fe^{3+}/desferrioxamine mesylate, and control (no additions).

only radical species tested that caused no conformational change, as assessed by proteolytic susceptibility. The alteration in conformation was confirmed by fluorescence studies: tryptophan fluorescence is greatly decreased by OH/O_2 (tryptophan fluorescence after 10 $OH^{\bullet}/O_2$ was ~ 55% of the control) whereas $OH^{\bullet}$ produces a relatively small decrease. Fluorescence decreases in BSA may be due to either tryptophan oxidation or solvent exposure of the 'buried' second tryptophan residue. Denaturation of BSA in 5 M-guanidinium chloride (in which fluorescence of tryptophan groups is comparable) reveals a 30% decrease in relative fluorescence after 10 OH/O_2. Diminution of fluorescence thus results from both conformational change and tryptophan oxidation.

Protein fragmentation by H_2O_2

We also tested the ability of H_2O_2 to fragment BSA and produce trichloroacetic acid-soluble peptides in the presence of Fe^{3+} and Cu^{2+} chelates (Fig. 6). 'Free' copper (actually bound to BSA and/or phosphate) is an effective catalyst, as are the Fe^{3+}- and Cu^{2+}–EDTA complexes. Fe^{3+}–DETAPAC and -desferrioxamine complexes, like H_2O_2 without further additions, are inert over the range of conditions employed.

DISCUSSION

Garrison (1968) proposed destruction of model peptides via labile iminopeptides generated from peroxy radicals. The iminopeptide intermediates can be hydrolysed under conditions conventionally used to displace ammonia from amides [1 M-H_2SO_4 for 3 h at 100 °C (Sokol *et al.*, 1965)]. More recently, Schuessler & Schilling (1984) observed that fragments of defined length were produced from BSA by radiolytically generated $OH^{\cdot}$. They proposed that peroxy radicals, produced randomly at the peptide α-carbon atoms, attacked residues of the imino acid proline, leading to specific sites of fragmentation, since fragment sizes were those expected if cleavage occurred only at proline residues. We have observed similar fragmentation and found that it can only be produced by $OH^{\cdot}$ in the presence of molecular O_2. Further, the inhibition of fragmentation by amino acid scavengers (rather than enhancement) would suggest that there is little transfer of damage from side-chain peroxy radicals to cleavage-sensitive sites.

We thus propose that $OH^{\cdot}/O_2$ must cause direct oxidation of the residue(s) crucial to integrity. Other amino acid side chains may protect the crucial residues, and this is consistent with the protection afforded by leucine and alanine. Iminopeptides [of the type proposed by Garrison (1968)] are either extremely labile or do not contribute to the specific fragmentation process, as we were unable to repair the protein (before SDS/polyacrylamide-gel electrophoresis) with $NaCNBH_3$ [a reagent selective for iminopeptide bonds (Jentoft & Dearborn, 1979)]. Rather surprising was the increase in free amino groups. This would not be predicted by Garrison's (1968) model and suggests that oxidation of crucial residue(s), perhaps proline, leads to spontaneous peptide hydrolysis. We have found that protein fragmentation also occurs in the presence of peroxidizing lipid, and this may implicate alkoxy radicals in such gross protein damage (S. P. Wolff & R. T. Dean, unpublished work).

Although $OH^{\cdot}/O_2$ is the only radical system capable of cleaving protein directly, several radical species (at low levels) are able to produce conformational changes in the protein which markedly enhance its susceptibility to enzymic hydrolysis. We thus suggest synergism between radical damage and decreased hydrolytic resistance. Treatment of haemoglobin, glomerular basement membrane and fibronectin with H_2O_2 has similarly been shown to render these substrates more susceptible to hydrolysis by a number of proteinases, including trypsin and elastase (Fligiel *et al.*, 1984). It has also been noted previously that the oxidation of histidine residues in glutamine synthetase *in vitro* renders the protein more susceptible to proteolysis (see Levine *et al.*, 1985). In agreement with the synergism suggested above, we have shown that the extent of radical flux in mitochondria correlates positively with the rate of intramitochondrial proteolysis (Dean & Pollak, 1985). These findings may have pathophysiological significance in extracellular components such as cartilage and also physiological relevance within cells.

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