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INEFFICIENT DEGRADATION OF OXIDIZED REGIONS OF PROTEIN MOLECULES

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We have previously shown that the intracellular half-life of endocytosed oxidized albumin is much longer than that of native albumin. We now report that the regions of oxidized albumin which contain oxidation products (carbonyls and fluorophores), are less readily released as small degradation products by cell-free proteolysis than is the molecule overall. We deduce that oxidized moieties in the polypeptide chain can confer localized resistance to enzymatic proteolysis. Such resistance to proteolysis may account for the intracellular accumulation of some endocytosed oxidized protein which we have previously observed.

KEY WORDS: Oxidized protein, proteolysis, hydrolysis, macrophage, oxidised, proteolytic resistance.

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INTRODUCTION

Several studies have shown that oxidized proteins are more readily degraded than native proteins in cell-free systems containing one or a limited number of proteinases.[1-4] In addition, during degradation of endocytosed oxidized bovine serum albumin (BSA) by intact macrophages overall catabolism was more rapid than for native protein.[5] Concomitantly, some of the oxidized protein accumulated within the cells, and had a long intracellular half-life. The amount which accumulated increased both with the extent of oxidation and with the time of incubation with cells. The estimated half life of the accumulated pool was more than 12 h, whereas that for native protein was less than 10 min.[5] Using BSA which had received 42 radicals per protein molecule, the amount of this oxidized protein that accumulated intracellularly (200 ng/mg cell protein) after 24 h continuous incubation with macrophages, was equal to the amount that was degraded.[5] Even after a subsequent 24 h "chase", 35% of the accumulated macromolecular oxidized albumin remained within the cells, suggesting that some components of the oxidized protein were especially resistant to proteolysis.

We have now examined the nature of oxidized protein which is less readily degraded. Two commonly used markers of oxidized protein are carbonyl groups, and a variety of fluorophores (excited between 300 and 400 nm) which are thought to be derived from aromatic residues on the proteins, particularly tryptophan.[6-10] We have compared the overall protein hydrolysis in a cell-free system modelling lysosomal proteolysis, with the rate of release of small fragments which contain these markers of oxidation. Regions of the molecule containing these oxidized moieties are less readily degraded than the bulk of the polypeptide. We suggest that

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their resistance to proteolysis may explain the observed long intracellular half life of oxidized protein as compared with native protein.

MATERIALS AND METHODS

Preparation of Oxidized Protein

Bovine serum albumin (10 mg/ml, w/v, Fraction V, A6793, Sigma, USA) was dissolved in 10 mM potassium phosphate buffer (pH7.2) and exposed to radicals generated by ^{60}Co radiolysis of H_2O (at Macquarie University, Sydney) while gassing continuously with O_2. Radiation doses of 0–11,000 Gy were used, which correspond as in previous work to 0–100 radicals per molecule of BSA.[2] Relatively high absolute radical doses were used in these experiments because high concentrations of albumin (10 times higher than in most previous work) were needed to permit the experiments defined below. Under these conditions, superoxide (O_{2-}) and hydroxyl (OH.) radicals are formed in approximately equal amounts.

For experiments determining the release of carbonyl and fluorophore-containing fragments, BSA which had received a dose of 42 radicals per molecule, was passed through a PD10 gel filtration column equilibrated with 10 mM potassium phosphate buffer, to remove low molecular weight carbonyls generated during radiolysis. Previous literature indicates that these include keto acids and peptide fragments.[11] These small carbonyls could contribute up to 25% of the total carbonyls present in the irradiated protein solution before gel filtration. Recovery of protein and fluorescence from the column was 90%.

After PD10 filtration, oxidized BSA contained approximately 2 moles carbonyls per mole-equivalent of 68 kDa albumin monomer. The fluorophores we measured in the oxidized protein have not been fully characterized, and may be quite diverse. After irradiation there was a 95% loss of tryptophan fluorescence in agreement with previous data.[2,5] Thus, if each oxidized tryptophan gave rise to a fluorophore, one would expect at least 2 fluorophores per molecule of protein. Our measurements of the fluorescence yield of likely fluorophores (such as kynurenine, dopa, dityrosine) indicate that this estimate of fluorophores per molecule is probably an underestimate.

Homogenate Preparation

To determine the proteolytic susceptibility of oxidized BSA components, we used macrophage homogenates as the source of enzymes thus bypassing the endocytosis step. This method enabled us to use larger amounts of BSA in which oxidized components were readily measurable.

Resident peritoneal macrophages were collected from QS mice and cultured overnight as previously described.[5,12] Cells were washed four times with phosphate buffered saline (PBS), carefully scraped off the dishes then centrifuged at 15,000 g for 10 min, resuspended in PBS and cell viability measured by trypan blue exclusion. Cells were further centrifuged then ruptured by resuspension of the cell pellet in H_2O at a concentration of $\sim 2 \times 10^7$ viable cells/ml, freezing at $-80°C$, and sonication for three periods of 10 seconds with equal intervals on ice, to provide a cell-free homogenate. Protein and peptide concentration were determined using the fluorescamine assay for free amines[13] and BSA Fraction V as a standard.

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Hydrolysis of Protein

Conditions were chosen to provide detectable oxidized moieties with minimal interference by cell homogenate components, for example, carbonyl molecules within cell nuclei. BSA (2 mg/ml) was incubated with macrophage homogenate (200 ug protein/ml) in 53 mM citrate buffer pH3.5 at 37°C in a total volume of 450 ul. Controls were incubated in buffer only. At each time point, samples were removed from the same tube for the measurement of protein and peptides, carbonyls or fluorescence, for both the whole system and for molecules soluble in 5% trichloracetic acid (TCA). To obtain the TCA-soluble fraction, an equal volume of 10% TCA was added to a sample and the mixture vortexed, left on ice for 15 min then centrifuged for 10 min at 16,000 g and the TCA-supernatant removed for analysis. Because free amino groups are generated during hydrolysis of protein by homogenate enzymes giving a gradual increase (~30%) in total amines over 8 h, the actual protein hydrolysis determined as a percentage of the total amines at each time point, has probably been underestimated. The majority of free amino groups are contributed by lysine side chains, and so their release into the TCA-supernatant gives a reasonable estimate of the proportion of peptide mass released into the supernatant; these estimates concur with those made independently by means of the bicinchoninic acid (BCA) protein assay[14] which, like the Lowry assay, is largely dependent on aromatic residues.

Carbonyl Assay

Total and TCA-soluble carbonyls were measured using 2, 4-dinitrophenylhydrazine (DNPH, Sigma, USA). An equal volume of 0.02% (w/v) DNPH in 1 M HCl was added to 150 ul of sample and the mixture incubated at 50°C for 30 min then cooled. A 300 ul aliquot of 30% (w/v) KOH was added and the absorbance read exactly 5 min after KOH addition in a Hitachi U-3210 spectrophotometer at 450 nm. An extinction coefficient of $2.4 \times 10^4\,M^{-1}\,cm^{-1}$ was used[11,15] as this represents an average for a variety of different carbonyl compounds under alkaline conditions. BSA contained fatty acids but comparison with fatty acid-free BSA in separate experiments showed no evidence that these contributed to detectable carbonyls measured with DNPH.

Measurement of Fluorophores

Fluorescence of native and oxidized BSA was measured in 1 cm pathlength cuvettes in a Hitachi F-4010 spectrofluorimeter using 5 nm slit widths. Wavelengths of 332 excitation and 416 emission were chosen, because these gave the maximum fluorescence for BSA exposed to 42 radicals per molecule. However, another fluorescent peak with about 90% of the intensity of the main peak, was also evident at about 360 nm emission (excitation 332 nm) which suggests the presence of more than one fluorophore. Several different reported fluorophores, as yet incompletely defined, may contribute to the fluorescence spectrum in this region.[8,10]

Increasing the pH has been reported to increase the intensity of a fluorophore in metal/hydrogen peroxide oxidized human serum albumin by >600% in this region.[9] However, between the pH range of 3.5 and 9.9, the fluorescence intensity (excit. 332, emiss. 416 nm) in our oxidized BSA samples varied by only ≤15%. Nevertheless, all fluorescence measurements within each experiment were made at the same pH.

Release of TCA-soluble fluorescent molecules was compared with hydrolysis of

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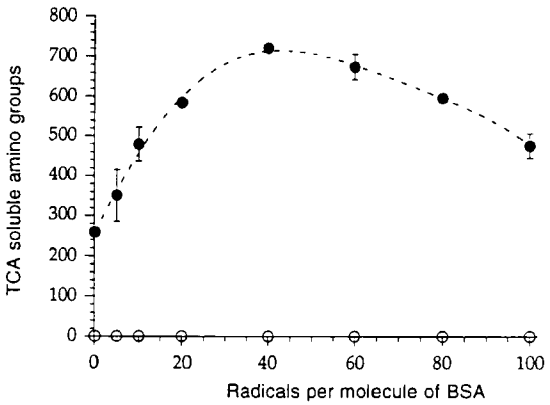

FIGURE 1 Hydrolysis of oxidized BSA by Trypsin. Irradiated BSA (0–100 radicals per BSA molecule) was incubated at 5mg-ml with trypsin (Sigma, Type XIII) at 2ug-ml in 10mM phosphate buffer pH 7.2 at 37°C for 1h. The increase in TCA-soluble protein-amino groups was measured. Determinations at 0h (open symbols) and 1 h (closed symbols) are shown. Fluorescence units are arbitrary

total protein. For fluorophore measurement, samples from the total incubation solution, and from the TCA-soluble supernatant were adjusted to pH 7.4 by the addition of minimal volumes of 3 M Tris, pH 7.4, before measurement of fluorescence (excit. 332, emiss. 416 nm).

Because preliminary experiments showed that TCA had the potential to quench by about 40% the fluorescence of BSA which had been acid hydrolysed to amino acids, we chose a second method to verify these results. Samples of the incubation mixture were concentrated in Centricon 10 concentrators (Amicon, Australia), M_r exclusion $< 10{,}000$, in a Hitachi Himac centrifuge (4500 g, 4°C). After 20 min centrifugation, approximately half the added volume had passed through as effluent. Effluent samples were measured for the appearance of peptides and fluorescence. For fluorophore measurement, as all samples were at the same pH in incubation buffer (pH 3.5), fluorescence was measured without further pH adjustment.

Data Presentation

Data are expressed as the mean of two, or the mean of three replicates +/−S.D., from single experiments representative of several experiments.

RESULTS

Susceptibility of Oxidized Albumin to Proteolysis

Figure 1 shows that oxidized BSA (0–100 radicals per molecule) is more susceptible to proteolysis by trypsin than native BSA (measured using fluorescamine) as found in previous *in vitro* experiments with individual proteinases.[1,2,4] An almost identical curve was obtained using the BCA assay. This data also shows that more extensive oxidation can lead to a gradual reduction in this increased proteolytic susceptibility as observed previously.[1,3]

We chose for further experiments albumin which had received a dose of 42 radicals

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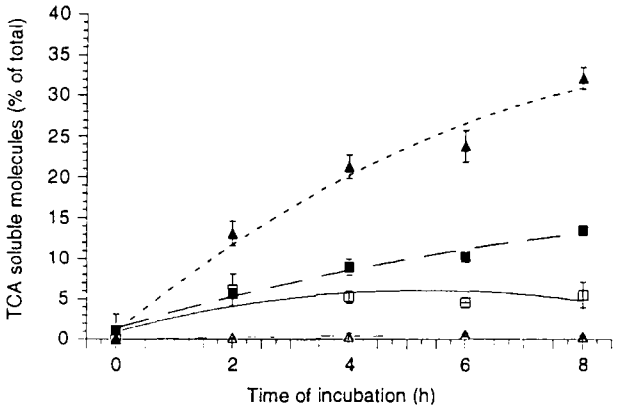

FIGURE 2 Release by hydrolysis of protein-amino groups and protein-carbonyls by macrophage homogenate. 2mg-ml oxidized BSA (42 radicals per molecule of BSA) was incubated with macrophage homogenate (200 ug protein-ml) as described in Methods. Total and TCA-soluble protein and carbonyls were measured at each time point and release of each expressed as % of the total. Protein hydrolysis: open triangles , BSA in buffer; closed triangles, BSA + homogenate. Carbonyl release: open squares, BSA in buffer; closed squares, BSA + homogenate.

per molecule, because it was the form of albumin most susceptible to trypsinolysis. However, in contrast, there was no enhancement in the macrophage homogenate hydrolysis of oxidized protein between 0–100 radicals per molecule, after 4 h incubation at acid pH (data not shown). We used incubation conditions of acid pH because earlier work showed that hydrolysis of BSA by lysed macrophages was higher at acid than neutral pH[5], and also because these are the conditions to which an endocytosed protein is exposed. In this system, a multiplicity of lysosomal hydrolases are active[16] rather than a single proteinase.

Comparison of Proteolytic Generation of Carbonyl-containing Fragments with Overall Protein Hydrolysis

For this and subsequent experiments we used a model lysosomal proteolysis system, in which macrophage cellular membrane barriers were broken and substrate molecules were exposed to the cellular degradative enzymes at acid pH.

Figure 2 shows that during 8 h incubation under such conditions, up to 32% of the total protein is progressively hydrolyzed to TCA soluble peptides by macrophage homogenate, as measured by the generation of small molecules containing amino groups. In contrast, the net release of low molecular weight carbonyls by enzymatic hydrolysis, is much slower, reaching only 8% after 8 h hydrolysis.

In this experiment and a range of others, we found that under these incubation conditions, the total carbonyl content of the incubations was constant throughout the period of incubation. Even when extensive hydrolysis (96%) of the protein was achieved with trypsin (Type 1, 45 ug/mg BSA) in a prolonged incubation (21 h), the recovery of carbonyls remained essentially constant.

Because each protein substrate molecule in this system contained on average two carbonyl groups (see methods), we conclude that those areas of individual albumin molecules which contain carbonyl functions are less susceptible to proteolysis than the surrounding polypeptide regions. However, even carbonyls in these areas of the

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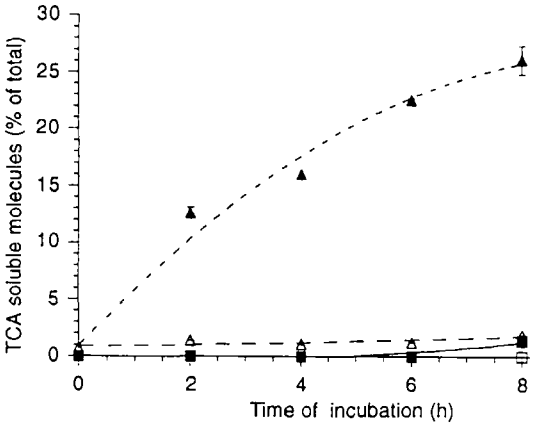

FIGURE 3 Release by hydrolysis of protein-amino groups and protein-fluorophores by macrophage homogenate. Release was measured as described in Fig 2 and in Methods.Protein hydrolysis: open triangles, BSA in buffer; closed triangles, BSA + homogenate. Fluorophore release: open squares, BSA in buffer; closed squares, BSA + homogenate.

molecules were released to the extent of 89% as TCA-soluble fragments during extensive trypsinolysis (as above). This confirms that the DNPH reaction is comparably sensitive for carbonyls in small and large molecules.

Comparison of Proteolytic Generation of Fluorophore-containing Fragments with Overall Protein Hydrolysis

Figure 3 shows that release of fluorescent molecules is also very slow compared with the overall hydrolysis of the protein as measured by release of amino groups. While 26% of the total protein was released as TCA-soluble molecules in 8 h, only 2% of the fluorescent molecules were released. Other studies demonstrated that TCA quenched the fluorescence of oxidized BSA which had been acid hydrolysed. Therefore, we confirmed the result of Figure 3, using an alternative analytical procedure for measurement of low molecular weight products of BSA hydrolysis utilizing Centricon 10 filtration (see Methods), This method indicated that whereas 18.1 ± 1.3% of the total protein was released as small molecules ($< 10{,}000\ M_r$), only 1.8 ± 0.3% of the fluorescent components were released.

As for the carbonyl assay, the validity of the fluorophore analysis was confirmed by the demonstration that after extensive trypsinolysis (96% of protein TCA-soluble) the recovery of fluorophores was essentially unchanged. We again conclude that areas of the albumin molecules containing the fluorophores are less susceptible to proteolysis than the surrounding polypeptide. In this case also, extensive trypsinolysis could release 85% of the fluorophores as TCA-soluble fragments.

DISCUSSION

Our data show that two categories of oxidized moieties in oxidized BSA, carbonyls and fluorophores, are less readily released in low molecular weight fragments than are other regions of the molecule. Since there are several carbonyls and fluorophores

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per protein molecule, we deduce that regions of individual polypeptides which contain the oxidized species, are less readily hydrolyzed than are other regions of the molecule. Our observations have an earlier parallel in the work of Dillon who found an endopeptidase-resistant fluorescent fraction isolated from human cataractous lenses.[17]

Several fluorophores excited at about 325 nm and with an emission band in the region 400–440 nm have been observed in various oxidized proteins.[7,8,10,18,19] These have been attributed to either N-formylkynurenine[7,8,10] or dityrosine[18,19] by comparison of fluorescence spectra with those of compounds known to exhibit fluorescence in the same regions. We have recently identified protein-bound DOPA (2,3-dihydroxyphenylalanine) as amongst these fluorophores.[20,21] However, the exact nature of the other fluorophores has not yet been determined.

Since we are using a complex mixture of proteinases acting under conditions of lysosomal pH, we envisage that many of the intracellular proteinases are inefficient in degrading oxidized regions of protein molecules. Perhaps this is because some of the oxidized amino acids fit less readily into the substrate binding sites of the proteinases than do their native counterparts. The reported resistance of oxidized alanine dipeptides to proteolysis by aminopeptidases and carboxypeptidases supports this theory.[22] Presumably the reduced substrate affinity in a localized area overcomes the usual accelerating effect of protein unfolding upon susceptibility to hydrolysis by proteinases.[23,24] Unfolded but chemically unmodified regions of individual molecules in oxidized protein, may still therefore be degraded more rapidly than their counterpart regions in native molecules under some conditions (Fig. 1).

The biological implications of these results are interesting. It has been proposed[25,26] that the accumulation of oxidized protein observed in diseases of ageing is due to age-related defects in the processes which would normally prevent the accumulation of any oxidized protein (e.g. antioxidants, degradation systems). However these proposals are not based on data concerning the metabolism of oxidized protein by intact cells; indeed such proposals are in conflict with data on protein catabolism by ageing cells.[27,28]

In contrast, our studies suggest that accumulation of oxidized proteins may be due to oxidative modifications which decrease the proteolytic susceptibility of some portions of the oxidized protein. In the case of long-lived scavenger cells such as resident macrophages, the rate of accumulation will depend upon the relative amounts of oxidized protein present, as well as the rates of uptake (endocytosis) and removal (degradation plus exocytosis). As degradation of endocylosed protein is largely determined by substrate susceptibility to lysosomal enzymes, this may be rate-limiting. Processes involved in the degradation of specific components of oxidized proteins need to be further investigated for intact cells with radioactive tracers.

Accumulation of oxidized proteins may be biologically important in diseases of ageing such as atherosclerosis, because such an accumulation may directly impair cell function. Indeed if some of the recently described reactive protein oxidation products[20] accumulate, then further damage to other cellular macromolecules in their vicinity may ensue.[6,29]

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Accepted by Professor B. Halliwell

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