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Effects of oxidative stress on some physicochemical properties of caeruloplasmin

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We report the effects of oxidative stress generated by low-intensity u.v. irradiation (366 and 254 nm), dialysis against ascorbate and isolated stimulated neutrophils on some physicochemical properties of caeruloplasmin. Low-intensity u.v. irradiation resulted in a loss of ferroxidase activity and 610 nm absorption, changes previously reported to occur during storage and manipulation of caeruloplasmin. These alterations were found to correspond to aggregation of the protein, induction of visible fluorescence (excitation, 360 nm; emission, 454 nm), changes in c.d. spectra which were indicative of alterations in protein conformation, loss of half-cystine, tryptophan and tyrosine content and loss of immunoreactivity. The changes in the far-u.v. c.d. spectrum of caeruloplasmin were more pronounced than those observed for u.v.-irradiated IgG. Similar c.d. changes and induction of fluorescence were observed following dialysis of caeruloplasmin against ascorbate or exposure to stimulated neutrophils. It is concluded that the lability of caeruloplasmin may arise from oxidative modification, in addition to the previously described susceptibility of this protein to proteolysis.

INTRODUCTION

The blue oxidase caeruloplasmin (ferroxidase-I; EC 1.16.3.1) is the major copper-containing protein of human serum. The functional properties of caeruloplasmin *in vitro* have led to suggestions that its role *in vivo* is that of a serum antioxidant (Stocks *et al.*, 1974) or a catalyst of iron mobilization from reticuloendothelial cells by virtue of its 'ferroxidase' activity (Osaki *et al.*, 1966). However, despite a considerable amount of work on the structure and function of this acute phase protein, its true physiological role is unclear. Studies have been hampered by the pronounced lability of caeruloplasmin during isolation and storage (Kasper & Deutsch, 1963; for a review see Ryden, 1984). Ryden (1971) has shown that proteolysis is responsible for an artifactual appearance of the 'subunits' reported by others (e.g. Kasper & Deutsch, 1963; Freeman & Daniel, 1973). In addition, several observations suggest that caeruloplasmin is susceptible to oxidative attack.

(1) Caeruloplasmin undergoes changes during storage and manipulation that appear to be distinct from those produced by proteolysis, e.g. a decrease in absorption at 610 nm, loss of copper with aggregation of the apoprotein (Ryden, 1984). Thus exposure of caeruloplasmin to trypsin results in fragments which largely retain 610 nm absorption, copper content and oxidase activity, despite the cleaving of 10–23 peptide bonds (Kasper, 1967).

(2) Caeruloplasmin scavenges the superoxide anion radical ($O_2^{-\cdot}$) stoichiometrically (Goldstein *et al.*, 1979).

(3) Several functional activities of caeruloplasmin can be inactivated by exposure to a flux of oxygen free radicals generated by the hypoxanthine/xanthine oxidase system (Winyard *et al.*, 1984). The presence of seven copper atoms per caeruloplasmin molecule might render the protein susceptible to a copper-dependent generation of the highly reactive hydroxyl radical ($OH^{\cdot}$) by a Fenton-type reaction at the metal sites (Samuni *et al.*, 1981).

(4) Storage of blue oxidases is associated with the induction of visible fluorescence (excitation, 330 nm; emission, 430 nm) (Avigliano *et al.*, 1983), indicative of the oxidation of aromatic amino acids (Lunec *et al.*, 1985).

Although the products of proteolytic attack on caeruloplasmin have been the subject of intensive investigation, the effects of oxidative stress on caeruloplasmin are poorly characterised. Low-intensity u.v. irradiation has previously been used as a model of oxidative stress and aging in protein solutions (Avigliano *et al.*, 1983; Lunec *et al.*, 1985). Since the copper-catalysed autoxidation of ascorbate results in oxygen radical formation (Shinar *et al.*, 1983), we have compared the properties of u.v.-irradiated caeruloplasmin with those of the protein following dialysis against ascorbate, a procedure used to prepare copper-depleted caeruloplasmin for spectroscopic studies. Furthermore, some studies have indicated that caeruloplasmin may be inactivated in certain inflammatory diseases, e.g. pulmonary emphysema (Galdston *et al.*, 1984) and rheumatoid arthritis (Gutteridge *et al.*, 1984). Therefore, we have characterized the effects of oxygen radicals generated by isolated stimulated peripheral blood neutrophils.

Abbreviations used: PMA, phorbol myristate acetate; PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Materials

Purified human caeruloplasmin (twice crystallized, lyophilized) was purchased from Andard-Mount (London) Ltd., Middx., U.K. This preparation had an A_{610}/A_{280} ratio of 0.044, a copper content of 0.32% (6.75 mol/mol of protein) and gave a single band of M_r approx. 135000 on SDS/polyacrylamide-gel electrophoresis at pH 8.6 in the presence of dithiothreitol. The oxidase activity, tested at 23 °C with ferrous ions as substrate, was 1.2 units/mg of protein [ferroxidase assay of Johnson *et al.*, (1967); see below]. One unit was defined as the activity that catalyses the oxidation of 1 μmol of ferrous ions/min at pH 6.0 in a 1 ml reaction volume. Once caeruloplasmin was dissolved for each experiment it was immediately dialysed against 40 mM-KH_2PO_4/K_2HPO_4 (pH 7.4), containing 0.15 M-NaCl, to remove the mannitol which is added to the commercial preparation, and stored at 4 °C. All experiments were carried out within 1 week, since the A_{610} decreases during storage (see Ryden, 1984). The following reagents were purchased from Sigma: phorbol myristate acetate (PMA), cytochrome *c* (type III, from horse heart), superoxide dismutase (from bovine erythrocytes), catalase (from bovine liver), kynurenine, 5-hydroxytryptophan, methionine sulphoxide, methionine sulphone and cysteic acid. PMA was dissolved in dimethyl sulphoxide and stored in portions at −70 °C. Dulbecco's phosphate buffered saline, without calcium (PBS) and 'mono-poly' resolving medium (Ficoll–Hypaque) were purchased from Flow Laboratories. Apotransferrin was obtained from Behring–Hoechst and Chelex-100 resin from Bio-Rad. All other chemicals were obtained from BDH and were AnalaR grade.

Low-intensity u.v. irradiation

Caeruloplasmin and IgG (approx. 1.5 mg/ml) were dissolved in 40 mM-phosphate buffer/0.15 M-NaCl, pH 7.4. Volumes of 1.2 ml were irradiated (366 nm plus 254 nm source) aerobically in matched quartz cuvettes, 1 cm^2 in cross-section, at a distance of 6 cm from the light source. The light source at 254 nm (emission in the 160–280 nm range) was a Sylvania G8T5-8W bulb with an average light intensity of 10.5 W/cm^2 at 1 m. The light source at 366 nm (emission in the 320–380 nm range) was a Sylvania F8T5/BLB-8W bulb with an average light intensity of 17 W/cm^2 at 1 m.

Stimulated human peripheral blood neutrophils

Normal human neutrophils were separated from fresh heparinized whole blood by Ficoll–Hypaque density centrifugation, and cultures were set up in 96-well microtitre plates as previously described (Brailsford *et al.*, 1985). Appropriate rows of wells contained a final concentration of 8×10^5 cells/ml and superoxide dismutase (36 μg/ml), catalase (500 μg/ml), mannitol (50 mmol/l), or thiourea (50 mmol/l). PMA (final concn. 100 μg/ml) was used to activate the cells. Other wells contained cells plus either PBS or dimethyl sulphoxide in PBS as controls. Cytochrome *c* solution ± superoxide dismutase was added to half of the wells to test for $O_2^{-\bullet}$ production (absorption coefficient of reduced cytochrome *c* at 550 nm = 21.1×10^3 $M^{-1} \cdot cm^{-1}$). To the remaining wells was added a solution of human caeruloplasmin, to give a final concentration of 0.6 mg/ml. Following the incubation period, supernatants containing caeruloplasmin were aspirated from the wells and centrifuged at 1000 *g* for 10 min to remove any remaining cell debris.

Dialysis of caeruloplasmin against ascorbic acid

Caeruloplasmin (approx. 1 mg/ml) was dialysed for 24 h at 4 °C against successive changes of 1.2 M-sodium acetate buffer, pH 5.2, containing 5 mg of crystalline ascorbic acid/ml (Kasper & Deutsch, 1963). The ascorbic acid was removed by dialysis against the pH 5.2 buffer and finally the sample was dialysed against successive changes of 40 mM-phosphate buffer/0.15 M-NaCl, pH 7.4.

High performance liquid chromatography

Caeruloplasmin samples were applied to a TSK G3000SW column. Elution was at 1 ml/min with a mobile phase consisting of 0.067 M-KH_2PO_4/KOH buffer (pH 6.8), containing 0.1 M-KCl. The elution of free-radical-altered caeruloplasmin products was monitored with a fluorimeter (Gilson Instruments). An *o*-phthaldehyde filter allowed detection of protein peaks with a maximum excitation and emission of 360 and 454 nm, respectively. Detection of proteins at 280 nm was performed simultaneously using a Gilson spectrophotometer. Unless otherwise specified, the term 'visible fluorescence' may be taken to refer to excitation at 360 nm and emission at 454 nm. The ratio of visible fluorescence to A_{280} (VF/A_{280}) was calculated from the integrals of the fluorescence and absorption peaks (monomer + aggregates). Integrals were obtained by means of an Apple IIe computer and Gilson software interfaced to the h.p.l.c. system.

Ferroxidase activity

Ferroxidase activity was measured by the method of Johnson *et al.* (1967). All reagents were prepared in Chelex-100-treated distilled water. Briefly, 100 μl of sample was added to a solution containing 250 μl of 2.0% (w/v) apotransferrin, 170 μl of 1.2 M-acetate buffer, pH 6.0, and 270 μl of Chelex-treated distilled water. The reaction was started by the addition of 300 μl of 4.0×10^{-4} M-ferrous ammonium sulphate, freshly prepared in deaerated water. The rate of formation of the transferrin–Fe_2 complex, catalysed by caeruloplasmin, was monitored at 460 nm.

Optical spectra

C.d. spectra were recorded on a JASCO J-40CS CD spectrometer. The protein concentration (using $A^{1\%}_{1\,cm}$ at 280 nm = 14.9) (Kasper & Deutsch, 1963) was kept at approx. 0.7 mg/ml throughout in 20 mM-phosphate buffer/0.075 M-NaCl, pH 7.4, and the near-u.v. and far-u.v. regions required cells of optical pathlength 1 cm and 0.2 mm, respectively. C.d. was expressed as the molar c.d. absorption coefficient, using a mean residue weight of 113. Absorption spectra were measured prior to c.d. measurement on a Cary 17 spectrophotometer. The β-sheet content of caeruloplasmin was estimated from the far-u.v. c.d. spectra (Greenfield & Fasman, 1969).

Amino acid analysis

This was carried out with the technical assistance of Dr. John Fox, Macromolecular Services Unit, Birmingham University. Acid hydrolysis of protein was performed in sealed tubes in 6 M-HCl at 110 °C for 24 h.

Oxygen was removed from the sample by degassing. The hydrolysate was then evaporated to dryness and redissolved in 0.1% formic acid before injection onto the column (Aminex A-8; 20 cm × 0.46 cm; Bio-Rad). Leucine was used as the internal standard. The retention times of commercial preparations of 5-hydroxytryptophan, kynurenine, cysteic acid, methionine sulphoxide and methionine sulphone were ascertained. On the detection of new peaks which appeared to correspond to these oxidized derivatives, the hydrolysed protein sample was 'spiked' with the commercial preparation to confirm identical retention times. Methionine sulphoxide, when hydrolysed by the above procedure, was recovered as 94% methionine sulphoxide and 6% methionine, calculated by employing the colour factor for methionine sulphoxide reported in the Results section. For tryptophan, hydrolysis of the protein was in 4 M-$Ba(OH)_2$.

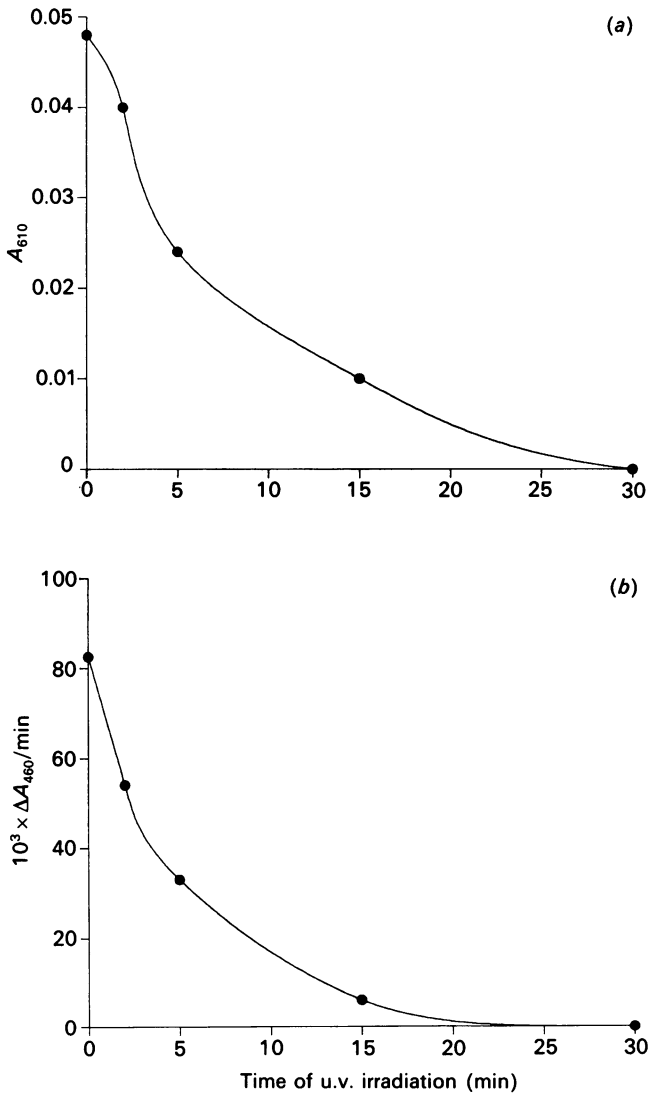

Fig. 1. Oxidative inactivation of caeruloplasmin by timed intervals of exposure to u.v. irradiation (366 nm + 254 nm source)

(*a*) Absorption at 610 nm; (*b*) ferroxidase activity determined by the method of Johnson *et al.* (1967). Data represent the means of duplicate analyses.

Radial immunodiffusion

The immunochemical reactivity of caeruloplasmin was measured using single radial immunodiffusion plates obtained from Hoechst Pharmaceuticals.

RESULTS

Low-intensity u.v. irradiation, exposure to oxygen radicals generated by activated neutrophils, storage and dialysis against ascorbate produced similar characteristic changes in certain physicochemical properties of caeruloplasmin, as assessed by a number of different techniques.

Oxidative inactivation and destruction of constituent amino acids

The irreversible loss of A_{610} and ferroxidase activity on u.v. irradiation (Fig. 1) is associated with the destruction of half-cystine, tryptophan and tyrosine residues (Fig. 2). Destruction of these amino acids after exposure of caeruloplasmin to 60 min of u.v. irradiation was calculated as 67%, 30% and 10%, respectively. These changes correspond to an average destruction per molecule of 9.4 half-cystines, 5.4 tryptophans and 6.3 tyrosines. None of the other amino acids were significantly altered. Inspection of the amino acid elution profile revealed the simultaneous formation of a small new peak corresponding to kynurenine (confirmed by spiking with a pure sample). Using a colour factor for kynurenine of 0.28, it was calculated that virtually all of

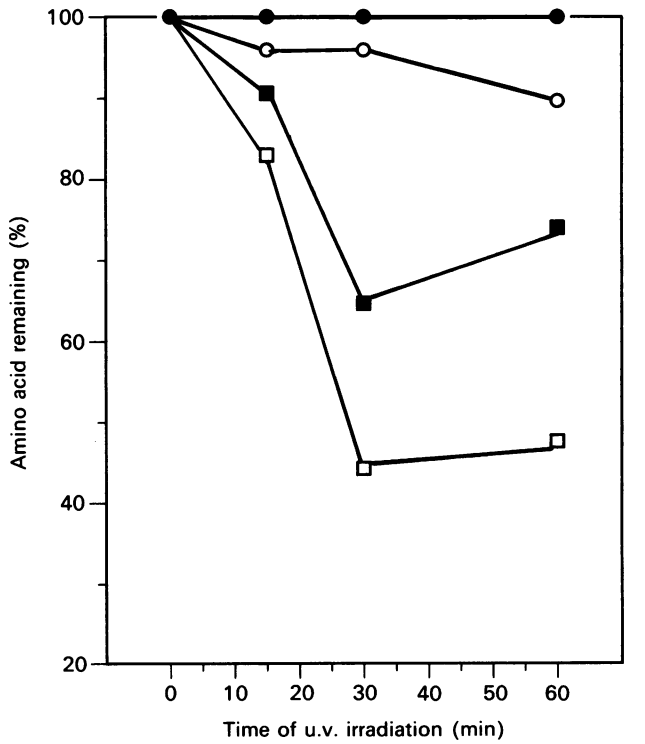

Fig. 2. Time course of destruction of constituent amino acids in caeruloplasmin by u.v. irradiation

□, Half-cystine; ○, tyrosine; ■, tryptophan; ●, leucine (internal standard). Data represent the means of duplicate analyses. All other amino acids were not significantly altered over the 60 min duration u.v. irradiation.

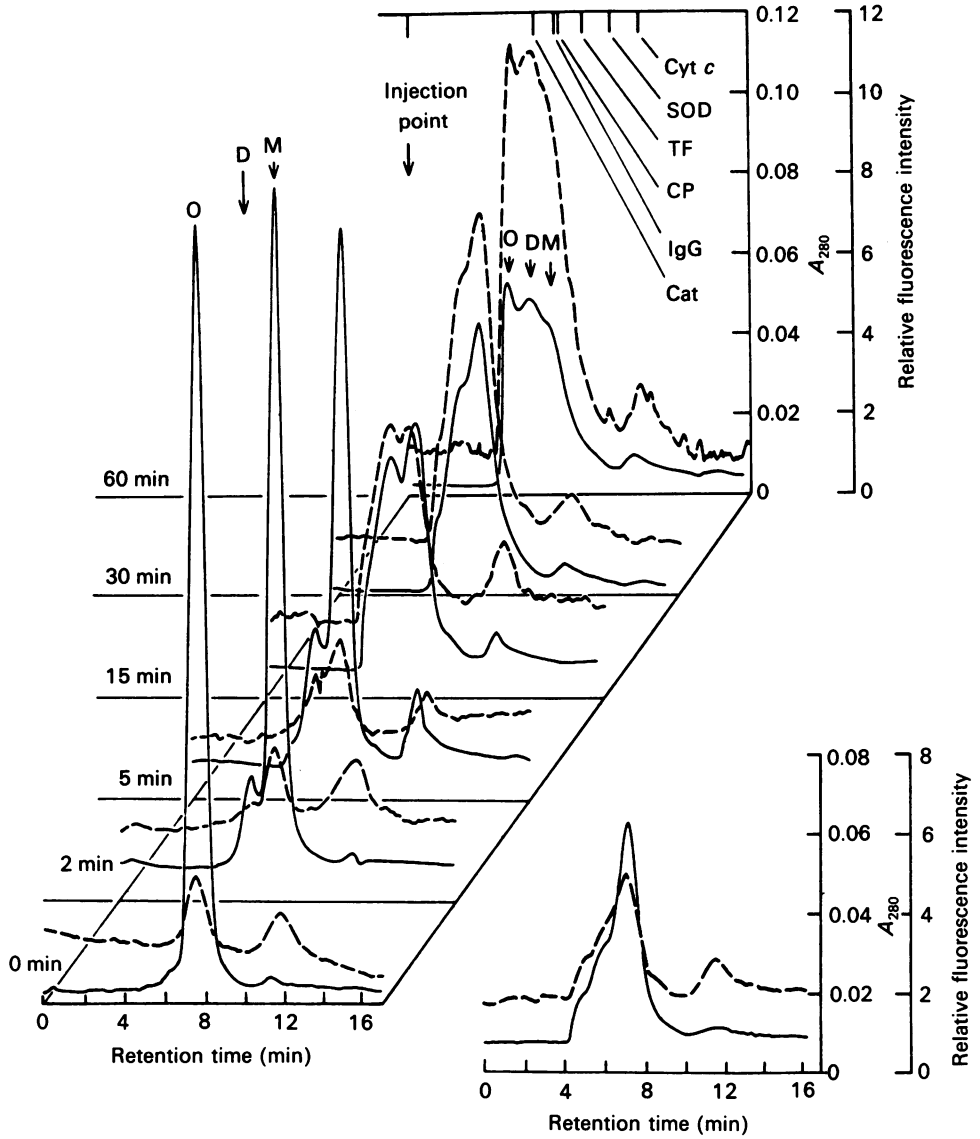

Fig. 3. Fluorescent monomeric and oligomeric reaction products of caeruloplasmin generated during timed intervals of exposure of caeruloplasmin to u.v. irradiation

Elution of caeruloplasmin products was monitored simultaneously for: ——, absorbance at 280 nm; – – –, visible fluorescence (excitation, 360 nm; emission, 454 nm). The fluorescence detector was set at range 20×: the maximum setting available was 100×. M, monomeric caeruloplasmin; D, dimers; O, oligomers. Inset: elution profile of reaction products of caeruloplasmin dialysed against ascorbate, according to the method of Kasper & Deutsch (1963). M_r calibration of the TSK G3000SW column was performed by monitoring the elution time of pure standards of cytochrome *c* (Cyt *c*; M_r 12400), superoxide dismutase (SOD; M_r 32000), transferrin (TF; M_r 80000), immunoglobulin G (IgG; M_r 150000), caeruloplasmin (CP; M_r 132000) and catalase (Cat; M_r 230000).

the tryptophan lost in u.v.-irradiated caeruloplasmin is recovered as kynurenine on amino acid analysis. In confirmation of this, no new peaks (including 5-hydroxytrytophan) were detected in the tryptophan analyses which followed alkaline hydrolysis. The traces of kynurenine on amino acid analysis may reflect the formation of *N'*-formylkynurenine in the protein that has been hydrolysed to kynurenine by acid hydrolysis (Dilley & Pirie, 1974). Significant formation of cysteic acid was not detected by amino acid analysis: presumably u.v. irradiation results in its conversion to other unidentified products (Creed, 1984). There was a dose-dependent decrease in immunochemical reactivity (as detected by radial immunodiffusion) with increasing periods of irradiation (results not shown). This change was relatively small and, after 10 min of irradiation, the diameter of the precipitate ring had decreased by only 16% relative to the native protein.

Caeruloplasmin was analysed within 24 h of a 1 h incubation with PMA-stimulated isolated human peripheral blood neutrophils (8×10^5 cells/ml; $O_2^{-\cdot}$ production 24.9 nmol/h per ml). Compared with controls, this did not result in significant changes in A_{610}, immunoreactivity or amino acid composition (results not shown). Dialysis of caeruloplasmin against ascorbic acid resulted in partial losses of absorbance at 610 nm

and oxidase activity: the A_{610}/A_{280} ratio fell to 0.012 and the ferroxidase activity to approx. 0.09 units/mg of protein. Thus, as expected (Kasper & Deutsch, 1963), not all of the blue copper is reduced by this procedure. As reported by a number of investigators (Kasper & Deutsch, 1963; Kasper, 1967; Ryden & Bjork, 1976; Ryden, 1984), storage of caeruloplasmin resulted in a loss of absorption at 610 nm. Substantial changes in amino acid composition were also found during sample aging, where 18 months' storage of caeruloplasmin at −20 °C resulted in an 86% loss of half-cysteine and an 84% loss of methionine, with formation of cysteic acid and methionine sulphoxide.

Induction of visible fluorescence and protein aggregation

When caeruloplasmin was exposed to u.v. irradiation, stimulated neutrophils, was dialysed against ascorbic acid or when it was stored, the protein became characteristically fluorescent (excitation, 360 nm; emission, 454 nm) and aggregated. This was demonstrated by h.p.l.c. of protein samples, based on size exclusion, with simultaneous u.v. and fluorescence detection. Fig. 3 shows the visible fluorescence and u.v. (A_{280}) elution profiles produced by irradiating caeruloplasmin for up to 60 min and by dialysing the protein against ascorbate. The profiles demonstrate the successive production of fluorescence in monomeric caeruloplasmin ('M' in Fig. 3), dimers ('D') and oligomers ('O') of $M_r > 10^6$. The possibility cannot be excluded that some of the material eluting from the column more rapidly is unfolded protein, although this seems unlikely. Early oligomerization appears to involve dimers, but after 60 min of irradiation a substantial proportion of high-M_r oligomers had formed (Fig. 3). A highly fluorescent low-M_r peak was also present, but its area was not reproducible on repeated loading of a given sample. The ratio of the integral of the visible fluorescence peaks (VF; excitation, 360 nm; emission, 454 nm; monomer plus aggregate) to the integral of the u.v. absorption peaks (A_{280}; monomer plus aggregate) has been computed and plotted against time of u.v. irradiation (Fig. 4). Following dialysis against ascorbate, the same caeruloplasmin preparation showed qualitatively similar changes (Fig. 3, inset): the ratio VF/A_{280} was increased to 0.69, which corresponds to the effect of about 10 min of u.v. irradiation.

H.p.l.c. of caeruloplasmin exposed to neutrophils also revealed an increase in monomer fluorescence. In a typical experiment the $O_2^{-\cdot}$ flux in the presence and absence of PMA was 29.9 and 3.0 nmol of $O_2^{-\cdot}$/h per ml, respectively. Following incubation with unstimulated cells, or with PMA alone, the VF/A_{280} ratio of caeruloplasmin was 0.04. After incubation with PMA-stimulated cells this ratio increased to 0.22 (results not shown). Induction of fluorescence was inhibited by superoxide dismutase (99%) and thiourea (94%), but less effectively by mannitol (28%). It was not possible to test the effect of catalase because the presence of this protein interfered with the h.p.l.c. method.

A change in visible fluorescence on storage, reported by Avigliano *et al.* (1983) for other blue oxidases, is also seen for caeruloplasmin: 3 weeks storage at 4 °C resulted in an increase of 60% in the ratio VF/A_{280} corresponding to the monomer plus aggregate peaks separated on h.p.l.c. Proteolysis of caeruloplasmin by trypsin and elastase (results not shown) did not cause the induction of fluorescence in either the monomer or peptide fragments, as assessed by h.p.l.c. with simultaneous fluorescence detection.

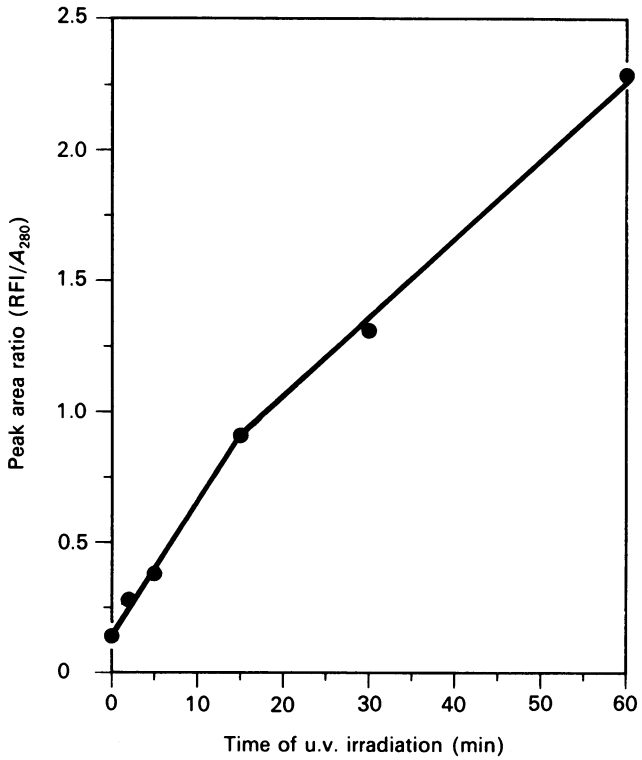

Fig. 4. Time course of visible fluorescence generation in caeruloplasmin by u.v. irradiation

Values are calculated by integration of monomer + aggregate peaks shown in Fig. 3. RFI, relative fluorescence intensity (excitation, 360 nm; emission, 454 nm).

Loss of β-sheet structure and unfolding of the protein

The visible c.d. spectrum of caeruloplasmin (results not shown) was similar to that in previous reports (Freeman & Daniel, 1978; Herve *et al.*, 1981; Noyer & Putnam, 1981). Fig. 5 shows the c.d. spectra of native and u.v.-irradiated caeruloplasmin in the far- and near-u.v. regions. In the far-u.v. spectrum (Fig. 5*a*), the negative band at 220 nm is somewhat less intense than the positive band at 200 nm. The extremum at 200 nm is also present in plastocyanin (Draheim *et al.*, 1986) and in that protein is due to the presence of an appreciable β-pleated sheet content (Manning & Woody, 1986). Using Greenfield and Fasman's method of calculation we estimate the content of β-sheet to be 60–65%, which is higher than that previously reported (Hibino *et al.*, 1969; Herve *et al.*, 1981; Freeman & Daniel, 1978; Noyer & Putnam, 1981). The α-helix content is estimated to be less than 10%. When caeruloplasmin is exposed to u.v. irradiation, the trough at 220 nm becomes less intense, broadens and is displaced to lower wavelengths (215 nm after 60 min of irradiation). Simultaneously, the positive extremum at 200 nm is considerably reduced, the peak failing to reach positive values after 30 min of irradiation. There is a blue shift in the 210 nm crossover of the native protein (to 206 nm after 15 min of irradiation). The isosbestic point at approx. 215 nm indicates that two major conformational states dominate the spectrum. The c.d. changes in the far-u.v. region are indicative of loss of β-sheet content.

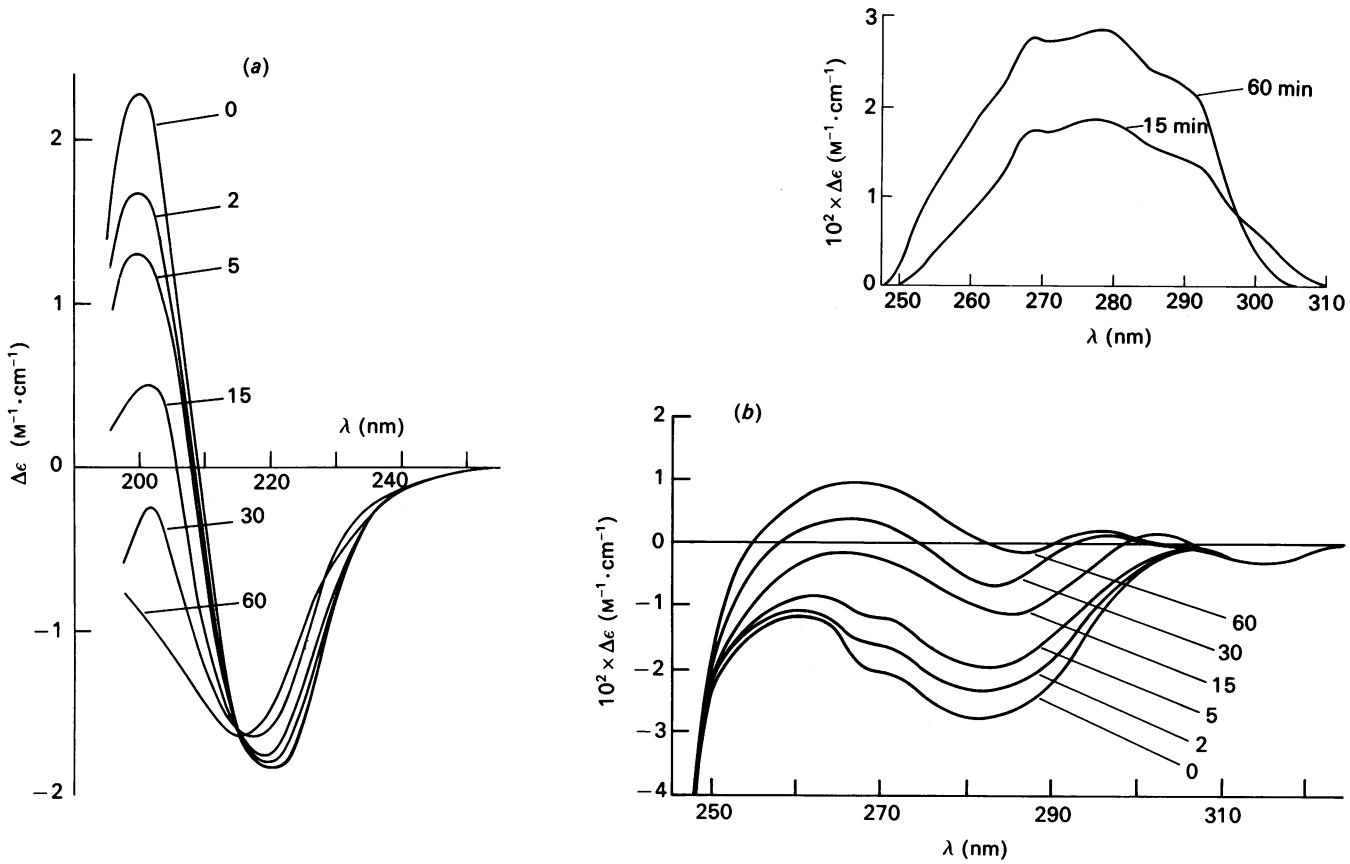

Fig. 5. C.d. spectra of caeruloplasmin exposed to timed intervals of u.v. irradiation

(*a*) Far-u.v. region; (*b*) near-u.v. region (inset: difference spectra of caeruloplasmin after 15 and 60 min irradiation minus spectrum of native caeruloplasmin). Numbers adjacent to each spectrum represent the time (min) of irradiation. Spectra were recorded in 20 mM-phosphate buffer/0.075 M-NaCl, pH 7.4.

The effect of u.v. irradiation on the near-u.v. c.d. spectrum of caeruloplasmin is shown in Fig. 5(*b*). The extremum in the region of 260 nm is considerably enlarged and undergoes a red shift (to 266 nm after 15 min of irradiation). The 280 nm negative band decreases in intensity and shifts to longer wavelengths (285 nm after 60 min irradiation). After 15 min of irradiation parts of the spectrum reach positive values and after 60 min irradiation a positive band is clearly visible at 292 nm, which Noyer & Putnam (1981) assigned to an 'L_b' tryptophan band in caeruloplasmin. The change in sign of many of the near-u.v. c.d. bands is likely to result from altered tertiary structure, although the chemical modification of some tryptophan side chains may also make a contribution. The near-u.v. c.d. difference spectra of caeruloplasmin after irradiation for 15 and 60 min are shown in the inset to Fig. 5(*b*). The difference spectra are similar to the near-u.v. c.d. spectrum of tryptophan, indicating that the partial unfolding of u.v.-irradiated caeruloplasmin involves the perturbation of tryptophan residues, as in the lens proteins (Andley & Chapman, 1986). Changes in molar c.d. absorption coefficient at 280 nm and 200 nm with time of irradiation are presented in Fig. 6. The largest changes in c.d. were observed during the first 15 min of irradiation. The far-u.v. and near-u.v. c.d.. spectrum of caeruloplasmin is also altered by several months' storage at −20 °C (results not shown).

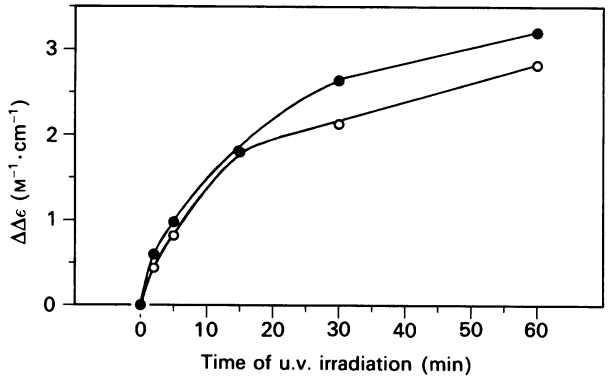

Fig. 6. Change in ellipticity at 200 and 280 nm of caeruloplasmin exposed to timed intervals of u.v. irradiation.

●, Change in molar c.d. absorption coefficient at 200 nm; ○, $10^2 \times$ change in molar c.d. absorption coefficient at 280 nm, relative to native protein. Values are calculated from Fig. 5.

We compared the changes in the c.d. spectrum of u.v.-irradiated caeruloplasmin with those in irradiated IgG. The far-u.v. and near-u.v. regions of the c.d. spectrum of native IgG and IgG exposed to 10 min of u.v. irradiation are shown in Fig. 7. No c.d.-detectable secondary struc-

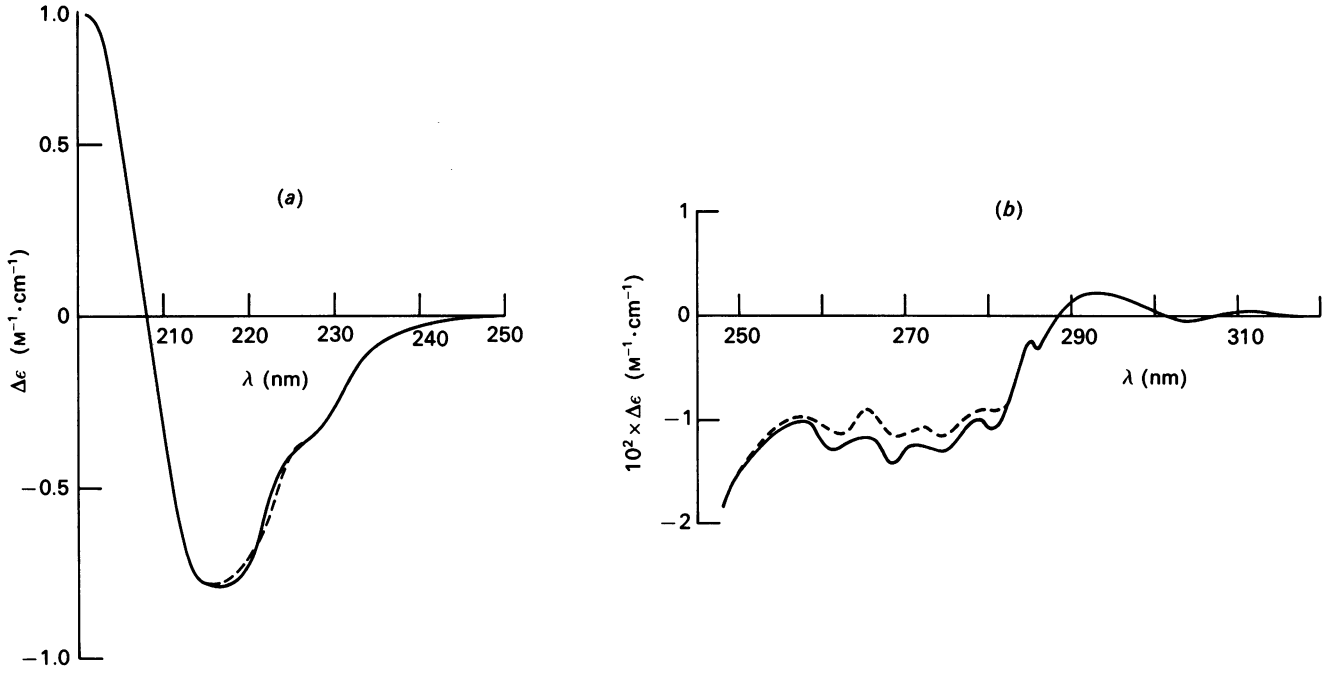

Fig. 7. C.d. spectra of native IgG and IgG exposed to 10 min of u.v. irradiation

(*a*) Far-u.v. region; (*b*) near-u.v. region. Spectra were recorded under the conditions given in the legend to Fig. 5. ——, Native IgG; – – –, IgG exposed to 10 min of u.v. irradiation.

ture changes were induced by u.v. irradiation, although there were some minor changes in the 260–280 nm region. This contrasts markedly with caeruloplasmin.

Dialysis of caeruloplasmin against ascorbate at low pH is a standard procedure for the removal of type 1 copper (Kasper & Deutsch, 1963). These conditions were found to induce appreciable changes in the c.d. spectrum in both the far-u.v. and near-u.v. regions (Fig. 8). The alterations resemble those seen with u.v. irradiation (Fig. 5), suggesting that it is the removal of some copper that affects the secondary and tertiary structure.

The far-u.v. and near-u.v. c.d. spectra of caeruloplasmin following incubation with neutrophils ± PMA for 1 h are shown in Fig. 9. Small but significant changes are observed in both regions, being similar to those which occurred in u.v.-irradiated and ascorbate-treated caeruloplasmin. The above interpretation of the u.v. light-induced c.d. spectral changes also applies here.

DISCUSSION

Physicochemical properties of free-radical-altered caeruloplasmin

Storage of caeruloplasmin at 4 °C or −20 °C results in a loss of oxidase activity and a decrease in its blue colour (Ryden, 1984). U.v. irradiation, which has been employed as a model of aging (Avigliano *et al.*, 1983), induces similar changes (Aprison & Hanson, 1959; this paper). The effects on caeruloplasmin of u.v. irradiation are likely to be due to the combination of photobleaching of copper chromophores and photodynamic events, e.g. the formation of reactive oxygen metabolites, such as H_2O_2, $O_2^{-\cdot}$ and $OH^{\cdot}$, resulting in irreversible inactivation. We have also detected parallel irradiation-induced alterations in other physicochemical properties such as visible fluorescence, c.d. spectra and amino acid content. Avigliano *et al.* (1983) suggested that the visible fluorescence of several blue oxidases may be related to an aging process involving tryptophan oxidation (although caeruloplasmin was not studied). They observed an increase in the visible fluorescence of albumin following u.v. irradiation.

Recently, Lunec *et al.* (1985) concluded that visible fluorescence formation in proteins specifically characterizes free-radical-induced damage and is derived from the products of $OH^{\cdot}$ attack on tryptophan residues. Our ability to inhibit fluorescence induction in the neutrophil system by addition of superoxide dismutase or thiourea is consistent with this conclusion. Several of the major fluorescent oxidation products of tryptophan that are generated by free-radical reactions have been tentatively identified as 5-hydroxytryptophan, kynurenine and *N*-formyl-L-kynurenine (Roshchupkin *et al.*, 1979; Sun & Zigman, 1979). From the amino acid analyses and visible fluorescence changes, it can be putatively concluded that, in our experiments, cysteine is oxidized to cysteic acid (or other products in the case of u.v. irradiation; Creed, 1984) and tryptophan to kynurenine and possibly other fluorescent products. These products are the same as those that have been identified in many other proteins following oxidative attack both *in vitro* and *in vivo*, e.g. lens proteins (Dilley & Pirie, 1974), IgG (Lunec *et al.*, 1985) and α_1-antitrypsin (Wong & Travis, 1980). In caeruloplasmin, the changes in tryptophan are confirmed by our spectral studies: the loss of monomer absorption at 280 nm, induction of visible fluorescence and the resemblance of the difference c.d. spectrum in the near-

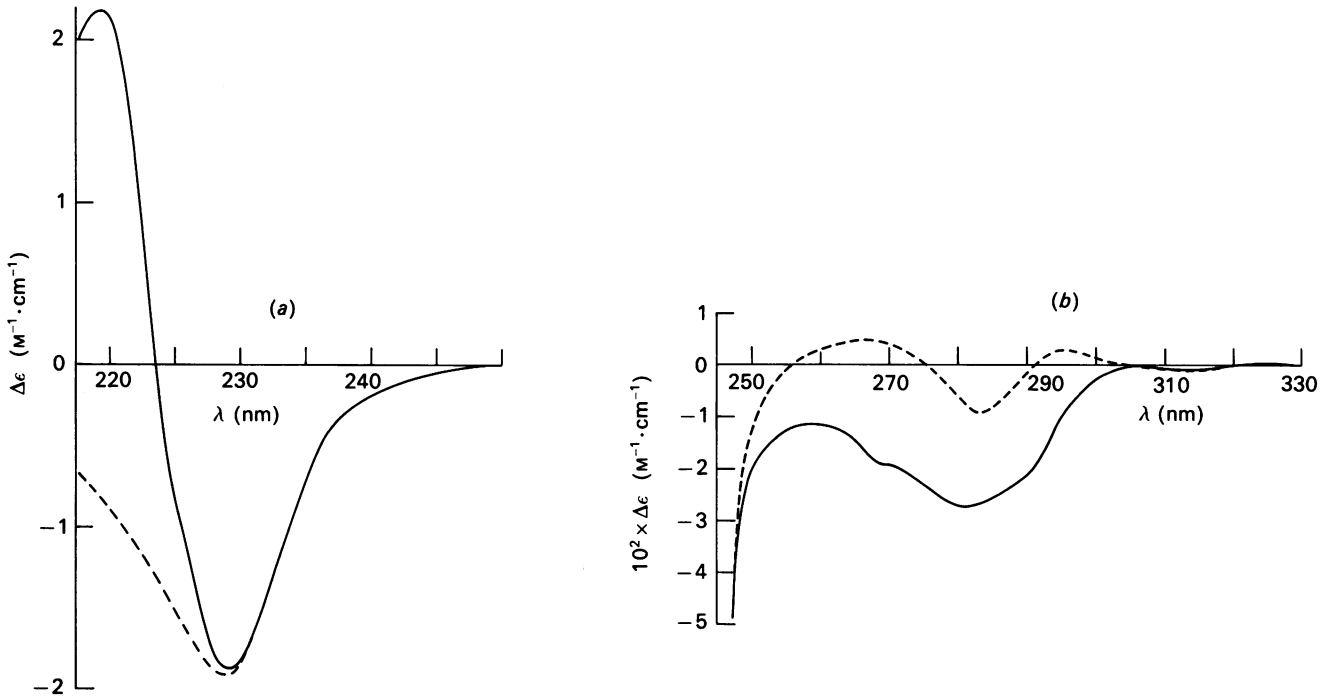

Fig. 8. C.d. spectra of native caeruloplasmin and ascorbate-treated caeruloplasmin

(*a*) Far-u.v. region; (*b*) near-u.v. region. Caeruloplasmin was dialysed against ascorbate according to the procedure of Kasper & Deutsch (1963). Spectra were recorded under the conditions given in the legend to Fig. 5. ——, Native caeruloplasmin; – – –, ascorbate-treated caeruloplasmin.

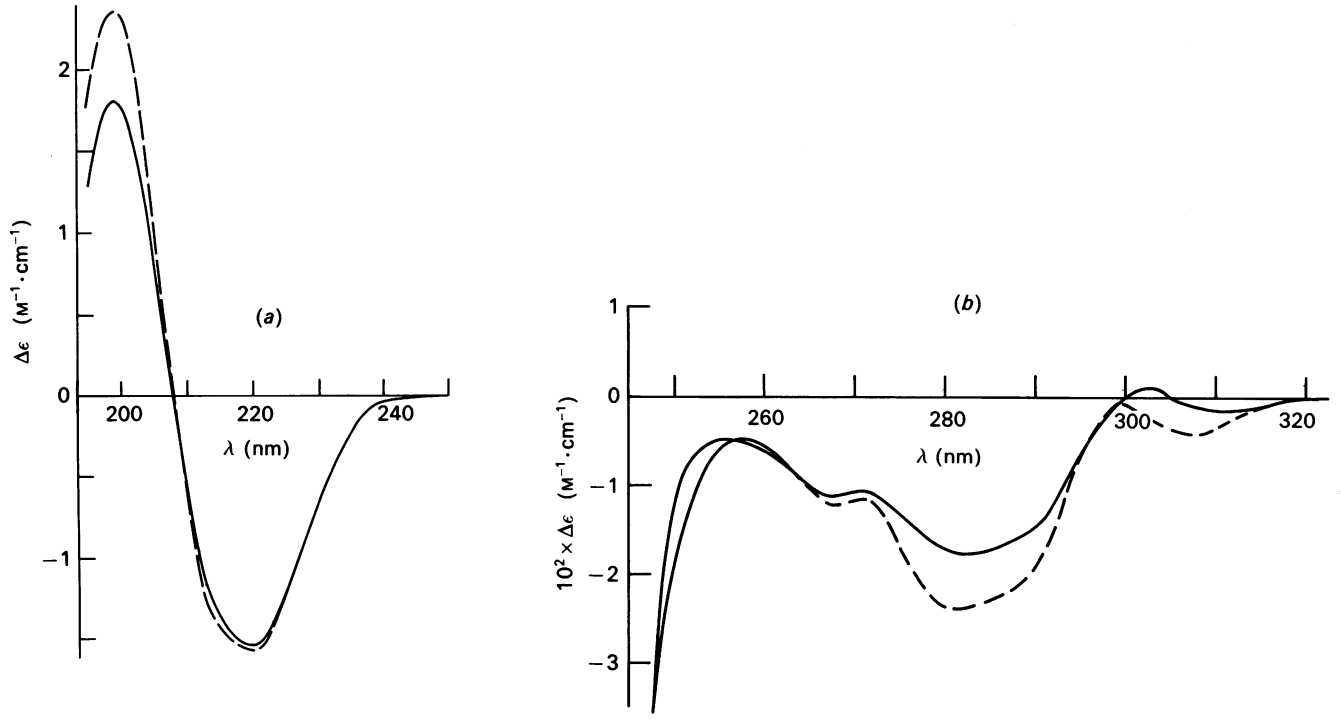

Fig. 9. C.d. spectra of caeruloplasmin exposed to PMA-stimulated and resting neutrophils

(*a*) Far-u.v. region; (*b*) near-u.v. region. ——, Spectrum of caeruloplasmin incubated with PMA-stimulated neutrophils (8×10^5 cells/ml) for 60 min at 37 °C, where the $O_2^{-\cdot}$ flux was 24.9 nmol/h per ml; – – –, spectrum of caeruloplasmin exposed to the same concentration of resting neutrophils for the same time interval, where the $O_2^{-\cdot}$ flux was 3.5 nmol/h per ml. The spectrum of caeruloplasmin incubated with PMA alone was not significantly different from the latter spectrum. Spectra were recorded in the medium used during incubation of the protein with neutrophils (see the Materials and methods section).

u.v. region to that of tryptophan. The far-u.v. c.d. spectra reveal that there is a concomitant change in the gross conformational structure of caeruloplasmin.

The increase in protein aggregates (dimers and oligomers) during irradiation could be due to the formation of intermolecular disulphide bridges, exposure of hydrophobic regions on the protein following the perturbation of tryptophan residues, or dityrosine crosslinking. Using size exclusion h.p.l.c., we observed protein aggregates in the 'native' protein sample ($A_{610}/A_{280} = 0.044$) irradiated for 2 min. Freeman & Daniel (1973) also observed such aggregates of caeruloplasmin and found that exposure to 2-mercaptoethanol eliminated the protein aggregate bands on sedimentation velocity pictures and SDS/polyacrylamide-gel electrophoresis, indicating that the aggregates were formed by intermolecular disulphide bonding.

In the c.d. spectra of u.v.-irradiated, ascorbate-treated and neutrophil-treated caeruloplasmin there were changes in both the far-u.v. and near-u.v. regions, compared with the native protein (see below). In contrast, proteolysis of caeruloplasmin made no significant difference to the c.d. spectrum (Noyer & Putnam, 1981). These observations indicate that oxidation as well as proteolysis can be an important means of modification of caeruloplasmin. Orr (1967) found that incubation of catalase with low concentrations of ascorbate resulted in u.v. absorption spectral changes which were not linked to changes in the activity of the enzyme and speculated that the oxidation of a small number of labile aromatic residues contributed to an absorption change out of all proportion with their concentration. A similar situation may exist in the neutrophil system, explaining the absence of significant changes when assessed by A_{610}, oxidase activity and amino acid analysis, despite the fluorescence and c.d. changes.

Removal of type 1 copper by dialysis of caeruloplasmin against ascorbate

We found that a preparation of caeruloplasmin dialysed against ascorbate had characteristics (visible fluorescence and protein aggregates) indicative of oxidative damage. This suggests that when ascorbate treatment has been used to facilitate spectroscopic studies of the copper types 2 and 3 (e.g. Dawson *et al.*, 1979), a change in the environment of these copper types might have occurred. In contrast to our results, Calabrese & Carbonaro (1986*a*) reported that H_2O_2 could reoxidize the ascorbate-reduced type 1 copper ions without inactivation of caeruloplasmin. However, in a different experiment Calabrese & Carbonaro (1986*b*) did observe the inactivation of caeruloplasmin by H_2O_2. A solution of caeruloplasmin was bleached by alkaline pH and H_2O_2 was added. When the pH was brought back to 7.4, there was a transient recovery of colour, followed by irreversible bleaching.

The changes in the far- and near-u.v. c.d. spectra of u.v.-irradiated and ascorbate-treated caeruloplasmin appear to be similar to those brought about by dialysis of caeruloplasmin against KCN, pH 5.5 (used for the preparation of apoprotein). For example, in the far-u.v. c.d. spectrum of cyanoapo-caeruloplasmin, the crossover at 212 nm (in the porcine protein) moves to 205 nm (Hibino *et al.*, 1969) and the 220 nm trough becomes less intense and much broader (Noyer & Putnam, 1981). As KCN lacks redox properties, these similarities indicate that the c.d. spectral changes in the far-u.v. region observed with irradiated caeruloplasmin may be the result of the loss of copper ions (see below). However, the dialysis procedure typically involves an incubation period of several days at 4 °C (Broman, 1964), conditions likely to cause oxidative damage.

Secondary structure estimates from c.d. studies of caeruloplasmin

We estimate the content of β-sheet in native caeruloplasmin to be 60–65%. Previous reports have been somewhat lower, varying between 31% and 46% (Herve *et al.*, 1975; Freeman & Daniel, 1978; Noyer & Putnam, 1981). It is also apparent, however, that u.v. irradiation profoundly alters the c.d. spectrum, including a marked decrease in the size of the peak at 200 nm, indicating a decrease in the β-sheet content of the protein. Our estimate of β-sheet content may be higher because the caeruloplasmin preparation used showed little evidence of oxidative attack, i.e. a high absorption at 610 nm, little sign of aggregation and only a small amount of visible fluorescence.

Mechanism of oxidative inactivation of caeruloplasmin

Samuni *et al.* (1981) suggested a 'site specific' mechanism of oxygen radical attack on a copper-containing protein, which involves the reduction of the protein-bound Cu(II) by $O_2^{-\cdot}$ to yield the Cu(I)–protein complex. This reduced complex reacts with H_2O_2 in a Fenton-type reaction to form secondary $OH^{\cdot}$ radicals locally. The short-lived $OH^{\cdot}$ reacts with a molecule very close to its site of formation, such as the copper ligand cysteine. An analogous mechanism has been suggested by Shinar *et al.* (1983) for the inactivation of acetylcholine esterase by ascorbate and copper.

In addition to reacting with copper ligands, $OH^{\cdot}$ will react with other nearby residues, among which tryptophan appears to be particularly susceptible (Lunec *et al.*, 1985). For example, Marx & Chevion (1985) noted a decrease in the intrinsic fluorescence (excitation, 290 nm; emission, 340 nm) of albumin exposed to copper(II) and ascorbate, indicative of the loss of tryptophan and/or other aromatic amino acids. In our experiments, dialysis of caeruloplasmin against ascorbate resulted in the induction of visible fluorescence (Fig. 3, inset), which indicates the oxidation of tryptophan residues. In the neutrophil system, our inability to inhibit fluorescence formation by addition of mannitol supports the 'site specific' mechanism, since the protective effect of $OH^{\cdot}$ scavengers is substantially lessened in such a system (Samuni *et al.*, 1981). Thiourea may be more effective because additionally it has the ability to chelate copper (Halliwell & Gutteridge, 1986).

Protein-bound Cu(II) 'sensitizes' co-ordinating ligands to damage by u.v. irradiation (Spikes & Straight, 1981). U.v. irradiation (inclusive of far-u.v. wavelengths) of caeruloplasmin results in the rapid irreversible loss of its blue colour (Fig. 1*a*) and the dissociation of the copper (Aprison & Hanson, 1959). On aerobic u.v. irradiation of the solution of caeruloplasmin at 254 nm the major absorbing chromophores will be the constituent tryptophan residues, resulting in photoionization of

tryptophan and the production of H_2O_2 (Grossweiner, 1984):

$$\text{Trp} \xrightarrow{\text{u.v.}} \text{Trp}^{+\cdot} + e^-$$

$$O_2 + e^- \rightarrow O_2^{-\cdot}$$

$$2O_2^{-\cdot} + 2H^{+\cdot} \rightarrow H_2O_2 + O_2$$

The Fenton-type reaction may then take place at the reduced copper sites. Caeruloplasmin contains four cysteine and five cystine residues. The cysteines putatively involved in copper binding (Cys-1021, -680, -319 and possibly -221) are likely to be rapidly oxidized. From our amino acid analysis data about 70% of the half-cystine residues are oxidized after 60 min of u.v. irradiation, which corresponds to four copper-binding cysteines plus six half-cystines involved in disulphide bridges. Ryden's model of the catalytic site of caeruloplasmin (Ryden, 1982) contains four out of the total of seven copper atoms per molecule (Ryden, 1982), i.e. a type 1 (blue) copper, a type 2 (nonblue) copper and two type 3 (spin-coupled) coppers. In this model, the type 1 copper and possibly the type 2 copper are involved in the interaction of more than one peptide strand. Moreover, many of the residues liganding these coppers probably form part of an exposed loop (Ryden, 1984). Thus the maintenance of extended β structure in this region may be dependent on the presence of these two coppers. Assuming folding is similar in all three copper-containing domains, this would also apply to the domain containing the second type 1 copper. The rapid oxidation of the copper-binding cysteines, resulting in the dissociation of their copper atoms, could explain the relatively large early loss of β-sheet structure. Our u.v. c.d. spectrum of native caeruloplasmin indicates that caeruloplasmin contains 60–65% β-sheet structure. The corresponding spectrum of caeruloplasmin subjected to 15 min of irradiation indicates that about 7% of the β-sheet has been lost. Since caeruloplasmin contains 1046 amino acids this corresponds to the disruption of 65–70 residues in the caeruloplasmin molecule, which corresponds to about 20 residues per domain or about three β-sheet strands per domain. It seems likely that part of the β-sheet loss corresponds to the disruption of the exposed loop referred to above and that this movement in turn would expose tryptophan residues, giving rise to the observed changes in the near-u.v. region of the c.d. spectrum.

Five tryptophan residues (out of the 18 residues per molecule) are oxidized after 60 min irradiation. Tyrosine residues are lost to a lesser extent. The mechanism of oxidation of these aromatic residues may involve direct attack by $OH^{\cdot}$ (Lunec *et al.*, 1985) or the reaction of triplet-excited tryptophan with O_2 (Singh *et al.*, 1984). During the period between 15 and 60 min irradiation, oxidation of cystine and tryptophan residues will lead to the further destabilization of the protein tertiary structure which is further reflected in a loss of β-sheet content.

Relative susceptibility of caeruloplasmin to photo-oxidation

Comparison of h.p.l.c. profiles of u.v.-irradiated IgG (Fig. 1 of Lunec *et al.*, 1985) and caeruloplasmin (Fig. 3) shows that visible fluorescence induction and aggregation is greater in the cuproprotein. Lunec *et al.* (1985) noted the loss of half-cystine and tryptophan residues in u.v.-irradiated IgG: 1 h of u.v. irradiation in a system which was identical to that used in this study resulted in reductions of 37% and 17%, respectively, with smaller losses in tyrosine and lysine residues. These changes are about half of those seen in caeruloplasmin (70% and 30%, respectively). The greater loss of half-cystine in caeruloplasmin may reflect the presence of cysteine residues.

In contrast to caeruloplasmin, the β-pleated sheet content of IgG is not affected by u.v. irradiation. Thus, appreciable protein backbone changes only occur in caeruloplasmin. Tertiary structure alterations are also more pronounced in caeruloplasmin than in IgG (compare Figs. 5*b* and 7*b*). As in the case of IgG, u.v. irradiation of lens proteins, all of which have a high content of β-sheet structure, does not cause major secondary structure changes, despite changes in the near-u.v. c.d. spectrum (Andley & Chapman, 1986; Mandal *et al.*, 1986). Thus the gross conformational structure of caeruloplasmin is more susceptible to oxidative modification than either IgG or the lens crystallins.

Implications of caeruloplasmin oxidation for laboratory manipulation and physiological processes

Whilst limited proteolysis of caeruloplasmin does not result in marked changes in its blue colour, oxidase activity (Kasper, 1967), or c.d. spectra (Noyer & Putnam, 1981), oxidative stress has a profound effect on these parameters. A number of other properties of this protein might be influenced by oxidative modifications *in vitro*, e.g. the stoichiometry of the type 2 copper. Quantitative estimations first showed type 2 copper to be 40–45% of total e.s.r.-detectable copper, but in measurements on carefully prepared protein this value drops to 33% (see Ryden, 1984). Syed *et al.* (1982) and Evans *et al.* (1985) contend that it may not be present at all *in vivo*. The variation is at least in part explained by the history of the sample (see Ryden, 1984), but does not seem to be accounted for by proteolysis (Deinum & Vanngard, 1973). The copper atoms in the new environment produced by oxidative modification of caeruloplasmin (i.e. type 2 copper?) could possess superoxide dismutase activity, explaining the conflicting reports as to whether caeruloplasmin possesses this activity (compare Plonka *et al.*, 1980, with Bannister *et al.*, 1980). The early overestimates of the M_r of caeruloplasmin may well have been due to aggregation of oxidized protein, whilst the visible fluorescence associated with caeruloplasmin and other blue copper proteins, which has previously been attributed to their contents of copper ions or carbohydrate (see Avigliano *et al.*, 1983), may indicate the susceptibility of these proteins to site-specific oxidation at their copper sites. Finally, we should like to recall that Weiss & Linder (1985) have reported a new copper transport protein, transcuprein, which binds 10–15% of rat plasma copper. The M_r of transcuprein, 270000, is consistent with the identity of this protein as a caeruloplasmin dimer.

From this study it appears that caeruloplasmin is relatively susceptible to oxidative stress compared with IgG and the lens crystallins, which have both been shown to be oxidatively damaged *in vivo* (Lunec *et al.*, 1985; Garner & Spector, 1980). *In vivo*, at the inflammatory site, both oxygen radicals and proteases are released by activated neutrophils. Oxygen radicals and proteases can act synergistically to modify proteins (Wolff & Dean,

1986). The possibility that the enzymic properties of caeruloplasmin change as a result of specific chemical modification needs investigation.

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REFERENCES

Andley, U. P. & Chapman, S. F. (1986) Photochem. Photobiol. **44**, 67–74

Aprison, M. H. & Hanson, K. M. (1959) Proc. Soc. Exp. Biol. Med. **100**, 643–647

Avigliano, L., Sirianni, P., Morpurgo, L. & Finazzi-Argo, A. (1983) FEBS Lett. **163**, 274–276

Bannister, J. V., Bannister, W. H., Hill, H. A. O., Mahood, J. F., Wilson, R. L. & Wolfenden, B. S. (1980) FEBS Lett. **118**, 127–129

Brailsford, S., Lunec J., Winyard, P. G. & Blake, D. R. (1985) Free Radical Res. Commun. **1**, 101–109

Broman, L. (1964) Acta Soc. Med. Uppsala **69** (Suppl. 7), 3–85

Calabrese, L. & Carbonaro, M. (1986*a*) Biochem. J. **238**, 291–295

Calabrese, L. & Carbonaro, M. (1986*b*) in Free Radicals and Arthritic Diseases: Topics in Aging Research in Europe vol. 11 (Swaak, A. J. G. & Koster, J. F., eds.), pp. 115–119, EURAGE, Rijswijk

Creed, D. (1984) Photochem. Photobiol. **39**, 577–583

Dawson, J. H., Dooley, D. M., Clark, R., Stephens, P. J. & Gray, H. B. (1979) J. Am. Chem. Soc. **101**, 5046–5053

Deinum, J. & Vanngard, T. (1973) Biochim. Biophys. Acta **310**, 321–330

Dilley, K. J. & Pirie, A, (1974) Exp. Eye Res. **19**, 59–72

Draheim, J. E., Anderson, G. P., Duane, J. W. & Gross, E. L. (1986) Biophys. J. **49**, 891–900

Evans, R. W., Madden, A. D., Patel, K. J., Gibson, J. F. & Wrigley, S. K. (1985) Biochem. Soc. Trans. **13**, 627–629

Freeman, S. & Daniel, E. (1973) Biochemistry **12**, 4806–4810

Freeman, S. & Daniel, E. (1978) Biochim. Biophys. Acta. **534**, 132–140

Galdston, M., Levytska, V., Schwartz, M. S. & Magnusson, B. (1984) Am. Rev. Respir. Dis. **129**, 258–263

Garner, M. & Spector, A. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1274–1277

Goldstein, I. M., Kaplan, H. B., Edelson, H. S. & Weissmann, G. (1979) J. Biol. Chem. **254**, 4040–4045

Greenfield, N. & Fasman, G. D. (1969) Biochemistry **8**, 4108–4116

Grossweiner, L. I. (1984) Curr. Eye Res. **3**, 137–144

Gutteridge, J. M. C., Hill, C. & Blake, D. R. (1984) Clin. Chim. Acta **139**, 85–90

Halliwell, B. & Gutteridge, J. M. C. (1986) Arch. Biochem. Biophys. **246**, 501–514

Herve, M., Garnier, A., Tosi, L. & Steinbuch, M. (1975) Biochim. Biophys. Acta. **405**, 318–323

Herve, M., Garnier, A., Tosi, L. & Steinbuch, M. (1981) Eur. J. Biochem. **116**, 177–183

Hibino, Y., Samejima, T., Kajiyama, S. & Nosoh, Y. (1969) Arch. Biochem. Biophys. **130**, 617–623

Johnson, D. A., Osaki, S. & Frieden, E. (1967) Clin. Chem. **13**, 142–150

Kasper, C. B. (1967) Biochemistry **6**, 3185–3197

Kasper, C. B. & Deutsch, H. F. (1963) J. Biol. Chem. **238**, 2325–2337

Lunec, J., Blake, D. R., McCleary, S. J., Brailsford, S. & Bacon, P. A. (1985) J. Clin. Invest. **76**, 2084–2090

Mandal, K., Bose, S. K. & Chakrabarti, B. (1986) Photochem. Photobiol. **43**, 515–523

Manning, M. C. & Woody, R. W. (1986) Biophys. J. **49**, 296a

Marx. G. & Chevion, M. (1985) Biochem. J. **236**, 397–400

Noyer, M. & Putnam, F. W. (1981) Biochemistry **20**, 3536–3542

Orr, C. W. M. (1967) Biochemistry **6**, 3000–3006

Osaki, S., Johnson, D. A. & Frieden, E. (1966) J. Biol. Chem. **241**, 2746–2751

Plonka, A., Metodiewa, D., Zgirski, A., Hilewicz, M. & Leyko, W. (1980) Biochem. Biophys. Res. Commun. **95**, 978–984

Roshchupkin, D. I., Talitsky, V. V. & Pelenitsyn, A. B. (1979) Photochem. Photobiol. **30**, 635–643

Ryden, L. (1971) FEBS Lett. **18**, 321–325

Ryden, L. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 6767–6771

Ryden, L. (1984) in Copper Proteins and Copper Enzymes, vol. 3 (Lontie, R., ed.), pp. 37–100, CRC Press, Boca Raton, FL

Ryden, L. & Bjork, I. (1976) Biochemistry **15**, 3411–3417

Samuni, A., Chevion, M. & Czapski, G. (1981) J. Biol. Chem. **256**, 12632–12635

Shinar, E., Navok, T. & Chevion, M. (1983) J. Biol. Chem. **258**, 14778–14783

Singh, A., Antonsen, S. A., Koroll, G. W., Kremers, W. & Singh, H. (1984) in Oxygen Radicals in Chemistry and Biology (Bors, W., Sarah, M. & Tait, D., eds.), pp. 491–500, Walter de Gruyter, Berlin

Spikes, J. D. & Straight, R. (1981) in Oxygen and Oxy Radicals in Chemistry and Biology (Rodgers, M. A. J. & Powers, E. L., eds.), pp. 421–424, Academic Press, London

Stocks, J., Gutteridge, J. M. C., Sharp, R. J. & Dormandy, T. L. (1974) Clin. Sci. Mol. Med. **47**, 223–233

Sun, M. & Zigman, S. (1979) Photochem. Photobiol. **29**, 893–897

Syed, M. A., Coombs, T. L., Goodman, B. A. & McPhail, D. B. (1982) Biochem. J. **207**, 183–184

Weiss, K. C. & Linder, M. C. (1985) Am. J. Physiol. **249**, E77–E88

Winyard, P. G., Lunec, J., Brailsford, S. & Blake, D. R. (1984) Int. J. Biochem. **16**, 1273–1278

Wolff, S. P. & Dean, R. T. (1986) Biochem. J. **234**, 399–403

Wong, P. S. & Travis, J. (1980) Biochem. Biophys. Res. Commun. **96**, 1449–1454

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