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Age-related Changes in Human Ceruloplasmin

EVIDENCE FOR OXIDATIVE MODIFICATIONS*

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Human plasma or serum from donors of age comprised between 15 and 95 years was analyzed for paramagnetic and total copper content, as well as for immunoreactive ceruloplasmin content and oxidase activity. All parameters were essentially unaltered, except the paramagnetic copper content, which increased 2-fold upon aging. A dramatic change of the electron paramagnetic resonance spectrum due to ceruloplasmin occurred in individuals over 65 years old and was associated with both an increase of the type 1 copper signal intensity and the appearance of new resonances of a type 2 copper species.

Ceruloplasmin was isolated from either young or old donors. Spectroscopic analyses of the isolated proteins confirmed the tendency of type 1 copper to stay reduced in the "young" and oxidized in the "old" protein. The type 2 copper signal observed in most young ceruloplasmin samples was different from the species invariably present in the old protein. The magnetic parameters of the latter species were more consistent with a partially reduced trinuclear copper site.

***In vitro* limited proteolysis resulted in identical fragmentation patterns and kinetics in both proteins. However, changes of the net electric charge were detected in the fragments of the protein isolated from aged individuals, which exhibited a carbonyl content of 0.6 mol of carbonyl/mol of protein. The same pattern of modifications, including a higher carbonyl content (0.65 *versus* 0.2 mol of carbonyl/mol of protein), could be reproduced by exposure of the young protein to the metal-catalyzed oxidation system iron/ascorbate. These results suggest that during aging ceruloplasmin is subjected to oxidative modifications which are likely to be the source of conformational changes around the copper sites leading to an intramolecular electron rearrangement among the various copper sites.**

Ceruloplasmin (CP),[1] the blue oxidase present in all vertebrates, is the major copper-containing protein of human plasma. Little is known about the biological function of the protein, for which both a role in copper transport (Cousins, 1985) and a catalytic activity toward a variety of substrates, including iron (Frieden, 1980), have been proposed. A series of studies has led to the proposal that CP plays a role in protecting against oxygen radical-mediated damages in plasma (Halliwell and Gutteridge, 1986), as well as in other exposed tissues like lung (Fleming *et al.*, 1991). However, conclusions have also been reached that CP is susceptible to oxidative modification *in vitro* (Winyard *et al.*, 1989) and that this may add to the susceptibility toward proteolytic attacks in determining the particular lability of the protein. Such lability has been a major obstacle in the study of the physicochemical characteristics of the protein. It is well known that once purified, CP easily undergoes conformational changes that lead to irreversible modifications of its spectroscopic properties, in particular those detected by EPR spectroscopy. Many investigations have pointed out the particularly labile conformation of the type 2 copper site, one out of the three distinct classes of copper-binding sites present in this protein (Malmstrom *et al.*, 1975), including also the type 1, mononuclear intensely blue site, and the type 3, binuclear site EPR-undetectable because of magnetic pairing. Different opinions, concerning the nature and numbers of the copper sites in human CP, are present in the literature (Rydén, 1984 and references therein). The EPR signal of the type 2 center would be an intrinsic feature of human CP, both in serum and in the purified protein (Rylkov *et al.*, 1991). In a series of studies carried out on ceruloplasmins from various sources, except human, it has been possible to show that type 2 copper is not detectable by EPR, possibly because of unique magnetic interactions with the type 3 copper pair, in the native state. These magnetic interactions easily break down, leading to disclosure, to a various extent, of type 2 copper signals in the EPR spectrum. This has been shown to occur upon storage of the protein or during redox cycles involving electron transport from donor substrates to oxygen (Calabrese *et al.*, 1988, 1989). In this latter case, however, the type 2 signal behaves as a transient and possesses slightly different magnetic parameters. Thus, it appears that type 2 copper can be considered an elective spectroscopic monitor for the transition of the protein toward altered, stable, conformational states. Type 3 copper constitutes, along with the type 2 copper site, the oxygen binding site. It has been implicated in the blue (400–450 nm) fluorescence exhibited by CP as well as by other multinuclear blue oxidases upon excitation at around 330 nm (Wynn *et al.*, 1983). Storage of the protein, low intensity UV irradiation, or *in vitro* induced oxidative stress have been reported to enhance the intensity of such fluorescence in CP, which has been therefore tentatively attributed to oxidation products of either half-cystine, tryptophan, or tyrosine resi-

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[1] The abbreviations used are: CP, ceruloplasmin; CP_Y, CP isolated from plasma or serum of young donors; CP_O, CP isolated from plasma or serum of old donors; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-sulfonate) of ammonium; DNPH, 2,4-dinitrophenylhydrazine; ELISA, enzyme-linked immunosorbent assay; T, tesla.

dues or to modified carbohydrates (Lunec *et al.*, 1985; Winyard *et al.*, 1989; Avigliano *et al.*, 1983).

There is increasing evidence in the literature that aging is a complex phenomenon involving a functional impairment of proteins at the molecular level. A major mechanism for aging has been invoked to be the covalent, irreversible modification of proteins, often associated with oxidative stress. It is well established that inactive or less active forms of several enzymes accumulate in cells during aging (Gershon and Gershon, 1970; Stadtman, 1986, 1988) and that many of these enzymes are among those that have been shown to be sensitive to metal-catalyzed oxidative modifications (Oliver *et al.*, 1987b). A positive relationship between cellular age and amount of carbonyl groups has been reported for human erythrocytes and cultured fibroblasts (Oliver *et al.*, 1987a), for human lenses (Garland *et al.*, 1988), as well as for rat hepatocytes (Starke-Reed and Oliver, 1989). In other cases, aging has been interpreted in terms of a conformational drift of a protein structure toward more stable states with possibly impaired functionality (Sharma *et al.*, 1980; Rothstein, 1984).

Past studies, based on either immunological or enzymatic determinations, have pointed out that only minor quantitative changes of CP occur upon aging in humans (Lyngbye and Krøll, 1971) and rats (Semsei, 1991). No report, however, has so far appeared on age-related structural modifications of CP. In this paper, we describe the results obtained by studying human CP directly in the plasma of healthy donors aged 15–95 years, and we have compared the properties of CP in the plasma with those of the purified protein. The existence of altered forms of CP in aged plasma, possibly deriving from oxidative modifications of the primary structure of the protein, was demonstrated.

EXPERIMENTAL PROCEDURES

Materials—All reagents were of analytical grade and were used without further purification, except chloroethylamine (Carlo Erba), which was recrystallized before use. Sepharose 4B was from Pharmacia LKB Biotechnology Inc. Human plasmin, bovine trypsin and chymotrypsin, bovine serum albumin, Tween 20, streptavidin-peroxidase conjugate, peroxidase substrate ABTS, and polystyrene culture plates (96 wells/plate) were from Sigma. Antiserum to human CP was from Calbiochem. Horseradish peroxidase was from Boehringer Mannheim.

Sample Collection—Human blood samples of healthy donors were collected from laboratory volunteers or from guests of Casa di Riposo "Baratto" (Schio, Italy). Sera and plasmas were separated from noncitrated and citrated blood, respectively, right after blood collection. Separation was achieved by centrifugation at 3,000 rpm for 15 min.

Preparation of Derivatized Sepharose—Sepharose was derivatized by a modified protocol of a published procedure (Calabrese *et al.*, 1988). 100 ml of the resin was suspended in 70 ml of 5 N NaOH under constant stirring in an ice bath, and 7 ml of epichlorohydrine followed by 0.5 g of $NaBH_4$ were added. The mixture was then incubated for 1 h at 40 °C and for 2 h at 60 °C. The resin was subsequently washed on a Buchner funnel with water until the pH reached neutrality and was then resuspended in 70 ml of 2 N NaOH containing 0.25 g of $NaBH_4$. The suspension was either heated at 95 °C for 45 min or subjected to three boiling-cooling cycles, then sequentially washed with 1 liter of 1 N NaOH containing 5 g of $NaBH_4$ and water until the pH reached neutrality. Excess borohydride was then removed by washing with 500 ml of diluted acetic acid (pH 3–4) followed by water to restore neutral pH. 100 ml of activated Sepharose was suspended in 70 ml of 10 N NaOH under constant stirring at 60 °C, and 100 ml of 100% (w/v) chloroethylamine was slowly added. At this stage, care was taken to keep the pH high (10–12) during the coupling reaction by the addition of 10 N NaOH. The resin was then incubated for 2 h at 70 °C, washed with water to remove unreacted material and to lower the pH to neutrality, and finally suspended in buffer, ready for use. Each batch of derivatized Sepharose was stored at 4 °C and sterilized with 0.1 N NaOH before use. Maximal binding capacity was about 8–10 mg of CP/ml of resin.

Isolation of Human Ceruloplasmin—CP was purified from human plasma by single step passage on derivatized Sepharose, with minor modifications of the procedure already used for the isolation of various other ceruloplasmins (Calabrese *et al.*, 1988, 1989; Musci *et al.*, 1990) The whole purification procedure was carried out at 4 °C, unless otherwise stated. Citrated fresh or frozen plasma from healthy volunteers was used immediately after withdrawal or thawing. The plasma was supplemented with 20 mM ϵ-aminocaproic acid and batchwise applied as such to the resin for 15 min. Because of the limited amount of serum available, CP_O was invariably purified from pools of 30–50 sera, withdrawn from donors aging 80–95 years, to obtain about 30 ml of serum to be processed. On the other hand, CP_Y was always purified from plasma or serum of individual donors, except for those samples utilized as control in fluorescence and electrophoretic analyses, and in carbonyl content measurements. In these cases, analogous pools (donors' age, 30–45 years) were made. Typically, for 200 ml of plasma from an individual donor, 20 ml of the chromatographic material (previously equilibrated in 50 mM phosphate buffer pH 7.0) were used. After filtering away the plasma, the resin (which was now pale blue) was washed with 300 ml of 80 mM phosphate buffer, pH 7.0, and 100 ml of 100 mM phosphate buffer, pH 7.0, both containing 0.1 M ϵ-aminocaproic acid, to remove nonspecifically bound proteins. A further washing with 100 ml of 120 mM phosphate buffer, pH 7.0, which caused a 20–40% loss of impure CP, was followed by elution of 50–60 μM pure (>95%) CP with 200 mM phosphate buffer, pH 7.0. When contaminants > 10% were still present, the protein could be purified by passage on quaternary amino ethyl Sephadex, in 30 mM phosphate buffer, pH 7.4, and elution with phosphate buffer 500 mM, pH 7.0. The entire purification procedure took from 20 to 100 min, depending on the starting amount of plasma.

ELISAs—All steps used a volume of 100 μl/well and were carried out at room temperature, unless otherwise indicated. Polystyrene wells were coated overnight at 4 °C with 1 μg of antibody, in phosphate-buffered saline, pH 7.0, containing 0.15 M NaCl and Tween 20, 0.5%, v/v. The antibody solution was then removed and the wells washed three times with phosphate-buffered saline plus Tween 20, 0.5%, v/v. This buffer was used for all subsequent washing steps. A 30-min incubation at 37 °C with 1% bovine serum albumin followed, to ensure complete saturation of unoccupied centers of sorption. After three washings, the immobilized antibody was then incubated for 1 h with either a standard, prepared by adding 5 μM purified human CP to CP-depleted plasma (prepared by passage on derivatized Sepharose) or with plasma or serum. The following dilutions in phosphate-buffered saline plus Tween 20, 0.5%, v/v, were used in all cases: 1:200,000; 1:64,000; 1:48,000; 1:32,000; 1:25,000; 1:16,000. Three more washings were performed, and 1 μg of biotinylated antibody (prepared after DiSilvestro *et al.*, 1988) were added. After 1 h the antibody was removed, the wells were washed three times and incubated with streptavidin-peroxidase conjugate (1:250 in phosphate-buffered saline plus Tween 20, 0.5%, v/v) for 30 min. The solutions again were removed, the wells washed three times, and ABTS, containing 1 mM H_2O_2, was added. The reaction was stopped after 30 min by the addition of 50 μl of 0.4% NaF, and the A_{405} was measured.

Determination of Protein Concentrations—Protein concentration was determined either according to Lowry *et al.* (1951) or by the biuret method (Goa, 1953). The latter method yielded values invariably lower by about 10%.

Copper Content Measurements—Total copper in purified CP samples was determined by the chemical method of Brumby and Massey (1967). The stoichiometry of copper atoms/protein molecule was estimated on the basis of protein concentrations measured by the biuret method. Total copper in sera and plasmas was measured by atomic absorption spectroscopy on a Perkin-Elmer-Cetus Instruments 3030 spectrometer equipped with a graphite furnace. The atomization temperature was 2,600 °C. Samples were diluted 5–10-fold in 0.2% nitric acid and kept at room temperature for 24 h before measurement.

Limited Proteolysis—Limited proteolysis of CP was achieved by incubation with plasmin, trypsin, or chymotrypsin (1:15 enzyme:substrate ratio) in 30 mM phosphate buffer, pH 7.0, for up to 30 min at room temperature. The reaction was stopped by the addition of 0.1 mM phenylmethylsulfonyl fluoride.

Electrophoretic Analyses and Western Blot—Disc electrophoresis in native conditions or in the presence of SDS was run according to Davis (1964) and Laemmli (1970), respectively. Two-dimensional electrophoresis was carried out by a modification of the method of O'Farrell (1975). Isoelectric focusing in the presence of 9 M urea was performed in a Pharmacia Multiphor II apparatus on ultrathin (0.5-

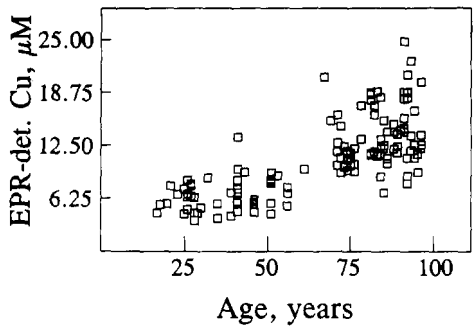

FIG. 1. **Plot of the intensity of the EPR copper signal of human plasma as a function of donor's age.** The EPR spectra were collected with the following settings: microwave power, 20 milliwatts, microwave frequency, 9.115 GHz, modulation amplitude, 1 mT, temperature, 100 K.

mm) gel slabs in a 2.5% ampholyte gradient from pH 4.0 to 6.5. Gel strips were cut and equilibrated 10 min in water and 10 min in 0.1% SDS-containing sample buffer and then electrophoresed on 12% SDS-polyacrylamide gel slabs and stained overnight with Coomassie Brilliant Blue R-250. Protein samples were denatured prior to loading by incubation for at least 30 min at room temperature in 9 M urea containing 80 mM dithiothreitol and 100 mM glycylglycine.

Western blot was carried out according to Sato *et al.* (1990), except that bovine serum albumin was used as blocking agent and detection of immunoreactive material was achieved by using the Extravidin Staining Kit (Sigma).

Oxidation of CP—Purified human CP was incubated with 25 mM ascorbate in the presence of 0.1 mM ferric chloride for 2 h at 25 °C. Ascorbate and iron were then removed by repeated passage on Sephadex G-25 (Amici *et al.*, 1989). EDTA was never added to the mixture to avoid chelation and abstraction of copper from the protein. Carbonyl content was measured by reaction with DNPH according to Oliver *et al.* (1987a).

Activity Measurements—Oxidase activity toward *o*-dianisidine was measured by the method of Schosinski *et al.* (1974). Activities were converted into CP concentrations by comparison with the oxidase activity of the standard used for ELISA determinations.

Spectroscopic Measurements—X-band low temperature EPR spectra were recorded with a Varian E-9 spectrometer, equipped with a Stelar temperature controller and interfaced to a Stelar Prometheus Data System for computer analysis and handling of the data. Paramagnetic copper content was estimated by double integration *versus* a Cu-EDTA standard. Optical measurements were performed on a Perkin-Elmer-Cetus Instruments 330 spectrophotometer, equipped with a Haake model G temperature controller. Fluorescence spectra were run on a Fluoromax ISA spectrometer. Bityrosine, used as a reference compound in fluorescence measurements, was enzymatically prepared according to Gross and Sizer (1959).

RESULTS

Ceruloplasmin in Human Plasma or Serum—About 100 human specimens of plasma or sera, from subjects 15–95 years old, were collected, and their EPR spectrum was measured under standard conditions (100 K, 20 milliwatts).[2] Pregnant women, women under hormonal therapy, and all subjects bearing any sort of major disease were not included in this screening. In particular, donors over 60 years old were in generally good health, apart from minor pathologies typical of the old age. Fig. 1 shows a plot of the intensity of the multiline EPR signal around $g = 2$, due to CP copper, as a function of age. The amount of EPR-detectable serum copper was stable for individuals up to 65 years old and then sharply increased, with a 2-fold enhancement factor between younger and older subjects. To ascertain whether the EPR changes upon aging were only quantitative, or rather involved qualitative modifications, all spectra were singularly analyzed, and the spectral line shape was found to fall into three groups, corresponding to distinct ranges of donor's age: 15–40 years ("young"), 40–65 years ("middle-aged"), and 65–95 years ("old"). The EPR spectra of all individuals of the same age group were computer-averaged, and the resulting curves are presented in Fig. 2. *Spectrum a*, from plasma of young donors, was composed by four clear resonances in the parallel region and by a single line in the perpendicular region of the spectrum. The splitting (about 7.5 mT) between the four hyperfines was typical of type 1 (blue) copper centers (Malmstrom *et al.*, 1975). In *spectrum b*, a broad resonance around 0.263 T started to become visible. In the average EPR spectrum of old individuals (*spectrum c*), an analogous resonance, centered at a slightly higher magnetic field, was well resolved. Resonances at these field positions are generally considered to arise from the first hyperfine line of copper ions in a type 2 (nonblue) site geometry (Malmstrom *et al.*, 1975). Thus, compared with the young spectrum, the old ceruloplasmin shows two prominent features: a different line shape related to the appearance, upon aging, of a signal of type 2 copper, and a nearly doubled overall intensity.

Over half of the copper ions are not detectable by EPR in native CP and only become EPR-detectable, giving type 2 copper signals, during redox processes or upon denaturation of the protein. The observed positive correlation between paramagnetic copper content in serum and donor's age might therefore originate either from an augmented content of paramagnetic copper per molecule or from an absolute increase of the amount of circulating CP. To distinguish between these two possibilities, sera of young and old individuals (middle-aged ones were disregarded, being essentially similar to younger individuals) were measured for (i) their content of immunoreactive CP, by ELISA; (ii) their total copper content; and (iii) their oxidase activity, using *o*-dianisidine as substrate. Purified human CP was used as reference in all measurements. Various samples of CP, purified from either young or old subjects (see next section), including freshly prepared or stored proteins, were reintroduced, at known concentration, into plasma previously depleted of CP, and these solutions were used as standards to make sure that a quantitative

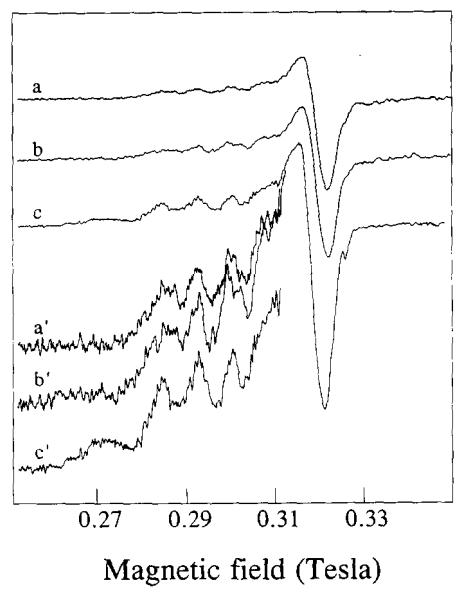

FIG. 2. **Averaged EPR spectra of plasmas belonging to different age groups.** *a*, 15–40 years old; *b*, 40–65 years old; 65–95 years old. *Spectra a'* and *b'* are 8-fold amplifications, *spectrum c'* is amplified 4-fold. Experimental settings were the same as in Fig. 1.

[2] It has been recently shown (Musci *et al.*, 1992) that plasma and serum of the same individual have identical EPR spectra.

comparison could be made. All standards gave identical results on ELISA, indicating that the polyclonal antibody used could not discriminate between different conformations of CP.

The results are summarized in Table I, which also reports data for paramagnetic copper, evaluated by double integration of the EPR spectra. All parameters, except paramagnetic copper, appear to be only slightly affected upon aging, ruling out an increase of the amount of circulating CP as the source of the different EPR spectra of old individuals. On the other hand, CP shows the same efficiency as an *in vitro* oxidase, both in young and in old plasma. This result is consistent with previous findings on the independence of oxidase activity of CP from the spectroscopic properties of the protein (Calabrese *et al.*, 1989).

Thus, the essentially unaltered total copper content and immunoreactive protein values between young and old plasma strongly suggest that the increase of paramagnetic copper upon aging is caused by conversion of EPR-silent copper into EPR-detectable copper. However, allowing for a 10% higher total copper concentration in older *versus* younger subjects (Table I) and considering, for the human protein, a stoichiometry of two type 1 centers for each type 2 site (Deinum and Vänngard, 1973), a 65% increase of paramagnetic copper would be expected, as due to the appearance of a signal originating from one type 2 copper atom. An even lower (45%) increase would be attained when three blue ions/CP are considered (Ortel *et al.*, 1984). Therefore, the disclosure of type 2 copper cannot, by itself, completely account for the over 100% increase in paramagnetic copper upon aging. A possible explanation is that copper ions, other than type 2, become EPR-detectable in sera from old individuals. The type 3 pair, in its half-oxidized, or half-met form (Solomon *et al.*, 1987), could give rise to an EPR signal, thus contributing to the increase of the first hyperfine line of the type 2 copper. A careful analysis of the spectra in Fig. 1 and of the data of Table I allows us, on the other hand, to assess that an increase of the type 1 copper signals takes place upon aging, besides the appearance of type 2 copper resonances. This suggests that part of the type 1 copper of CP may stay reduced in the plasma of young and reoxidize in the plasma of old individuals.

Different results were obtained when the comparison was made between subjects belonging to the same age group. In this case, a linear, positive relationship was found to exist between EPR-detectable copper and either oxidase activity or immunoreactive CP. Fig. 3 shows the results pertaining to the old individuals. Results from young subjects were essentially identical. These relationships demonstrated that the differences in paramagnetic copper content were caused by quantitative changes of CP, independent of the state of the protein. Thus, different mechanisms are responsible for the variation

TABLE I

Summary of properties of CP in human serum for different classes of age

Age	EPR[a]	ELISA	Total copper	Activity[b]
years	*μM*	*μM*	*μM*	*μM*
15–40	6.21 ± 1.90	3.81 ± 1.1	17.8 ± 2.8	3.72 ± 1.92
40–65	6.93 ± 1.68			
65–95	13.5 ± 3.75	3.65 ± 1.05	19.4 ± 3.2	3.69 ± 0.93

[a] Values represent concentrations of paramagnetic copper, estimated by double integration of the spectra *versus* a Cu-EDTA standard.

[b] Activity rates have been converted into CP concentrations, by comparison with the oxidase activity of a standard at known CP concentration.

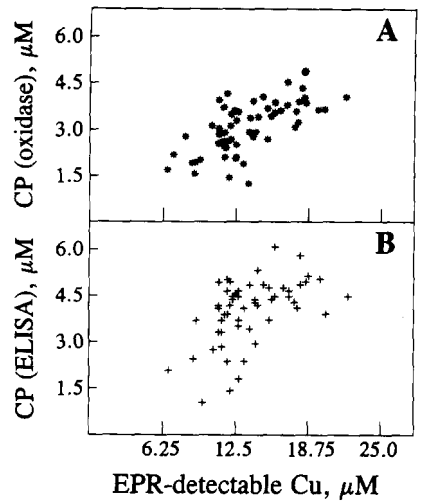

FIG. 3. **Relationship between oxidase activity (*panel A*) or immunoreactive CP (*panel B*) and intensity of the EPR copper signal of plasmas belonging to old donors (65–95 years old).**

of the EPR spectrum of plasma CP: quantitative changes occur inside the same age group, and qualitative modifications of the protein are observed when individuals from distinct age categories are compared. This was further corroborated by analyses of sera from young pregnant women, who are known to have higher plasma levels of CP (Flynn, 1980). Although EPR intensities were in the range of those observed for old subjects, the line shape fully reproduced that typical of young individuals (data not shown).

Purified Human Ceruloplasmin—In the attempt to explain the mechanisms of age-related changes of CP, the spectroscopic properties of the isolated protein were carefully examined. Human CP was rapidly purified from either young (20–40 years, individual or pooled plasma, CP_Y) or old (80–95 years, pooled plasma, CP_O) donors with the procedure described under "Experimental Procedures." The protein was > 95% pure in both cases, as revealed by SDS-polyacrylamide gel electrophoresis, which showed only the 130-kDa band and a faint band at 110 kDa, caused by a fragment typically resulting from limited proteolytic cleavage. This result is analogous to that obtained in whole plasma,[3] indicating that the purified protein was representative of the state of CP in the parent plasma. Electrophoretic patterns in native conditions were also similar, suggesting a similar conformation and a negligible extent of aggregation. Mean values of 5.78 and 5.61 copper atoms/protein were obtained for CP_Y and for CP_O, respectively, indicating a stoichiometry of six metal ions/molecule, in good accord with the literature (Rydén and Bjork, 1976). The discrepancy between this value and the average stoichiometry (about 5) found directly in the plasma may be because of the presence, in the latter case, of a fraction of metal-free CP (Holtzman and Gaumnitz, 1970). It is worth of notice that the CP standards used for the ELISA determinations, where the purified protein had been reintroduced into CP-depleted plasma, all gave values very close to six copper atoms/molecule, indicating that the apo component was lost during the purification step.

The state of the copper binding sites in the purified proteins

[3] Actually, the antibodies used in these experiments were able to recognize even samples of CP which had been partially digested with trypsin or plasmin, as well as the individual fragments generated by these treatments. This property was used to reveal, by Western blot analysis, that CP is mainly present as a 130-kDa single chain in the plasma, with only a minor (about 10%) fraction of the 110-kDa.

was evaluated by both optical and EPR spectroscopy. The optical spectra of the two ceruloplasmins were similar; however, the absorbance at 610 nm, expressed as molar extinction/total copper, was 1,200 M^{-1} cm^{-1} in CP_Y and 1,580 M^{-1} cm^{-1} in CP_O. To test for the presence of reduced type 1 copper, both proteins were treated with ferricyanide. Only CP_Y was affected by the treatment, with its molar extinction/total copper eventually reaching the value of 1,600 M^{-1} cm^{-1}. This value is consistent with that assigned in the past to the human protein purified by harsher and longer isolation procedures (Deinum and Vänngard, 1973). The EPR spectra of both CP_Y and of CP_O showed the presence of resonances assigned to type 1 and 2 copper (Fig. 4), with the following major differences. In CP_Y, the height of the first hyperfine of type 2 copper varied from sample to sample, as shown in *spectra a–c* of Fig. 4, representative of the line shape found in different samples, all purified under identical conditions. The increase was paralleled by an increment of the intensity of the overall signal, which accounted for a paramagnetic copper content of 38, 40, and 43%, respectively. Treatment with ferricyanide induced a noticeable increase of only the type 1 copper EPR signal in CP_Y, with the paramagnetic fraction of the copper reaching about 55% of total copper (data not shown), confirming that a fraction of blue copper is reduced in CP_Y but not in CP_O, in analogy with what observed on the respective sera. *Spectrum a* is representative of "best" preparations (2 out of 15), with goodness implying an EPR-silent type 2 copper. *Spectrum d* (Fig. 4), which reports the line shape of sample *a* after concentration on Centricon YM30 and 1 month of storage at −20 °C, suggests in fact that degradative phenomena are the source of the appearance of the type 2 copper signal in the purified protein. *Spectra a–c* apparently reproduce the trend observed for CP in the plasma of different age

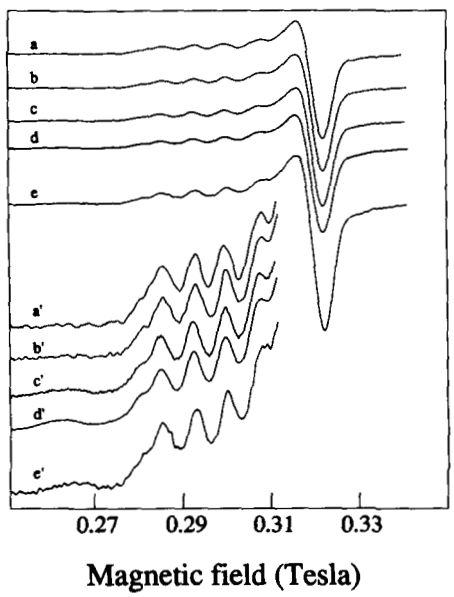

FIG. 4. **EPR spectra of various CP samples in 150 mM phosphate buffer, pH 7.0.** *Spectra a–c* are from young single individuals and show different amounts of EPR-detectable type 2 copper. The three samples had been purified with identical isolation procedures. *Spectrum d* represents the sample shown in *spectrum a* which had been stored for 1 month at −20 °C and then concentrated on Centricon YM30. The spectra are normalized on the $g = 2$ peak-to-peak intensity. *Spectrum e* is from a pool of sera from old (>80 years) individuals and is shown normalized for protein concentration on *spectrum d*. Experimental settings are as given in Fig. 1. Primed spectra are 8-fold amplifications, except *spectrum e'*, which is amplified 6-fold.

groups (see Fig. 2). A close examination of the spectra in Figs. 2 and 4, however, reveals that the type 2 copper of the protein purified from young plasma may not be the same species that emerges during aging in old plasma, as evidenced by the low field hyperfine line, broadened and shifted at higher magnetic fields in this latter sample. This is even more evident when we consider the EPR spectrum of CP_O, which shows, as expected, a higher intensity of the signal on a protein concentration basis, besides the shifted position of the type 2 copper first hyperfine line (Fig. 4, *spectrum e*). Various samples of CP_O obtained from three different pools of old sera were examined, but no spectral variability was found. Moreover, no appreciable changes of the EPR line shape were noticed upon storage of the protein. As far as the spectroscopic features of the copper sites are concerned, CP_O seemed to be "locked" in a conformation distinct from those available to CP_Y. In the attempt to elucidate the mechanism(s) responsible for such differences, different approaches were followed. Since CP is known to be highly labile toward known proteases, and an increased proteolytic susceptibility may be considered a sensitive index of protein modification (Oliver *et al.*, 1987a; Davies *et al.*, 1987b), the susceptibility to limited proteolysis was checked. Fragmentation patterns apparently undistinguishable, as judged by SDS-polyacrylamide analyses, were obtained in CP_Y and in CP_O with either trypsin, plasmin, or chymotrypsin. The kinetics of cleavage, evaluated in the time interval of 5–60 min, were also similar. In particular, the pattern obtained after digestion for 30 min with plasmin was fully consistent with that already reported for human CP (Ortel *et al.*, 1984), with major fragments with M_r 92,000, 26,000, 25,000, and 19,000 and minor components of M_r 116,000, 67,000, and about 50,000 (results not shown). To identify possible changes in the electric charge, the two proteins were analyzed by two-dimensional gel electrophoresis under denaturing conditions. This analysis revealed the presence of a major (95%) spot in both uncleaved CP_Y and CP_O (corresponding to the 130-kDa form), with pI values of 5.72 and 5.75, respectively, and a minor, more acidic species identical in the two proteins. Multiple components were present in the digested proteins. Nearly all fragments exhibited a different behavior in CP_Y *versus* CP_O (Fig. 5). Although the 19-kDa fragment was slightly ($\Delta pI \sim 0.04$) shifted to higher pI values, both the 92- and the 26-kDa exhibited multiple components scattered over a larger pI interval because of the appearance of more acidic species ($\Delta pI \sim 0.15$ and ~ 0.05, respectively) in CP_O *versus* CP_Y.

To establish whether such changes were caused by spontaneous phenomena such as sequence- or structure-facilitated deamidation phenomena (Kossiakoff, 1988) or to exogenous processes such as the oxygen radical-mediated conversion of amino acids to spectroscopically detectable species (Stadtman, 1990a), the formation of carbonyl derivatives and of visible fluorescence were monitored. The *left panel* at the *bottom* of Fig. 5 shows the optical absorbance formed upon reaction of CP with DNPH, revealing the presence of hydrazone adducts of carbonyls, in CP_Y (*curve a*) and in CP_O (*curve b*). The carbonyl content of CP from old individuals could be estimated to be about 0.6 mol of carbonyl/mol of CP, *versus* a value of about 0.2 for the protein purified from the plasma of young donors. It is important to point out that these values were independent of the spectroscopic properties of the samples, *i.e.* the detectability of type 2 copper, and were unaffected by previous storage and manipulation of the protein sample. On the other hand, when the CP_Y sample was incubated with ascorbate and iron in the presence of oxygen, a mixture known to induce oxidative damages in proteins, its carbonyl content

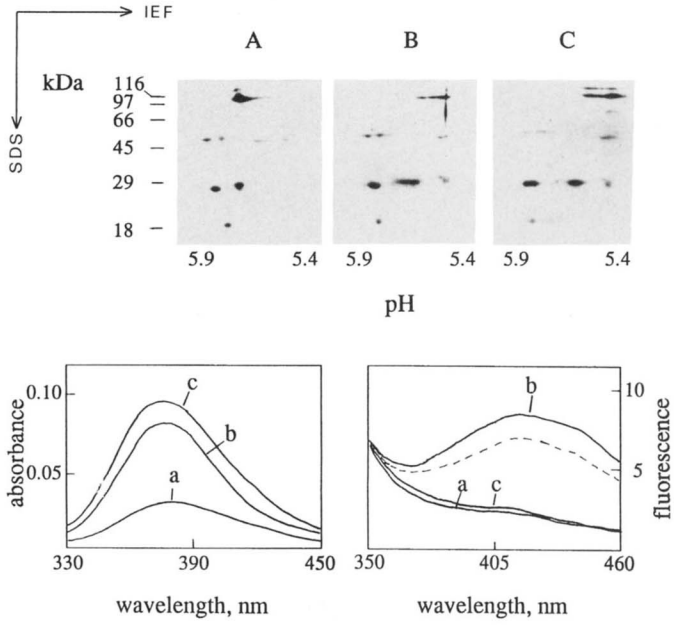

FIG. 5. **Analysis of oxidative modifications in purified human CP.** *Upper panel*: two-dimensional gel electrophoresis of CP_Y (*panel A*), CP_O (*panel B*), and CP_Y after incubation with iron and ascorbate (stressed CP_Y) (*panel C*). See "Experimental Procedures" for further details. *Lower left panel*: absorption spectra of DNPH-reacted CP_Y (*a*), CP_O (*b*), and stressed CP_Y (*c*), representing the carbonyl content of the various samples. The final protein concentration was 0.9 mg/ml. All spectra were corrected for protein blank absorption. *Lower right panel*: emission fluorescence spectra of CP_Y (*a*), CP_O (*b*), and stressed CP_Y (*c*). The *dashed curve* represents the spectrum of a sample of manipulated CP_Y (see "Results" for details). Excitation wavelength was 325 nm. The protein was 2.35 mg/ml in 150 mM phosphate buffer, pH 7.0. The fluorescence intensity is expressed in arbitrary units.

was raised to 0.65 mol of carbonyl/mol of CP (*curve c*). The two-dimensional electrophoretic pattern of the latter sample, shown as *lane C* in the *upper* part of Fig. 5, indicated that the protein moiety had undergone charge modifications similar to those observed in CP isolated from sera of old donors.

A different behavior was observed when the fluorescence spectra of all of these samples were measured to characterize the state of the aromatic residues in our ceruloplasmins (*right panel* in the *bottom* part of Fig. 5). As can be seen, CP_Y displayed a negligible fluorescence in the 350–450 nm region, when excited at 325 nm (*curve a*), and CP_O exhibited a broad emission band, with a maximum at 410 nm and a shoulder at longer wavelength (*curve b*). The oxidatively "stressed" sample did not show any alteration, remaining similar to the parent CP_Y (*curve c*). Moreover, storage and/or manipulation of the sample was able to induce the blue fluorescence in CP_Y, although to a variable extent. As an example, the emission spectrum of the CP_Y sample the EPR spectrum of which had been shown as *curve d* in Fig. 4 is reported as a *dashed line* in Fig. 5.

These results indicated that the increase of blue fluorescence of ceruloplasmin was not directly related to the process of aging *in vivo* but may rather derive from changes of the protein moiety which occur upon storage. However, since CP_O appeared to be more prone than CP_Y to undergo such modifications, attempts were made to identify the residue(s) implied in this phenomenon. Considering that oxygen radical-induced bityrosines are generally involved in the fluorescence emission over 400 nm (Davies *et al.*, 1987a), the line shapes of both the excitation and the emission spectra of fluorescent CP samples were carefully analyzed and compared with those of authentic bityrosine produced by enzymatic conversion of tyrosine by horseradish peroxidase. Both the excitation and the emission patterns of CP displayed at least two components, at 325 and 350 nm, and at 410 and 440 nm, respectively, at variance with the model compound, which exhibited a single peak centered at 410 nm. Moreover, the fluorescence of CP was found to be unchanged between pH 5.5 and 8.5. A strong pH dependence in this pH interval is considered a most characteristic feature of bityrosine, at least in oligopeptides, because of the low ionization constant of one of the two phenols (Prutz *et al.*, 1983).

DISCUSSION

Direct evidence is presented in this paper that human ceruloplasmin undergoes, upon aging of the organism, modifications of the protein moiety, which dramatically affect the state of prosthetic copper sites. The data obtained in this work also show that this process is clearly distinct from that occurring on the isolated protein *in vitro*. The various copper binding sites of ceruloplasmin seem to be the elective spectroscopic probes of the ongoing modifications of the protein molecule and are, in this respect, more sensitive markers of the state of the protein, when compared with other properties, such as enzymatic activity, immunological properties, or susceptibility to proteolysis.

The blue copper-containing sites are the only magnetic probes of human CP in the adult life span (15–65 years). The conformation of the molecule is such that other centers are not detectable by EPR. In particular, type 2 copper seems to be EPR-silent, and this property is retained also in some batches of the protein purified from young donors. The nature of type 2 copper centers in human CP has been the object of many conflicting reports. Evidence has been repeatedly presented that EPR-detectable type 2 copper is a feature of human CP even in serum (Deinum and Vänngard, 1973; Rylkov *et al.*, 1991). Recent findings from our laboratory have rather suggested that, in this latter case, it was indeed copper lost by CP and associated to albumin (Musci *et al.*, 1992). Actually, the finding that human CP can also be purified in a form that lacks an EPR-detectable type 2 copper is a major breakthrough in the history of the purification of this protein. A structural basis for this property may reside in the strong interactions of this center with the type 3 copper pair, possibly to form a trinuclear cluster, as already suggested for other ceruloplasmins displaying similar properties (Calabrese *et al.*, 1988, 1989; Musci *et al.*, 1990). The maintenance of these interactions appears particularly critical. Both the aging process and the changes occurring *in vitro* on the isolated protein invariably induce the appearance of resonance(s) typical of a type 2 copper species in the EPR spectrum. However, the type 2 copper signal of old plasma CP is different from that seen in some batches of the purified protein from young individuals (Fig. 4, *curves b–d*). Although the magnetic parameters ($A_{//}$, $g_{//}$) cannot be precisely measured, it is evident that the $M_I = -3/2$ hyperfine line lies at higher magnetic fields in the case of the old protein, either purified or in the plasma (Fig. 2, *curve c*, and Fig. 4, *curve e*). The disclosure of the type 2 copper signal which takes place upon isolation of the protein from young plasma occurs at variable extent and apparently does not correlate with modifications of the other copper sites. Since all young plasmas were spectroscopically homogeneous, a straightforward explanation for the differences among purified samples of CP is that the purification procedure itself, although rapid and mild, can induce conformational rearrangements of the protein backbone, which lead to appreciable modifications of the geometry of the type 2 copper

binding site. This is not surprising since CP, in particular the human protein, has always been considered a particularly labile system, which easily undergoes profound spectroscopic modifications (Rydén, 1984).

The conformational rearrangements of the protein backbone which lead to the modification of the state of the type 2 site appear to be monitored by the enhancement of the fluorescence of the protein in the blue region, although a clear-cut relation between the two phenomena was not evident. This will be discussed later on.

The other striking property of CP_Y is that a considerable fraction ($\geq$ 30%) of type 1 copper is in the reduced state, as suggested by the increase of both A_{610} and blue hyperfine lines upon reaction with ferricyanide. This property has never been mentioned before for human CP. However, these data are not really in contrast with the literature, when we consider that longer purification procedures probably induced reoxidation of blue centers, along with the appearance of the type 2 signal (Deinum and Vänngard, 1973). The property appears to be related to the functioning of the protein *in vivo*, as confirmed by the EPR spectra of young plasma, where the blue component is weaker with respect to that observed in old sera. This is furher confirmed by the high value of the optical extinction and by lack of reaction of CP_O with ferricyanide.

The type 2 copper species present both in old sera and in the corresponding purified protein is certainly related to this phenomenon. In fact, the presence of this peculiar signal is invariably accompanied by a maximal intensity of both optical and EPR signals of type 1 copper. It is known that in multinuclear copper units, like those present as copper pairs in laccase, tyrosinase, and hemocyanin (Solomon *et al.*, 1987), or as a trinuclear copper cluster in ceruloplasmin (Calabrese *et al.*, 1988), the selective reduction of one out of two metal ions in dimeric units (Solomon *et al.*, 1987), or of two out of three in trimeric units (Calabrese *et al.*, 1988), gives rise to type 2 copper signals very similar to that observed in CP_O.

In view of these considerations, a reasonable conclusion would be that the type 2 copper species present in the spectrum of CP_O, both purified and in the serum, represents a partially reduced trinuclear unit, originating from an intramolecular electron transfer from type 1 copper, which would be, for this reason, oxidized at its maximal extent.

The question now is which mechanism(s) govern(s) these modifications. It is clear that no gross changes of the protein occur upon aging, with respect to the size (aggregation and/or fragmentation) and to the conformation of the molecule. Neither CP_Y nor CP_O is appreciably proteolyzed in the respective sera or after purification. Both proteins can be recognized and proteolytically degraded *in vitro* by a series of proteases with identical fragmentation patterns and with comparable kinetics. However, the charge differences found in the resulting fragments allow us to hypothesize the presence of multiple modified sites as the structural basis of the observed differences between CP_Y and CP_O. Different mechanisms are known to mediate modifications of side chains of amino acid residues in proteins. The presence of DNPH-reactive carbonyls has been interpreted as a selective marker of oxidative damage (Stadtman, 1990b). A considerably higher content of carbonyl residues is found in CP_O *versus* CP_Y. The number of DNPH-reactive residues can be raised in CP_Y by subjecting the protein to a metal-catalyzed oxidative attack by ascorbate and iron, and the figure after treatment (0.65 mol of carbonyl/mol of CP) reaches about the same value found in CP_O as such (0.6). More important, the electrophoretic pattern evolves toward that of CP_O when the protein is treated with ascorbate and iron. Altogether, these results strongly suggest that the changes of CP upon aging may be caused by oxidative modifications. Moreover, the complex pattern of modified fragments indicates that these changes involve multiple target sites. There is an apparent discrepancy between the number of fragments which are affected by aging (at least three) and the number of amino acid residues found modified according to the carbonyl measurements. However, it has to be remembered that in addition to carbonyl derivatives, some amino acid residues are converted to other, and often more acidic, species upon oxidative attack and that there are residues that are never oxidized to carbonyl derivatives, like methionine and cysteines (Stadtman, 1990b and references therein). Oxidative modifications of the glucidic moiety of the protein, leading to release of terminal sialic residues, as shown to occur in IgG (Griffiths and Lunec, 1989) cannot, in principle, be ruled out for fragments of CP_O. In particular, the 26- and the 92-kDa fragments are both generated by cleavage of the N-terminal 116-kDa component allocating the four oligosaccharides chains (Ortel *et al.*, 1984). However, the more acidic nature of these fragments in CP_O *versus* CP_Y can be better explained by modifications of basic residues possibly yielding the corresponding carbonyl derivatives. The modification of the 19-kDa fragment cannot be due to the presence of carbohydrates, as no glycosylated site is present on this fragment (Ortel *et al.*, 1984). This fragment, which is at the C-terminal of the protein, has been postulated to possess ligands for different copper atoms. In particular, a cysteine ligand to type 1 copper is connected to two hystidine ligands of the trinuclear site in the sequence His-Cys-His (Rydén, 1982; Messerschimdt and Huber, 1990). The same sequence is present in glutamine synthetase, and it has been shown to be, in this latter case, the specific site of attack by mixed function oxidation systems, including iron/ascorbate (Farber and Levine, 1986). Although it is tempting to postulate that the same phenomenon might occur in CP, our data do not allow to assign the DNPH-reactive residue(s) to this fragment, possibly to a histidine of His-Cys-His, in analogy again with glutamine synthetase (Farber and Levine, 1986). However, many reasons support the idea that this part of the molecule might be involved in oxidative modifications, as already suggested. Since both ascorbate and iron are reducing substrates for the blue sites of CP, one can speculate that in case of excess of these reductants and in the presence of oxygen, a fraction of Fe(II) is not promptly reoxidized by CP, thus generating oxygen radicals. Residues at, or near to, the blue copper site and/or the trinuclear cluster would be attacked, with resulting oxidative damages apparently spread out all over the molecule. This would be consistent with the observed concomitant modification of the spectroscopic properties of both type 1 and the trinuclear cluster centers. The trinuclear cluster also comprises ligands located in the N-terminal region. An alternative hypothesis is that multiple binding sites for iron are present in CP and that they are all equally susceptible to oxidative modifications.

The fluorescence of CP_O appears not to be directly related to these events, since, as already pointed out, "worst" samples of CP_Y often display a rather strong fluorescence at around 400–450 nm and that, on the contrary, the ascorbate/iron system failed to induce an appreciable emission intensity in CP (see Fig. 5). The emission at 430 nm upon excitation at around 330 nm is a well known property of type 3 copper-containing proteins. There is, however, some confusion in the literature on the nature of the emitting fluorophore(s), which have been variably identified as the type 3 center itself, as modified aromatic side chains, or even as undissociated carboxylates (Wynn *et al.*, 1983; Avigliano *et al.*, 1983; Davies *et*

al., 1987a; Bacci *et al.*, 1983). In particular, storage and oxidative stress have been implicated in the onset of such fluorescence in human CP (Winyard *et al.*, 1989). Their results are apparently at variance with ours, however it should be emphasized that their stressed samples were also "stored" samples, since they had been kept in the thawed state for several days. The same authors identified the emitting fluorophores as oxidized products of the aromatic residues tryptophan and tyrosine. Among these, biphenol adducts like bityrosine have been implicated as major products of oxidative stress (Davies *et al.*, 1987a). However, bityrosine formation is known to occur when nonspecific oxidative attacks, like those exerted by γ-rays, are performed. Metal ion-catalyzed oxidation seems to be, in this respect, quite harmless (Stadtman, 1990b). Our experiments addressing the involvement of the bityrosine adduct in the fluorescence of CP, which were mainly based on comparison with model compounds, do not provide conclusive evidence. It should be remembered that the fluorescence parameters (energy, quantum yield) are strongly affected by the chemical environment surrounding the fluorophore (Lakowicz, 1983), which can be obviously different in CP with respect to low molecular weight compounds. Whatever is the mechanism underlying the production of fluorescence, conformational changes and/or oxidative attacks, it is clear that this property appears to monitor an *in vitro* aging of the isolated protein, rather than the *in vivo* process.

In conclusion, changes of oxidative nature can explain the spectroscopic differences observed between CP extracted from young and old individuals. To our knowledge, the results presented in this paper represent the first direct evidence of a protein oxidatively modified upon aging *in vivo*. The involvement of oxidative modifications of proteins in the aging process has been so far derived from the observation that inactive forms of several enzymes accumulate in cells during aging (Gershon and Gershon, 1970; Stadtman, 1986, 1988) and that many of these enzymes can be inactivated by metal-catalyzed oxidative stress carried out *in vitro* (Oliver *et al.*, 1987b). It remains to be established which one, of the many proposed functions of CP, is impaired upon aging.

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REFERENCES

Amici, A., Levine, R. L., Tsai, L., and Stadtman, E. R. (1989) *J. Biol. Chem.* **264,** 3341–3346
Avigliano, L., Sirianni, P., Morpurgo, L., and Finazzi-Agrò, A. (1983) *FEBS Lett.* **163,** 274–276
Bacci, M., Baldecchi, M. G., Fabeni, P., Linari, R., and Pazzi, G. P. (1983) *Biophys. Chem.* **17,** 125–130
Brumby, P. E., and Massey, V. (1967) *Methods Enzymol.* **10,** 473–474
Calabrese, L., Carbonaro, M., and Musci, G. (1988) *J. Biol. Chem.* **263,** 6480–6483
Calabrese, L., Carbonaro, M., and Musci, G. (1989) *J. Biol. Chem.* **264,** 6183–6187
Cousins, R. J. (1985) *Physiol. Rev.* **65,** 238–309
Davies, K. J. A., Delsignore, M. E., and Lin, S. W. (1987a) *J. Biol. Chem.* **262,** 9902–9907
Davies, K. J. A., Lin, S. W., and Pacifici, R. E. (1987b) *J. Biol. Chem.* **262,** 9914–9920
Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* **121,** 404–427
Deinum, J., and Vänngard, T. (1973) *Biochim. Biophys. Acta* **310,** 321–330
DiSilvestro, R. A., Barber, E. F., David, E. A., and Cousins, R. J. (1988) *Biol. Trace Elem. Res.* **17,** 1–9
Farber, J. M., and Levine, R. L. (1986) *J. Biol. Chem.* **261,** 4574–4578
Fleming, R. E., Whitman, I. P., and Gitlin, J. D. (1991) *Am. J. Physiol.* **260,** L68–L74
Flynn, A. (1980) in *Inflammatory Diseases and Copper* (Sorensen, J. R. J., ed) pp. 17–30, Humana, Clifton, NJ
Frieden, E. (1980) *Ciba Found. Symp.* **79,** 93–124
Garland, D., Russell, P., and Zigler, J. S., Jr. (1988) in *Basic Life Sciences* (Simic, M. G., Taylor, K. S., and Ward, J. F., eds) pp. 347–353, Plenum Press, New York
Gershon, H., and Gershon, D. (1970) *Nature* **227,** 1214–1216
Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **37,** 218–223
Griffiths, H. R., and Lunec, J. (1989) *FEBS Lett.* **245,** 95–99
Gross, A. J., and Sizer, I. W. (1959) *J. Biol. Chem.* **234,** 1611–1614
Halliwell, B., and Gutteridge, J. M. C. (1986) *Arch. Biochem. Biophys.* **246,** 501–514
Holtzman, N. A., and Gaumnitz, B. M. (1970) *J. Biol. Chem.* **245,** 2350–2353
Kossiakoff, A. A. (1988) *Science* **240,** 191–194
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp. 187–215, Plenum Press, New York
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
Lunec, J., Blake, D. R., McCleary, S. J., Brailsford, S., and Bacon, P. A. (1985) *J. Clin. Invest.* **76,** 2084–2090
Lyngbye, J., and Krøll, J. (1971) *Clin. Chem.* **17,** 495–500
Malmstrom, B. G., Andreasson, L. E., and Reinhammar, B. (1975) in *The Enzymes* (Boyer, P. D., ed) pp. 507–579, Academic Press, New York
Messerschimdt, A., and Huber, R. (1990) *Eur. J. Biochem.* **187,** 341–352
Musci, G., Carbonaro, M., Adriani, A., Lania, A., Galtieri, A., and Calabrese, L. (1990) *Arch. Biochem. Biophys.* **279,** 8–13
Musci, G., Bonaccorsi di Patti, M. C., Carlini, P., and Calabrese, L. (1992) *Eur. J. Biochem.* **210,** 635–640
O'Farrell, P. H. (1975) *J. Biol. Chem* **250,** 4007–4021
Oliver, C. N., Anh, B., Moerman, E. J., Goldstein, S., and Stadtman, E. R. (1987a) *J. Biol. Chem.* **262,** 5488–5491
Oliver, C. N., Levine, R. I., and Stadtman, E. R. (1987b) *J. Am. Geriatr. Soc.* **35,** 947–956
Ortel, T. L., Takahashi, N., and Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 4761–4765
Prutz, W. A., Butler, J., and Land, E. J. (1983) *Int. J. Radiat. Biol.* **44,** 183–196
Rothstein, M. (1984) in *Molecular Basis of Aging* (Roy, A. K., and Chatterjee, B., eds) pp. 209–234, Academic Press, Orlando, FL
Rydén, L. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 6767–6771
Rydén, L. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, L., ed) vol. 3, pp. 37–100, CRC Press Inc., Boca Raton, FL
Rydén, L., and Bjork, I. (1976) *Biochemistry* **15,** 3411–3417
Rylkov, V. V., Tarasiev, M. Y., and Moshkov, K. A. (1991) *Eur. J. Biochem.* **197,** 185–189
Sato, M., Schilsky, M. L., Stockert, R. J., Morell, A. G., and Sternlieb, I. (1990) *J. Biol. Chem.* **265,** 2533–2537
Schosinski, K. H., Lehmann, H. P., and Beeler, M. F. (1974) *Clin. Chem.* **20,** 1556–1563
Semsei, I. (1991) *Gerontology* **37,** 199–207
Sharma, H. K., Prasanna, H. R., and Rothstein, M. (1980) *J. Biol. Chem.* **255,** 5043–5050
Solomon, E. I., Allendorf, M. D., Kau, L. S., Pate, J. E., Spira-Solomon, D., Wilcox, D. E., and Porras, A. G. (1987) *Life Chem. Rep.* **5,** 37–89
Stadtman, E. R. (1986) *Trends Biochem. Sci.* **11,** 11–12
Stadtman, E. R. (1988) *J. Gerontol. Biol. Sci.* **43,** B112–B120
Stadtman, E. R. (1990a) *Free Rad. Biol. Med.* **9,** 315–325
Stadtman, E. R. (1990b) *Biochemistry* **29,** 6323–6331
Starke-Reed, P. E., and Oliver, C. N. (1989) *Arch. Biochem. Biophys.* **275,** 559–567
Winyard, P. G., Hider, R. C., Brailsford, S., Drake, A. F., Lunec, J., and Blake, D. R. (1989) *Biochem. J.* **258,** 435–445
Wynn, R. M., Sarkar, K. S., Holwerda, R. A., and Knaff, D. B. (1983) *FEBS Lett.* **156,** 23–28