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Oxidative Aggregation of Ceruloplasmin Induced by Hydrogen Peroxide is Prevented by Pyruvate

M'HAMMED AOUFFEN[a], JOANNE PAQUIN[a], ALEXANDRA FURTOS[b], KAREN C. WALDRON[b] and MIRCEA-ALEXANDRU MATEESCU[a,*]

[a]*Department of Chemistry and Biochemistry, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Qué., Canada H3C 3P8;*
[b]*Department of Chemistry, Université de Montréal, C.P. 6128, Succ. Centre-ville, Montréal, Qué., Canada*

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Ceruloplasmin (CP) is a blue copper glycoprotein with multiple physiological functions including ferroxidase and oxidase activities. CP is also an important serum oxygen free radical (OFR) scavenger and antioxidant, exerting cardioprotective and antifibrillatory actions. Although it has been reported that CP activities can be inhibited by OFR, the intimate mechanism of this inactivation is still not clear. Exposure of bovine CP to H_2O_2 induced inactivation of the protein as well as structural alterations as indicated by loss of protein bands by SDS-PAGE. Both phenomena were H_2O_2 concentration and time dependent. HPLC gel filtration and capillary electrophoresis analysis of CP treated with H_2O_2 revealed an aggregation of the protein. Quantification of dityrosine formation by fluorescence indicated the involvement of dityrosine bridging, which could be responsible for aggregation of CP under oxidative attack. Oxidative damage to CP under H_2O_2 treatment was completely prevented by pyruvate, suggesting that the association of CP with antioxidants could extend the range of the protective action of this protein.

Keywords: Ceruloplasmin; Hydrogen peroxide; Metal-catalysed oxidation; Aggregation; Dityrosine bridges; Pyruvate

Abbreviations: AE: aminoethyl; AU: absorbance unit; CP: ceruloplasmin; CE: capillary electrophoresis; DPD: N,N-diethyl-p-phenylenediamine; EDTA: ethylenediamine tetraacetic acid; HPLC: high performance liquid chromatography; OFR: oxygen free radicals; PDA: *p*-phenylenediamine; RFU: relative fluorescence unit; SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis

INTRODUCTION

Ceruloplasmin (CP) is a multifunctional α_2-globulin (132 kDa) produced by liver and secreted as a blue copper glycoprotein. It is also synthesised by non-hepatic tissues such as lung and central nervous system.[1] CP is abundant in plasma, being present at 150–200 mg/l in normal conditions, and is considered as an acute-phase reactant-protein since its concentration can reach 900 mg/l in inflammatory conditions. Ceruloplasmin is considered as responsible for the transportation of more than 95% of the circulating copper. As an enzyme (EC 1.16.3.1) CP exhibits oxidase and ferroxidase activities. Because it catalyses the oxidation of Fe^{2+} to Fe^{3+}, CP plays an essential role in iron metabolism and mobilisation.[2] CP is also an important serum oxygen free radical (OFR) scavenger and antioxidant.[3] Furthermore, CP was previously found to exert cardioprotective[4] and antifibrillatory[5] actions.

CP activities can be inhibited by non-aggressive agents such as citrate[6] and ascorbate,[7,8] or aggressive agents/conditions able to induce structural alterations, such as azide,[9,10] nitric oxide[11] and pH variation.[8] OFR can also inactivate and structurally modify CP. In humans, particularly in elderly people, CP was reported to be subjected to oxidative modifications, as revealed by a dramatic change of its electron paramagnetic resonance spectrum.[12] However, the exact mechanism of CP alteration by OFR is still not well documented. It is known that OFR can induce oxidative damages to many macromolecules *via* Fenton-like reactions in the presence of metal ions.[13] It has been reported that exposure of CP to hydrogen peroxide induced

*Corresponding author. Tel.: +1-514-987-4319. Fax: +1-514-987-4054. E-mail: mateescu.m-alexandru@uqam.ca

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a release of free copper ions from the protein, which can generate hydroxyl (˙OH) radical species, enhancing thus the oxidative damage.[14,15] This can be supported by the fact that bathocuproine, a copper chelator, or hydroxyl radical scavengers, like mannitol, abolished the OFR induced inactivation of CP.[14] Ceruloplasmin was also reported to undergo fragmentation by exposure to H_2O_2, and the *in vitro* hyperglycosylation of the protein enhanced this damage.[15] Beside inactivation, non-enzymatic fragmentation or increased susceptibility to proteolytic degradation,[16] oxidative damages to a protein can involve reticulation. While previous studies reported an inactivation and fragmentation of CP under H_2O_2 treatment,[14,15] the present work shows that bovine CP can also undergo covalent aggregation upon such treatment and that pyruvate, a known scavenger of H_2O_2, prevents both inactivation and reticulation of the protein.

MATERIALS AND METHODS

Materials

N,N-diethyl-p-phenylenediamine (DPD) and sodium pyruvate, were from Sigma–Aldrich Canada Ltd. (Oakville, Ont., Canada). Commercial chemicals were reagent grade and used without further purification. Aminoethyl (AE)-agarose is a chromatographic material obtained by the treatment of 300 ml agarose beads (Superose 12) with 130 g 1-chloro-2-ethylamine (chlorohydrate) in alkaline medium, under conditions previously described.[17,18] Trypsin-agarose was prepared by coupling 100 mg (2500 BAEE units/mg) trypsin (Sigma–Aldrich) to 100 mg activated CNBr-Sepharose, as described by the manufacturer (Pharmacia Biotech, Canada).

Purification of Bovine CP

CP was purified from bovine serum as described by Wang *et al.*,[17] and Mateescu *et al.*,[18] using a fast single-step affinity chromatography on an aminoethyl (AE)-agarose column equilibrated in 10 mM potassium phosphate (KPi), pH 7.4. CP was adsorbed on this material in the equilibration buffer and eluted with 100 mM KPi, pH 7.4. The final preparation was electrophoretically homogeneous, with a value of $A_{610\,nm}/A_{280\,nm}$ of 0.04 or higher. CP was stored at −20°C, in 100 mM KPi, pH 7.4, until use.

Exposure of CP to H_2O_2

A volume of 500 µl CP (1 µg/µl in 50 mM KPi, pH 7.4) was treated with H_2O_2 (5 mM final concentration) for various times (0–24 h) at 37°C. In another set of experiments, the protein was incubated for 24 h at 37°C with various concentrations of H_2O_2 (0–10 mM) in the presence or absence of 5 mM sodium pyruvate, a H_2O_2 scavenger. In all cases, at the end of treatments, an excess of sodium pyruvate (10 mM) was added to remove residual H_2O_2, and then samples were stored at −20°C, until analysis.

Trypsinolysis of CP

A partially proteolysed CP was obtained by incubating 1 mg of native CP in 0.5 ml of 50 mM KPi, pH 7.4, for 24 h at 37°C, with 0.5 ml of trypsin–agarose beads. The protein digest was then separated from the beads by filtration and stored at −20°C until SDS-PAGE analysis. Complete trypsinolysis of CP for capillary electrophoresis (CE) was achieved by incubating CP with trypsin (2500 U BAEE/mg protease) at a mass ratio of 100:1 in 50 mM KPi, pH 7.4, for 24 h at 37°C; the reaction was stopped by freezing at −20°C.

SDS-PAGE Analysis

Gel electrophoresis was done with a Protean II cell (Bio-Rad, Mississauga, Ont., Canada). Briefly, various samples of CP preparations were reduced and denatured in sample buffer (2% SDS, 10% sucrose, 0.005% bromophenol blue and 5% β-mercaptoethanol in 50 mM Tris–HCl, pH 6.8), then heated for 5 min at 95°C. Aliquots of 10 µl of reduced samples were resolved by SDS-PAGE on a 10% polyacrylamide gel, using Tris–glycine solution as running buffer. The resulting gel was stained with Coomassie Blue to reveal protein bands and analysed by densitometry, using a Personal Densitometer SI (Sunnyvale, CA, USA) and an IPLab Gel P Software (Vienna, VI, USA).

Capillary Electrophoresis

Zonal capillary electrophoresis (CE) was performed at 25°C on a Beckman P/ACE System 5000 (Beckman, Brea, CA, USA) with a 50 µm (I.D.) × 75 cm uncoated fused silica capillary, and 50 mM KPi, pH 5.2, as running buffer. The applied voltage was fixed at 20 kV. Before each run, the capillary was rinsed 2 min with 1 M sodium hydroxide, 2 min with nanopure water and 2 min with the running buffer. Samples were each injected under low pressure for 5 s and detection was done at 212 nm.

HPLC Gel Filtration Analysis

Native and H_2O_2 treated CP were analysed by gel filtration HPLC using a Biosep SEC S-3000 (Phenomenex®, CA, USA) column and a Varian

HPLC apparatus equipped with a UV/Vis detector (280 nm). The separation buffer contained 0.1 M Na_2SO_4, 0.1 M KH_2PO_4, 0.05% sodium azide, pH 6 and chromatography was run at a flow rate of 1 ml/min.

Tryptophan, Phenylalanine and Tyrosine Fluorescence Measurements

Fluorescence was measured using a spectrofluorimeter microplate reader (SPECTRA MAX Gemini, Molecular Devices, Sunnyvale, CA, USA). Samples (200 µl) of native or H_2O_2 treated CP were monitored for aromatic amino acid fluorescence using excitation and emission wavelengths of respectively 258 and 285 nm for phenylalanine, 275 and 305 nm for tyrosine, 280 and 350 nm for tryptophan, and 310 and 420 nm for di-tyrosine.

Monitoring of Dityrosine Formation

Quantification of dityrosine formation was carried out on 200 µl of different samples of native or H_2O_2 treated CP, in the presence or absence of 5 mM pyruvate. Each sample was excited at 310 nm and fluorescence monitored with the microplate reader at 420 nm (SPECTRA MAX Gemini).

Determination of CP Oxidase Activity

Oxidase activity was determined spectrophotometrically according to Osaki's method.[19] Briefly, 50 µl of CP samples untreated or treated with H_2O_2 were mixed with 0.6 ml of 100 mM KPi, pH 7.4; then a volume of 0.4 ml of 10 mM *p*-phenylenediamine substrate in 0.25 M sodium acetate buffer, pH 5.2, containing 5 mM EDTA, was added to start the reaction. Oxidation of the substrate was followed as Δ OD/min at 540 nm, and 25°C. Results were expressed as specific activity (enzyme units/mg protein). An enzyme unit was considered as the amount of CP enzyme required to increase the absorbency with one Absorbance Unit/min, in the conditions described above.

RESULTS

Incubation of CP with 5 mM H_2O_2 caused a progressive denaturation of the protein with time, as indicated by the gradual loss of the 132 kDa band by SDS-PAGE (Fig. 1a). Moreover, no band corresponding to CP itself or derived fragments was detected after 24 h of exposure to 5 mM H_2O_2. In contrast, when CP was digested by trypsin, four peptides with Mr ranging from 19 to 45 kDa were detected by SDS-PAGE analysis (Fig. 1a, CPp lane). Similar results were obtained when electrophoresis was run on a 8–15% gradient gel (not shown). The absence of additional bands for medium Mr CP fragments following the protein exposure to H_2O_2, suggested extensive fragmentation to small species (no CP fragments higher than 10 kDA) or modifications other than fragmentation, supposingly aggregation. Pyruvate (5 mM) prevented alteration of CP induced by H_2O_2 (Fig. 1b) suggesting a direct interaction between the protein and the oxidant. Since CP structural modification under H_2O_2 exposure was completed by 24 h of incubation, we considered this time as adequate treatment duration to study the effects of H_2O_2 concentration on the structural and functional properties of the protein. The disappearance of CP bands in SDS-PAGE upon exposure to H_2O_2 could be due to an extensive fragmentation of the protein with the generation of very small fragments that could have left the running gel, or to a cross-linkage of CP molecules with the retention of the cross-linked species within the stacking gel. It seems unlikely that CP molecules were lost through the formation of large irreversible insoluble precipitates since such precipitates were not detected.

Capillary electrophoresis (CE) has important applications in the analysis of protein purity, protein–protein and protein–effector interactions, and peptide maps since it allows the separation of small polar peptides and their detection with high sensitivity.[20] It was thus considered as a powerful method to reveal the presence of small fragments, if any, in H_2O_2 treated CP samples. Native CP

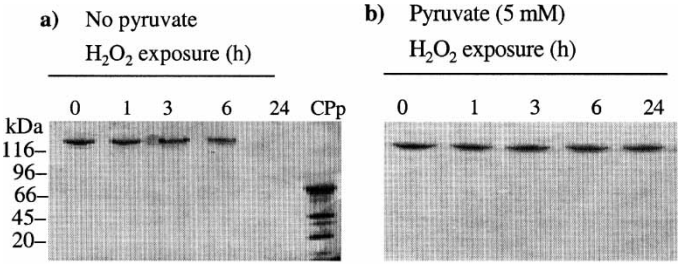

FIGURE 1 SDS-PAGE patterns of CP following exposure to H_2O_2. CP samples (1 mg/ml) were exposed for 0 to 24 h to 5 mM H_2O_2 at 37°C, in the absence (a) or the presence (b) of 5 mM pyruvate. Partially proteolysed CP (CPp) was obtained by the incubation of one-mg CP with trypsin-agarose beads in 50 mM KPi, pH 7.4, for 24 h at 37°C. Beads were removed by filtration. Gels were stained with Coomassie Blue.

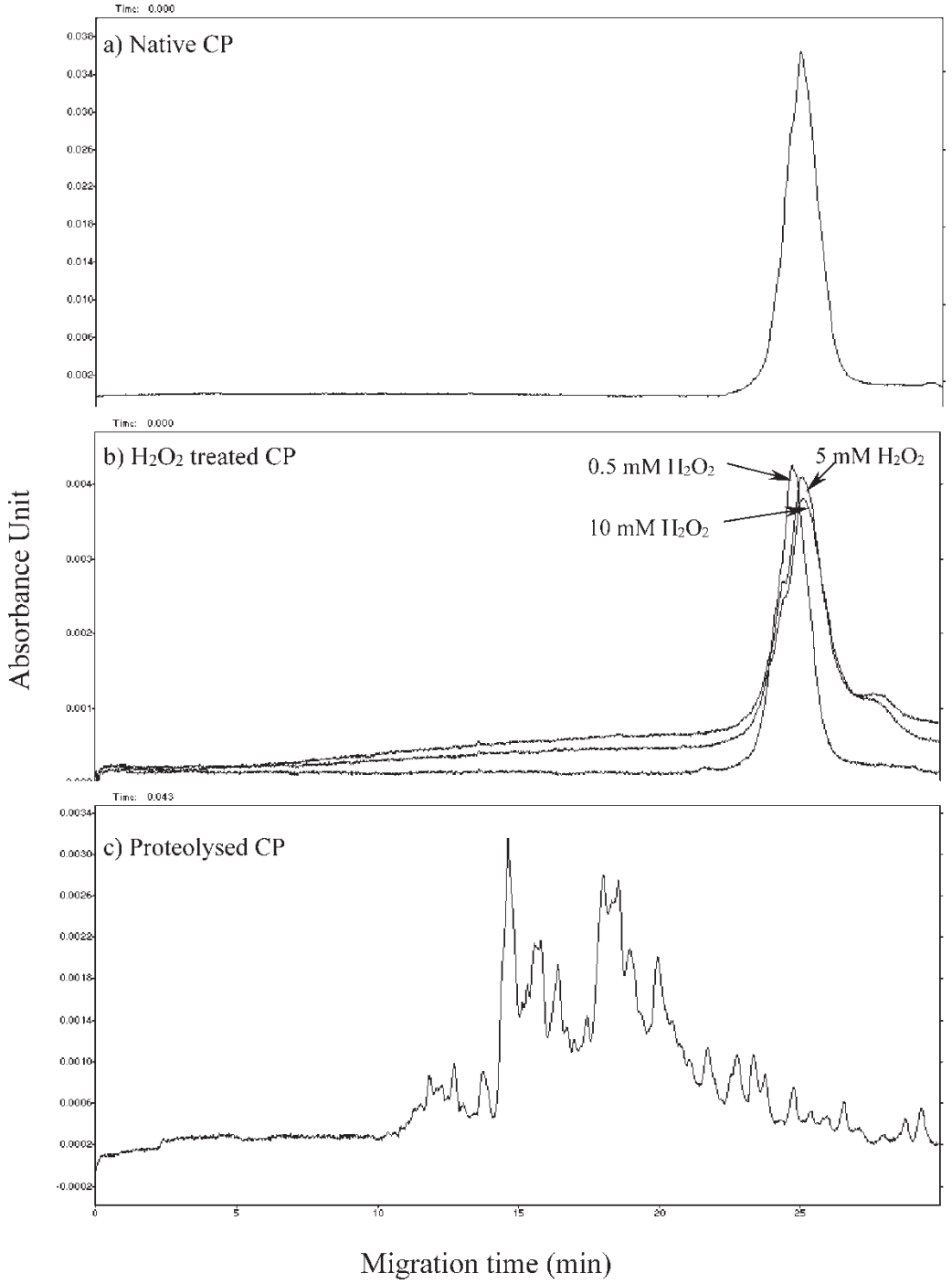

FIGURE 2 Capillary electrophoresis of native (a), H_2O_2-treated (b) and proteolysed CP (c). H_2O_2 treated CP samples were obtained as indicated in "Materials and Methods Section" and their behaviour by CE analysis was compared to that of native CP incubated for 24 h at 37°C in absence of H_2O_2. To obtain proteolysed CP, CP was subjected to complete trypsinolysis by incubation with trypsin at a ratio of 0.01 mg trypsin/mg CP in 50 mM KPi, pH 7.4, for 24 h at 37°C. The reaction was stopped by freezing at −20°C and analysed by CE. A control containing trypsin without CP gave no CE signal.

showed a unique peak migrating at 25 min (Fig. 2a), whereas CP exposed to increasing concentrations of H_2O_2 peaked at the same migration and exhibited a unique broader signal of decreased intensity (Fig. 2b), without being accompanied by the appearance of other peaks. In contrast to the oxidised CP samples, intensive proteolysis of CP with trypsin abolished the original CP signal and generated a collection of peaks eluting before 25 min and whose intensities were much smaller than that of native protein (Fig. 2c). These data indicate that treatment of CP with H_2O_2 did not release small fragments at detectable levels, and rather suggested that structural alteration of the protein could involve

aggregation. Indeed, unless it induces a large pI shift, the formation of dimers or larger aggregates often does not affect the migration time of a protein since this time is a function of the charge-to-mass ratio. The decreasing CP signals observed upon oxidation could be explained by a non-specific irreversible adsorption of the potential protein aggregates on the uncoated capillary walls, a phenomenon observed for large protein.[21]

The possibility that H_2O_2 induced aggregation of CP was investigated by HPLC gel filtration (Fig. 3). Pyruvate presented one peak at 8.49 min, which appeared to be stable and reproducible for all experiments (Fig. 3a–d). Hydrogen peroxide at increasing concentrations induced a decrease in the peak intensity of native CP (6.25 min). This decrease was accompanied by the appearance of a new broad peak (5.00–5.16 min) corresponding to higher Mr protein species, and the shift to larger sizes even increased with increasing concentrations of the oxidant agent (Fig. 3c,d). Calibration of the column with proteins ranging from 12 to 700 kDa led to estimate the Mr of the new CP species at 580 kDa, which would correspond to protein aggregates of about four to five molecules of CP. No additional fragments smaller than native CP were detected upon H_2O_2 treatment, in agreement with CE data. The results clearly indicate that oxidative conditions caused an aggregation of CP molecules.

Protein aggregates can result from the non-covalent association of polypeptides and/or their covalent crosslinkage *via*, for example, intermolecular dicysteine or dityrosine bridging. The formation of intermolecular cysteine–cysteine bridges as the sole mechanism of CP aggregation can be excluded since reducing denaturant SDS electrophoresis would not have conducted to the disappearance of the CP band on gel during H_2O_2 treatment (Fig. 1). This is in line with the fact that the four free cysteines in human CP are not exposed on the protein surface[22] and would thus be not easily accessible for cross-linking reactions. Dityrosine residues can result from metal ion-catalysed oxidation, a situation applicable to CP in view of its copper content. There was an important decrease in the fluorescence intensity of tryptophan and phenylalanine residues under H_2O_2 treatment (Fig. 4a,b), an observation that agrees with the participation of copper ions in H_2O_2-induced oxidation of CP.[14,15] The decrease in tryptophan and phenylalanine emission (Fig. 4a,b) was accompanied by a H_2O_2 concentration dependent and strong (up to 30-fold) increase in dityrosine fluorescence (Fig. 4d). The apparition of dityrosine structures upon H_2O_2 treatment of CP was correlated with that of aggregated forms of the protein (Fig. 3c,d and Fig. 4d), suggesting a crosslinking *via* intermolecular dityrosine bridging. In contrast to tryptophan and phenylalanine, fluorescence of tyrosine was increased up to 3 times versus the control (Fig. 4c). This increase can be explained by the transformation of phenylalanine into *m*- and *o*-tyrosine residues

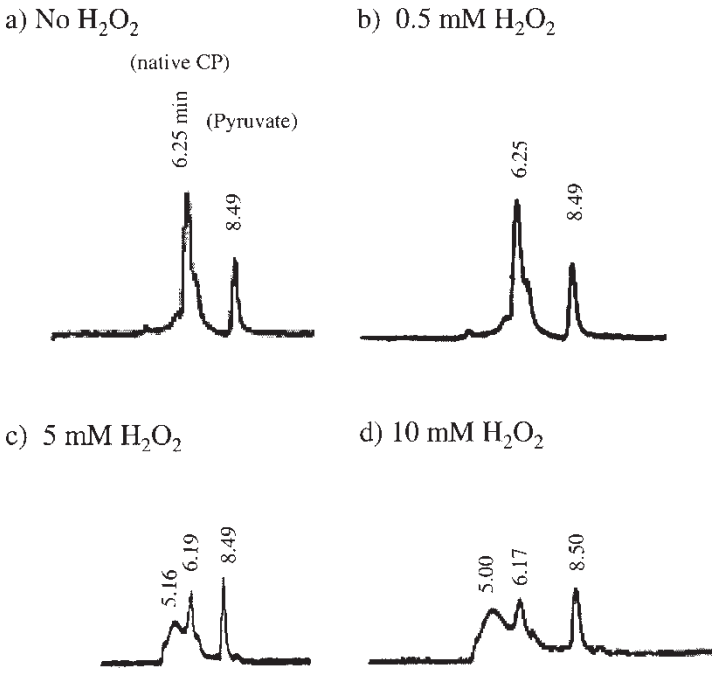

FIGURE 3 HPLC profiles of native CP (a) and CP treated with various concentrations of H_2O_2, for 24 h, at 37°C (b–d). CP was used at a final concentration of 1 mg/ml for treatment with H_2O_2. At the end of treatment, an excess of pyruvate (10 mM) was added to stop the reaction.

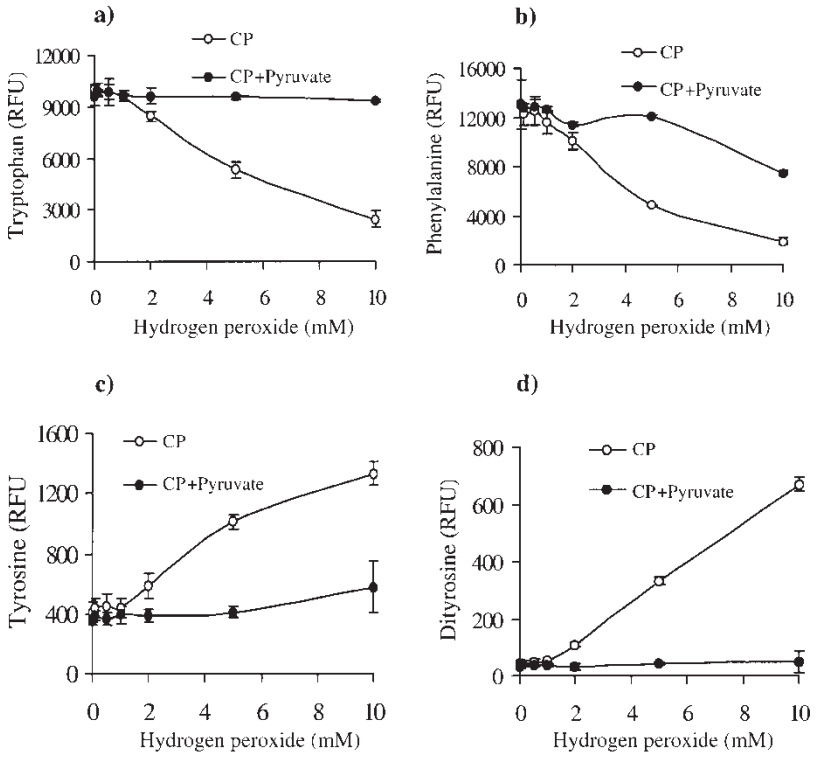

FIGURE 4 Intrinsic fluorescence measurements of native CP and of CP treated with various concentrations of H_2O_2 in the absence or presence of 5 mM pyruvate. Tryptophan (a), Phenylalanine (b), Tyrosine (c) and Dityrosine (d) fluorescence. Results are expressed as the means ± S.D. for three independent studies.

upon hydroxyl radical attack.[23] All fluorescence changes induced by H_2O_2 were almost completely inhibited by pyruvate (Fig. 4).

In addition to aggregation, H_2O_2 induced a significant loss in CP oxidase activity in a concentration dependent manner (Fig. 5). Almost complete inactivation occurred following exposure to 10 mM H_2O_2. At 2 mM H_2O_2, the loss in activity accounted for more than 50% while the fluorescence of aromatic amino acid residues were modified by not more than 10% (Figs. 4 and 5), suggesting that inactivation could precede aggregation. As in the case of the structural alteration of CP by H_2O_2, pyruvate protected CP oxidase activity against inactivation. Even at a concentration of H_2O_2 as high as 10 mM, about 62% of initial CP activity was preserved in the presence of the antioxidant (Fig. 5). The preventive effect of pyruvate on H_2O_2 induced loss of CP oxidase activity and native structure indicates a direct interaction of the oxidant with the protein.

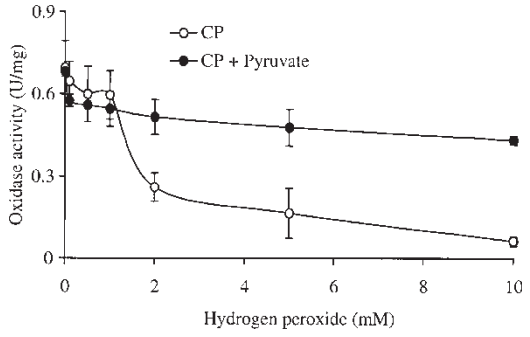

FIGURE 5 Effect of H_2O_2 on CP oxidase activity. CP samples (1 mg/ml) were treated with various concentrations of H_2O_2 (0–10 mM) for 24 h, at 37°C, in the absence or presence of 5 mM pyruvate. At the end of treatment, an excess of pyruvate (10 mM) was added to remove residual H_2O_2, and CP was assayed for its oxidase activity. Results are expressed as the means ± S.D. for three independent studies.

DISCUSSION

Metal-catalysed oxidation systems (Fenton type reactions) can inactivate a wide range of proteins. Metallo-proteins, such as the antioxidant enzymes SOD,[24] catalase,[25] glutathione peroxidase[26] and CP,[14,15] are considered to be particularly sensitive to these systems since they contain copper and iron metal ions in their structure. Two groups have recently studied the effect of H_2O_2 on CP,[14,15] using conditions that we have reproduced in this work. Analysis of the reaction products by electrophoresis showed a disappearance of the native CP bands and no generation of protein fragments,[14,15] results similar to those reported in Fig. 1. The authors concluded that the loss of CP signals was due to fragmentation but not observing fragment release.[14,15] The disappearance of CP signals remained intriguing to us. A loss of Coomassie staining as a consequence of aromatic amino acid

oxidation can be considered, but in our conditions tyrosine (generated by radical hydroxylation of phenylalanine, Fig. 4c) and dityrosine converted from tyrosine, via a radical process (Fig. 4d), still conserve an aromatic structure. Furthermore, dityrosine gives a good reaction (even higher absorbency than tyrosine) with Coomassie reagent (not shown). These observations, allowed us to postulate that H_2O_2 can induce the aggregation of CP molecules.

We investigated this possibility, using a combination of CE and HPLC gel filtration. The present study reports that H_2O_2 treatment did not release fragments from bovine CP, rather it caused extensive aggregation (Figs. 2 and 3). Since aggregation was accompanied by the formation of dityrosine bridges, as revealed by fluorescence measurements (Fig. 4), it is possible that intermolecular dityrosine cross-linkages were involved in the apparition of higher Mr CP species. In a recent report, Dean *et al.*,[27] indicated that oxidative attack of tyrosine residues could be responsible for inter-chain crosslinking of proteins. Similar aggregation of proteins *via* dityrosine bridging can be induced by gamma-irradiation.[28] Although the presence of small fragments was not detected following exposure of CP to H_2O_2, it cannot be concluded that no fragmentation had occurred. Fragments, if generated, could have remained linked together (i.e. via dityrosine bridges). Beside intermolecular reticulation, there could exist non-covalent interactions between polypeptide chains within CP aggregates since preparations obtained after 24 h-treatment with 10 mM H_2O_2 still contained CP species migrating as native CP by HPLC gel filtration (Fig. 3c,d; peak at 6.17–6.19 min). However, there was no band having the Mr of the native protein by SDS-PAGE with a similar treatment (Fig. 1a, 24 h-lane). It is possible that H_2O_2-induced intramolecular cross-linkages prevented unfolding of the protein in the presence of SDS and interfered with electrophoretic migration. Overall, this is the first study showing that high concentrations of H_2O_2 induce the aggregation of CP. While the apparition of dityrosine residues is compatible with the formation of intermolecular cross-links within the aggregates, the exact contribution of intramolecular and intermolecular interactions in the generation of these structures may be difficult to assess. The decreased fluorescence of phenylalanine and tryptophan induced by H_2O_2 treatment of CP (Fig. 4a,b) fits well with the proposal of Choi *et al.*,[14] that oxidative damages to CP and fragmentation under H_2O_2 treatment may result from the direct interaction of H_2O_2 with the labile copper on the protein to produce hydroxyl radical. Our study suggests aggregative effects, probably as results of the propagation of oxidative effects induced by this radical. It was found that H_2O_2 oxidative modifications of the primary structure of proteins such as albumin[23] and α-synuclein,[29] catalysed by Cu(II) ions, involved tryptophan oxidation and dityrosine formation.

H_2O_2 treatment not only caused structural alteration of CP but also inhibited its oxidase activity. More than half-inactivation of oxidase activity was achieved with 2 mM H_2O_2, a concentration reported as sufficient to inactivate several metalloenzymes such as SOD,[30] ascorbate peroxidase,[31] and cytochrome c oxidase[32] or peroxidase.[33] Extensive inactivation occurred at concentrations of H_2O_2 lower than those causing structural changes in fluorescence (Figs. 4 and 5). It is possible that a tyrosine residue present near or at the active site, or important for catalysis, could be more accessible to oxidation than other tyrosines in CP. Recently, we have shown that oxidase activity was more susceptible to H_2O_2 in native CP than in its deglycosylated counterpart, suggesting the involvement of a modified carbohydrate moiety in oxidative damage to the protein.[34]

Kang *et al.*,[35] recently showed that exposure of human CP to 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), which generates peroxyl radicals, also led to the protein aggregation, loss of ferroxidase activity and release of copper ions. These data and our results suggest that oxidative modification of CP can be generated by various reactive oxygen species.

Pyruvate, a known scavenger of H_2O_2, protected CP from structural alteration and functional inactivation by the oxidant. Pyruvate has been found to exert antioxidant properties in several models such as hepatocyte cultures exposed to H_2O_2[36] and isolated rat hearts submitted to ischemia-reperfusion.[37] It was also shown that H_2O_2 reacts directly with pyruvate to induce its own oxidative decarboxylation.[38] Pyruvate can be wisely associated to CP to enhance the overall antioxidant activity. For example, pyruvate could protect CP against oxidative damages that seem to affect the protein with aging[12] or in several pathologies related to oxidative stress such as the respiratory distress syndrome.[39] Aspects of these associations will be dealt with in a follow-up study.

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