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Oxidation of Cu,Zn-superoxide Dismutase by the Myeloperoxidase/Hydrogen Peroxide/Chloride System: Functional and Structural Effects

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This study investigated the functional and structural effects of bovine Cu,Zn-superoxide dismutase (Cu,Zn-SOD) oxidation by the myeloperoxidase (MPO)/hydrogen peroxide (H_2O_2)/chloride system and reagent hypochlorous acid (HOCl). Exposure to HOCl led to a fast inactivation accompanied by structural alterations. The residual SOD activity depended on the reactants concentration ratio and on the exposure time. The concomitant high consumption of HOCl indicated the presence of multiple targets on the protein. As assessed by SDS/PAGE, HOCl caused the dissociation of the protein into protomers at 16 kDa stable to both SDS and reducing conditions. Results from isoelectric focusing gels showed that exposure to HOCl induced the formation of modified protein derivatives, with a more acidic net electric charge than the parent molecule, consistent with the presence of additional ions observed in the electrospray ionization mass spectra. The reaction of protein with HOCl resulted in changes in protein conformation as assessed by the UV fluorescence and oxidation of the unique methionine and tyrosine, chlorination of several lysines with formation of chloramines. There was no significant formation of dityrosine and carbonyl groups. Exposure to high levels of HOCl resulted in complete enzyme inactivation, loss of additional lysine, histidine and arginine residues and coincident detection of weakly bound zinc and copper using 4-pyridylazaresorcinol. Collectively, the results suggest that the decrease of the dismutase activity is probably related to both dissociation into protomers and unfolding due to extensive oxidative modifications of amino acids.

Keywords: Myeloperoxidase; Superoxide dismutase; Cu,Zn-SOD; HOCl

Abbreviations: MPO, myeloperoxidase; SOD, superoxide dismutase; PAR, 4-(2-pyridinylazo) resorcinol

INTRODUCTION

Activation of neutrophils and monocytes results in the formation of reactive oxygen species such as superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). The release of the heme-enzyme, myeloperoxidase (MPO) during the respiratory burst[1,2] catalyzes the formation of HOCl from the H_2O_2-dependent oxidation of chloride ions.[3] HOCl is considered to play a major role as a potent microbicidal agent in immune defense[4,5] but the excessive production of HOCl causes tissue damage.[6,7] Indeed, HOCl reacts with a wide range of oxidizable biomolecules containing thiols, nitrogen compounds or unsaturated double carbon bonds, as reviewed in Ref. [8]. They correspond to the main targets of HOCl oxidation in proteins,[9–12] nucleotides[13–17] and unsaturated lipids and cholesterol.[18–21]

Cellular protection against the reactive oxygen species involves an elaborate antioxidant defense system, including superoxide dismutases.[22] Cu,Zn-superoxide dismutase (Cu,Zn-SOD) has been shown to reduce injury to reperfused ischemic heart, brain

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and others tissues.[23,24] This enzyme, located in the cytosol of eukaryotic cells, is a homodimer of molecular weight approximately 32 kDa, each subunit containing one copper ion and one zinc ion in the active site.[25] It catalyzes the dismutation of superoxide anion through the cyclic reduction and oxidation of the active site copper and produces molecular oxygen and H_2O_2.[26] Whereas the rate constant for the uncatalyzed dismutation reaction depends strongly on the pH of the solution and is about $5 \times 10^5\,M^{-1}\,s^{-1}$ at physiological pH, the reaction catalyzed by bovine erythrocyte Cu,Zn-SOD is relatively independent of pH in the range 5.3–9.5, with a rate constant for reaction of $O_2^{\bullet -}$ with the active site of $2 \times 10^9\,M^{-1}\,s^{-1}$.[27,28]

X-ray diffraction studies at 2 Å resolution[29] combined with amino acids sequence analysis[30] indicate that each subunit contains a binuclear cluster with the active copper and zinc ions bridged by a common ligand, the imidazolate of histidine 61. In the oxidized enzyme, three other imidazole rings of histidine 44, 46 and 118 and one water molecule are ligated to Cu^{2+}, whereas the remaining ligands of Zn ion are the imidazole rings of histidine 69 and 78 and the carboxylate of aspartate 81.

Any antioxidant enzyme introduced to a site of inflammation will be susceptible to attack by the oxidants. For example, it has been reported that Cu,Zn-SOD is irreversibly inactivated by its product H_2O_2[31,32] and is very sensitive to peroxynitrite[33] and HOCl.[34,35] Two independent groups[34,35] have studied the effect of exposure of Cu,Zn-SOD to HOCl. The first report showed that HOCl inactivated Cu,Zn-SOD although more slowly than catalase and glutathion peroxidase.[34] The latter one[35] reported that exposure of Cu,Zn-SOD to HOCl free reagent or generated by stimulated neutrophils resulted in the formation of catalytically active new forms of SOD. Because of these conflicting results, we decided to reexamine in detail the oxidation of Cu,Zn-SOD by HOCl and to investigate the structural and functional alterations of SOD. Therefore, we have attempted to relate the HOCl-induced loss of SOD activity with structural changes in the enzyme active site (Cu^{2+} binding) and the amino acids of the protein. Collectively, the results suggest that the decrease of the dismutase activity is probably related to both dissociation into protomers and unfolding due to extensive oxidative modifications of amino acids.

MATERIALS AND METHODS

Chemicals and Reagents

Superoxide dismutase from bovine erythrocytes (5000 U/mg, purity index of 98%), hypoxanthine, xanthine oxidase, methionine and cytochrome c were purchased from Sigma Chemical Co. (St Louis MO, USA). Fresh solutions of $HOCl/OCl^-$ ($pK_a = 7.6$)[36] were prepared by dilution of commercial NaOCl in 10 mM NaOH before use. The hypochlorite concentration was determined spectrophotometrically at 292 nm ($\varepsilon = 350\,M^{-1}\,cm^{-1}$) after dilution with 10 mM NaOH.[36] All other chemicals used were of the highest grade available and were prepared daily in distilled deionized water. All experiments were performed in 50 mM phosphate pH 7.5.

MPO Characteristics

MPO was purified from human polymorphonuclear neutrophils by sequential heparin agarose affinity and anion-exchange chromatographies as previously described in Ref. [37]. The enzyme preparation used in this study exhibited a purity index (A_{428}/A_{279}) equal to or higher than 0.81. The MPO concentration, expressed as heme-concentration, was determined spectrophotometrically ($\varepsilon_{430} = 89\,mM^{-1}\,cm^{-1}$).[38]

Spectral Measurements

Absorbance spectra, repetitive scans and kinetic absorbance measurements were performed with a Perkin–Elmer lambda 40 spectrometer interfaced with a computer allowing the collection, manipulation and analysis of the data with the corresponding appropriate software.

Protein Determination

Protein concentration for the unmodified SOD, expressed as a dimeric enzyme unless stated otherwise, was determined at 258 nm using an extinction coefficient of $10.3\,mM^{-1}\,cm^{-1}$.[39] Concentration of HOCl-modified samples were determined using the dye-binding method of Bradford[40] with Coomassie Blue G-250. A commercially available kit (Bio-Rad, Richmond, CA, USA) was used with bovine serum albumin as a standard.

Oxidative Treatment of SOD

When SOD was oxidized by the MPO system, to 1.1 μM SOD (0.03 mg/ml) in a 50 mM phosphate buffer at pH 7.5 and 100 mM NaCl, six additions of 80 μM H_2O_2 were made at 10–15 min intervals at 37°C. MPO 19 nM was added at the start and subsequently at every second addition of H_2O_2. At alternate additions of H_2O_2, 15 μM ascorbate was added to recycle inactivated MPO.[41] Aliquots were removed at 10-min intervals, diluted 100-fold, and assayed for SOD activity, as described below.

When reagent NaOCl was used to oxidize SOD it was added as single bolus. The reaction was

carried out at various HOCl concentrations from 25 to 900 μM, different SOD concentrations and for various incubation times at 37°C in 50 mM phosphate at pH 7.5. The oxidation reaction was stopped using 1 mM methionine and SOD was separated from reagents on a PD-10 Sephadex column. Then, samples were kept at 4°C and assayed as detailed below.

SOD Activity Measurement

Activity was determined by the cytochrome c-xanthine oxidase method.[26] A 20 μM cytochrome c solution was reduced by $O_2^{\bullet -}$ generated by 1 mM hypoxanthine and 0.01 U/ml xanthine oxidase (2.6×10^{-8} M). SOD was directly diluted to 10 nM into the assay solution before starting the recording of ferricytochrome c monitored at 550 nm ($\varepsilon_{550\,nm} = 21\,mM^{-1}\,cm^{-1}$). The residual SOD activity was calculated as followed:

$$\text{residual activity } (\%) = (v1 - v2)/(v1 - v3) \times 100$$

where $v1$ is the reduction rate of ferricytochrome c alone by $O_2^{\bullet -}$, $v2$ the reduction rate of cytochrome c in the presence of HOCl-treated SOD and $v3$ is the reduction rate of cytochrome c in the presence of native SOD.

SDS-PAGE Electrophoresis

Slab gels of 12% (w/v) polyacrylamide were run essentially according to Laemmli.[42] Samples of SOD and molecular mass standards were diluted in Laemmli buffer containing 5% β-mercaptoethanol, 2% SDS, 5% glycerol, 0.065% bromophenol blue in 65 mM Tris buffer, pH 6.8. Heating of the samples for 10 min at 95°C was performed to detect the monomeric form of the enzyme. The migration of the samples was carried out in a Tris/glycine buffer, pH 8.3, containing 0.5% SDS. Gels were developed with Coomassie blue procedures.

Isoelectric Focusing Electrophoresis

Experiments were performed at 15°C on a Multiphor II apparatus (Amersham Pharmacia) using precasted Ampholines PAG plates gels (245 × 110 × 1 mm) with pH range of 3.5–6.5. The pH values on the gels were calibrated using the pI markers (Amersham Pharmacia) as standards. Gels were stained with Coomassie blue.

Electrospray Ionization Mass Spectrometry

All MS measurements were carried out in positive-ion mode on a triple quadrupole instrument (Micromass Ltd, Altrincham, UK) equipped with an atmospheric pressure ionization electrospray source and were performed in collaboration with Drs B. Coddeville and G. Ricart (CCM/Spectrométrie de masse/Université des sciences et technologies de Lille, Villeneuve d'Ascq, France). A mixture of polypropylene glycol was used to calibrate the quadripole mass spectrometer. The samples were dissolved in acetonitrile/water (50/50) containing 0.2% of formic acid at a concentration of 10 pMole μl^{-1}. Injections and analyses were performed and detailed in Ref. [43].

Detection of Chloramines Formation

5-Thio-2-nitrobenzoic acid (TNB) was used to quantify the formation of chloramines. TNB was prepared from commercial DTNB (5,5′-dithio-bis-2-nitrobenzoic acid) (Sigma) using the method described in Ref. [44]. A 20 mM $NaBH_4$ was added to a solution of 1 mM DTNB in 50 mM phosphate buffer pH 7.0, containing 5 mM EDTA. The mixture was then heated at 37°C for 30 min. After treatment with HOCl and separation from any excess HOCl, SOD at 1 μM was mixed to 100 μM TNB. The concentration of TNB remaining after reaction with SOD-derived chloramines was determined at 412 nm using an absorbance coefficient of $14.15\,mM^{-1}\,cm^{-1}$.[45]

Carbonyl Measurements

2,4 Dinitrophenylhydrazine (DNPH) (Merck) was used to detect the formation of carbonyl groups. Samples of SOD (2 mg/ml) were treated in parallel with 10 mM DNPH in 2.5 M HCl (200 μl) or with HCl alone. After 1 h at room temperature and in the dark, the protein was precipitated with 10% TCA and centrifuged for 10 min at 3000 rpm. The obtained pellet was washed with acetronitrile to eliminate unreactive DNPH and then diluted in 6 M guanidine hydrochloride. The spectra were recorded between 200 and 500 nm, using the sample treated with HCl alone as a reference. The carbonyl contents was quantified at 375 nm using an absorbance coefficient of $22\,mM^{-1}\,cm^{-1}$.[46]

PAR Chelation Assay for Zinc and Copper Content

Zinc and copper present in HOCl-treated SOD samples were quantified by the colorimetric procedure using 4-(2-pyridinylazo)resorcinol (PAR).[47] The reaction was performed in the presence of 170 μM PAR and 2.8 μM SOD sample in 50 mM phosphate buffer giving after reactions a final pH 7.4 as measured by a standard pH electrode. The change in absorbance due to Cu^{2+}- and Zn^{2+}-PAR complex formation was monitored by repetitive scans performed between 600 and 350 nm at a rate of 240 nm/min and found to be essentially instantaneous. The amount of zinc release upon treatment

by HOCl was calculated by adding 1 mM of nitrilotriacetic acid which chelates zinc more tightly than PAR but cannot remove copper from the higher affinity Cu^{2+}-PAR complex. The decrease in absorbance at 500 nm corresponds to Zn^{2+}-nitrilotriacetic acid complex formation as previously reported[48] and quantified at this pH using $\varepsilon_{500\,nm} = 56.7\,mM^{-1}\,cm^{-1}$. The amount of copper release was calculated by the subsequent addition of 1 mM EDTA added to chelate copper from Cu^{2+}-PAR complex quantified at this pH using $\varepsilon_{500\,nm} = 37\,mM^{-1}\,cm^{-1}$.[48] Reaction solutions containing all components except SOD were subjected to PAR assay. Typically, zinc and copper contamination amounted to less than 1% of that from SOD denatured by incubation for 2 h at 37°C in a 6 M guanidium chloride solution at pH 7.8.

Fluorescence Measurements

Protein fluorescence emission spectra were recorded from 280 to 400 nm on a Hitachi F-4010 spectrofluorimeter following excitation at 260 nm, with a maximum emission wavelength at 305 nm. The fluorescence of a 3 μM SOD solution was measured in buffer. The assays were calibrated by means of a calibration curve generated in the same medium with tyrosine and phenylalanine, the only fluorescent naturally occurring amino acids. Dityrosine production was assayed by recording the fluorescence emission spectra of dityrosine from 550 to 350 nm following excitation at 320 nm and measured at its maximum 410 nm. The assay was calibrated using a standard curve constructed in the same medium with authentic dityrosine. Dityrosine standard was prepared using the HRP-catalyzed oxidation of tyrosine by H_2O_2[49] previously described.[50] Its concentration was monitored spectrophotometrically at 315 nm using $\varepsilon = 5\,mM^{-1}\,cm^{-1}$ at pH 7.5.[51]

Analysis of the Amino Acids Composition

The amino acids composition of oxidized SOD was established by M. Courteau at the Sequence Laboratory of the Institut de Biologie et Chimie des Protéines (CNRS, Lyon, France). The samples were introduced into a reactor containing a mixture of HCl 6N/TFA/thioglycolic acid (2 V/1 V/5%) under a nitrogen flux. Acid hydrolysis of peptidic bonds was carried out for 45 min at 150°C in a Water Pico Tag station. Samples were then dried under vacuum and diluted in a Beckman buffer. Then, the amino acids were separated on an ion exchange column and eluted with a gradient of buffers differing by their ionic strength, pH values and temperature. After elution, the amino acids were revealed with ninhydrine at 135°C and monitored at 570 nm, or 440 nm for proline derivatives.

RESULTS

Inactivation of SOD by MPO Activity

Treatment of SOD with MPO, in the presence of 100 mM chloride and repetitive additions of H_2O_2, resulted in time-dependent inactivation (Fig. 1, curve a). H_2O_2 was added in aliquots of 80 μM to ensure a complete conversion to HOCl.[52] After a total addition of 480 μM H_2O_2 followed by 1 h incubation, a complete loss of activity was observed. Addition of methionine, the HOCl scavenger, known to be one of the amino acids which reacted the most rapidly with HOCl,[53–55] to incubation mixture exerted a protective effect against inactivation by scavenging HOCl (Fig. 1, curve b), thus, suggesting that the inactivation of SOD by the MPO system was mostly linked to HOCl production.

Kinetics of Inactivation by HOCl

Indeed, replacement of the MPO system with reagent HOCl resulted in comparable effects (Fig. 2). The extent of such oxidative inactivation depended from the stoichiometric ratio used between HOCl and SOD, being more pronounced at higher ratio. Plots of remaining activity vs. incubation time revealed an exponential dependence on incubation time over a time period of 1 h. A complete loss of SOD activity was observed for a 300:1 molar ratio of HOCl to SOD (hereafter referred to as molar ratio of HOCl) after an exposure to HOCl of 10 min (Fig. 2, curve c), or a 200:1 HOCl molar ratio after 1 h exposure (Fig. 2, curve b). In contrast, at 1 h 40% activity remains for a 67:1 molar ratio (Fig. 2, curve a). For a constant incubation time of 1 h, there is a linear relation

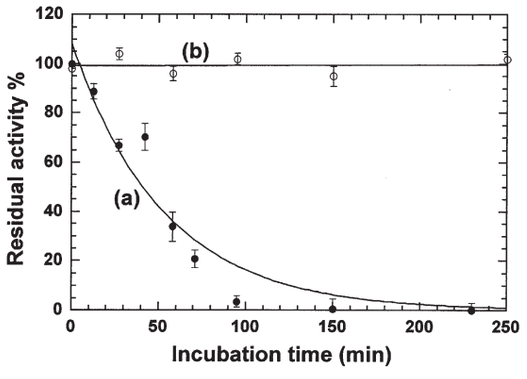

FIGURE 1 SOD activity following exposure to myeloperoxidase/H_2O_2/Cl^- system in the absence (curve a) or presence of 1 mM methionine (curve b). Increasing concentrations of H_2O_2 (6 × 80 μM) were added to 19 nM myeloperoxidase in 50 mM phosphate buffer pH 7.5, 100 mM NaCl and 3 μM SOD at 37°C. Aliquots were removed at the times indicated, diluted 100-fold to perform SOD activity assays as described in "Materials and Methods" section. Assays were performed in duplicate. The continuous line (a) was drawn according to a mono-exponential process with rate constant of $0.019\,min^{-1}$, $R^2 = 0.93$.

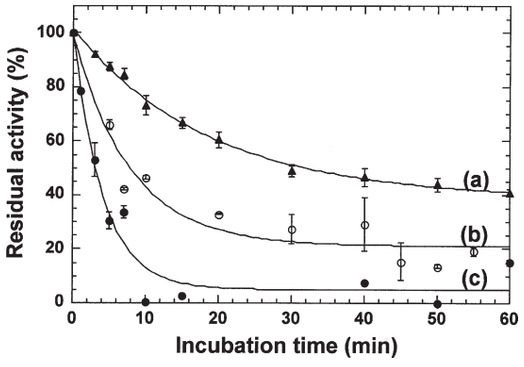

FIGURE 2 Effect of incubation time with HOCl on the SOD activity. SOD samples were incubated at 37°C in 50 mM phosphate buffer pH 7.5 with various HOCl concentrations and tested for activity at the times indicated as outlined in "Materials and Methods" section. Incubation conditions were the following: (a) SOD 3 μM, HOCl 200 μM ($k_a = 0.05\,min^{-1}$, $R^2 = 0.99$); (b) SOD 1 μM, HOCl 200 μM ($k_b = 0.13\,min^{-1}$, $R^2 = 0.94$); (c) SOD 3 μM, HOCl 900 μM ($k_c = 0.24\,min^{-1}$, $R^2 = 0.96$). Data points represent the means of triplicate measurements and the error bars indicate standard deviations. The continuous lines were drawn according to a mono-exponential process with rate constants k_a, k_b, k_c, respectively.

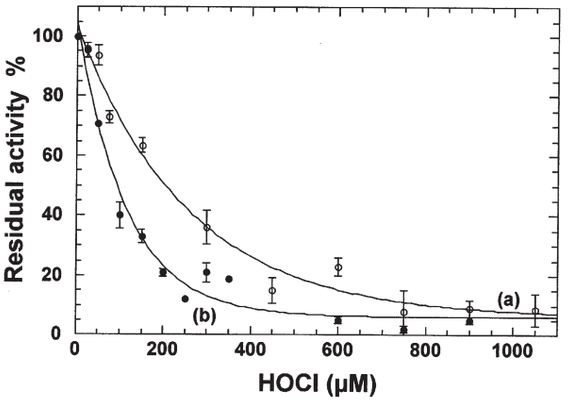

FIGURE 3 Effects of HOCl concentrations on the residual activity. Samples of SOD at concentration of 3 μM (a) and 1 μM (b) were incubated for 30 min at 37°C in 50 mM phosphate buffer pH 7.5 with various concentrations of HOCl. Aliquots were removed at the time indicated to test the SOD activity as described in "Materials and Methods" section. Assays were performed in duplicate and each experiment was performed two times. The error bars indicate standard deviations.

between the residual SOD activity and the HOCl/SOD molar ratio (data not shown). We concluded that reagent HOCl was a suitable model for MPO/H_2O_2/Cl^- oxidation of SOD and used it for two reasons in all subsequent experiments. First, it allowed determination of stoichiometries of oxidative changes in relation to the molar amounts of HOCl added. Second, it avoided the complex kinetic pattern of the chloration reaction catalyzed by MPO which could have complicated the results if this enzyme had been used.[6]

Effect of HOCl Concentration on SOD Activity

At a convenient incubation time of 30 min, addition of HOCl rapidly inactivated SOD in a concentration-dependent manner (Fig. 3). The HOCl concentration required for 50% inactivation (IC_{50}) showed a marked dependence on the initial SOD concentration. The IC_{50} for HOCl was 200 μM when the concentration of SOD was 3 μM (Fig. 3, curve a) in agreement with the value of Fig. 2, curve a. A complete loss of catalytic activity was observed for a HOCl concentration higher than 600 μM, i.e. for a molar ratio >200. A 3-fold decrease in SOD concentration decreased the HOCl IC_{50} to 80 μM. These results confirmed that the residual activity is highly dependent both on the initial concentration ratio of HOCl/SOD and the duration of exposure to HOCl.

Consumption of HOCl in the Presence of SOD

To study the interaction between HOCl and SOD UV-visible spectra of HOCl/OCl^- were recorded before and after incubation in the presence of SOD at 37°C. Figure 4 showed the absorbance changes of HOCl at 292 nm, characteristic wavelength of the hypochlorite form. After 30 min incubation in the absence of SOD, a spontaneous 20% decrease of the absorbance level initially measured was noticed at 292 nm (Fig. 4, curves a and b). In contrast, the presence of SOD led to the total disappearance of the HOCl absorbance (Fig. 4, curve c), suggesting that SOD scavenged HOCl. The comparison of curves b and c in Fig. 4 allowed to quantify the amount of HOCl which reacted with SOD. A linear relation between the amount of trapped HOCl and the initial concentration of HOCl could be established showing that 68% of each HOCl concentration was trapped by SOD (data not shown). Thus, the complete loss of

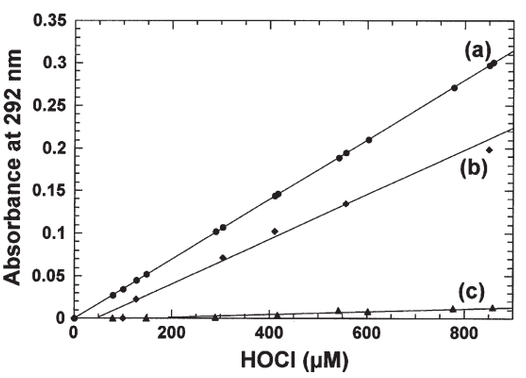

FIGURE 4 HOCl consumption in the presence of SOD. (a) Initial absorbance level of HOCl samples at 292 nm in the absence of SOD, (b) absorbance level after 30 min incubation at 37°C in the absence of SOD, (c) absorbance level after 30 min incubation in 50 mM phosphate buffer pH 7.5 at 37°C in the presence of 3 μM SOD. The spectra were scanned from 400 to 200 nm at 240 nm/min.

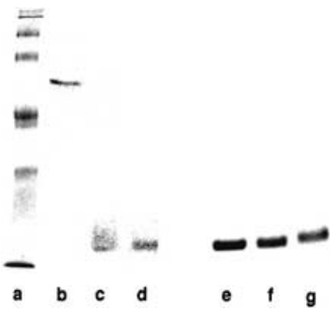

FIGURE 5 SDS-PAGE profiles of SOD exposed to HOCl. SOD solutions (30 μM or 1 mg/ml) were incubated for 30 min at 37°C with HOCl molar equivalents in 50 mM phosphate buffer pH 7.5. SOD samples were diluted in Laemmli buffer containing 5% β-mercaptoethanol and 2% SDS and subjected to electrophoresis using a 3 μg of protein/lane. From left to right, lane (a) contains the color molecular mass standards (Sigma Chemical Co., St Louis MO, USA) as follows from bottom to top: α-lactalbumin (15 kDa), soybean trypsin inhibitor (26 kDa), carbonic anhydrase (33 kDa), ovalbumin (47 kDa), albumin (98 kDa), β-galactosidase (135 kDa) and myosin (240 kDa). Lanes (b)–(d) contain samples not denaturated by the boiling treatment: (b) control SOD; (c) and (d) SOD incubated with 120 and 240 HOCl molar equivalents, respectively. Lanes (e)–(g) same samples boiled for 10 min at 95°C.

activity of a 3 μM SOD solution, obtained for 600 μM HOCl, corresponded to the scavenging of 400 μM HOCl by selective targets on the protein.

Structural Modifications of the Protein in Presence of HOCl

Changes in the molecular mass distribution of SOD following exposure to HOCl was examined by polyacrylamide gel electrophoresis and electrospray ionization-mass spectrometry. SOD is a dimer in the native form. As judged by denaturing gel electrophoresis (SDS-PAGE) shown in Fig. 5, SOD migrated mainly with an apparent molecular mass of 64 kDa (lane b), when the sample was not boiled before application. This value previously obtained by Abernethy *et al.*[56] was explained as the result of anomalous binding between an incompletely denatured protein and SDS. The monomer of SOD was detected at 16 kDa only after boiling the sample for 10 min at 95°C (lane e). After exposure to HOCl and under classical denaturing conditions a unique band at 16 kDa was detected (Lanes e–g). When the samples were not boiled before application, after exposure to 120 and 240 HOCl equivalents (lanes c and d), respectively, the band at 64 kDa was lost and there was a corresponding increase in material at 16 kDa. In conclusion, HOCl treatment as well as the boiling treatment induced the formation of a denaturated molecule and SOD dissociated into protomers. No significant aggregation and fragmentation of the protein were detected.

To further study the molecular structure of SOD oxidized by HOCl, the samples were subjected to electrospray ionization mass analyses. The positive-ion chemical ionization mass spectra of control SOD and HOCl-treated SOD yielded series of protonated molecular ions $(M + nH)^+$ with the same m/z values but differing by their intensities (Fig. 6). However, after exposure to HOCl a noisier mass spectrum was obtained and a shift of the molecular ions towards higher m/z values was observed (Fig. 6b). Deconvolution of the control SOD mass spectra showed a subpeak corresponding to a molecular mass of 15,588 Da (Fig. 6a inset,) consistent with the value of 15,600 published for the monomer from the amino acids composition.[30] The monomeric form of the enzyme was still detected in HOCl-treated SOD at 15,587 Da (Fig. 6b, inset), with additional species of calculated masses 15,619; 15,690; 15,729 and 15,755. The increase in molecular mass of 32, 103, 142 and 168 Da suggested the addition of one, three, four and five chloride atoms. These results, taken together, strongly support the conclusion that chlorination occurred randomly and led to several modified protein molecules differing by the number of bound chloride atoms.

Following treatment with HOCl, changes in the overall electrical charge of SOD were revealed by analysis of isoelectric focusing gels, which confirmed that the SOD molecule became significantly more acidic. As shown in Fig. 7, the pI of the native protein was determined at 4.8 as expected.[57] Upon HOCl concentration increase, new more negative forms of the enzyme appeared and the native form at pI 4.8 disappeared at a 50 molar ratio. At higher HOCl molar ratio a predominant form of SOD accumulated at an isoelectric point of 4.1 in association with faint bands at 4.0 and 3.7. Such charge changes were indicative of alteration in the primary structure which affected overall electrical charge.

Zn and Cu Release from HOCl-treated SOD

To determine if zinc and copper ions were released after incubation of SOD with HOCl the 4-(2-pyridylazo)resorcinol (PAR) assay was used.[48] The changes in binding Zn and Cu lagged behind the loss in activity of SOD (Fig. 8). The equivalent of 0.4 Zn and 0.2 Cu atom per SOD was released at a 100-fold excess of HOCl, a concentration of HOCl which caused half-inactivation of SOD. The concentration of HOCl producing essentially total inactivation (approximately 750 μM), i.e. a 250-fold excess of HOCl resulted in the release of 1.5 Zn and 0.7 Cu atom per dimeric enzyme.

Aromatic Amino Acids Modifications

Tyrosine and phenylalanine are the only fluorescent naturally occurring amino acids. Solutions of tyrosine and phenylalanine showed that the fluorescence of the protein observed at 300 nm after

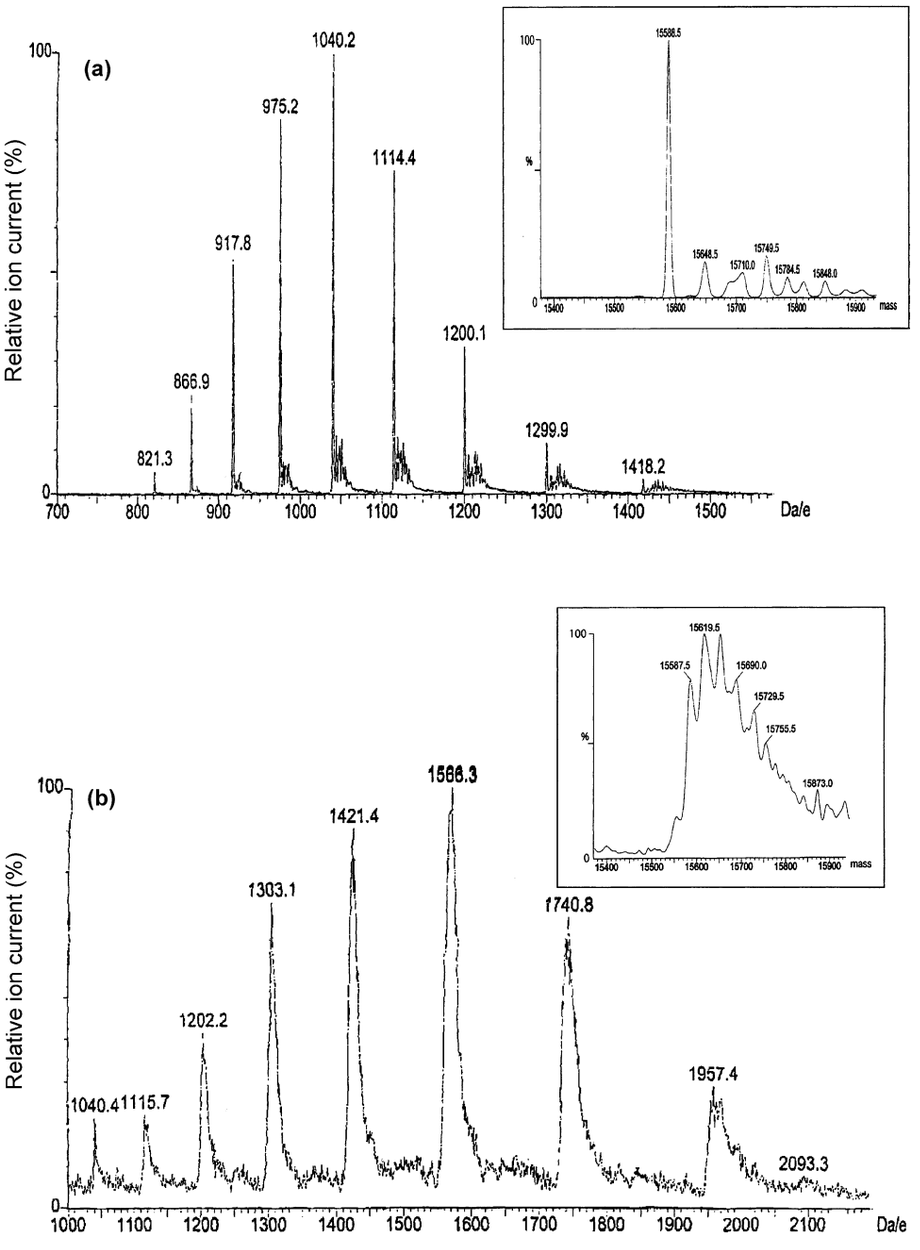

FIGURE 6 Electrospray ionisation mass spectra of SOD performed in positive mode. (a) Mass spectrum of native SOD. The inset shows the corresponding deconvoluted mass spectrum demonstrating a protein mass of 15588.5 daltons. (b) Mass spectrum of SOD treated by a 100-fold molar excess of HOCl. The inset shows the corresponding deconvoluted mass spectrum associated to a peak of 15587.5 daltons and a series of higher protein masses from 15619 to 15941 daltons.

excitation at 260 nm was related to 1.7 tyrosines per dimer. Thus, the fluorescence was mainly due to the two tyrosine residues and very few to the 8 phenylalanines known to be present in a dimer.[30] The observed fluorescence quenching was directly dependent upon HOCl concentration with a IC_{50} for a 200 μM HOCl, corresponding to a 67-fold excess HOCl (Fig. 9). In fact the fluorescence variation paralleled the loss of activity (Fig. 3 curve a) and was stabilized at a HOCl molar ratio higher than 200. These results confirmed changes in protein conformation as already suggested by results from SDS/PAGE and isoelectric focusing electrophoresis. However, the formation of dityrosine was not demonstrated.

Determination of Chloramines Formation

The decrease in the protein charge state suggested the reaction of HOCl with positively charged amino acids. The lysine residues are well known to react very rapidly with HOCl leading to the formation of

FIGURE 7 Effect of HOCl exposure on the net charges of SOD samples. Solutions of 3 μM SOD were treated with increasing concentrations of HOCl for 30 min at 37°C in 50 mM phosphate buffer pH 7.5 and analyzed for net charge determination by isoelectric focusing gel electrophoresis (pH 6.5–3.5). From left to right, protein bands corresponded to control SOD, lane (a), and to SOD incubated with 8.3-, 16.7-, 50-, 100-, 150- or 300-fold molar excess of HOCl, respectively, lanes (b)–(g). The right-hand lane (h) contained the following pI markers (Amersham-Pharmacia) from bottom to top: aminoglucosidase (pI 3.5), methyl red dye (pl 3.75), glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β lactoglobulin A (pI 5.2) and bovine carbonic anhydrase B (pI 5.85).

chloramines.[58] Using the oxidation of 5-thio-2-nitrobenzoic acid (TNB) to monitor chloramines formation, Fig. 10 showed that chloramines were detected in a dose-dependent manner with the quantity of HOCl added per mol SOD. At a 100 molar ratio of HOCl the yield of the reaction corresponded to 10 chloramines formed per SOD. The reaction of HOCl with the side-chain ε-amino groups confirmed a decrease of the positive charge of the protein, in agreement with results from electrospray ionization mass spectrometry and electrofocusing experiments.

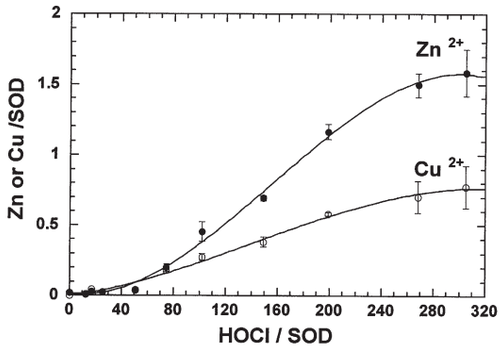

FIGURE 8 Metals release from SOD upon HOCl treatment. SOD samples at 3 μM were incubated for 30 min at 37°C with various HOCl molar ratio in 50 mM phosphate buffer pH 7.5. Zinc and copper release was determined by the PAR assay as outlined in "Materials and Methods" section. The concentration of Zn^{2+} present in the SOD samples was determined by the change in absorbance at 500 nm following NTA addition, and that of Cu^{2+} was determined by the change in absorbance at 500 nm following EDTA addition. Assays were performed in duplicate and each experiment was performed two times. The error bars indicate standard deviations.

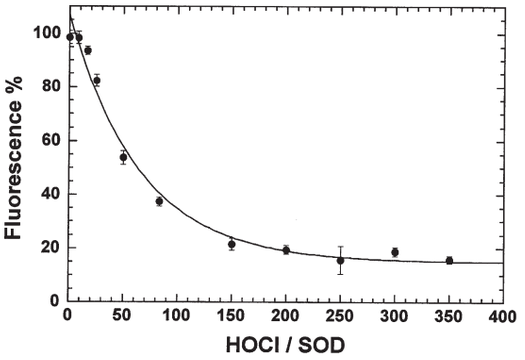

FIGURE 9 Effect of HOCl exposure on the SOD fluorescence. Variations of fluorescence of a 3 μM SOD solution after addition of various HOCl molar ratio. Fluorescence measurements were made at 300 nm after excitation of the protein at 260 nm. Assays were performed in duplicate and each experiment was performed two times. The error bars indicate standard deviations.

Determination of Protein Carbonyl Content

Previous studies have shown that chloramines, resulting from HOCl reaction on lysine residues, are slowly hydrolyzed into aldehydes and ammonia.[12,59] The carbonyl derivatives, thus obtained, are considered as early oxidation markers for protein oxidation in inflammatory tissues.[60] In the present study, the reaction of 2,4 dinitrophenylhydrazine, DNPH, with protein carbonyls to form protein hydrazones was used to detect the potential increase of the number of carbonyl residues after exposure of SOD to HOCl.[46] There is no significant modification of the number of carbonyls in SOD (0.56 ± 0.06 nmol/mgprotein) and HOCl-treated SOD (0.61 ± 0.03 nmol/mgprotein) at a 300 molar ratio. Therefore, this result was in agreement with no further oxidation of the chloramines.

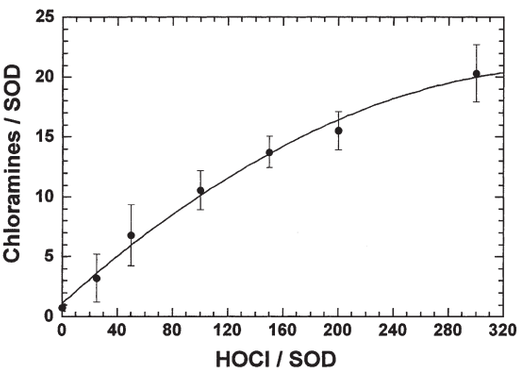

FIGURE 10 Formation of chloramines on exposure to HOCl. Samples of SOD 3 μM were incubated with 25–250-fold molar excess of HOCl for 30 min at 37°C in 50 mM phosphate buffer pH 7.5. After SOD separation from excess reagents by chromatography on a PD10 column, the assay was performed by addition of 1 μM HOCl-treated SOD to a 100 μM TNB solution and absorbance changes due to chloramine formation were monitored at 412 nm. Results are means ± SD for duplicate determinations.

Changes in the Primary Structure of HOCl-treated SOD

SOD that had been exposed to HOCl was submitted to amino acids analysis following acid hydrolysis of the modified proteins. Table I shows the amino acid composition of both control the and modified enzymes. The values obtained for control SOD, each subunit containing 151 amino acids, were in agreement with the known sequence and amino acid composition.[30] Amino acid contents of the modified enzymes showed significant difference in methionine, aromatic amino acids, positively charged amino acids. The methionine residue known to be one of the amino acids which reacted the most rapidly with HOCl[53–55] disappeared from a 100 molar ratio of HOCl. The loss of the unique tyrosine residue (Tyr-108) of a subunit was found in agreement with the loss of fluorescence. The decrease in the content of positively charged lysine and histidine residues confirmed the mass spectrometry and electrofocusing results. Moreover, they occurred in a HOCl concentration-dependent manner. In contrast, arginine residues were significantly modified from a 300 molar ratio of HOCl. It had to be noticed that 3-chlorotyrosine, a possible product of the reaction of chlorination of tyrosine by HOCl, eluted at the same retention time as histidine and, thus, could not be quantified. Aliphatic amino acids and hydroxy amino acids were not significantly modified. All these results confirmed that HOCl treatment mediated several attacks on specific amino acids of the protein.

Time Courses of Changes in SOD Activity and Chloramines Content

To provide better insight into the mechanism of SOD inactivation upon HOCl treatment, the time courses of changes in SOD activity and chloramine concentration were established at a 200 molar ratio of HOCl (Fig. 11). The SOD inactivation actually proceeds through an apparently first-order process ($k_a = 0.14\,min^{-1}$, $R^2 = 0.92$) in agreement with the data shown in Fig. 2, curve b. Parallel experiments

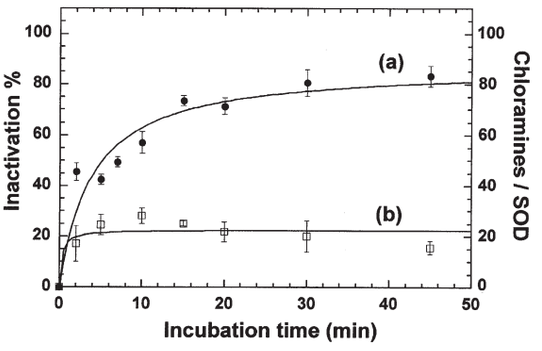

FIGURE 11 Comparative time-courses for SOD inactivation (a) and chloramine formation (b). Samples of SOD at concentration of 3 μM were incubated at 37°C in 50 mM phosphate buffer pH 7.5 with HOCl at a molar ratio of 200. The reaction was stopped at the time indicated by addition of 1 mM methionine and SOD was separated from reagents on a PD-10 Sephadex column. Aliquots were assayed for SOD activity and chloramine formation as described in "Materials and Methods" section. Data points represent the means of triplicate measurements and the error bars indicate standard deviations.

TABLE I Changes in amino acids composition of Cu,Zn-SOD treated with HOCl

			SOD treated by HOCl‡			
Amino-acids	Literature value*	Control SOD† ($n = 4$)	HOCl/SOD = 25 ($n = 2$)	HOCl/SOD = 100 ($n = 2$)	HOCl/SOD = 250 ($n = 1$)	HOCl/SOD = 300 ($n = 2$)
Asp¶	17	18.5 ± 0.3	21.2 ± 1.7	21.7 ± 0.3	26.8	21.8 ± 0.7
Thr	12	10.5 ± 0.3	10.3 ± 0.8	10.2 ± 0.9	7.5	5.2 ± 2.2
Ser	8	7.3 ± 0.1	7.2 ± 0.3	7.3 ± 0.6	6.5	5.8 ± 0.2
Glu§	11	13.2 ± 0.3	13.4 ± 0.1	14.4 ± 0.7	16.1	16.8 ± 1.3
Pro	6	6.4 ± 0.2	5.8 ± 0.3	5.6 ± 1.1	4.3	5.3 ± 0.1
Gly	25	29.5 ± 1.6	29.3 ± 1.1	30.9 ± 0.9	34	32 ± 0.3
Ala	9	10.3 ± 0.3	10.8 ± 0.6	12.0 ± 0.7	12.2	13.2 ± 0.3
Val	15	10.6 ± 0.3	9.9 ± 0.9	10.7 ± 1.1	9.7	11.6 ± 0.6
Met	1	0.9 ± 0.2	0.7 ± 0.2	0	0	0
Ile	9	5.8 ± 0.4	4.9 ± 0.7	6 ± 0.6	5.9	5.5 ± 0.5
Leu	8	8.7 ± 0.5	9.0 ± 0.3	8.3 ± 1.4	8.2	8.4 ± 1.0
Tyr	1	0.6 ± 0.1	0.6 ± 0.2	0.1 ± 0.1	0	0
Phe	4	5.6 ± 0.7	5.9 ($n = 1$)	4.5 ± 1.0	5.3	5.1 ± 0.3
His	8	7.0 ± 0.1	6.9 ± 0.6	7.2 ± 0.7	5	3.0 ± 0.1
Lys	10	10.8 ± 0.2	8 ± 0.6	5.5 ± 0.9	3.9	5.1 ± 0.1
Arg	4	4.2 ± 0.4	3.7 ± 0.4	3.7 ± 0.2	3.6	1.7 ± 0.3

Amino acids composition of a control SOD and HOCl-modified SOD determined from acid hydrolytic procedures described in "Materials and Methods" section. Values are means of n independent determinations. * Values deduced from known amino acids sequence of bovine erythrocyte Cu,Zn-SOD.[30] † Values obtained for control SOD. ‡ Values obtained for SOD treated at the indicated HOCl/SOD molar ratio for 30 min at 37°C. ¶ Included asparagine residus. § Included glutamine residus.

observed at the level of chloramines reveal an initial rapid formation of chloramines of small amplitude. An important point is that chloramine formation reaches its maximal value at a short incubation time where 50% activity is lost. On longer exposure to HOCl, in the time range greater than 10 min, inactivation further increases up to 80% while chloramine level remains constant at a value close to the one observed previously in the HOCl-dose responses experiments.

DISCUSSION

Our results indicate that oxidative modification of SOD by HOCl generates a modified protein. To our knowledge, this is the first report in which SOD inactivation by HOCl has been correlated with amino acids modifications and metals release. Previous studies have only examined the inactivation of the enzyme without description of the structural modifications of the protein induced by HOCl.[34,35] We report that the inactivation of SOD also occurred with purified MPO and was dependent on MPO and HOCl production since it was inhibited by methionine. The complex kinetic pattern of the chlorination reaction has been well established in the literature. The halogenation reaction is favored at acidic pH when the halide concentration is low relative to H_2O_2, and at neutral pH when the concentration of H_2O_2 is low relative to halide.[61,62] At plasma concentrations of chloride ion, when using a NaCl to H_2O_2 ratio of 10^3 the chlorinating activity of MPO is optimal at pH 6.[52,63] Under the chosen physiological conditions for pH (7.5) and chloride concentration (100 mM) complications arise from inhibition of MPO with loss of oxidizing equivalents available for SOD damage. Thus, the use of the MPO system as an enzymatic HOCl-producing system for studying protein damage has no advantage over direct use of the reagent HOCl, but is of interest for what it may reveal about MPO activity *in vivo*. Another advantage of choosing reagent HOCl and pH = 7.5 was that this pH corresponds to the pK_a value of HOCl and allows to monitor $^-$OCl absorbance peak at 292 nm.

We show that the inactivation of SOD is highly dependent on the HOCl molar ratio and on the duration of exposure. Indeed, the IC_{50} value for HOCl increased with SOD concentration simply because more oxidant was required to inactivate 50% of a larger target concentration even with a 1:1 reaction stoichiometry. This dependence of the IC_{50} for HOCl on SOD concentration is indicative of a more generalized target concentration phenomenon. SOD inactivation has been said to be a slow process,[34] we report that SOD was inactivated by 50% after a 8 or 30 min incubation time depending on the SOD concentration used in the presence of a constant HOCl concentration. This indicates that the oxidant effect can be fast as it is a function both of concentration and time.

Upon oxidation or chlorination of amino acid residues by HOCl, changes in protein conformation could occur altering the structure of the enzyme and the interactions between the anionic substrate and copper sites, thus, explaining the inactivation of the enzyme. Indeed, subsequent to prolonged exposure to HOCl, the dimeric enzyme was dissociated into protomers by SDS without boiling treatment, thus, indicating the absence of new covalent linkage. Newly formed lower molecular weight protein bands were not observed. Direct fragmentation of proteins has been observed following exposure to certain oxidants. Fragmentation of Cu,Zn-SOD was observed by glycation reaction[64] and by H_2O_2 exposure.[32] The mechanism implies lateral chain scission or peptide bond cleavage at proline residues.[65–68] In the HOCl-oxidized Cu,Zn-SOD, no significant carbonyl groups were generated and no proline loss was detected. These results confirmed that no cleavage occurred repeatedly at an oxidation site of SOD.

Interestingly, oxidatively modified proteins tend to form aggregates due to increased hydrophobic interactions and covalent cross-links. In the present study, no evidence of aggregation was observed which could be attributed to new intermolecular covalent bonds. This is consistent with the absence of dityrosine detection, which can account for subunit cross-linking and higher molecular mass complexes on reducing gels,[60,63,69] despite the disappearance of the tyrosine residue present in the SOD subunit.

X-ray studies[29,70] showed that the two identical subunits in a dimer were mainly stabilized by hydrophobic interactions associated with light hydrophilic ones. Indeed, the dissociation of the native enzyme into subunits by SDS was known to be forced to completion under drastic conditions leading to the complete denaturation of the protein either by prolonged preincubation at 37°C[56] or prolonged preincubation with 8 M urea[71] and did not require a reductant due to the absence of inter-subunits disulfide cross-links. Our data revealed that HOCl exposure facilitated the dissociation and was accompanied by the progressive inactivation of the enzyme. In the monomer subunit, the loss of activity was previously noticed upon treatment with detergents[72,73] or site-directed substitutions of hydrophobic residues at the dimer interface.[74] However, no significant modification of the content of Ileu and Ala residues mostly implicated in the stabilization of the dimer was evident in the amino acid analysis (Table I).

In the present study, evidence for extensive oxidative protein modification was provided by

the presence of an excess of negative charges on the newly generated protein species upon HOCl treatment. Indeed, for low HOCl molar ratio (≤ 100), the reaction of HOCl implied amino acids fully exposed to the solvent. A typical example was the disappearance of the only tyrosine residue in the sequence, Tyr-108, present at the protein surface as indicated from analyses of X-ray diffraction structures at 2 Å resolution downloaded from the Protein Data Bank.[75] In addition, among other solvent-exposed residues, a decrease in lysine residues was noticed, together with the conversion of lysine to chloramines. These results are in agreement with data from deconvoluted mass spectra showing, upon HOCl incubation, the formation of new molecules of molecular masses differing from that of SOD by the number of bound chloride atoms. In addition, the conversion of lysine to chloramines can account for the generation of a protein species with a more acidic pI in agreement with our results. Correlatively, these changes in electrostatic interactions provide an altered tertiary structure, as confirmed by changes in UV fluorescence, and can contribute to a rearrangement of the solvent-exposed hydrophobic dimer interface, thus, facilitating the dissociation into protomers noticed from a 100 molar ratio of HOCl.

The decrease in dismutase activity is very likely explained, firstly by reduction of the number of encounters between enzyme and substrate and secondly by modifications of amino acids residues implicated at the copper active site. Indeed, the relationships between SOD structure and its catalytic activity are well established. Structural information from X-ray crystallography[29,70] reveals the existence of a solvent access channel, of four highly conserved charged residues represented by Lys-120, Lys-134, Asp/Glu-130 and Glu-131 which provide the electrostatic guidance of the superoxide anion to the active site.[76,77] Their involvement in the catalytic activity has been postulated on the basis of results obtained with single and double mutants of the charged residues.[78,79] Furthermore, within the solvent access channel to the active site, Arg-141 is positioned within 6 Å of the copper and plays a crucial role in the catalytic activity because it correctly docks the superoxide anion near the active metal.[76,77,80] Other small anions such as cyanide, azide, halides and phosphate were also known to have easy access to the channel and bind to the active site copper or Arg-141.[75,81–83] Of interest, at pH 7.5 HOCl is 50% deprotonated to $^{-}$OCl,[36] so the reactions described in the present study can involve either or both species.

Accordingly, hypochlorite anions will also gain access inside the active channel. This interpretation accounts for our results showing a decrease in lysine content and their conversion to chloramines accompanying decrease of SOD activity. If the modified lysine residues were located in the channel the elimination of the charge on ε-amino group by chlorination would produce a less positive electrostatic potential at the entrance of the channel, thus affecting $O_2^{\bullet -}$ diffusion and decreasing the enzyme-substrate encounter. At higher molar ratio of HOCl, the complete enzyme inactivation was achieved and 2 arginine and 5 histidine residues per subunit had disappeared. The conformation integrity of the enzyme molecule was so destroyed that zinc and copper became accessible from the outside to chelating agents. These results suggest that the binding sites of Cu^{2+} and Zn^{2+} in SOD are partly destroyed by treatment with HOCl. Indeed, four histidine residues have been known to locate at the active site channel and coordinate with Cu^{2+} and 3 histidine residues with Zn^{2+}[29,70,77] So far, we have not identified oxidized histidine residues generated in the HOCl-oxidized Cu,Zn-SOD. However the concomitant decrease in histidine and arginine content and the partial liberation of Zn^{2+} and Cu^{2+} could not be ruled out.

To improve the understanding of the mechanism of SOD inactivation and its correlation with modifications of the various reactive sites of the protein by HOCl, a first approach was to compare kinetics of chloramine formation and SOD inactivation. The present results show that an initial 50% decrease of SOD activity can be related to the formation of chloramines. Indeed, the second-order rate constant for the reaction of HOCl with the lysine side chain was determined in the range of $5 \times 10^3\ M^{-1}\ s^{-1}$ at 22°C.[84] However, as SOD inactivation further proceeds without changes of the chloramine yield, this result suggests that reaction of HOCl with less reactive sites occurs and further damages the structure of the SOD active site. A detailed comparison of the time-courses of HOCl reaction with selected reactive sites is currently in progress.

Superoxide is generated by a number of cell types and pathways at sites of inflammation where HOCl is also formed. In addition, it is well established that $O_2^{\bullet -}$ counteracts the inhibitory effects of H_2O_2 on MPO chlorinating activity by reducing compound II back to native enzyme and thereby, boots the MPO-dependent production of HOCl.[63] It would be the reason why $O_2^{\bullet -}$ enhances HOCl production by stimulated human neutrophils.[85] From this, it follows that HOCl formation by neutrophils could be inhibited by SOD and this may be a factor in producing its anti-inflammatory properties. However, on the basis of the results presented here, our study demonstrates that SOD could act also by scavenging free HOCl and/or HOCl generated by the MPO system and the resulting modified protein lost its dismutase activity. Whereas substantial amounts of MPO activities have been detected in

various tissues during acute and chronic inflammatory diseases thus, contributing to tissue damage,[86] few data are available concerning the location and activities of SOD, except for extracellular Cu,Zn-SOD. Recently, an extracellular Cu,Zn-SOD has been identified in atherosclerotic vessels[87] and catalytically active MPO has been localized in human atherosclerotic lesions.[7,88,89] Therefore, it might be predicted that in inflammatory conditions HOCl scavenged by SOD, would induce a loss of the ability of SOD to protect the vascular cell surface from oxidative stress and increase the pathogenesis of inflammation.

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