Changes in the histidine residues of Cu/Zn superoxide dismutase during aging

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Abstract Cu/Zn-Superoxide dismutase activity (Cu/Zn-SOD) was studied in liver from 3- and 24-month-old rat. A significant decrease of enzyme activity in liver of the aged rat was found. Various amino acid residues and protein carbonyl groups (CO) were measured in purified young and old enzyme. It was found that the 'old' enzyme had one histidine fewer and higher CO content than the 'young' Cu/Zn-SOD. Inactivation 'in vitro' of purified commercial bovine erythrocyte Cu/Zn-SOD led to a decrease in the enzymatic activity, an increase in the CO and one histidine residue modified. A similar behavior between aging and oxidation was suggested.

Key words: Cu/Zn superoxide dismutase; Protein oxidation; Carbonyl group; Aging

1. Introduction

Alterations in a large number of enzymes are found in tissues of old animals [1–3]. Many of these changes result in the accumulation of the inactive or less active forms of the enzymes [2,3]. It has been proposed that the loss of activity is due to the oxidative inactivation of the protein with the consequent increase in carbonyl groups [4]. The most susceptible amino acid residues to oxidation during aging are histidine and lysine [2,3,5].

Oxidative damage has been implicated as one of the principal causes of age-related oxidative inactivation of enzymes [6]. Such effect is, at least partially, associated with superoxide radicals [7], which seems to be increased in aging [8].

The main mechanism of defence against the toxic effect of superoxide radical is its dismutation catalyzed by SOD [7]. It has been reported that Cu/Zn-SOD specific activity is decreased during aging in some animals and in various tissues [9]. Cu/Zn-SOD is also inactivated by oxidation in vitro [10,11] leading to the loss of one histidine residue.

In this paper we show that during aging, activity of Cu/Zn-SOD decreases in rat liver and there is an accumulation of inactive or less active forms. The most susceptible amino acid residues to oxidation, as well as carbonyl groups, have been studied in both Cu/Zn-SOD from young and old animals, and in oxidized 'in vitro' commercial Cu/Zn-SOD. The present study demonstrates that the 'old' enzyme has one histidine fewer than the young one. The results suggest that the decrease of specific activity of Cu/Zn-SOD during aging could be due to the chemical modification of the histidine residues, probably caused by oxidation.

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2. Materials and methods

2.1. Chemicals

^{125}I was purchased from ICN (Barcelona, Spain). Sepharose 4B CNBr was obtained from Pharmacia LKB Biotechnology (Barcelona, Spain). Biochemicals were from Boehringer-Mannheim (Barcelona, Spain) or Sigma (Alcobendas, Madrid).

2.2. Animals

Young (3 months) and old (24 months) Wistar rats were used. They were maintained on a standard laboratory diet with free access to food and water.

2.3. Measurement of enzyme activity

Cu/Zn-SOD was assayed according to Mc.Cord and Fridovich [12]. Protein concentrations were determined by the method of Lowry et al. [13].

2.4. Purification of Cu/Zn-SOD

Cu/Zn-SOD from liver was purified according to the method previously described by Kohtaro and Burr [14].

2.5. Preparation of antibody

Antiserum to the enzyme was prepared by pure enzyme inoculation of female New Zealand rabbits by the technique of Chauser [15].

2.6. Purification of rat liver Cu/Zn-SOD from young and old rats by immunoaffinity

Liver homogenate was dialyzed in 5 mM potassium phosphate buffer, pH 7.2, 0.1 mM EDTA and 5 μM PMSF. The dialyzed supernatant was applied onto an immunoaffinity column (1.5 × 6 cm, Sepharose 4B CNBr activate), prepared with antibodies against Cu/Zn-SOD, equilibrated with 5 mM potassium phosphate buffer (pH 7.2), 0.1 mM EDTA and 5 μM PMSF. The enzyme was eluted from the column with a 5–50 mM linear gradient of potassium phosphate buffer.

2.7. Antibody iodination

The antibody was radioactively labelled with ^{125}I by the chloramine-T method according to Greenwood et al. [16].

2.8. Immunoprecipitation of the Cu/Zn enzyme

Immunoprecipitation experiments were carried out by incubating 100 mU Cu/Zn-SOD with increasing volumes of iodinated antibody (0 to 100 μl). The mixture was adjusted to a constant volume with 1.7% Triton X-100 and 150 mM NaCl. The mixtures were allowed to react for 1 h at 37°C and overnight at 4°C. The reaction mixture was then centrifuged at 12,000 × g for 10 min and the remaining SOD activity was assayed in the supernatant. The precipitates were washed twice with 150 mM NaCl. The radioactivity incorporated into the precipitate was counted in a Gamma cord II.

2.9. Carbonyl groups determination

Determination of carbonyl groups in the enzyme eluted from immunoaffinity was carried out in aliquots of this preparation as described by Lenz et al. [17].

2.10. Chemical modification

Sulphydryl groups were determined by the method of Ellman [18]. Arginine residues were determined by the method described by Yamasaki [19]. Histidine residues were quantified using the diethylpyro-

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carbonate method [20]. Lysine residues were determined by the method of Tuengler and Pfletderer [21].

2.11. Oxidative modification of commercial Cu/Zn-SOD

Bovine erythrocyte Cu/Zn-SOD (Sigma Chemical Co.) was purified using a Rotofor unit (Bio-Rad). The purified fractions were oxidized using a metal-catalyzed oxidation system (MCO) as described by Levine [22]. Briefly, the purified SOD was incubated in potassium phosphate buffer, pH 7.2, containing 25 mM ascorbate, 0.1 mM $FeCl_3$ at 37°C. At various times, aliquots were withdrawn from the reaction mixture. These aliquots were thoroughly dialyzed against 5 mM potassium phosphate buffer, pH 7.2, 0.1 mM EDTA and 5 μM PMSF, with several changes of buffer. Both enzymatic activity and carbonyl groups were determined in these aliquots.

3. Results

3.1. Age-dependent changes in specific activity and amount of hepatic Cu/Zn-SOD

The specific activity of Cu/Zn-SOD was determined in homogenates of liver from young (3 months) and old (24 months) rats. In old rats the specific activity decreased 15% (Table 1). The amount of Cu/Zn-SOD at both ages was also studied by immunotitration. The polyclonal antibodies used were prepared against purified young rat liver-SOD. The amount of antiserum needed to inactivate 100% of the activity was higher in older animals (127%), which suggests that there is accumulation of a less active form of SOD during aging.

3.2. Changes in amino acid residues of the Cu/Zn-SOD in young and old rats

In order to investigate the possible mechanism underlying the loss of Cu/Zn-SOD specific activity during aging, several amino acid residues were quantified in SOD preparations purified by immunoaffinity chromatography from young and old rat liver. The results show that there are no age-dependent changes in all the amino acid residues studied, except for histidine (Table 2). Thus, the 'old' enzyme showed one histidine residue fewer than the 'young' enzyme. Comparing these results with those obtained in denatured protein, it can be concluded that this histidine residue is present in a domain accessible to diethyl pyrocarbonate. Similar results were previously found in our laboratory for malic enzyme [2], and suggest that the decrease in the histidine number in the Cu/Zn-SOD could be due to a chemical modification of this residue, probably caused by oxidation.

3.3. Carbonyl groups content in 'young' and 'old' Cu/Zn-SOD

Oxidative modification typically causes inactivation of enzymes and also the introduction of carbonyl groups into amino acid side chains of the proteins. In order to understand the nature of Cu/Zn-SOD inactivation during aging, carbonyl groups content was determined in purified preparations of Cu/Zn-SOD. As can be seen in Table 1, the content of carbonyl groups increased 49% in old rats.

Table 1
Specific activity, immunoprecipitable amount and carbonyl groups content of liver Cu/Zn-SOD in young and old rats

Age	Specific Activity (U/mg protein)	cpm	Carbonyl Groups (mol CO/mol SOD)
3 months	14.3 ± 0.69	3771 ± 329	0.976 ± 0.016
24 months	12.2 ± 0.30*	4810 ± 125*	1.457 ± 0.120*

Results are mean ± S.E.M. of five determinations. Carbonyl groups were determined in purified preparations of SOD. *Significantly different ($P < 0.025$, Student's t-test).

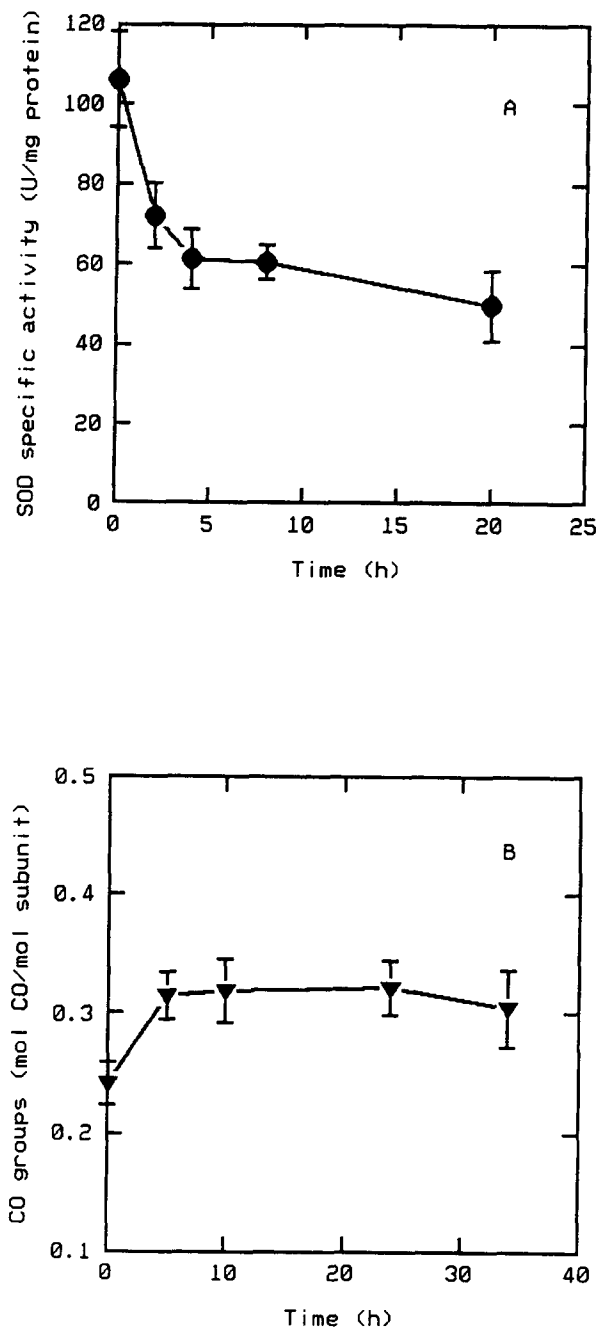

Fig. 1. Effect of oxidation on commercial bovine erythrocyte Cu/Zn-SOD. (A) Specific activity (units/mg protein). (B) Carbonyl groups content. Specific activity and carbonyl groups were determined as described in section 2. Each point represents the mean ± S.E.M. of five determinations.

3.4. 'In vitro' inactivation of commercial Cu/Zn-SOD with MCO system

The relationship between Cu/Zn-SOD specific activity decrease and oxidation was studied by incubating purified commercial Cu/Zn-SOD with an MCO system. As shown in

Fig. 1, a rapid loss of enzymatic activity was found after 8 h of incubation. Thus, oxidation with the MCO system led to a decrease of 43% in the specific activity. After this period of time, the decrease tended to reach a plateau. Carbonyl groups were determined in the same samples. The CO content increased 30% in the the 34-h oxidized SOD with respect to the non-oxidized. It is therefore likely that the variations in specific activity of SOD preparations reflect the amount of oxidized, inactive enzyme present. In addition, the oxidative modification alters only a single histidine residue in the Cu/Zn-SOD (Table 3).

4. Discussion

In the present study an age-dependent decrease in the specific activity of Cu/Zn-SOD from rat liver was observed. This decrease has also been described by other authors in liver and other tissues [9]. Cu/Zn SOD is an important scavenger of free radicals and shows a good positive correlation with the life span in mammals [23]. Therefore, the reduction in Cu/Zn-SOD activity as a function of age could result in an impaired protection against the toxic effects of free radicals, (such as the superoxide anion), and thus might lead to severe cellular damage. Indeed, the superoxide free radical has been suggested to be an important agent implicated in the aging process [8].

The decrease in enzymatic activity is not associated with a decrease in the amount of protein (Table 1). Thus, immunoprecipitation experiments showed that the amount of immunoprecipitated Cu/Zn-SOD in old tissue is greater than in young tissue extracts. This suggests that there is an accumulation of altered protein in the liver during aging.

It is currently assumed that the decrease in activity during aging is due to a post-translational modification of the enzyme. To test this possible alteration, the carbonyl groups content, as well as the quantification of several amino acid residues, was determined using the Cu/Zn-SOD of young and old rats purified by immunoaffinity. The carbonyl groups are produced by oxidation of some amino acid side residues such as proline, arginine, lysine and histidine [24]. Our results show that carbonyl groups content increased (49%) in old enzyme. Similar results have been found in erythrocytes, eye lenses, hepatocytes [25] and fibroblasts [4]

To test the possible modification by oxidation of Cu/Zn-SOD amino acids, the amino acid residues most susceptible to oxidation were quantified in young and old Cu/Zn SOD. The results show that there is no difference between the concentrations of cysteine, arginine and lysine in the Cu/Zn-SOD from young and old rats. However, a statistically significant difference was obtained among the histidine residues (Table 2). The 'old' protein has one histidine fewer that the 'young', and this residue is accessible in the native protein. This suggest sthat the decrease in Cu/Zn-SOD activity could be produced by the 'loss' of or change in one histidine residue in the protein.

This possibility is supported by the finding that 'in vitro' oxidization of commercial SOD with ascorbate led to a loss in its activity and to an increase in the carbonyl groups content. The increase in carbonyl groups could be due to products such as 2-oxo-histidine as has been described by Uchida and Kawakishi [26]. A loss of one histidine residue was also found (Table 3). This suggests that the decrease in enzymatic activity found in Cu/Zn-SOD could be produced by the oxidation of one histidine residue of the native enzyme. Other authors have also inactivated Cu/Zn-SOD by oxidation 'in vitro' [10] and it has also been reported that this inactivation was accompanied by the loss of one or more histidine residues [11].

Some authors have shown that other enzymes are inactivated by oxidation 'in vitro', most of them requiring a divalent cation for activity [10,26] The oxidation 'in vitro' of glutamine synthetase, phosphoglycerate quinase, enolase, glucose-6-P dehydrogenase or malic enzyme is also associated with the loss of one histidine residue per enzyme subunit [2,10,22]. Moreover, activity of some of these enzymes decreases during aging, as in the case of malic enzyme [2], in which one fewer histidine was measured in old protein. In other cases the oxidation 'in vitro' of protein led to a loss of lysine residues, as occurs for 6-phosphogluconate dehydrogenase [3]. Besides, some data point to the possibility that the amino acid residues modified 'in vitro' by oxidation and during aging process are the same in the sequence of the protein.

It can be concluded that there is similarity between the inactivation process by oxidation 'in vitro' and the inactivation produced during aging. Therefore we suggest that the loss of amino acid residues found in 'old' enzymes could be produced by an increase in the oxidative environment of cells in aging with the consequent oxidation of the most susceptible residues.

Table 2
Amino acid residues per mol of liver Cu/Zn-SOD purified from young and old rats

Residue	Sample of Cu/Zn-SOD	mol residue/mol SOD	
		3 months	24 months
-SH	Native	2.03 ± 0.15	1.87 ± 1.14
	Denatured	2.95 ± 0.36	3.09 ± 0.08
Arginine	Native	2.66 ± 0.36	2.62 ± 0.19
	Denatured	4.94 ± 0.68	4.78 ± 0.26
Histidine	Native	5.05 ± 0.13	4.02 ± 0.34*
	Denatured	9.07 ± 0.36	7.50 ± 0.15*
Lysine		10.99 ± 1.89	11.32 ± 1.14

SOD was denatured with 6 M guanidinium chloride. Results are mean ± S.E.M. of five determinations. *Significantly different ($P < 0.025$, Student's t-test).

Table 3
Amino acid residues per mol of commercial Cu/Zn-SOD from bovine erythrocytes purified using a Rotofor unit

Residue	Sample of Cu/Zn-SOD	mol residue/mol SOD
-SH	Non oxidized	1.76 ± 0.02
	Oxidized	1.74 ± 0.04
Arginine	Non oxidized	2.56 ± 0.52
	Oxidized	2.31 ± 0.27
Histidine	Non oxidized	8.07 ± 0.65
	Oxidized	6.68 ± 0.42*
Lysine	Non oxidized	9.24 ± 0.46
	Oxidized	8.99 ± 0.76

Results are mean ± S.E.M. of five determinations. *Significantly different ($P < 0.025$, Student's t-test).

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