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REVIEW

Alterations in Superoxide Dismutase Isozymes by Methylmercury

Y. Kumagai,[1]* S. Homma-Takeda,[1] M. Shinyashiki[2] and N. Shimojo[1]
[1] Department of Environmental Medicine, Institute of Community Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305, Japan
[2] Graduate School Doctoral Program in Medical Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305, Japan

A study of alterations in two mammalian superoxide dismutases (SODs), Cu,Zn-SOD and Mn-SOD, caused by methylmercury has been reviewed. Mechanisms for the isozyme-selective decrease in MnSOD activity by the organometallic compound observed *in vivo* have been extensively examined by experiments with purified enzyme preparations. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

Methylmercury (MeHg) is a commonly encountered form of environmental mercury because of its widespread use and its biomethylation by aquatic organisms. MeHg damages the central nervous system[1] because it readily penetrates the blood–brain barrier.[2] Exposure to the organomercurial results in 'Hunter–Russell syndrome', characterized by constriction of visual fields, tremor, hypomnesia, and impairment of speech and hearing. The toxicity of mercury compounds is thought to be due, in part, to their high affinity for cysteine thiol functions.[3,4] Based on the results of numerous studies, the involvement of oxidative stress in MeHg toxicity is being recognized.[1,5] For example, exposure of animals or nerve cells to MeHg results in stimulation of lipid peroxidation[6,7] and formation of reactive oxygen species.[8,9] Mercury compounds have also been found to decrease the activity of antioxidant enzymes such as superoxide dismutase (SOD) or glutathione peroxidase.[10–13] The nervous system is exquisitely sensitive to peroxidative damage since it is rich in oxidizable substrates such as lipids and catecholamines.[14] For these reasons, the effects of MeHg on antioxidant enzymes have received considerable attention.

SOD (EC 1.15.1.1), which dismutes the superoxide anion with a rate constant of 2×10^9 M^{-1} s^{-1} at 25 °C[15] has a pivotal role in protecting cells from free-radical damage.[16] The toxicity of superoxide radicals is thought to be due to involvement in a metal-ion-catalyzed Haber–Weiss reaction, the final product of which is the highly reactive hydoxyl radical. Mammalian cells contain two types of SODs: one contains copper and zinc (Cu,Zn-SOD) and is localized in the cytosol and in the intermembrane space of mitochondria; the other contains manganese (Mn-SOD) and is found in the matrix of mitochondria.[16] No sequence homology is apparent between Cu,Zn- and Mn-SOD. During SOD catalysis, the copper or manganese is thought to be reversibly oxidized and reduced by successive encounters with superoxide to yield molecular oxygen and hydrogen peroxide by the following reaction (Eqns [1]–[3]):[17,18]

$$E\text{–}M^{n+} + O_2^{\bullet -} + H^+ \leftrightarrows E'\text{–}M^{(n-1)+} + O_2 \quad [1]$$

$$E'\text{–}M^{(n-1)+} + O_2^{\bullet -} + H^+ \leftrightarrows E\text{–}M^{n+} + H_2O_2 \quad [2]$$

$$2O_2^{\bullet -} + 2H^+ \leftrightarrows O_2 + H_2O_2 \ (M^{n+} = Cu^{2+}, Mn^{3+}) \quad [3]$$

Since the observation that exposure of animals to MeHg causes a decrease in total superoxide

† Correspondence to: Y. Kumagai.

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dismutase activity of mouse brain has been reported recently,[12] oxidative-damage-inducing free radicals seems to play a role in the neurotoxicity of the organomercurial. However, mechanistic details of SOD inhibition caused by organomercurials are still poorly understood. Our approach to this question was to examine the effects of MeHg on gene expression, protein content and SOD activities of mouse brain to determine whether MeHg affects the transcriptional or post-transcriptional (protein synthesis, protein itself) stage of each SOD isozyme to cause loss of enzyme activity. This review briefly summarizes our findings.

STUDIES *IN VIVO*

It is probable that there are multiple factors affecting MeHg cytotoxicity such as the oxidation of protein SH, alterations in cellular glutathione (GSH) levels and possible tissue peroxidation. When MeHg chloride ($10\ mg\ kg^{-1}$) was administered subcutaneously to mice, the total mercury content in the brain increased with time and peaked at about two days after exposure ($1.89 \pm 0.20\ \mu g$ per g of tissue).[19] This level was maintained for about four days, after which the brain mercury declined. Under these conditions, MeHg caused an induction of oxidative stress as evidenced by a decrease in protein- and nonprotein-sulfhydryl content of mouse brain; the changes in both SH concentrations were biphasic.[19] As shown in Table 1, exposure of mice to MeHg decreased mouse brain Mn-SOD activity selectively whereas Cu,Zn-SOD activity was not appreciably altered by metal exposure.[20, 21] Levels of mRNA and protein for Mn-SOD were not markedly affected by MeHg administration, suggesting that reduction of Mn-SOD activity by the metal was not due to effects on transcription and translation. A similar, but not significant, decrease in pulmonary Mn-SOD activity was also observed after exposure of mice to mercury vapor; in this case, a significant increase in the Cu,Zn-SOD activity of mouse lung, resulting from the enhancement of protein synthesis, was noted.[22] In a preliminary study, however, subcutaneous injection of $HgCl_2$ ($1\ mg\ kg^{-1}$) showed an opposite effect to that of mercury vapor, i.e. enhancement of Mn-SOD protein in mouse brain and kidney, but not in lung and liver.[23] Thus tissue and isozyme differences in alterations of SOD gene regulation caused by mercuric compounds should be considered. Because Mn-SOD mRNA is induced by lipopo-

Table 1 Changes (% of control) in gene expression, protein synthesis and enzyme activity of mouse brain SOD isozymes after exposure to methyl mercury

Time after exposure	mRNA content[a]		Protein content[b]		Enzyme activity[c]	
(days)	Cu,Zn-SOD	Mn-SOD	Cu,Zn-SOD	Mn-SOD	Cu,Zn-SOD	Mn-SOD
1	83	78	141	131	113±8	92±11
2	109	121	133	117	109±4	95±1
5	85	84	112	93	102±9	61±8*
12	—	—	—	—	105±2	83±11

MeHgCl ($10\ mg\ kg^{-1}$, s.c.) was injected into mice. mRNA content, protein content and SOD activities were determined by Northern blotting, Western blotting and acetylated cytochrome *c* reduction in the absence and presence of KCN, respectively. Northern blot was probed with a ^{32}P-labeled human Cu,Zn-SOD cDNA, human Mn-SOD cDNA and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). mRNA levels were quantified densitometrically relative to the intensity of the GAPDH signal. For immunoblot analysis, bands which are immunoreactive against anti-mouse Cu,Zn-SOD and Mn-SOD IgG were quantified by densitometer.

[a, b] Quantitative data are presented only for signals in the linear range of the membrane and the densitometer. The data are expressed as a ratio of Cu,Zn-SOD or Mn-SOD/GAPDH. Numbers represent the average, where given, from at least two to four independent experiments.

[c] Each value is the mean ± SD of three animals. Control enzyme activities for Cu,Zn- and Mn-SOD of mouse brain were 7.81 ± 0.64 and 3.65 ± 0.34 units per mg of protein, respectively.

* Significantly different ($P < 0.01$) compared with control.

lysaccharide, tumor necrosis factor, interleukin-1 or interleukin-6,[24–26] inorganic mercury may affect regulation of these cytokines and thereby increase protein content.

Mercury compounds increased the susceptibility of target tissue to damage by oxidative stress by producing reactive oxygen species and depleting glutathione.[27] The fact that gene expression of γ-glutamylcysteine synthetase, the rate-liming enzyme for glutathione synthesis, was increased by MeHg has also been reported.[28] Thus, induction of Cu,Zn-SOD, Mn-SOD or γ-glutamylcysteine synthetase could be considered as an initial adaptive response to mercury-promoted lung damage involved in oxidative stress. Yee and Choi[12] showed that continuous injection of MeHg ($2.5\ mg\ kg^{-1}$) daily for 3, 7 and 14 days into C57BL/6J mice resulted in an induction of oxidative stress, evidenced by increases in suproxide radical and hydrogen peroxide and a decrease in total SOD activity in the brain. The observation that both isozymes were affected differs from the observations reported here. The discrepancy may be due to differences in the strain used or the amount of mercury injected.

STUDIES *IN VITRO*

Since the effect of MeHg on mouse Mn-SOD *in vivo* indicated that selective inhibition of SOD activity was attributable to direct modification by the metal, purification of Cu,Zn- and Mn-SOD from mouse liver was attempted. To do this, we have developed an efficient method for isolation of Cu,Zn-SOD from mammalian tissue to obtain the highly purified enzyme.[29] The procedure utilizes the stability of the metal-binding protein at acidic pH and in methanol, and has been performed in the presence of a large excess of ammonium sulfate. The efficient isolation of Cu,Zn-SOD from tissue is due to selective extraction by a salting-out effect at pH 5 rather than by liquid–liquid partition at the isoelectric pH (Y. Kumagai *et al.*, unpublished observations). Although purification of Cu,Zn- and Mn-SOD from mouse liver has previously been reported,[30] specific activities of the final enzyme preparations were relatively low (Cu,Zn-SOD, $2600\ units\ mg^{-1}$; Mn-SOD, $2300\ units\ mg^{-1}$), suggesting that both preparations could contain other proteins or could release metals from active sites of SOD isozymes during purification. Cu,Zn-SOD obtained by our method possessed approximately two moles of copper and zinc per subunit and gave a single band with a specific activity of $5464\ units\ mg^{-1}$ on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Also, pure Mn-SOD purified by a modification of the method of Taniguchi and coworkers[31] had a specific activity of $3882\ units\ mg^{-1}$ protein.

The results of direct interaction of purified mouse SOD isozymes with mercuric compounds is shown in Table 2.[21, 32] When a fixed quantity of each SOD isozyme (10 units) was incubated with 0.2 mM mercuric compounds, inorganic mercury suppressed the activities of purified Cu,Zn- and Mn-SOD in a time-dependent manner but exhibited a different inhibitory effect on each. For example, the activity of Mn-SOD was completely abolished at 24 h after reaction, whereas 63% of Cu,Zn-SOD activity still remained even after the same time period. These phenomena were also observed when either Cu,Zn- or Mn-SOD (1 μM) was incubated with MeHg at a ratio of 1:600 for 24 h.[21] MeHg caused a facile reduction in Mn-SOD activity but not in that of Cu,Zn-SOD after 24-h incubations, although 1-h incubation of SOD isozymes with MeHg resulted in a slight enhancement of these enzyme activities. In contrast, dimethylmercury (Me_2Hg) caused little decrease in Mn-SOD activity. *N*-Ethylmaleimide, which is known to modify thiol groups, had relatively low potency in inhibiting Mn-SOD activity, but the suppression of the activity was not remarkable compared with MeHg, suggesting that not only covalent modification via sulfhydryl functions but also the resulting alteration in structure may be required for a loss of enzyme activities. The loss of activity casued by inorganic mercury and MeHg was blocked by preincubation with glutathione.[20, 32] The results indicate that these mercuric compounds fall into three distinct groups; inorganic mercury is more effective than MeHg and Me_2Hg is ineffective. The simplest explanation for this grouping is based upon sites available for sulfhydryl binding, the inorganic mercury can bind two molecules of cysteine, the organic R–Hg only one, and dimethylmercury none.

To assess the involvement of mercury interaction with reactive amino-acid, and the differential affinity for metal binding to SOD, a simple method for detection of mercury bound to protein has recently been developed in our

laboratory.[33] Briefly, the reaction mixtures of each SOD isozyme, in the absence and presence of mercuric compounds, are applied to isoelectric focusing agarose-gel electrophoresis to separate the unreacted mercury from enzyme. Then, the metal binding is determined by synchrotron radiation X-ray fluorescence (SR–XRF) line analysis. By use of this method, digestion of protein with strong acid prior to elemental analysis and use of radio-labeled compounds are unnecessary. Exposure of mouse Cu,Zn-SOD isozyme to inorganic mercury resulted in a change in isoelectric point (pI) from 5.8 for the native form to a pI of 6.2, 6.4 and 6.5 (Fig. 1A, lane 3).[32] The binding of mercuric ion, which could not be removed by electrophoresis, was observed in all the charged isomers as shown in Fig. 2 (left-hand side, B). However, metal was not detected in the Cu,Zn-SOD that had reacted with MeHg or inorganic mercury in the presence of glutathione (Fig. 2, left-hand side, C and D). In contrast, Mn-SOD (pI = 8.1–8.3), after incubation with inorganic mercury, did not migrate because the protein was insoluble, probably due to aggregation caused by mercury binding, during electrophoresis (Fig. 1B, lane 3). Interestingly, apparent binding of MeHg to Mn-SOD was observed (Fig. 2, right-hand side, C), whereas similar bonding Cu,Zn-SOD was not (Fig. 2, left-hand side, C).[21] Such metal binding was abolished by pretreatment with glutathione (Fig. 2, right-hand side, E). Furthermore, experiments using an HPLC gel filtration column revealed that exposure to inorganic mercury

Table 2 Alterations in purified mouse Cu,Zn- and Mn-SOD activities caused by mercuric compounds

	Enzyme activity (% of control)			
	Cu,Zn-SOD		Mn-SOD	
Addition	1 h	24 h	1 h	24 h
None	100	100	100	100
$HgCl_2$	66 ± 5*	63 ± 1**	54 ± 13**	0
GSH[a] + $HgCl_2$	90 ± 15	86 ± 11	95 ± 17	113 ± 9¶
MeHg[b]	152 ± 15**	90 ± 13	129 ± 15*	37 ± 10**
GSH + MeHg[b]	—	—	—	71 ± 5**, ¶
NEM[c]	95 ± 8	79 ± 6*	101 ± 3	71 ± 2**
Me_2Hg	89 ± 6	77 ± 12*	109 ± 6	93 ± 17

Cu,Zn- and Mn-SOD were purified from mouse liver. Each highly purified enzyme showed a single band on 12% SDS-PAGE. Cu,Zn- and Mn-SOD, which are distributed in mouse brain, reacted immunologically with the antibodies raised against these purified enzyme preparations obtained from liver (Kumagai *et al.*, unpublished observation). A two-stage incubation method was employed to monitor the effects of mercuric compounds on SOD activity. In the first stage, incubation mixtures (0.1 ml) consisted of each SOD isozyme (10 units), 0.2 mM mercuric compounds, 0.2 mM NEM or 2 mM GSH in 50 mM potassium phosphate buffer (pH 7.8). Incubations were performed at 25 °C for 1 or 24 h. After reaction, aliquots (2.5–15 μl) were transferred to the second incubation mixture (1.5 ml), which is composed of 10 μM cytochrome *c*, 33 nM xanthine oxidase, 50 μM xanthine (sodium salt) and 50 mM potassium phosphate buffer (pH 7.8)–0.01 mM EDTA, to determine SOD activity. Under these conditions, the xanthine oxidase was unaffected by the mercuric compounds used. Each value is the mean ± SD of three determinations.

[a] GSH, glutathione.

[b] Chloride form was used.

[c] NEM, *N*-ethylmaleimide.

Significantly different compared with control: * $P < 0.05$; ** $P < 0.01$; the condition in the absence of GSH: ¶ $P < 0.01$.

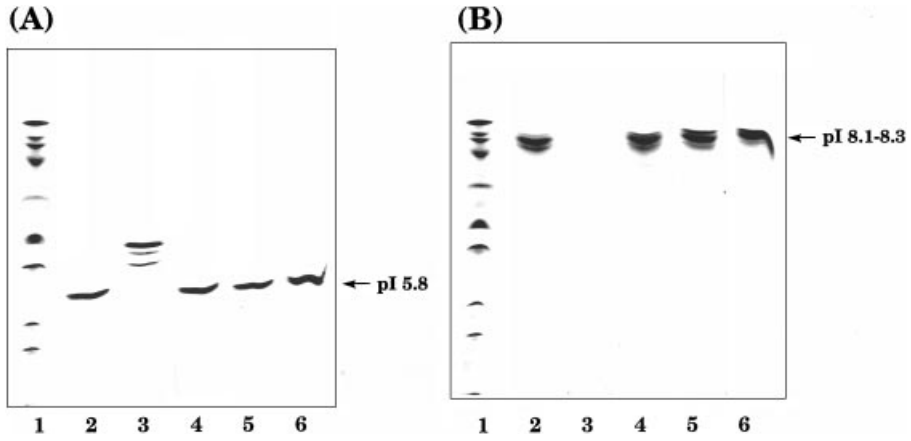

Figure 1 Isoelectric focusing–agarose gel electrophoreses of reaction mixtures of mouse SOD isozymes with mercuric compounds. After incubation of each SOD isozyme (180 units) with 0.2 mM $HgCl_2$ or MeHgCl in the absence and presence of 2 mM GSH (24 h, 25 °C), each sample (10 μg) was placed to a slab agarose gel (9.5 cm × 8.8 cm, pH 3.5–9.5). (A) Cu,Zn-SOD; (B) Mn-SOD. Lanes: 1, standards; 2, SOD; 3, SOD + $HgCl_2$; 4, SOD + $HgCl_2$ + GSH; 5, SOD + MeHgCl; 6, SOD + MeHgCl + GSH. The standards for pI calibration consisted of amyloglucosidase (3.50), Methyl Red (3.75), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin (acidic band) (6.85), horse myoglobin (basic band) (7.35), lentil lectin (acidic band) (8.15), lentil lectin (middle band) (8.45), lentil lectin (basic band) (8.65) and trypsinogen (9.30).

caused dissociation of Mn-SOD, but not Cu,Zn-SOD, into its subunits.[32] By contrast, the peak corresponding to Cu,Zn-SOD was unaltered by MeHg but that for Mn-SOD decreased drastically and the decrease in the peak correlated with the loss of enzyme activity.[21]

In other experiments, with bovine Cu,Zn-SOD, the decrease in the activity caused by inorganic and organo-mercurials has been examined.[34] Incubation of bovine Cu,Zn-SOD with higher concentrations of $HgCl_2$ resulted in a fragmentation of the enzyme followed by its precipitation, whereas that with MeHg did not, suggesting that the Cu,Zn-SOD easily undergoes structural changes and aggregation in the presence of inorganic mercury. Equal amounts of copper and zinc ions were released by incubation of bovine Cu,Zn-SOD (12 μM) with 2 mM $HgCl_2$, but not by incubation with MeHgCl for 24 h. Under these conditions, although ratios of inorganic and MeHg bound to Cu,Zn-SOD were 34 and 3 mol per mol of enzyme, respectively, the enzyme activities were inhibited by 93% (inorganic mercury) and 49%. Taken together, the data indicate that an alteration in structure caused by covalent attachment of inorganic mercury to bovine Cu,Zn-SOD and the resulting release of copper and zinc ions from the active site partially contributes to the activity loss. However, the MeHg-mediated enzyme inhibition probably involves an alternative mechanism.

PROPOSED MECHANISM

Figure 3 shows sketches of the conformation of mouse Cu,Zn-SOD and Mn-SOD subunits, presumed from amino-acid sequence determinations,[35–37] three-dimensional structure studies[38–41] and cDNA clonings.[42, 43] In the case of Cu,Zn-SOD, each 153-residue subunit is composed primarily of eight antiparallel β-strands that form a flattened cylinder, plus three external loops of non-repetitive structure.[38] The active-site copper ion on the enzyme is at the bottom of a deep channel on the outside of the β-barrel between two large loops.[39] The copper ion, which is liganded by His-120, -46, -63 and -48 with an uneven tetrahedral distortion from square planar geometry, exposes about 5.2 Å to a 1.4 Å radius probe, but the zinc ion, which is liganded by His-63, -71 and -80 and by Asp-83, is completely buried:[38, 39] Cu,Zn-SOD, which is dimeric in its native form, possesses sulfhydryl residues Cys-57 and -146, present as disulfide, and Cys-6 as a free group (Fig. 3).[44] Because the Cys-6, which lies within the β-barrel structure, is ordinarily not exposed to solvent,[45] the single

thiol is unreactive in the native conformation.[46] Such reasons may be involved in the poor reactivity of Cu,Zn-SOD with MeHg. In the case of Mn-SOD, the 198 residues of each subunit fold into three strands of antiparallel β-sheet, seven α-helices, seven connecting structures, and the N- and C-termini.[41] A direct attack of MeHg on the manganese ion (liganded to His-26, -74 and -163 and to Asp-159), which functions in catalysis by alternate reduction and reoxidation,[17] producing effective loss of the enzyme activity has been proposed; but it seems unlikely because the critical metal ions on Mn-SOD, which is a tetrameric enzyme, are close to the subunit–subunit interface.[41] A crystallographic study on the structure of human Mn-SOD at 2.2 Å resolution reveals that the SH groups of both Cys-140 and Cys-196 are exposed at the subunit surface.[41] Experiments consisting of thiol titration with Ellman's reagent, alkylation by iodoacetamide, and purification of digested fragments of human Mn-SOD by thiol-Sepharose column chromatography, followed by amino-acid sequence determination, indicate that Cys-196, but not

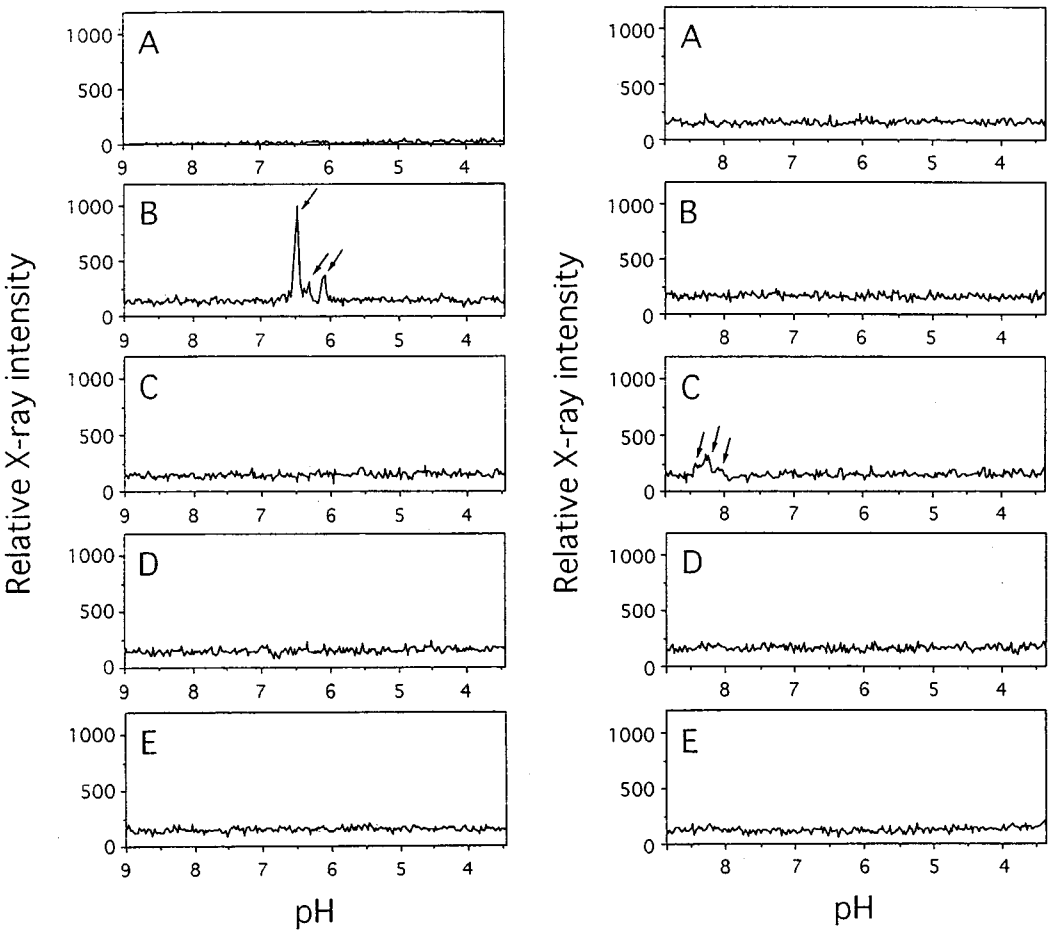

Figure 2 Plots of X-ray intensity data of mercuric compounds bound to mouse Cu,Zn-SOD and Mn-SOD. Left-hand side: A, Cu,Zn-SOD; B, Cu,Zn-SOD + $HgCl_2$; C, Cu,Zn-SOD + MeHgCl; D, Cu,Zn-SOD + $HgCl_2$ + GSH; E, Cu,Zn-SOD + MeHgCl + GSH. Right-hand side: A, Mn-SOD; B, Mn-SOD + $HgCl_2$; C, Mn-SOD + MeHgCl; D, Mn-SOD + $HgCl_2$ + GSH; E, Mn-SOD + MeHgCl + GSH. Incubations were carried out under the conditions described in Fig. 1. SR-XRF line analysis was performed by irradiating the sample with monochromatic X-rays (energy, 16 keV; beam size, 3 mm width, 0.3 mm high) step-scanning it (0.3 mm/step) along the vertical direction and measuring the X-ray fluorescence intensity with a Si(Li) solid-state detector (counting time, 20 s per point). The X-ray intensity data of the mercury L_β fluorescence lines were processed with a personal computer and the result was shown as a plot of normalized data which were calculated by converting the maximum value of the X-ray intensity to 1000. Arrows indicate mercury contents.

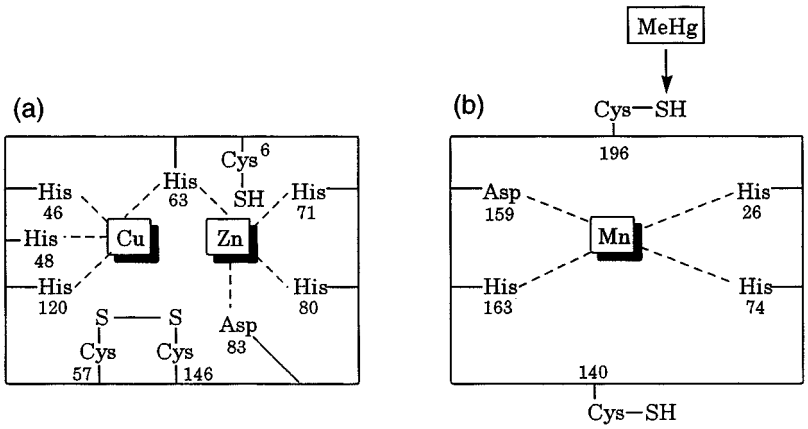

Figure 3 Sketches of structure assignments for mouse (a) Cu,Zn-SOD and (b) Mn-SOD subunits. Arrows indicate possible SH group which undergo modification by MeHg.

Cys-140, is the reactive sulfhydryl.[47] Thus we feel that the most likely explanation is that modification of accessible SH groups on Mn-SOD appears to be involved, as shown in Fig. 3: in other words, differences between Cu,Zn-SOD and Mn-SOD in the availability of reactive residues with which MeHg reacts could play an important role in the loss of enzyme activity.

We also found a species difference in the binding capacity of MeHg to the Cu,Zn-SOD molecule.[48] The ability of MeHg to bind bovine Cu,Zn-SOD did correlate with its ability to inhibit SOD activity, whereas no binding of the organomercurial to mouse Cu,Zn-SOD was noted. It is also argued that binding of mercury to SH functions is not the sole basis for MeHg-mediated decrease in mouse Mn-SOD activity because *Escherichia coli*, which does not possess cysteine residue,[49] was affected by MeHg (Y. Kumagai *et al.*, unpublished observations). These findings suggest that the involvement of reactive amino-acid residues other than sulfhydryl groups in the interaction of Cu,Zn-SOD with MeHg shuld be considered, as speculated previously by others.[50, 51]

Our findings indicate that both SOD isozymes predominantly undergo modification by inorganic mercury compared with MeHg. The release of copper ion during incubations at a 1:167 ratio of bovine Cu,Zn-SOD to $HgCl_2$ for 24 h was observed, whereas MeHg causes no copper release but does decrease the enzyme activity. It appears that the mercuric ion is too bulky to approach the active site of the enzyme because of the narrowness of the channel (<4 Å) above the copper ion.[39] Presumably the phenomenon may result from a drastic structure change caused by covalent binding of mercury to Cu,Zn-SOD.

In conclusion, although inhibition or activation of a variety of enzymes by mercury compounds has been reported,[52–57] our findings indicate that inorganic mercury is an inhibitor of both SOD isozymes *in vitro* whereas Mn-SOD, but not Cu,Zn-SOD, is susceptible to modification by MeHg *in vivo* and *in vitro*. Mitochondria consume over 90% of the cell's oxygen and active oxygen species are generated during normal cellular metabolism in the organelle by the respiratory chain.[58] SOD acts as a primary defense against oxidative stress, e.g. in post-ischemic reperfusion of organs,[59, 60] tissue damage,[61, 62] acute and chronic inflammation and ionizing radiation,[63] by scavenging superoxide. Therefore the MeHg-mediated loss of Mn-SOD, which is present in mitochondria, could be involved in the neurotoxicity.

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