Alternative Origin for "Gain-of-Function" by Mutant SOD Enzyme and for Conformational Change of Normal Prion Protein

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Gain-of-function of Mutant SOD, Hydroperoxo-copper(II) Complex, Amyloid-β-peptide

Capillary electrophoresis and ESI-Mass spectrometry methods have revealed that a hydroperoxo-copper(II) complex with (tpa) (=tris(2-pyridylmethyl)amine) reacts with carbonic anhydrase or amyloid beta-peptide (1–40) as a nucleophile to induce the conformational change of the protein structure, while the Cu(bdpg)-complex ((bdpg)=N,N-bis(2-pyridylmethy)-beta-alanineamide) acts as an electrophile toward the proteins to degrade them under the same experimental conditions. This will lead to suggest that enhanced nucleophilic attack by a copper(II)-peroxide adduct to peptide bonding may be one of the serious origins for the "gain-of-function" by mutant superoxide dismutase and for conformational change of normal prion protein.

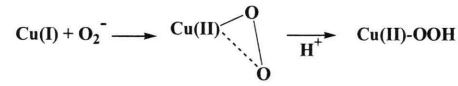
Introduction

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, is a motor neuron degenerative disease. (Brown 1995) About 10 percent of cases are familial, and 20 to 25% of familial ALS (FALS) cases are associated with dominantly inherited mutations in SOD1, the gene that encodes human CuZn-SOD. Initial studies of the FALS-associated CuZnSOD mutants appeared to demonstrate reduced enzymatic activity, however, subsequent studies with transgenic mouse and cell culture models of FALS indicated a dominant, gain-of-function effect of the FALS-associated CuZnSOD mutants. However, the precise origin for "gain-of-function" in the mutant CnZnSOD enzyme is not clear at present. (Pozas *et al.*, 1993; Rabizadeh *et al.*, 1995; Estevez *et al.*, 1999).

In order to give a clear elucidation on this problem, we have started the investigation on the reaction mechanism of the CuZnSOD, and on the reactivity on a (hydroperoxide)-copper(II) species. Generally, it has been recognized that SOD reaction may proceed as follows: (Holm *et al.*, 1996):

$$Cu(II) + O_2^- \rightarrow Cu(I) + O_2 \; ; \; Cu(I) + O_2^- \rightarrow Cu(II) + O_2^{2-}.$$

The peroxide ion formed in the last reaction is decomposed by catalase or glutathione peroxidase. We have proposed that the second reaction should proceed by an inner-sphere mechanism, i.e., *via* the formation of a copper(II)-peroxide adduct (see below). (Nishida *et al.*, 1992; Nishida *et al.*, 1994).



In the wild-type CuZnSOD, the formed peroxide ion is removed form the copper(II)-site and is decomposed quickly. But, it seems quite likely that the copper(II)-OOH species described above may live for a long time in the mutant SOD, because of the altered environment around the copper ion (Deng *et al.*, 1993), and the peroxide ion is more activated through interaction with organic groups nearby. (Nishida and Nishino, 2001) These should be origin for the enhanced oxidative activity observed for the mutant SOD. In order to understand the reactivity of a copper(II)-hydroperoxide adduct we have prepared many copper(II) compounds with the ligands illustrated in Fig. 1, and compared the reactivity of these compounds in the presence of hydrogen peroxide. As the results, we have observed that the reactivity of the copper(II) complex is highly dependent on the ligands used (tpa, bdpg, bdpe, and dpgt in Fig. 1) in the presence of hydrogen peroxide (Okuno *et al.*, 1997; Kobayashi *et al.*, 1998; Nishino *et al.*, 1999; Ishi-

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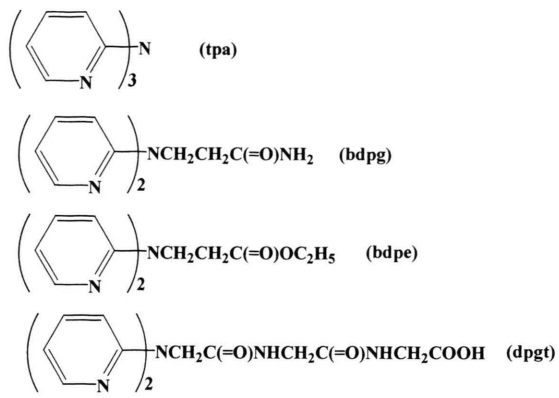

Fig. 1. Chemical structures and abbreviations of the ligands cited in this paper.
(dpgt): N,N-bis(2-pyridylmethyl)triglycine; (bdpe):N,N-bis (2-pyridylmethyl)-β-alanineethylester.

kawa *et al.*, 1998; Nishino and Nishida, 2001) This strongly suggests that the change in the coordination environment around the copper atom give a remarkable effect on the oxidative activity of the peroxide molecule in the Cu(II)-OOH species.

In this study we have investigated the effect by hydrogen peroxide on the interaction between carbonic anhydrase (CA) or amyloid beta-peptide (1–40) and copper(II) compounds by means of capillary electrophoresis and mass-spectral methods, and found that copper(II)-(tpa) complex acts as a nucleophile toward both CA and amyloid beta-peptide (1–40) in the presence of hydrogen peroxide, which may be consistent with the recent results by DFT calculations (Nishida and Nishino, 2001; Nishino *et al.*, 2001). This may give an alternative idea to understand the "gain-of-function" of the mutant CuZnSOD enzyme.

Experimental

Materials

The copper(II) compounds, $Cu(bdpg)Cl_2$, $Cu(bdpe)ClClO_4$, and $Cu(dpgt)ClClO_4$ (Okuno *et al.*, 1997; Kobayashi *et al.*, 1998; Nishino and Nishida, 2001) and binuclear zinc(II) complex with H(HPTP), $Zn_2(HPTP)(CH_3COO)(ClO_4)_2$ were obtained according to the published methods; where (H(HPTP) denotes N,N,N′,N′- tetakis(2-pyridylmethyl)-2-hydroxy-1,3-diaminopropane. (Nishida *et al.*, 1999; Nishino *et al.*, 2001) Carbonic anhydrase (mixture of I and II: C 3934; abbreviated as CA) and amyloid beta-peptide (1–40) were purchased from Sigma.

Capillary electrophoresis

Capillary electrophoregrams of the solution were obtained with a Beckman/Coulter P/ACE MDQ and with a CAPI-3200 (Otsuka Electronics Co., Osaka): in the case of Beckman/Coulter P/ACE MDQ, temperature, 25 °C, buffer solution, 25 mM tris-HCl (tris=tris(hydroxylmethyl)aminomethane, pH 8.2); sample injection, 0.5 psi (35.1 g/cm^2) 5 sec; 20 kV; column, eCAP TM I. D. 75 μm, O. D. 375 μm; detection 214 nm. Three solutions (1 μl) were mixed and the electrophoresis measurements were done on these solutions; protein, 1 mg/1 ml solution; hydrogen peroxide, 1/100 M solution; metal complex, 1 mM solution and the solution was eluted with buffer solution (25 mM, Tris). In the case of CAPI-3200, experimental conditions employed were essentially the same as those as described for the P/ACE MDQ, but capillary column (purchased from Otsuka Electronics Co., uncoated column, I. D. 75 μm, O. D. 375 μm) and injection method are different, and the solution was eluted with buffer solution (tris, 10 mM).

Electrospray mass spectrometry

ESI-Mass spectra were obtained with an API 3000 triple quadrupole mass spectrometer at Institute for Molecular Science, Okazaki, Japan. Experimental conditions are as follows: Amyloid β-peptide (1–40) (100 μg) was dissolved in water (100 μl), and H_2O_2 solution (0.1–2% solution; 100 μl) was added to the above solution. To this solution (20 μl), was added 2 μl of a copper(II) complex solution (1/350 M solution).

Results and Discussion

In Fig. 2A, capillary electrophoregram (hereafter abbreviated as CE) of amyloid beta-peptide (1–40) (hereafter abbreviated as Aß (1–40); the peaks at retention time around 5 min are due to Aß(1–40)) obtained by P/ACE MDQ is shown. The results obtained by the P/ACE MDQ are essentially the same as those by the CAPI-3200, but in some cases remarkable difference was observed, especially in the case where Cu(tpa) complex was used (not shown). When $Cu(bdpg)Cl_2$

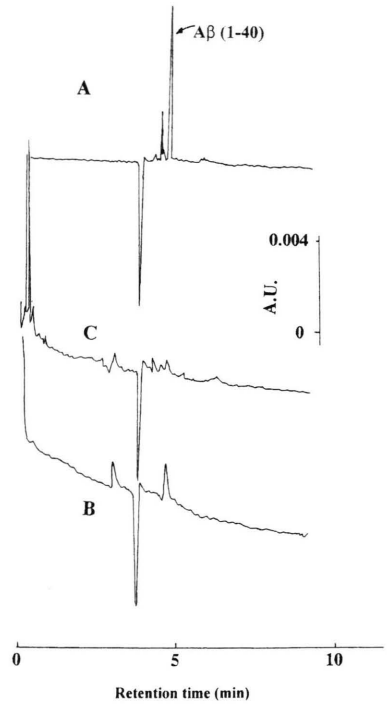

Fig. 2. Capillary electrophoregram (abbreviated as CE; obtained by P/ACE MDQ) of A: Aß (1–40) B: CE measured at 15 minutes after $Cu(bdpg)Cl_2$ and H_2O_2 were added to Aß (1–40). C: CE measured at 30 minutes after $Cu(bdpg)Cl_2$ and H_2O_2 were added to Aß (1–40).

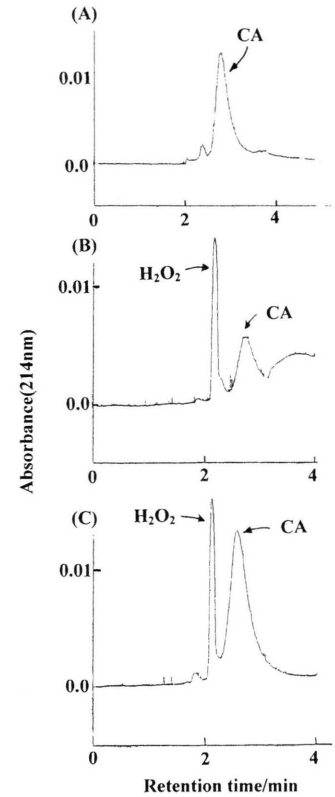

Fig. 3. Capillary electrophoregram (abbreviated as CE; obtained by CAPI-3200) of A: CE of CA(carbonic anhydrase) and $Cu(bdpe)ClClO_4$, B: CE measured immediately after $Cu(tpa)ClClO_4$ and H_2O_2 were added to CA. C: CE measured at 60 minutes after $Cu(tpa)ClClO_4$ and H_2O_2 were added to CA.

and hydrogen peroxide were added to this solution, the decrease of the peak intensity was detected as illustrated in Figs. 2B and 2C, which is consistent with the results obtained in the ESI-Mass spectrometry (see later), and with the fact that $Cu(bdpg)Cl_2$ complex can degrade albumin in the presence of hydrogen peroxide (Ishikawa *et al.*, 1998). This should be due to high electrophilic nature of the peroxide adduct of Cu(bdpg) system as described in the previous papers (Okuno *et al.*, 1997; Kobayashi *et al.*, 1998).

In the CE of carbonic anhydrase (CA), peaks due to CA are observed around 2.7–3.2 min region, and this pattern is not affected by the addition of $Cu(bdpe)ClClO_4$ as illustared in Fig. 3(A). But, the addition of $Cu(tpa)ClClO_4$ to CA solution induces remarkable change in CE to give the profile, similar to that shown in Fig. 3(B); reduction of peak height by the CA itself, and appearance of new broad signals in the range 3–6 min, but in this case no further change was detected within three hours; the appearance of the peaks around in 3–6 min may be due to conformational change of the original CA protein through interaction with Cu(tpa) species (Wadworth *et al.*, 1999). It should be noted here that the presence of hydrogen peroxide in this system induces the gradual change of the spectrum of Fig. 3(B) to give the CE profile similar to that of the original one within an hour (see Figs. 3(C) and 3(A)). This may indicates that a Cu(II)-OOH species formed in the solution effects the conformational change of the species at the retention time 3–6 min in Fig. 3(B) to those similar to the original protein. Electrophoregrams obtained by P/ACE MDQ are similar to those obtained by CAPI-3200, but in this case at 30–50 min after hydrogen peroxide was added, many signals were observed in the range around 10 min and these peaks disappeared after 60 minutes

without the decreasing of peak height due to CA itself. These indicate that no degradation of CA occurs in the presence of $Cu(tpa)Cl^+$ and hydrogen peroxide, but undoubtedly some interaction occurred among the Cu(tpa)-complex, hydrogen peroxide and CA. Similar facts were also observed in the system containing amyloid β-peptide(1–40), $Cu(tpa)ClClO_4$ and hydrogen peroxide.

In Fig. 4, ESI-Mass spectrometry of the solution containing Aß (1–40), and hydrogen peroxide are illustrated. In our experiments, detected species must be lower than m/z = 3000 because of the instrumental requirements. The major peaks at m/z = 1083.4 and 843.6 in trace A in Fig. 4 are attributed to $(Aß\ (1–40)+4H^+)^{4+}/4$, and $(Aß\ (1–39)+5H^+)^{5+}/5$, respectively. Appearance of the latter signal indicates that the fragmentation of Aß (1–40) has occurred in the measurement. The addition of H_2O_2 solution (0.1% solution) to the Aß peptide solution has induced the gradual appearance of new signals at m/z = 1087.4 and 846.8 (see traces A~C in Fig. 4), which should be due to the formation of Aß with Met(O) (Watson *et al.*, 1998); this is well consistent with the fact that Aß (1–40) is oxidized at sulfur atom of methionine 35 in the presence of hydrogen peroxide, and was confirmed by the use of $H_2{}^{18}O_2$ (92% purity; see Supplementary data: signals at m/z = 1087.4 and 846.8 shift to m/z = 1087.8 and 847.0, respectively).

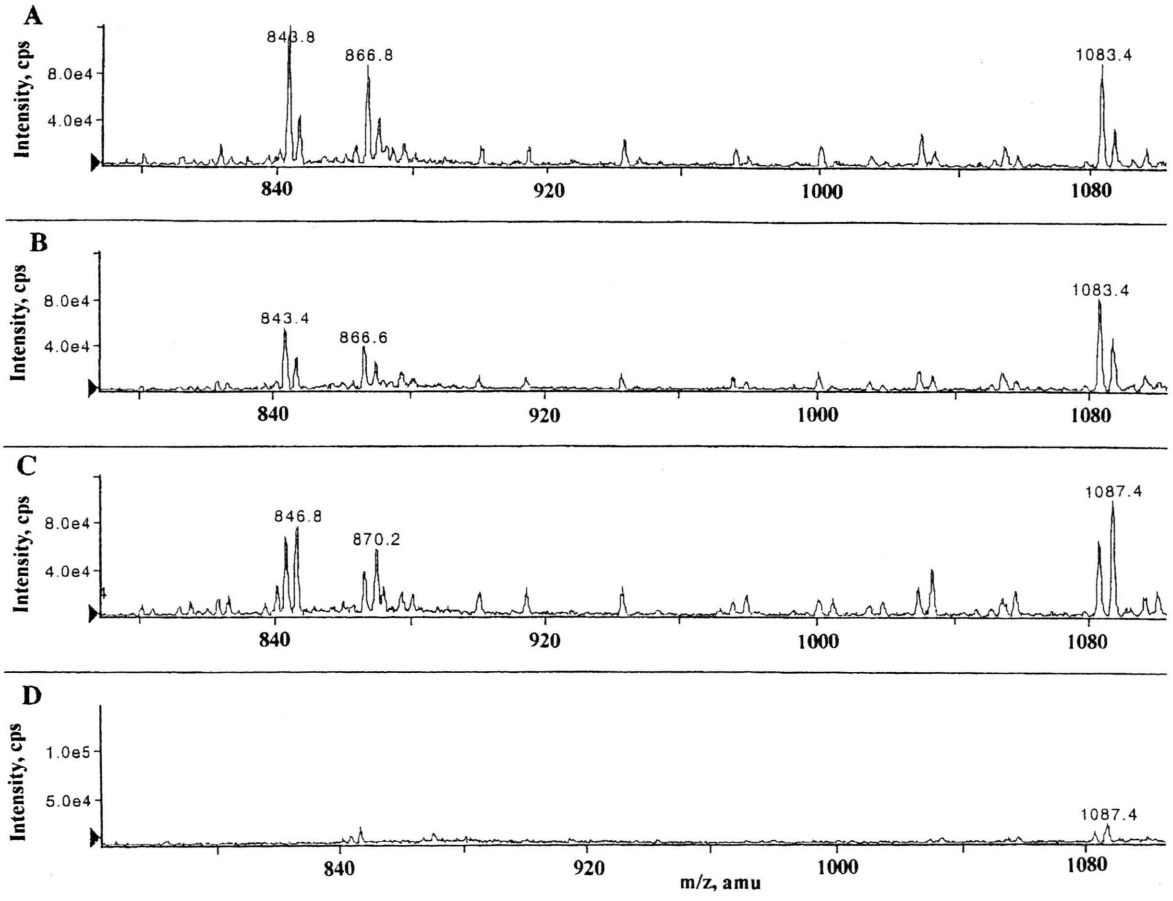

Fig. 4. ESI Mass spectra of the solution containing Aß (1–40), and hydrogen Peroxide (0.1% solution). (e4 and e5 represent $\times 10^4$ and $\times 10^5$, respectively)
A: 2 hours after hydrogen peroxide (0.1% solution) was added to Aß solution. (**Solution-A**)
B: 1 hours after **Solution-A** was prepared.
C: 2 hours after **Solution-A** was prepared.
D: 1 hours after $Cu(bdpg)Cl_2$ was added to **Solution-A**.

Hereafter we will call the trace A solution in Fig. 4 as **Solution-A**. We have observed that the mass-spectral changes of Aß (1–40) in the presence of hydrogen peroxide as illustrated in traces A~C in Fig. 4 are not effected by the presence of $Cu(tpa)Cl^+$ and $Zn_2(HPTP)^{2+}$ compounds in the solution, on the other hand, the degradation of the Aß (1–40) by $Cu(bdpg)Cl^+$ complex is remarkable as illustrated in the Fig. 4D. The results obtained by means of ESI-Mass spectrometry seem to be consistent with the above conclusion based on the capillary electrophoregrams.

Based on these facts, it seems reasonable to assume that a (hydroperoxo) adduct of the Cu(tpa) complex binds CA through electrostatic interaction, which is promoted by the high electron density at the terminal oxygen atom of the peroxide adduct (Nishida and Nishino, 2001), and this interaction induces some chemical changes, not the cleavage or degradation, rather conformational change of the protein structure, giving CE similar to that observed at the initial stage. Thus, the present results strongly suggest that the enhanced nucleophilic attack by the copper(II)-peroxide adduct to the peptide bonding may be one of the serious origins for the "gain-of-function" in the mutant SOD enzyme, and also for conformational change of the normal prion protein (conversion of PrP^C into PrP^{Sc}), especially in the case of mutant PrP^C (Caughey, 2001), observed in mad cow disease (Prusiner, 1998; Wadsworth *et al.*, 1999).

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