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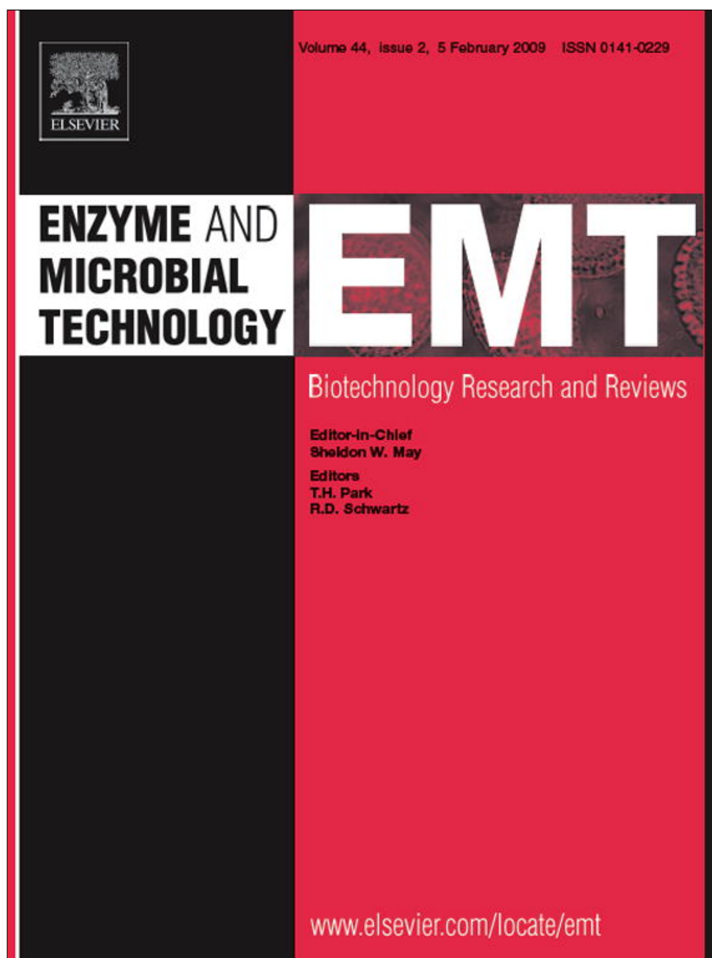

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Liposome membrane can act like molecular and metal chaperones for oxidized and fragmented superoxide dismutase

Le Quoc Tuan, Hiroshi Umakoshi, Toshinori Shimanouchi, Ryoichi Kuboi *

Department of Chemical Science and Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama, Toyonaka, Osaka 560-8531, Japan

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ABSTRACT

A mechanism for liposome-recruited activity of oxidized and fragmented superoxide dismutase (Fr.-SOD) [Tuan LQ, Umakoshi H, Shimanouchi T, Kuboi R. Liposome-recruited activity of oxidized and fragmented superoxide dismutase. Langmuir 2008;24:350–4] was further investigated, focusing on the secondary structure of Fr.-SOD. Liposome membrane was found to assist the conformational change of Fr.-SOD and reactivate the enzymatic activity, like molecular and metal chaperones. The loss of SOD activity and its secondary structure was observed during 6 h oxidation in 2 mM hydrogen peroxide. The contents of the α-helix and β-sheet structures in the oxidized and fragmented SOD (2 μM) were increased only in the presence of 10 μM Cu^{2+} and Zn^{2+} together, or in the presence of 2 mM POPC liposomes. The mixture of all of these elements (fragmented SOD and POPC liposomes with Cu^{2+} and Zn^{2+}) gave not only the increase of the α-helix and β-sheet contents but also the mediation of the high SOD-like activity.

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1. Introduction

Cu,Zn-superoxide dismutase (SOD) is a metalloenzyme that contains copper and zinc at its active site and catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide [1]. However, under oxidative stress conditions, SOD was fragmented into small debris [2,3], which did not have enzymatic activity [4–6]. Some researchers have attempted to correlate H_2O_2-induced loss of SOD activity [4,7] with the structural changes in its active site [8,9], including increased proteolytic susceptibility [10–12]. The inactivation of SOD is halted by some substrates [6,13] and by assisting with liposome prepared from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) [8].

It has recently been reported that the liposome membrane can recruit the oxidized SOD fragment on its surface to produce a complex that has enzyme activity [14]. Among the fragments generated after SOD oxidation [15,16], some specific peptides contain its active site (∼5 and ∼10 kDa) [9] although these fragments have no SOD-like activity. A mechanism of recruitment (or recognition) of Fr.-SOD on membrane was considered to involve the recognition and folding of peptides by hydrophobic interaction, in which liposomes perform a molecular chaperone-like function [17–19]. The specific peptide purified from reverse-phase HPLC was recovered on the liposome membrane and expressed SOD-like activity [14]. However, the structural conformation of SOD fragments derived from oxidation has not been adequately clarified yet. It is therefore important to clarify the mechanism of conformational change and/or refolding of the oxidized and fragmented SOD on the liposome membrane with the addition of metal ions as a modification of the active center.

The objective of this study is to clarify the role of the liposome membrane in the reconstruction of the secondary structure of the recruited SOD fragment, including its active sites, as well as in the enhanced binding of metal ions. The results in the reactivation of the SOD fragment indicated that the liposome membrane could act as molecular and metal chaperones.

2. Materials and methods

2.1. Materials

Bovine erythrocyte Cu,Zn-SOD (EC 1.15.1.1), purchased from Sigma–Aldrich (St. Louis, MO, USA) with a specific activity of 4470 U/mg (product no. S2515-30KU, lot no. 125K740), was used without further purification. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and was used for liposome preparation. All other reagents of analytical grade were purchased from Wako Pure Chemical (Osaka, Japan).

2.2. Liposome preparations

The POPC liposome was prepared by using the following procedure according to the previous work [8,17]. The phospholipid powder was dissolved in chloroform/methanol. After the solvent was evaporated, the resulting thin film was dried

Abbreviations: SOD, Cu,Zn-superoxide dismutase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Fr.-SOD, fragmented SOD.

* Corresponding author. Tel.: +81 6 6850 6285; fax: +81 6 6850 6285.
E-mail address: kuboi@cheng.es.osaka-u.ac.jp (R. Kuboi).

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for at least 2 h under a vacuum. The lipid film was hydrated by pure water to form the multilamellar vesicles (10 mM as a final concentration of POPC). The solution of the multilamellar vesicle was frozen in dry ice-ethanol (−80 °C) and incubated in the water bath above the phase-transition temperature. The above freezing–thawing treatment was repeated five times and was then passed through two stacked polycarbonate filters of 100-nm pore size by using an extrusion device to adjust the liposome size.

2.3. Treatment of SOD and fragmented SOD

Cu,Zn-SOD (2 μM) was incubated with H_2O_2 (2 mM) in 50 mM phosphate buffer (pH 7.4) at 37 °C for various periods. The enzymatic activity and protein concentration of Fr.-SOD were determined after the incubation of SOD with H_2O_2. The SOD, totally oxidized into fragments for 12 h in 2 mM H_2O_2, was ultrafiltered by a 5 kDa molecular cutoff filter to remove the non-specific peptides and free copper. Retentate was then filtered by a 10 kDa molecular cutoff filter to separate specific peptide from mixture and obtain the potentially active fragment (fragmented SOD) containing the active sites according to the previous report [14]. Permeate was collected for further analyses. By using this technique, 25% of 10 kDa fragments in molecular mass of total SOD were recovered (2 μM). The 10 kDa fragments filtrated from ultrafiltration were incubated with POPC liposomes (2 mM) and metal ions (Cu^{2+} and Zn^{2+}) 10 μM for each of several alternatives of POPC or metal ions in phosphate buffer at 37 °C for 12 h. The enzymatic activity of the complex of liposomes, Fr.-SOD, and metal ions was determined. Structural change in fragmented and recruited SOD was observed by CD spectra.

2.4. Ultrafiltration of H_2O_2-treated SOD

Ultrafiltration using an Ultrafree-MC filter (Millipore, Billerica, MA, USA) with a molecular mass cutoff of 5 and 10 kDa was applied for fragmentation analysis as described above. SOD, after being treated by H_2O_2, was applied to an ultrafiltration tube and centrifuged at 15,000 rpm for 30 min at room temperature. Aliquots were then analyzed by reverse-phase HPLC, and their enzymatic activity was determined. The filter with a molecular mass cutoff of 3 kDa was also applied to remove small debris and free copper released from active site in oxidation process by hydrogen peroxide. The retentate fraction was then used for the further experiments. It was confirmed that the fragmented SOD recovered in the fraction had no activity and Cu ion could not be detected by using the calcein binding method (described below) in the fraction.

2.5. Metal binding analysis

After co-incubation fragmented SOD with liposomes and metals, the complex of liposomes, fragmented SOD and metals was applied into the ultrafiltration by a 3 kDa molecular cutoff filter to remove the free metals. The free copper in permeate solution was determined by the fluorescent decrease of calcein (a fluorescent probe has excitation and emission at 495 and 515 nm, respectively) when copper interacted with calcein. Fluorescence of calcein was measured by fluorescent spectrophotometer (JASCO FP 6500; Jasco, Tokyo, Japan). Langmuir's equation was applied to calculate the copper binding by liposomes and fragment peptide complex

$$q = \frac{q_m KC}{1 + KC}$$

where q and C are metal adsorption and equilibrium concentration of metal, respectively. q_m is maximal adsorption and K is the equilibrium constant.

2.6. Circular dichroism analysis

The secondary structure of SOD was determined using circular dichroism analysis (J-720W spectrometer; JASCO, Tokyo, Japan) at 37 °C. A 0.1 cm quartz cell was used for the measurement, and the CD spectra were recorded from 190 to 250 nm. Samples contained 2 μM SOD in 50 mM potassium phosphate buffer with or without 2 mM POPC liposomes. It was preliminarily confirmed that liposomes had no effect on the secondary structure of SOD. All CD measurements were carried out using the following parameters: 1 nm bandwidth, 50 nm/min run speed, 1 nm step size, 10 s response times, and an average of five runs. The contents of the secondary structure of the SOD and its fragments were analyzed through the fitting analysis of the CD spectra based on the previous data [20].

2.7. SOD activity

A highly water-soluble tretrazolium salt, WST-1 [2-(4-lopophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], produces a water-soluble formazan dye upon reduction with a superoxide anion, where the rate of the reduction with O_2^- is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD. The absorption spectrum of WST-1 formazan was measured at 450 nm, and the SOD activity as an inhibition activity can be quantified through the decrease in color development by setting the activity value of 2 μM native SOD as 100% and that without sample as 0% [21]. It was confirmed that the solution of Cu/Zn and/or POPC liposomes showed no SOD-like enzymatic activity as control experiment. A possible contribution of the free copper itself in the SOD activity was also assessed by the EDTA addition experiment. After the complex of liposome, fragmented SOD, and Cu/Zn complex was recovered by ultrafiltration, the addition of EDTA to the above solution did not affect the SOD-like activity of complex at all. The above negative control shows that the free Cu ions have no SOD-like enzymatic activity.

2.8. Statistical analysis

Results are expressed as mean ± standard deviation (S.D.). All experiments were performed at least in triplicate. Data distribution was analyzed, and statistical differences were evaluated using Student's *t*-test. A P value of <0.05% was considered significant.

3. Results and discussion

The oxidation and fragmentation of protein by oxidants concerned to the loss of its tertiary and secondary structures [22]. SOD has shown to be fragmented by hydrogen peroxide, depending on the oxidation time [2,14], where the enzyme activity of SOD also decreases in correlation with its fragmentation. The conformational change of SOD, incubated with 2 mM hydrogen peroxide, was first analyzed by circular dichroism (CD) spectra. During the oxidation, the peak of SOD gradually shifted to 198 nm from 209 nm, showing that the decreases in α-helix and β-sheet contents involved the increase of random coil contents (Fig. 1a). These results also demonstrate the structural change in SOD under oxidative conditions, where the α-helix contents totally disappeared after 6 h oxidation, as well as by the slight decrease in the β-sheet (Table 1).

The effect of the addition of POPC liposomes and Cu–Zn ions on the secondary structure of the Fr.-SOD was investigated. After the Fr.-SOD was collected by ultrafiltration (using a Millipore Ultrafree-MC filter with a molecular mass cutoff of 10 kDa), the effect of the additives on the secondary structure contents was investigated. In the presence of both Cu–Zn ions and POPC liposomes, both α-helix and β-sheet contents increased, showing the similar contents of the secondary structure of native SOD. The calculated results support the positive role of POPC liposomes and metal ions on the reconstruction of the secondary structure of the Fr.-SOD. The obtained results indicated that the addition of POPC liposomes, together with Cu and Zn ions, to the Fr.-SOD reconstruct its secondary structure and further induced the SOD-like activity. The conformational change of fragmented SOD into a native SOD-like structure was observed in the presence of POPC liposomes and copper and zinc ions (Fig. 1b). This process occurs because of the interaction of damaged protein with the membrane and its refolding [23,24]. However, the construction of the conformation of the potentially active peptide on the membrane surface is essential in order to induce the activity of inactive and Fr.-SOD. The CD spectra also show that the metal coordination of the SOD fragments to histidines described previously [14] induce β-sheet formation in the above conditions (Fig. 1b). CD spectra analysis revealed that the folding of the SOD fragments was significantly enhanced when both POPC liposomes and metal ions were added. Consequently, the structure of Fr.-SOD returned to a conformation similar to that of native SOD. The increase in α-helices of the fragments in the presence of only metal ions or only POPC liposomes shows that there was a partial reconstruction of the active site by metal ions or liposomes, respectively. However, in the presence of both metal ions and POPC liposomes, the α-helix contents increased significantly, from 0 to 4.4%. Similarly, β-sheet also increased mildly in separate settings of experiments with metal ions, POPC liposomes, or both (Table 1). The increases in α-helix and β-sheet contents in the Fr.-SOD could result from the folding and conformation of fragment SOD on the surface of POPC liposomes in the presence of metal

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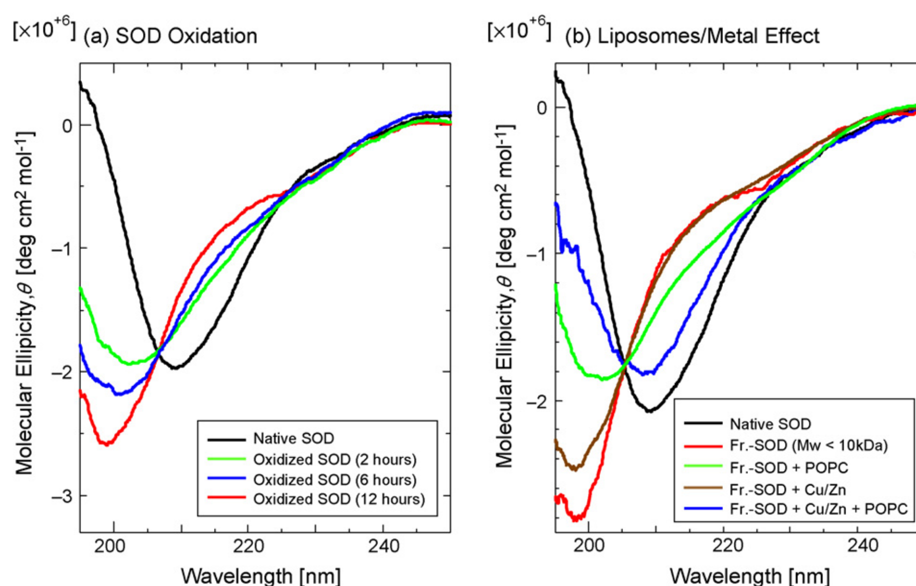

Fig. 1. Circular dichroism spectra of (a) SOD under oxidation and (b) Fr.-SOD with or without metal ions and POPC liposomes. SOD 2 μM was oxidized and fragmented by 2 mM H_2O_2 in phosphate buffer solution at pH 7.4. (a) Significant change in the secondary structure of SOD was observed under oxidative stress conditions (2 mM H_2O_2, 2, 6, and 12 h incubation). (b) The secondary structure of SOD lost under oxidative conditions (2 mM) was recovered by the addition of both metal ions (10 μM) and POPC liposomes (2 mM POPC, 100 nm).

ions. It is considered that both the refolding of Fr.-SOD and binding of metal ions to Fr.-SOD with the contact of POPC liposomes occurred cooperatively. Additionally, the reaction rate of these processes can depend on the addition order of substances. In a separate experiment, when liposome was added to suspension of Fr.-SOD before the addition of metals, the recovery of enzymatic activity reached 52% in 6 h. The obtained results indicated that supplementation with POPC liposomes, metal ions together, contributes to the reconstruction of new active sites similar to the original one.

The SOD-like activity of these samples was also analyzed (Fig. 2). The high enzymatic activity was obtained under the above conditions to induce the recovery of the secondary structure of the fragments. Data obtained from control experiments in both CD analysis and activity measurement also indicated that the refolding occurred mildly in the presence of metal or POPC liposomes only. Significantly, the liposome membrane was thus found to play an important role in reconstructing the secondary structure of the active center of the oxidized SOD with the assistance of metal ion binding. The interaction of copper and Fr.-SOD in presence of liposome membrane is considered to be involved in histidine residues of SOD fragments [25–28]. The Cu binding to Fr.-SOD with or without POPC liposomes was investigated at different initial concentrations of copper (Fig. 3). In the presence of POPC liposomes, the binding of copper to Fr.-SOD was found to be enhanced to higher value than that of control (without POPC liposomes). This result clearly shows that the binding of copper to fragments is stabilized by POPC liposomes. Based on the fitting analysis of the adsorption curve using Langmuir isotherm, the capacity of copper adsorption (q_{max}) on the Fr.-SOD with POPC liposomes was estimated as approximately four times that without the liposomes although no

Table 1
Calculated contents of the SOD secondary structure under oxidative conditions (Fig. 1a) and the Fr.-SOD with or without Cu/Zn and POPC liposomes (Fig. 1b).

Condition	Contents of secondary structure (%)[a]			
	α-Helix	β-Sheet	β-Turn	Random coil
Native SOD	7.4	61.7	4.4	26.5
Oxidized SOD (2 h)[b]	1.9	52.4	8.6	37.0
Oxidized SOD (6 h)[b]	0	52.9	8.4	37.7
Oxidized SOD (12 h)[b]	0	44.7	10.7	44.6
Fragmented SOD[c]	0	36.7	15.0	48.3
+Cu/Zn[d]	1.8	37.7	14.1	46.3
+POPC[e]	2.5	48.6	9.0	39.9
+Cu/Zn and POPC[d,e]	4.4	54.8	9.6	31.3

[a] Contents of secondary structure was calculated from the fitting-analysis of CD spectra (Fig. 1) using Spectrum Manager (JASCO, Japan) based on the standard data reported by Chen et al. [20].

[b] Oxidization of 2 μM SOD was performed in the presence of 2 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.4).

[c] The fragment SOD (2 μM) was collected by the ultrafiltration (a molecular mass cutoff filter of 10,000 Da), just after SOD oxidation for 12 h.

[d] Final concentration of both Cu and Zn was set at 10 μM.

[e] 2 mM POPC liposomes (100 nm).

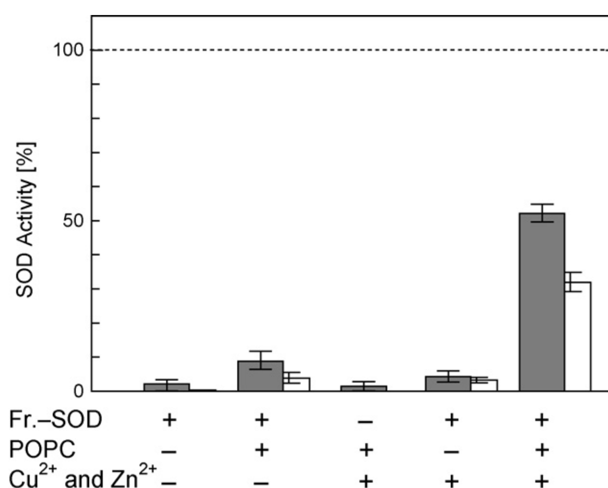

Fig. 2. SOD activity of Fr.-SOD with and without metal ions (Cu^{2+} and Zn^{2+}) and POPC liposomes. Fragmented SOD was co-incubated with POPC liposomes (2 mM POPC) and copper and zinc (10 μM for each) in phosphate buffer solution pH 7.4 at 37 °C in 12 h. *Filled rectangle*: Fr.-SOD recovered by ultrafiltration; *open rectangle*: data from previous report (recovered from RP-HPLC) [14]. All of the data represent the mean ± S.D. (n = 3–5).

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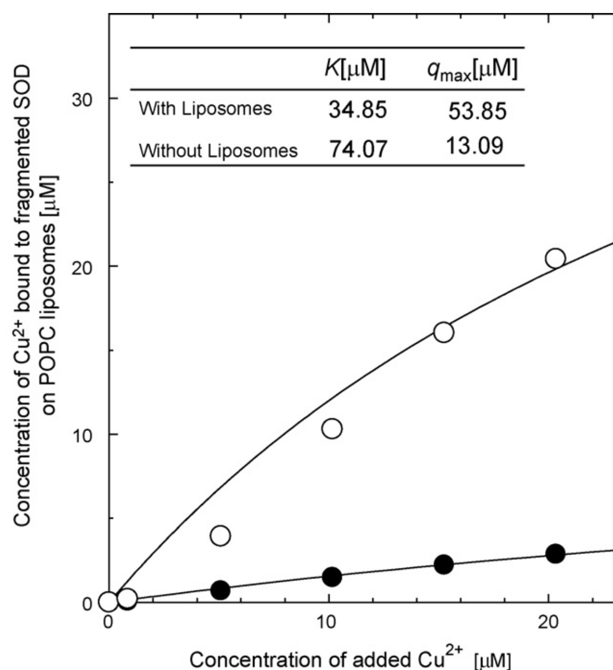

Fig. 3. Cu adsorption behaviors on Fr.-SOD in the presence (○) or in the absence (●) of POPC liposomes. A 2 μM Fr.-SOD was mixed with 2 mM POPC liposomes and metal ion mixture (Cu^{2+} and Zn^{2+}) at various concentrations. Langmuir isotherm was applied for the fitting of the adsorption curve. The capacity of Cu adsorption on the Fr.-SOD with POPC liposome was estimated to be approximately four times that without the liposome.

adsorption was observed on POPC liposome itself (data not shown). Under the above conditions, the SOD-like activity of the fragment increased from 0% (inactive) to 52%. In previous studies, when SOD was exposed to an appropriate concentration of hydrogen peroxide, histidine in the active site was oxidized into 2-oxo-histidine, and 34% of the total histidine was oxidized [5,29]. The mode of copper coordination with peptides depends on the number of copper ions bound, and copper ions are coordinated by multiple histidine imidazole groups [30,31]. Therefore, the reactivation of Fr.-SOD in the obtained results only reached an SOD-like enzymatic activity of 52%. These findings show that SOD fragments can acquire new metal binding sites by interacting with liposomes, and can also acquire a new secondary structure similar to that of native SOD. The folding of the Fr.-SOD in the presence of metals on the liposome surface implies that the membrane is an essential material in repairing damaged proteins and enzymes. Recent studies have demonstrated that the reactivation of enzymatic activity of Fr.-SOD on the liposome membrane involved to the liposome properties [33]. The interaction between SOD fragments and liposomes has reported to be related with the characteristics of both fragments and liposomes, in which hydrophobicity, hydrogen bond stability and surface charge were the key parameters. Such interactions mediated the suitable conformation of fragments on the liposome surface, resulting in the reconstruction of structure and activity of Fr.-SOD.

The relationship between the conformation of Fr.-SOD and SOD-like enzymatic activity in the presence of liposome and metal ions was further investigated by measuring the SOD activity and α-helix contents as shown in Fig. 4. When the concentration of metal ions increase from 0 to 10 μM (Fig. 4a), the α-helix increases from 0 to 4.4%. It was also confirmed that the POPC liposome showed no activity only in the presence of Cu and Zn without Fr.-SOD. However, if the concentration of metals was more than 10 μM, there was a precipitation of Fr.-SOD, representing the increase of sample turbidity. This may be due to the Fenton-like reaction by free copper ions in solutions in the case of the higher metal concentration, resulting in the production of hydroxyl radical, which in turn amplifies the damage to the fragments [10]. As shown in Fig. 4b, the relationship between SOD-like activities to α-helix contents was investigated. The obtained result indicates that the conformation of Fr.-SOD, represented by the α-helix content, contributed to SOD-like activity of fragmented and recruited SOD. Recent conformational analyses have also indicated that the structural insights into the binding and structuring process induced by peptide–membrane interaction [32]. The increase of copper binding to Fr.-SOD in the presence of liposome membrane can explain that the fragments contained the histidine-rich residues. Data obtained from amino acid sequence analysis showed that the percentage of histidine residue in Fr.-SOD was more that that in native SOD [33]. Furthermore, histidine residues have reported to possess a strong affinity with copper [23,31,34,35] and can control the redox activity of copper [36].

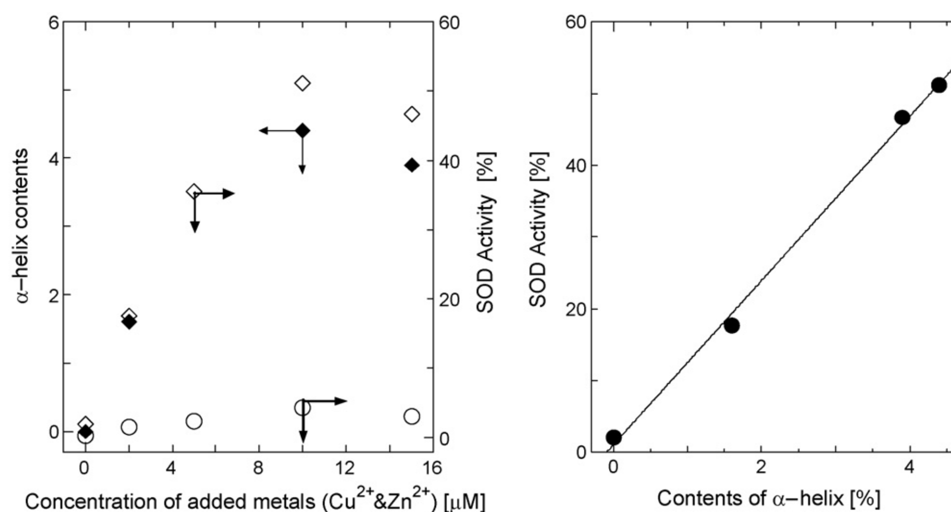

Fig. 4. (a) The relative leaner of the SOD activity and α-helix contents of recruited SOD on the range of copper and zinc concentration. A 2 μM Fr.-SOD was co-incubated with 2 mM POPC liposomes and metal ion mixture (Cu^{2+} and Zn^{2+}) at various concentrations (0–15 μM) in phosphate buffer solution pH 7.4. (b) The correlation of α-helix contents (♦) and SOD-like activity (◊) of liposome-fragmented SOD complex with the concentrations of added metal ions. Control experiments with metal ions only on SOD-like activity (○).

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In previous findings, some kinds of copper chaperone have reported to transport copper ions to apo-SOD to form fully mature SOD structures [37]. The process of dimerization requires either disulphide formation or metal coordination, whereas full catalytic function requires both [38]. Thus, in the correlation of fragment refolding with enzymatic activity, either the liposomes or metal ions independently induced the refolding of Fr.-SOD. The former played a significant role in the refolding process, as shown by CD spectra measurement (Fig. 1b). However, in both cases, the enzymatic activity did not increase significantly (Fig. 2). Otherwise, the presence of both metal ions and liposomes contributed not only to the fragment refolding but also to the recovery of enzymatic activity of the Fr.-SOD. The refolding of SOD fragments in our results involved the interactions of metal ions and Fr.-SOD on the liposome surface (Fig. 3). Furthermore, the metal binding capacity of a oxidized and fragmented SOD peptide can roughly be estimated as more than five [35] in contract that of a natural SOD (one in a subunit) which supported by the copper chaperone [39]. These results demonstrate that the POPC liposomes could act as an enhancer of copper binding similarly in the case of metal chaperone. Consequently, the structure of Fr.-SOD was reconstructed as well as functionally reactivated in the presence of liposome membrane. The increases in α-helix (Fig. 4) and β-sheet in the process of the recovery of the secondary structure and the SOD-like activity imply that Fr.-SOD interacted with POPC liposomes on its membrane surface. The SOD-like activity of the oxidized and Fr.-SOD has recently reported to be modulated, depending on the surface characteristics of liposome [33], implying the significance of the potential function of liposome itself.

It is concluded that, as a total process of the liposome-fragment recruitment and enzymatic reactivation, the liposome membrane is considered to induce a series of the potential functions such as (i) the recognition of specific SOD fragment [14,33], (ii) the enhancement of metal chelation (Fig. 3), (iii) the formation of secondary structure (Figs. 1 and 4) and (iv) the induction of SOD-like activity (Fig. 2) [14,33] as would occur with the molecular and metal chaperones. Based on the obtained results, the liposome-based mimics of enzyme could be designed by using the potential functions of liposome ((i)–(iv)).

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