STUDY ON PREPARATION AND CHARACTERIZATION OF ANTIOXIDATIVE LIPOZYME

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STUDY ON PREPARATION AND CHARACTERIZATION OF ANTIOXIDATIVE LIPOZYME

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Preface

This dissertation work was conducted under the supervision of Professor Ryoichi Kuboi at the Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University from 2004 to 2008.

The objective of this thesis is to clarify a final defense system against the oxidative stress in the biological system, especially concerning to the antioxidative role of "liposome" itself induced under oxidative stress condition. The interest is focused on (1) Preparation of potential SOD (superoxide dismutase) LIPOzyme from "build-up" and "beak-down" approaches: recruitment of synthetic ligand or oxidized/fragmented superoxide dismutase on liposome membranes, (2) Characterization of SOD-LIPOzyme prepared based on liposome-recruited activity of oxidized and fragmented SOD, and (3) Preparation of bifunctional LIPOzyme co-induced under oxidative stress. A mechanism of liposome functions in recruitment of oxidatively stressed peptide and its folding to active center-like structure is studied in relation to the damage state of membrane. The SOD/CAT (catalase) LIPOzyme preparation is selected as a case study. The LIPOzyme prepared by the damaged liposome membrane and peptide continuously displays not only SOD-like activity but also CAT-like activity.

The author hopes that this research would contribute to clarification of the "potential" role of liposome membranes in the effective utilization of bio-resources produced under the stress condition. The application of toxicological fail-safe mechanism in biological system opens a new research frontier of Membrane Stress Biotechnology and Bio-Envrionmental Chemistry.

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Summary

Antioxidative stress-response functions of the biological cells can be induced and utilized for survival when the cells were exposed to oxidative stress condition. From the conventional viewpoint of molecular biology, the specific genes, such as *sod* or *cat*, are expressed under the oxidative stress condition to protect the cell from the reactive oxygen species (ROS) through the expression of antioxidative enzymes eliminating ROS, such as superoxide dismutase (SOD) and catalase (CAT). However, there are some biological cells, which defect the gene for the antioxidative enzymes, though they still survive under the oxidative stress condition. Such a contradictional mismatch between genotype and phenotype in the biological system could sometimes be caused by the potential functions of the biological membrane induced under the oxidative stress condition. In this study, the potential functions of phospholipid bilayer membrane (liposome) were systematically studied, focusing on the membrane recruited activity on its surface via the use of the minimal elements i.e. liposomes, metal ions, and the fragments of SOD.

In chapter I, the roles of membrane in reactivation of oxidatively-damaged enzyme were demonstrated to clarify the membrane function in biological systems. Under oxidative stress, SOD was observed to be fragmented, leading to enzymatic inactivation, and the loss of an α-helix neighboring its active center. The H_2O_2-treated SOD, which lost their activity depending on stressed condition, was reactivated on the added POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) liposomes, resulting in the increase of enzymatic activity. When strong stress induced fragmentation of SOD, produced specific peptide fragments could interact with POPC liposomes. Liposome membrane was found to assist the conformational change of oxidized and fragmented superoxide dismutase (Fr.-SOD) and accelerate the adsorption of metal ions on the fragment to give the original SOD-like activity. 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 induction of the high SOD-like activity. In this case, the POPC liposomes could act like molecular and metal chaperones for the stress-damaged peptides, resulting in the creation of a new SOD-like active center, i.e. the antioxidative LIPOzyme that continues to express the SOD-like enzymatic activity under the oxidative conditions.

In chapter II, characterization of the liposome recruited activity, LIPOzyme activity, was made by studying the effect of lipid composition of liposome and by analyzing the possible elemental steps of the formation of LIPOzyme. The specific peptide fragment, originated from SOD, was first analyzed, and its sequence was identified. The oxidized SOD fragment was found to be displayed on the liposome surface through the following three steps: (1) binding (recognition), (2) refolding, and (3) reactivation on the membrane. Such interaction was considered to be related with the characteristics of the both fragments and liposomes caused by the combination of electrostatic, hydrophobic interactions, and hydrogen bonding between the peptide and liposome membrane. A strategy to elucidate the liposome-induced antioxidative activity by recruiting the minimal elements has finally been established, focusing on the "state of stressed membrane" (the membrane activity and toxicity).

In chapter III, conversion and regulation of the antioxidative function of the liposome were attempted as a case study of the strategy described in chapter II. Surprisingly, the CAT-like activity, which decomposes H_2O_2, could be induced on the liposome surface which displays the SOD-fragments-metal ions (Cu/Zn) complex. Actually, after the fragment-metal complex was recovered by liposome membrane, under continuous oxidative stress of hydrogen peroxide, membrane could regulate both enzyme-like activities. The LIPOzyme or the membrane-recruited SOD fragment possessed not only SOD-like enzymatic activity but also CAT-like activity, resulting in the decomposition of all the ROS in the biological system. The SOD-like activity and CAT-like activity were found to be modulated depending on the state of the membrane. Unexpected response of living cell (algal cell) such as survival under the strong oxidative stress could be related to such membrane-recruited antioxidative activities of the LIPOzyme.

The obtained results demonstrated the potential roles of membrane in recognition, recruitment, modification, and regulation of active sites of the LIPOzyme. The present results are applicable in modulation of artificial enzyme to control the oxidative agents and in the deeper understanding of the cellular response against oxidative stress.

GENERAL INTRODUCTION

What's oxidative stress? Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the biological cell, including proteins, lipids, and DNA (Pederson and Aust, 1973; Que et al., 1980). The oxidative stress is exposed to the cells as a result of one of three factors: (i) an increase in oxidant generation, (ii) a decrease in antioxidant protection, or (iii) a failure to repair oxidative damage. Cell damage is induced by reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and so on (Uauy et al., 1998), which are either free radicals, reactive anions containing oxygen atoms, or molecules that can either produce free radicals or are chemically activated by themselves. The main damage to cells results from the ROS-induced alteration of macromolecules such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA. Additionally, oxidative stress and ROS have been occasionally implicated in disease states, such as Alzheimer's disease (Manton et al., 2004), Parkinson's disease (Gorell et al., 1999), cancer (Hayes, 1997), and ageing (Fridovich, 1978), but they may also be important in prevention of ageing by induction of a process named mitohormesis. However, the ROS can be beneficial, as they are used by the immune system as a way to attack and kill pathogen and they are also used in cell signaling (Thannickal and Fanburg, 2000).

Oxidative stress is often caused by the redox potential of metal ions in a biological system (Valko et al., 2005). Some metals are severely toxic and widely found in environment. Recent studies indicate that the transition metals act as catalysts in the oxidative reactions of biological macromolecules. Redox-active metals may cause an increase in production of ROS such as hydroxyl radical (HO^*), superoxide radical ($O_2^{\cdot-}$) or hydrogen peroxide (H_2O_2). The effect of toxic metals to cell and its belongings was reviewed and presented in **Figure 1** by Ercal and his co-workers (Ercal et al., 2001), in which mechanisms involved in toxic metals induced oxidative damage were mentioned. Interestingly, metals are essential for life activity because of their capacity in acting as an intermediary in the transfer of electrons. The metals are found in the prosthetic groups of enzymes that participate in processes such as cellular respiration, ion transport and metabolism, and neutrotransmitter synthesis, etc. However, metals may cause the increase of the oxidative damage to lipids, proteins, and DNA and contribute to neurodegenerative disorders at high metal concentration, i.e copper (Gaetke, et al., 2003). It is generally accepted that copper toxicity is a consequence of the generation

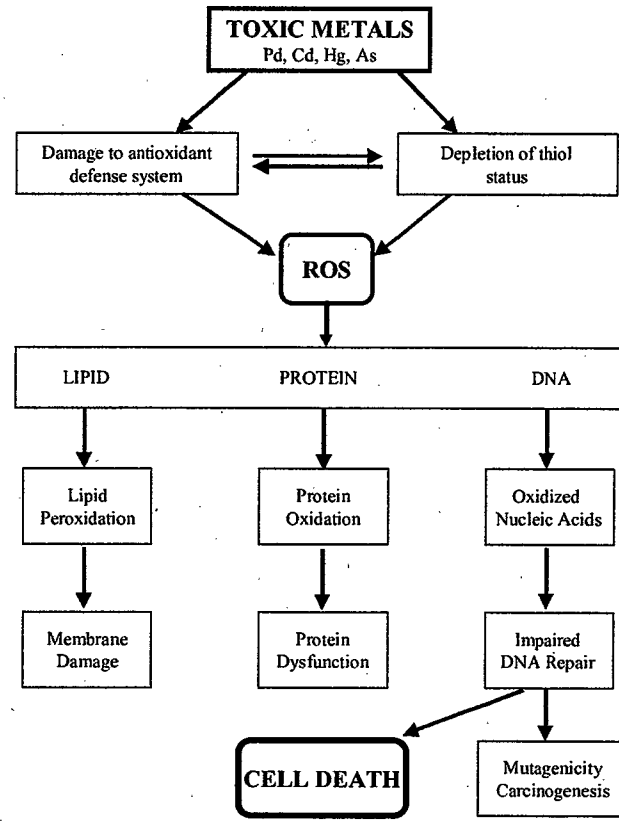


Figure 1 Possible mechanisms for metal-induced oxidative stress (Ercal et al., 2001)

of ROS by copper ions via Fenton or Haber–Weiss reactions (Lloyed et al., 1997). Copper ions display high affinity for thiol and amino groups occurring in proteins. Thus, specialized proteins containing clusters of these groups transport and store copper ions, hampering their potential toxicity (Bull et al., 1994). This mechanism, however, may be overwhelmed under copper overloading conditions, in which copper ions may bind to thiol groups occurring in proteins non-related to copper metabolism (Fernandes et al., 1991). Indiscriminate binding of copper may lead to damaging consequences to protein structure, modifying their biological functions. Cu (II) at toxic concentrations is known to bind to free thiols (e.g., glutathione) and other functional groups (e.g., -SH) of enzymes and may also replace metals that are constituents and the active centers of enzymes, cofactors, or other biomolecules in order to prevent the ROS formation and, sometimes, to utilize them as an active center formation (Sani et al., 2001)

How is the response of biological cells against the oxidative stress? Oxidative damage to cellular membranes plays an important role in the pathobiology of both chronic and acute tissue injury (McCord, 1985). For example, the unsaturated fatty acids present in the membrane (phospholipids, sterols, glycolipids, and glycerides), and in transmembrane proteins containing oxidizable amino acids, are particularly susceptible to free radical damage (Downey, 1990). Increased membrane permeability caused by lipid peroxidation and oxidation of structurally important proteins can disrupt transmembrane ion gradients and cellular metabolic processes. Lipid peroxidation of cellular membranes has also been implicated in various disease

processes, including cardiac ischemia-reperfusion injury (Meeson et al., 1982). From the viewpoint of the molecular biologist, such a harmful oxidative stress could be well modulated by the expression of the specific gene, such as *sod* and *cat*, which can be translated to superoxide dismutase (SOD) that can convert O_2^- to H_2O_2 and catalase (CAT) that can decompose the H_2O_2 (Deisseroth and Dounce, 1970). It is considered that the modulation of gene expression of both SOD and CAT within a cell could be a definite response of biological cells (Karaman et al., 2008). However, there are some exceptional examples, in which one cannot explain the mechanism of the cellular response by using the antioxidative genes: In the sod-deficient *B. subtilis* cells, the antioxidative protection was observed depending on the concentration of the additional Mn (II) ions, implying that the antioxidative functions could be elucidated by the metal ions and cellular components (Inaoka et al., 1999). The cellular response is thus not as simple as we expected from the genome information and the other components of the cell should be considered to understand its response. .

A biomembrane can sensitively experience oxidative stress condition. In a biological cell, each membrane type contains a specific set of protein-receptor and enzymes but the base of every membrane is a biomolecular layer of lipids that performs in each membrane two functions: (1) a barrier for ions and molecules, and (2) structural base for functioning of receptors and enzymes. In all living cells, biological membrane functions as a barrier that divides the cell from the environment and the internal cell volume into the comparably isolated "compartments". Lipid bilayer includes numerous built-in protein molecules and molecular complexes. Those

possess the properties of selective "channels" for ions and molecules. Others are "pumps" transferring actively ions through membrane (Schwartz, 1971). On the membrane there are some proteins and protein groups that are disposed in a definite order and create systems of electron transfer (Gray and Winkler, 1996), energy accumulation in the form of ATP (Proverbio and Hoffman, 1977), regulation of intracellular processes by hormones coming in from outside and intracellular mediations (Kurtz, 1986), recognizing of other cells and foreign proteins, light reception, mechanical effects, etc (Bretscher and Raff, 1975). However, under oxidative stress, such a mechanism will break down. Then, the lipid peroxidation induces membrane disturbance and damage and its products are known to induce the generation of various cytokines and cell signaling. Cholesteryl esters (CEs) and phosphatidylcholines (PCs) were more readily oxidized by free radical in plasma than in organic solution under similar conditions (Yoshida et al., 2003). The studies with *S. cerevisiae* have demonstrated that susceptibility to heavy metal-induced plasma membrane permeabilization and toxicity is markedly dependent on plasma membrane fatty acid composition. Both cadmium-induced and copper-induced plasma membrane disruptions were markedly accelerated in *S. cerevisiae* enriched with polyunsaturated fatty acids (Howlett et al., 1997). The role of LOOH as reductants for Cu (II) and to play a concomitant role in establishing the redox cycle between Cu (II) and Cu (I) in the promotion of lipid peroxidation. A role for LOOH-dependent reduction of Cu (II) to Cu (I) can only be demonstrated if the metal exhibits catalytic behavior in the lipid-peroxidation system and in the absence of alternative reductants such as amino

acids or α-tocopherol (Patel et al., 1997).

The roles of "phospholipid membrane" in biological systems have been studied and lipid bilayers are the model for many experiments about the interaction of protein and liposome membrane. The phospholipids bilayer as a solvent influencing the structure and function of membrane protein is well established. Lipids can act as molecular chaperones in the protein folding. Molecular chaperones facilitate the folding of proteins by interacting non-covalently with nonnative folding intermediates and not with either the native or totally unfold protein (Dowhan et al., 2004). The interaction of lipids and partially folded lactose permease during refolding was found to be dependent on both the chemical properties of the individual lipid molecules and the collective properties of phospholipids mixture (Bogdanov et al., 1999). The liposome, which is composed of unsaturated phospholipids, has previously been used as a model biological membrane. The liposome has a chaperon-like function to assist the refolding of protein, in which, denature lysozyme interacted with the liposome membrane to be refolded and returned to the native form (Kuboi et al., 1997; Yoshimoto and Kuboi, 1999). Based on the above results, lipids can function as molecular chaperones that specifically mediate the folding of proteins, thereby extending the definition of chaperones to other biomolecules in addition to protein (Bogdanov and Dowhan, 1999).

The interaction of membranes and peptides also results in the translocation of peptides into membranes. The translocation of proteins across the membrane is one of the importantly biological functions (Hardy and Randall, 1989; Arkowitz and

Bassilana, 1994). In translocation process, the peptide forms an amphipathic helix in lipid bilayers, which essentially lies parallel to the membrane surface (Bechinger et al., 1993; Matsuzaki et al., 1994), imposing a positive curvature strain on the membrane (Wieprecht et al., 1997; Matsuzaki et al., 1998). Several helices together with several surrounding lipids then form a membrane-spanning pore comprising a dynamic, peptide-lipid supramolecular complex (toroidal pore), which allows not only ion transport but also rapid flip-flop of the membrane lipids (Matsuzaki et al., 1996; Ludtke et al., 1996). Upon the disintegration of the pore, a fraction of the peptide molecules stochastically translocate into the inner leaflet (Matsuzaki et al, 1995). While assisting with the liposomes, the characteristics of proteins, especially enzymes become stable. A clear example is that catalase was encapsulated in the liposome to improve the stability of its tetrameric structure and activity (Yoshimoto et al., 2007). The studies on the effect of liposome-encapsulated superoxide dismutase in cytokine production have been conducted. The liposome-encapsulated superoxide dismutase induced the augmentation of tumor necrosis factor from peripheral blood leucocytes in the cancer treatment (Shibuya-Fujiwara, et al., 2001). The above obtained results proved that membrane could stabilize and regulate the enzymatic activities in biological systems. Additionally, the liposome-encapsulated drug can be applied in fields such as medicine and pharmacology.

In the protection of membrane from peroxidation by ROS, liposome membrane can assist with the proteins or peptides. The experiments have previously been conducted to evaluate the oxidation of lipid bilayer by copper. The obtained

results indicated that the unsaturated lipid bilayer was easy to be oxidized by copper. However the addition of Aβ slightly decreased the amount of ROS generation and the lipid peroxidation. Aβ inhibits the ROS generation by interacting with the immobilized Cu (II) to protect the liposome from oxidation (Nagami, PhD thesis, 2005). The Aβ was found to form the metalloenzyme-like coordination structure on the liposome surface. The effect of liposome on the metalloenzyme-like function Aβ-Cu complex has been successfully investigated. The activity of this complex was depending on the physical properties of liposome membrane because the interaction of the Aβ-Cu complex with membrane (Yoshimoto et al., 2005). From the gained results, the interaction of membrane and proteins or peptides on the membrane surface prevented both membrane and proteins from the further oxidation by ROS.

SOD Functions as an "Antioxidative Modulator". Our study on the role of membrane in protection and recruitment of the oxidatively-damaged enzyme is an attempting investigation of the "potential" function of membrane under stress condition. Actually, under normal conditions, ROS are cleared from the cell by the action of superoxide dismutase (SOD) (McCord, J. M; Fridovich, I., 1969), catalase (Bunkler, 1992; DeJong et al., 2007), or glutathione peroxidase (Hayes, et al., 1999). Thus, a close relationship between ROS production, antioxidant defense impairment, perioxidative membrane damage and inflammatory or degenerative pathological processes could be assumed (Pamplona et al., 2002; Bengtsson, 2001) as shown in **Figure 2**. Superoxide dismutase which is an intracellular enzyme vital to every cell in the body is a metalloenzyme containing copper and zinc, and sometimes manganese or

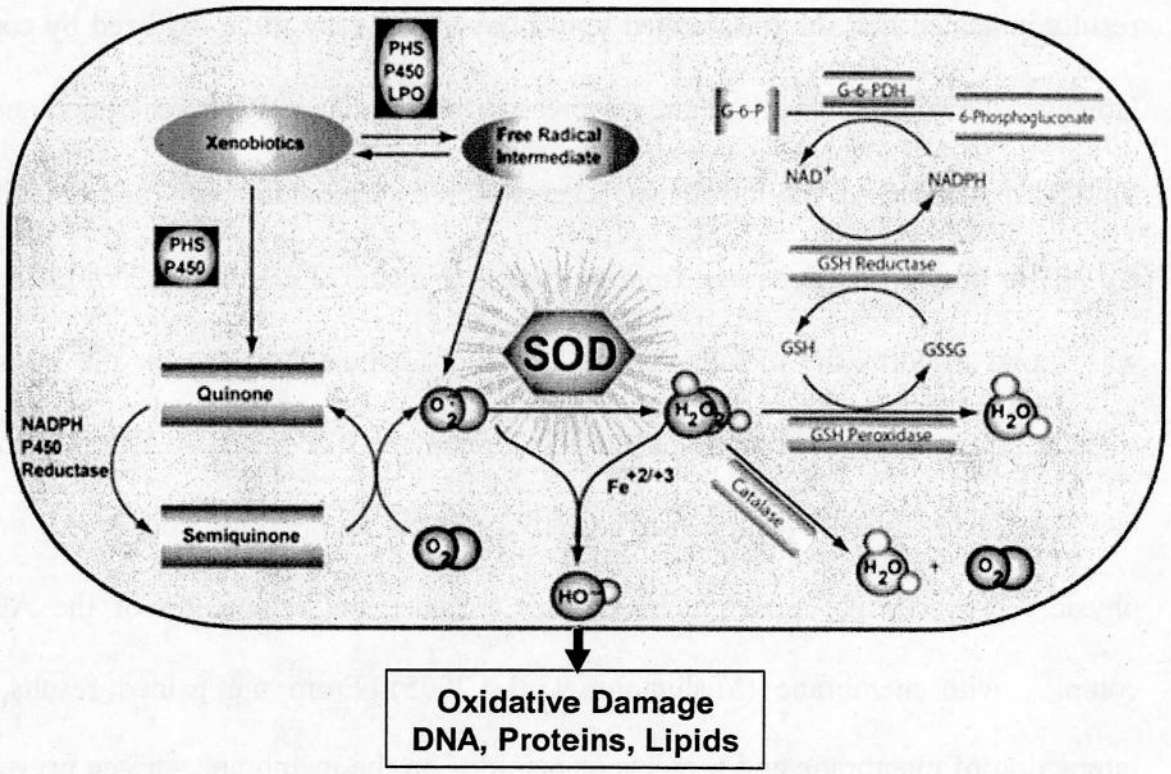


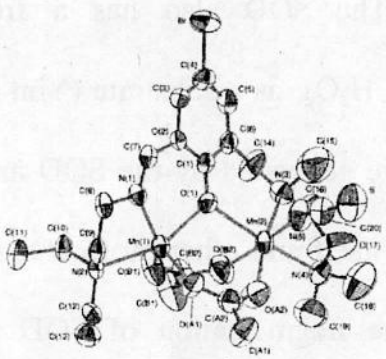
Figure 2 Conventional understanding on mechanisms in cell detoxification of ROS by antioxidants (Pamplona et al., 2002; Bengtsson, 2001)

iron, at the active site. SOD plays an important role in the protection of all aerobic biological systems against oxygen toxicity and the free radicals derived from oxygen, catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (McCord and Fridovich, 1969). In *in vitro* experiments, it has previously been reported that the enzyme is inhibited by one of the reaction products, hydrogen peroxide, in the absence of catalase (Hodgson and Fridovich, 1975). Some researchers have attempted to relate the H_2O_2-induced loss of enzymatic activity of SOD (Salo et al., 1990; Sankarapandi and Zweier, 1999) with the structural changes in the enzyme active site (Cu^{2+} binding) (Ramirez et al., 2005), including increased proteolytic susceptibility (Davies, 1987; Blech and Borders 1983; Choi et al., 2000), fragmentation (Sato et al., 1992), and

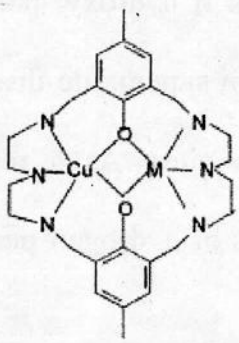
oxidation of the active site (Bray et al., 1974). The SOD also has a free radical-generating function that utilizes its own product, H_2O_2, as a substrate (Yim et al., 1993; Kim and Kang, 1997). If hydroxyl radicals are generated by the SOD and H_2O_2 system, they can react with superoxide dismutase itself and other molecules in the vicinity of their generation sites. As a result, the fragmentation of SOD is elucidated at high concentrations of hydrogen peroxide (Nagami et al., 2005; Choi et al., 1999; Kwon et al 1998).

SOD activity was modulated on membrane surface. In a biological system, the SOD seems to be moderately controlled on the biomembrane. For example, the extracellular SOD is known to be anchored to biomembranes by binding with heparin in the membranes, although the amount of extracellular SOD expressed is significantly lower as compared with that of intracellular SOD (Enghild et al, 1999). The extracellular SOD may thus have a strong resistance to oxidation and may maintain the suitably oxidative conditions outside the cell. The role of biological membrane during SOD activity modulation should be clarified, especially under oxidative stress conditions. In an *in vitro* approach, Nagami *et al.* have recently reported that SOD retains 80% of its initial activity in the presence of liposomes after incubation with hydrogen peroxide for 24 hours (Nagami et al., 2005). Under relatively mild stress conditions (less than 2 mM hydrogen peroxide), the liposomes were shown not only to prevent oxidation of SOD but also to maintain its activity (Nagami et al., 2005).

How one could mimic the enzyme function by using the ligands? There are several approaches on the design and development of the enzyme mimics (**Figure 3**) (Kirby et

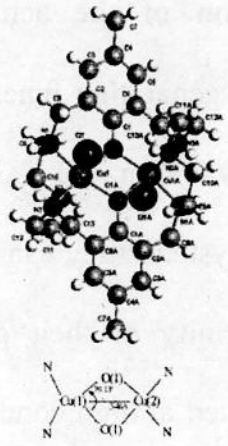


Chikako et al., J. Chem. Soc.
Dalton Trans. 1994, 7, 1097 – 1103.

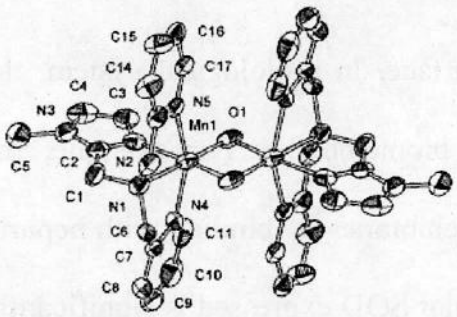


M = Zn(II) and Mn(II)

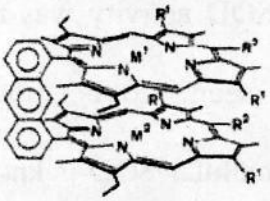
Gao et al., Inorg. Chimi. Acta
2001, 325, 164–170.



Gao et al., Inorg. Chimi. Acta
2003, 346, 32-42.



Triller et al., Inorg. Chem. 2002,
41, 5544-5554.



R^1=n-C_6H_{13}, R^2=p-C_6H_4,
M^1=H_2, M^2=$Mn^{3+}Cl$

Naruta et al., J. Am. Chem. Soc.
1991, 113(9) 3595 – 3596.

Figure 3 Examples of previous enzyme mimics

al., 1996; Batinic-Harber et al., 1998). The metal-ligand complex, modified from the suitable chemical structures of the ligand and metals, catalyze the enzymatic activity (Guner et al., 1999; Naruta et al., 1991 and 1997; Gerasimchuk et al., 1999). The SOD-like active site has been reported to be induced by using the specific peptide and metal ions (copper and zinc), in which the formation of macrochelate via the coordination of the imidazole residue was suggested. The complex of specific peptide and metal ions displayed the SOD-like activity (Boka et al., 2004). A model biomembrane (liposome), with the specific properties, has been used as a platform to create the catalytic site (Nagami et al., 2004; Walde, 2006; Murakami et al., 1996). The

metal-ligand complex on the liposome surface possessed enzyme-like activity, such as SOD (Nagami et al., 2004 and 2005; Umakoshi et al., 2008) and cholesterol oxidase (Yoshimoto et al., 2005). The artificial enzyme was considered to be stable under the strong stress condition, resulting in the display of its enzyme-like activity with the high efficiency. Interestingly, the complex could be regulated by the membrane properties of the liposome, as well as affording functional elements on the liposome surface. The above enzyme-like function of liposome, which can be defined as "LIPOzyme" (Kuboi et al., 2006), can be utilized for the design of the artificial enzymes.

In this thesis, the potential functions of phospholipid bilayer membrane (liposome) were systematically studied, focusing on the membrane recruited activity on its surface via the use of the minimal elements i.e. liposomes, metal ions, and the fragments of SOD.

In chapter I, a SOD LIPOzyme was prepared by utilizing the membrane roles in recognition and reactivation of oxidatively-damaged enzyme in order to clarify the membrane function in biological systems. Under oxidative stress, SOD was observed to be fragmented, leading to enzymatic inactivation and the loss of an α-helix neighboring its activity center. The H_2O_2-treated SOD, which lost their activity at different incubation times, was significantly reactivated only by adding POPC (1-palmitoyl-2-oleoyl-*sn*- glycero-3-phosphocholine) liposomes, resulting in the increase of enzymatic activity. The fragmentation of SOD produced some specific peptide fragments that can interact with POPC liposomes. Liposome membrane was also found to assist the conformational change of oxidized and fragmented superoxide

dismutase (Fr.-SOD) and accelerate the adsorption of metal ions on its fragment to give the original SOD-like activity. 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 induction of the high SOD-like activity. In this case, the POPC liposomes could act like molecular and metal chaperones for stress-damaged peptides, resulting in the creation of a new SOD-like active center that continues to express the original enzymatic activity under the oxidative conditions.

In chapter II, a mechanism of the LIPOzyme displaying mechanism was investigated by studying the effect of lipid composition of liposome and by analyzing the possible elemental steps of the liposome-recruited activity. The specific peptide, originated from SOD, was first analyzed, resulting that the peptide sequence of the peptide was identified. By using the peptide, the liposome-recruited activity of the oxidized SOD fragment was found to be displayed on the liposome surface through the following three steps: (1) binding (recognition), (2) refolding, and (3) reactivation (See below). Such interaction was considered to be related with the characteristics of the both fragments and liposomes caused by the combination of electrostatic and hydrophobic interaction, and hydrogen bonding between the peptide and liposome membrane. A strategy to elucidate the liposome-induced antioxidative activity by recruiting the minimal elements has finally been established, focusing on "state of stressed membrane" (the membrane activity and toxicity), based on the above findings.

In chapter III, the antioxidative LIPOzyme displaying both SOD and CAT was attempted to be prepared as a case study of the strategy described in chapter 2.

Surprisingly, the CAT-like activity, which decomposes H_2O_2, was induced on the liposome surface which displays the SOD-fragments and metal ions (Cu and Zn). The SOD-like activity and CAT-like activity were found to be modulated, depending on the stressed state of the membrane. Moreover, after fragment was actively and structurally recovered by liposome membrane under continuously oxidative stress of hydrogen peroxide, membrane can regulate the enzyme-like activities. As a result, the membrane-recruited SOD possessed not only SOD-like enzymatic activity but also CAT-like activity, resulting in the decomposition of all the ROS in the biological system (proposal in **Figure 4**). The expression of bifunctional enzyme-like activity of membrane-recruited SOD (LIPOzyme) demonstrated that the role of membrane in regulation of recruited enzyme on the liposome surface. Unexpected response of living cell (algal cell) such as survival under the strong oxidative stress could be related to such membrane-recruited antioxidative activities of the enzymes.

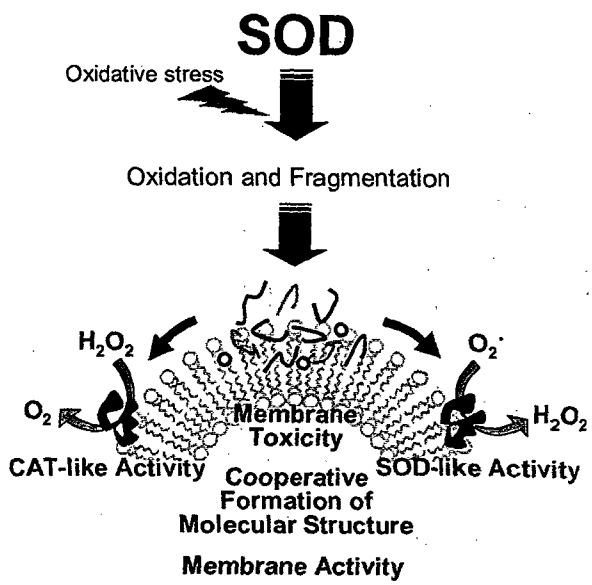


Figure 4 A schematic Illustration of Bifunctional Activity of SOD-recruited Liposome Complex.

The obtained results demonstrated the potential roles of membrane in recognition, recruitment, modification, and regulation of active sites of enzyme from damaged peptides. The present results are prospectively applicable in modulation of artificial

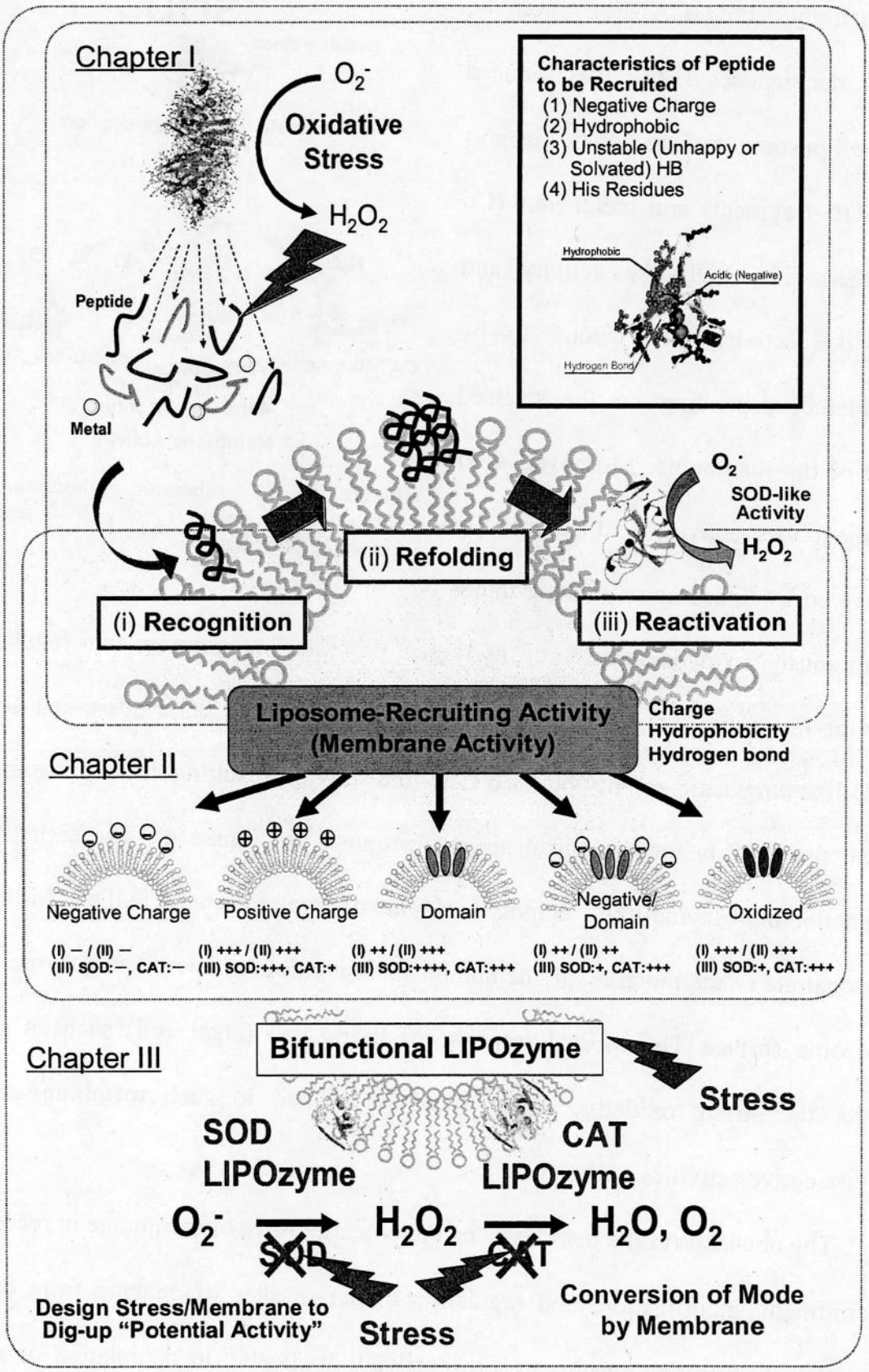


Figure 5 Framework of the present study

Chapter I

Preparation of SOD LIPOzyme with Build-Up and Beak-Down Approaches: Recruitment of Synthetic Ligand or Oxidized/Fragmented Superoxide Dismutase on Liposome Membrane

- SOD LIPOzyme from "Build-up" Approach: Dodec-His Modified Liposome with Cu/Zn
- Characterization of Properties of Ligand-Modified Liposome
- Metal Adsorption Behaviors on Ligand-Modified Liposome
- SOD-like Activity of Metal/Ligand-Modified Liposome
- SOD LIPOzyme from Break-Down Approach: Liposome-Recruited Activity of SOD under Oxidative Stress Condition.
- Effect of Hydrogen Peroxide on the Enzymatic Activity and Oxidation of Superoxide Dismutase: Role of Liposome in Protection of SOD from the Oxidation
- Binding of Specific Peptide Containing Active Site on Membrane Surface.
- Conformational Change of Native SOD under Oxidative Stress and Fragmented SOD in the Presence of Liposomes
- Reactivation of Fragmented SOD and Modification of Active Site on Liposome Surface.

Chapter II

Characterization of SOD LIPOzyme Prepared with Break-Down Approach

- Reactivation and Adsorption of Oxidized and Fragmented SOD Recruited on Various Liposomes
- Characterization of Oxidized and Fragmented Peptide Recruited on Liposome Surface
- Liposome Can Recognize the Specific Fragments of Fragmented SOD through Integrated Interaction Theory.
- Membrane Toxicity and Its Functions under Stress Condition.
- Analysis of Elemental Process of SOD LIPOzyme Activity.
- Comparison of SOD LIPOzyme with Previous SOD Mimics

Chapter III

Preparation of Antioxidative LIPOzyme based on Modification of Peptide Fragment under Stress Condition

- Active Center for Cu,Zn-SOD and Mn-CAT.
- The CAT-like Activity of the Liposome-Recruiting SOD Fragment.
- Stabilization of SOD/CAT LIPOzyme under Continuously-Exposed Oxidative Stress.
- Conformational Change of Fragmented SOD on Liposome under Stress Condition.
- Summary and Comparison of SOD and CAT Activities

Figure 6 Flow chart of present study

enzyme to control the oxidative agents and in the deeper understanding of the cellular response against oxidative stress.

The framework and flow chart of the present study is schematically shown in **Figures 5** and **6**. The obtained results in this work are summarized in the General Conclusion section. Suggestions for Future Work are described as an extension of the present thesis.

Chapter I

Preparation of SOD LIPOzyme with Build-Up and Beak-Down Approaches: Recruitment of Synthetic Ligand or Oxidized/Fragmented Superoxide Dismutase on Liposome Membrane

1. Introduction

Superoxide dismutase, SOD, which is an intracellular enzyme vital to every cell in the body, is a metalloenzyme containing copper and zinc, and sometimes manganese or iron, at the active site. SOD plays an important role in the protection of all aerobic biological systems against oxygen toxicity and the free radicals derived from oxygen, catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide (McCord and Fridovich, 1969).

The overexpression of Cu,Zn-SOD *in vivo* has been reported to be related to the negative responses of cells such as cell death (Amstad et al., 1994), the enhancement of lipid peroxidation (Elroy-Stein et al., 1986), reduced transport of biogenic amines (Epstein et al., 2001), and decomposition of brain neurons (Ceballos-Picot, et al., 1991). In *in vitro* experiments, it has previously been reported that the enzyme is inhibited by one of the reaction products, hydrogen peroxide, in the absence of catalase (Hodgson and Fridovich, 1975). Some researchers have attempted to relate the H_2O_2-induced loss of enzymatic activity of SOD (Salo et al., 1990;

Sankarapandi and Zweier, 1999) with the structural changes in the enzyme active site (Cu^{2+} binding) (Ramirez et al., 2005), including increased proteolytic susceptibility (Davies, 1987; Blech and Borders, 1983; Choi et al., 2000), fragmentation (Sato et al., 1992), and oxidation of the active site (Bray et al., 1974). The Cu,Zn-SOD also has a free radical-generating function that utilizes its own product, H_2O_2, as a substrate (Yim et al., 1993; Kim and Kang, 1997). If hydroxyl radicals are generated by the Cu,Zn-SOD and H_2O_2 system, they can react with superoxide dismutase itself and other molecules in the vicinity of their generation sites (Goldstone et al., 2006). As a result, the fragmentation of SOD is induced at high concentrations of hydrogen peroxide (Nagami et al., 2005; Choi et al., 1999; Kwon et al., 1998). The fragments produced by H_2O_2 include the active site (~5 and ~10 kDa) and the non-active site (smearing between 3 and 16 kDa) (Ramirez et al., 2005).

In a biological system, the SOD seems to be moderately controlled on the surface of biomembrane. For example, the extracellular SOD is known to be anchored to biomembranes by binding with heparin in the membranes, although the amount of extracellular SOD expressed is significantly lower as compared with that of intracellular SOD (Enghild et al., 1999). The extracellular SOD may thus have a strong resistance to oxidation and may maintain suitable oxidative conditions outside the cell.

The role of biological membrane during the modulation of SOD activity should be clarified, especially under oxidative stress conditions. In an *in vitro* approach, Nagami *et al.* have recently reported that Cu,Zn-SOD retains 80% of its initial activity in the presence of liposomes after incubation with hydrogen peroxide for 24 hours (Nagami et al, 2005). Under relatively mild stress conditions (less than 2 mM hydrogen peroxide), the liposomes were shown not only to prevent oxidation of SOD but also to maintain its activity (Nagami et al., 2005).

It has recently reported that the liposome membrane can recruit the oxidized SOD fragment on its surface to produce a complex that has enzyme activity (Tuan et al., 2008). Among the fragments generated after SOD oxidation (Ookawara et al., 1992; Kurahashi et al., 2001), there are some specific peptides contain its active site (~5 and ~10 kDa) (Ramirez et al., 2005), although these fragments have no SOD-like activity. The recruiting mechanism of fragmented SOD on membrane was considered to involve the refolding and conformation of peptides by hydrophobic interaction, in which liposomes perform a molecular chaperone-like function (Yoshimoto et al., 1999 and 2000; Kuboi et al., 2000). One of the specific peptides purified from reverse-phase HPLC was recovered on membrane and expressed SOD-like activity (Tuan et al., 2008). However, the structural conformation of SOD fragments derived from

oxidation has not been adequately clarified yet. It is therefore important and essential 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, as well as the enzyme activity with the support of the liposome membrane under strong oxidative stress.

Artificial enzymes that mimic the above antioxidative enzymes and show high stability need to be designed to efficiently eliminate the ROS, such as superoxide and hydrogen peroxide. Much effort has been devoted to the design and development of artificial antioxidative enzymes (Kirby, 1996). Mn-porphyrin (Mn-PyP) complex is known to be an effective material for the design of artificial antioxidative enzymes that possess either SOD activity (Batinic-Harber et al., 1998). However, it has been reported that Mn-PyP catalysis demonstrates relatively low activity in both SOD in comparison with natural enzymes, although several efforts have been made to control their activities through the modulation of the metal ion distance in a ligand molecule by its suitably designed chemical structures (Naruta et al., 1997, Gerasimchuk et al., 1999). Mn-PyP in the monomer state has often been utilized for the design of artificial SOD, as described in a series of previous reports (Batinic-Harber, 1997, Ohtsu et al., 2000, Michiels et al., 1994). Although there are other types of artificial SODs or SOD

mimics (Nagami et al., 2004 and 2005; Umakoshi et al., 2008), the strategy of the design of the SOD mimics is mainly retained to "reproduce an active center of enzyme precisely" by using the well-designed ligand.

A model biomembrane, liposome, is a possible candidate to provide a sophisticated platform to reproduce the catalytic centers of enzymes (i.e. SOD). Such a liposome herewith possesses several benefits in the regulation of catalytic activity, where it can provide (i) a nano hydrophobic environment, (ii) a stress-responsible character, (iii) a microdomain structure, and (iv) membrane-membrane interactions. Some researchers have reported the effectiveness of the use of a model biomembrane (liposome) as a platform to immobilize the functional catalytic center (Nagami et al., 2004, Walde, 2006, Murakami, 1996). Enzyme-like activity, such as that of SOD (Nagami et al., 2004, Nagami et al., 2005, Tuan et al., 2008) and cholesterol oxidase (Yoshimoto et al., 2005) has already been regulated by the membrane properties of the liposome, as well as affording functional elements on the liposome surface. The above enzyme-like function of liposome, which can herewith be denoted as "LIPOzyme" (Kuboi et al., 2006) can be utilized for the design of the artificial enzymes through the "Break-Down Approach" and from the "Build-Up Approach" (**Figure 1-1**).

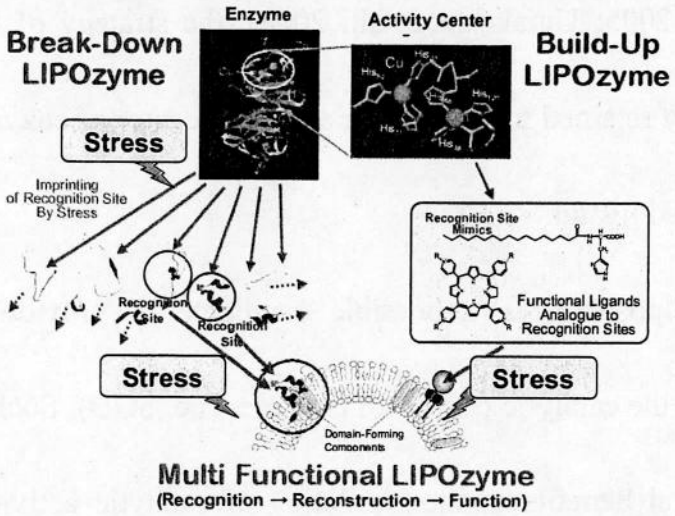


Figure 1-1 Schematic Illustration of LIPOzyme

In this chapter, the LIPOzyme has been prepared by using two kinds of approaches (**Figure 1-1**), focusing on the preparation of SOD LIPOzyme. First: "Build-up" type approach has been employed. The SOD LIPOzyme was first prepared by using the acylated amino acid, which is compatible with the amino acids constructing the active center of SOD. After the characterization of membrane properties of ligand-modified liposome, the SOD-like activity was investigated. Second: "Break-down" type approach has been employed. The recruitment of peptide fragments of oxidized SOD (without enzymatic activity) on membrane surface and the reactivation of inactive enzyme by the addition of POPC liposomes was investigated, together with the reconstruction of the secondary structure the enhanced binding of metal ions. Based on these results, the effectiveness of the LIPOzyme has been shown as a summary of this chapter.

2. Materials and Methods

2.1. Materials.

Bovine Erythrocyte Cu,Zn-SOD (EC 1.15.1.1), purchased from Sigma Aldrich with a specific activity of 4470 U/mg (product No. S2515-30KU and lot No. 125K740), was used without purification. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC: T_m = 42°C), 1,2-dimyristoyl-*sn*-glycero- 3- phosphocholine (DMPC: T_m = 23°C), and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC: T_m = 0°C), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. (AL, USA) and was used for the liposome preparation described somewhere (Bangham et al., 1974). Dodecanoyl-Histidine (Dodec-His) and Pyrene-dodecanoyl-histidine (Py-Dodec-His) was synthesized and prepared according to the modified method described in the previous literature (Yasuhara et al. 2006). All the other reagents of analytical grade were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2 Preparations of Dodec-His modified Liposome.

The liposomes modified with Dodec-His were prepared by using the following procedure according to the previous work (Nagami et al., 2005; Yashuhara et al., 2006; Umakoshi et al., 2008). Phospholipid and Dodec-His (with a molar ratio 10:1) were dissolved in chloroform/methanol (lipid concentration: 2 mM). After the solvent

was evaporated, the resulting thin film was dried for at least 2 hours under a vacuum. The lipid film was hydrated by 50 mM potassium phosphate buffer to form the multilamellar vesicles. The solution of the multilamellar vesicle was treated by ultrasonication (15 min, 80 W, 5 ml) on ice (4 °C) to obtain the liposome with the size of 30-nm.

2.3 Characterization of Membrane Fluidity.

The membrane fluidity of liposomes was determined by using a hydrophobic fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Lentz, 1993). DPH was added to the solution of liposome-copolymers or liposome-proteins at a final concentration of 1 μM. The fluorescence polarization of the DPH probe in the liposome membrane was measured at a wavelength of 360 nm for the excitation and 430 nm for the emission. The fluorescence intensity of the DPH was measured by using the fluorescent spectrophotometer (JASCO FP-777, Japan). The degree of fluorescence polarization (P) was calculated using the following equation:

$$\frac{1}{P} = \frac{(I_{//} + I_{\perp})}{(I_{//} - I_{\perp})}$$

where $I_{//}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel (//) and perpendicular (⊥) to that of the exciting beam, respectively. The term "fluidity" is inversely proportional to the degree of fluorescence polarization of the probe; that is, the 'membrane fluidity' of the interior of the membrane was defined by (1/P) of DPH.

2.4 Characterization of Ligands Cluster on the Liposome Surface.

It has been reported that the pyrene molecules with different clustering state (monomer and dimer) shows the different fluorescence spectra. The clustering state of the Dodec-His molecules on the liposome membrane was evaluated by using the Pyrence-conjugated acylated-Histidine (Py-Dodec-His). The liposome was modified with Py-Dodec-His in replace of Dodec-His at molar ratio of 10%. The fluorescence spectra of the Py-Dodec-His modified liposome were measured by using the fluorescent spectrophotometer (JASCO FP-777, Japan) at the excitation wavelength of 350 nm. The spectra of fluorescence emission were recorded. The relative clustered state of the ligands was assessed as a ratio of the fluorescence at excimer and that at monomer (E/M ratio) (Nagami et al., 2005).

2.5 Metal Adsorption on Dodec-His Modified Liposome.

The metal adsorption experiment was carried out by shaking the liposome suspension in the presence of metal ion at 25 °C for 24 h. After the above operation, the liposome and metal ion were separated by ultralfiltration (Ultra free MC; Millipore) at the specific centrifugal condition (5000 $\times g$ for 10 min). The concentration of metal ion in the filtered solution was measured by using fluorescent indicators for metal ion, such as Phen Green or Bio.

2.6. Analysis of Cu,Zn-SOD fragmentation by H_2O_2.

Cu,Zn-SOD (2μM) was incubated with H_2O_2 (2mM) in phosphate buffer (pH 7.4) at 37 °C for various periods. The enzymatic activity and protein concentration of

fragmented SOD were determined after the incubation of SOD with H_2O_2. SDS-PAGE technique was used for analyzing SOD fragmentation.

For the 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 (Peskin et al., 2000). It was confirmed that the solution of Cu/Zn and/or POPC liposomes showed no SOD-like enzymatic activity as the control experiments. 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 100 μM 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.

The BCA Protein Assay Kit was used to determine protein concentrations. The protein was precipitated in a cold acetone solution to separate it from contaminants so

that a more accurate estimation of protein content in the sample could be obtained. The mixture was then centrifuged at 15,000 rpm for 20 min. Pellets solubilized in 50 µl H_2O were added to 1000 µl of BCA reagent solution and incubated for 30 min at 37°C, after which the absorbance at 562 nm was measured. A standard curve was set up to analyze the protein concentrations (Smith et al., 1985).

For SDS-PAGE analysis, aliquots of the sample were solubilized with the denaturing buffer (0.25 mM Tris-HCl, 8% SDS, 40% glycerol, 20% L-mercaptoethanol, 0.01% bromophenol blue) and were boiled at 100 °C for 10 min before electrophoresis. An aliquot of each sample was subjected to SDS-PAGE as described by Laemmli (Laemmli, 1970) using a slab gel (PhastGel High Density, acrylamide < 0.2%). The gels were stained with 0.2% Coomassie brilliant blue R-250. The amount of protein was quantitatively characterized by the staining intensity using the method described by Shaw (Shaw et al., 2003) and Scion Image software on the website http://www.scioncorp.com/.

2.7. Ultrafiltration of H_2O_2-Treated SOD

Ultrafiltration using a Millipore Ultrafree-MC filter with a molecular mass cut-off of 10 kDa or 50 kDa was applied for fragmentation analysis. SOD after being treated by H_2O_2 was applied to the ultrafiltration tube and centrifuged at 15,000 rpm for 30 min at room temperature. The retentate portion had fragments larger than 50

kDa in MW, which also bound liposomes (vesicles 100 nm in diameter), and the permeate portion had fragments smaller than 10 or 50 kDa in MW (called as free fragment or free SOD). Both were then analyzed to determine SOD activity and by reverse-phase HPLC. The filter with a molecular mass cutoff of 3 kDa was applied to remove free copper released from active site.

As a control experiment, it was confirmed that all the activity of native SOD (32 kDa) was detected in the "permeate" solution across the ultrafiltration membrane, and neither measurable protein concentrations nor the activity were detected in the "retentate" solution. As a negative control experiment, the oxidized SOD solution was also treated by ultrafiltration, resulting in both the SOD activity and measurable protein concentrations being detected only in the "permeate" solution. These results of the control experiments indicated that the oxidized SOD did not form large aggregates at more than 50 kDa. The mass balance of protein concentration was checked to confirm both SOD and fragmented SOD was not adsorbed on the ultrafiltration membrane.

For the reverse-phase HPLC, a Shimadzu HPLC system equipped with an FCV-10AL pump and a DGU-20A_3 degasser, and both an SPD-10A UV-VS detector and an LC-10AD liquid chromatograph were used. Elution profiles were monitored at

220 nm on the UV detector. The mobile phase of acetonitrile and water (v/v: 7/3) with a flow rate of 1 ml/min was applied at 25^{0}C. A STR ODS-M column (0.46 x 15cm), in which the particle surface was octadecylated, was used throughout this study.

2.8. Circular Dichroism Analysis.

The secondary structure of SOD was determined using circular dichroism analysis (J-720W spectrometer; JASCO, Tokyo, Japan) at 37 ^{0}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.

2.9. Statistical Analysis.

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

3. Results and Discussion

3.1 SOD LIPOzyme from "Build-up" Approach: Dodec-His Modified Liposome with Cu/Zn

It is important and needed to design and develop the liposome catalysis, which can be defined as LIPOzyme (Kuboi et al., 2006), demonstrating the activity of the SOD. As shown in **Figures 1-2(a)** and **(b)**, the active center of SOD is known to consist of the Cu and Zn coordinating with six His residues and one Asp residue. In this study, it has been investigated whether the molecular assembly of simple and minimal elements (His and Cu/Zn) on the liposome could induce the SOD-like activity (**Figure 1-2(c)**). After the liposome modified with dodecanoyl-His (Dodec-His, **Figure 1-2(d)**) was prepared, the basic characteristics of the

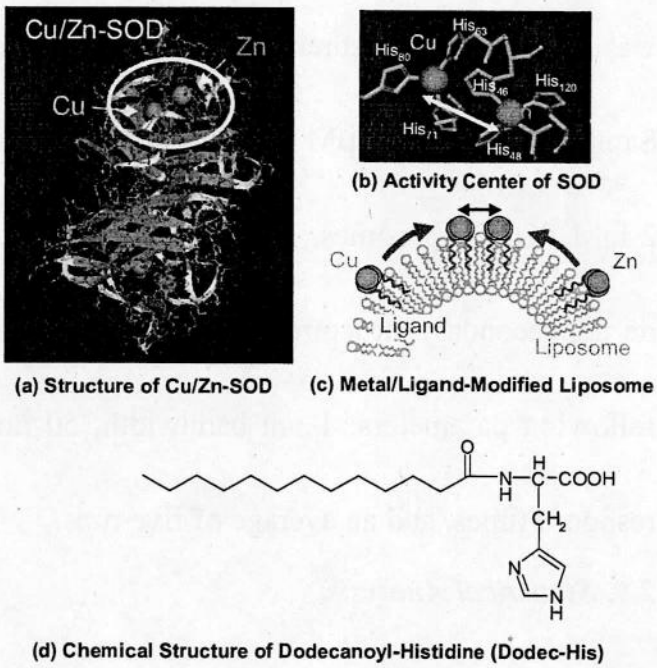


Figure 1-2 Schematic Illustration of SOD LIPOzyme Using Metal/Ligand-Modified Liposome. (a) and (b) show the structure of Cu/Zn-SOD and that of its active center (Data from Protein Data Bank (ID=1CBJ, http://www.rcsb.org/pdb/) were visualized by using ViewerLite (http://www.accerlrys.com). (c) The schematic illustration of metal/ligand modified liposome which could be utilized for the design of the SOD LIPOzyme. (d) The chemical structure of N-dodecanoyl-Histidine (Dodec-His) used as a ligand to be modified.

Dodec-His modified liposome and the adsorption behaviors of metal ions (Cu and Zn) on it were systematically investigated.

3.2 Characterization of Properties of Ligand-Modified Liposome

The clustered state of the ligands with hydrophobic tails has been reported to be dependent on the phases of liposome (Nagami et al., 2005, Umakoshi et al., 2008). The membrane properties of Dodec-His modified liposome, such as membrane fluidity and ligand clustering, were first characterized.

Figure 1-3(a) shows the temperature dependence of the membrane fluidity, analyzed by DPH, of different type of liposomes. In the case of DLPC liposome, the value of membrane fluidity was gradually increased with the increase of the temperature and was reached to the saturated value at more than 40 °C. A

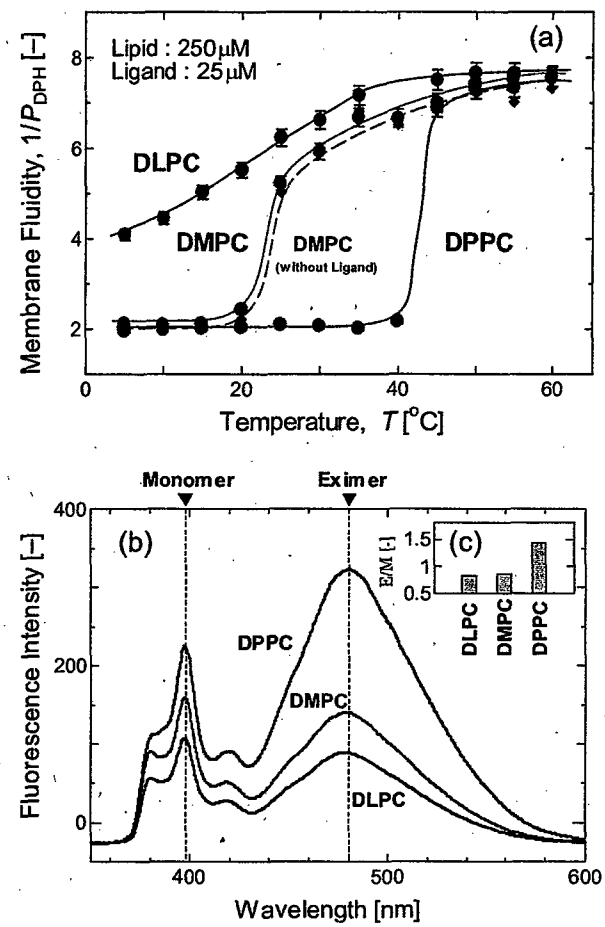


Figure 1-3 Characterization of Membrane Properties of Dodec-His Modified Liposome. (a) Temperature dependence of the membrane fluidity of the Dodec-His modified liposome prepared with different lipids (DLPC, DMPC, and DPPC). DPH was added at the molar ratio of 1:250 against lipid concentration and was used as a fluorescence probe of the membrane fluidity. (b) Fluorescence spectra of the Py-Dodec-His in different types of liposome at 25 °C. (c) The relative fluorescence of eximer and monomer (E/M ratio) in different liposomes (DLPC, DMPC, and DPPC).

distinct phase transition behavior was observed in the case of DMPC and DPPC liposomes, where the value sharply increased at the temperature of 22 °C and 43 °C, respectively. It has been reported that the phase transition temperatures of the DLPC, DMPC, and DPPC liposomes are 0 °C, 23 °C, and 45 °C, respectively (Umakoshi et al., 2008). The observed temperature for the phase transition of liposome from gel phase to liquid-crystalline phase was well corresponding with that of the previous report although a slight difference in the value of the membrane fluidity between the liposome and ligand modified-liposome was observed. It was thus found that the phase transition behaviors of the liposome was not significantly affected by the modification of the liposome with the 10mol% Dodec-His.

The clustered state of the Dodec-His molecules on the liposome membrane was further studied by using the Py-Dodec-His as its probe molecule. **Figure 1-3(b)** shows the fluorescence spectra of the Py-Dodec-His-modified liposomes at 25 °C, where two major peaks were observed at 398 nm and 480 nm. It has been reported that the Pyrene molecule shows the different fluorescence values depending on the clustering state of the molecules (Nagami et al., 2005, Hinderliter et al., 2001). The fluorescence at 398 nm and 480 nm corresponds with the extents of the monomer and dimer (excimer), respectively. The relative fluorescence of the excimer and monomer (E/M) of the probe molecules in different liposomes was shown in **Figure 1-3(c)**. Although the E/M ratio in DLPC and DMPC liposomes at liquid-crystalline state was small, the value was increased in the case of DPPC liposomes because of the increase of the excimer extent.

It has been previously shown that the Hexadecyl-porphyrin (HPyP) molecules could undergo their clustering on the liposome surface, depending on the physical state of the lipid membrane, where the clustering of the molecules was distinctly observed in the case of liposome at gel phase (Umakoshi et al., 2008, Tuan et al., 2008). The clustering of acylated-tryptophan, which has similar structure as Dodec-His, has reported to be observed on the lipid membrane surface at the gel phase (Yasuhara et al., 2006). It has been reported that the fatty acid, which has a hydrogen donor and accepter, also show the clustering behaviors on the lipid membrane at the gel phase because of the hydrogen bond formation between the molecules in the hydrophobic environment of the liposome membrane (McLean and Hagaman, 1992). The present ligand has the hydrogen bond donor and accepter in the amide region of the molecule as shown in **Figure 1-2(d)**. According to the previous report on the molecular arrangement of the lipid membrane with different physical states (Heller et al., 1993), the lipid bilayer membrane at liquid crystalline phase could intake more water molecules inside the lipid membrane as compared with that at gel phase, suggesting that the former has the less hydrophobic or hydrated surface.

It is considered that the Dodec-His molecules could induce their clustering because of the hydrogen bond formation of the amide group in the lipid membrane which can provide a relatively dehydrated environment.

3.3 Metal Adsorption Behaviors on Ligand-Modified Liposome

A minimal requirement to elucidate the metalloenzyme-like activity is the metal adsorption on the surface of the ligand-modified liposome. The adsorption of metal

ions, such as Cupper (Cu) and Zinc (Zn), on the Dodec-His modified liposome was further investigated at 25 °C by varying the type of liposomes (i.e. DLPC, DMPC, and DPPC).

Figures 1-4(a) and **(b)** show the dependence of the Cu and Zn concentration on the amounts of adsorbed metal ions on the Dodec-His-modified liposome at 25 °C. The adsorbed amounts of the metal ions increased with the increase of the metal concentration and were varied depending on the type of liposomes, where the maximal adsorption was observed in the case of DPPC liposomes. The adsorbed amounts of Cu were, in general, higher than those of Zn when the same type of liposome was used. A Langmuir-type isotherm was herewith applied for the analysis of the adsorption behaviors and the theoretical curve

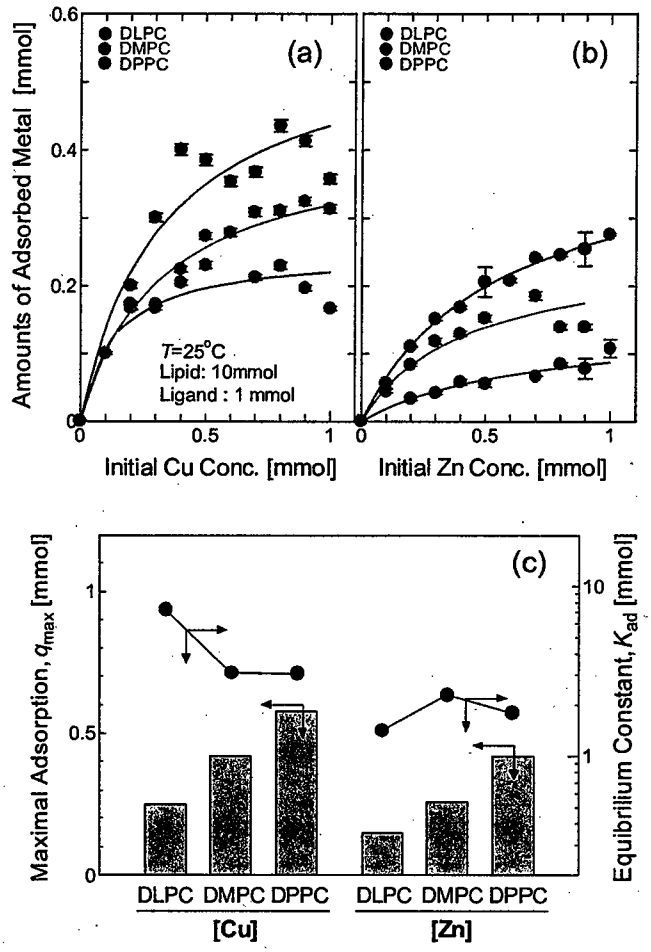


Figure 1-4 Adsorption Behaviors of Cupper and Zinc on Dodec-His modified Liposomes. Adsorption was performed at 25 °C for 12 hours in the solution of liposome composed of 10 mM lipid and 1mM Dodec-His with 0.1-1 mM copper or zinc. (a) and (b), respectively, show the adsorption isotherm of Cu and Zn on Dodec-His modified liposomes. The curve was calculated based on the Langmuir-type adsorption isotherm equation. (c) shows the maximal adsorption (q_{max}) and equibrium constant (K_{ad}), which determined from the fitting calculation of (a) and (b).

was also shown in **Figures 1-4(a)** and **(b)**. The parameters obtained from the fitting analysis, such as q_{max} and K_{ad}, were summarized in **Figure 1-4(c)**. The results show that the adsorption constant (K_{ad}) of Cu was 2-4 folds higher than that of Zn. In both cases of the Cu and Zn adsorption, the q_{max} value was found to increase with the increase of the membrane fluidity ($1/P$) at 25 °C, where the q_{max} values of Cu and Zn on Dodec-His-modified DPPC liposomes were, respectively, 0.56 and 0.41 mmol against 1.0 mmol ligand.

The adsorption of transition metal ions on the liposomes modified with the metal affinity ligands harboring carboxyl group and thiol group in the structure, where similar adsorption behavior on the ligand modified liposome was obtained in both cases of Cu and Zn and the adsorption was governed by the hydrogen-bond network of the ligand and the phosphate group of the lipid membrane (Nagami et al., 2004). Different from the extraction behaviors in organic/water two-phase systems, the higher selectivity of the Cu or Zn adsorption was then observed (Nagami et al., 2004). In the present experimental system, the ligand used, Dodec-His, has some possible chelating groups in the hydrophilic region of the ligand, such as carboxyl group and imidazole group (**Figure 1-2(d)**). Among them, the pKa values of the carboxyl group and imidazole group were, respectively, determined as 9.1 and 5.6 through the pH titration experiment if the Dodec-His was modified into the lipid membrane (data not shown), implying that the metal ion could preferably coordinate with imidazole group. At the neutral pH region, it was also confirmed that the UV absorbance at 260nm, derived from imidazole group, was strongly affected by adding the metal ions to the

Dodec-His modified liposome, showing that the imidazole group of the ligand could mainly contribute to the metal adsorption on the Dodec-His-modified liposome.

It seems that the difference in the adsorption behaviors of metal ions could be closely related to the environmental condition neighboring the coordination group of the ligand molecule. Especially, in the case of the DPPC liposomes which shows the gel phase and higher clustering nature of the Dodec-His, the adsorption capacity of both Cu and Zn was maximal. In the case of the natural Cu/Zn-SOD, the Cu and Zn were coordinated with imidazole groups of six His residues and carboxyl group of an Asp (McCord and Fridovich, 1969) and its activity center was well wrapped by the relatively hydrophobic amino acid residues (Pinto et al., 1997). It is generally known that the hydrogen bond or metal-ligand coordination could be stabilized in the dehydrated (hydrophobic) environment (Chakraborty and Patel, 1996). Considering its hydrophobic (or non-hydrating) environment of the surface of DPPC liposomes (Heller et al., 1993), the cluster-formation of the ligands and, furthermore, the metal-ligand complex could be stabilized on the surface of the liposome.

It is thus concluded that a similar coordination structure, by the imidazole group and carboxyl group of the Dodec-His, was formed on the surface of the liposomes and could be utilized for the construction of the mimics of the active center of SOD.

3.4 SOD-like Activity of Metal/Ligand-Modified Liposome

Based on the above results of the basic characteristics of membrane and the metal adsorption on it, the SOD-like activity of the metal/ligand-modified liposome was finally investigated.

The SOD activity of the liposomes modified with Dodec-His and metal ion (Cu or Zn) was determined as the IC_{50} value and the value was summarized in **Table 1-1**. In the case of Zn/Dodec-His modified liposome, the SOD-like activity was not

Table 1-1 Comparison of IC_{50} Values of SOD and Liposome-Recruited SOD

Enzymes / LIPOzyme			Metal / Ligand Ratio [-] (*1)	IC_{50} [μM] (*2)	
[SOD LIPOzyme] (This Study) (*3)					
<Ligand>	<Lipid>	<Metal>			
Dodec-His	DLPC	Cu	0.19	25	[4.8]
Dodec-His	DMPC	Cu	0.24	56	[13.4]
Dodec-His	DPPC	Cu	0.35	9.0	[3.2]
Dodec-His	DLPC	Zn	0.06	n.d.	
Dodec-His	DMPC	Zn	0.13	n.d.	
Dodec-His	DPPC	Zn	0.19	45	[8.7]
[Others for Comparison]					
Dodec-His	DPPC	Cu/Zn	0.36 (Cu: 0.24, Zn: 0.12)	18	[6.5] (*4)
His (*5)	POPC	Cu	-	n.d.	
His (*5)	POPC	Zn	-	n.d.	
His (*5)	POPC	Cu/Zn	-	3200	(*6)
HPyP	DMPC	Mn	1.0	0.35	[0.35] (*7)
[Enzyme]					
Cu/Zn-SOD (Native)			n.d.	0.034	

*1 Relative Ratio of Adsorbed Metal on Ligand. The value was calculated based on Langmuir isotherm equation with parameters calculated in Fig.3(c).
*2 IC_{50} values were evaluated from the SOD inhibitory activity at different concentration by using the NBT method. The values inside the square brackets show the IC_{50} values recalculated based on the ratio of the adsorbed metal on the ligands.
*3 Initial concentration of metal was adjusted to 0.5 mM
*4 Cu/Zn mixture was applied at the initial concentration of Cu (0.25 mM) and Zn (0.25 mM)
*5 SOD-like activity was measured just after 10 mM Histidine was mixed with 1 mM Cu and/or 1 mM Zn in the presence of POPC liposomes.
*6 The enzymatic activity of SOD was lost within 12 hours although it was kept for at least two hours.
*7 Previous data (H. Umakoshi *et al.*, *Langmuir*, **24**, 4451 (2008))
n.d.: The effective SOD activity was not detected owing to the lower value in inhibitory activity (less than 20%)
-: No data

observed in the case of DLPC and DMPC liposomes and that for DPPC was low (IC_{50} = 45 μM). The relatively higher value in SOD-like activity was observed in the case of Cu/Dodec-His modified liposome as compared with that of Zn/Dodec-His modified liposome. Although the IC_{50} value of DLPC and DMPC was similar with those of Zn /Dodec-His modified DPPC (25 μM and 56 μM), the SOD activity was maximal in the case of the DPPC (IC_{50} = 9 μM). The obtained IC_{50} values were furthermore corrected based on the net amounts of the adsorbed metal on the Dodec-His modified liposome (**Figure 1-4**) and were shown inside the square brackets. It was found that the DMPC liposomes with mixed phase of gel and liquid crystalline showed the lowest activity and that was the highest in the case of DPPC liposomes modified with Cu and Dodec-His.

The obtained SOD-like activity of Cu/Dodec-His modified DPPC liposomes was compared with those of other type of liposome catalysis and natural SOD. The addition of both Cu and Zn has previously reported to increase the SOD-like activity of the oxidized and fragmented SOD on the surface of liposome (Tuan et al., 2008). Although the addition of both Cu and Zn was investigated, the activity level was not significantly affected (IC_{50} = 8.7 μM). In order to compare the role of acyl group modification, the SOD-like activity of His and Cu/Zn with liposome was measured similarly in the case of previous study (Costanzo et al., 1993), resulting that the enzymatic activity level was approximately one-thousand times lower than the present results and the SOD-like activity was not stable for time incubation. The above results show that the effectiveness of the His-metal complex on the surface of lipid membrane.

However, the SOD-like activity of Cu/Dodec-His modified DPPC liposomes was 10 times lower than that of hydrophobically-modified Mn-porphyrin (Nagami et al., 2005). The value was also much lower (5% of native) than that of native SOD. It has been reported that the molecular recognition function of the liposome itself could be achieved by a combination of the non-specific interactions of the lipid surface (Kuboi and Umakoshi, 2006). Although the level of the SOD-like activity of the present liposome is retained to the lower level, its tuning could be performed through the modulation of the structure of active center complex and the regulation of the substrate recognition on the structure based on the basic characteristics of liposome surface induced under the stress condition.

It was thus shown that the simple ligand-modified liposome can create a metal complex similar to the active center of SOD although the activity level of SOD was not so significantly. Although SOD activity was successfully induced on the liposome surface, another approach should be employed from the viewpoint of the SOD activity level.

3.5 SOD LIPOzyme from Break-Down Approach: Liposome-Recruited Activity of SOD under Oxidative Stress Condition.

The SOD LIPOzyme has been prepared from the Break-Down Approach (**Figure 1-5**). The peptide fragment of H_2O_2-treated Cu,Zn-superoxide dismutase (SOD) was shown to be reactivated with liposomes prepared by POPC liposome. The H_2O_2-treated SOD, which lost its activity at different incubation times, was dramatically reactivated only by adding POPC liposomes, resulting in 1.3-2.8 times

higher enzymatic activity. A comparison of the fractions detected in reverse-phase chromatography shows that specific SOD fragments are able to contribute to the reactivation of oxidized and fragmented SOD in the presence of POPC liposomes. The liposomes can recruit the potentially active fragment of SOD among the lethally damaged SOD fragments to elucidate the antioxidative function. The results and discussion on this SOD LIPOzyme with "Break-Down" approach have been shown as follows.

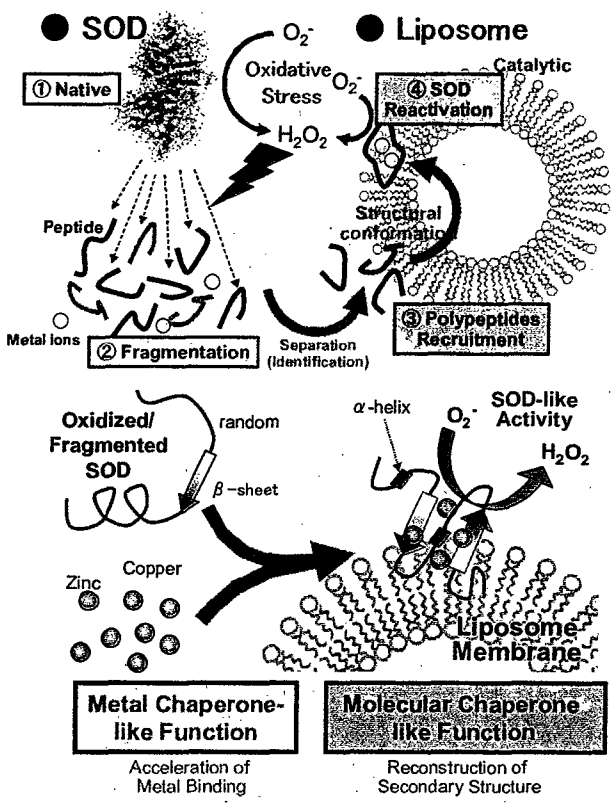


Figure 1-5 SOD LIPOzyme Using Liposome-Recruited Activity

3.6 Effect of Hydrogen Peroxide on the Enzymatic Activity and Oxidation of Superoxide Dismutase: Role of Liposome in Protection of SOD from the Oxidation

In order to investigate the basic tendency of the conformational change and fragmentation of SOD under the oxidative stress condition, the effects of hydrogen peroxide on the enzymatic activity and fragementation of SOD were first investigated. **Figure 1-6** shows the responses of SOD as a function of H_2O_2 concentration:

fragmentation of SOD (Enghild et al, 1999) and the α-helix contents of SOD (Nagami et al., 2005). The α-helix neighboring the active center of SOD was shown to be destroyed with increased H_2O_2 concentrations and was completely lost at more than 2 mM (**Figure 1-6**). Simultaneously, the fragmentation of SOD was observed from the data of SDS-PAGE analysis according to a previous report (Enghild et al., 1999), resulting in an estimated 75% of the SOD being fragmented at more than 2 mM. A similar result (81% fragmentation) was also obtained by the SDS-PAGE analysis of the H_2O_2-treated SOD (**Figure 1-6**: half black-white circle). In contrast, the fragmentation of SOD reached only 46% in the presence of the POPC liposomes, despite the higher concentrations of H_2O_2 (2mM) (**Figure 1-6**: half black-white square). The above results show that the POPC liposomes can positively act on SOD under the oxidative stress condition to protect the enzyme from being inactivated (Nagami et al., 2005), inducing conformational change, and fragmentation.

The activity of SOD fragments in the presence of POPC liposomes was further studied by analyzing the time course of SOD activity during its oxidation and after liposome addition. The liposome-assisted activity of SOD has previously been modeled as the SOD-liposome interaction with the "partially-denatured SOD," which loses the α-helix content neighboring the activity site of SOD (as observed in the

circular dichroism spectra analysis) (Nagami et al., 2005). However, the fragmentation of SOD was herewith observed in addition to the inactivation and loss of secondary

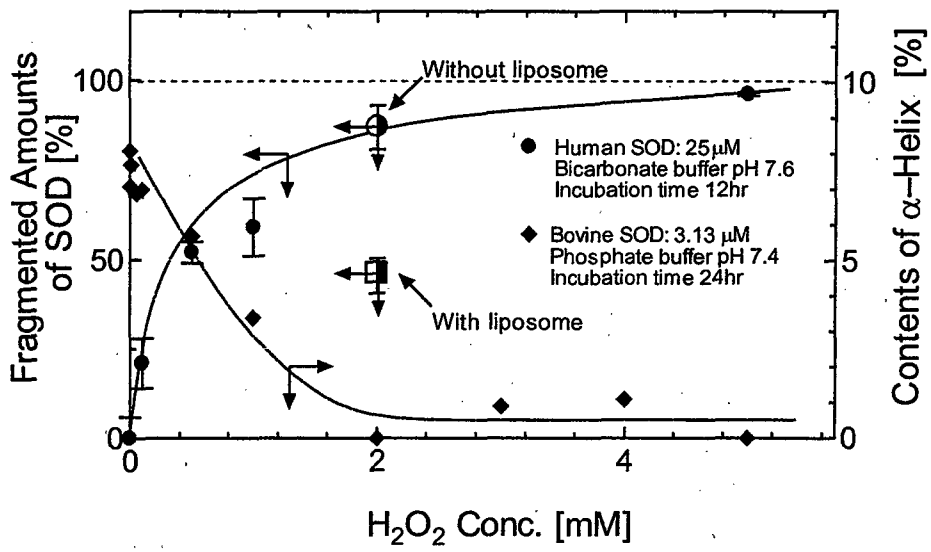


Figure 1-6 Dependence of H_2O_2 Concentrations of the Fragmentation of SOD Determined from SDS-PAGE analysis. Data of fragmented amounts of SOD (closed circle) (Choi et al., 1999) and α-helix contents (closed square) (Nagami et al., 2005) were replotted from previous studies. Fragmentation of SOD was conducted by the incubation of 2 μM bovine SOD, in presence of 2 mM POPC (haft black-white square) or absence of POPC liposomes (haft black-white circle), phosphate buffer pH 7.4, time incubation 24 h. The reaction was stopped by adding catalase to a final concentration of 100 μg/ml, and an aliquot was analyzed by SDS-PAGE. The bands of stained-gel of SDS-PAGE analysis were evaluated by Scion Image Software relying on the density of images (see Experimental Section). Data represent the mean ± S. D. (n=3-5).

structure, especially, at H_2O_2 concentrations more than 2 mM (**Figure 1-6**). It is hypothetically considered that some kinetic intermediates of SOD are formed during the oxidation of SOD with H_2O_2 and that these fragments are able to display the activity through its interaction with the liposome membrane. The oxidation of SOD and its interaction with liposomes were separately analyzed by employing their

sequential treatment. After 2μM SOD was incubated with 2mM H_2O_2 (preoxidation), 2mM POPC liposomes was added to the reaction bath at 2h, 6h, and 12h (liposome addition). The results in **Figure 1-7(a)** show the time course of the relative SOD activity during preoxidation and after liposome addition. In the absence of liposomes, the SOD activity was drastically reduced and reached saturated values (less than 20%) in approximately 7 hours. When the liposomes were added 12 hours after the preoxidation, the decrease in SOD activity (15%) was reversed and reached 40% (2.66 times more). Similarly, the SOD activity in different preoxidation periods was also increased by the addition of liposomes from 70% to 90% (1.28 times) and from 22% to 60% (2.72 times) for liposome addition at 2 and 6 hours, respectively, after the preoxidation (**Figure 1-7(b)**).

It has been reported that the α-helix content of SOD is lost by H_2O_2 in both the absence and presence of liposomes (Nagami et al., 2005). Our data show that SOD fragmentation can also occur in the presence of H_2O_2, as previously reported (Enghild et al., 1999), resulting in the inactivation of enzymatic activity of SOD (Uchida and Kawakishi, 1994; Blech and Borders, 1983). During the oxidation, there are some possibly temporal intermediates such as (i) SOD with a partially destroyed conformation (nonfragmentation), (ii) specific fragments, and so on. The above two

possible states can be related to the "liposome-assisted activity" which was varied according to the preoxidation time in the above sequential treatment (**Figure 1-7(b)**).

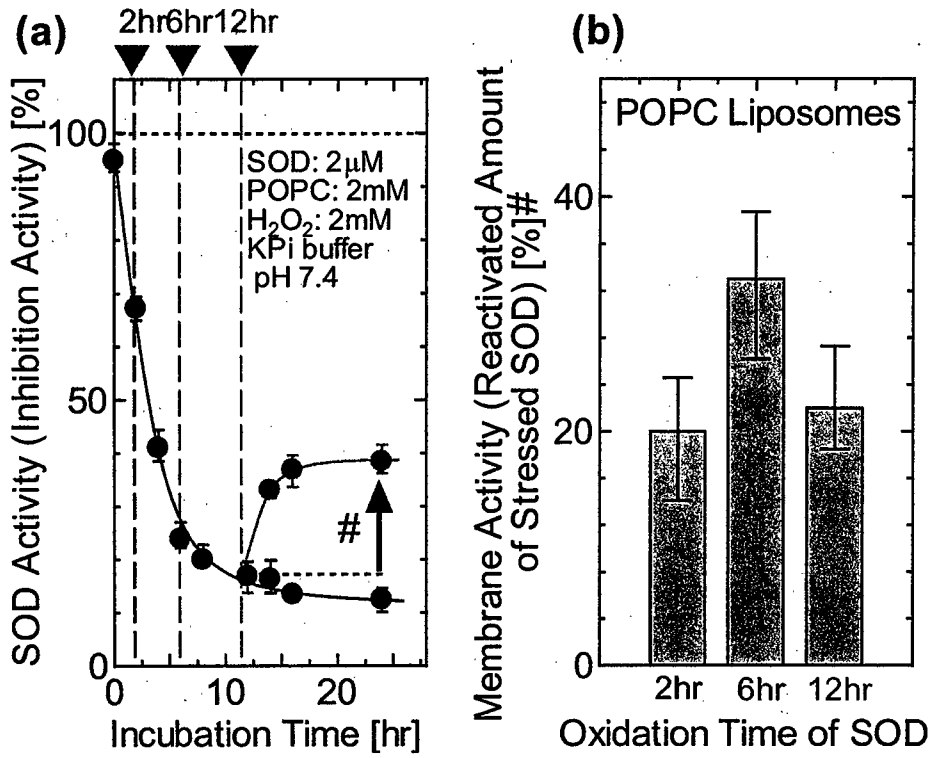


Figure 1-7 Response of SOD Activity after Sequential Treatment of Preoxidation and Liposome Addition. (a) Time course of SOD Activity. A sample of 2.0 μM SOD was treated with 2 mM H_2O_2 in the phosphate buffer (pH 7.4) for 24 h at 37^0C for the appropriate time (2, 6, 12 hours) as a preoxidation treatment, and 2 mM POPC liposomes was added to the solution. The filled black circle is the control (without POPC); the filled green circle is the recovery of SOD activity after liposome addition. (b) Summary of the Increase in SOD Activity after Liposome Addition followed by Preoxidation. All the data represent the mean ± S. D. (n=3-5).

At lower levels of oxidative stress [i. e. a short treatment time (2 hours) at 2 mM H_2O_2 or treatment at lower concentrations], the POPC liposomes can interact with the partially destroyed SOD and similarly protect it from further oxidation, as described previously (Nagami et al., 2005). This function of the POPC liposomes is

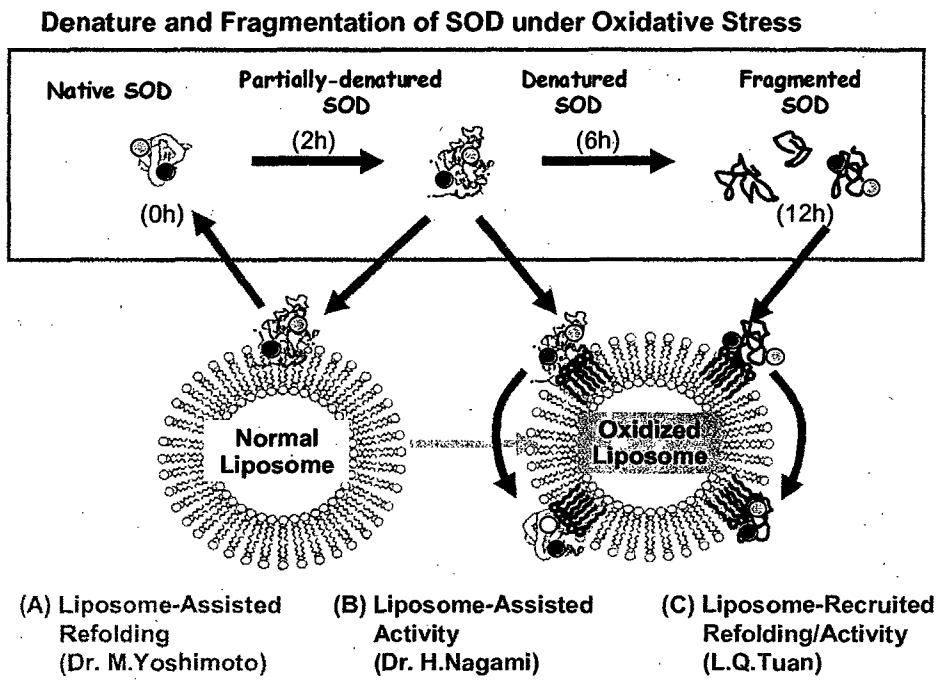


Figure 1-8 Conceptual Illustration of Liposome-Recruited Activity of SOD under Oxidative Stress. (i) Conformationally-changed intermediate could be refolded on the surface of liposome. (ii) Partially-fragmented intermediate could interact with liposome and its activity was kept. (iii) Fragmented SOD intermediate could be recruited on liposome surface and form a new structure.

supported by the suppression of fragmentation in the presence of liposomes (**Figure 1-6**). At higher oxidation levels (12 hours of preoxidation), the specific fragments containing the active site of SOD are able to be recruited on the liposomes and its conformation can be repaired for the continuous expression of SOD-like function. Further exposurement of the SOD under strong oxidative stress (more than 2mM or long oxidation time) could induce the fragmentation of the peptide into further smaller pieces, resulting in the less reactivation effect in spite of the presence of liposome

(further discussed in Chapter III). As schematically shown in **Figure 1-8**, it is thought that both effects are elucidated at 6 hours preoxidation, resulting in a maximal value in the increase of SOD activity after liposome addition (**Figure 1-7(b)**). In the following investigations, the possibly specific fragment of SOD at the higher levels of oxidative stress was further determined.

3.7 Binding of Specific Peptide Containing Active Site on Membrane Surface.

The binding of the peptide fragment of SOD on the liposomes was confirmed through ultrafiltration analysis. After the addition of liposomes, followed by preoxidation, the sample was treated by ultrafiltration (50-kDa molecular cut-off filter), which enables us to separate the liposomes and the fragments bound on it from the non-bound fragments (**Figure 1-9(a)**). The SOD activity and protein concentrations were measured in both "permeate" and "retentate" solution after the ultrafiltration experiment. The lower activity of SOD (25~45% of total SOD activity) was observed in the "permeate" solution and, further, both the activity and specific activity of SOD decreased with increases in the preoxidation time (**Figure 1-9(b)**). The majority of the SOD activity was detected in the "retentate" solution, while the SOD activity was reduced by increasing the preoxidation time (**Figure 1-9(c)**). However, the specific activity of the SOD in the "retentate" solution was not changed, regardless of the

increase in the preoxidation time in contrast to that in "permeate" solution. Furthermore, the relative ratio of SOD activity in "retentate" to that in "permeate" solution increased with increases in the preoxidation time (**Figures 1-9(b)(c)**). The above phenomenon clearly shows that the liposomes can gather specific fragments of oxidized SOD and reactivate the specific fragment on the liposome membrane. Nagami *et al.* have previously reported that the liposome-assisted activity of H_2O_2-treated SOD is maintained by the interaction between the POPC liposomes and oxidized SOD (Nagami et al., 2005). It has also been demonstrated in a series of previous works that liposomes can perform a molecular chaperone-like function to assist in the refolding of partly denatured proteins (Yoshimoto et al., 1999 and 2000; Kuboi et al., 2000). However, the SOD was found to be irreversibly fragmented with H_2O_2 in just a few hours (**Figure 1-7(a)**). Another possible explanation based on the molecular chaperone-like function of the liposomes and the obtained results is the assemblage and conformational rearrangement of the intermediates of SOD on the liposome surface under a stress condition. When the POPC liposomes were added to the SOD treated with H_2O_2, the liposome membrane can recruit the fragments of oxidized SOD, including the specific residues of the active sites and nonactive parts,

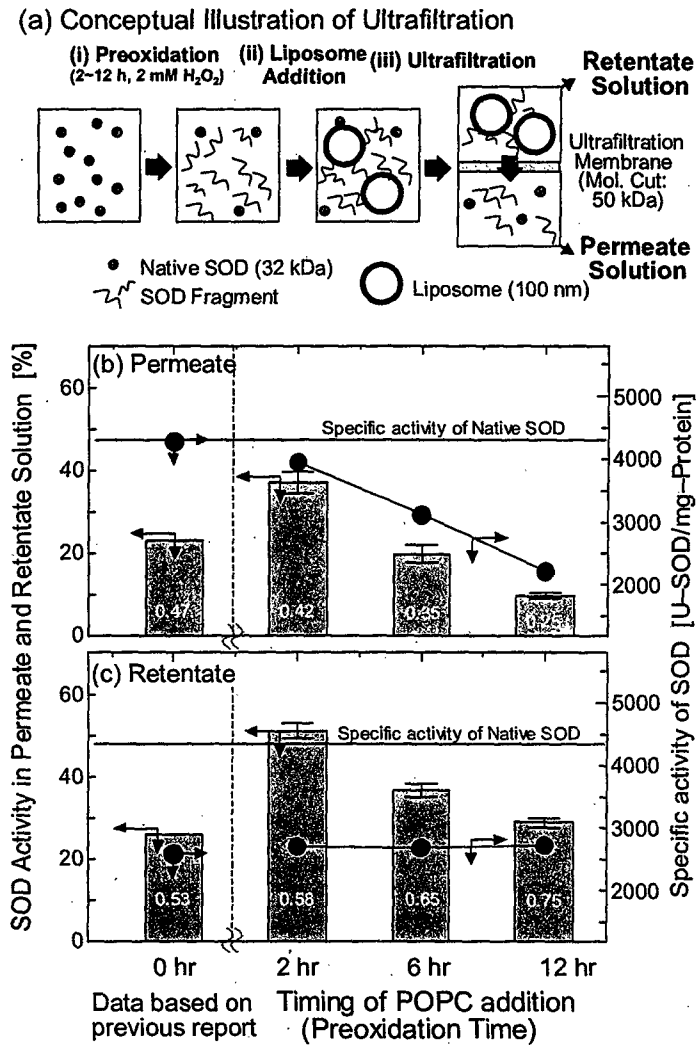


Figure 1-9 Oxidized SOD Activity and specific activity with POPC Liposomes before/after the ultrafiltration operation. (a) Conceptual Scheme of Preoxidation, Liposome Addition, and Ultrafiltration Treatments. A sample of 2.0 μM SOD was incubated with 2 mM H_2O_2 for the appropriate time (2, 6, 12 hours) in phosphate buffer (pH 7.4). After 2 mM POPC liposomes was added to the solution, the final aliquots of 24-hour incubation were subjected to ultrafiltration to separate the permeate solution, including the smaller molecules less than 50 kDa and the retentate solution including the larger molecules or liposomes. In the preliminary experiments, native SOD (32 kDa) and its oxidized molecules were confirmed to be included in "permeate solution". (b) and (c) show the SOD activity and specific activity of SOD in the permeate and retentate solutions, respectively. The samples treated by this ultrafiltration were further used for the RP-HPLC analysis (**Figure 1-10**).

and modulate its conformation, leading to the display of new SOD-like activity (**Figure 1-7**).

Normal and oxidized forms of SOD after ultrafiltration were further analyzed by reverse-phase HPLC. **Figure 1-10A** shows the chromatograms of (a) normal SOD, (b) oxidized SOD solution, and (c) "permeate" SOD solution after ultrafiltration of a preoxidized and liposome-treated sample. The results indicate that some oxidized species such as P2, P3, and P4 were formed after the oxidation of SOD. It has been reported that a 10-kDa fragment including the active center and other fragments (5 kDa) of SOD can be formed by H_2O_2 addition due to the fragmentation of SOD (Ramirez et al., 2005). The active site contains four His residues with Cu^{2+} and Zn^{2+}, catalyzing the dismutation of toxic superoxide radical into molecular oxygen and hydrogen peroxide without significant energetic cost (Branco et al., 2006). A UV spectra analysis of the fragmented SOD separated by HPLC indicated that the fragmented SOD contains histidine residues or other residues with aromatic rings. The detected peaks in oxidized SOD correspond to the oxidized fragments reported previously. Among the above fragments, the P4 fragment is thought to be relatively hydrophobic, judging from its long retention in an octadecyl-column. As shown in **Figure 1-10A(c)**, a part of P3 and the whole P4 fraction were not detected in the

"permeate" solution after ultrafiltration, showing that these fragments interact with the liposome membrane and continue to function as SOD together with liposomes because they contained active-site fragments. For further investigation, the P4 fragment of SOD, recovered from the RP-HPLC fraction, was co-incubated with the POPC liposomes together with Cu and Zn ions at 10 μM for supplementation of metals to modify the active site. Similarly, the SOD activity was found to be increased to 32% when the P4 fragment (inactive) was incubated with POPC and metal ions. Otherwise, the enzymatic activity of this fragment has nearly increased to 4% in control experiments with the addition of Cu^{2+} and Zn^{2+} or POPC only (**Figure 1-10B**). The specific conformation of the P4 fragment with the POPC liposomes was also observed through circular dichroism analysis (discussed in following sections). Binding of the P4 fragment with liposomes and its resulting conformational change can elucidate the re-activation of the inactive SOD fragment.

From previous studies (Ramirez et al., 2005; Nagami et al., 2005; Choi et al., 1999) and our present results, we can conclude that the specific fragment (P4), which has a hydrophobic site and a catalytic site of approximately 10 kDa, still displays the original SOD-like function after recruitment on the liposome membrane surface. The

above results also imply the possible significant role of the membrane, as a fail-safe function, in the biological system during the response under lethal stress conditions.

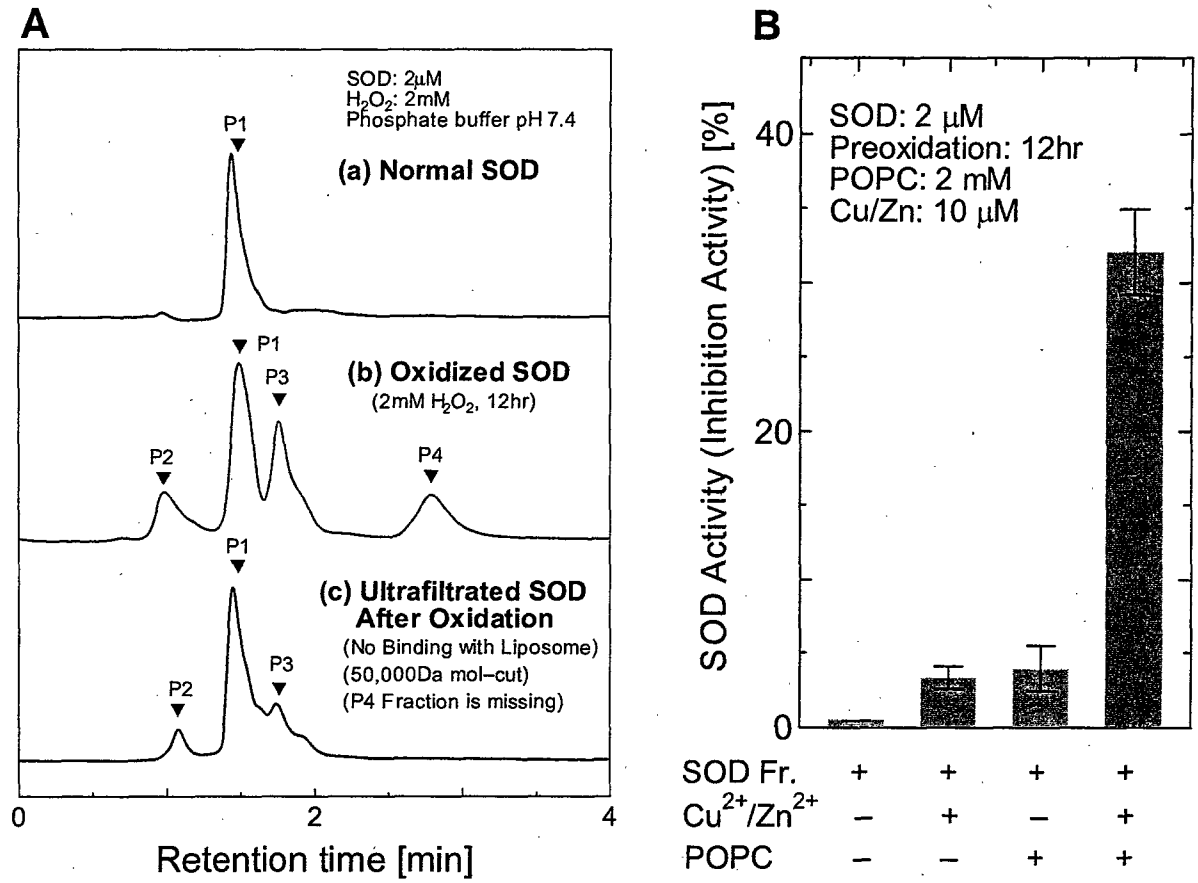


Figure 1-10 A. C18-RP-HPLC Analysis of SOD and Oxidized SOD. The above samples were compared with that of oxidized SOD after ultrafiltration. P1: native SOD; P2, P3 and P4: derived from fragmentation. A part of P3 and all of P4 were kept by the liposomes after filtration. Solvent of acetonitrile and water (v/v: 7/3) was applied for running RP-HPLC at 25^0C with a flow rate of 1 ml/min and a wavelength of 220 nm for absorption. **B.** SOD Activity of a Specific Fragment (SOD Fr.) Recovered by RP-HPLC with and without POPC liposomes and metal ions. All the data represent the mean ± S. D. (n=3-5).

3.8 Conformational Change of Native SOD under Oxidative Stress and Fragmented SOD in the Presence of Liposomes

The oxidation and fragmentation of protein by oxidants concerned to the loss of its secondary and tertiary structures (Davis and Delsignore, 1987). SOD becomes fragmented by hydrogen peroxide, depending on the oxidation time (Choi et al., 1999; Tuan et al., 2008). SOD's enzyme activity also decreases in correlation with the fragmentation. The conformation of SOD, incubated with 2 mM hydrogen peroxide, was first analyzed by CD measurement. The peak 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 (**Figure 1-11a**). These results also demonstrate the structural change in SOD under oxidative conditions, which is expressed by the decrease in α-helix, whose total disappearance was observed after 6 h oxidation, as well as by the slight decrease in the β-sheet.

Interestingly, the addition of POPC liposomes, Cu and Zn ions together, to the fragmented SOD recovered its secondary structure and further induced SOD-like activity. The conformational change of fragmented SOD into a native SOD-like structure was observed in the presence of POPC liposomes and Cu and Zn ions (**Figure 1-11b**). This process occurs because of the interaction of damaged protein with the membrane and its refolding (Brown et al., 2004; Myari et al., 2001, Kuboi et

al, 2004). The CD spectra also show that the metal coordination of the SOD fragments to histidines induces β-sheet formation in the above conditions. CD spectra analysis revealed that the refolding of the SOD fragments occurred significantly when both

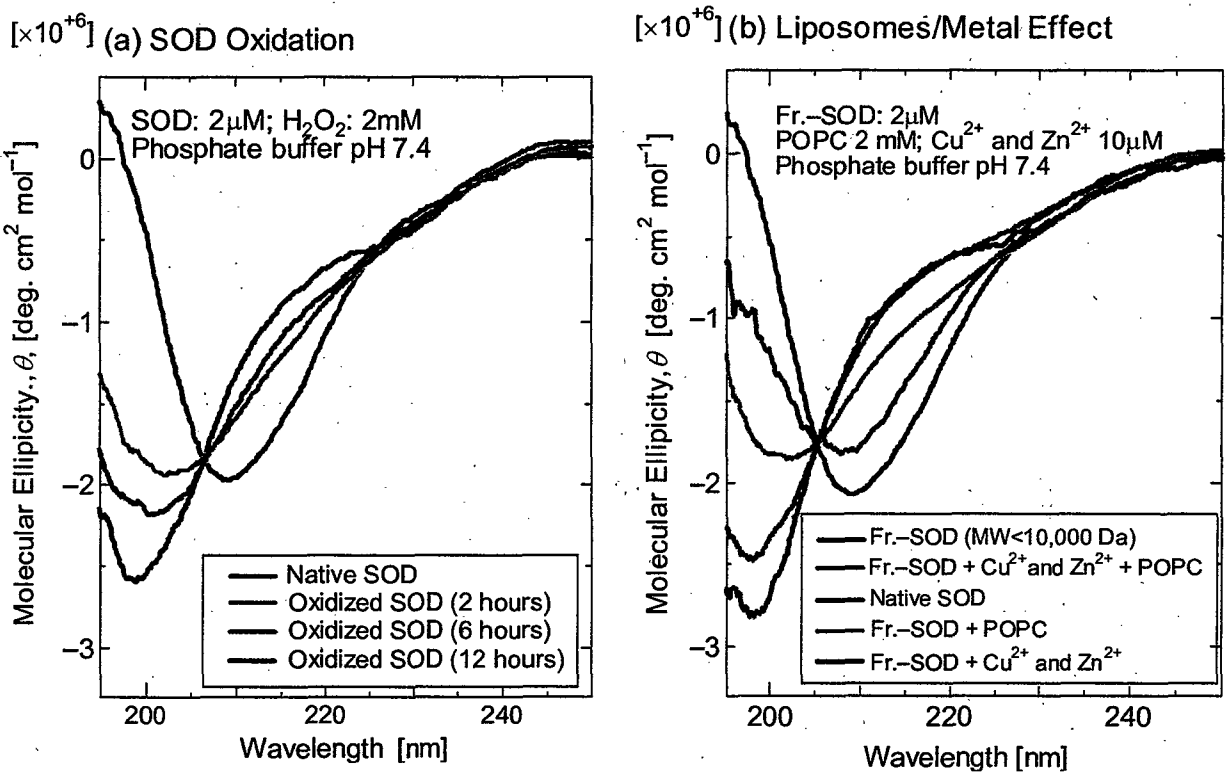


Figure 1-11 Circular dichroism spectra of (a) SOD under oxidation and (b) fragmented SOD with or without metal ions and POPC liposomes. (a) Significant change in the secondary structure of SOD was observed under oxidative stress conditions (2 mM H_2O_2, 2, 6, and 12 h incubation). (b) The secondary structure of SOD lost under oxidative conditions (2 mM) was recovered by the addition of both metal ions (10 μM) and POPC liposomes (2 mM, 100 nm).

POPC liposomes and metal ions were added. Consequently, the structure of fragmented 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 grew significantly, from 0 to 4.4%. Similarly, β-sheet also increased in separate settings of experiments with metal ions, POPC liposomes, or both. The increases in α-helix and β-sheet contents in the fragmented SOD results from the folding and conformation of fragment SOD on the surface of POPC liposomes in the presence of metal ions. Supplementation with POPC liposomes, metal ions together, contributes to the reconstruction of new active sites similar to the original one.

3.9 Reactivation of Fragmented SOD and Modification of Active Site on Liposome Surface.

The SOD-like activity of these samples was also analyzed (**Figure 1-12**), resulting in the high enzymatic activity 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 Cu and fragmented SOD in presence of liposome membrane is considered to be involved in histidine residues of SOD fragments (Casella et al., 1983; Dean et al., 2003; Buryak and Severin, 2004; White and Holcombe, 2007).

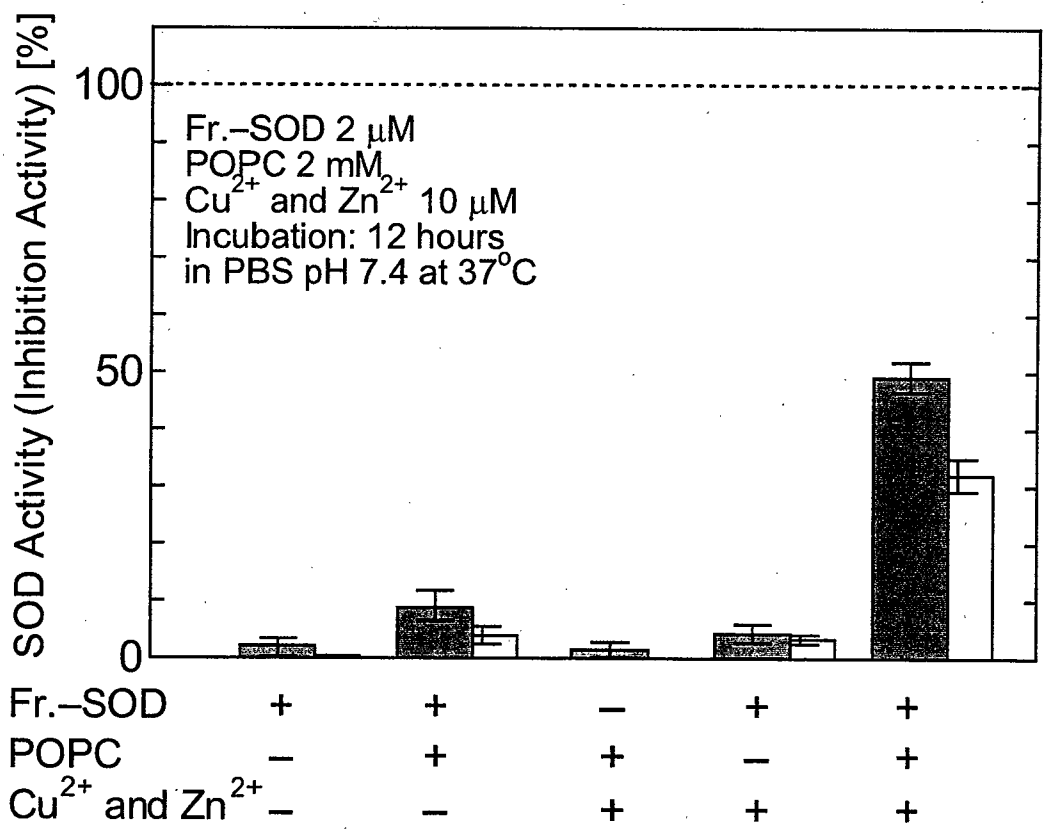


Figure 1-12 SOD activity of fragmented SOD with and without metal ions (Cu^{2+} and Zn^{2+}) and POPC liposomes. Filled rectangle: Fr.-SOD recovered by ultrafiltration, Open rectangle: Data from previous report (recovered from RP-HPLC) (Tuan et al., 2008). All of the data represent the mean ± SD (n = 3 – 5).

The Cu binding to fragmented SOD with or without POPC liposomes was investigated at different initial concentrations of Cu (**Figure 1-13**). In the presence of

POPC liposomes, the binding of copper to fragmented SOD was found to be higher than that of control (without POPC liposomes). This result clarifies that the binding of Cu to fragments is stabilized by POPC liposomes. Based on the fitting analysis of the adsorption curve using Langmuir isotherm, the capacity of Cu adsorption (q_{max}) on the fragmented SOD with POPC liposomes was estimated as approximately four times that without the liposomes. 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 (Uchida and Kawakishi, 1994; Lewisch and Levine, 1995). The mode of Cu coordination with peptides depends on the number of Cu ions bound, and Cu ions are coordinated by multiple histidine imidazole groups (Wells et al., 2006; Aronoff-Spencer et al., 2000). Therefore, reactivation of fragmented SOD in our 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 fragmented SOD in the presence of metals on the liposome

surface implies that the membrane is an essential tool in repairing and recovering damaged proteins and enzymes.

The relation between the conformation of fragmented SOD and SOD-like enzymatic activity in the presence of liposome and metal ions, further investigated by measuring the SOD activity and α-helix contents, was shown in **Figure 4**. This result

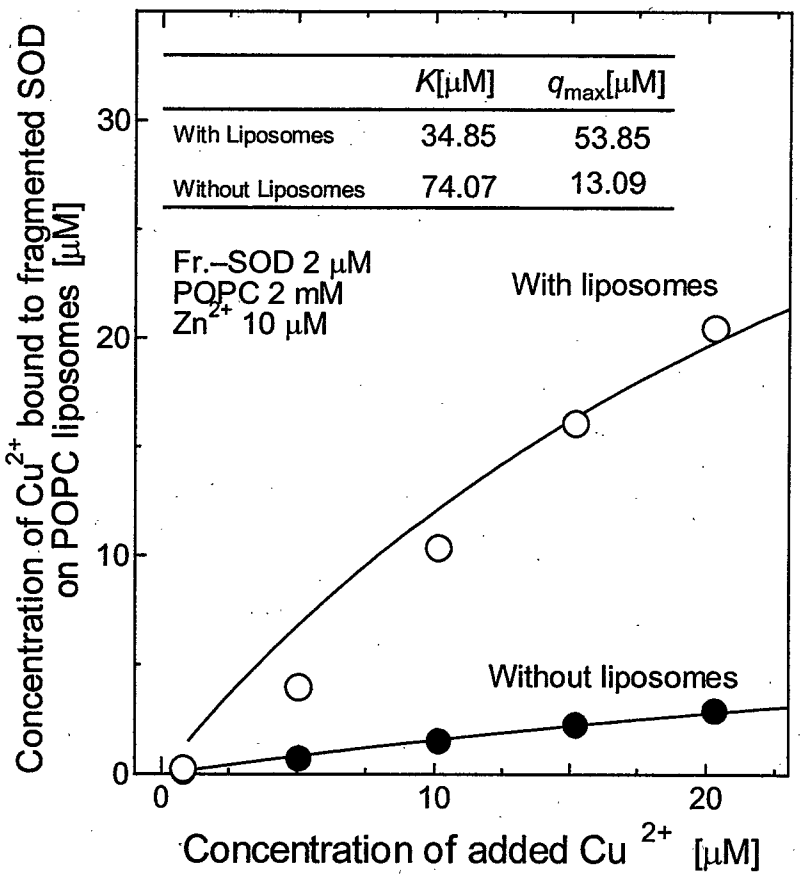


Figure 1-13 Cu adsorption behaviors on fragmented SOD in the presence (○) or in the absence (●) of POPC liposomes. A 2 μM fragmented SOD was mixed with 2 mM POPC liposomes and metal ion mixture (Cu^{2+} and Zn^{2+}) at various concentrations. The Langmuir isotherm was applied for the fitting of the adsorption curve. The capacity of Cu adsorption on the fragmented SOD with POPC liposome was estimated to be approximately four times that without the liposome.

indicates that the conformation of fragmented SOD, representing by the α-helix content, contribute to SOD-like activity of fragmented and recruited SOD. Recent conformational analyses have indicated that the structural insights into the binding and structuring process induced by peptide-membrane interaction (Dong et al., 2007). The increase of copper binding to fragmented SOD in the presence of liposome membrane to promote the α-helix contents can be explained that the fragments contained the histidine-rich residues (data not shown) which have a strong affinity with copper (Belosi et al., 2004; Burns et al., 2003) and can control the redox activity of copper (Nakamura et al., 2007).

In previous findings, some kinds of copper chaperone transport copper ions to apo-SOD to form fully mature SOD structures (Argirova et al., 2003). The process of dimerization requires either disulphide formation or metal coordination, whereas full catalytic function requires both (Hörnberg et al., 2007). Thus, in the correlation of fragment refolding with enzymatic activity, either the liposomes or metal ions independently induced the refolding of fragmented SOD. The former played a significant role in the refolding process, as shown by CD spectra measurement (**Figure**

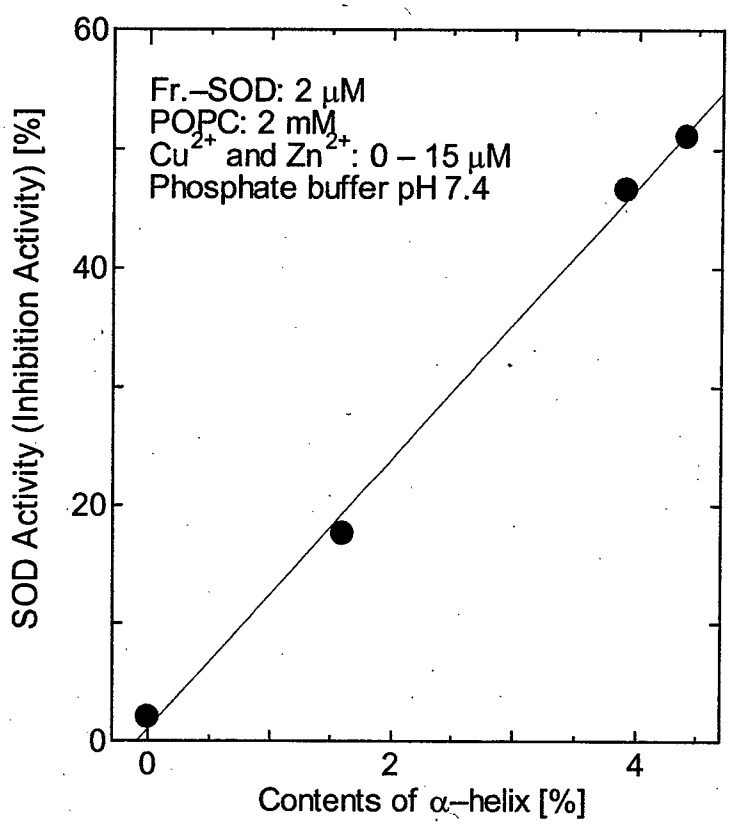


Figure 1-14 Correlation of the SOD activity and α-helix contents of recruited SOD on the range of copper and zinc concentration. A 2 μM fragmented SOD was co-incubated with 2 mM POPC liposomes and metal ion mixture (Cu^{2+} and Zn^{2+}) at various concentrations ((0 – 15 μM).

1-11(b)). However, in both cases, the enzymatic activity did not increase significantly (**Figure 1-14**). Otherwise, the presence of both metal ions and liposomes contributed not only to fragment refolding but also to the recovery of enzymatic activity. The refolding of SOD fragments in our results involved the interactions of liposomes and metal ions with fragmented SOD. Therefore, the complex of liposomes and metal ions can be considered a copper chaperone for fragmented SOD. These results demonstrate that the binding of Cu to fragmented SOD was promoted by POPC liposomes (**Figure**

1-13). Consequently, the fragmented SOD was structurally recovered as well as functionally reactivated in the presence of liposome membrane. The increases in α-helix (**Figure 1-11**) and β-sheet in the process of the recovery of the secondary structure and the SOD-like activity imply that fragmented SOD interacted with POPC liposomes on the membrane surface.

4. Conclusion

SOD LIPOzymes were prepared based on "Build-Up" type and "Break-Down" type approaches.

SOD LIPOzyme with "Build-Up" Approach

The liposome modified with simple ligand and metal ions shows the superoxide dismutase-like activity. The membrane fluidity of various liposomes modified with the functional ligand (Dodecanoyl-His; Dodec-His) and the clustering of the ligand on the liposome surface were first characterized, showing that the clustering of Dodec-His could be induced on the liposome surface at gel-phase. The capacity of adsorption of Cu and Zn was found to be increased, dependending on the type of liposome, resulting in the maximal adsorption in liposome prepared by 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at gel state and with higher

ligand clustering state. As a result on the SOD-like activity of the metal/ligand-modified liposome, the SOD-like activity was found to be induced by using the above liposomes although its activity level is not so high.

SOD LIPOzyme with "Break-Down" Approach

The peptide fragment of H_2O_2-treated Cu,Zn-superoxide dismutase (SOD) was found to be reactivated with POPC liposomes. The fragmentation of SOD occurred under oxidative stress of hydrogen peroxide, as well as SOD inactivation and the loss of an α-helix neighboring its activity center. The H_2O_2-treated SOD, which lost their activity at different incubation times, was dramatically reactivated only by the addition of POPC liposomes, resulting in 1.3~2.8 times higher enzymatic activity. The ultrafiltration analysis of H_2O_2-treated SOD co-incubated with liposomes shows that some specific peptide fragments of the oxidized SOD can interact with POPC liposomes. The comparison of the fractions detected in reverse-phase chromatography shows that specific SOD fragments are able to contribute to the re-activation of oxidized and fragmented SOD in the presence of POPC liposomes. The liposomes can recruit the potentially active fragment of SOD among the lethally damaged SOD fragments to elucidate the antioxidative function.

Liposome membrane played an important role not only in the refolding process of fragmented SOD but also in modification of the active sites of fragments, as would occur with molecular and metal chaperones. Liposome membrane was found to assist the conformational change of oxidized and fragmented superoxide dismutase (Fr.-SOD) and accelerate the adsorption of metal ions on its fragment to give the original SOD-like activity. The loss of SOD activity and its secondary structure was observed during 6 h oxidation in 2 mM hydrogen peroxide solution. The secondary structure of fragmented and recruited SOD, analyzed by Circular Dichroism showed that the full conformation of fragmented SOD occurred on the membrane surface. 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 induction of the high SOD-like activity. Significance in the Cu binding to the oxidized and fragmented SOD was observed in the presence of POPC liposomes. These results show that the POPC liposomes can act like molecular and metal chaperones for stress-damaged peptides, resulting in the

creation of a new SOD-like active center that continues to express the original enzymatic activity under the oxidative conditions.

The above two examples of SOD LIPOzyme preparation show that the significance of the liposome as a core material of the biomimetic catalysis.

Chapter II

Characterization of SOD LIPOzyme Prepared with Break-Down Approach

1. Introduction

The interaction between membrane and fragmented SOD was discussed in chapter I, in which membrane recognized fragments originated from the oxidation of SOD (step-i), refolded (step-ii) and, as a result, reactivated to give a SOD-like enzymatic activity (step-iii). The mechanism of this interaction was represented by the conformational changes of fragmented SOD through the modification of new active site on the membrane surface. However, the above three steps could be affected by the characteristics of "membrane itself". The interaction of liposome membrane and fragmented SOD should be further clarified by investigating the effect of various liposome membranes. A liposome membrane has shown to recruit the oxidized SOD fragment on its surface to produce a complex that has enzyme activity in chapter I. Among the fragments generated after SOD oxidation (Ookawara et al., 1992; Kurahashi et al., 2001), some specific peptides contain its active site (~5 and ~10 kDa) (Ramirez et al., 2005) although these fragments show a quite low SOD-like activity. The recruiting mechanism of fragmented SOD on membrane was considered to involve the recognition and refolding of such specific peptides by hydrophobic, electrostatic interaction, as well as microdomain formation, in which liposomes perform a molecular chaperone-like function (Yoshimoto and Kuboi, 1999; Yoshimoto et al., 2000; Kuboi et al., 2000). The liposome-SOD fragment complex needs to be

characterized, focusing on the characteristics of the membrane, in order to establish its rational preparation method.

Much effort has been devoted to the design and development of artificial antioxidative enzymes (Kirby, 1996; Batinic-Harber et al., 1996). A model biomembrane, liposome, is a possible candidate to provide a common platform for different catalytic centers of SOD. Such a liposome herewith possesses several benefits in the regulation of catalytic activity, where it can provide (i) a nano hydrophobic environment, (ii) a stress-responsible character, (iii) a microdomain structure, and (iv) membrane-membrane interactions. Some researchers have reported the effectiveness of the use of a model biomembrane (liposome) as a platform to immobilize the functional catalytic center (Nagami et al., 2004; Walde, 2006; Murakami et al., 1996). Enzyme-like activity, such as that of SOD (Nagami et al., 2004 and 2005; Tuan et al., 2008) and cholesterol oxidase (Yoshimoto et al., 2005), has already been regulated by the liposome properties, as well as affording functional elements on the liposome surface. The above enzyme-like function of liposome, which can herewith be defined as "LIPOzyme" (Kuboi et al., 2006), can be utilized for the design of the artificial enzymes.

In this chapter, the oxidized and fragmented superoxide dismutase being recruited on the liposome surface and representing the SOD-like enzymatic activity was further characterized for the preparation of "break-down" type LIPOzyme. The relation between the enzymatic activity of recruited SOD and the charge and fluidity of membranes was first investigated. The adsorption of fragmented peptide on the

membrane and SOD-like activity was observed. Based on characteristics of the identified peptide fragment, a mechanism on the interaction of fragmented and oxidized SOD with various liposomes was finally discussed.

2. Materials and Methods

2.1 Materials.

1,2-dipalmitoyl-*sn*-glycero-3- phosphocholine (DPPC: T_m = 42^{0}C), 1,2-dimyristoyl-*sn*-glycero- 3- phosphocholine (DMPC: Tm = 23^{0}C), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC: T_m = 0^{0}C), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and were used for liposome preparation. Dodecanoyl-Histidine (Dodec-His) and Pyrene-dodecanoyl-histidine (Py-Dodec-His) was synthesized and prepared according to the modified method described in the previous literature (Yasuhara et al., 2006). 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. All other reagents of analytical grade were purchased from Wako Pure Chemical (Osaka, Japan).

2.2 Preparations of Liposomes.

The lipids were dissolved in chloroform/methanol (10 mM lipid prepared will be diluted to 2 mM for each experiment). POPC/Chl 28% was composed from POPC and cholesterol with 28% mole in comparison to POPC. After the solvent was

evaporated, the resulting thin film was dried for at least 2 hours under a vacuum. The lipid film was hydrated by 50 mM potassium phosphate buffer to form the multilamellar vesicles. 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. Characterization of Membrane Fluidity.

The membrane fluidity of liposomes was determined by using a fluorescence probe, the hydrophobic probe, DPH (Lentz, 1993). DPH was added to the solution of liposome-copolymers or liposome-proteins at a final concentration of 1 μM. The fluorescence polarization of the DPH probe in the liposome membrane was measured at a wavelength of 360 nm for the excitation and 430 nm for the emissions. The fluorescence intensity of the DPH was measured by using the fluorescent spectrophotometer (JASCO FP-777, Japan). The degree of fluorescence polarization (P) was calculated using the following equation:

$$\frac{1}{P} = \frac{(I_{//} + I_{\perp})}{(I_{//} - I_{\perp})}$$

where $I_{//}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel (//) and perpendicular (⊥) to that of the exciting beam, respectively. The term "fluidity" is inversely proportional to the degree of fluorescence polarization of the probe; that is, the 'membrane fluidity' of the interior of the membrane was defined by (1/P) of DPH.

2.4. Analytical methods

Analysis of Cu,Zn-SOD fragmentation by H_2O_2. Cu,Zn-SOD (2 μM) was incubated with H_2O_2 (2 mM) in phosphate buffer (pH 7.4) at 37°C for 12 h. The enzymatic activity and protein concentration of fragmented SOD were determined after the incubation of SOD with H_2O_2. The SDS-PAGE technique was used to analyze SOD fragmentation.

For the 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 (Peskin and Winterbourn, 2000).

The BCA Protein Assay Kit was used to determine the protein concentrations. The protein was precipitated in a cold acetone solution to separate it from contaminants, so that a more accurate estimation of protein content in the sample could be obtained. The mixture was then centrifuged at 15,000 rpm for 20 min. Pellets solubilized in 50 μl H_2O were added to 1000 μl of BCA reagent solution and incubated for 30 min at 37 °C, after which the absorbance at 562 nm was measured. A standard curve was set up to analyze the protein concentrations (Smith et al., 1985).

Ultrafiltration of H_2O_2-Treated SOD. Ultrafiltration using an Ultrafree-MC filter (Millipore, Billerica, MA, USA) with a molecular mass cutoff of 10 kDa was applied

for fragmentation analysis. 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 applied to remove free copper released from active site.

For the reverse-phase HPLC, a Shimadzu (Kyoto, Japan) HPLC system equipped with an FCV-10AL pump, a DGU-20A3 degasser, an SPD-10A UV-VS detector, and an LC-10AD liquid chromatograph was used. Elution profiles were monitored at 220 nm on the UV detector. The mobile phase of acetonitrile/water (v/v 7/3) with a flow rate of 1 mL/min was applied at 25 °C. An STR ODS-M column (0.46 cm x 15 cm), in which the particle surface was octadecylated, was used throughout this study.

Treatment of SOD and fragmented SOD. The SOD, totally oxidized into fragments for 12 h in 2 mM H_2O_2, was ultrafiltered by a 10 kDa molecular cutoff filter to obtain the potentially active debris (fragmented SOD) containing the active sites (Tuan et al., 2008; Ramirez et al., 2005). The 10 kDa fragments filtrated from ultrafiltration were incubated with POPC 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, fragmented SOD, and metal ions was determined. Amino acid sequences were analyzed by Protein Institute.

2.5. Statistical Analysis.

Results are expressed as means ± standard deviation (SD). 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

3.1. Reactivation and Adsorption of Oxidized and Fragmented SOD Recruited on Various Liposomes

The liposome has reported to recruit the oxidized and fragmented SOD on its surface and induce the SOD-like activity as described in chapter I. The fragmented SOD was also shown to acquire its secondary structure through the coordination with metal ions on the surface of the liposome. The effect of liposome types on the liposome-recruited activity was investigated.

Figure 2-1(a) shows the SOD-like activity of the fragmented SOD (2 μM) with Cu and Zn (10 μM) in the presence of various types of liposomes (2 mM lipid). In the presence of POPC liposomes, the SOD activity was approximately 50%, which is compatible with the previous results in chapter I. The value was reduced depending on the type of zwitterionic lipid (DMPC and DPPC). As shown in **Figure 2-1(b)**, the membrane fluidity was reduced in correspondence with the decrease of the SOD activity. It has been reported that the phase transition temperature of the POPC, DMPC, and DPPC liposomes are -4 °C, 23 °C, and 45 °C, respectively (Umakoshi et al., 2008), where the POPC liposomes exists in a liquid crystalline phase at the temperature tested

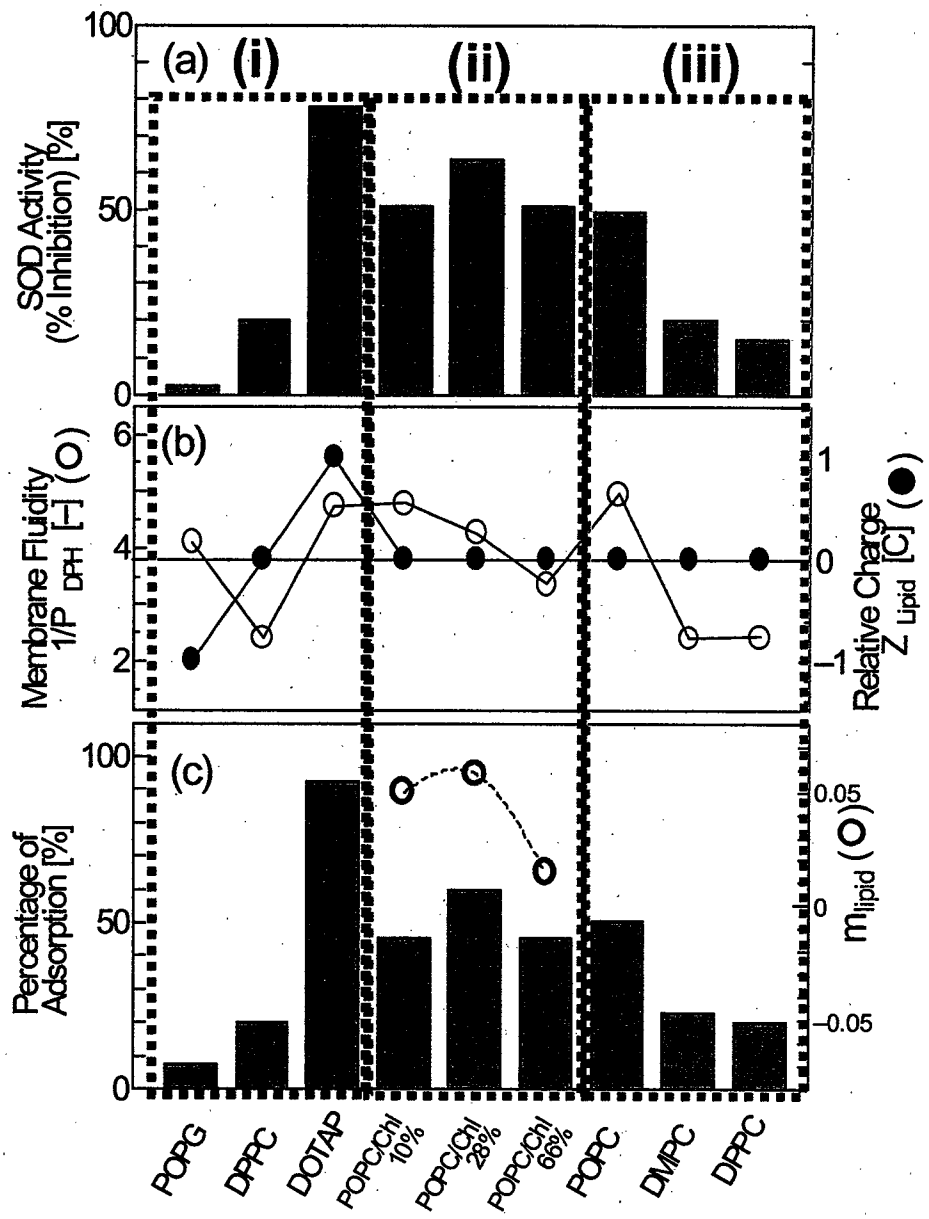


Figure 2-1 Effect of Liposome Type on Recovered Activity of Fr.-SOD and Its Adsorption on the Liposome. (a) Recovered Fr.-SOD (2 μM) was treated with 10 μM Cu^{2+} and Zn^{2+} with various liposomes (2 mM lipid concentration). The SOD activity was shown as a percentage of inhibitory activity. (b) The basic characteristics of liposome membrane, such as membrane fluidity measured by DPH (○) and relative charge of the hydrophilic head group of the lipid molecules (●). (c) The percentage of the adsorption of Fr.-SOD on the surface of liposome. After mixing the Fr-SOD and liposome, the non-adsorbed Fr.-SOD was recovered by ultrafiltration. The percentage of adsorption was determined from the mass-balance of the protein concentration before and after the ultrafiltration.

here. It has been also reported that the membrane fluidity of the liposome could increase the hydrophobicity of the liposome surface (Kuboi et al., 1997; Umakoshi et al., 1998). The above tendency of the SOD enzymatic activity could be related to the hydrophobic interaction between the fragmented peptides and liposome membrane.

As shown in **Figure 2-1(a)**, the SOD activity was further increased in the case of the POPC/Ch liposome, resulting in the 62% activity, although the membrane fluidity was not significantly increased (**Figure 2-1(b)**). It has been reported that the POPC/Ch liposomes possess microdomain structures on its surface and the microdomain structure shows higher affinity with the peptide components (Taylor and Roseman, 1996) owing to the hydrogen bond stabilization of the peptide on the membrane (Yoshimoto et al., 2005). It has been reported that the stability of the hydrogen bonds inside the peptide could be modulated with the wrapping hydrophobic residues (Fernandez and Berry, 2003). The stability effect of the hydrogen bond in liposome membrane, m_{lipid}, has already been analyzed by using the immobilized liposome chromatography (Yoshimoto, 2004) and also liposome dielectric dispersion analysis (Shimanouchi, 2005), based on the hydrogen bond stability of protein reported by Fernandez. The m_{lipid} values of POPC/Chl was shown in **Figure 2-1(c)**, resulting that the values of peptide adsorption and SOD-like activity were well corresponding with the m_{lipid} values. The hydrogen bonds, which contribute to the conformational change of fragment and its interaction with liposome, could be enhanced in the hydrophobic environment provided by the liposome membrane.

The effect of the charged liposomes was also investigated by adding the POPG

and DOTAP liposomes. An opposite effect on the liposome addition was obtained. The SOD-like enzymatic activity was reached to 78% in the presence of DOTAP while the value was only 3% in the case of POPG liposomes (right keys in **Figure 2-1(a)**). The calculated net charge of head group of a lipid molecule at this experimental condition was shown in **Figure 2-1(b)**. Different from the above liposomes (POPC, DMPC, DPPC, and POPC/Ch), the surface of POPG liposomes has been negatively charged owing to the phosphate group and that of DOTAP has been positively charged by the contribution of the tetra-ammonium group. As can be seen in the chemical structure of the PC-lipids, it also has a positively charged choline-group together with the phosphate group, implying that the edge of membrane surface has positively-charged group. It has been reported that the liposome could interact with the negatively charged biomacromolecule (i.e. RNA) because of their electrostatic interaction (Janas et al., 2006). The above results and the previous findings show that the fragmented SOD to be recruited on the surface of liposome because of the electrostatic interaction between them.

In all the condition of various liposomes, the adsorbed amounts of the fragmented SOD on the liposome were analyzed by using the ultrafiltration technique as shown in **Figure 2-1(c)**. The percentage of the adsorption was well related to the variation of the SOD-like activity. The adsorption behaviors of the SOD fragment show that the "liposome-recruited activity of the oxidized and fragmented SOD" could be derived from the interaction (recognition) of the fragmented peptides with the

liposome surface, implying that the interaction was governed by the combined effect of the electrostatic interaction, hydrophobic interaction (Carpenter et al., 1997; Rankin et al. 1998) and hydrogen bonds. The fragment to be recruited on the liposome surface could have a negative charge, hydrophobic surface and unstable hydrogen bonds not wrapped by hydrophobic environment. Although this mechanism has not been clarified yet in detail, the role of membrane is to display the potentially-harboring functions of polypeptide. In the case of denatured SOD, denatured SOD itself can be refolded into the native state by its global minimum of stabilization energy. In this case, the liposome membrane plays a supporting role to accelerate the refolding rate. In present study, fragmented SOD potentially shows several local minimum state of stabilization energy, where it itself cannot be refolded. Therefore, the liposome membrane could provide a global (or local) minimum of stabilization energy, which give an appropriate structure to induce a suitable activity in the given environment. That is why specific fragment has to interact with membrane to be stabilized. The driving force for this interaction and the conformational change is hydrogen bond stability in hydrophobic environment. Fragmented SOD has unstable hydrogen bonding and liposome has fluctuated hydrogen bond stability depending on the membrane composition (especially POPC/Cholesterol), resulting in the interaction of

fragmented SOD and liposome to require the stable hydrogen bonding in hydrophobic environment. If the fragmentation could occur, the protein skin layer could be removed and protein becomes naked (ex.Fr-SOD). The naked peptide has many desolvated hydrogen bonds on its surface, but it shows the certain conformation depending on the surrounding environment. If the liposome could exist there, it provides a local minimum of stabilization energy because the surface has a slight hydrophobic potential, which could interact in a long distance, against bulk water phase. The bound peptide could affect the liposome surface because of its fluctuation nature. The complex could induce another local (or global) minimum for both structures. In the case of water soluble protein, its surface turned to hydrophobic on the liposome surface and it was released from surface (Folding or Translocation). If it has lower hydrogen bond stability (i.e. amyloidgenic protein), it could be stabilized on the surface (possible destiny: LIPOzyme, membrane protein, initiator of fusion, etc.). The combination of the non-specific interaction could convert to the specific interaction because of the dynamic and stress-responsive nature of the liposome with nano-scale interface.

However, the recruited SOD-like activity was not always followed in this phenomenon. For example, the interaction of oxidized POPC and fragmented SOD

was also investigated (to be discussed in chapter III). In this condition, the adsorption of fragmented SOD to membrane was so high although the recruited SOD-like activity was low. The above results imply that there could be, at least, two steps such as (i) peptide adsorption and (ii) reconstruction of its conformation to induce the liposome-recruited enzymatic activity. A mechanism of the above liposome function is now under investigation.

3.2 Characterization of Oxidized and Fragmented Peptide Recruited on Liposome Surface

Based on the above results, the oxidized and fragmented peptide recruited on the liposome surface was furthermore characterized. The molecular weight of the oxidized and fragmented SOD was analyzed by using the SDS-PAGE. **Figure 2-2(a)** shows the CBB-stained image of the analyzed gel. In the case of the native

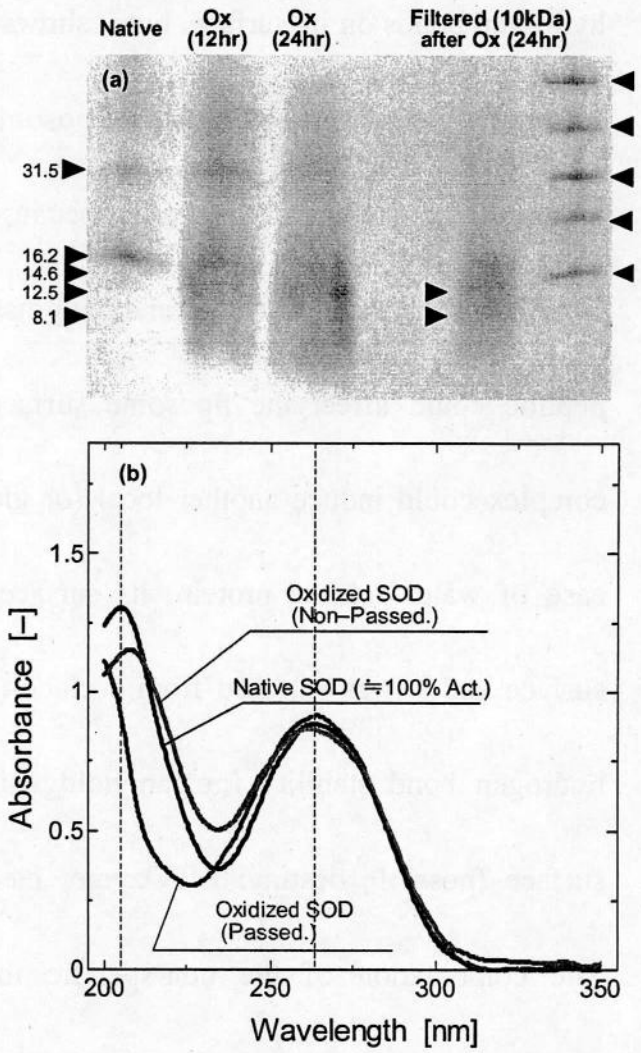


Figure 2-2 Basic Characteristics of Fr.-SOD. (a) The CBB-stained image of SDS-PAGE analyzed gel. The native SOD, oxidized SOD (12 and 24 hours) and recovered SOD were applied for the SDS-PAGE analysis. (b) The UV spectra of the native SOD, oxidized SOD and the recovered SOD

SOD, the dimer (31.5 kDa) and monomer (16.2 kDa) were observed (Lane 1). By treating the SOD with 2 mM H_2O_2, such original bands were gradually lost and small fragments were newly formed (Lane 2 and 3). The liposome recruited fragment recovered finally shows two main bands at 12.5 kDa and 8.1 kDa and also show the much small fragments below these bands (Lane 4). The molecular weight of the finally recovered fragments could range from 8 kDa to 10 kDa. The UV spectra of the recovered SOD were contrasted with the native one as shown in **Figure 2-2(b)**. Similarly in the case of the native SOD, a strong peak was observed at 260 nm, showing that the SOD fragment could possess the His and aromatic groups in its structure. The comparison of the absorbance at 205 nm shows that the SOD fragment could be abundant in the amino acids harboring the carboxyl groups as compared with

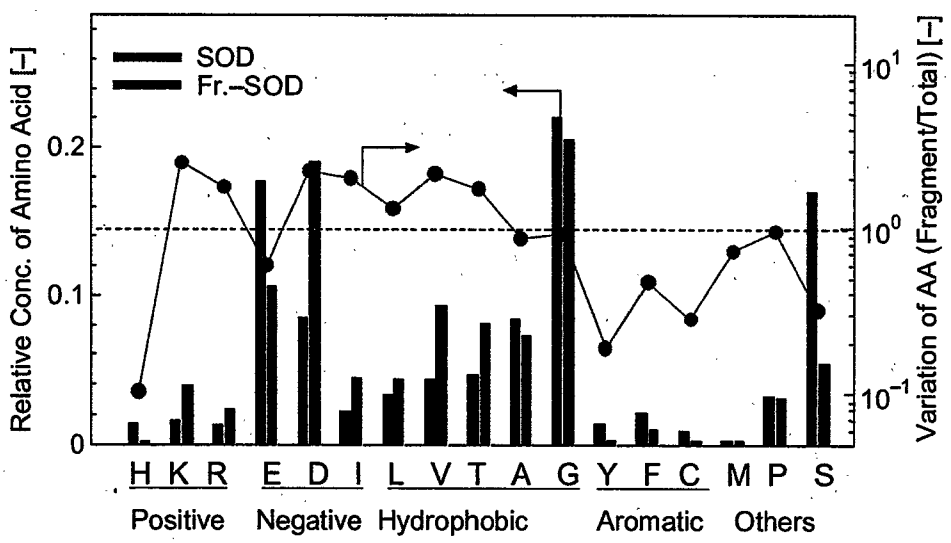


Figure 2-3 Relative Concentrations of Amino Acids of Native SOD and Fr.-SOD. The amino acid composition was determined by reverse-phase HPLC after the hydrolysis of the peptides in NaOH solution (Analysis in Protein Institute: See experimental Section). The variation of relative concentration of each amino acid was shown as the relative ratio of the amino acid concentration of Fr.SOD against the native SOD.

the simply-oxidized fragment. The amino acid composition of the native SOD and the fragmented SOD was further investigated. **Figure 2-3** shows the relative concentration of the 18 amino acids of native SOD (black keys) and the fragmented SOD (red keys). The variation of amino acid of fragmented SOD against the native one was also shown as a relative ratio of these values. Although the decrease in the relative concentration of His residue was observed, the increase of the charged amino acids and hydrophobic amino acids was observed. The above results show that the fragmented peptide has a charged and hydrophobic surface and also possess the His residues although its absolute amounts were lower than those of the original one.

3.3. Liposome Can Recognize the Specific Fragments of Fragmented SOD through Integrated Interaction Theory.

The surface characteristic of the fragmented SOD was finally discussed based on the above results and also the basic database of the native SOD. The structure data of the Cu/Zn-SOD (PDB ID: 1CBJ) were used for the discussion in **Figure 2-4**. **Figure 2-4(a)** shows the position of the His residues in the SOD structure, where the 6 His residues contribute to the formation of its activity center among 8 residues. The position of the secondary structure of SOD was shown as solid lines in **Figure 2-4(b)**, where β-sheet structure is mainly distributed along them except for the α-helix structure neighboring to its activity center. The relative score on the secondary structures, predicted by Chou and Fasman method, was also shown as curves in **Figure 2-4(b)**. Although the predicted score in general matched with the actual secondary structure in the case of β-sheet or β-turn, some unmatched domains were observed in focusing on the predicted α-helix value; where the relative scale was high

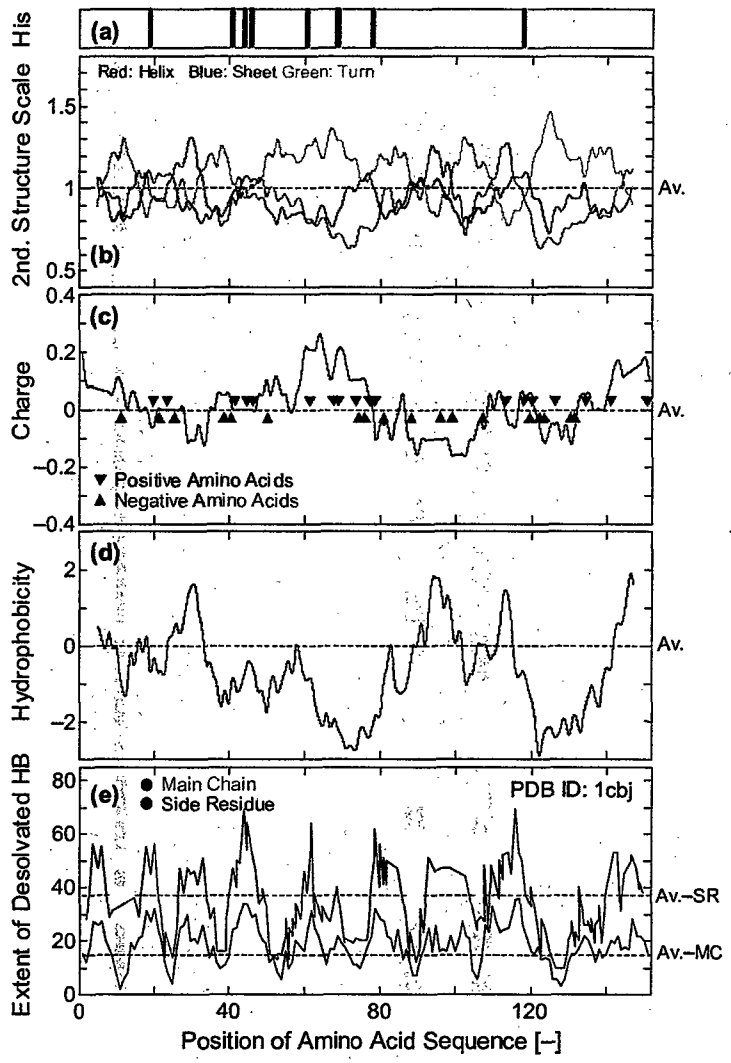


Figure 2-4 Basic Characteristics of SOD at Any Position along Amino Acid Sequence from N- to C-Terminal. All the data were analyzed based on structure data of domain A of native SOD registered in Protein Data Bank (http://www.rcsb.org/, PDB ID: 1CBJ). (a) The position of His and (b) secondary structure (red: α-helix, blue: β-sheet, green: β-turn: Curve; prediction by Chou and Fasman Method; Bold Line in the top; actual secondary structure). (c) The moving-average of the surface net charge of neighboring nine amino acids by assuming the Glu and Asp show -1 and His, Arg, and Lys show +1. (d) The moving-average of the surface net charge of neighboring nine amino acids by using the hydrophobicity scale reported previously (Kyte J. and Doolittle R.F., *J. Mol. Biol.*, **157**, 105 (1982)). (e) The extents of desolvation of hydrogen bond by hydrophobic residues in main chain and side residue of SOD, where the hydrogen bonds at any position are stabilized in the case of the higher extents of desolvated hydrogen bond (Fernandez, A. amd Berry, R.S., *PNAS*, **100**, 2391 (2003) / Fernandez, A. amd Scott, R., *Biophys. J.*, **85**, 1914 (2003)). Yellow-colored bands in (b)-(e) shows the domain with both negative charge and high hydrophobicity and blue-colored bands shows that with low stability of hydrogen bonds among the yellow regions.

in Nos.18-30, 74-80, 95-100, 92-124 although the actual structure was β-sheet or β-turn structure. **Figure 2-4(c)** shows the relative values of the surface charge of the side residues of the amino acids, where the negative charge were clustered in the N-terminal region (No. 10-40) and middle region (No. 70-90). The hydropathy plot was shown in **Figure 2-4(d)**, where the hydrophobic residues were assembled in some domains along the amino acid sequence (Nos.1-12, 22-38, 81-120, and 130-152). As shown in **Figure 2-4(e)**, the stability of the hydrogen bonds of the main chain and side residue were also plotted based on the previous method on the calculation of the hydrogen bond-wrapping (Fernandez and Berry, 2003; Fernandez and Scotty, 2003). There are some valleys in the curve of hydrogen bond stability of both main chain and side residues. The results obtained in the previous section show that the SOD fragment has negative charge and hydrophobic surface (**Figure 2-1**). Among the possible sequences of the SOD, the amino aside residues harboring the above two characteristics were picked up and were shown as yellow-colored bands. It has been reported that the proteins with under-wrapped hydrogen bonds could favorably interact with the lipid membrane (Fernandez and Berry, 2003). Among the yellow-colored bands, the specific domains with low stability of the hydrogen bond were further shown as blue-colored bands.

Although the fragment recruited on the liposome has not been specified yet, it is suggested that the parts of the SOD shown as yellow- and blue-colored bands in. **Figure 2-4** could be candidates of its fragment considering the data on the SOD-like activity recruited on the various types of liposomes. Liposome membranes that can

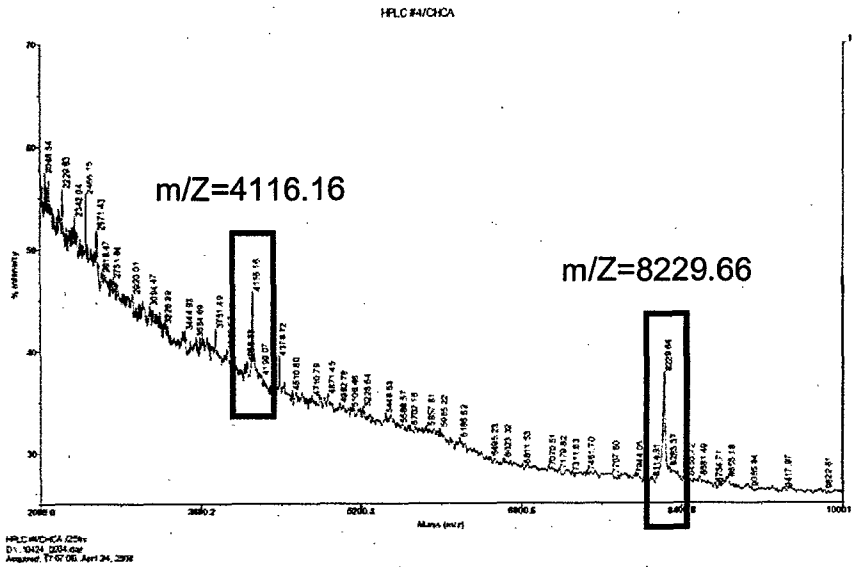


Figure 2-5 Mass Spectra of SOD fragment to be recruited on the surface of POPC membrane.

recruit the SOD fragment could have potentials to recognize the peptide with unstable hydrogen-bond (**Figure 2-4(e)**), considering the potential ability to form the α-helix of the peptide (unmatched region of predicted and practical secondary structure in **Figure 2-4(b)**). The above results also imply that the obtained fragment characteristics could provide useful information on the "molecular-recognition function" of the lipid membrane itself.

In order to justify the above analysis of the amino acid sequence based on the physicochemical parameters, the peptide recruited on the liposome surface was recovered and was further analyzed by using the mass spectra. **Figure 2-5** shows the mass spectra of the peptides to be recruited on liposome by using RP-HPLC. Peaks were detected at *m/Z*= 8229.64(H^+) and *m/Z*=4116.16($2H^+$), resulting in the exact mass of 8229.64. The amino acid sequence to satisfy the above *m/Z* was searched in Cu/Zn-SOD via sequence identification tool on the ExPacy web (http://us.expasy.org/tools/findpept.html). The amino acid sequences identified by

exact mass are shown as follows

70 80 90 100 110
(L)SKKH|GGPKDEERHV|GDLGNVTADS|NGVAIVDIVD|PLISLSGEYS|

120 130 140
IIGRTMVVHE|KPDDLGRGGN|EESTKTGNAG|SRLAC(G) in 1CBJ.pdb

As discussed above, the fragmentation of SOD produced the rational specific and nonspecific peptide. The hydrolytic mechanism of SOD by hydrogen peroxide was explained by the interaction of free copper released from active site (Uchida and Kawakishi, 1994; Sato et al., 1992). When Cu was released without chelators, copper rebinds to unknown residues in SOD, where Fenton-like reaction occurs in the presence of excess H_2O_2 and produces hydroxyl radical. Hydroxyl radical then reacts with protein to form a protein backbone-centered radical and induces non-active site fragmentation of SOD (Sato et al., 1992; Chevion, 1988; Kocha et al., 1997; Hawkins and Davies, 2001). The oxidation occurred specifically at Cys6, Cys55, and Cys144 in backbone, His46 and His44 in active site and Pro64 neighboring to the active site (Kurahashi et al., 2001) to produce the nonspecific and specific fragments, respectively. The obtained SOD fragment, described in the above section, was the peptide from Ser57-Cys144, where both edges of its fragment were neighboring to the H_2O_2-sensitive Cys residues as described in the previous paper. Our results on the recruited SOD fragment are well corresponding with the previous findings. The possible response of the SOD molecule was schematically shown in **Figure 2-6**. Under the oxidative stress condition, the conformation of some portions of SOD was partly destroyed and Cu ions were released from the molecule. If the liposome could coexist

with the partly-damaged form, the SOD conformation could be refolded (Yoshimoto, 1999) or it could be activated through its interaction with the liposome (Nagami, 2005) (**Figure 1-8**). However, owing to the formed hydroxyl radical caused by the released Cu ions, the specific parts of the peptide backbone could be attacked, where the Cys residues in the random coil structure being exposed to the SOD surface would easily be fragmented. In this case, the "primary intermediates" (Fr-1, Fr-2, and Fr-3) under oxidative stress could be formed. Liposome could recognize the specific peptide among the primary intermediates. Under the strong oxidative stress (i.e. higher concentration of H_2O_2; >2mM: **Figure 1-6**), the further fragmentation could occur and small peptides could be formed because the buried residues were exposed to the surface owing to the 1st oxidation, resulting that the liposome cannot reactivate them. The peptide to be recruited on the surface liposome could thus be predicted by considering the oxidative stress-sensitive residues.

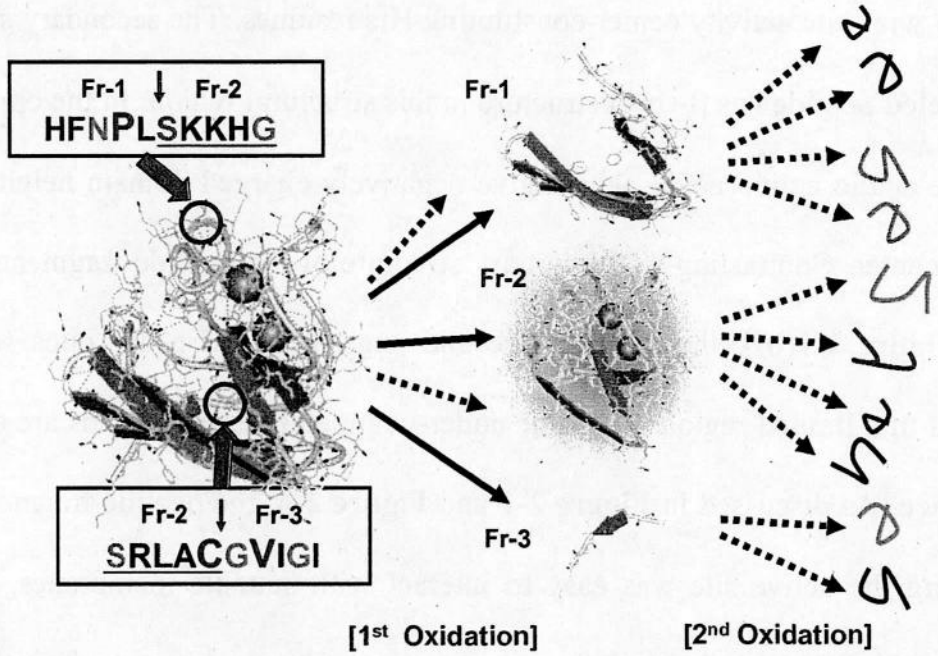


Figure 2-6 Modulation of SOD fragmentation by hydrogen peroxide.

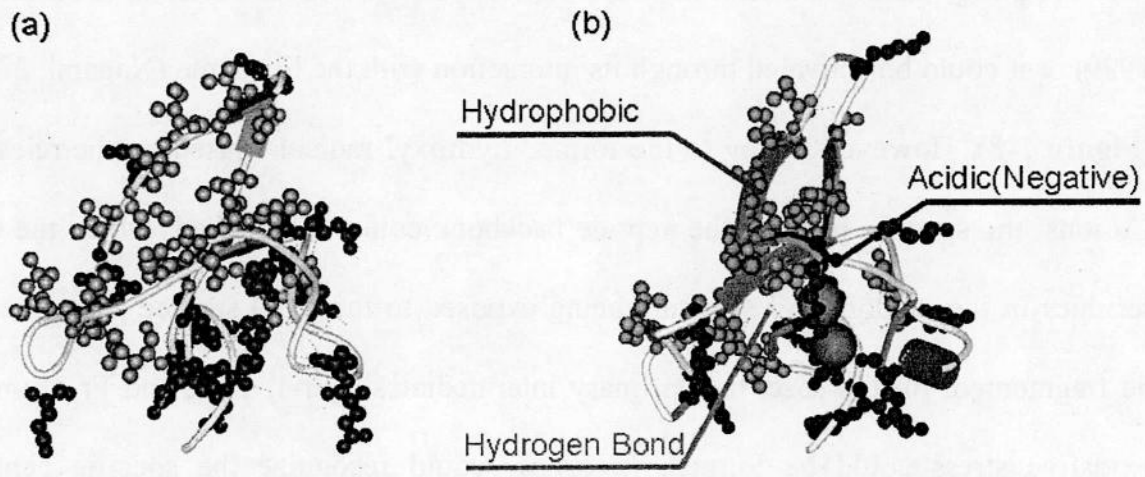


Figure 2-7 Tertiary structure of the identified peptide predicted based on SwissModel(a) and cut view from the native structure of native Cu,Zn-SOD(b)

The tertiary structure of above amino acid sequence was modeled in SwissPlot and was shown in **Figure 2-6(a)**, together with characteristics of the above sequences (**Figure 2-6(b)**). One side of the peptide fragment has hydrophobic surface which seems to wrap the activity center-constituting His residues. The secondary structure of the modeled peptide has β-sheet structure in this structural region. In the opposite side, there are acidic amino acids, which give negatively charged domain neighboring the activity center. Contrasting to the tertiary structure of the peptide fragments in native SOD (**Figure 2-7(b)**), the hydrophobic and negatively charged groups seem to be clustered in a limited region and some under-wrapped hydrogen bonds are existing on the surface. As discussed in **Figure 2-1** and **Figure 2-4**, the peptide fragment of SOD containing the active site was easy to interact with suitable membranes, especially, DOTAP and POPC/cholesterol liposomes as mentioned above, to be refolded and

reactivated. The above results show that the identified peptide is well corresponding with the phenomena on the liposome-recruited activity.

3.4. Membrane Toxicity and Its Functions under Stress Condition.

The change of membrane fluidity in the interaction of typical liposomes and the peptide fragment was discussed to point out the differences between normal membrane and damaged membrane (toxic membrane). The discussion focused the four kinds of liposome; (1) POPC as a control, (2) DOTAP, positively charged liposome, (3) POPC/cholesterol, domain forming membrane, and (4) Ox-POPC, toxic membrane. The scheme of membrane structure was described in **Figure 2-8**. The membrane fluidity of DPH fluorescent probe, buried deep within the acyl chain region of the bilayer, has reported to reflect the acyl chain mobility and chain order in the hydrophobic core (Lentz, 1989, 1993). The obtained results shown in **Figure 2-9** indicated that there was a decrease in membrane fluidity of liposome while liposome

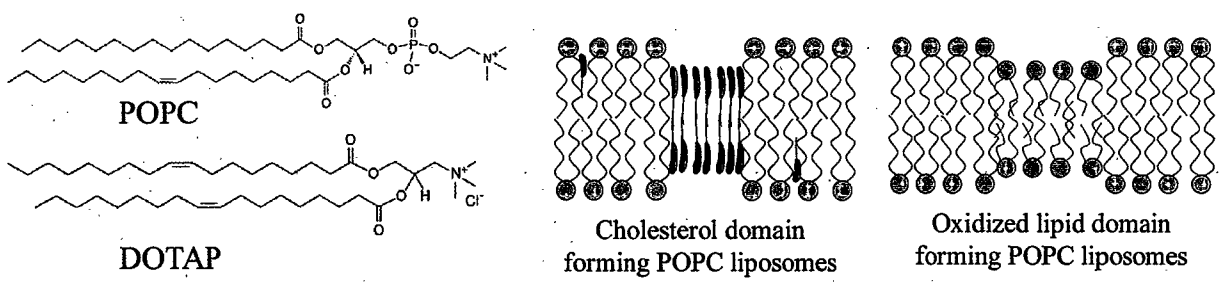


Figure 2-8 Typical lipids and domain formation models.

had a contact with the peptide fragment. Such decreases are typically observed with insertion of hydrophobic part of peptide into a membrane (Hoyt and Gierasch, 1991).

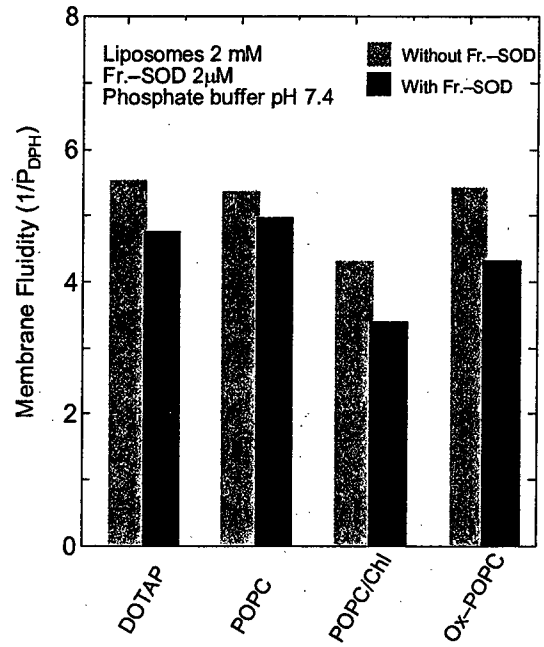


Figure 2-9 The change in membrane fluidity of typical liposomes in the interaction with the peptide fragment.

Among the standard liposomes which have been used, the above liposomes such as (2), (3) and (4) have different characteristics from the standard liposomes. In the case of (2) DOTAP, it basically has a positively charged surface and can therefore be utilized as a gene vector of DDS because of its electrostatic interaction with the negatively charged cell membrane. In the case of (3) POPC/cholesterol, it has been reported that the microdomain structure could form on its surface via hydrogen-bond formation between the hydroxyl-group of cholesterol and phosphate group of lipid. Recent results show that the hydrogen bond of the POPC/Ch liposomes is not stable and can be stabilized via the interaction with proteins possessing their unstable hydrogen bonds (Yoshimoto, 2005). In the case of (4) oxidized POPC, the aldehyde group could be formed inside the hydrophobic tails and, similarly in the case of

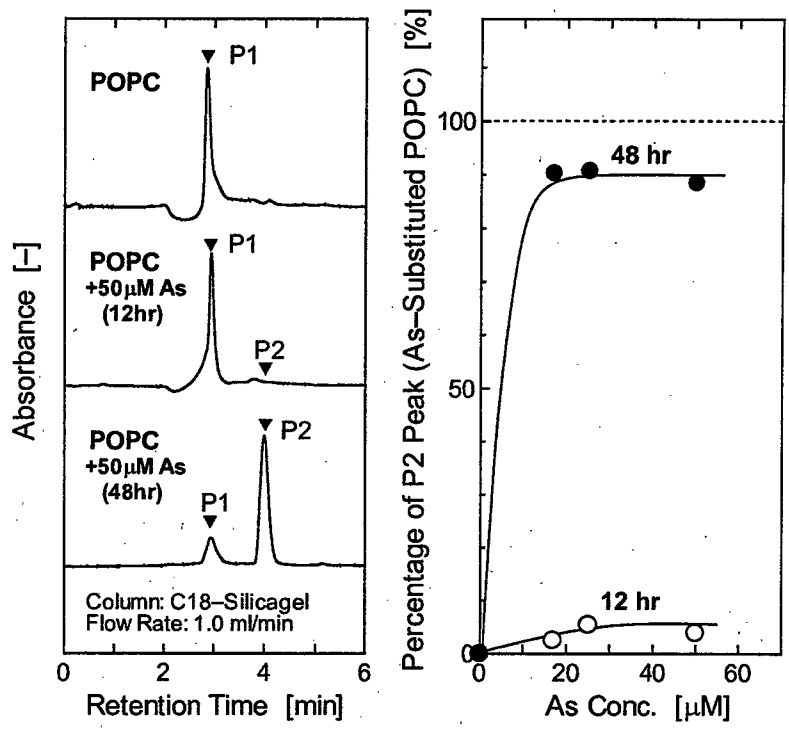


Figure 2-10 RP-HPLC analysis of arsenic-substituted POPC. POPC 5 mM was incubated with arsenate 50 μM at 30^0C in phosphate buffer (pH 7.3). POPC-bound arsenic lipids were detected by an RP-HPLC. Elution profiles were monitored at 254 nm on the UV detector. The mobile phase of acetonitrile/water (65/35 v/v) with a flow rate of 1 mL/min was applied at 30^0C. An Inertsil ODS-SP column (0.46 cm x 2.5 cm) was used throughout this study. P1 represents POPC. P2 represents a product of the reaction between POPC and arsenic.

POPC/Ch, the hydrogen bond of liposome membrane could be destabilized. Based on the above results, all the above liposomes have "abnormal characteristics" in surface charge for DOTAP and in hydrogen-bond stability for POPC/Ch and Ox-POPC.

There are some other possibilities of such a membrane abnormality. Especially, when the metal ions were added in the system, the membrane properties could be modulated through (i) its binding to membrane surface (Ca, Ni, Cu, Fe, Zn, etc), (ii) substitution of atom (As, Hg, etc), and (iii) the oxidation of membrane species via

redox turnover of the metal ions (Cu, Fe, Mn, etc) (Nagami et al., 2005). One possible example of the effect of metal on such an abnormal state of membrane is "As". As ion has both (ii) and (iii), resulting in the variation of the nature of phospholipid (**Figure 2-10**). It has recently been reported that a new series of cationic phospholipids characterized by a cationic charge with a phosphorus or arsenic atom is being developed (Stekar, 1995 and Guenin, 2000). The transfection activities of new cationic As-phospholipids were studied *in vitro* in different cell lines (HeLa, CFT1, and K562) and *in vivo* using a luciferase reporter gene. It was also demonstrated that cation substitution on the polar domain of cationic phospholipids (N replaced by P or As) resulted in a significant increase in transfection activities in both the *in vitro* and *in vivo* assays, as well as a decrease in cellular toxicity (Floch et al., 2000). In this way, the As-treatment of the lipid (liposome) could also provide "abnormalization" of liposome surface (Tuan et al., 2008).

From the above results of membrane toxicity (membrane abnormalization), the variation of a toxic state of membrane is considered to be related with a protective and resistant mechanism of membrane under strong stress condition via the induction membrane-recruited activity to display the LIPOzyme activity. Consequently, membrane can adopt the severe stress to produce the adaptive functions to protect its

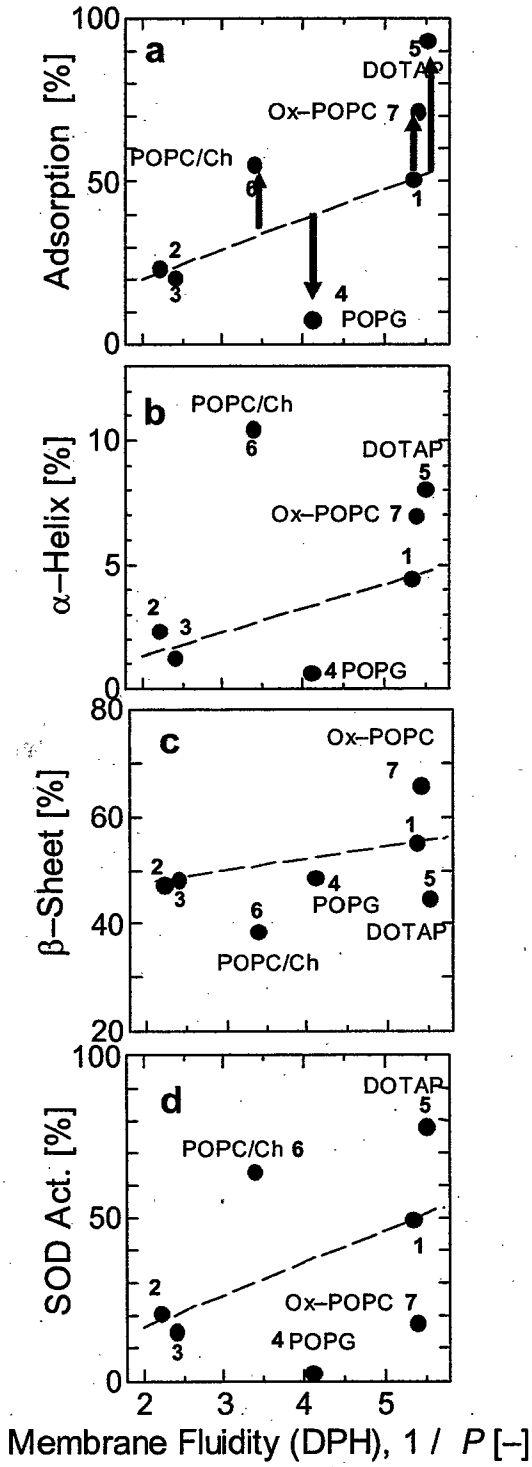


Figure 2-11 Plot of Membrane Fluidity and Elemental Steps of LIPOzyme activity

interiors from the lethal damage in biological system. The membrane toxicity could, in the future, be studied by using the "liposome"-based analytical method, such as (metal affinity-) immobilized liposome chromatography and membrane chip.

3.5. Analysis of Elemental Process of SOD LIPOzyme Activity.

The liposome-recruited activity of the oxidized and fragmented SOD was found to consist of the following steps: (i) peptide fragment binding, (ii) folding of the fragment on the liposome surface, and (iii) activity center formation on the surface. These elemental steps on the liposome-recruited activity were further investigated by studying their relationship. **Figure 2-11** shows the relationship between membrane fluidity,

measured by DPH, and (a) peptide binding, (b) contents of α-helix, (c) those of β-sheet, and (d) SOD-like activity. The data seem to be scattered although there are some trend of the relationship.

In the case of peptide binding (**Figure 2-11(a)**), it seems that there is a linear relationship among the PC liposomes (Nos.1-3), showing that the binding of peptide on the PC surface was basically dependent on the membrane fluidity of liposome and (local) hydrophobicity of the peptide. In the case of positively charged liposomes (No.5), the binding was enhanced because of the additional electrostatic interaction between the negatively charged domain of the peptide and the positively charged liposome surface. When the liposome surface was negatively charged, the peptide binding was suppressed because of the electrostatic repulsive force among them (No.4). POPC/Ch (No.6) and Ox-POPC liposomes (No.7) are exceptional for the above tendency. It has been reported that the POPC/Ch liposome shows higher affinity against the low ρ proteins, which has unstable hydrogen bonds in its main peptide structure, because of the microdomain formation on the liposome surface and change the hydration state of the microdomain surface (Yoshimoto, 2004). In the case of oxidized-POPC liposomes, the acyl chain of the lipid molecule could form the aldehyde group on the edge of the lipid, resulting in the perturbation of oxidized domain of the membrane and the formation of hydrogen bond donor inside the hydrophobic environment (Shimanouchi, 2005). If the stability of the hydrogen bonds of membrane itself was varied, the peptide with lower stability of the hydrogen bonds could easily be bound on the surface because of the SOD fragment properties,

identified in **Figure 2-7**, has lots of "under-wrapped" hydrogen bonds on its surface. Although the precise situation of both membranes was different, the decrease of the hydrogen bond stability of the liposome surface could be related with this increase of the binding of the peptide. As a short summary on the first elemental process, the peptide binding was found to be controlled by the integrated interactive forces of (i) electrostatic, (ii) hydrophobic interactions and (iii) hydrogen bonds.

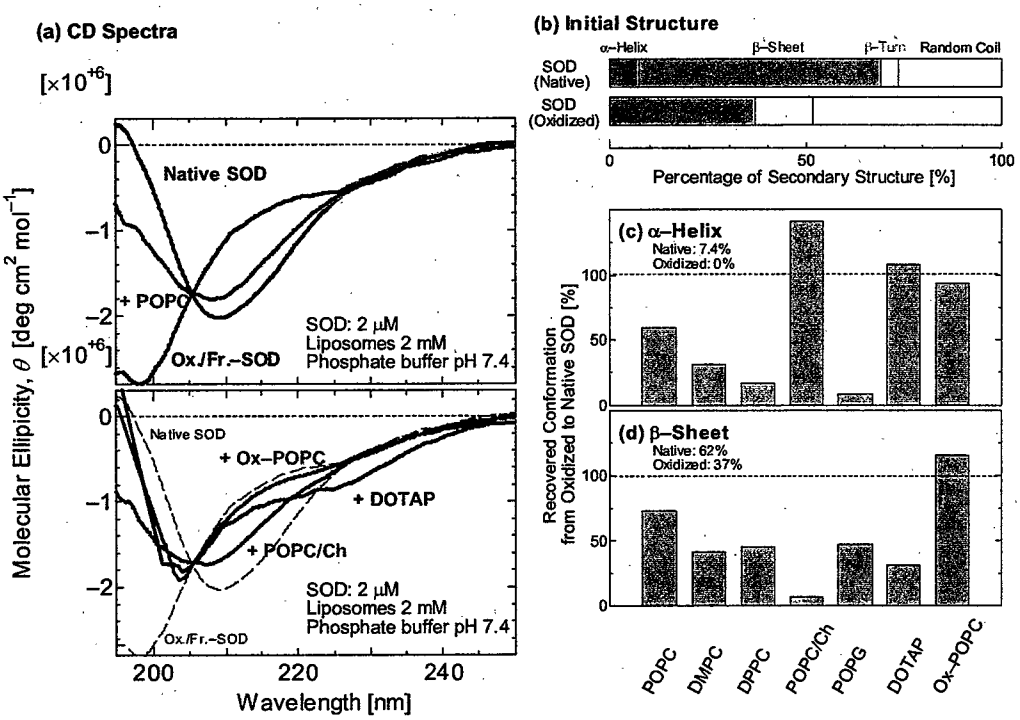


Figure 2-12 Recovery of Secondary Structure of Oxidized and Fragmented SOD by the Addition of Liposome. (a) CD Spectra of Oxidized and Fragmented SOD with Various Kinds of Liposome. (b) Percentage of Secondary Structure of Native SOD and Oxidized SOD. Recovery of Secondary Structure of (c) α-Helix and (d) β-Sheet after Liposome Addition (100% and 0% of Secondary Structure was respectively set at contents of Native and Oxidized SOD).

The recovery of secondary structure of fragmented SOD by the addition of liposomes was analyzed. The result indicated that α-helix contents increased in the case of POPC, POPC/Chl, DOTAP liposomes, resulting in the increase of SOD-like

activity of LIPOzyme (**Figure 2-12**). However, in the case of Ox-POPC liposomes, the α-helix contents increased but SOD-like activity of this complex did not increase. It could be due to the strong interaction of fragment and Ox-POPC liposomes and induction of the other function (to be discussed in chapter III).

In the case of folding process (**Figures 2-11(b)(c)**), the data of the contents of secondary structure, such as α-helix and β-sheet, were normalized by using the binding data in order to eliminate the effect of the difference of the peptide binding. **Figures 2-13** show the relationship between membrane fluidity and percentage of

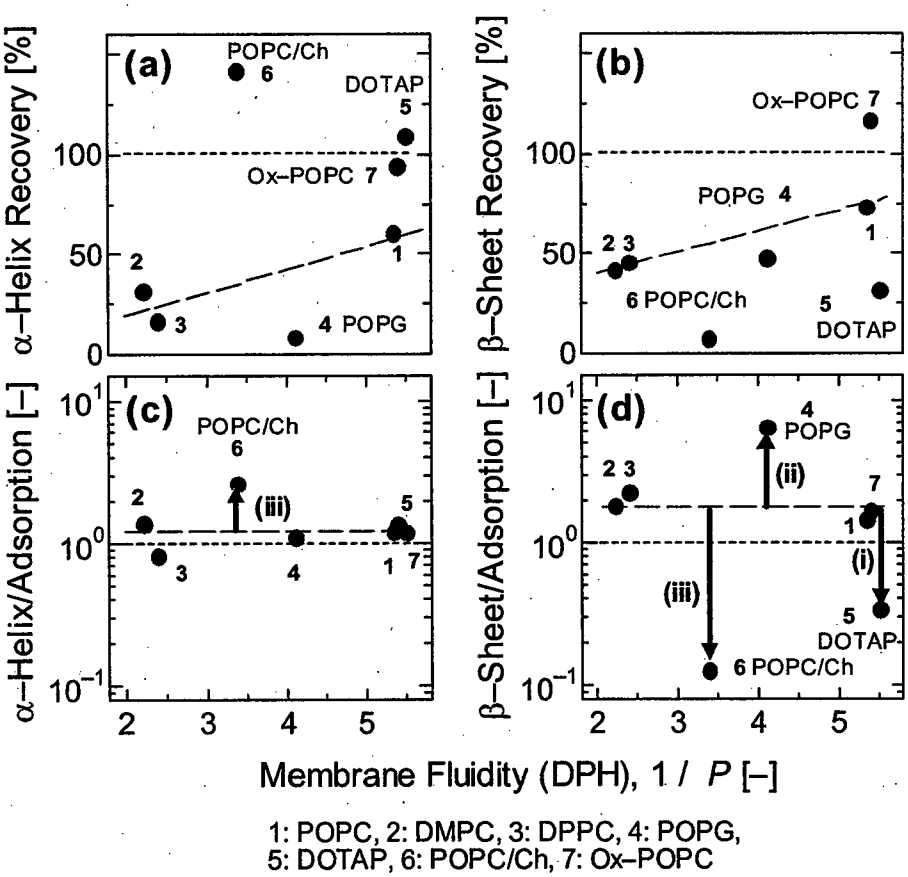


Figure 2-13. Relationship between Membrane Fluidity and Percentage of Recovery of Secondary Structure of (a) α-Helix and (b) β-Sheet after Liposome Addition. (c) and (d) show the normalized value of percentage of secondary structure of the fragmented peptide.

recovery of secondary structure by liposomes. If the folding of the peptide could correspond with its adsorption, the data would be clustered on the dotted line (**Figure 2-13(c) and (d)**). In the case of the α-helix (**Figure 2-13(c)**), only one exceptional data was observed in the case of POPC/Ch liposomes, showing that the POPC/Ch surface can enhance the α-helix formation. In the case of the β-sheet (**Figure 2-13(d)**), there are three exceptions: decrease in POPC/Ch and DOTAP liposome and increase in POPG liposome. The folding of the recruited peptide was thus considered to basically depend on the binding of the peptide and there are three exceptions; (i) POPC/Ch increase α-helix and decrease β-sheet, (ii) POPG increase β-sheet, and (iii) DOTAP decrease the β-sheet.

The last step is the formation of activity center to induce the SOD-like activity. The data of **Figure 2-11(d)** were similarly normalized by the peptide adsorption as shown in **Figure 2-13(c)** and **(d)**. The data of PCs and DOTAP (Nos.1-3, 5) were scattered on a single line, showing that these liposomes similarly bind and fold the peptide to induce the expected SOD enzymatic activity. In the case of POPC/Ch liposome, the data was shifted positively (No.6), probably caused by the conformational modulation (increase of α-helix and decrease of β-sheet). On the contrary, POPG liposome negatively affected to the SOD-like activity, implying that the excess formation of β-sheet could inhibit the activity center formation on the surface. Unexpected from the binding and secondary structure, the oxidized POPC

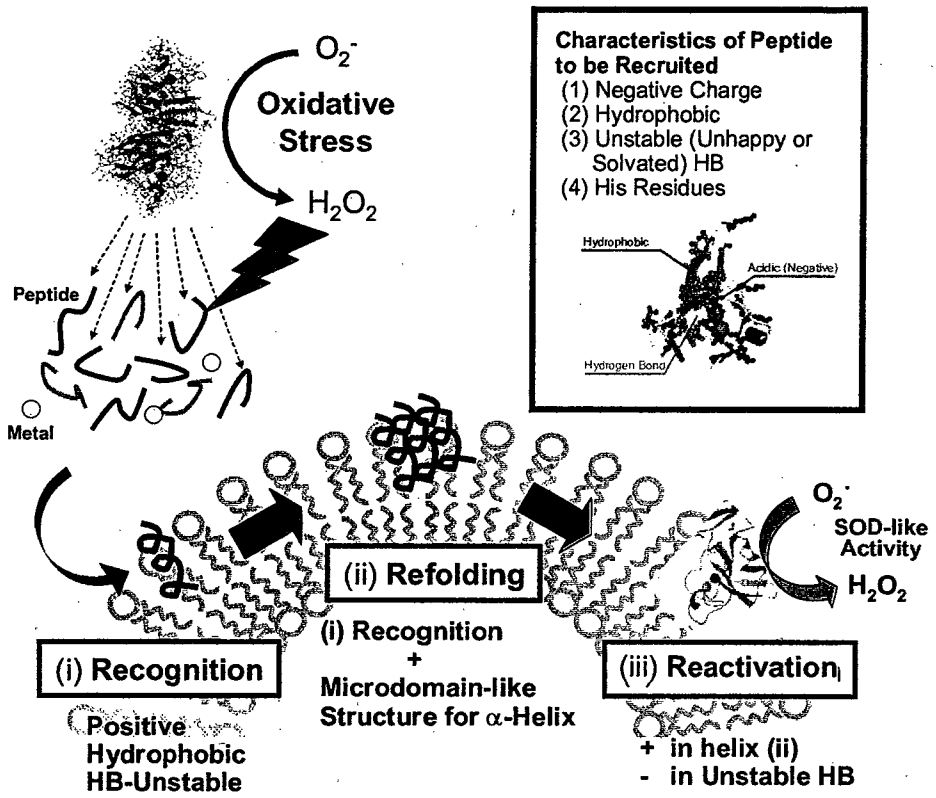


Figure 2-14 Summary of Display of SOD LIPOzyme Based on SOD Fragment

liposomes also induce the negative effect on the SOD activity, implying the different effects of the oxidized POPC on the activity center formation. Although some points should further be investigated, the total process of the liposome-recruited activity was summarized and schematized in **Figure 2-14**.

Based on the above results on the liposome-recruited activity of oxidized and fragmented SOD, the strategy for the design of the antioxidative LIPOzyme was established and was shown in **Figure 2-15**. After one would select the peptide library, the LIPOzyme optimization could be performed by checking the (i) binding, (ii) folding and (iii) active site formation. In the case of SOD-LIPOzyme, the peptide mixture of the oxidized and fragmented SOD was used and the processes (i)-(iii) were examined by using the standard liposomes which differs in hydrophobicity, surface

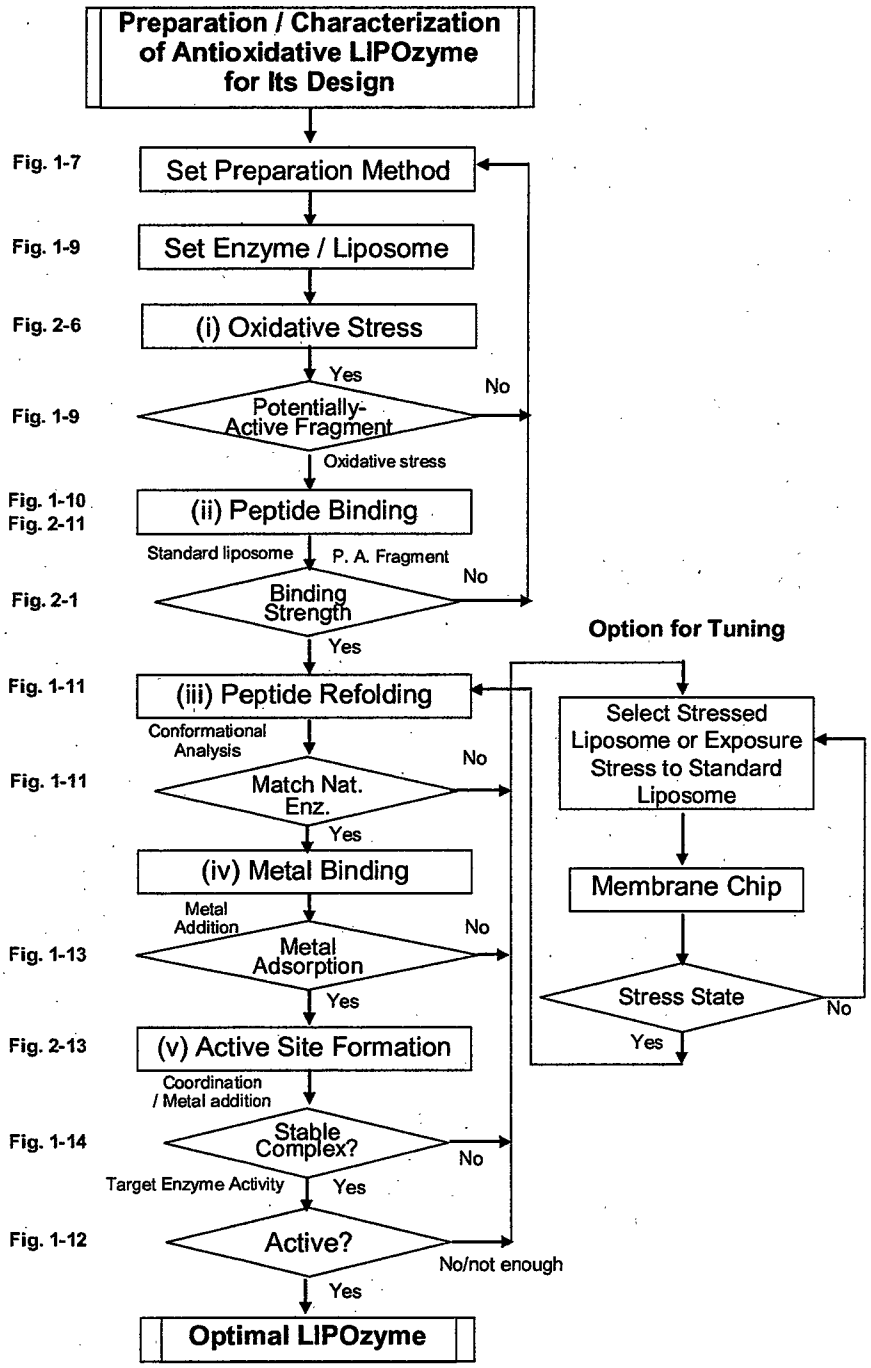


Figure 2-15 Summary of Preparation / Characterization of Antioxidative LIPOzyme

3.6 Comparison of SOD LIPOzyme with Previous SOD Mimics

The SOD activity level is compared among natural SOD and its mimics as shown in **Figure 2-16**. There are several ligands in order to induce the SOD-like activity. Ohtsu et al have already shown the highest SOD activity level (Ohtsu et al., 2000). In the case of "Build-Up" type LIPOzyme, the maximal SOD activity was approximately 5%, which is more than conventional oligopeptide mimics and is less than the above Fukuzumi's SOD. On the contrary, the SOD activity level was significantly increased when the SOD LIPOzyme was prepared by using the "Break-Down" type approach, resulting in the more than 78% (Fr.-SOD/DOTAP) of native SOD, showing the significance of the LIPOzyme design using break-down approach. However, to obtain the optimal lipozyme, the POPC/Chl 28% is the best choice because of its stability under continuously oxidative stress (Chapter III).

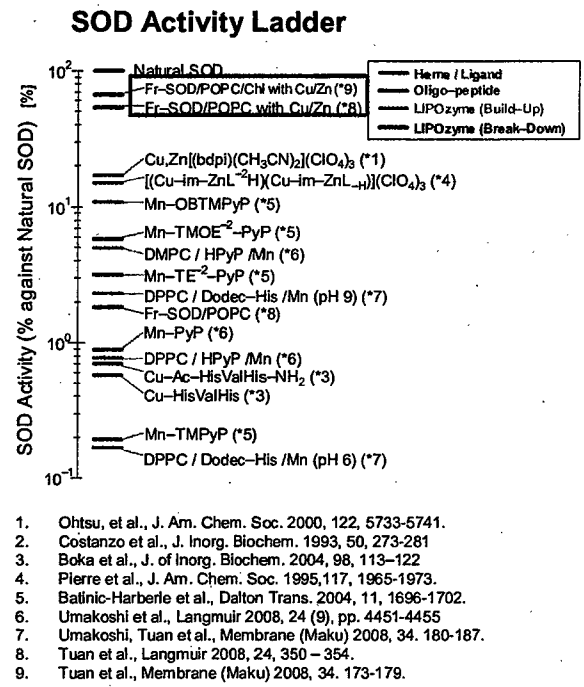


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Figure 2-16 Ladder for SOD Activity

4. Conclusion

The preparation of SOD LIPOzyme from (i) "build-up" and (ii) "break-down" approaches was described in chapter I, resulting that the higher efficiency was

obtained in the case of the latter case. The method to prepare the LIPOzyme was shown by clarifying the membrane characteristics and the elemental processes of liposome-recruited activity (binding, refolding and reactivation). First, the addition of zwitterionic liposomes with high membrane fluidity or that with positive charge was found to increase the SOD-like enzymatic activity of fragmented SOD although the negatively charged liposome has no effect on its activity. The analyzed characteristics of the peptide, together with the above findings, imply that the liposome-recruited activity of the fragmented SOD was related to the recognition of the SOD fragment by the liposome caused by the combination of electrostatic and hydrophobic interaction, and hydrogen bonding between the peptide and liposome membrane. The ligand-modified membrane also possessed the SOD-like enzymatic activity. It was easily considered that the modification of active site from the suitable ligand and liposomes requires the coordination of the imidazole group and carboxyl group of the Dodec-His on the membrane surface. The previous studies indicated that no systematic investigation of the possible role of specific lipids or the chemical properties of membrane lipids on the yield of refolding, formation of refolding intermediates, or alteration of the refolding pathway was included in these studies. Membrane protein folding is more complex than interaction with the lipid bilayer approximated as a simple hydrophobic core bounded by water interfaces (Bogdanov and Dowhan, 1999). The obtained results provided an evidence that the damaged membrane (Ox-POPC), domain formed membrane (POPC/Ch) possessed the different characteristics from the others. Therefore, they can easily recognize the damaged peptide (fragment derived from the oxidation of SOD), then refold the damaged peptide fragment. The becoming

damage is considered to be related with the function of membrane to resist and tolerance to the lethal stress such as toxic stress of heavy metals. The damaged membrane in resistance to the toxicants and in recruitment of fragmented SOD will be discussed more detail in the following sections, particularly focusing on the regulation of enzyme-like activities of LIPOzyme on the membrane surface under continuously oxidative stress. The method to prepare the LIPOzyme was finally summarized as a scheme based on the above findings.

Chapter III

Preparation of Antioxidative LIPOzyme based on Modification of Peptide Fragment under Stress Condition

1. Introduction

The membrane activity in recruitment and reactivation of damaged peptide was discussed and clarified in the previous chapters through the case study of the SOD LIPOzyme preparation using the oxidized and fragmented SOD. In process of recruitment of "potentially-functional" elements, a membrane was shown to act as a metal and molecular chaperone for the specific fragment of SOD, resulting in the recognition, conformational changes, and its reactivation to induce new SOD-like enzyme. The role of the membrane activity and membrane toxicity has to be studied in relation to the creation of a new enzyme complex displaying the bifunctional enzymatic activities to repair the stressful environmental condition. A "membrane toxicity" can herewith be defined as the toxic state of membrane under toxic stress or oxidative stress as schematically shown in **Figure 3-1**. The membrane toxication could be induced when the membrane was

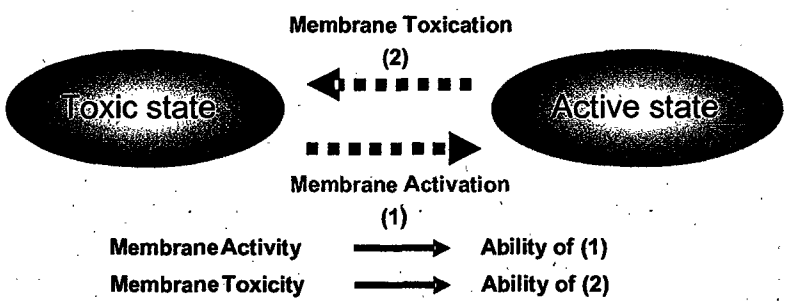

Figure 3-1 Definition of membrane toxicity and membrane activity.

exposed to the above stress condition. The destruction of the membrane structure and its functions could induce the cell death. The membrane could have an ability to inversely activate its toxic state and to repair the toxic or stressed environment surrounding the membrane.

Some examples of the "possible" activity of the membrane in a biological system have been reported in the previous researches. In biological systems, the membrane can act as a physical boundary to pack various kinds of bioelements needed to maintain the cellular homeostasis and, also, an active interface that protects the interiors from the damages caused by ROS (Pamplona et al., 2002). The accumulation of toxic materials induces the loss of the membrane integrity, and an increase in permeability to protons and ions (Duan et al., 2003). In that case, the state of membrane becomes toxic and may affect its surrounding environment. However, organisms, especially microorganisms, have some mechanisms occurring on the membrane to monitor the toxic stress. The adaptation of membrane to toxic stress or oxidative stress is necessary for cells in environment. The previous study relating to membrane toxicity indicated that the interactions of the ROS with hydrophobic parts of the cell could play an important role in the mechanism of the toxic action (Shirai, 1987). With respect to the adaptation to toxic effects of lipophilic compounds, these

compounds are not relevant. Changes in the hydrophobicity of the cell wall may be of more interest. It has been shown that bacteria with hydrophobic cell walls have a higher affinity for hydrophobic compounds than do bacteria with more-hydrophilic cell walls (Jarlier and Nikaido, 1994; van Loosdrecht et al., 1990). In previous report, under toxic stress of copper, one major mechanism of copper toxicity towards microorganisms is the disruption of plasma membrane integrity, where the variation of the fatty acid composition of the plasma membrane was found to dramatically alter the susceptibility of *Saccharomyces cerevisiae* to Cu^{2+} toxicity (Avery et al., 1996). The membrane seems to become tolerant with the stresses, especially oxidant stress and toxic stress, through the induction of possible potential functions of the membrane itself.

Antioxidative enzymes are commonly known to act against such oxidative stress. As mentioned in the above sections, the ROS has been reported to cause various kinds of damage, such as membrane lipid peroxidation, DNA oxidation, and enzyme inactivation. In those cases, the role of antioxidants was mentioned as an eliminator of ROS. SOD, which is one of the most important antioxidative enzyme, catalyzes the dismutaion of the

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Figure 3-2 The reactive elimination of reactive oxygen species by SOD and CAT

superoxide ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) and molecular oxygen. Hydrogen peroxide is also an oxidant needs to be cleared from the living organism. Therefore, catalase (CAT) further converts hydrogen peroxide into water and molecular oxygen. These sequential reactions to eliminate the ROS can be summarized in the **Figure 3-2.**

***(1) Superoxide Dismutase*:** SOD outcompetes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. The reaction of superoxide with non-radicals is spin forbidden. In biological systems, this means that its main reactions are with itself (dismutation) or with another biological radical such as nitric oxide (NO). The superoxide anion radical ($O_2^{\cdot-}$) spontaneously dismutes to O_2 and hydrogen peroxide (H_2O_2) quite rapidly (~10^5 M^{-1} s^{-1} at pH 7). SOD is biologically necessary because superoxide reacts even faster with certain targets such as NO radical, which makes peroxynitrite. Similarly, the dismutation rate is second order with respect to initial superoxide concentration. Thus, the half-life of superoxide is actually quite long at low concentrations (e.g. 14 hours at 0.1 nM), although it is very short at high concentrations (e.g. 0.05 seconds at 0.1 mM). In contrast, the reaction of superoxide with SOD is first order with respect to superoxide concentration. Moreover, superoxide dismutase has the fastest turnover number (reaction rate with its substrate) of any known enzyme (~10^9 M^{-1} s^{-1}) (Klug-Roth, et al., 1972, 1973; Imply and

Fridovich, 1991). The above clear descriptions of SOD reflect that SOD is an indispensable antioxidant in all living organisms. ***(2) Catalase:*** Besides of the SOD, CAT also plays a role at the end of the ROS elimination by converting hydrogen peroxide into water. CAT is a common enzyme found in nearly all living organisms. Active site of catalase mainly contains Fe or Mn within the hem group (Kono and Fridovich, 1983; Shima et al., 2001; Honor et al., 2006). Catalase also has one of the highest turnover rates of all enzymes; one molecule of catalase can convert millions of molecules of hydrogen peroxide to water and oxygen per second (Beer and Sizer, 1953; Maehly and Chance, 1954). The combination of SOD and CAT in biological system is necessary.

A "membrane" under the oxidative stress could display the above antioxidative functions such as SOD and CAT in the replace of the natural enzymatic system in order to repair its toxic state and, also, the toxic environment. One of the responses of the membrane (liposome) is the SOD LIPOzyme, prepared through the recognition, folding and activation of the specific fragment of oxidized SOD as described in chapter I and chapter II. One of the antioxidative machinery could be supported by such a "membrane activity". The above functions of membrane itself could further be applied to the preparation of the biomimetic materials, such as

artificial enzymes or enzyme mimics, which have different kinds of functions. In the conventional approach, some researchers have attempted to investigate the roles of SOD and CAT by using the covalent conjugates between the vascular wall glycosaminglycan chondroitin sulfate (CHS) and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) (Maksimenko et al., 2004). The conjugation of SOD and CAT by CHS, as the result, represented both enzymatic activities with a high effectiveness. The membrane-incorporated or -encapsulated enzymes have been conducted to maintain the lifespan of enzyme *in vitro* experiments (Turrens et al., 1984; Niwa et al., 1985, Michelson and Puget, 1986), as well as in implication of disease treatment (Petelin et al., 2000; Freeman et al., 1985; Ledwozyw, 1991; Thibeault et al., 1991) by the transfer of enzymes into the disease body. Based on the characteristics of the membrane, one also designated and controlled the enzymatic activity of membrane-carried enzymes (Yoshimoto et al., 2007). In the above examples, there are some unsuccessful aspects such as (i) less efficiency, (ii) the difficulty in design, and (iii) the low stability of the catalysis. It is expecting that new type of bi-enzymatic mimics could be prepared by utilizing the "membrane activity", clarified in the previous chapters.

In this chapter, the bifunctional enzyme which expresses the simultaneously

SOD-like and CAT-like activity was prepared by utilizing the response of membrane itself under the additionally or strongly oxidative stress of hydrogen peroxide, focusing on the LIPOzyme preparation based on "membrane toxicity". The oxidized liposomes and cholesterol liposomes were found to play a significant role in LIPOzyme displaying process, including recruitment, reactivation and regulation of SOD fragments derived from the oxidation and fragmentation of SOD. In this case, oxidized and cholesterol liposomes are considered as the toxic membranes. It is expected that these toxic membranes could have a significant potential. To elucidate several "anti-stress" functions, in converting superoxide to hydrogen peroxide (SOD-like activity), and then, hydrogen peroxide to water and oxygen (CAT-like activity). It has been shown that the complex represents a potential function which continuously converts hydrogen peroxide to water and oxygen to protect SOD LIPOzyme complex from the further oxidation, although the CAT-like activity of enzyme complex is not so high in comparison to native catalase,.

2. Materials and methods

2.1 Materials

Bovine Erythrocyte Cu,Zn-SOD (EC 1.15.1.1), purchased from Sigma Aldrich with a specific activity of 4470 U/mg (product No. S2515-30KU and lot No.

125K740), was used without purification. Catalase, obtained from Sigma Aldrich in crystalline form with a specific activity of 10,000 units/mg, was used without purification. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (POPG), 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) all purchased from Avanti Polar Lipids, Inc. (AL, USA) were used for the liposome preparation. All the other reagents of analytical grade were purchased from Wako Pure Chemicals (Osaka, Japan).

2.2 Preparations of Liposomes

The lipids were dissolved in chloroform/methanol (10 mM lipid prepared will be diluted to 2 mM for each experiment). POPC/Chl 28% was composed form POPC and cholesterol with 28% mole in comparison to POPC. After the solvent was evaporated, the resulting thin film was dried for at least 2 hours under a vacuum. The lipid film was hydrated by 50 mM potassium phosphate buffer to form the multilamellar vesicles. 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. Preparation of oxidized liposomes was processed in following steps. Pre-incubation 2 mM POPC with 2 mM H_2O_2 and 1 mM

Cu^{2+} in 24 h. After oxidation of POPC, a purification of oxidized POPC (ox-POPC) was carried out by chloroform:methanol:water (2:1:0.8 v/v) as described somewhere (Bligh and Dyer, 1959). Ox-POPC liposomes made from purified POPC lipids were experimentally conducted with Fr.-SOD as the same case of POPC liposomes in recruitment of fragmented SOD.

2.3 SOD Activity Analysis

For the 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 (Peskin et al., 2000).

2.4 CAT Activity Analysis

The CAT activity was measured by Catalase Assay Kit though the reduction of hydrogen peroxide by the enzyme complex. This assay method is based on the measurement of the hydrogen peroxide substrate remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen (catalatic pathway)

and then this enzymatic reaction is stopped with sodium azide. An aliquot of the reaction mix is then assayed for the amount of hydrogen peroxide remaining by a colorimetric method (Fossati et al., 1980). The colorimetric method uses a substituted phenol (3,5-dichloro-2-hydroxybenzenesulfonic acid), which couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase (HRP) to give a red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonatep-benzo-quinone-monoimine) that absorbs at 520 nm.

2.5 Circular Dichroism Analysis.

The secondary structure of SOD was determined using circular dichroism analysis (J-720W spectrometer; JASCO, Tokyo, Japan) at 37 ^{0}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.

2.6 Treatment of SOD and Fragmented SOD.

The SOD, totally oxidized into fragments for 12 h in 2 mM H_2O_2, was ultrafiltered by a 10 kDa molecular cutoff filter to obtain the potentially active debris (fragmented SOD) containing the active sites (Ramirez et al., 2005; Tuan et al., 2008). The 10 kDa fragments filtrated from ultrafiltration were incubated with POPC 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 ^{0}C for 12 h. The enzymatic activity of the complex of liposomes, fragmented SOD, and metal ions was determined. Structural change in fragmented and recruited SOD was observed by CD spectra.

2.7 Statistical Analysis.

Results are expressed as means ± standard deviation (SD). 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

3.1 Active Center for Cu,Zn-SOD and Mn-CAT.

The active sites of SOD and CAT were attempted to be mimicked to investigate the possible formation of bifunctional LIPOzymes. the active site of

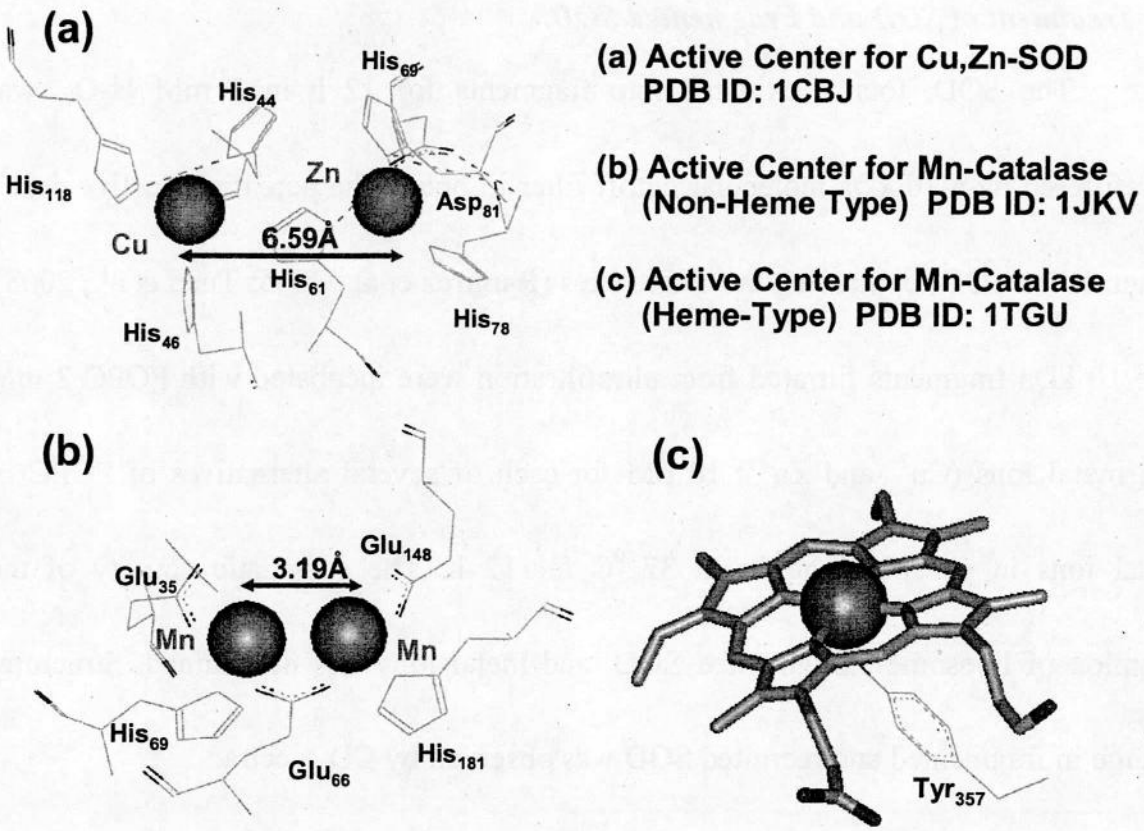

Figure 3-3 Active Center of Cu,Zn-SOD and Mn-Catalase

LIPOzyme. The active centers of the SOD and CAT were first studied based on the structural data of these enzymes (**Figures 3-3**). SOD has a bimetal active center consisting of the six His residues and one Asp residue, which coordinate copper and zinc (**Figure 3-3(a)**). Mn-CAT also has a bimetal active center of Mn-Mn, coordinated by three Glu residues and two His residues (**Figure 3-3(b)**). In contrast to the active center of heme-type catalase (**Figure 3-3(c)**), the distance of bimetals is well regulated in both SOD and CAT, where the former distance (6.59Å) is wider than that of the latter (3.19 Å). The basic information of the above enzymes was also studied in relation to the secondary structures, charge, hydrophobicity, and hydrogen bond

stability as shown in **Figure 3-4**. The yellow bands show the position of amino acid residues consisting of the active center of Cu,Zn-SOD and Mn-CAT, where the relatively-uniform characters are observed in the amino acid residues consisting of active center. The above amino acid residues show (i) positive charge, (ii) hydrophilic, and (iii) stable hydrogen bond nature although the other amino acids neighboring to them or between them show opposite characteristics, such as (i)' negative charge, (ii)' hydrophobic, and (iii)' unstable hydrogen bond nature. In practice, the oxidized and fragmented SOD (Fr-SOD), which has shown in chapter II, has the above latter characteristics of (i)'-(iii)' and further has a hydrophobic surface (region clustering hydrophobic residues) on the back of the Cu/Zn binding sites and negatively charged

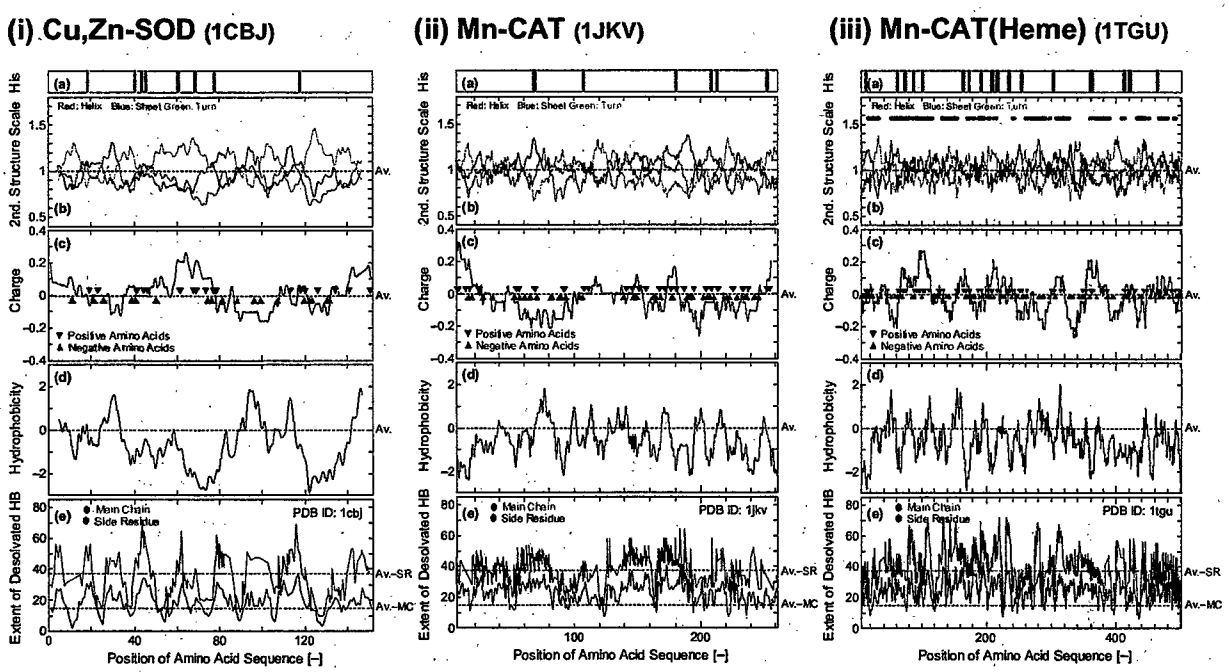

Figure 3-4 Basic Characteristic Profiles of Cu,Zn-SOD and Mn-Catalase

surface on the same side of the metal binding sites. The above characteristics positively affect the induction of SOD-like activity because the Fr-SOD could be bound on the liposome surface through the integrated interaction and could also be

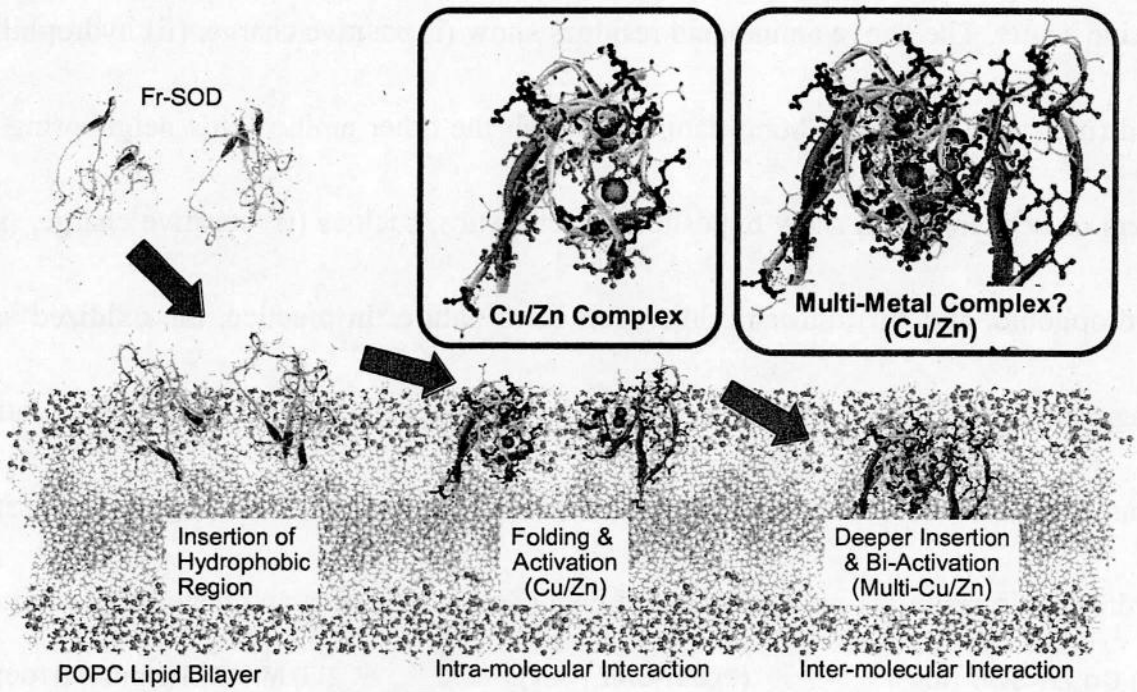

Figure 3-5 Speculative Illustration of Possible Destiny of Fr-SOD in Membrane

folded by the stabilization effect of hydrogen bond in the hydrophobic environment inside the lipid membrane. Such a series of process could be modulated depending on the characteristics of the membrane itself as described in chapter II (**Figures 2-1** and **2-8**). The speculative illustration of the possible actions of Fr-SOD in membrane has been shown in **Figure 3-5**. Although only the SOD-like activity was focused in chapter I and II, the inter-molecular complex of the Fr-SOD inside the membrane could also be

formed and the membrane characteristics could be dynamically changed under the stress condition. In such cases, it is possible that the bimetal complex between the Fr-SOD and another Fr-SOD was formed on the surface. In chapter II, the membrane fluidity was shown to be reduced especially at the interior of the lipid membrane (**Figure 2-8**) and there are some mismatch between the secondary structure and SOD activity (**Figure 2-11**). The above results may imply that there is an existence of the "inter-molecular" complex of Fr-SOD, which could induce the "CAT"-like activity. Although there are many reports on the SOD mimics by using the biomimetic synthetic ligands or oligopeptides, few examples of SOD-like model complexes have been investigated as catalysts to convert the hydrogen peroxide into water and molecular oxygen. A statement has been confirmed from various results that a good SOD-model complex should first be a good CAT-like model complex (Gao et al., 2001).

The Mn catalase (Non-Heme) has reported to be a hexamer of the α-helix-rich subunit as shown in **Figure 3-6(a)**. The active center composed on bimetal complex of Mn (**Figure 3-3(b)**) is surrounded by the α-helix structures which are abundant in the hydrophobic residues (**Figure 3-6(b)**). If the possible bi-metal

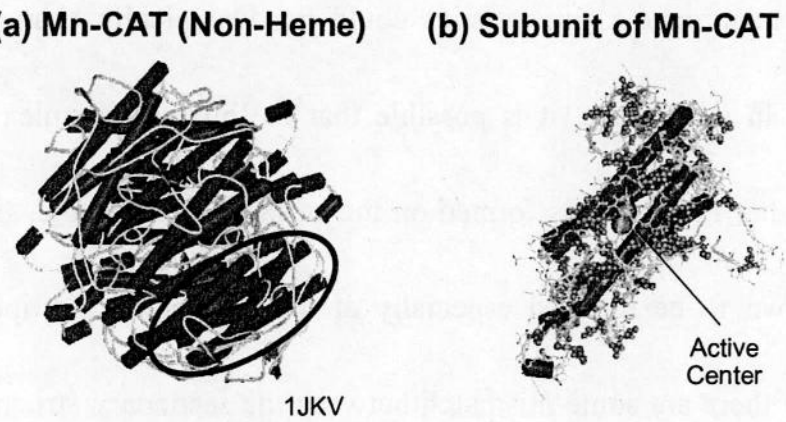

Figure 3-6 Structure of Mn-Catalase (Non-Heme)

complex could be formed on the membrane (**Figure 3-5**), the induction of CAT-like activity has been achieved. Similarity of the amino acid sequence between the CAT and the SOD fragments, which were identified in chapter II and includes the active center forming amino acids, was studied as summarized in **Table 3-1**. The results showed that the similar conformation of the peptide, possibly forming active center of

Table 3-1 Partially-matched Alignment of Amino Acids between SOD and CAT.

SOD (PDB: 1CBJ)		CAT (PDB: 1JKV)	
92-102	VAIVDIVDPLI	67-77	MA**H**VEMISTMI
127-131	GNEE	63-66	GTE**E**
17-82	LGNVTADSNGV	23-39	LGGQWG**E**TTGM
74-79	DEERHV	65-70	E**E**MAHV
102-109	ISLSGEYSIIGRTMVVHE	131-148	VTSSGNLVADMRFNVVR**E**
84-89	NVTADS	144-149	NVVR**E**S
80-86	GDLGNVT	31-37	GQWG**E**TT
128-136	GNEESTKTG	30-38	GGQWG**E**TTG
75-78	EERH	178-181	ETQ**H**

* Blue font: Matched Residue, Bold/Underlined: Active Center

Mn-CAT, could also be induced in the case of oxidized fragment of SOD. In practice, the obtained fragment (Residue 66-144) is consisted of the random coil and two β-sheets in the native SOD structure. However, according to the secondary structure prediction (Chou and Fasman Method), there are some residues which tend to form the α-helix structure and with relative hydrophobic nature (for example, 75-80, 104-110, 112-122 in **Figure 3-4(i)**). The bimetal complex of Cu, to show the CAT-like activity, could be formed by forming the α-helix structure inside the membrane and inter-molecular complex of the SOD fragments.

The co-induction of both SOD and CAT is also effective from the viewpoint of the biofunctional catalysis design. The SOD-like activity of LIPOzyme has already shown to be obtained in variations of values depending on the liposome properties (chapter II). From the "Build-Up" type approach, the ligand-modified liposomes possessed the "biofunctional" enzyme-like activities (SOD and CAT) on the same spot on the liposome surface (Umakoshi et al., 2008). The design of catalase from ligand and metals has been conducted by several researchers by using the ligand complex with the Cu (II) (Gao, 2001) and Mn (II) (Sakiyama et al., 1993; Larson and Pecoraro, 1991; Mathur et al., 1987). However the catalase enzymatic activity of those complexes was not so high in comparison with the native catalase, resulting from the

slow reaction rate for ligand-modified catalase.

In the following, taken together with the view point of membrane activity in incorporation with the peptide fragment and metal ions (chapter I and chapter II), the strong stress condition was applied to check the activity of LIPOzyme in elimination of ROS by the addition of hydrogen peroxide into the formed LIPOzyme suspension. Interestingly, the LIPOzyme has possessed the CAT-like enzymatic activity by clearing the added hydrogen peroxide.

3.2 CAT-like Activity of the Liposome-Recruiting SOD Fragment.

After fragmented SOD was recruited on the liposome surface, the CAT-like activity of the liposome-recruited SOD complex was investigated. All conducted samples possess the CAT-like enzymatic activity which converted added hydrogen peroxide to water and oxygen. The results were shown in **Figure 3-7.** In previous report, the interaction of fragmented SOD and liposomes was differently depending on the characteristics of liposomes. Among the experimented liposomes, the interaction between DOTAP and Ox-POPC with fragmented SOD was the strongest. Their SOD-like enzymatic activities are significantly different, 77.6% and 17.15% for DOTAP and Ox-POPC, respectively. Otherwise, the CAT-like activities of these complexes was opposite, $1.4x10^3$ and $10.5x10^3$ $mol_{H2O2}/min/ mol_{catalyst}$ for DOTAP and

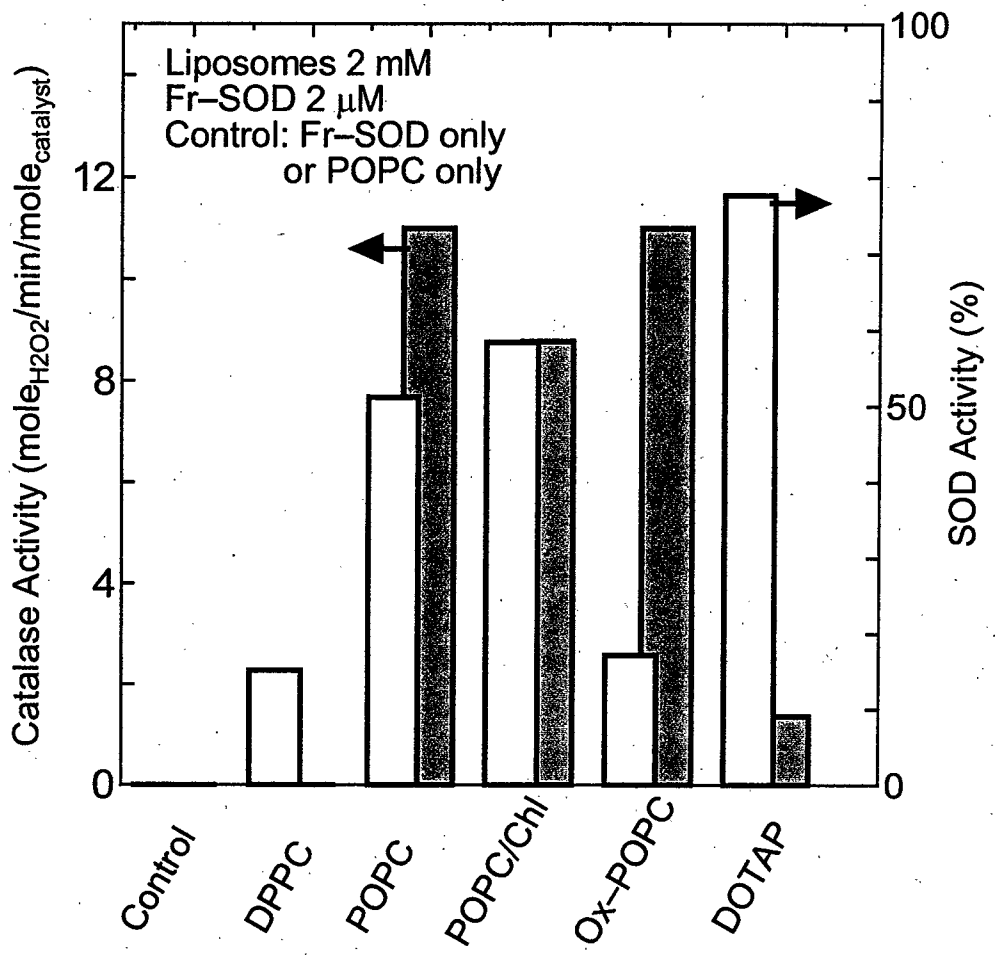

Figure 3-7 SOD-like (a) and CAT-like (b) activity of enzyme complexes under continuously oxidative stress. Hydrogen peroxide was initially added to solution of enzymes and periodically 1 h in 24 h. CAT-like activity was measured through the reduction of hydrogen peroxide. The shown values of SOD-like enzymatic activity are initial values before the addition of hydrogen peroxide. H_2O_2 added was totally cleared because of CAT-like activity after 1h of incubation.

Ox-POPC, respectively. In the case of POPC/Ch and POPC, the CAT-like activity was also high. The results indicated that the interaction between neutral liposomes and fragmented SOD mediated the high CAT-like activity. On the contrary, the interaction of DOTAP liposomes and fragmented SOD induced a less CAT-like enzymatic activity.

This may be due to the formation of CAT-like active site on the membrane surface or the conversion of active sites from SOD-like enzyme to CAT-like enzyme occur on the surface of neutral liposomes easier than that on the positively charged liposomes. The interaction of fragment and DOTAP liposomes mainly occurred on the membrane surface due to the electrostatic interaction, resulting in no insertion of peptide in the hydrophobic region to induce the CAT-like activity as well as stabilization of enzyme. The lipid oxidation by added hydrogen peroxide also plays a role in induction of CAT-like activity. The DPPC liposome, which exists at "gel-phase", is a control experiment (**Figure 3-7**). The complex of DPPC liposome and SOD-fragment induced SOD-like activity but no CAT-like activity when strong stress of hydrogen peroxide was applied. The results demonstrated that the change of characteristics of hydrophobic region of liposome enhanced the deeper insertion of fragment into membrane, resulting in the induction of CAT-like activity and stabilization of SOD-LIPOzyme. The control experiments with 10 μM copper and 2 mM POPC liposomes only were proven that copper did not affect the decomposition of hydrogen peroxide as previously reported (Yoshimoto et al. 2007).

There was not significant interaction of fragmented SOD and POPG (negatively charged liposomes) due to the conflict of their charges. However, the

cholesterol-containing POPG liposomes had a contact with fragmented SOD, resulting in the reactivation of SOD-like enzymatic activity with the value of 30%. Such interaction also mediated the CAT-like activity under continuously exposed-oxidative stress. In another experiment on domain formation effect, the CAT-like activity of POPG/Ch-fragments complex reached $8.6x10^3$ $mol_{H2O2}/min/ mol_{catalyst}$, being equal to the value of POPC/Ch-fragment complex, although POPG liposomes have not significantly interacted with the peptide fragment due to the electrostatic force of negative charge. Thus, the POPG/Ch-fragment complex has been formed and stable under strongly oxidative stress. The obtained results indicated that the domain formation could play a supporting role in the interaction between the liposome membrane and fragmented SOD. In previous study, the novel marcocyclic heterodinuclear catalase-like model complex with Cu and Zinc as the active site was successfully synthesized by Gao and co-workers. The analysis has been shown that the CAT-like activity dismuted the substrate H_2O_2 molecules through a homo-cleaving pathway. The reaction rate was estimated through the O_2 release from the CAT-like reaction. The CuZn-ligand CAT-like complex can decompose H_2O_2 with a reaction rate $9x10^3$ $mol_{H2O2}/min/mol_{catalyst}$ (Gao et al. 2001). With another aspect, the ligand-modified liposomes have both CAT-like and SOD-like functions on the

catalytic site on the membrane surface. The obtained results indicated that the characteristics of membrane also affect the modification of active sites and expression of enzyme-like activity. In previous report, DMPC liposomes itself had a CAT-like activity with an enzymatic activity of $1.4x10^0$ $mol_{H2O2}/min/mol_{catalyst}$ and POPC liposomes also possessed CAT-like activity but lesser than DMPC liposomes. The mechanism concerns to the decomposition of hydrogen peroxide depended on the physicochemical properties of the lipid head group region (Yoshimoto el al., 2007).

Briefly, both membrane and artificial complex serve as a CAT-like activity, but reaction rate was very low in comparison to native catalase. The obtained result in present study can be considered as the acceleration effect of the reaction rate in comparison with the previous results. The important expectation is that, with specific characteristics of membrane surface, liposome-recruited SOD in the present study had CAT-like activity that prevents the newly formed complex from the stress of hydrogen peroxide.

3.3 Stabilization of SOD/CAT LIPOzyme under Continuously-Exposed Oxidative Stress.

The stability of the SOD LIPOzyme was studied under the strong oxidative stress. Hydrogen peroxide was initially added to the enzyme solution and periodically 1 h for totally 12 h. Catalase activity was measured through the reduction of hydrogen

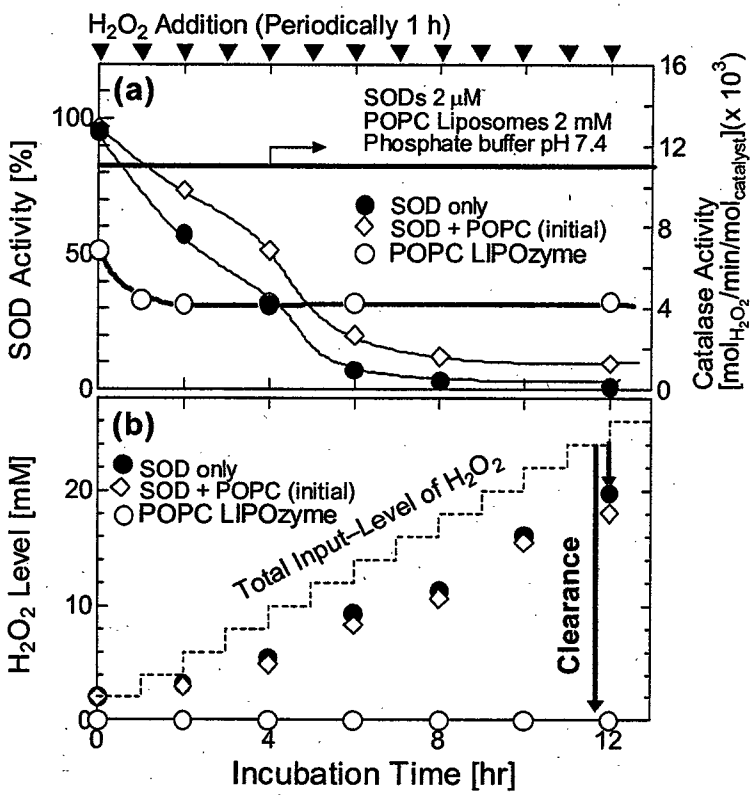

Figure 3-8 Time Course of SOD Activity of SOD, SOD with POPC Liposome, and SOD LIPOzyme under Periodical Exposurement of Oxidative Stress. 2mM H_2O_2 was added for every one hour and the sample solution was kept at 37°C. (a) shows the SOD activity of the sample and (b) shows the H_2O_2 level in the system. The dotted line in (b) shows the theoretical concentration based on the assumption that the H_2O_2 was not decomposed.

peroxide. Two mM of H_2O_2 added to suspension of the liposome-recruited SOD complex were cleared within 1 h by the induction of CAT-like activity of these complexes. Thus, the continuous addition of hydrogen peroxide was conducted to check both CAT-like activity and the stability of complexes. As shown in **Figure 3-8**, in the beginning, the SOD-like activity of complexes reduced. It is maybe because of the adaptation time of complexes to respond to strong oxidative stress.

After the adaptation process, the complexes become durable in SOD-like enzymatic activity and continue displaying the CAT-like activity. This phenomenon

indicates that the complexes of liposomes and fragmented SOD were stable under strong oxidative stress while native SOD and mixture of SOD and POPC liposome (initially) are feasible to be oxidized and fragmented. Native catalase has been reported to decrease the CAT-like enzymatic activity in a series of experiments to evaluate the enzymatic activity of the liposome-encapsulated catalase (Yoshimoto et al., 2007). The interaction between membrane and molecular enzymes maintains the stability of enzyme, especially under oxidative stress. The stability of bifunctional enzyme in the present study results from the characteristics of interaction, such as hydrophobicity, electrostatics and hydrogen bond stability. In the case of DOTAP liposomes, the interaction of liposome and fragmented SOD was strong in the recovery of SOD-like activity. However, in the continuous stress exposure (strong stress for long time), this interaction becomes unstable, resulting in the less CAT-like activity and the decrease of the SOD-like activity after only 3 h of oxidative stress of hydrogen peroxide (SOD-like activity was changed from 78% to 17%) . In the case of the Ox-POPC and POPC/Chl liposomes, the SOD-like activity was also kept while CAT-like activity was expressed simultaneously. Among the experimented liposomes, Ox-POPC was the most stable. It seemed not to be affected by the oxidative stress

condition, representing the high stability of SOD-like activity. All complexes, except for Ox-POPC liposomes, mildly declined their SOD-like activity.

Table 3-2 Kinetic parameter of CAT-like activity for LIPOzyme and other catalase

Enzyme	Substrate	Catalase*			LIPOzyme**		
		K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
POPC LIPOzyme	H_2O_2	16.39	7.5×10^3	4.5×10^5	16.39	5.97×10^5	3.6×10^7
POPC/Ch LIPOzyme	H_2O_2	99.59	67.29×10^3	3.4×10^5	99.59	26.8×10^5	2.7×10^7
OxPOPC LIPOzyme	H_2O_2	80	99.5×10^3	4.0×10^5	99.5	31.87×10^5	3.2×10^7
DMPC LIPOzyme	H_2O_2	25.7	20	7.7×10^2	25.7	7.97×10^2	3.1×10^4
POPG LIPOzyme	H_2O_2	No activity			No activity		
Horse liver catalase	H_2O_2	1.23	1.25×10^5	10.2×10^7	This study		
$[Mn(bpia)(\mu\text{-}OAc)]_2(ClO_4)_2$	H_2O_2	31.5	1.1×10^3	3.4×10^4	Triller et al., Inorganic Chemistry **2002**, *41*, 5544 – 5554.		
T. thermophilus catalase	H_2O_2	83	2.6×10^5	3.1×10^6	Shank et al., Biochemistry **1994**, *33*, 15433 – 15436.		
$[Mn(III)(2\text{-}OH\text{-}5\text{-}Cl\text{-}salpn)]_2$	H_2O_2	47	16	3.4×10^2	Gelasco and Pecoraro, JACS **1993**, *115*, 7928 – 7929.		
Horse liver catalase	H_2O_2	1100	3.8×10^7	3.5×10^7	Nagy et al., J. Biol. Chem. **1997**, *272*, 31265 – 31271.		

* Calculation base upon the peptide concentration
** Calculation base on the liposome concentration. There are approximately 80 fragments adsorbed on 1 liposome.

The above results imply that the hydrophobic interaction, especially in the case of damaged membrane (Ox-POPC) and damaged peptides, plays an important role in recruitment of fragmented SOD and in create a newly enzymatic function (CAT-like activity). The CAT-like activities of LIPOzyme was summarized in **Table 3-2**. The CAT-like activities of ligand modified complex and native catalase are also shown to make a visible comparison between LIPOzyme and the other catalases. The level of CAT-like activity of the LIPOzyme was found to be higher than that of previous CAT-mimics although the value was not so high in comparison with native catalase. If one liposome is considered as a molecule (there are 80 peptides adsorbed on a liposome), the CAT-like activity of LIPOzyme will be equal to native catalase.

Table 3-3 The membrane fluidity of liposomes in the interaction of liposome and fragmented SOD.

Conditions	Membrane fluidity (1/P, DPH)		Membrane fluidity (1/P, TMA-DPH)	
	Without Fr-SOD	With Fr-SOD	Without Fr-SOD	With Fr-SOD
DOTAP	5.52	4.75	4.3	4.15
POPC/Chl	4.3	3.39	2.88	2.62
Ox-POPC	5.41	4.32	4.25	4.18
POPC	5.36	4.97	3.1	3.12

This interaction of liposomes and peptide fragment to mediate the CAT-like activity was also expressed through the change of membrane fluidity. The obtained results are shown in **Table 3-3**. In all conducted liposomes, the membrane fluidity of the surface (TMA-DPH) was not change when liposomes interacted with the fragmented SOD. On the contrary, that of hydrophobic region (DPH) decreased when they interacted with fragmented SOD. The above results imply that the hydrophobicity plays an important role in their interaction which is stable under stress condition, expressing the SOD-like and CAT-like activity. A little decrease of the fluidity of membrane surface (TMA-DPH) occurred with DOTAP liposomes. The results show that the electrostatic interaction is the key factor affecting the contact of this liposome and fragmented SOD. As a consequence, this interaction was not stable under continuously oxidative stress after the recruitment of fragmented SOD, representing the low CAT-like activity and the decrease of SOD-like activity. The domain

formation also affected the liposome-fragmented SOD interaction. It was confirmed that the liposome displaying the SOD fragment was stably and highly active in CAT-like and SOD-like activity in both recruitment and stabilization.

3.4. Conformational Change of Fragmented SOD on Liposome under Stress Condition.

The conformational change of the SOD fragment was studied among the possible states of LIPOzyme. The CD spectra of fragmented SOD, recruited SOD, and CAT-like complex exposed in further oxidation stress were shown in **Figure 3-9**. The increase of α-helix contents from fragmented SOD to final form indicated that the conformational change occurred significantly. The rearrange of contents of the secondary structure on the membrane surface mediates the

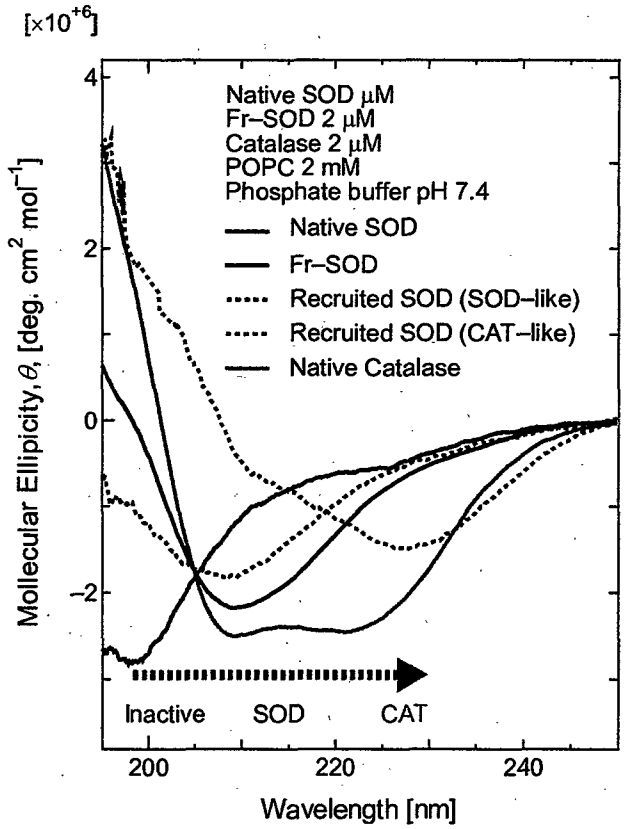

Figure 3-9 Secondary structures of SODs and Catalase in the relation of SOD-CAT-like activity. Recovered SOD was formed under oxidative stress of hydrogen peroxide (H_2O_2). H_2O_2 was added periodically 1 h in 24 h to complex of POPC liposomes and fragmented SOD, after this complex was recruited to have SOD-like activity.

suitable adaptation of recruited-SOD fragment under the given oxidative stress, resulting from conformational change from β-sheet to α-helix (**Table 3-4**). It is maybe a mechanism to form more active sites on the membrane surface to control and monitor the stress with the best efficiency. The tolerance for oxidative stress could be considered as an adaptation of biomolecules in a "stressful" condition. The modification of liposome-recruited SOD under continuously oxidative stress induced the refolding of damaged fragments and rearrangement of recruited ones to mediate a

Table 3-4 The contents of secondary structure of SODs and catalase.

Condition	Contents of Secondary Structure [%]			
	α-helix	β-sheet	β-turn	Random coil
Native SOD	7.4	61.7	4.4	26.5
Fr-SOD	0	36.7	15	48.3
Re-SOD (SOD)	4.4	54.8	9.6	31.3
Re-SOD (CAT)	55.5	0	26.6	18.0
Native Catalase	40.5	11.0	17.9	30.5

CAT-like structure. The interaction of fragmented SOD and oxidized POPC liposomes and cholesterol-containing POPC liposomes was too strong to induce the decrease of β-sheet on the membrane surface and the increase of α-helix in intermembrane layers. As a result, the change of obtained conformation of recruited-SOD fragments occurred

and the CAT-like structure was induced after the continuous exposure to oxidative effect. The variation of UV absorbance of the SOD/CAT LIPOzyme before and after the addition of the hydrogen peroxide also shows that the variation of structure of metal-ligand complex, together with the secondary structure of neighboring residues (data not shown). Thus, this structure not only ensures the CAT-like activity expression but also maintains SOD-like activity.

3.5. Summary and Comparison of SOD and CAT Activities

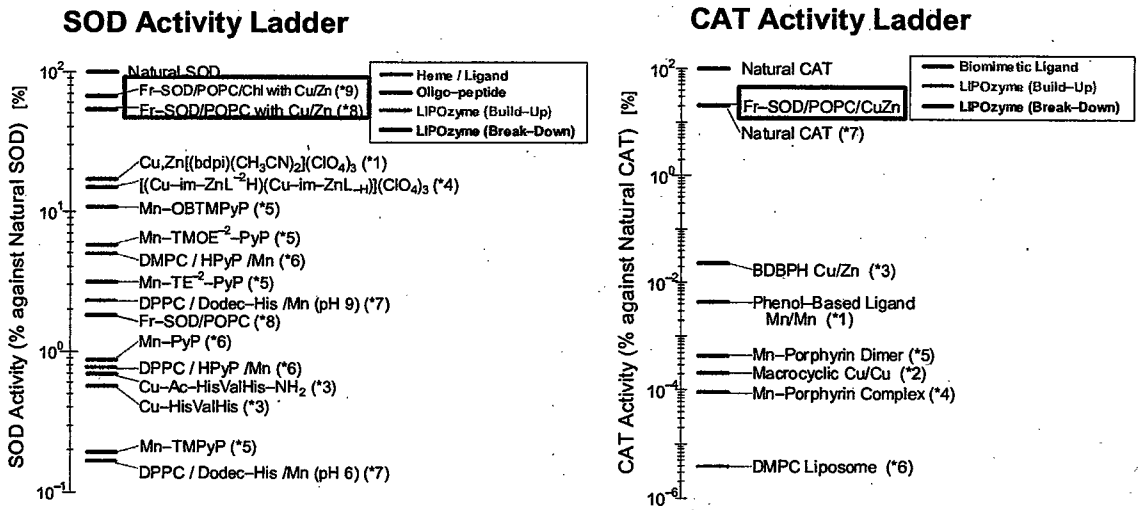

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Figure 3-10 Ladder for Comparison of SOD and CAT Activities

The SOD-like and CAT-like activity of Fr-SOD recruited on the liposome with Cu and Zn ions were summarized as a ladder in **Figure 3-10**. If one could select

the conventional biomimetic approach or "Build-Up" type approach of LIPOzyme for the design of the artificial enzyme, their activity was not equal to the natural enzymes. However, one could get the higher activity of SOD by selecting the "break-down" type approach for LIPOzyme preparation. Furthermore, one could display bicatalytic centers for SOD and CAT on the single liposome surface. This type approach based on the potentially-possessing functions of "liposome" itself (LIPOzyme preparation method) could be a new pioneer technique to mimic the natural enzyme function.

4. Conclusion

It is concluded that the membrane-recruited SOD possessed the CAT-like activity under continuously oxidative stress. The DOTAP liposomes expressed the weak CAT-like activity although it had a high SOD-like activity. The neutral liposomes and modified liposomes with the cholesterol show their stable and high enzymatic activity in stress condition, leading to the expression of both SOD-like and CAT-like activity with high values. Among all the experimented membranes, the damaged membranes (Ox-POPC) have a strong interaction with damaged peptides derived from the oxidation and fragmentation of SOD. Although the complex of Ox-POPC-fragmented SOD obtained the mild SOD-like activity, it represented high CAT-like activity and was very stable under continuously oxidative stress. The role of damaged membrane in recognition of damaged peptides is to actively recover the non-sense peptide to be useful materials and to induce its conformational change. The oxidative stress in conducted experiments also plays a significant role in mediating the new complex of liposomes and fragmented SOD to produce a novel function, to show

CAT-like enzymatic activity. The membrane was thus shown to induce both SOD and CAT activities as a response against the oxidative stress by using and converting the "potentially-active" peptide fragment on the membrane surface. The above results also show that the LIPOzyme activities could rationally be induced based on the membrane toxicity together with the environmental stress condition.

General Conclusion

The potential role of membrane in recognition, recruitment, and reactivation of damaged materials as well as regulation of antioxidants was characterized in order to create a new value, the active center mimics of enzyme, on the liposome surface (LIPOzyme) under oxidative stress. The membrane for recognition and reactivation of the stress-exposed peptides on the membrane surface was first designed, where the oxidized and fragmented SOD (superoxide dismutase) was recruited and refolded to continuously induce the enzyme-like activity (SOD and CAT (catalase)). The above LIPOzyme activity was found to be displayed on the membrane via at least three steps: (1) binding (recognition), (2) refolding, and (3) reactivation. All steps of the "LIPOzyme display" were found to concern to both characteristics of membranes and fragments, both in which were exposed to stress condition. The hydrophobicity, charge and hydrogen bond stability were proved to act as driving forces for the interaction (binding/recognition) between membranes and peptides during the LIPOzyme display processes. The display of bifunctionally enzymatic activities (both SOD and CAT) of the recovered complex of liposomes and fragmented SOD significantly concerns to the oxidatively damaged membrane (Ox-POPC liposomes), resulting in the elucidation of its CAT-like activity and higher stability of LIPOzyme activities than that of standard liposome under continuously oxidative stress. The protective role of membrane appears visibly in the damage of membrane via its antioxidative role. Such LIPOzyme activities displayed on the damaged membranes have been promisingly applied in clinic and in monitoring the oxidants of environment. The interaction of the

membranes and fragments has been finally developed (i) to design the active site of enzymes from the damaged fragments and (ii) to control and monitor the oxidants in environment. As a result, the target of LIPOzyme in production of antioxidative enzyme has been firstly approached.

In chapter I, the SOD LIPOzyme was prepared by using the potential function of liposome itself from "Build-up" and "Break-down" approaches.

Build-Up Type LIPOzyme

The liposome modified with simple ligands and metal ions presents the SOD-like activity. The membrane fluidity of various liposomes modified with the functional ligand (Dodecanoyl-Histidine: Dodec-His) and the clustering of the ligand on the liposome surface were first characterized, showing that the clustering of Dodec-His could be induced on the liposome surface at gel-phase. The capacity of adsorption of Cu and Zn was found to be increased, depending on the type of liposome, resulting in the maximal adsorption in liposome prepared by 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) at gel state and with high ligand clustering state. As a result on the SOD-like activity of the metal/ligand-modified liposome, the SOD-like activity was found to be induced by using the above liposomes although its enzymatic activity is not so high.

Break-Down Type LIPOzyme

The peptide fragment of H_2O_2-treated SOD was found to be reactivated with POPC liposomes. The fragmentation of SOD occurred under oxidative stress of hydrogen peroxide, as well as SOD inactivation and the loss of an α-helix neighboring

its activity center. The H_2O_2-treated SOD, which lost their activity at different incubation times, was significantly reactivated only by the addition of POPC liposomes, resulting in 1.3~2.8 times higher enzymatic activity. The ultrafiltration analysis of H_2O_2-treated SOD co-incubated with liposomes shows that some specific peptide fragments of the oxidized SOD can interact with POPC liposomes. The comparison of the fractions detected in reverse-phase chromatography indicates that the specific SOD fragments are able to contribute to the reactivation of oxidized and fragmented SOD in the presence of POPC liposomes. The liposomes can recruit the potentially active fragment of SOD among the lethally damaged SOD fragments to elucidate the antioxidative function. Liposome membrane played an important role not only in the refolding process of fragmented SOD but also in modification of the active sites of enzyme, as would occur with molecular and metal chaperones. Liposome membrane was found to assist the conformational change of oxidized and fragmented superoxide dismutase (Fr.-SOD) and accelerate the adsorption of metal ions on its fragment to give the original SOD-like activity. The loss of SOD activity and its secondary structure was observed during 6 h oxidation in 2 mM hydrogen peroxide. The secondary structure of fragmented and recruited SOD showed that the full conformation of fragmented SOD occurred on the membrane surface. 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 induction of the high SOD-like activity. Significance in the Cu binding to the oxidized and fragmented SOD was observed in the presence of POPC liposomes. These results show that the POPC liposomes can act like molecular and metal chaperones for stress-damaged peptides, resulting in the creation of a new SOD-like active center that continues to express the original enzymatic activity under the oxidative conditions.

The interaction of oxidized and fragmented SOD with liposomes has been reported in chapter I to mediate the original SOD-like enzymatic activity. In chapter II, the break-down type LIPOzyme was further characterized through the effect of membrane properties on their recruited activity of oxidized and fragmented SOD. The addition of zwitterionic liposomes with high membrane fluidity or that with positive charge was found to increase the SOD-like activity of fragmented SOD while the negatively charged liposome has no effect on the enzymatic activity of peptide fragment. The SOD-like activity was found to be strongly dependent on the adsorbed amount of the peptides on the liposome surface. The analyzed characteristics of the peptide, together with the above findings, imply that the liposome-recruited activity of the fragmented SOD was strongly related to the recognition of the SOD fragment by the liposomes caused by the combination of electrostatic and hydrophobic interaction, and hydrogen bonding between the peptide and liposome membrane. The DOTAP and POPC/Ch liposomes were significantly considered as active membranes when they recruit and reactivate the fragmented SOD with the high efficiency. Otherwise, negatively charged liposomes (POPG) almost did not have a contact with the

fragmented SOD, thus they could not refold it. Particularly, the Ox-POPC interacted with fragment strongly, representing the high percentage of adsorptive fragment on the membrane surface and also refolding although the SOD-like activity is not so high. The higher stability of SOD-LIPOzyme in Ox-POPC in contrast to DOTAP is considered to be dependent on the insertion of the peptide into the hydrophobic region of the membrane. The enzymatic activity of the complex also depended on both properties of liposome and ligand and characteristics of liposome, which could control the enzymatic activity of LIPOzyme. A scheme for the preparation of LIPOzyme was finally proposed based on the obtained results.

In chapter III, antioxidative LIPOzyme (SOD/CAT LIPOzyme) was prepared through the recruitment, refolding and regulation of the oxidized and fragmented SOD. Among the conducted liposomes in the experiments, cholesterol-containing POPC liposomes and oxidized POPC liposomes (damaged membranes) have a strong interaction with damaged peptides derived from the oxidation and fragmentation of SOD, resulting in the easy conversion of enzyme-like function under continuously oxidative stress condition. The complex of liposomes and fragmented SOD became stable under oxidative stress condition, except for DOTAP liposome-fragmented SOD. The role of toxic membrane in recognition of damaged peptides is to actively recover and structurally change them to be useful materials. The oxidative stress in conducted experiments also plays a significant role in mediating the new complex of liposomes and fragmented SOD to produce a novel function, obtaining CAT-like activity. The LIPOzyme plays an important role in the clearance of ROS because the eliminating

activities of ROS could occur mainly on the surface of the liposome membrane. The current research is a promising model for future applications in antioxidation.

The obtained results finally indicate that the reciprocal conversion of enzymatic activities mediated the diversity of membrane function in recognition, recruitment and reactivation of damaged enzyme to modify new enzyme-like complexes (LIPOzyme) under oxidative stress. Moreover, the mediation of membranes, under oxidative stress condition, played an important role in protection of the interiors of membrane from the lethal damages. The use of membrane functions in controlling and monitoring stresses is essential and vital in the production of enzymes for industrial applications with safety, low cost and energy.

Suggestions for the Future Works

1. Recruitment of "Potentially-Active Peptides" for other LIPOzyme Display.

The fragmented SOD was recruited and actively recovered by the liposome membrane. The mechanism of this process was found to be related to the interaction of liposomes and fragmented SOD, in which liposomes recognize, refold and reactivate the fragments due to the characteristics of both fragments and liposomes. The damaged enzyme or protein seems to be hydrophobic and to have unstable hydrogen bonds. Therefore, the familiar properties of liposomes, especially hydrophobicity, induce the suitable interaction between them. As a result, liposome can easily recover the damaged enzyme and protein structurally and actively. After the recruitment, the complex of liposome and recovered enzyme becomes stable under stronger and longer oxidative stress. A variety of applications of liposome-recruited enzyme (LIPOzyme) in regulation of oxidants was prospectively considered because of its stability under continuously stress condition. Such a strategy to prepare the LIPOzyme can be applied to the display of the other types of enzyme-like activities. The LIPOzyme has some benefits such as (i) high stability, (ii) high efficiency, and (iii) multi-function. By recruiting the "potentially-active peptides" from the various peptides originated from

some bioresources, the bio-environment friendly-bioreactor that could display the suitable enzymatic functions on the surface in response to the environmental toxicity can be designed and developed in the future.

2. Tuning-up of LIPOzyme Activity with Synthetic Ligands of Peptide and Their Combination

The specific peptide originated from the oxidation of SOD was determined to contain the active site. The important objective is to modify the active site from unusable substances to create a new active site on the membrane surface. The amino acid sequences mimics from the fragmented SOD should be synthesized and applied to the modification of active site, together with metal ions, on the membrane. Membrane could be considered as a carrier which has the spaces containing more active sites than those of free native enzymes in solution. Thus, the enzymatic activity of complex of membrane-fragments increases the reaction rate and resists to the stress condition. The combination of the peptide-displayed LIPOzyme or "break-down" type LIPOzyme with the "build-up" type LIPOzyme could also be achieved to show the multifunctional activities because of embryonic nature of the liposome membrane and its wider surface area. The design of such fused LIPOzyme with stable multi-functions could positively affect the design and development of the possible LIPOzyme reactors

described above.

3. More studies by using damaged membrane or toxic membrane in recruitment of toxic materials.

The effect of toxicant (arsenate) to biological membrane was conducted and achieved the initial results. In this work, arsenate induced the increase of membrane fluidity under mild concentration of arsenic and the cell death or the membrane disruption under high concentration of arsenic.

When cells are exposed to arsenate, their life activities are immediately affected by various biochemical reactions, such as the binding of arsenic to membranes and the substitution of arsenic for phosphate or the choline head of phospholipids in the biological membranes. The effects of arsenate on the life activities of algae (*Chlorella vulgaris*) depend on arsenate concentrations and exposure times. Algal cells and the artificial membranes (liposomes) exposed to arsenate could induce the increase of membrane fluidity. In the presence of arsenate, the membranes were fluidized due to the binding and substitution of arsenate groups for phosphates or the choline head on the their membrane surface. The phenomenon in turn enhanced the membrane permeability and led to the accumulation of arsenic and subsequent cell death. At high concentrations of arsenate, the liposome or cell membranes were destroyed, resulting

in liposome disruption or cell death, respectively. The experiments with POPC liposomes with arsenate once confirmed the chemical interactions happening on the liposome surface. That was one of various mechanisms occurring in the interaction of toxic metals with biomembrane. According to the view point of previous report (Floch et al., 2000), it is initially suggested that arsenate can replace the choline group more easily than the phosphate group of POPC molecules of the biomembranes. It is thus considered that arsenic toxicity mainly affects cell membrane and model membrane (liposome). However, in some cases, the membranes play a positive and protective role in the removal of toxicants, because the removal activities of toxicants happen mainly on the membranes. In practice, arsonolipids and arsonoliposomes (a kind of toxic membrane) have been applied in the treatment of cancer cells (Gortzi et al., 2002 and 2003). The obtained results contribute to the explanation of interaction mechanism between membrane and toxicants, especially arsenic compounds. The correlation of membrane activity and membrane toxicity was proposed to diversify the role of membrane in biological systems. In some case, toxic membrane becomes a useful tool in detecting the toxic materials. The synthesis of arsenic-containing lipids and liposomes was proposed to clarify the role of toxic membrane in controlling the toxicants and damaged fragments derived from lethal stresses.

Appendix

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Arsenic (V) induces a fluidization of algal cell and liposome membranes

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ABSTRACT

Arsenate is one of the most poisonous elements for living cells. When cells are exposed to arsenate, their life activities are immediately affected by various biochemical reactions, such as the binding of arsenic to membranes and the substitution of arsenic for phosphate or the choline head of phospholipids in the biological membranes. The effects of arsenate on the life activities of algae *Chlorella vulgaris* were investigated at various concentrations and exposure times. The results demonstrated that the living activities of algal cells (10^{10} cells/L) were seriously affected by arsenate at a concentration of more than 7.5 mg As/L within 24 h. Algal cells and the artificial membranes (liposomes) were exposed to arsenate to evaluate its effects on the membrane fluidization. In the presence of arsenate, the membranes were fluidized due to the binding and substitution of arsenate groups for phosphates or the choline head on the their membrane surface. This fluidization of the biological membranes was considered to enhance the transport of toxicants across the membrane of algal cells.

1. Introduction

Arsenic is one of the most significant global environmental toxicants. Humans can be exposed to arsenic through the intake of air, food, and water. Trace amounts of arsenic in drinking water can endanger human health, mainly because arsenic compounds in drinking water occur in inorganic forms which are toxic (Abernathy et al., 2003). Arsenic in natural waters mainly as inorganic arsenite and arsenate. Organic arsenicals also occur in natural, but their toxicities are much lower than inorganic arsenic (Chen et al., 1994; Li et al., 1995).

In living cells, arsenic exists in the states of +5, +3, 0, and −3. It can form alloys with metals and covalent bonds with carbon, hydrogen, oxygen, and sulfur (Ferguson and Gavis, 1972). Because its biochemical properties are similar to those of phosphate, arsenate can replace phosphate in energy transfer phosphorylation reactions, resulting in the formation of adenosine diphosphate (ADP)-arsenate instead of adenosine triphosphate (ATP) (Gresser, 1981). However, the arsenate concentration required for the formation of ADP-arsenate is reported to be as high as 0.8 mM (Moore et al., 1983). Arsenic is well known for its ability to induce the production of superoxide (Barchowsky et al., 1999; Lynn et al., 2000). If excess superoxide is produced in the pancreatic cells, an impairment of insulin secretion is expected (Tseng, 2004). With respect to biological membranes, there are some reports indicating that arsenic compounds affect the structure and functions of cell membranes, especially those of human erythocytes (Zhang et al., 2000; Winski et al., 1997; Winski and Carter, 1998).

The final purpose of the current study was to examine the effects of low concentrations of arsenate at different exposure times on the life activities of algal cells and on liposome, the "artificial" membrane system discovered more than 40 years ago by Bangham et al. (1965) that has become a versatile tool in biology, biochemistry and medicine. The cell membrane is a diffusion barrier which protects the cell interior. Therefore, the change of its structure and functions results in the disturbance inside or outside membrane. In the present study, to more deeply understand the molecular mechanisms of the interaction between arsenic and biomembrane, we exposed the cell membranes of algae and artificial membranes (liposomes) to arsenate. By using the fluorescence microscope, spectrofluorometer and the reverse-phase high performance liquid chromatography, as well as the atomic absorption spectrometry analyzing bound arsenic on the membrane, we initially determine the interaction between arsenate and biological membranes. The results showed that arsenate changed the characteristics of the membrane by substituting phosphate or the choline head of

Abbreviations: As, arsenic; As (V), arsenate; As (III), arsenite; POPC, 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine; Calcein, 3,3-bis [*N*,*N*-bis(carboxymethyl)-aminomethyl] fluorescein.

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phospholipid molecules on the biomembrane. Fluidization of the membrane or increase of the membrane fluidity, permitting the toxicants to move into and/or be released from the cells, was also investigated under arsenic toxic stress. Based on the results, the toxic effect of arsenate on the artificial and cell membrane was finally discussed.

2. Materials and methods

2.1. Materials

Arsenate solution with a concentration of 60% was purchased from Wako Pure Chemicals (Osaka, Japan). $FeCl_3$ from Sigma–Aldrich was used for the comparative experiments to estimate the toxic strength of arsenic (V) and iron (III). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and was used for liposome preparation. Calcein 3,3-bis [*N*,*N*-bis(carboxymethyl)-aminomethyl] fluorescein, purity 98%) was obtained from Wako (Osaka, Japan).

Algal cells of *C. vulgaris*, purchased from Wako Pure Chemicals, were used after a purification procedure in which the cells were centrifuged at 800g for 5 min and the supernatant was removed. The other reagents were of analytical grade.

2.2. Liposome preparation

POPC was used to produce liposomes. In brief, the phospholipid was dissolved in chloroform, the solvent was evaporated under vacuum, and the resulting dry thin film was dried for 2 h under vacuum at room temperature. The lipid film was hydrated by dispersion in 50 mM phosphate buffer (pH 7.3) to form multilamellar vesicles. The multilamellar vesicle suspension was frozen in dry ice-ethanol (−80 °C) and was dispersed above the phase transition temperature for five cycles. The resulting suspension was passed through two stacked polycarbonate filters of 100 nm pore size by using an extrusion device.

For successful separation of all membrane lipids, algal cells were ground to fine powder in the presence of liquid nitrogen. While cells were still frozen, methanol–chloroform–water (2:1:0.8, v/v) (Bligh and Dyer, 1959) was added in a ratio of one part tissue to three parts solvent. The extraction was continued until the sample completely lost its color. After filtration, the cleared extract was transferred into a separation funnel. A 5 ml volume of chloroform and water was added for complete separation of the two phases. The chloroformic fraction was collected and evaporated to dryness using a vacuum evaporator at 40 °C. The dry residue was then dissolved in 0.2–0.5 ml chloroform. The above lipids were analyzed to determine the membrane-bound arsenic.

2.3. Toxic effect of arsenate on algal cell membranes

Pure cells were incubated with different arsenate doses to evaluate the effects of arsenic toxicity on living cells. Algal cells of *C. vulgaris* with a density of 10^{10} cells/L were incubated in Proteos medium, a modified form of Bristol medium (Nichols, 1973), with various concentrations of arsenate (H_3AsO_4) under neon lights with an intensity of 3000 lux at 30 °C.

The cells were incubated for 24 h, and the effects of the arsenate concentration on the living cells were investigated by measuring fluorescence intensity with a synchronous scan as an index of the number of living cells (Liu et al., 2005). At the same time, the optical density at 650 nm of media solution was measured in combination with counting the number of cells in a Quadrate chamber.

The time-dependence of the toxicity of arsenate on cell viability was then examined. Algal cells were incubated with different concentrations of arsenate for 12, 24, and 48 h, and cell viability was determined by measuring the fluorescence intensity.

2.4. Effect of arsenate on model cell membranes (liposomes)

In order to clarify the mechanism of the cellular toxicity of arsenic, the structure of the compound formed between arsenic and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) liposome was examined. POPC (5 mM) in 50 mM phosphate buffer was incubated at 30 °C for 12 or 48 h with the following molar ratios of arsenate to POPC: 1/300, 1/200, and 1/100. The pH value was kept at 7.3. POPC without arsenate was also incubated as a control. The change in the binding structure between POPC and arsenic according to the incubation time was investigated by running reverse-phase high performance liquid chromatography (RP-HPLC) to detect arsenic bound to POPC liposomes in the incubated solutions.

2.5. Fluidization of membranes of POPC liposomes and algal cells under arsenic toxicity

Fluidization increases the permeability of membranes, permitting the toxicants to move into and/or be released from the cells. In this study, we investigated changes in permeabilization by arsenate dosage in both algal cells and POPC liposomes using two parallel experiments.

Cell membrane permeability under a toxic condition induced by arsenate was examined by means of the accumulation of calcein, a fluorescent probe, in algal cells of *C. vulgaris*. Algal cells were incubated in Proteos medium with MES buffer containing 7.5 mg As/L of arsenate and without arsenate as a control. Calcein (0.05 mM) was added to the medium as a fluorescent probe. The concentration of calcein in the media solution, which was analyzed periodically during the incubation, was calculated to estimate the transport of calcein into the cells.

A series of experiments was conducted to examine the fluidization of POPC liposomes that had been treated with arsenate. Calcein was entrapped in POPC liposomes, which had a diameter of 100 nm prior to incubation. Arsenate (50 μM as a final concentration) was added to liposome solution (5 mM of POPC) at 30 °C. The change in the concentration of calcein, which was released from the liposomes by fluidization of the membrane structure into the solution, was measured according to exposure time. Triton X-100 at a concentration of 1% was added to the solution to release all the calcein entrapped in liposome membranes (Felix et al., 2002).

2.6. Analytical methods

A Shimadzu reverse-phase HPLC (RP-HPLC) system equipped with an FCV-10AL pump and DGU-$20A_3$ degasser was used, as were a SPD-10A UV–vis detector and LC-10AD liquid chromatograph. Elution profiles were monitored at 254 nm on the UV detector. The mobile phase of acetonitrile/water (65/35 v/v) with a flow rate of 1 mL/min was applied at 30 °C. An Inertsil ODS-SP column (0.46 cm × 2.5 cm) was used throughout this study. POPC-bound arsenic lipids were detected by an RP-HPLC.

Free arsenic and bound arsenic was detected by hydride generation atomic absorption spectroscopy (HG-AAS). A Varian SpectrAA 220 Atomic Absorption instrument (with software SpectrAA 220 ver. 10) connected with Varian Vapor Generation Accessory, VGA-77, was used. An heated oven containing a Varian quartz hydride absorption cell was connected to the hydride system and placed in the optical path of the instrument. With HG-AAS, the minimum concentration of arsenic for the possible detection is 1 ppb.

Appendix

In the exposure experiments, the concentration of calcein in the supernatant was determined after centrifugation at 2200g by a fluorescent spectrophotometer (JASCO FP 6500; Jasco, Tokyo, Japan) at wavelengths of 495 nm and 515 nm for excitation and emission, respectively.

2.7. Statistical Analysis

Results are expressed as the mean ± standard deviation (SD). All experiments were performed at least in triplicate. The data distribution was analyzed, and statistical differences were evaluated using the Student *t*-test. A *P* value of <0.05 was considered to be significant.

3. Results and discussion

3.1. Toxic effect of arsenate on algal cell membranes

The toxic effect of arsenate on the algal cells was first investigated. Fig. 1a shows the color change of the algal cell suspension induced by 24 h of incubation of algal cells with arsenate. Fig. 1b indicates the spectra of fluorescence intensity after 24 h of incubation with various concentrations of arsenate. Increases in the arsenate concentration resulted in decreases in fluorescence intensity, which implied the death of algal cells. Arsenate attached to the cell membrane to produce reactive oxidative species, which easily oxidize lipids and membrane proteins and which could result in cell destruction (Delnomdedieu et al., 1995). Although there are many previous reports on the binding of metal ions on algae, few efforts have been made to characterize the complex of metal ions and algae. The photodegradation of estrogens and photoproduction of hydroxyl radicals in algae solutions with (or without) Fe (III) were observed in previous work (Liu et al., 2003, 2004). Therefore, experiments were conducted with arsenic (V) and iron (III) to compare their toxicities (Fig. 1c). The above results with arsenate and iron (as a control experiment) indicated that arsenate was more toxic than iron under the same experimental conditions. By destructing the cell membrane (images obtained from the fluorescence microscope), arsenate induced cell death, which appears after only a short incubation time and increases drastically in experimental period (24 h).

Fig. 2 shows the cell viability after 24 h of incubation with various concentrations of arsenate. The viability of cells decreased sharply as the arsenate concentrations increased. Under a fluorescence microscope, cell aggregation, in which the cells were still alive, was observed at arsenate concentrations between 30 mg As/L and 45 mg As/L, while a cell disruption process, indicating cell death, was observed at arsenate concentrations of 60 and 75 mg As/L.

According to our experimental results, even a low concentration of arsenate adversely affected the viability of algal cells. A 75 mg As/L of arsenate induced cell destruction and 45 mg As/L of arsenate induced cell aggregation after 24 h of incubation. This acute toxicity can be explained by membrane fluidization, which enhanced the permeability of the membrane, leading to high accumulation of arsenate in the cells.

The viability of algae was also examined by incubating algal cells with different concentrations of arsenate for a time-dependence of exposure. The results shown in Fig. 2 indicate that the viability of algae was affected not only by the arsenate concentration but also by the incubation time under toxic conditions. An experiment was conducted to determine the site on which arsenate was adsorbed. After incubation of alga with arsenic, the lipid component of the algal membrane was extracted and was analyzed by an atomic absorption spectrometry device to determine the percentage of the total arsenic that had accumulated on the membrane. Fig. 2b shows that the algal membrane adsorbed an increasing amount of arsenic along with the increasing arsenic concentration in the culture solution. The membrane is the main place where adsorption reactions of nutrients occur both passively

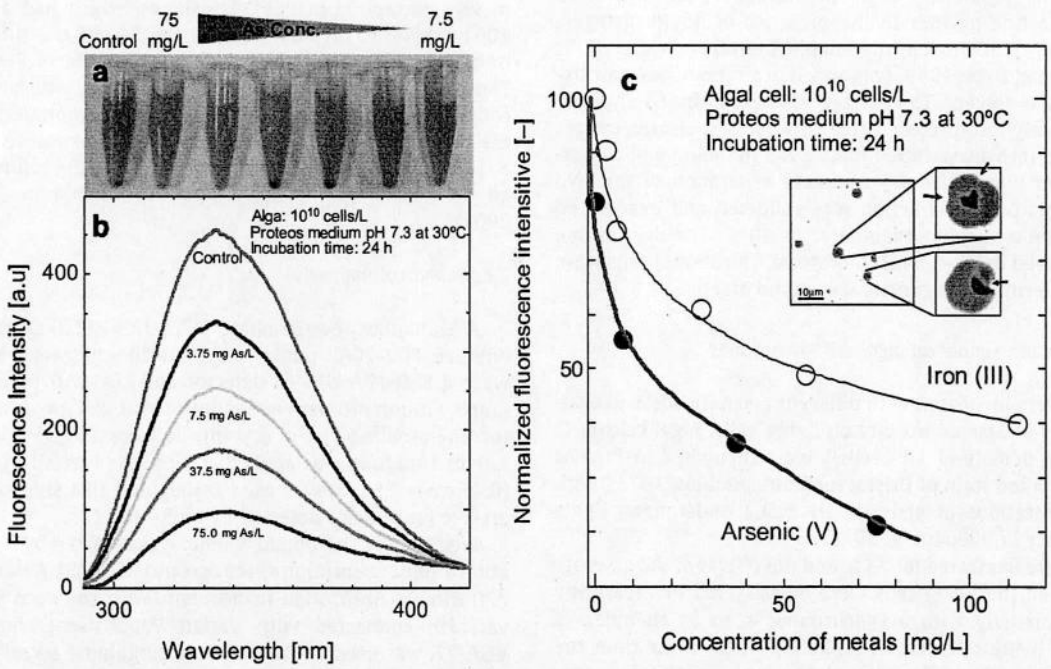

Fig. 1. Effects of arsenic (V) on the synchronous-scan fluorescence intensity of 10^{10} cells/L *C. vulgaris* solution with As (V). Control (without As) and with 3.75, 7.5, 37.5, and 75.0 mg As/L of arsenic (V) and with Fe (III) as a comparison. Algal cells were incubated with arsenic at 30 °C for 24 h. (a) The color change of algal solution at different concentrations of arsenic. (b) The fluorescent intensity of algal solution. (c) The effect of toxicants on algal culture and disruption of cell membrane under toxic stress of arsenate.

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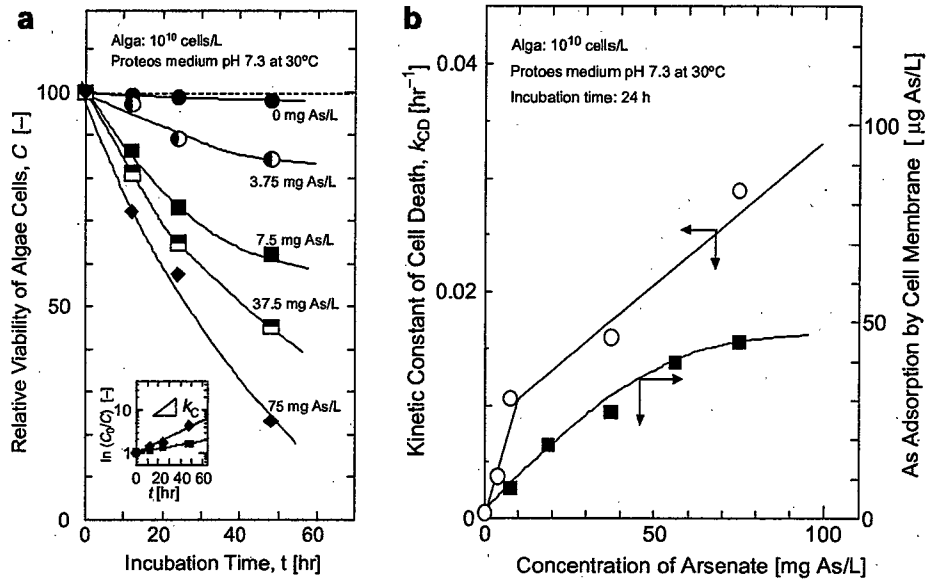

Fig. 2. (a) Cell viability as a function of time and arsenic concentration. (b) Kinetic constant of cell death (○) and arsenic adsorption on the cell membrane (■). After incubation of cells with arsenic, the cell membranes were extracted (Section 2) to determine the arsenic amount on biomembranes.

and actively. Remarkably, it is not the toxicant itself which induces cell death, but rather the cell membrane, following its adsorption of arsenic. Thus, an increase in the arsenic concentration resulted in an increase in cell death (Fig. 2a). However, in some cases, the membrane plays an important role in removal of toxicants from the environment. One of the essential removal mechanisms is the binding of arsenic on the membrane. This mechanism is an indispensable part of the interactions between toxicants and the cell membrane. To demonstrate this phenomenon, an artificial membrane model (liposome) was used in the following experiments for the reaction with arsenate.

3.2. Effect of arsenate on model cell membranes (liposomes)

The cell membrane can be considered a "frontier" facing the attack of toxicants (Zhang et al., 2000). In order to better understand the interactions between the membrane and toxicant, experiments were carried out using POPC liposomes and arsenate. The results indicated that the liposomes were fluidized and disrupted by arsenate, which was regarded as evidence that arsenic bound to the liposomes and affected them directly. However, the chemical binding with POPC molecules might occur after they are loosely attached to liposomes. The relatively high level of membrane-bound arsenic at the beginning of the total process implies a rapid binding of arsenate from the bulk solution to the membrane. The release after rapid binding might also result from the transfer of arsenic from kinetically favored binding sites in the membrane to more thermodynamically stable ones in both the membrane and cytosol (Winski and Barber, 1995). In a recent report about As (III), it was suggested that the arsenite anion may form a hydrogen bond directly with the PO_4^- group of dimyristoylphosphatidylethanolamine (DMPE) molecules in competition with hydrating water molecules as well as amino groups. Reduction of the effective PE–PE head group interaction should leave the phosphorus group free, and hence its mobility should increase as well as the interfacial area of lipid. Thus, there is a direct insertion of arsenic into the head group (Suwalsky et al., 2007).

Fig. 3a and b show the results of RP-HPLC analysis for the POPC solutions after 12 h and 48 h incubation, respectively. In Fig. 3a, the first peak (P1) was detected at 2.7 min, which represented the POPC, while the second peak (P2) shifted to 4.0 min. This shift indicates the change in the binding structure of arsenic to the POPC molecule. One of the possibilities is that arsenate molecules bound to liposomes by a hydrogen bond or electrostatic force, and then were chemically entrapped in liposomes by the substitution of arsenate for the phosphate group or choline group of POPC molecules, as described above, and this process was accelerated by 48 h incubation (Fig. 3b). It has recently been reported that a new series of cationic phosphonolipids characterized by a cationic charge with a phosphorus or arsenic atom is being developed (Stekar et al., 1995 and Guenin et al., 2000). The transfection activities of new cationic As-phosphonolipids were studied *in vitro* in different cell lines (HeLa, CFT1, and K562) and *in vivo* using a luciferase reporter gene. It was also demonstrated that cation substitution on the polar domain of cationic phosphonolipids (N replaced by P or As) resulted in a significant increase in transfection activities in both the *in vitro* and *in vivo* assays, as well as a decrease in cellular toxicity (Floch et al., 2000).

In a previous report, arsenate was shown to replace phosphate in many biochemical reactions, because two compounds have similar structures and properties (Dixon, 1997). For example, arsenate reacts *in vitro* with glucose and gluconate (Gresser, 1981 and Lagunas, 1980) to form glucose-6-arsenate and 6-arsenogluconate, respectively. Glucose-6-arsenate is a substrate for glucose-6-phosphate dehydrogenase and can inhibit hexokinase, as does glucose-6-phosphate (Lagunas, 1980). Arsenate can also replace phosphate in the sodium pump and the anion exchange transport system of human red blood cells (Kenney and Kaplan, 1988). However, the concentration of arsenate required for such reactions is high and not physiologically relevant. Furthermore, these effects may happen only in acute intoxication and may not be effective in subjects chronically exposed to low-dose arsenic.

When binding, arsenate interacts with phospholipid by a hydrogen bond or electrostatic force only. In the case of substitution, arsenate replaces the phosphate or choline groups of POPC molecules. Consequently, arsenate was able to change some of the characteristics of both the artificial and algal membranes in the present study.

Appendix

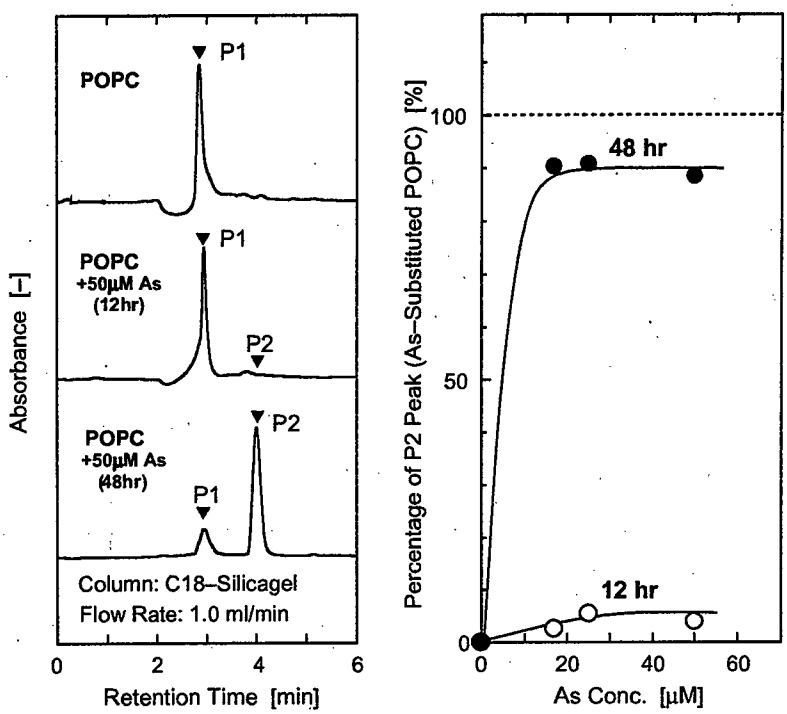

Fig. 3. RP-HPLC analysis of arsenic-substituted POPC. POPC 5 mM was incubated with arsenate 50 μM at 30 °C in phosphate buffer (pH 7.3). POPC-bound arsenic lipids were detected by an RP-HPLC. Elution profiles were monitored at 254 nm on the UV detector. The mobile phase of acetonitrile/water (65/35 v/v) with a flow rate of 1 mL/min was applied at 30 °C. An Inertsil ODS-SP column (0.46 cm × 2.5 cm) was used throughout this study. P1 represents POPC. P2 represents a product of the reaction between POPC and arsenic.

3.3. *Fluidization of membranes of POPC liposomes and algal cells by arsenic toxicity: a mechanism for cell death by arsenic toxicity at low arsenate concentration*

Fig. 4 shows the release of calcein from the POPC liposomes after exposure to 50 μM arsenate. Fifty percent of the total amount of calcein was released in the first 1 h of incubation, and the remainder was continuously released over the remainder of the 24 h experimental period. On the other hand, no calcein release was observed in the control experiment without arsenate. This is possibly because of the membrane fluidization of the POPC liposomes under arsenic toxicity. When liposome membranes become

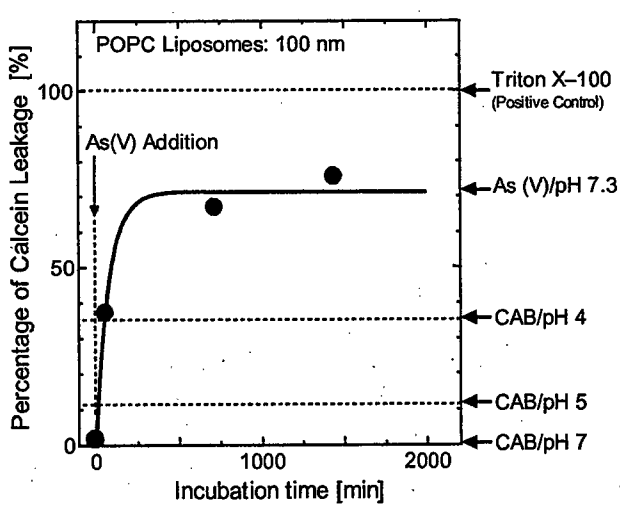

Fig. 4. Arsenate induced the fluidization of POPC liposome resulting in the release of calcein from the inside to the outside of the liposome membranes. Calcein was captured inside the liposomes. Free calcein was washed by phosphate buffer, and then arsenate was added to the liposome solution. The release of calcein indicated that arsenate induced membrane fluidization. Previously reported results on the effect of bovine carbonic anhydrase (CAB) and triton X-100 on membrane fluidization were plotted (Kuboi et al., 2004) for purposes of comparison.

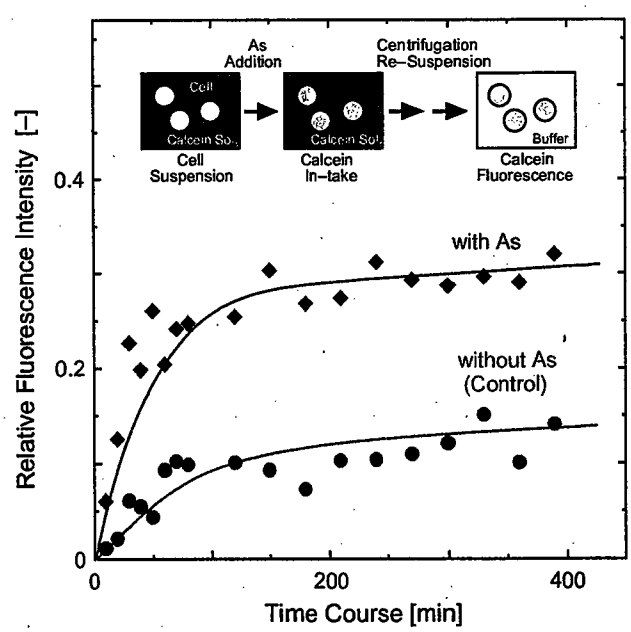

Fig. 5. Calcein accumulation in algal cells due to fluidization of the algal membrane. The cells were incubated in a Proteos medium with (♦) and without arsenic (●), and the fluorescent probe. Calcein adsorption by algal cells under arsenic toxic stress was analyzed periodically during the reaction.

Appendix

fluid or are disrupted, entrapped calcein is released into the environment, resulting in increased fluorescence intensity. The results show that arsenate induced membrane fluidization. A previous study was conducted to compare the toxicity of arsenate with that of other substances such as bovine carbonic anhydrase and triton X-100 (Kuboi et al., 2004). Like this previous study, our present results underscore that arsenate is one of the most toxic elements.

To examine whether arsenate induces biomembrane fluidization, experiments were conducted with the exposure of algae to arsenate and calcein. The results are shown in Fig. 5. The permeability of fluorescent probe (calcein) into cells initially occurred quickly. The process of calcein adsorption into cells reached a state of balance even though the concentration of calcein outside the cells was still higher than that inside. The accumulation of calcein in the algal cells was dependent on the incubation time. The enhanced accumulation of calcein by increasing exposure to arsenate was considered to have been induced by fluidization of the cells, leading to an increase in membrane permeability.

In inducing cell death, arsenate initially fluidizes the membrane and then destroys it. The adsorption of arsenate by the cell membranes through passive or active mechanisms also can lead to generation of reactive oxygen species (Yamanaka et al., 1989, 1990) that oxidize lipids, proteins and genes (Delnomdedieu et al., 1995). Cell death is the inevitable consequence. The information obtained in the present study is necessary for application of algae to the treatment of toxicants (heavy metals).

4. Conclusion

The present study demonstrates that low concentrations of arsenate adversely affected algal cells. Arsenate with more than 7.5 mg As/L induced cell aggregation and death after 24 h. This acute toxicity induced the fluidization of the membrane, which in turn enhanced the membrane permeability and led to the accumulation of arsenic and subsequent cell death. Arsenate fluidized liposomes as well as cell membranes. At high concentrations of arsenate, the liposomes or cell membranes were destroyed, resulting in liposome disruption or cell death, respectively. When arsenate bound to the POPC liposomes, binding sites occurred at the phosphate head of POPC or choline of POPC liposomes. Arsenic (V) was also substituted for the phosphate or choline group of POPC molecules as well. According to the view point of previous report (Floch et al., 2000), we initially suggest that arsenate can replace the choline group more easily than the phosphate group of POPC molecules of the biomembranes. Thus, we considered that arsenic toxicity mainly affects cell membrane and model membrane. However, in some cases, the membranes play an important role in the removal of toxicants, because the removal activities of toxicants happen mainly on the membranes. The current research is a promising model for future applications to the treatment of arsenic contamination.

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Appendix

Arsonoliposomes Enhanced the SOD-LIPOzyme Activity

The arsonoliposomes play a role in recruitment of superoxide dismutase (SOD) fragment. POPC liposomes were incubated with the various concentrations of arsenate from 75 to 7500 ppb as arsenic (V) in 24 h to produce arsonoliposomes. The arsonoliposomes (2 mM) were incubated with fragmented SOD (2 μM) in 12 h. The obtained results showed that (1) the increase of arsenate adsorption by liposome membrane and (2) arsonoliposomes highly reactivated the SOD activity of fragmented SOD from no activity to maximum 93% at 2250 ppb of arsenic. The results indicated that toxic membrane was easy to interact and reactivate the damage enzyme (fragmented SOD).

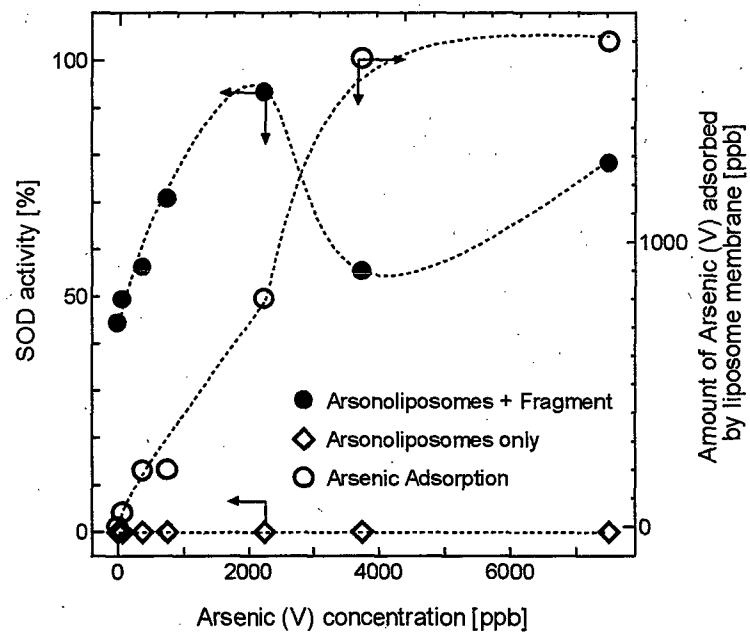

Figure 1 The adsorption of arsenic (V) by POPC liposomes and SOD-like activity of arsonoliposomes and fragmented SOD complex

Experimental

1. POPC liposomes (5 mM POPC) were incubated with As (V) at various concentrations from 75 to 7500 ppb and control (0 ppb) in 24 h. After 24 h of incubation with arsenic, arsonoliposomes were collected by using the ultrafiltration method with a 50 kDa cut-off filter.
2. After 2 mM arsonoliposomes were incubated with 2 μM fragmented SOD in 12 h, SOD-like activity of complex was measured.
3. Adsorption of As on liposomes was analyzed by using atomic absorption spectrometry (AAS) to measure free arsenic in solution after an incubation of POPC liposomes with arsenic (V)

Nomenclature

(1/P)	Membrane fluidity of liposome	[-]
(E/M)	Ratio of fluorescence intensity of an eximer/monomer of pyrene	[-]
A_x	Absorbance at x nm	[-]
$I_{//}$	Fluorescence intensity of parallel to the excited light	[-]
$I_{\perp}$	Fluorescence intensity of perpendicular	[-]
IC_{50}	50% inhibition concentration of superoxide dismutase	[μM]
K	Equilibrium constant of metal adsorption	[μM]
OD_x	Optical density at x nm	[-]
q_{max}	Adsorption capacity	[mol/mol-peptide]
T_m	Phase transition temperature	[^{0}C]

List of Abbreviations

CAT	Catalase
EDTA	Ethylen diamine tetraacetic acid (metal chelator)
DLPC	1,2-dilauroyl-*sn*-glycero-3-phosphocholine
DMPC	1,2-dimyristoyl-*sn*-glycero- 3- phosphocholine
Dodoc-His	Dodecanoyl-Histidine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	1,2-dipalmitoyl-*sn*-glycero-3- phosphocholine
Fr.-SOD	Fragmented SOD
Ox-POPC	Oxidized POPC
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPC/Chl	POPC/cholesterol
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt)
Py-Dodec-His	Pyrene-dodecanoyl-histidine
ROS	Reactive oxygen species
SOD	Bovine erythrocyte Cu,Zn-SOD

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1. Le Quoc Tuan, Hiroshi Umakoshi, Toshinori Shimanouchi, and Ryoichi Kuboi, Liposome-Recruited Activity of Oxidized and Fragmented Superoxide Dismutase. *Langmuir*, **24**, 350–354 (2008).

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