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Production and removal of superoxide anion radical by artificial metalloenzymes and redox-active metals

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Generation of reactive oxygen species is useful for various medical, engineering and agricultural purposes. These include clinical modulation of immunological mechanism, enhanced degradation of organic compounds released to the environments, removal of microorganisms for the hygienic purpose, and agricultural pest control; both directly acting against pathogenic microorganisms and indirectly via stimulation of plant defense mechanism represented by systemic acquired resistance and hypersensitive response. By aiming to develop a novel classes of artificial redox-active biocatalysts involved in production and/or removal of superoxide anion radicals, recent attempts for understanding and modification of natural catalytic proteins and functional DNA sequences of mammalian and plant origins are covered in this review article.

Introduction

Empirically, people have been aware that increased intake of antioxidants in the form of fruits and vegetables may reduce the risk of chronic diseases, chiefly of cancer.1 Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism as well as in cellular responses to xenobiotics, cytokines, and bacterial invasion; and oxidative stress in living cells of both animal and plant origins.2 Today, natural plant products are viewed as potential candidates for the cancer chemoprevention due to their antioxidant, anti-inflammatory and antitumor activities and, therefore, intake of such antioxidants are necessary for controlling degenerative reactions produced by ROS and nitrogen species in vivo.3

On the other hand, generation of ROS is useful for various medical, engineering and agricultural purposes namely, clinical modulation of immunological mechanism,2,4 enhanced degradation of organic compounds released to the environments,5 removal of microorganisms for the hygienic purpose,6 and agricultural pest control both directly acting against pathogenic microorganisms and indirectly via stimulation of plant defense mechanism represented by systemic acquired resistance and hypersensitive response.7,8

In this review article, recent approaches for understanding and modification of natural catalytic proteins and functional DNA sequences of mammalian and plant origins are covered, aiming to develop a novel classes of artificial redox-active biocatalysts involved in production and/or removal superoxide anion radicals (O$_2^{•-}$).

In general, catalysts (Cs) can be defined as the set of different types of elements, molecules or compounds as follows:

$$\{Cs\} = \{\{OCs\}, \{ICs\}, \{BCs\}\} \quad (1)$$

where OCs, ICs, and BCs are organic catalysts, inorganic catalysts, and biocatalysts, respectively.9 OCs can be represented by biological and bio-inspired organic molecules such as guanidine-type10 or amino acid proline-type catalysts,11 and ICs can be represented by inorganic molecules or complex such as metal-based catalysts.12 While the natures of OCs and ICs can be clearly defined and described based on their chemical properties, the category of BCs simply implies the origins but not the natures of these catalysts. It is trivial that the set [BCs] can be divided into 2 subsets as follows:

$$\{BCs\} = \{\{Es\}, \{Ns\}\} \quad (2)$$

where Es and Ns are enzymes and nucleozymes, respectively.
In fact, $E_s$ and $N_s$ can be further confirmed as follows:

\[
\{E_s\} \in \{\{Cs\} \cap \{\text{proteins } \cup \text{ peptides}\}\}
\]
\[
\{N_s\} \in \{\{Cs\} \cap \{\text{RNA } \cup \text{ DNA}\}\}
\]

Moreover, the sets of $\{E_s\}$ and $\{N_s\}$ can be further divided into subsets as follows:

\[
\{E_s\} = \{\{E_{s,s}\}, \{E_{s,s}\}\}
\]
\[
\{N_s\} = \{\{N_{s,s}\}, \{N_{s,s}\}\}
\]

where $E_{s,s}$ and $N_{s,s}$ are natural enzymes and nucleozymes, respectively, which can be found in or produced by living organisms; and $E_{s,s}$ and $N_{s,s}$ are artificial enzymes and nucleozyme, respectively, which are now newly designed or engineered in the laboratory. Among the $E_s$, many portion of both $E_{s,s}$ and $E_{s,s}$ reportedly bind catalytically active metals directly or indirectly (by possessing prosthetic groups such as iron-centered hemes), to form the center of catalytic reactions within the molecules. Thus, catalytic activities of natural and artificial metal-binding enzymes can be attributed to the behaviors of bound metals. Therefore, it is natural to obtain the following proposition, $P(E_s)$.

\[
P(E_s) = \exists \{E_s\} \in \{Ics\}
\]

This type of enzymes should be categorized and termed as metalloenzymes. According to recent reviews on metalloenzymes, an artificial metalloenzyme can be designed de novo by arranging the peptidic sequence composed of 20 natural amino acids. Basically, such de novo designs of metalloproteins can be achieved freely designing the amino acid sequences capable of binding metal ions. In order to artificially design or modify the catalytic peptides or proteins, it is much easier to learn from the catalytically active peptidic motifs within the naturally existing active enzymes or proteins as the platforms of engineering.

As reported by Yeung et al., modification of myoglobin (Mb) is one of successful cases in engineering of semi-natural metalloenzyme. Accordingly, natural Mb was re-designed into a functional nitric oxide reductase, by newly forming a non-heme iron binding site in the distal pocket of Mb. Presence of such natural, semi-natural and artificial metalloenzymes consists the elements of conceptual subset of bio-originated catalysts within the set of $\{Ics\}$ fulfilling the proposition (7):

\[
P(E_s) = \exists \{E_s\} \in \{Ics\}.
\]

Similarly to the cases of metalloenzymes, we have been seeking for the cases of metallonucleozymes, in which catalytic activities of natural and artificial metal-binding nucleic acids (DNAs and RNAs) can be attributed to the behaviors of bound metals. Therefore, it is also natural to obtain the following proposition, $P(N_s)$.

\[
P(N_s) = \exists \{N_s\} \in \{Ics\}
\]

In the processes to revise recent progresses, we would like to highlight some examples of metallonucleozymes which may be supporting the examples (8).

Two propositions listed above (7, 8) can be combined and generalized as follows:

\[
P(BC_s) = \exists \{BC_s\} \in \{Ics\}
\]

By analogy to the cases of metal-binding $BG$s, we can assume that catalytic actions of some $BG$s can be attributed to the catalytic mode of actions similar to $OC$s, since proline, one of natural amino acids composing proteins, is now consider as an active catalyst. Thus, following proposition can be arisen.

\[
P(BC_s) = \exists \{BC_s\} \in \{OCs\}
\]

This proposition will be discussed at the end of this article.

Non-biological catalysts producing $\text{O}_2^{\bullet\bullet}$

As an example of $\{Ics\}$ in definitive proposition (1), we have studied the mechanism of $\text{O}_2^{\bullet\bullet}$ production by titanium dioxide ($\text{TiO}_2$)-based sono-photocatalytic (SPC) system. Recently, a variety of ultraviolet (UV)-driven photochemically active catalysts designated as photocatalysts consisting of $\text{TiO}_2$ has been developed and applied for hygiene and antimicrobial purposes. The likely mechanism of such catalysts involves the generation of ROS on the surface of the catalysts as expected (not fully proven) from the previously proposed models. However, no attempt to confer long-lasting chemical properties to the waters (e.g., preparation of waters rich in ROS) has been reported, except for our model, despite of the increasing demands for the use of photocatalysts in various environments including the use in aqueous phase.

Our attempts were firstly, to obtain the data scoring the rate of $\text{O}_2^{\bullet\bullet}$ production by both sono- and photo-catalytic manner, and secondly, to testify if the sono-photochemical priming of oxygen saturated water results in continuous release of $\text{O}_2^{\bullet\bullet}$ in the system lasting at least for a half hour or not. For above purposes, a novel water conditioning SPC apparatus (Figs. 1A and B) equipped with sheets of $\text{TiO}_2$-coated photocatalytic fibers were applied for the preparation of ROS-containing water. The apparatus used in the demonstration was equipped with $\text{TiO}_2$-coated fibers and UV-A (360 nm) bulbs enabling the excitation of $\text{TiO}_2$, and also with 2 ultrasonic wave (USW) generating devices to assist the reaction of interest. When required, $\text{O}_2$ gas was supplied to the system through artificial lung (thus minimizing the impacts of air bubbles) connected with a magnet/roller pump. Monitoring of the dissolved $\text{O}_2$ level was necessarily required for enabling the optimal generation of $\text{O}_2^{\bullet\bullet}$ in the water (5 L) maintained at ca. 20°C and circulated at 20 L/min.

In the model experiments, we detected the generation of hydroxyl radicals ($\text{HO}^{\bullet\bullet}$) (Fig. 1C) and $\text{O}_2^{\bullet\bullet}$ (Fig. 1D) as the key members of ROS generated in the water circulated in SPC chambers, through electron spin resonance (ESR) spectroscopy and chemiluminescence (CL) assays, respectively. For CL assays, $\text{O}_2^{\bullet\bullet}$-specific CL probe Cypridina luciferin analog (2-Methyl-6-phenyl-3,7-dihydromidazo[1,2-a]pyrazin-3-one; designated as CLA) was used. For ESR, a spin trapping agent, DMPO (5, 5-Dimethyl-1-pyrroline-N-Oxide) that readily forms an adduct.
with HO\(^*\) was used. Note that CL reflecting the generation of O\(_2\)\(^{**}\) can be detected in the water transferred out of the water circulating system, suggesting that some intermediate species or precursors of O\(_2\)\(^{**}\) can be produced for certain length of time (Fig. 1D). In contrast, HO\(^*\) was detected only when the trapping agent, DMPO was circulated inside the SPC chambers. DMPO added to the water manually sampled out of the apparatus did not provide the signal for HO\(^*\) (data not shown).

One likely use of the conditioned water is controlling of the biological responses of living plant cells since it has been well documented that various physiological and biochemical events during the plant life cycle, such as germination of seeds, induction of defense mechanism against pathogenic microorganisms and adaptation to severe environments, are controlled by ROS.\(^{24}\) It has been shown that expression of a number of redox-induced stress-related genes is regulated through calcium signaling in plant cells.\(^{8}\) Therefore, pre-treatments of plants with redox-modifying agents that target the calcium channel opening may result in induction of anti-oxidative capacity (conferring by expression of ROS-responsive genes).

We proposed an attempt for conferring anti-oxidative capacities to plants for protecting the damaging impacts of photochemical oxidants or other oxidative stress, by novel water-processing technology which provides redox-active water designed for stimulating the expression of redox-related and defense-related genes in plants, through pre-treatments with moderate oxidative stresses.

To assess if the level of ROS produced in the photochemically conditioned water attained and remained at the level actively inducing the responses of living plant cells, we tested the responses of tobacco cell suspension culture (BY-2, expressing aequorin gene) to SPC-conditioned water. Presence of O\(_2\)\(^{**}\) in the conditioned water-treated cell suspension culture was detected with the CL of CLA (Fig. 2A). Similarly, addition of SPC-conditioned water to tobacco BY-2 cells expressing aequorin gene resulted in increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) (Figs. 2B and C) and slight increase in cell death (Fig. 2D). Similarly, we have shown that addition of nano-sized particles of TiO\(_2\) induces the generation of ROS and also an increase in [Ca\(^{2+}\)]\(_c\) in living tobacco cells.\(^{25}\)

In addition, we observed that treatment of plants of ROS-sensitive tobacco cultivar (Bel-W3) with the SPC-conditioned water resulted in induced expression of PR1a, a ROS-responsive gene known to be involved in pathogenesis response in plants (Fig. 2E). Our proposal to use this novel water-treatment technology based on SPC has potential for vaccinating the plants, thus conferring the resistance to oxidative stresses as illustrated in Figure 2F.

**Learning from the chemico-biological models in plants (1): plant peroxidase as model enzyme**

Living plants are sources of a number of enzymes involved in metabolism (production and removal) of ROS.\(^{7}\) In higher plants, 2 major mechanisms are known to be responsible for production of ROS, namely one involving NADPH oxidases\(^{27}\) and one involving peroxidases.\(^{28}\) A research group in Geneva University metaphorically described that plant enzymes belonging to

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**Figure 1.** Generation of ROS by SPC processes. (a, a’) Water conditioning SPC apparatus. Two exPCAW1.2s (WIPO No: WO10/032765; K2R Inc., Kitakyushu, Japan) connected in tandem (A) and the diagram of water conditioning system (a’). (B) Scanning electron microscopic image of the catalytic fiber which was fabricated by finely coating TiO\(_2\) over alumina layer of aluminum fibers. (C) Detection of DMPO-OH signal with ESR reflecting the generation of HO\(^*\). (D) Monitoring of long lasting redox activity in the SPC-processed water using O\(_2\)\(^{**}\)-specific CL probe, CLA. (E) Quenching of CLA-CL by Tiron, a scavenger of O\(_2\)\(^{**}\). (F) Synergistic impact of UV and USW in generation of O\(_2\)\(^{**}\). Images were obtained and modified from.\(^{17}\)
Peroxidases (EC 1.11.1.7) display multiple functions more than a ‘Swiss army knife’. In the conventional peroxidase cycle, hydrogen peroxide (H₂O₂) is used as the common acceptor of electrons (e⁻), and a variety of compounds function as e⁻-donating substrates. Therefore, regulation of the level of H₂O₂ and oxidation of a wide range of substrates are the likely functions of plant peroxidases. Furthermore, diversified roles for plant peroxidases also include concomitant generation of ROS coupled to oxidation of phenolics such as salicylates, and aromatic amines such as phenylethylamine. As above, it is now accepted that plant peroxidases are capable of catalysis leading to generation of ROS, chiefly O₂⁻, through oxidation of key substrates through H₂O₂-dependent conventional peroxidase cycle involving the cyclic formation of redox active enzyme intermediates known as Compounds I and II (path 3→5→4→3 in Fig. 3). Generation of O₂⁻ via peroxidase cycle involves the H₂O₂-dependent generation of intermediate radical species such as phenoxyl radicals or aromatic amine radicals which could be detected by ESR spectroscopy by using ascorbic acid as a spin trap.
In addition to aforementioned e\(^{-}\) acceptor-driven (\(H_2O_2\)-requiring) conventional peroxidative cycle, there is an alternative mechanism for generation of \(O_2^{2-}\) in an e\(^{-}\) donor-dependently driven manner, which should be described as the oxygenation or oxygenase cycle of plant peroxidase (path 3\(\rightarrow\)2\(\rightarrow\)6\(\rightarrow\)3 in Fig. 3). The most widely known substrate for this \(H_2O_2\)-independent cycle is indole-3-acetic acid (IAA), the principal form of natural auxin in higher plants.\(^{35-38}\)

We view that the role of IAA in POX-catalyzed generation of \(O_2^{2-}\) is one of effective e\(^{-}\) donors converting native enzyme into ferrous intermediate in the oxygenation cycle (Fig. 3, upper half). IAA-dependent reduction of native enzyme (with heme at \(Fe^{III}\)) into ferrous intermediate (with heme at \(Fe^{II}\)) is immediately followed by a series of reaction proceeding under the atmospheric \(O_2\)-rich condition, by which, unstable ferrous complex can be readily converted to \(O_2\)-bound form of enzyme intermediate known as Compound III in which the state of heme iron can be described as \(O_2\)-heme-\(Fe^{II}\) or \(O_2^{2-}\)-heme-\(Fe^{III}\).\(^{39}\) Then, gradual decay of this complex into native enzyme at heme-\(Fe^{III}\) state accompanies the release of \(O_2^{2-}\) (Fig. 3, upper half) as confirmed with IAA-stimulated horseradish peroxidase (HRP) using \(O_2^{2-}\)-specific CL probe, CLA.\(^{37}\)

Interestingly, medical application of HRP-labeled antibodies and IAA, novel \(O_2^{2-}\)-generating agents enabling cancer-targeted cell death induction, has been proposed based on the views that transient formation of [POX-IAA-\(O_2\)] complex results in robust release of \(O_2^{2-}\).\(^{37}\) To date, 3 approaches have been reported, namely, HRP-conjugated immuno-labeling of cancer-related molecules,\(^{4,40}\) expression of recombinant HRP in mammalian cells,\(^{41}\) and modulation of the IAA-induced reaction using a fungal molecule.\(^{38}\)

Assuming that the hypothetical model mechanism proposed in Figure 3 (upper half) is correct, we should be able to screen or identify some effective e\(^{-}\) reducing agents which target the native enzyme to trigger the onset of oxygenation cycle in plant POXs, eventually leading to a robust and long-lasting burst of \(O_2^{2-}\) production. After testing a wide range of chemicals, we observed that free ferrous ion (\(Fe^{II}\)) acts as a novel inducer of \(O_2^{2-}\) production in aid of plant POX, possibly by behaving as an effective e\(^{-}\) donor for \(Fe^{III}\)-to-\(Fe^{II}\) conversion of heme in a model POX, HRP. The aim of the present section is to share our novel finding on the Fe-driven \(O_2^{2-}\) production mechanism involving HRP.

In plants, physiological significance of such peroxidase-mediated redox reaction could be found in the defense mechanism against microbial attacks. Recently, we have proposed a likely role for non-biological inorganic factors such as free ferrous ion\(^{42}\) and nitric oxide\(^{43}\) in reduction of ferric native enzyme into ferrous intermediate protein which readily produces \(O_2^{2-}\) through the mechanism involving the formation and decay of Compound III (Path 3\(\rightarrow\)2\(\rightarrow\)6\(\rightarrow\)3 in Fig. 3, upper half), thus, possibly contributing to the plant defense mechanism. By making use of the oxygenase cycle in plant peroxidase, modulation of the enzyme activities by doping-chemicals without supplementation of any substrate is one of the artificial regulatory modes of peroxidase.

![Figure 3. Hourglass model that summarizes the inter-conversions among active and inactive forms of peroxidase intermediates. The model was simplified based on earlier works.\(^{28,43,106}\) The numbers on the black balls indicate the formal oxidation states of enzyme and its intermediates. Formation of radical intermediate (A*) further participates in formation of \(O_2^{2-}\). This model dissecting 2 distinct cycles initiated by interaction of native POX with acceptors and donors of e\(^{-}\), is often referred to as the hourglass model due to its shape.\(^{28,29,106}\)](image)
Accordingly, one of key factors required for plant survival under O₃ toxicity was shown to be endogenous peroxidase, since the level of ROS determines the onset of localized apoptotic cell death in O₃-exposed cells.

In addition to plant peroxidases, small peptides behaving similarly to peroxidases reportedly participate in the plant responses to O₃. As one of O₃-induced plant responses, expression of OI genes have been identified in saltbush (Atriplex canescens) and few other plants. OI gene inducers include some additional chemical and environmental factors such as SO₂ and water deficit, suggesting the multiple roles for these genes. By No and his colleagues, 2 isotypes of OI peptides, OI2–2 (158 amino acids) and OI14–3 (119 amino acids), were identified. These peptides possess a common repeat unit (8–10 times repeated in tandem), consisting of hexa-amino acids (Y-G-H-G-G-G). OI peptides are considered to be putative members of glycine-rich proteins designated as GRPs, due to the presence of above repeat unit. The secondary structures of most GPRs and OI peptides are rich in β-pleated sheets. Suggests that there is some similarity between prion proteins (PrPs) from animal systems and plant GRPs including OI peptides.

Recently, it was suggested that the hexa-repeat in OI peptides acts as a metal-binding motif and synthesized model peptides with the hexa-repeat found in OI peptides are highly active in generation of O₂*, upon addition of H₂O₂ to the copper-loaded peptides. Interestingly, in the above report, possible mechanism of the reaction and biological consequence of the reactions catalyzed by OI peptides were discussed by analogy to the action of octarepeat peptides derived from human prion protein (PrP) as discussed later.

Role of His and Tyr residues in redox-active peptides

As described above, plant-derived OI peptides containing PrP-like repeated sequence consisted of a His- and Tyr-containing hexa-repeat unit repeated for 8–10 times in tandem. Accordingly, the repeat unit found in the O₂-induced peptides shows H₂O₂-dependent O₂* generating activity by using self-Tyr residues as phonoic substrates required for peroxidative process, thus simply converting H₂O₂ to O₂* without requirement for additional phenolics or amine substrates. As mentioned above, 2 OI peptides, OI2–2 and OI14–3, share the identical repeat motif (G-G-G-Y-G-H) which is repeated for 9 and 7 times, respectively. The each repeat unit in OI peptide was shown to form a complex with transition metals, chiefly copper ion in the physiological pH range based on the spectroscopic studies by measuring the cyclic dichroism (CD) and nuclear magnetic resonance (NMR). Yokawa et al. have assessed the O₂* generating activity of metal-binding motif derived from plant OI peptides, by chemically synthesising a series of peptides, namely G-G-G-Y-G-H, Y-G-H-G-G-G, H-G-G-Y-G, and G-G-G-F-G-H. To prevent the peptide from forming self circularization, amino and carboxyl terminal of each peptide was acetylated and amidated, respectively.

Accordingly, the peptide with His residue located at the carboxyl terminal (G-G-G-Y-G-H) showed the highest activity and that with N-terminal His residue (H-G-G-G-Y-G) showed no activity. Therefore, not only the presence, but also the location of His residue is one of critical requirement for the reaction. Similar observation confirming the role for His residue have been reported for the model His-containing Cu-binding peptides as discussed in the below sections of this article.

By analogy to the putative role for Tyr residue as intra-molecular substrates within some redox active enzymes such as cyclooxygenase and ribonucleotide reductases, in which corresponding reactions proceed via the formation of a tyrosyl radical, the importance of Tyr residue in the repeat unit in OI peptides was assessed. Accordingly, a Tyr-to-Phe (Y-to-F) substituted mutant peptide (G-G-G-F-G-H) was synthesized and used for comparison. As predicted, the Cu/G-G-G-F-G-H complex showed no activity for the generation of O₂* after the addition of H₂O₂. On the other hand, by supplementation of free Tyr as a substrate into the reaction mixture containing Cu/G-G-G-F-G-H complex, the H₂O₂-dependent O₂* generating activity was regained. Requirement for free Tyr or Tyr residue in H₂O₂-dependent O₂* generating reaction suggest that tyrosyl radical (one form of phenoxy radicals) is transiently formed for one-electron reduction of O₂ similarly to the plant peroxidase reaction H₂O₂-dependently generating O₂* by coupling to oxidation of phenolics (such as salicylic acid) to form phenoxy radicals.

Finding and defining the metal-binding and catalytic motifs within chicken prion proteins

There is a set of proteins which could not be defined by proposition (3)

{Es} ∈ \{\{Cs\} ∩ \{proteins ∪ peptides\}\}

The case of prion proteins (PrPs) and derived small peptides could be one such example. Generally, PrPs and derived peptides are not considered as enzymes at present, although they are either proteins or peptides having catalytic nature (proposition 11).

{PrPs} ∈ \{\{\{Cs\} ∩ \{proteins ∪ peptides\}\} \{Es\}\}

By admitting that there are proteins or peptides (both natural and artificial) with catalytic activity which can be considered as element of \{BCs\} in a broad sense as defined below (proposition 12), the phenomena observed with plant OI-peptides and animal PrPs belonging to novel class of BCs can be compared with conventional BCs such as plant peroxidases.

P(novelBCs) = \exists \{\{proteins ∪ peptides\} ∈ \{\{BCs\} \{Es ∪ Ns\}\}\}

Actually, the kingdoms of plants and animals are rich in such small peptidic metalloenzymes, belonging to BCs in a broad sense, catalyzing the generation of O₂*.

The criteria for consisting a minimal peroxidase-like small peptides is the presence of His-rich motifs required for binding to metals (chiefly copper), and free and/or peptide-bound substrates. Similarly, recent studies have shown that peptides
derived from human PrP mediates the production of $O_2^{-}$ through oxidation of substrates such as aromatic monoamines or phenolics (mostly, neurotransmitters and their analogs). Upon binding to copper at 4 different putative copper-binding motifs (Fig. 4A), PrP and derived peptides may gain the catalytic activities as our earlier works have revealed that PrP-derived copper-binding peptides catalyze the generation of $O_2^{-}$ in peroxidase-like manner involving $H_2O_2$ as e$^-$ acceptor and aromatic amines or phenols as the e$^-$ donors.

Actions of Cu-bound PrPs are of great importance from the engineering point of view in order to design the novel peptidic metalloenzymes. Apart from engineering purpose, but viewing from the biological and medical points, the importance of metalloproteins in neurobiology has been suggested both as oxidants and antioxidants in neurodegenerative processes in animals. Cu is an essential trace element in most living organisms but its redox reactivity often leads to the risk of oxidative damage to the cells and tissues, as observed in the neurodegenerative diseases such as ‘prion’ disease and Alzheimer, Menke’s and Wilson’s diseases all occurring via disorders of Cu metabolism. Especially, Alzheimer disease and prion disease are 2 of known major conformational diseases, as documented to date.

Deposition of abnormal protein fibrils is a common pathological feature observed in “protein conformational” diseases, including prion dementias and Alzheimer, Parkinson and motor neuron diseases. Generation of ROS is now considered as one of key events required for development of conformational diseases. In the cases of accumulation of $\alpha$-synuclein in Parkinson disease and accumulation of $\beta$-amyloid in Alzheimer disease, the evidence for involvement of ROS, chiefly $H_2O_2$ and derived HO$^*$, in the neurodegenerative mechanisms have been documented, suggesting that pathogenesis of such neurodegenerative diseases could be attributed to the generation and damaging impacts of ROS which eventually stimulates the formation of abnormal protein aggregates.

PrPs are the only known causative agents for transmissible spongiform encephalopathies in mammalian brains. A number of studies have shown that PrPs can form a group of Cu-binding proteins possibly involved in redox reactions as human PrP has 4 Cu-binding sites in the “octarepeats” region (PrP 60–91) in which amino acid sequence P-H-G-G-G-W-G-Q appears 4 times in tandem and each repeat possibly binds single $Cu^{2+}$ at physiological neutral and basic range of pH. Similarly, in chicken PrP, the Cu-binding motif analogous to the octarepeats are known as hexa-repeats in which each repeat consist of the 6 amino acids, H-N-P-G-Y-P. In chicken PrP, His residues in hexa-repeat are considered to play a key role in anchoring of Cu.

Figure 4. Copper-binding motifs in human PrP. (A) Putative copper-binding domains present in human PrP. Four peptide sequences corresponding to copper-binding motifs in PrP. (1) The most characterized Cu-binding sequence repeated 6 times in bovine and 4 times in human, mouse and ovine. (2) An additional Cu binding sequence found in the basic region in human and mouse PrPs immediately after the octarepeats. (3) PrPs from mammals and chicken show similar sequences. (4) Well characterized PrP helix 2 sequence. Recently proposed overlapping motifs in the human PrP’s octarepeat region required for binding to metals. (i) X-X-H motif suggested to bind Tb in the Tb-fluorescence assay. (ii) H-G-G-W motif suggested to bind Cu in the peptide-fluorescence assay. $M^{n+}$, metal cations. Histidine residues possibly involved in anchoring of copper are marked with asterisks.

PrPs are the only known causative agents for transmissible spongiform encephalopathies in mammalian brains. A number of studies have shown that PrPs can form a group of Cu-binding proteins possibly involved in redox reactions as human PrP has 4 Cu-binding sites in the “octarepeats” region (PrP 60–91) in which amino acid sequence P-H-G-G-G-W-G-Q appears 4 times in tandem and each repeat possibly binds single $Cu^{2+}$ at physiological neutral and basic range of pH. Similarly, in chicken PrP, the Cu-binding motif analogous to the octarepeats are known as hexa-repeats in which each repeat consist of the 6 amino acids, H-N-P-G-Y-P. In chicken PrP, His residues in hexa-repeat are considered to play a key role in anchoring of Cu.

Note that both His and Tyr residues can be found in the chicken PrP’s hexa-repeat unit. As Tyr-containing peptides could be a target of the redox reaction catalyzed by metal-containing proteins or peptides involved in peroxidative and ROS generating reactions, we synthesized 6 peptides corresponding to Cu-binding region (hexa-repeat) of chicken PrP and examined its catalytic activity for the generation of $O_2^{-}$. Each of 6 peptides synthesized (N-P-Y-Y-P-H, P-G-Y-P-H-N, G-Y-P-H-N-P, Y-P-H-N-P-G, P-H-N-P-G-Y, and H-N-P-G-Y-
P) contains both His residue possibly anchoring Cu ion and Tyr residue possibly behaving as a substrate were used for assessing the \( \text{O}_2^{**} \)-generating activity using the \( \text{O}_2^{**} \)-specific CL of CLA.\(^{70}\) As a results, the generation of \( \text{O}_2^{**} \) was observed in the presence of hexapeptide, copper and \( \text{H}_2\text{O}_2 \) without addition of any phenolic substrate since Tyr-residue on the hexapeptide possibly behaves as a substrate for the reaction.

To conclude that requirement of Tyr residue on the peptides in \( \text{H}_2\text{O}_2 \)-dependent generation of \( \text{O}_2^{**} \), we also tested the mutation of Tyr residues into Phe residues in 2 model peptides (i.e. N-P-G-E-P-H and E-P-H-N-P-G). As expected, the Y-to-F substitution mutant peptides showed complete loss of \( \text{H}_2\text{O}_2 \)-dependent generation of \( \text{O}_2^{**} \). Furthermore, we confirmed that supplementation of free Tyr to the reaction mixture containing the Y-to-F mutant peptides results in production of \( \text{O}_2^{**} \). It is conclusive that similarly to plant OI-peptide, both the presence and positions of His and Tyr residues in chicken PrP's hexarepeat units are highly important for the catalytic modes leading to generation of \( \text{O}_2^{**} \).

Catalytic activity in human prion-derived peptides

To date, key involvement of trace elements, chiefly of Cu, in prion disease has been well documented.\(^{71,73}\) Until recently, 2 opposing roles for Cu-bound PrPs have been proposed and discussed, namely the role of bound copper as an anti-oxidant element and contrary as a pro-oxidant element enhancing the neurodegenerative process.\(^{74}\) In both cases, Cu-binding sequences highly preserved in PrP's play key roles in generation of ROS.\(^{72}\)

A series of works conducted by our group suggested that 4 distinct peptide sequences corresponding to 7 putative copper-binding sites containing metal anchoring His residues (His61, His69, His77, His85, His96, His111, and His187) in human PrP function as catalytic motifs active for \( \text{O}_2^{**} \)-generation through reactions with aromatic monoamines.\(^{57}\) Furthermore, phenol-dependent \( \text{O}_2^{**} \)-generation catalyzed by several PrP-derived copper-binding peptides was recently assessed using various phenolics as substrates such as free phenolics, free Tyr, solubilized polymers with phenolic groups (i.e. polyvinyl phenol which is a polymer with multiple phenolic groups, chain length, 12–58 mer,\(^{9}\) and Tyr residues on peptide chains.\(^{58}\) Since supplementation of \( \text{H}_2\text{O}_2 \) is required for oxidation of amines or phenols by these copper-centered peptides, the modes of reactions were considered to be peroxidase-like.\(^{57}\\)\(^{57}\)

Among PrP-derived and related Cu-binding motifs ever examined, \( \text{H}_2\text{O}_2 \)-dependent \( \text{O}_2^{**} \)-generating activity was most active in a truncated helical sequence (V-N-I-T-K-Q-H-T-V-T-T-T-T) which is highly analogous to original (wild-type) PrP's helical sequence.\(^{57}\\)\(^{57}\)

Two distinct metal-binding motifs overlaid in the PrP octarepeat region

Our earlier studies have revealed that His residues (at least single His) are required for anchoring copper on PrP-derived peptides,\(^{57,76}\) and consequently, the catalytically active copper-binding motif within PrP-derived peptides was determined to be X-X-H, where X can be any amino acids followed by His residue.\(^{9,77}\)

In human PrP, His96 is located between G-G-G-T and S-Q-W-N sequences. To examine the positional effect of His on the catalytic activity in the derived peptides, comparison of the His-started H-S-Q-W-N pentapeptide and the His-ended G-G-G-T-H pentapeptide was carried out.\(^{53}\) While reaction with tyramine (given as model substrate) and G-G-G-T-H peptide resulted in robust production of \( \text{O}_2^{**} \), the H-S-Q-W-N peptide showed no catalytic activity. By assuming that G-T-H motif within the G-G-G-T-H pentapeptide is one of X-X-H motif derivatives, experimental comparison of the catalytic activities among G-G-G-T-H pentapeptide and shorter derivatives (G-G-T-H and G-T-H) were performed and the data obtained clearly suggested the importance of the N-terminal glycolylic acid elongation for manifesting the maximal reductox activity in C-terminal His anchored peptides.

Example of artificial enzyme based on X-X-H motif was developed,\(^{78}\) demonstrating that Cu-binding peptides with X-X-H motif conjugated to organic materials could form a novel class of biosensing and bioengineering tools. Accordingly, a tripeptide, Gly-Gly-His (G-G-H, one of X-X-H motif derivatives) was introduced onto the glycidyl methacrylate-grafted porous hollow fiber membrane made on the polyethylene platform by radiation-induced graft polymerization. After loading of Cu\(^{2+} \) on the membrane, CL assay was performed to testify the catalytic activity of the membrane, generating \( \text{O}_2^{**} \) upon addition of \( \text{H}_2\text{O}_2 \) and tyramine as the paired substrates. Inokuchi et al.\(^{79}\) have studied the minimal motifs required for binding of metals within human PrP, by assessing (1) the peptide-dependent quenching of Tb\(^{3+} \) fluorescence and (2) the Cu\(^{2+} \)-dependent quenching of intrinsic fluorescence in human PrP octarepeat-derived peptides. Nobel assays based on the quenching of Tb-fluorescence by interacting peptides emphasized the role of His-ended peptides sharing X-X-H motif. The obtained data clearly supported the view that an intact X-X-H motif located at C-termini of peptides, is desirable as the site of metal chelation. In the case of human PrP's octarepeat unit, P-Q-H motif rather than N-terminal H-G-G-G-W pentapeptide was shown to be active in metal binding.

Empirically, N-terminal His-started oligo-peptides derived from human PrP have been used as models for Cu-binding in earlier in vitro studies. These study suggested that the actual least motif in the octarepeats necessarily required for binding of Cu consists of 5 amino acids H-G-G-G-W\(^{80}\) or 4 amino acids H-G-G-G.\(^{68}\)

Interestingly, the role of His-started motif (H-G-G-G-W) was supported by the Cu-dependent peptide fluorescence quenching assay.\(^{79}\) Among the octapeptide sequences examined, the P-H-G-G-W-G-Q peptide was shown to be the most sensitive to the low Cu concentration although this sequence lacks the presence of intact metal-binding X-X-H motif.

Taken together, in the mammalian PrP octarepeat regions, in which P-H-G-G-G-W-G-Q is repeated for 4 (human) to 6 (bovine) times, 2 distinct metal binding motifs, namely X-X-H motif and H-G-G-G motif, could be overlaid by sharing common His residue and thus co-existed (Fig. 4).
Role of Tyr residues within human PrP, the likely targets of catalysis

Human PrP-derived catalytic model peptides all showed requirements for addition of aromatic substrates in order to produce $O_2^{•−}$ in the presence of $H_2O_2$. While the studies with the Cu-binding motifs in plant OI-peptides and chicken PrP are strongly indicating that Tyr residues presented on the peptide chains are the likely target of redox relay eventually converting $H_2O_2$ into $O_2^{•−}$. Since free Tyr (among the active phenolics examined) is a good substrate for human PrP-derived catalytic short peptides, we assume that the modes of catalytic actions among the plant-derived, chicken-derived and human-derived copper-binding peptides described above may not differ much.

In case of non-peptidic free Tyr given as a model substrate, the presence of phenolic moiety, but not the amino and carboxyl groups, was shown to be important in the interaction with Cu-bound PrP-derived peptide. Therefore, it is tempting to test if the Tyr residues flanking on peptidic chains or proteins function as putative targets of human PrP’s copper-binding motifs.

In fact, human PrP possesses several Tyr-residues being exposed to the external media and events involving such Tyr residue may play a pivotal role in development of prion dementias, as recent reports suggested that helix H1 of human PrP and its 2 flanking loops (highly rich in Tyr residues) are subjected a transition into a β sheet-like structure during forced conformational conversion of the intrinsic cellular form of PrP (PrP$^+$) into the scrapie form (PrP$^S$) (Bertho et al., 2008). By definition, conversion of the PrP$^C$ into PrP$^{C^+}$ is a fundamental event observable upon onset of prion disease development. Yokawa et al. have reported an attempt to testify if the Tyr residues on PrP or derived peptides can be used as the substrate for a human PrP-derived Cu-bound catalytic peptide. In their experiments, the Cu-bound V-N-I-T-K-Q-H-T-V-T-T-T-T helical peptide was used as a model catalyst $H_2O_2$-dependently producing $O_2^{•−}$. On the other hand, the tested putative substrates include (1) tyrosyl-tyrosyl-arginine tripeptide (Y-Y-R) which appears twice in the Octapeptide derived from human PrP, we have designed a series of novel peroxidative biocatalysts as discussed below.

By analogy to G-G-G-T-H, a phenol-oxidizing catalytic pentapeptide derived from human PrP, we have designed a series of simplified model peptides (G$_n$H$_n$ series peptides) which are composed of oligoglycyl chains ended with C-terminal His. To test the importance of the elongated N-terminal glycyll chain and anchoring His residue, both G$_n$H$_n$ series peptides varied in N-terminal glycyll chain length ($n$ = 2, 3, 4, 5 and 10) and oligoglycyl peptides lacking His ($G_n$ series) were synthesized.

As expected, $G_n$ series showed no catalytic activity since these sequence lack the motif for binding to catalytically important Cu$^{2+}$. Within the $G_n$H$_n$ series, catalytic activity of the minimal Cu-binding motif (G$_2$H$_2$ tripeptide) was hardly detected despite the Okobira model. Probably, in the Okobira model, in addition to G-G-H sequence, the supporting chains of glycylid methacrylate on which G-G-H is grafted may playing a role similarly to N-terminal elongating oligo-Gly chain. In G$_n$H$_n$ series, G$_n$H tetrapeptide showed a detectable increase in production of $O_2^{•−}$, confirming the importance of N-terminal Gly elongation. It can be generalized that the catalytic performance in G$_n$H$_n$ series can be ca. 3-fold enhanced with single amino acid elongation (addition of N-terminal Gly residue, allowing elongation from G$_2$H to G$_4$H, G$_5$H to G$_7$H, and G$_7$H to G$_9$H). However, further elongation from G$_7$H hexapeptide to G$_9$H undecapeptide resulted in only ca. 3-fold of enhancement suggesting that the requirement for the N-terminal elongation is nearly fulfilled. These data suggest that the presence of the C-terminal His is the primary requirement for catalytic performance, and N-terminal elongation contributes to the enhancement of the catalytic activity.

Although involvement of Cu and generation of ROS are analogous to tyrosinase which oxidizes Tyr and polyphenols with concomitant release of $O_2^{•−}$, the roles played by $H_2O_2$ are largely different in the $G_n$H$_n$ series metalloenzymes. While $H_2O_2$ is often regarded as an inhibitor of the tyrosinase reaction, the $G_n$H$_n$ series metalloenzymes require the presence of $H_2O_2$ as cosubstrate. On the other hand, plant peroxidases are shown to be active in generation of $O_2^{•−}$ upon oxidation of various phenolics and monoamines in the presence of $H_2O_2$. suggesting that the modes of reactions catalyzed by PrP-derived peptides and artificial $G_n$H$_n$ series metalloenzymes are analogous to the modes of peroxidase reactions.

Among hydroxybenzoic acids (HBAs) and benzoic acid (BA), 2-HBA (salicylic acid) and BA were shown to be poor substrates for $O_2^{•−}$-generating reactions catalyzed by G-G-G-T-H pentapeptide and $G_n$H$_n$ series metalloenzymes. In contrast, 3-HBA
and 4-HBA were shown to be good substrates, suggesting that the presence of phenolic moieties with m- or p-positioned OH group is required for generation of \( \mathrm{O}_2^{**} \), notably differed from the plant peroxidase which favors 2-HBA.\(^{56}\) When dihydroxybenzoic acids (DHBA) were used as model substrates, inutility of o-positioned OH group was also observed using 2,6-DHBA. However, PrP-derived peptides (G-G-G-T-H and V-N-I-T-K-Q-H-T-V-T-T-T-T), plant OI-peptides, and G\(_5\)H series metalloenzymes showed \( \mathrm{O}_2^{**} \)-generating activity upon addition of other DHBAs with o-positioned OH (2,3-DHBA, 2,4-DHBA, and 2,5-DHBA), indicating that the presence of o-positioned OH does not interfere with the roles for active OH groups at \( m \)- and \( p \)-positions.\(^{53,58}\)

**Kinetic analysis performed with G\(_5\)H series metallopeptides**

G\(_5\)H hexapeptide, one of G\(_5\)H series artificial enzyme was selected for kinetic analyses based on the \( \mathrm{O}_2^{**} \)-specific CL with Cla.\(^{53}\) By calibrating the yield of \( \mathrm{O}_2^{**} \) using KO\(_2\) as standard, the rate of \( \mathrm{O}_2^{**} \) production proceeding in the presence of tyramine was assessed. With Lineweaver-Burk analysis, \( K_m \) and \( V_{\text{max}} \) for the G\(_5\)H hexapeptide-catalyzed production of \( \mathrm{O}_2^{**} \) in the presence of tyramine were determined to be 0.42 mM and 0.12 mmol/mg peptide/min, respectively.

**Enhanced thermo-stability in novel metalloenzymes**

It has been reported that prion-infected brain tissues or homogenates hardly lose their infectivity even after severe heat treatment\(^{82}\) and repeated freezing and thawing.\(^{83}\) Yokawa et al\(^{84}\) have hypothesized that redox activities reflected by the generation of ROS in PrP-derived Cu-binding peptides may also show thermo-stability, therefore, PrP-derived peptide may strive through heating and/or repeated freezing and thawing cycles. It is noteworthy that tyramine-dependent \( \mathrm{O}_2^{**} \)-generating activity found in the peptides corresponding to the Cu-binding motifs in human PrP (G-G-G-T-H, V-N-I-T-K-Q-H-T-V-T-T-T-T), showed extreme thermo-stability surviving under heat-incubation (90°C, 100 min), autoclaving, and repeated freezing/thawing cycles, despite most enzymes and proteins are sensitive to high temperature and repeated freezing.

Newly designed metallo-peptides, G\(_5\)H hexapeptide and G\(_{10}\)H undecapeptide were also used for thermo-stability assessment.\(^{53}\) When the peptides (G\(_5\)H and G\(_{10}\)H) were incubated in the absence of copper, any loss of the catalytic activity was observed even after thermal denaturing treatments. In contrast, Cu-bound form of G\(_5\)H hexapeptide showed some extent of the loss in catalytic activity (ca. 20%) after autoclaving (121°C, 20 min) but this peptide survived the 100 min of heating at 90°C and 10-time repeated freezing and thawing cycles.

**Signal transduction-sensitive artificial enzyme**

Protein phosphorylation is associated with most cell signaling and developmental processes in eukaryotes. Owing to the introduction of phosphate, a bulky and highly charged group, the phosphorylating events often results in a drastic changes in the properties of proteins (mostly enzymes), eventually modulating the activity of enzymes or protein-protein interaction properties.\(^{85}\)

In order to design a novel (probably first) signal transduction-sensitive artificial enzyme, a chimeric bio-catalyst designated as ErkG\(_5\)H (Figs. 5A and B) was constructed,\(^{15}\) by fusing G\(_5\)H, a PrP-inspired metalloenzyme\(^{53}\) with Tyr-containing Erk1/2 MAP kinase (MAPKK) substrate sequence (erk1/2 residues, 182–187 MAPKK phosphorylation sites) consisting of F-L-T-E-Y-V-A.\(^{86}\) Using this molecule, Tyr residue-assisted conversion of H\(_2\)O\(_2\) to \( \mathrm{O}_2^{**} \) was successfully achieved without supplementation of phenolic substrates (Fig. 5C and D), due to the presence of a single Tyr-residue vicinal to Cu-binding catalytic motif (G\(_5\)H).

Then, ErkG\(_5\)H, consisting of (i) Tyr-containing substrate mimic region and (ii) the catalytic region, was used to study the impact of amino acid phosphorylation, which could be the first demonstration of the phosphorylation-regulated artificial enzyme.\(^{15}\) Within short peptide sequence F-L-T-E-Y-V-A-G-G-G-G-H, 2 amino acid residues, namely, Thr and Tyr residues, can be the sites of phosphorylation since the original sequence was derived from the phosphorylation domain within MAPKK.\(^{86}\)

In order to examine the phosphorylation-sensitivity in ErkG\(_5\)H molecule, \( \mathrm{O}_2^{**} \)-generating catalytic activities in non-phosphorylated and phosphorylated peptides (Thr-phosphorylated, Tyr-phosphorylated, and Thr and Tyr double phosphorylated) were compared.\(^{15}\) Catalytic activity of ErkG\(_5\)H was completely lost in the double phosphorylated sample (Figs. 5C and D). Even by single phosphorylation at Tyr residue alone, catalytic activity of ErkG\(_5\)H was mostly lost. In contrast, single phosphorylation at Thr residue resulted in partial inhibition only. Positional impact of phosphorylation determining the catalytic activity is summarized in Figure 5E. This work provided the first implication that phosphorylation-controllable artificial enzyme can be synthesized.

**Interaction between DNA and redox-active metals**

It is well known that oxidative damage to genomic DNA is promoted in the presence of ROS such as HO*, which can be generated via Fenton-type or Harbor-Weiss-type reactions in the presence of the Cu and Fe ions.\(^{88}\) It is also known that the metal-mediated oxidative damage to DNA is further enhanced by coexisting ascorbic acid or H\(_2\)O\(_2\).\(^{88}\) Such oxidative DNA fragmentation and subsequent chromosomal dysfunction may play key roles in apoptotic cell death mechanisms in mammalian cells.\(^{89}\) Through the DNA-degrading reactions in the system containing Cu(II) + H\(_2\)O\(_2\) or Cu(II) + ascorbate, the production of a large amount of HO* at physiological pH condition via Harbor-Weiss-like reaction has been recorded, by monitoring the level of 8-hydroxyguanosine which is a reliable biomarker for HO*-dependent oxidative damage to guanosine residues on DNA.\(^{88}\)

It is known that Cu\(^{2+}\) strongly binds to the guanosine and cytidine bases at physiological pH, eventually perturbing the A-T base pairs and disrupting the double-helical structure of DNA.\(^{90}\) In addition, specific regions on DNA, so called Z-DNA structure-like micro-domains, show much higher affinity to binding of Cu\(^{2+}\), especially at the base guanine.\(^{91}\)
HO\textsuperscript{•} generated in the biological system hardly migrates even a short distance in aqueous phase due to its hyper-reactivity toward neighboring molecules such as water molecules, suggesting that HO\textsuperscript{•} generated at the site vicinal to DNA likely results in enhanced degradation of or damages to DNA. Accordingly, the complex between Cu and aforementioned Z-DNA domains readily results in oxidative damaging to DNA chains.\textsuperscript{91}

Aiming at prevention of the Cu-mediated DNA damages, which is highly important from a gerontological point of view, Yokawa et al.\textsuperscript{92} have reported their attempt in which copper-binding tripeptides with X-X-H motif found within PrP-derived catalytic peptides, prevented the degradation of DNA proceeding in the presence of Cu(II) + H\textsubscript{2}O\textsubscript{2} and Cu(II) + ascorbate. The likely mechanism of peptide-mediated prevention of DNA degradation may involve the chelation of the Fenton catalysts such as Fe and Cu ions.

According to Kageenishi et al., PrP-derived redox-inert copper-binding octapeptide, K-T-N-M-K-H-M-A, effectively protected the plant cells from the toxicity of copper which initiates the apoptotic cell death development in plant cells.

Protection of plant cells by oligo-DNA interacting with metals

Treatments of living plants with excess of transition metals reportedly coincide the production of, molecular responses to, and cellular damages by ROS members such as O\textsubscript{2}\textsuperscript{•−}, H\textsubscript{2}O\textsubscript{2} and HO\textsuperscript{•}, possibly via direct e\textsuperscript{−} transfer involving metal cations, formation of catalytic complex with natural chelating agents such as small peptides, or as a consequence of metal-mediated inhibition of metabolic reactions.\textsuperscript{93,97} It has been well documented that homeostasis and signaling crosstalk involving ROS and other signaling events such as the changes in [Ca\textsuperscript{2+}], are crucial to plant responses and adaptation to abiotic environmental factors threatening the plants, thus determining the fate of plant cells under stressful conditions.\textsuperscript{46–48,92,98–100}

We have previously testified the effect of a copper-binding PrP-derived peptide (Fig. 4A–3) on protection of tobacco BY-2 cells from Cu toxicity.\textsuperscript{77} By analogy, Iwase et al.\textsuperscript{101} have shown that oligo DNAs derived from copper-binding motifs function as effective plant cell-protecting agents preventing the toxicity of copper. Accordingly, addition of GC-rich double-stranded DNA fragments (such as CGCGCG hexamer), prior to treatment with copper ions, effectively blocked both the copper-induced calcium...
signaling event and resultant programmed cell death. It is known that GCGCGC DNA hexamer is one of minimal GC-rich Z-DNAs.\textsuperscript{102}

In case of plant cell protection by Cu-binding peptides, ca. 5 to 10-fold higher concentrations of peptides compared to that of Cu\textsuperscript{2+} were required for significantly blocking both the Cu\textsuperscript{2+} influx and cell death induced by copper, simply due to removal of copper with excess of Cu-binding molecule.\textsuperscript{77} In contrast, DNA-based protection of plant cells from Cu toxicity requires much lower concentrations of oligo-DNA (between 1/10 and 1/3 of Cu\textsuperscript{2+} concentration) suggesting that there would be alternative mechanism of plant protection performed by added DNA fragments.

Designing the DNA-based catalytic molecules removing O$_2^*$

In addition to simple Cu\textsuperscript{2+} removal model, the DNA-Cu complex examined was shown to possess the O$_2^*$-scavenging catalytic activity, suggesting that DNA-mediated protection of the cells from Cu toxicity is due to removal of O$_2^*$\textsuperscript{−}.\textsuperscript{101} For assessing the catalytic activity hidden in the Cu-binding DNA oligomers, 4 different sources of O$_2^*$\textsuperscript{−} was employed, namely, (i) short pulse of O$_2^*$\textsuperscript{−} increase by injection of potassium superoxide (KO$_2$, dissolved in dry DMSO), (ii) slow release of O$_2^*$\textsuperscript{−} in the presence of copper ions and H$_2$O$_2$ (through known path for Cu\textsuperscript{2+}-mediated conversion of H$_2$O$_2$ to O$_2^*$\textsuperscript{−} via HO$_2$), (iii) addition of SPC-activated O$_2^*$\textsuperscript{−}-rich medium which was passed through the SPC reactor equipped with TiO$_2$-coated alumina fiber, ultra violet light source, and ultrasonic generator designated as Ex-PCA\textsubscript{W} (as described in Fig. 1), and (iv) xanthine oxidase.

Through analysis of O$_2^*$\textsuperscript{−} removal by DNA-Cu complex, the mode of DNA-Cu complex was highly similar to the catalytic mode of SOD-like molecules (unpublished results). Kinetic analysis evaluated the $V_{\text{max}}$ for the O$_2^*$\textsuperscript{−}-degrading action of the DNA-Cu complex, performed in the presence of various concentrations of KO$_2$ or SPC-dependently produced O$_2^*$\textsuperscript{−}, to be between 1.155 and 1.675 nmol / mg DNA / sec.

It is conclusive that the primary role of GC-rich oligo DNA is the trapping of toxic copper ions. Furthermore, upon binding to Cu, such metalonucleic acid complex may show catalytic activity for removal of O$_2^*$\textsuperscript{−}. As the $V_{\text{max}}$ Values obtained from independent assays fall in similar range, we now understand that GC-rich double-stranded DNA forms some catalytic complex for removal of O$_2^*$\textsuperscript{−}. This type of molecules could be a good model for designing novel metal-centered artificial nucleozymes, thus fulfilling the criterion predicted by definitive proposition (8):

$$P(NS) = \exists \{NS\} \in \{ICs\}$$  \hspace{1cm} (8)

**Organic catalyst-like biocatalysts**

Propositions (1) to (6), (12) and (13) are merely definitive. Predictions by propositions (7) to (9), of the cases of biocatalysts acting upon binding to catalytically active metals, such as copper-centered metallo-enzymes, peptides and nucleic acids, were examined and proved through discussion up to here, thus confirming the generalized proposition (9).

Contrary, our knowledge on the cases fulfilling the proposition (10) is yet to be covered.

$$P(BCs) = \exists \{BCs\} \in \{OCs\}$$  \hspace{1cm} (10)

As this proposition predicts that there could be biocatalysts showing catalytic activity due to the action of guanidine-like or amino acid Pro-like catalytic domains without involvement of the action of metals. In addition, the model presented in a series of classical works by Kunitake and his colleagues on imidazole-containing enzyme-like catalytic polymers could be also considered.\textsuperscript{103,104}

Interestingly, Pro-containing octarepeat-peptides from human PrP shows self-catalytic generation of O$_2^*$\textsuperscript{−}, showing spiky CLA-CL upon mixture with media lacking metals. Since this phenomenon can be silenced by replacing Pro with other groups (Inokuchi et al., unpublished results), this topic may provide more clues to the development of OC-type BCs in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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