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Electrostatic and Steric Interaction between Redox Polymers and Some Flavoenzymes in Mediated Bioelectrocatalysis

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Abstract

H$_2$O$_2$-generating oxidase/peroxidase (POD)-based mediated biosensors are very useful to minimize interference, but require suitable mediators which work well only for POD but not against the oxidase. Pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[Fe(CN)$_5$]), PVI[Os(dcbppy)$_2$Cl] (dcbppy = 4,4’-dicarboxy-2,2’-bipyridine) and PVI[Os(dmebpy)$_2$Cl] (dmebpy = 4,4’-dimethyl-2,2’-bipyridine) have been utilized to investigate the interaction with four kinds of H$_2$O$_2$-generating oxidases: glucose oxidase, sarcosine oxidase, choline oxidase (ChOD) and lactate oxidase. The mediated bioelectrocatalytic activities of the redox polymers for the enzymes have been determined by cyclic voltammetry in the presence of the substrates. The highly negatively charged PVI[Fe(CN)$_5$] shows practically no mediating activity against the four flavoenzymes, but strong one to POD. On the other hand, PVI[Os(dmebpy)$_2$Cl] with neutral ligands shows a high activity for the oxidases except ChOD. The mediating activity of PVI[Os(dcbppy)$_2$Cl] with negatively charged ligands is much smaller than that of PVI[Os(dmebpy)$_2$Cl]. These results reveal that electrostatic repulsion and steric hindrance are enhanced by using negatively charged polymers to realize minimum activity against the oxidases.
Keywords: Pentacyanoferrate-bound polymer; Osmium complex; Flavoenzymes; Peroxidase; Surface electrostatic potential; Electrostatic repulsion.
1. Introduction

Various types of transition metal redox polymers such as Os and Ru complexes and ferrocene derivatives have been developed for co-immobilization of enzymes to construct mediated electron transfer (MET) bioelectrocatalytic systems over the past two decades [1-3]. These electroconductive polymers are conspicuous due to their unique properties over diffusional mediators: they can be covalently bound to enzymes by crosslinkers on electrode surface; they provide three-dimensional electrocatalytic systems which are not leachable but swollen in water to form redox hydrogels for MET between the redox center of enzymes and electrodes; they have so high density of the redox groups immobilized on electrode surface that they are more efficient than low molecular weight mediators in the electron transfer; moreover, transition metal redox polymers are more stable than quinone-containing polymers since reactive semiquinone radicals formed in the one-electron reduction of quinones easily react with thiols, amines, phenols and other functions [4-6]. These unique properties of metal redox polymers are very useful for MET-type biosensor and biofuel cell application [5, 7, 8]. In MET glucose biosensors, Os-containing polymers have frequently been utilized to shuttle electrons from the redox center of glucose oxidase (GOD) or flavin adenine dinucleotide (FAD)-dependent glucose dehydrogenase (GDH) to electrode, and show
higher stability and sensitivity than low molecular weight mediators [1, 9]. In MET bioelectrocatalysis, we have to consider the linear free energy relationships (LFER) of the electron transfer rate constant \(k\) between enzyme and mediator to the formal potential of mediator \(E^{°}\) [10-12]. The relation is given by Eq. (1) for the oxidation of the substrate,

\[
\log \frac{k_j}{k_i} = \beta \frac{nF}{2.303RT} \left( E^{°'}_j - E^{°'}_i \right)
\]  

(1)

where \(\beta\) is a proportional constant \((0 < \beta < 1)\), \(n\) is the number of electrons, \(F\) is the Faraday constant, \(R\) is the gas constant, \(T\) is absolute temperature, and the subscripts \(i, j\) indicate given mediators as a series of redox compounds with similar structure. In order to increase the current density, it is essential to use a mediator with a large \(k\) value and then with a more positive \(E^{°'}\) value, which leads to increase the oxidative interferences such as ascorbic acid and uric acid in physiological samples. Furthermore, \(O_2\) must sometimes be removed to avoid the competition with mediators in the case of oxidase, which would be difficult and not practical in real sample measurements.

In order to circumvent the problems, \(H_2O_2\)-generating oxidase/peroxidase (POD) bienzyme biosensors mediated by Os-containing polymers were developed [13]. Oxidase-based mediated biosensors coupled with POD allow the determination of \(H_2O_2\) generated from the oxidase such as GOD at low operating potentials around 0 V vs.
Ag|AgCl, with high sensitivity and stability, and elimination of the undesirable oxidation of interferences [14, 15]. However, there is one thing to be concerned that mediators may react with both of oxidase and POD. As shown in Scheme 1, the mediator oxidized by POD may be reduced not only at electrode but by oxidase, because most of oxidases show dehydrogenase activity to transfer the electron to artificial mediators. Such cross reaction causes a decrease in the electrochemical response of mediator reduction [13, 16]. Therefore, it is necessary to select an appropriate mediator with highly selective reactivity for POD but practically no reactivity for oxidase.

The $k$ value in MET bioelectrocatalysis also depends on the structure and charge of the mediator. In the case of PQQ-dependent GDH, the $k$ values of Os-complexes are much lower than those of quinone compounds at a given $E^{\circ}$ [12]. The main factor to cause the difference seems to be the steric effect, since the size of the Os-complex is much larger than that of the reactive site of the enzyme. In the case of GOD, the $k$ values of negatively charged inorganic and organic mediators are much lower than those of neutral quinone mediators with almost identical $E^{\circ}$ [17]. The electrostatic repulsion between negatively charged mediators and the active site of GOD is expected.

We expect in this work that such steric hindrance and electrostatic repulsion will be
enhanced by using redox polymers with large molecular weight and high density of negative charge. On the other hand, such negative effects seem to be minimized for POD, because the catalytic center of POD is located on the surface of the enzyme and the vicinity of the catalytic center is positively charged [18].

In order to verify our hypothesis, we focus on pentacyanoferrate-bound poly(1-vinylimidazole) (PVI[Fe(CN)$_5$]) (Fig. 1A) as a redox polymer mediator for oxidase/POD bienzyme sensors. The mediator has been synthesized for high MET activity with bilirubin oxidase (BOD) [19]. Os-complex-bound PVIs, PVI[Os(dmebpy)$_2$Cl] (dmebpy = 4,4’-dimethyl-2,2’-bipyridine) with neutral ligands (Fig. 1B) and PVI[Os(dcbbp)$_2$Cl] (dcbbp = 4,4’-dicarboxy-2,2’-bipyridine) with negatively charged ligands (Fig. 1C) are also used as references.
2. Experimental

2.1 Reagents

(NH₄)₂[OsCl₆], 4,4’-dimethyl-2,2’-bipyridine, and 1-vinylimidazole were purchased from Sigma-Aldrich Co. (USA). 2,2’-Bipyridine-4,4’-dicarboxylic acid, ethylene glycol, sodium hydrosulfite (Na₂S₂O₄), 2,2’-azobisisobutyronitrile (AIBN), diethyl ether, sodium pentacyanonitrosylferrate(III) dihydrate (Na₂[Fe(CN)₅(NO)]·2H₂O), glucose, sarcosine, choline chloride and L-lactate were obtained from Wako Pure Chemical Industries (Osaka, Japan). POD from horseradish (POD, 257 U mg⁻¹), GOD from Aspergillus sp. (100 U mg⁻¹), sarcosine oxidase from microorganism (SOD, 16.6 U mg⁻¹), choline oxidase from Alcaligenes sp. (ChOD, 16.9 U mg⁻¹) and lactate oxidase from microorganism (LOD, 101 U mg⁻¹) were from Toyobo Co. (Osaka, Japan). Substrate solutions and other enzyme solutions were prepared with a phosphate buffer solution (100 mM, pH 7.0). 1 M Na₂S₂O₄ was prepared with distilled water. Other chemicals were of analytical grade and used as received.

2.2 Synthesis of mediator-containing polymers

Os(dmebpy)₂Cl₂ and Os(dcbbpy)₂Cl₂ were synthesized as reported [20]. In brief, (NH₄)₂[OsCl₆] (0.57 mmol) and 2 equivalents of 4,4’-dimethyl-2,2’-bipyridine or 2,2’-bipyridine-4,4’-dicarboxylic acid were dissolved in 9 mL of ethylene glycol, heated
under reflux and stirring for 2 h in Ar. After cooling to room temperature, the solution was treated with 15 mL of 1 M Na$_2$S$_2$O$_4$ to reduce [Os(dmebpy)$_2$Cl$_2$]$^+$ or [Os(dcbbpy)$_2$Cl$_2$]$^+$ which might be formed during the synthesis, and then cooled in an ice bath for 30 min. The dark-violet precipitate was obtained after being washed by distilled water and diethyl ether.

Poly(1-vinylimidazole) (PVI) was prepared according to the literature [1]. Briefly, 6 mL of 1-vinylimidazole mixed with 0.5 g of AIBN was heated at 70 °C for 2 h under Ar with stirring. After cooling, the yellow precipitate was observed and re-dissolved with methanol, followed by adding the methanol solution dropwise to acetone under strong stirring. White PVI powder was obtained after filtering and drying.

PVI[Os(dmebpy)$_2$Cl] and PVI[Os(dcbbpy)$_2$Cl] were also prepared according to the literature [1]. The powder of synthesized Os(dmebpy)$_2$Cl$_2$ (66 mg, c.a. 0.105 mmol) or Os(dcbbpy)$_2$Cl$_2$ (85 mg, c.a. 0.105 mmol) and PVI (100 mg, c.a. 1.05 mmol) were dissolved in 100 mL of absolute ethanol. The mixture was heated at reflux and stirred for 3 days. The precipitate was obtained by adding the solution to diethyl ether under stirring. After filtering and drying, the precipitate was dissolved in 10 mL of the phosphate buffer solution (10 mM, pH 7.0) and stored at 4 °C. The final concentration
of the two polymers was about 18 mg mL$^{-1}$. The ratio of the imidazole unit in PVI to
the Os(dmebpy)$_2$Cl complex may be approximately 10 according to the literature [1].

PVI[Fe(CN)$_3$] was synthesized similar to the method in the literature [19]. In brief,
200 mg of Na$_2$[Fe(CN)$_3$(NO)]·2H$_2$O and 188 mg of PVI were dissolved in 50 mL of 0.6
M NaOH and were refluxed at 65 °C for 24 h. The mixture was dialyzed against
distilled water for 24 h to remove unreacted compounds. After centrifuged at 5000 g for
20 min 2 times to remove red precipitate, the suspension was vacuum freeze-dried at
$-40$ °C for 24 h to get PVI[Fe(CN)$_5$] powder. The ratio of the imidazole unit in PVI to
the Fe(CN)$_5$ complex was 4.3 as measured by elemental analysis. The stock solution of
PVI[Fe(CN)$_5$] was prepared by dissolving in 10 mM phosphate buffer at pH 7.0.

2.3 Electrochemical measurements

Electrochemical measurements were performed with glassy carbon (GC) electrodes
(3 mm, BAS) and carried out in the phosphate buffer solution (100 mM pH 7.0) at 25
°C with an electrochemical analyzer (BAS CV 50 W, BAS Inc., Japan). All potentials
are referred to Ag|AgCl|sat. KCl reference electrode in this work. Considering the fact
that the carboxyl group of PVI[Os(dcbbpy)$_2$Cl] is also crosslinked with poly(ethylene
glycol) diglycidyl ether, the redox polymers were used in soluble state without
immobilization on electrode surface. The final concentration of the Os-containing
polymers in the sample solution was one tenth of the Os-containing polymer stock solutions, while the final concentration of PVI[Fe(CN)₅] was 0.3 mg mL⁻¹ in the sample solution. The peak anodic currents of the diluted PVI[Os(dmebpy)₂Cl] and PVI[Os(dcbbpy)₂Cl] solutions were, respectively, 260 and 350 nA at a scan rate of 20 mV s⁻¹ in cyclic voltammetry. Supposing that the diffusion coefficients of the two Os-containing polymers are close to each other, the concentrations of Os²⁺/³⁺ in the sample solutions were in the same level. The activity of the metal redox polymer against enzyme was also evaluated by cyclic voltammetry.

2.4 Measurements of enzyme’s concentration

The concentrations of GOD, SOD and LOD were determined spectrophotometrically. The molar extinction coefficients of GOD, SOD and LOD were chosen as 13.0 mM⁻¹ cm⁻¹ at 450 nm [21], 12.2 mM⁻¹ cm⁻¹ at 454 nm [22] and 12.5 mM⁻¹ cm⁻¹ at 450 nm [23], respectively.
3. Results and discussion

The formal potentials ($E^\circ$) of PVI[Os(dmebpy)$_2$Cl], PVI[Os(dcbbpy)$_2$Cl] and PVI[Fe(CN)$_5$] were determined, respectively, as 0.156 V, 0.204 V and 0.213 V vs. Ag$|$AgCl by cyclic voltammetry. Their MET activities against GOD are shown in Fig. 2. The clear catalytic oxidation current of glucose was observed with PVI[Os(dmebpy)$_2$Cl] after addition of GOD (Fig. 2A), while the catalytic current with PVI[Os(dcbbpy)$_2$Cl] was much smaller than that with PVI[Os(dmebpy)$_2$Cl] (Fig. 2B). The MET activity can be quantitatively expressed by the bi-molecular reaction rate constant between enzyme and mediator, $k_{cat}/K_M$, where $k_{cat}$ is the catalytic constant of the enzyme and $K_M$ is the Michaelis constant for the mediator. The $k_{cat}/K_M$ can be easily evaluated from the slope of the linear relation between the limited catalytic current ($I_c$) and the total concentration of mediator ([M]) at $[M] < K_M$ [24],

$$I_c = n_M FA[M] \sqrt{(n_S/n_M)}D_M k_{cat}[E]/K_M$$  \hspace{1cm} (2)

where $n_M$ and $n_S$ are the number of electron of mediator and substrate, respectively, $A$ is the electrode surface area, $D_M$ is the diffusion coefficient of mediator, and $[E]$ is the concentration of enzyme. In this work, the $\sqrt{D_M}$ values for the redox polymers used were evaluated from the peak current ($I_P$) of cyclic voltammetry of the polymer.
solution in the absence of enzyme by assuming the reversible response of the mediator at a given scan rate ($\nu$) [25].

$$I_p = -0.4463n_M FA[M]V^{-n_M F\nu D_m / RT} \quad \text{(at 25°C)}$$  \hspace{2cm} (3)

The $k_{cat} / K_M$ was calculated from the $(I_c/I_p)^2$ at a given concentration of mediator.

$$k_{cat} / K_M = (I_c / 3.59I_p \sqrt{[E]})^2$$  \hspace{2cm} (4)

at $n_M = 1$, $n_S = 2$, and $\nu = 0.02 \text{ V s}^{-1}$ (for our experimental conditions). The result is summarized in Table 1, which indicates that the MET activity of PVI[Os(dmebpy)$_2$Cl] is approximately one order larger than that of PVI[Os(dcbbpy)$_2$Cl]. Since the $E^{\text{ox*}}$ and the size of the two Os-containing polymers are almost identical with each other, the large difference in the MET activity cannot be explained in terms of LFER, but some electrostatic effect is expected.

The active site of GOD, FAD, is in hydrophobic surroundings and is buried in the molecule [26]. Moreover, GOD has essentially negative electrostatic surface potential at pH 7 and the surface electrostatic potential of the channel to FAD is negative (Fig. S1) [27, 28]. Therefore, PVI[Os(dcbbpy)$_2$Cl] with the negatively charged ligand (–COO$^-$) is more difficult to reach the FAD center in GOD than PVI[Os(dmebpy)$_2$Cl] with the neutral ligand due to the repulsive electrostatic interaction. The results are consistent with the previous research that the rate constant of the electron transfer between GOD
and Os-complexes is strongly related to the charge of the Os complexes: 

\[ \text{Os(dmebpy)}_2(\text{pyNH}_3^+)(\text{imNH}_3^+) \text{(global charge }} \text{(the net charge of Os}^{3+} \text{ and ligands) } = +5, \text{ py } = \text{pyridine, im } = \text{imidazole) } > \text{Os(dmebpy)}_2(\text{py})(\text{imNH}_3^+) \text{(global charge } = +4) > \text{Os(dmebpy)}_2\text{Cl(}\text{pyNH}_3^+\text{)} \text{(global charge } = +3) \] [29] indicating the attractive electrostatic interaction between positively charged Os-complex and GOD [30, 31].

When PVI[Fe(CN)]_3 was used as a mediator, no clear catalytic current was observed in the GOD system (Fig. 2C), in spite of the fact that the size of PVI[Fe(CN)]_3 (7.2 \times 6.2 \text{ Å}^2) is approximately three times smaller than that of PVI[Os(dcbbpy)_2Cl] (11.2 \times 13.8 \text{ Å}^2) (Fig. S2) and the \(E^{\circ'}\) is almost identical with that of PVI[Os(dcbbpy)_2Cl] (Table 1). The extremely low MET activity of PVI[Fe(CN)]_3 against GOD can be interpreted by the increased repulsive electrostatic interaction between PVI[Fe(CN)]_3 and GOD, since the negative charge density of PVI[Fe(CN)]_3 is much higher than that of PVI[Os(dcbbpy)_2Cl].

It is well known that hexacyanoferrate ion is frequently utilized as a mediator of GOD-based MET-type glucose biosensor [32-34], although the MET activity is low [17]. The fact and the present result indicate that the polymerization of pentacyanoferrate increases repulsive electrostatic effect. The polymerization also seems to introduce the steric hindrance effect. As a result, we have successfully found a redox polymer
(PVI[Fe(CN)$_5$]) with practically no MET activity against GOD.

SOD is a kind of flavoenzymes; the global charge of SOD (pI = 4.9, Toyobo Co.) at pH 7 is negative but the surface electrostatic potential near the redox center is slightly positive (Fig. S3). Therefore, some MET activity against SOD might be expected even for negatively charged metal complex-containing polymers. However, no catalytic current was observed for both PVI[Os(dcbpy)$_2$Cl] and PVI[Fe(CN)$_5$]. In addition, the MET activity of PVI[Os(dmebpy)$_2$Cl] was extremely low compared to that in the GOD reaction (Table 1). The poor MET activity of these redox polymers seems to be ascribed to the steric hindrance in the reaction with SOD, since the FAD of SOD is deeply buried in the molecule; moreover, the path of the channel to the FAD with positive surface potential outside comprises hydrophobic residues [35]. The steric effect and electrostatic repulsion with the tunnel are enhanced by the polymerization. Anyway, PVI[Fe(CN)$_5$] as well as PVI[Os(dcbpy)$_2$Cl] may be utilized as a mediator of SOD/POD-based bienzyme sensors.

All the redox polymers used did not give any catalytic current for ChOD. The disappearance of the MET activity by the polymerization seems to be ascribed to the steric hindrance as in the case of SOD. The FAD is buried near the center of the subunit
of the homodimer and only 2.1% of the FAD surface area is exposed to the solvent [36], therefore even PVI[Os(dmebpy)$_2$Cl] is hard to react with the FAD of ChOD.

LOD with flavin mononucleotide (FMN) as the redox center was also examined. The surface electrostatic potential of the tunnel to the FMN of LOD is positive although the entrance is slightly negatively charged (Fig. S4). In addition, the FMN located on the bottom of the tunnel is somewhat accessible to solvent compared with the FAD in ChOD and SOD. Therefore, the steric hindrance does not seem to be so strong as SOD and ChOD, and the negatively charged electron acceptor may also react with LOD. Actually, the rate constant of LOD with ferricyanide ($5.7 \times 10^3$ M$^{-1}$s$^{-1}$ in pH7.5, 0.1 M PBS at 25 °C) is approximately one order larger than that of GOD ($3.2 \times 10^2$ M$^{-1}$s$^{-1}$ in pH7.0, 0.1 M phosphate/citrate at 25 °C) [17, 37].

As expected from the structural information, PVI[Os(dcbbpy)$_2$Cl] gave clear catalytic current in the presence of LOD and lactate (Fig. 3A) and even PVI[Fe(CN)$_5$] gave catalytic current although it was very small (Fig. 3B). However, the larger catalytic current was observed with PVI[Os(dmebpy)$_2$Cl]. The MET activity of these redox polymers is summarized in Table 1.

On the other hand, it was found that PVI[Fe(CN)$_5$] works as a good mediator to POD to reduce H$_2$O$_2$ as shown in Fig. 4. The protoheme redox center of POD locates
near the enzyme surface, the surface electrostatic potential near the protoheme is positively charged and the entrance channel into the protoheme for solvent is widely open (Fig. S5). Therefore, the high MET activity of PVI[Fe(CN)$_5$] to POD is reasonably understood. Similar situation is observed for PVI[Fe(CN)$_5$] to BOD [19], of which the type 1 redox center locates near the enzyme surface.
4. Conclusion

The MET activity of the three kinds of metal complex-containing polymers: PVI[Os(dmebpy)$_2$Cl], PVI[Os(dcbbp)$_2$Cl], and PVI[Fe(CN)$_5$] has been estimated for four flavoenzyme oxidases. The results have revealed that the MET reactivity of the negatively charged polymers is very low, which is strongly related to the electrostatic repulsive interaction between the local surface charge of the flavoenzymes and the polymer’s charge as well as the steric hindrance. Especially, in PVI[Fe(CN)$_5$], the polymerization of Fe(CN)$_5$ via PVI increases the negative charge density and then enhances the electrostatic repulsive effect. However, PVI[Fe(CN)$_5$] works as a good mediator for the POD reaction to reduce H$_2$O$_2$. This specific catalytic property of PVI[Fe(CN)$_5$] is very convenient to construct H$_2$O$_2$-generating oxidase/POD-based bienzyme biosensors.
References

[14] H. Sakai, R. Baba, K. Hashimoto, A. Fujishima, A. Heller, Local detection of


Web reference

Figure captions:

Scheme 1 The electron transfer profile of oxidase/POD bienzyme sensor system. The gray-colored arrows indicate the electron transfer path in negative interference due to dehydrogenase activity of oxidase.

Figure 1 Structures of (A) PVI[Fe(CN)$_5$], (B) PVI[Os(dmebpy)$_2$Cl] and (C) PVI[Os(dcbbpy)$_2$Cl].

Figure 2 Cyclic voltammograms of 500 mM glucose solution containing (A) PVI[Os(dmebpy)$_2$Cl], (B) PVI[Os(dcbbpy)$_2$Cl], or (C) PVI[Fe(CN)$_5$] in the absence (dash line) and presence (solid line) of GOD (39.2 U mL$^{-1}$) at a scan rate of 20 mV s$^{-1}$.

Figure 3 Cyclic voltammograms of 11.8 mM lactate solution containing (A) PVI[Os(dcbbpy)$_2$Cl], (B) PVI[Fe(CN)$_5$] in the absence (dash line) and presence (solid line) of LOD (40.4 U mL$^{-1}$) at a scan rate of 20 mV s$^{-1}$.

Figure 4 Cyclic voltammograms of a solution containing POD (205.6 U mL$^{-1}$) and PVI[Fe(CN)$_5$] in the absence (dash line) and presence (solid line) of 2 mM H$_2$O$_2$ at a
scan rate of 20 mV s$^{-1}$. 
Figure 1
Figure 2
Figure 3
Figure 4
Table 1 Values of log($k_{cat}/K_M$) for the substrate oxidation catalyzed by flavoenzymes with mediator-containing polymers

<table>
<thead>
<tr>
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<th>$E^\circ (V)$</th>
<th>GOD</th>
<th>SOD</th>
<th>ChOD</th>
<th>LOD</th>
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<td>PVI[Os(dmebpy)$_2$Cl]</td>
<td>0.156</td>
<td>7.1</td>
<td>4.3</td>
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<td>N/D</td>
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</tbody>
</table>
Supplementary Data

Electrostatic and Steric Interaction between Redox Polymers and Some Flavoenzymes in Mediated Bioelectrocatalysis

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All data of enzyme’s structures were from protein data bank (PDB) and generated on MDL® Chime.

Figure S1 Surface electrostatic potential of GOD. The arrow indicates the position of FAD. Red: negative potential, Blue: positive potential. (PDB: 1W4W)
Figure S2 Molecule model of imidazole-Fe[(CN)₅] (left) and imidazole-Os[(dcbbpy)₂Cl] (right) drawn on Spartan molecular modeling software (Wavefunction, Inc., USA). The sizes of Fe[(CN)₅] and Os[(dcbbpy)₂Cl] were estimated as 7.2 × 6.2 Å² and 11.2 × 13.8 Å² (height × width), respectively.

Figure S3 Surface electrostatic potential of SOD. The molecule in the blue circle represents FAD. (PDB: 3AD9)
**Figure S4** Overall structure of LOD (tetramer). Left: ribbon diagram of LOD. Pink ribbon and yellow ribbon represent alpha helix and beta sheet, respectively. The four molecules are the redox center, FMN. Blue arrow indicates the possible entrance for solvent to one of FMN. Right: surface electrostatic potential of LOD. The circle indicates the position of FMN in one of the subunits. (PDB: 2DU2)

**Figure S5** Overall structure of POD. Left: ribbon diagram of POD. The space filled molecule is the redox center, protoheme. Right: surface electrostatic potential of POD. Blue arrow indicates the possible entrance for solvent to the protoheme. (PDB: 1CF3)