Coupling of Redox and Structural States in Cytochrome P450 Reductase Studied by Molecular Dynamics Simulation

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Cytochrome P450 reductase (CPR) is the key protein that regulates the electron transfer from NADPH to various heme-containing monooxygenases. CPR has two flavin-containing domains: one with flavin adenine dinucleotide (FAD), called FAD domain, and the other with flavin mononucleotide (FMN), called FMN domain. It is considered that the electron transfer occurs via FAD and FMN (NADPH → FAD → FMN → monooxygenase) and is regulated by an interdomain open-close motion. It is generally thought that the structural state is coupled with the redox state, which, however, has not yet been firmly established. In this report, we studied the coupling of the redox and the structural states by full-scale molecular dynamics (MD) simulation of CPR (total 86.4 μs). Our MD result showed that while CPR predominantly adopts the closed state both in the oxidized and reduced states, it exhibits a tendency to open in the reduced state. We also found a correlation between the FAD-FMN distance and the predicted FMN-monooxygenase distance, which is embedded in the equilibrium thermal fluctuation of CPR. Based on these results, a physical mechanism for the electron transfer by CPR is discussed.

The high-energy electron in the reduced-form nicotinamide adenine dinucleotide phosphate (NADPH) provides the driving force for a large number of biochemical reactions in living organisms1. Cytochrome P450 reductase (CPR) is the key protein that receives the high-energy electron from NADPH and distributes it selectively to heme-containing monooxygenases such as cytochrome P4502 and heme oxygenase (HO)3; upon receiving the electrons from CPR, cytochrome P450 and HO become capable of metabolizing drugs2 and decomposing toxic free hemes4, respectively. Furthermore, CPR can be used in the anticancer therapy where anticancer prodrugs are locally activated by the reducing power of CPR5.

CPR has two flavin-containing domains, one with flavin adenine dinucleotide (FAD), called “FAD domain”, and the other with flavin mononucleotide (FMN), called “FMN domain”, and these two domains are connected by a “connecting domain”6. The FAD domain contains the NADPH binding site close to the embedded FAD cofactor, which is suited for the electron transfer (hydride transfer) from NADPH to FAD. The crystal structure by Wang et al.7 showed that CPR adopts a “closed form” where FAD and FMN cofactors are situated in close proximity to each other so that the electron transfer from FAD to FMN would become efficient. On the other hand, Hamdane et al. found structural polymorphism in the crystal structure of a mutant of CPR, where the FMN domain is positioned away from the FAD domain8, which is referred to as the “open form”. The open form appeared to be suited for the intermolecular interaction with monooxygenase, facilitating the electron transfer from FMN to the heme in the monooxygenase. Indeed, this mutant CPR was found to strongly interact with HO, and the crystal structure of the mutant CPR in complex with HO was solved by Sugishima et al.9 where CPR adopts an open form similar to that observed in the uncomplexed CPR. Recently, Freeman et al. reported the solution structure of another CPR mutant in complex with cytochrome c using small-angle neutron scattering (SANS)10, which indicated the same binding mode as observed in the crystal structure of the CPR-HO complex.

Therefore, CPR is considered to regulate the electron transfer from FMN to monooxygenases via the structural state change, most likely the open-closed-like interdomain rearrangement, that alters the binding affinity with the monooxygenases11. Importantly, the structural state of CPR is considered to be coupled with its redox...
state. Indeed, several experimental observations indicated that CPR adopts the closed state in the oxidized state whereas it adopts the open state in the reduced state. However, some experimental observations suggested that the redox-state-dependent structural change is only marginal compared to the structural change that is anticipated from the closed and the open forms as observed in the crystal structures. The coupling of the redox and the structural states in CPR, thus, has not yet been firmly established. To address this issue, we conducted molecular dynamics (MD) simulation and studied how the structural state of CPR is affected by its redox state. So far, only a few MD simulations have been done to study the structural state of CPR. To the best of our knowledge, the present study is the first report on full-scale MD simulation (total 86.4 μs) of CPR toward elucidating the coupling of the redox and the structural states.

Results
To investigate the coupling of the redox and the structural states, we first studied the domain-level open-closed-like structural change as inferred from the crystal structures. In Fig. 1A, the time courses for the distance between FAD and FMN domains are displayed; in this case, MD simulations were started from the closed form, and 16 independent 0.2-μs conventional MD (cMD) runs were conducted for each redox state (oxidized or reduced). cMD runs were followed by 1-μs accelerated MD (aMD) runs. In the cMD period, the average time course of the interdomain distance for the reduced state showed an immediate increase. However, it did not show further increase that is expected if CPR adopts the open form. To see whether or not CPR becomes more opened on a longer time scale, we employed the aMD technique after 0.2 μs. The time course data for the reduced state in the aMD period demonstrates that the interdomain distance did not increase but gradually decreased to the value of the closed form. The interdomain distance for the oxidized state also remained near the value of the closed form. These results indicate that the closed state of CPR is intrinsically stable. However, the probability distributions of the interdomain distance that were calculated using the data for the last 0.5 μs show that the distribution for the reduced state is shifted toward the open form compared to that for the oxidized state, indicating that there is a coupling of the redox and the structural states. To see the influence of the initial structure, we started the MD simulations from the open form. As seen in Fig. 1D, the interdomain distance for both the oxidized and the reduced states largely decreased in the cMD period (first 0.2 μs), and continued to decrease in the subsequent aMD period (0.2–0.6 μs) to the value of the closed form, again indicating the intrinsic stability of the closed state. However, the closed state observed in this case (Fig. 1D) was not the same as that observed in the simulations starting from the closed form (Fig. 1B,C).
the interdomain distance exhibited larger fluctuation in this case, so the coupling of the redox and the structural states was obscured.

We further analyzed the redox-state dependence of the inter-cofactor distance between FAD and FMN. Since it is the alloxazine rings in the cofactors that are directly involved in the electron transfer, we monitored the distance between the center of mass of the alloxazine ring of FAD cofactor and that of the FMN cofactor. In the cMD period (first 0.2 μs), the average inter-cofactor distance slightly increased in the reduced state, as was observed in the interdomain distance. In the subsequent aMD period, the average inter-cofactor distance in the reduced state further increased, which was not observed in the interdomain distance, and one trajectory came close to the value of the open form. Although the distribution of the inter-cofactor distance (Fig. 2B) was more widespread than that of the interdomain distance because of the thermal fluctuations of the bound cofactors, the redox-state dependence can be seen in the inter-cofactor distance as well as in the interdomain distance.

In addition to the redox state, the bound NADP⁺ is considered to have an influence on the structural state of CPR. We then examined the structural relaxation of CPR in the absence of the bound NADP⁺. In Fig. 3, the time courses and the probability distributions for the interdomain and inter-cofactor distances are shown for the oxidized and the reduced states. In the absence of the bound NADP⁺, the closed form was intrinsically stable in both the oxidized and the reduced states, as was the case in the presence of the bound NADP⁺. However, the redox-state dependence that was observed in the presence of the bound NADP⁺ disappeared in the absence of
the bound NADP$^+$, suggesting that the bound NADP$^+$ is involved in the coupling of the redox and the structural states. We hereafter focus our attention on the results in the presence of the bound NADP$^+$.

There is an expectation that the observed structural state of CPR in the reduced state, where the inter-cofactor distance is increased, may exhibit a suitability for the electron transfer from the FMN cofactor to the heme in monooxygenase. We then investigated the distance between the FMN cofactor and the heme using the complex structure of CPR and heme oxygenase (HO)$^9$. The CPR-HO complex structure indicates that the FMN cofactor that is buried in the closed form$^6,7$ is exposed to the interaction interface with HO, and both the FAD and FMN domains contribute to the interaction with HO. In Fig. 4, we show the correlation between the FAD-FMN distance and the FMN-heme distance by depicting 2D free-energy landscape. Since HO was not included in our MD simulation, we predicted the distance between the FMN cofactor and the heme using the CPR-HO complex structure$^9$ (see text), and the center-of-mass distance between the alloxazine ring of FMN and the heme in HO was used. The circle and the square represent the crystal structure of the closed$^7$ and that of the open form$^9$, respectively. MD snapshot structures (triangles in A and B) are shown in the right (the heme and HO are shown in purple; other coloring and the view angle are the same as in Fig. 1A).

Figure 4. Free-energy landscape depicted in the 2D space constructed by the FAD-FMN distance and the FMN-heme distance: (A) free-energy landscape for the oxidized state, and (B) that for the reduced state. The free energies were calculated by $-k_BT\ln P$, where $P$ represents the probability density obtained from the reweighted data for the last 0.5-μs aMD, and were adjusted so that the lowest free-energy value becomes zero. FMN-heme distance is a predicted one using the CPR-HO complex structure$^9$ (see text), and the center-of-mass distance between the alloxazine ring of FMN and the heme in HO was used. The circle and the square represent the crystal structure of the closed$^7$ and that of the open form$^9$, respectively. MD snapshot structures (triangles in A and B) are shown in the right (the heme and HO are shown in purple; other coloring and the view angle are the same as in Fig. 1A).
Redox states (1- to 3-electron reduced states) should be conducted, keeping in mind that a recent single-protein study. To obtain the whole spectrum of the redox-structural state coupling, MD simulations for the intermediate combination with the aMD technique, is expected to have captured the coupling of the redox and the structural state. NADP⁺ is consistent with the surface plasmon resonance study where NADP⁺ binding to CPR was shown to strengthen the binding affinity of CPR with HO, suggesting that CPR becomes opened upon NADP⁺ binding.

Discussion

Our MD simulation indicated that the closed state is intrinsically stable. Although the open form as found in the crystal structure was not observed in our MD simulation, the interdomain distance and the inter-cofactor distance between FAD and FMN showed a tendency to increase in the reduced state, suggesting that the structural state is coupled with the redox state. The redox-state dependent structural state has been investigated experimentally. It was shown that CPR predominantly adopts the closed state in the oxidized state and becomes opened in the reduced state. The observation in our MD simulation is in accord with these experimental observations, even though the observed tendency to open in the reduced state was rather weak in our MD simulation. The weak tendency to open in the reduced state, on the other hand, seems to be well in accordance with the recent FRET experiment by Kovrigina et al. In the present MD study, we considered the two end states of CPR: the fully oxidized state (FAD and FMN are both oxidized), and the fully (4-electron) reduced state (FAD and FMN are both 2-electron reduced). While the 1- to 3-electron reduced states are considered as the major redox states of CPR in vivo, the 4-electron reduced state can also be realized at high NADPH concentrations, which is the case in the experiment by Kovrigina et al. Therefore, the reduced state employed in our MD study is considered to be the same as in the FRET study. In addition, the recent SANS experiment by Freeman et al. indicated that the 4-electron reduced state is more opened (extended) than the 1- to 3-electron reduced states, whereas the 1- to 3-electron reduced states are more opened than the fully oxidized (0-electron) state. Therefore, our MD study is considered to capture the two end states of CPR in terms of the structural state as well. Although there still remains a concern about the statistical uncertainty, our MD simulation, with total 86.4 μs MD data in combination with the aMD technique, is expected to have captured the coupling of the redox and the structural states. To obtain the whole spectrum of the redox-structural state coupling, MD simulations for the intermediate redox states (1- to 3-electron reduced states) should be conducted, keeping in mind that a recent single-protein tracking experiment indicated that CPR in the 2-electron reduced state binds to a reductant partner (cytochrome P450) more strongly than CPR in the fully oxidized state and furthermore the binding constant is at the same level as in the fully reduced state.

With regard to the effect of the bound NADP⁺ on the structural state of CPR, the currently prevailing view is that CPR adopts the closed form in the NADP⁺-bound state, which was not observed in our MD simulation. On the other hand, our MD result that the interdomain distance slightly increased in the presence of the bound NADP⁺ is consistent with the surface plasmon resonance study where NADP⁺ binding to CPR was shown to strengthen the binding affinity of CPR with HO, suggesting that CPR becomes opened upon NADP⁺ binding.

Based on our MD results, we can envisage a physical mechanism of the electron transfer from CPR to monoxygenase as follows. Even if the coupling of the redox and the structural states is weak, it could be sufficient for the reduced CPR to initiate the interaction with monoxygenase to which electron is delivered. After the initial weak binding, the binding affinity could become stronger via induced-fit-like structural change of CPR toward the open form as seen in the CPR-HO complex crystal structure. It is noteworthy that the single-protein tracking experiment detected two binding states, weak and strong binding states, for the association between CPR and cytochrome P450. The principal modes (Fig. 5) suggest that the overall direction toward the structural state suitable for the monoxygenase binding and electron transfer is embedded in the equilibrium thermal fluctuation of CPR.

Then, what physical mechanism can explain the observed coupling of the redox and the structural state? Addressing this question is beyond the scope of the present study, so we just mention what could be the key. The surface of the FMN domain is largely polar, presenting bipolarity. Furthermore, we can find clear electrostatic complementarity between the FMN and the FAD domains, which leads us to expect that the electrostatic attraction between the two domains is the origin of the intrinsic stability of the closed state. Then, the redox state...
change, which is accompanied by the net charge change, could affect the electrostatic interaction between the FAD and the FMN domains. Binding of NADP$, which carries net negative charge, could also affect the interaction between the two domains, triggered by local rearrangement of the electrostatic bonds; Actually, such rearrangement of the electrostatic bonds involving Asp632, which is located near the binding site of NADP$ and was noticed in the recent structural studies37,38, was observed in our MD simulation (data not shown). In addition, the redox state change in FMN (and also in heme) should affect the electrostatic interaction between the FMN domain and monooxygenase39. From the viewpoint of electrostatics, proteins are regarded as dielectric materials. Then, CPR should exhibit dielectric response to the electrostatic inputs (in the present case, the redox-state change and NADP$ binding), as was found in the ATP-binding induced dielectric response of myosin30,31. The dielectric response causes the polarization charge on the domain surface, affecting the electrostatic interactions between the two domains and between CPR and monooxygenase. The atomic-level analysis for the dielectric response, which is caused by large-scale concerted rearrangement of the electrostatic bonds (called "dielectric allostery")30,31, will be done in our next study.

Considering that clear electrostatic complementarity also exists at the interface between CPR and HO$ and between CPR and cytochrome P45019,32,33, the rearrangement of the electrostatic interaction network in CPR should play the key role in the regulation of downstream protein binding and intermolecular electron transfer. Then, we have to remember that CPR and the downstream proteins are located on the membrane, and the membrane environment exerts substantial influence on the function of these proteins19,26,32,34. Collectively taking into account (i) the above-mentioned electrostatic complementarity, (ii) the fact that the membrane surface is largely polar and lipid molecules often contain charged head groups19, and (iii) anomalous dielectric property of water that could arise near the surface35, it is obviously important to investigate the electrostatic effect of the lipid membrane on the redox-structural state coupling of CPR and the association between CPR and the downstream proteins. MD studies of these proteins in the presence of the membrane18,19,32,33 are offering the important first steps toward elucidating the electrostatic effect of the membrane on this electron transfer machinery.

Methods

As the initial structure of the MD simulation, we used the two crystal structures of CPR, one in the closed form7 (PDB ID: 1AMO, chain A), and the other in the open form8 (PDB ID: 3ES9, chain A). Missing (or mutationally deleted) residues in the hinge and loop regions were complemented by MODELLER36, while the N-terminal transmembrane region (63 residues) were kept removed. The N- and C-termini were capped by the acetyl and the N-methyl-amide groups, respectively. His180, His403, and His615 were doubly protonated according to the 

\[ E^\alpha = \frac{1}{2} \alpha E_B - \frac{1}{2} E_B V(r) < E_B \]

\[ \Delta V(r) = \begin{cases} 0, & (V(r) \geq E_B) \\ \frac{(E_B - V(r))^2}{\alpha + E_B - V(r)}, & (V(r) < E_B) \end{cases} \]

where \( V \), \( E_B \), and \( \alpha \) denote the original potential energy, the threshold energy below which boosting (energy lifting) is turned on, and the parameter to determine the extent of boosting, respectively. According to the prescription39, we set \( E_B = 0.091 \times 10^5 \text{ kcal/mol} \) and \( \alpha = 0.005 \times 10^5 \text{ kcal/mol} \) for the dihedral potential boost, and \( E_B = -3.222 \times 3.239 \times 10^5 \text{ kcal/mol} \) and \( \alpha = 0.172 \times 10^5 \text{ kcal/mol} \) for the total potential boost. To obtain the canonical ensemble from the aMD trajectory, the statistical weight of an aMD snapshot is multiplied by \( \exp(\Delta V/k_B T) \) using the \( \Delta V \) value for the snapshot38 (\( k_B \) is the Boltzmann constant and \( T = 310 \text{ K} \)). The...
volume of the system was kept constant in the aMD period in consideration of the reduced virial due to the boost potential. To investigate the effect of the bound NADP⁺, we also conducted MD simulations in the absence of NADP⁺ (16 runs of 0.2-µs cMD followed by 1-µs aMD) for each redox state. All of the MD simulations were executed using AMBER12. All of the MD simulations that we conducted in the present study were summarized in Table 1.

Table 1. MD runs conducted in this study.

| Redox state | Initial structural state | MD method | MD length (µs) | #MD runs | Total length (µs) |
|-------------|--------------------------|-----------|----------------|----------|-----------------|
| Oxidized    | Closed                   | cMD       | 0.2            | 16       | 19.2            |
|             |                          | aMD       | 1.0            | 16       |                 |
| Reduced     | Closed                   | cMD       | 0.2            | 16       | 19.2            |
|             |                          | aMD       | 1.0            | 16       |                 |
| Oxidized    | Open                     | cMD       | 0.2            | 8        | 4.8             |
|             |                          | aMD       | 0.4            | 8        |                 |
| Reduced     | Open                     | cMD       | 0.2            | 8        | 4.8             |
|             |                          | aMD       | 0.4            | 8        |                 |
| Oxidized (no NADP⁺) | Closed           | cMD       | 0.2            | 16       | 19.2            |
|             |                          | aMD       | 1.0            | 16       |                 |
| Reduced (no NADP⁺) | Closed          | cMD       | 0.2            | 16       | 19.2            |
|             |                          | aMD       | 1.0            | 16       |                 |

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Author Contributions
M.T. designed the research. M.I., J.O. and T.S. prepared the simulation settings, and M.I. carried out MD simulations. M.I., J.O., T.S. and M.T. analyzed and interpreted the data. M.S. provided structural information. M.I. and M.T. wrote the manuscript. All authors checked and approved the manuscript.

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