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Review

Structure and function of NADPH-cytochrome P450 reductase and nitric oxide synthase reductase domain

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Abstract

NADPH-cytochrome P450 reductase (CPR) and the nitric oxide synthase (NOS) reductase domains are members of the FAD–FMN family of proteins. The FAD accepts two reducing equivalents from NADPH (dehydrogenase flavin) and FMN acts as a one-electron carrier (flavodoxin-type flavin) for the transfer from NADPH to the heme protein, in which the $FMNH^{\cdot}/FMNH_2$ couple donates electrons to cytochrome P450 at constant oxidation–reduction potential. Although the interflavin electron transfer between FAD and FMN is not strictly regulated in CPR, electron transfer is activated in neuronal NOS reductase domain upon binding calmodulin (CaM), in which the CaM-bound activated form can function by a similar mechanism to that of CPR. The oxygenated form and spin state of substrate-bound cytochrome P450 in perfused rat liver are also discussed in terms of stepwise one-electron transfer from CPR. This review provides a historical perspective of the microsomal mixed-function oxidases including CPR and P450. In addition, a new model for the redox-linked conformational changes during the catalytic cycle for both CPR and NOS reductase domain is also discussed.

Keywords: Mixed-function oxidase; NADPH-cytochrome P450 reductase; Nitric oxide synthase reductase domain; Cytochrome P450; Microsomal electron transfer system; Perfused liver; Flavin enzyme; Neutral semiquinone

It is now 50 years since Mason et al. [1], Hayaishi et al. [2], and Hayano et al. [3] independently demonstrated direct substrate incorporation of molecular oxygen. These pioneering experiments opened a new field in the area of biological oxidation. Molecular oxygen is metabolized by three broad classes of enzymes: (i) dioxygen transferases, (ii) mixed-function oxidases, and (iii) electron transfer oxidases [4]. Axelrod [5] first pointed out that microsomal deamination of amphetamine requires the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen. Enzymes of the liver microsomes are of major importance in the detoxification of many drugs and foreign organic compounds. In the 1950–1960s, these enzyme systems were shown to perform a variety of reactions such as the hydroxylation of aromatic compounds, side chain oxidation of barbiturates, and deamination of amines. These reactions were characterized as a mixed-function oxidation, in which the enzyme system catalyzes the consumption of one molecule of oxygen/molecule of substrate (SH); one atom of this oxygen molecule inserts into the product (SOH), while the other undergoes two equivalents of reduction Eq. (1).

$$\mathrm{NADPH} + \mathrm{SH} + \mathrm{O_2} + \mathrm{H^+} \rightarrow \mathrm{NADP^+} + \mathrm{SOH} + \mathrm{H_2O} \quad (1)$$

Xenobiotic compounds, which are foreign to living organisms (*Greek: xenos "stranger" biot "life"*), are metabolized by mixed-function oxidases [6]. These pioneering studies led to the discovery of a new microsomal electron transfer system, including NADPH-cytochrome

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P450 reductase (CPR) and cytochromes P450 (P450 or CYP).

In this review, I will discuss the most recent advances in understanding the function of CPR, which can donate an electron to microsomal P450s. The regulation of nitric oxide synthase reductase domain, which has a similar function to CPR, is also discussed.

Microsomal mixed-function oxidase containing P450

Mixed-function oxidation is based on the activation of molecular oxygen by the reductive splitting of dioxygen. In 1957, Mason [4] proposed a mechanism for this reaction: one oxygen atom of enzyme–oxygen complex, EO_2, is reduced to O^{2-} (H_2O), forming the oxygen-transferring intermediate, EO (Eqs. (2) and (3)).

$$EO_2 + DH \rightarrow EO + D^+ + OH^- \ (H_2O) \quad (2)$$

$$EO + AH \rightarrow E + AOH \quad (3)$$

where E, DH, and AH represent the enzyme, electron donor, and oxygen acceptor, respectively. In this mechanism, Mason proposed the positively charged oxygen-transferring species, $(FeO)^{3+}$ for EO, and mentioned "*Much work remains to be done in this field.*" Recently, the mechanism for the activation of dioxygen and O–O bond cleavage in enzymatic reactions has been addressed [7–9], but the precise oxidation states of iron and oxygen for $(FeO)^{3+}$ are not well understood at the enzyme level.

In 1962, Hashimoto, Yamano, Mason [10] demonstrated the "microsomal Fe_x signal" at $g = 2.25$ in liver microsomes, derived from a low spin ferric hemoprotein, which was preferentially reduced by NADPH. Using submicrosomal particles containing P450, Miyake et al. [11] demonstrated that microsomal Fe_x signal is derived from low spin ferric P450. Murakami and Mason [12] suggested that sulfide appears to be a ligand in low spin P450. In 1968, Lu and Coon [13] demonstrated that microsomal fatty acid hydroxylase requires three fractions containing P450, cytochrome c reductase activity, and phospholipids for activity. In 1973, Iyanagi and Mason [14,15] found an air-stable $g = 2$ signal in liver microsomes, which was increased by the addition of NADPH. This signal was derived from air-stable semiquinone of the NADPH-cytochrome c reductase, which was first identified by Horecker [16] as an NADPH-specific cytochrome c reductase. Furthermore, the enzyme was shown to contain one molecule of FAD and FMN per protein molecule, with each flavin performing an individual function. The spin concentrations of air-stable semiquinone and their spectra were compared with those of flavodoxin [14]. The data indicated that the enzyme contained a flavodoxin-type flavin. An iron sulfur protein with $g = 1.94$ signal could not be detected in liver microsomes [17]. These observations strongly suggested that NADPH-cytochrome c reductase participates as an electron carrier in microsomal electron transfer. In 1974, we postulated that this enzyme could directly reduce cytochrome P450 and we named it NADPH-cytochrome P450 reductase (CPR) [15].

CPR supplies reducing equivalents required by P450 isoenzymes, which are a large superfamily of mixed-function oxygenases or monooxygenases found in all three domains of life. Although there are numerous functional cytochrome P450 genes in mammals (e.g., 84 in rat, 87 in mouse, and 57 in human), there is only one CPR gene in each species. Thus, a single CPR is responsible for electron transfer to all the microsomal P450s. Knocking out the CPR gene in mice leads to embryonic lethality [18,19], and missense mutations cause disordered steroidogenesis, ambiguous genitalia, and Antley–Bixler syndrome [20,21]. Polymorphisms of this enzyme appear to affect xenobiotic metabolism, including P450 function.

Function of NADPH-cytochrome P450 reductase

The microsomal electron transfer system consists of two components, CPR and P450. The FMN domain of CPR has a similar function to that of the flavodoxins, which contain a single noncovalent-bound FMN prosthetic group, and can substitute for the low-potential ferredoxin during growth under low-iron conditions. The low-potential flavin, FAD, accepts two reducing equivalents from NADPH (dehydrogenase flavin) and the high-potential flavin, FMN, acts as a one-electron carrier (flavodoxin-type flavin) for the net two-electron transfer from NADPH to P450 [14,15,22]. The cDNA cloning [23] and crystallographic structure of CPR [24] confirmed that the protein has evolved by fusion of two ancestral genes encoding proteins related to ferredoxin-$NADP^+$ reductase (FNR) and flavodoxin. In 1991, Bred et al. [25] isolated the cDNA of neuronal nitric oxide synthase (nNOS), which is localized to neurons throughout the peripheral and central nervous system. The reductase domain of nNOS bears 58% sequence similarity with CPR. In the case of microsomal desaturase system, the cytochrome b_5 can function as a one-electron carrier. These electron transfer sequences suggest that a one-electron carrier is necessary for the sequential electron transfer from a two-electron donor (NAD(P)H) to a P450, which is a one-electron acceptor. In contrast, the mitochondrial system contains adrenodoxin reductase, a single subunit, mono FAD-containing enzyme, and adrenodoxin (adrenal ferredoxin), an iron ferredoxin type, which functions as an electron carrier from NADPH to P450 [26,27]. In general, the bacterial systems consist of three components, including a

ferredoxin. This fact suggests that the mitochondrial P450 electron transfer system in eukaryotes is derived from bacteria.

In the electron transfer systems (Fig. 1), the two electrons are transferred from NAD(P)H to the P450 in two sequential steps, and the P450 can activate molecular oxygen (O_2) by stepwise one-electron reduction. The introduction of the first electron involves the reduction of ferric P450 (Fe^{3+}) and binding of O_2 (Eq. (4)). The second electron addition enables the heterolytic O–O bond scission, in which the thiolate axial ligand of P450 promotes the cleavage of heme-bonded dioxygen by increasing the electron density of the iron atom [8,9,28]. This reaction is coupled with proton donation. In the case of P450eryF [29] and $P450_{CAM}$ [30], the proton is supplied from a hydrogen-bonding network including water molecule(s), forming the water and $(FeO)^{3+}$ species. Thus, the active oxygen-transferring species, $(FeO)^{3+}$, may be formed by a concerted cleavage in a ''push and pull'' effect mediated via the thiolate axial ligand and proton donation (Eq. (5)) [31]. Finally, one oxygen atom is inserted into the substrate (SH) (Eq. (6)).

$$Fe^{3+} + e^- + O_2 \rightarrow Fe^{2+} - O_2 \quad (4)$$

$$Fe^{2+} - O_2 + e^- + 2H^+ \rightarrow (FeO)^{3+} + H_2O \quad (5)$$

$$(FeO)^{3+} + SH \rightarrow Fe^{3+} + SOH \quad (6)$$

In principle, the sequence of electron transfer for mixed-function oxidases is summarized as follows: two-electron donor → dehydrogenase flavin → one-electron carrier → oxygenase (P450) (Fig. 1). In these electron transfer systems, the FMN domains of CPR and NOS can also function as a one-electron carrier. These electron transfer systems require the three functions: (i) dehydrogenase from NAD(P)H, (ii) one-electron carrier, and (iii) stepwise activation of molecular oxygen by an oxygenase. Therefore, a ''step down'' reaction from a two-electron donor to a one-electron acceptor is an essential step in biological oxidations.

The human genome project has identified 57 functional P450 genes: seven of them encode mitochondrial enzymes, all of which play key roles in sterol biosynthesis, and 50 encode microsomal enzymes [20,21]. Of the 50 microsomal enzymes, 20 participate in the biosynthesis of endogenous substrates, such as steroids and eicosanoids; 15 principally metabolize xenobiotic compounds; and 15 are orphan enzymes. CPR can donate electrons to 50 microsomal P450s, which are located in the endoplasmic reticulum. The NOSs are localized in the cytosolic compartment or membranes and are highly complex one-component systems. These enzymes can function as a dimer in which the oxygenase domain accepts electrons from the FMN of the reductase domain from the polypeptide partner. Electron transfer is regulated by a variety of mechanisms, including the binding of calmodulin (CaM), autoinhibitory motifs, protein–protein interactions with other proteins, and phosphorylation [32].

Redox potentials of NADPH-cytochrome P450 reductase and nitric oxide synthase reductase domain

CPR and nitric oxide synthase reductase (NOS) domain are typical members of the FAD–FMN family of proteins. In 1997, Kim and co-workers reported the three-dimensional structure of CPR [24]. The structure clearly indicates that the enzyme molecule is composed of four domains: the FMN-binding domain, the connecting domain, and the FAD- and NADPH-binding domains. The FMN-binding domain is similar to the structure of flavodoxin, whereas the two C-terminal dinucleotide-binding domains are similar to those of fer-

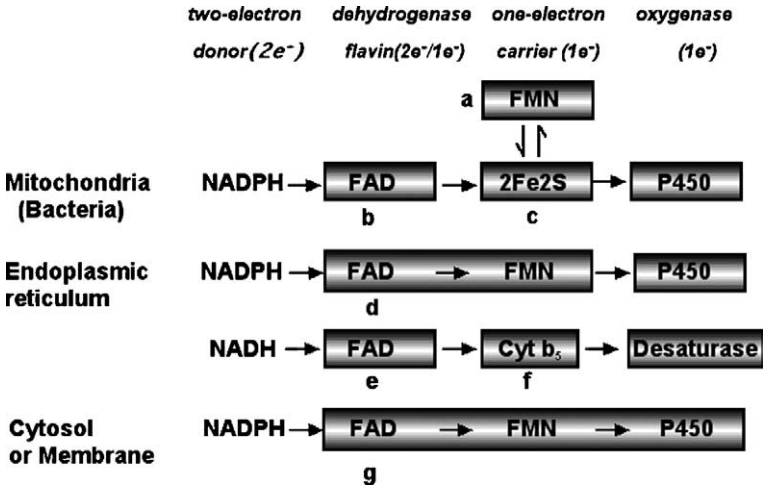


Fig. 1. The electron transfer systems in the mixed-function oxidases. (a) Flavodoxin; (b) ferredoxin reductase; (c) ferredoxin; (d) NADPH-cytochrome P450 reductase; (e) NADH-cytochrome b_5 reductase; (f) cytochrome b_5; and (g) nitric oxide synthase.

redoxin-$NADP^+$ reductase. The distance between the isoalloxazine components of the flavins is 4 Å, indicating direct electron transfer between the dimethylbenzene edge of the two flavins. In this case, the electron transfer rate constant is largely dependent on both the separation distance and the redox potential difference between the electron donor (FAD) and acceptor (FMN) [33]. The X-ray crystal structure of rat nNOS reductase domain, which lacks the CaM-binding domain, has been reported by Garcin et al. [34]. The overall structure is very similar to that of rat CPR [24]. However, the distance between the FAD and FMN moieties is less than 5 Å, and the position of the two flavins differs significantly from that of CPR. In CPR, the plane of the FAD and FMN molecules are inclined relative to each other at an angle of about 45°. This orientation could favor orbital overlap between the extended π-orbital systems of the prosthetic groups. However, in the nNOS reductase the relative orientation of FMN and FAD is different, resulting in reduced overlap compared with that of CPR, although direct electron transfer is possible at this distance [35].

The air-stable (neutral) semiquinone form is characteristic of both CPR and NOS reductase domains. This contains the oxidized flavin (FAD) and semiquinone form of FMN ($FMNH^{\cdot}$) in equimolar amounts. In the flavodoxin, the FMN semiquinone form is stabilized by the interaction with apoprotein. In the CPR and NOSs, the value of the semiquinone formation constant *K*s is larger than unity, indicating an increase in the separation between the oxidized/semiquinone couple ($E_{m.1}$) and semiquinone/fully reduced couple ($E_{m.2}$) in each flavin. This stabilization of neutral semiquinone is due to hydrogen bond formation between the polypeptide backbone of the protein and the N(5) of the reduced FMN, lowering its activity. A hydrogen bond from FMN is formed to the Gly^{141} in rat CPR, and Gly^{810} in rat nNOS, whereas from FAD it is formed to the Ser^{457} in rat CPR and Ser^{1176} in rat nNOS [24,34]. However, the fully reduced form is destabilized by constraints from the unfavorable planar conformation and its activity is increased. Therefore, this state has more activity than that of semiquinone.

The reduction potentials of the individual one-electron redox couples ($E_{m.1}$ and $E_{m.2}$) of FAD and FMN of CPR [15] are given in Fig. 2. In both FAD and FMN, the semiquinone formation constant is larger than unity. In the case of NOSs [36], the overall redox potentials are very similar, although the values for the FAD domains are greater than those of CPR. In both enzymes, the air-stable semiquinone form, $FAD/FMNH^{\cdot}$, has a relatively high reduction potential and low reactivity with electron acceptors. Therefore, the semiquinone/fully reduced couple ($FMNH^{\cdot}/FMNH_2$) could function as a one-electron carrier in the catalytic cycle for both the FAD–FMN pairs in CPR and NOSs. The couples ($FMNH^{\cdot}/FMNH_2$) of each enzyme are −270 mV for CPR, −245 mV for iNOS, −220 mV for nNOS, and −230 mV for eNOS, respectively. The first and second electrons from reductase to P450/or P450-like hemoprotein are sequentially transferred from these states, which can donate an electron at constant oxidation–reduction potential.

Redox states of cytochrome P450 in the perfused rat liver

The mechanism of mixed-function oxidation by P450s has been studied in isolated liver microsomes, liver slices, hepatocytes, purified reconstituted systems, and perfused liver. The direct measurements of P450 in perfused liver have proved important in the understanding of the redox states and the dynamics of P450 in intact cells [37,38]. Sies and Brauser [37] reported that

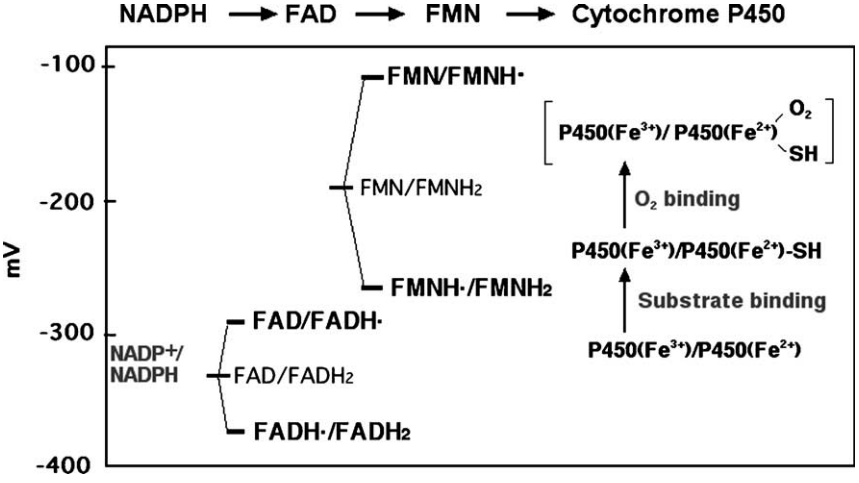
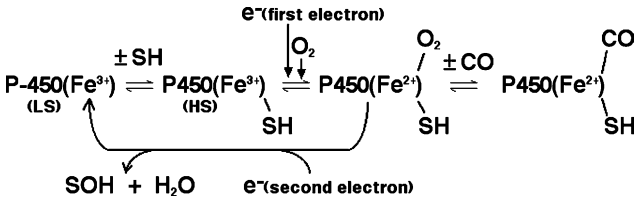


Fig. 2. The reduction potentials of the individual one-electron redox couples ($E_{m.1}$ and $E_{m.2}$) of FAD and FMN of CPR [15]. The redox potentials of cytochrome P450 are discussed in the text and [39–41].

the ratio of NADPH/NADPH + $NADP^+$ decreased from 0.8 to 0.7 in the liver of phenobarbital (PB)-treated rats when hexobarbital was added. Furthermore, the steady-state level of reduced P450–CO increased.

In 1981, we analyzed the spectral changes of P450-associated with mixed-function oxidation of drugs in the hemoglobin-free perfused rat liver, using reflectance spectrophotometry [39,40]. In the same livers, the oxidation–reduction state of pyridine nucleotide(s) and oxygen uptake were measured. Infusion of hexobarbital into livers from fed, PB-treated rats resulted in a decrease of pyridine nucleotide(s) fluorescence (oxidation), accompanied by an increase in oxygen consumption. In addition, reduced P450 was observed in the presence of 3% CO. Under these conditions, mixed-function oxidase activity is not significantly inhibited. The steady-state concentration of the reduced P450–CO complex is approximately 20% of the total P450 and is dependent on the concentration of substrate (hexobarbital) added. These results indicate that a proportion of reduced P450, which can combine rapidly with O_2 to yield the oxygenated form, can react with CO. The different spectrum observed in the presence of hexobarbital indicates the mixture of oxygenated- and CO-complexes. In the absence of exogenous substrate, the formation of the P450–CO complex is very minor, indicating that the resting oxidation–reduction state of P450 is almost oxidized and low spin in perfused rat liver. Interaction of the P450 with substrate results in a shift to the ferric high spin form, and the degree of conversion to the high spin state may regulate the mixed-function oxidase activity. These processes are shown in Scheme 1.

Sligar et al. [41] demonstrated that type I substrates increase the oxidation–reduction potential of microsomal P450. In the presence of hexobarbital, a positive shift of oxidation–reduction potential of about 75 mV was observed. The redox potential of substrate (hexobarbital)-bound P450 was determined to be $E_{m.7} = -237$ mV. This shift increased the rate of flow of the first electron from reductase to P450 in the reconstituted system [42,43]. The resulting reduced form is stabilized by binding O_2, causing its redox potential to increase, thereby allowing a second electron to be transferred from the CPR (Fig. 2).



Scheme 1. Proposed mechanism for mixed-function oxidation catalyzed by cytochrome P450 in the perfused rat liver [37,39,40]. SH represents a substrate and SOH the corresponding product. LS and HS represent low spin and high spin, respectively.

Function of the FAD–FMN pair

In 1969–1970, Iyanagi and Yamazki [44] demonstrated that microsomal NADPH-cytochrome P450(*c*) reductase catalyzes the one-electron reduction of quinones. In contrast, NAD(P)H quinone oxidoreductase (also called DT-diaphorase) catalyzes the two electron reduction of quinones [45]. These findings contributed to the elucidation of the mechanism of redox cycling, including the formation of the oxygen radical [46,47], and to the analysis of one-electron transfer between the two flavins in CPR and the NOS reductase domains. In CPR, iNOS, and nNOS reductase domains, the fully reduced FMN ($FMNH_2$) is the most active flavin species with quinones. In nNOS, one-electron reduction of quinones is activated by the binding of CaM, indicating the activation of interflavin electron transfer [48,49].

Interflavin electron transfer

In the reduction of the oxidized FAD–FMN pair by NADPH, interflavin electron transfer occurs in two steps: $FADH_2$–FMN to $FADH^{\cdot}$/$FMNH^{\cdot}$ and then $FADH^{\cdot}$/$FMNH^{\cdot}$ to FAD–$FMNH_2$ [50–53]. In CPR, iNOS, and nNOS reductase domains, the initial formation and decay of the semiquinone species also has been observed. In rat and human nNOS reductase domains, the rate of formation of semiquinone and its decay indicate that the generation of FAD and FMN semiquinones is significantly accelerated in the presence of CaM [54–56]. In CPR, NADPH (or $NADP^+$) binding is important in controlling the rate of internal electron transfer [57].

In the reduction of air-stable semiquinone by NADPH, interflavin electron transfer occurs from $FADH_2$/$FMNH^{\cdot}$ to $FADH^{\cdot}$/$FMNH_2$ (Fig. 3). The semiquinones of the FAD and the FMN in CPR and NOS reductase domain can be identified by characteristic absorption peaks at 630 nm [50] and 520 nm [55,56], respectively. Upon reduction of air-stable semiquinone in CPR, a decrease in absorbance at 630 nm is observed. This is a characteristic absorption peak of the FMN semiquinone [50,58]. The decrease in absorbance at 630 nm also occurs without any lag phase. In iNOS reductase domain, the increase at 520 nm also occurs without any lag phase. These observations indicate that the internal electron transfer between the two flavins in these enzymes is faster than between NADPH and FAD [55–57]. In the nNOS reductase domain, the rate of formation of FAD semiquinone significantly increases in the presence of CaM [55,56]. In contrast, interflavin electron transfer is comparatively slow in the eNOS reductase domain (Iyanagi et al., unpublished data).

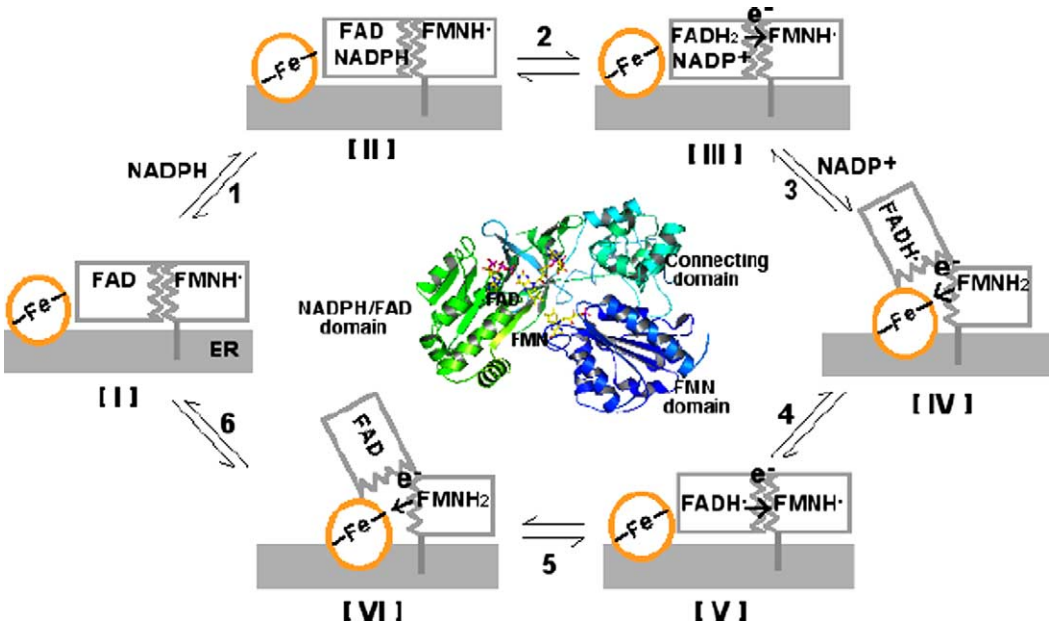


Fig. 3. Proposed model: redox-dependent domain movement of CPR and NOSs reductase domains. ER indicates the membrane of the endoplasmic reticulum. The X-ray crystal structure of CPR was cited from [24].

We have used menadione (MD) to study interflavin electron transfer and flavin semiquinone species during the catalytic cycle [58]. MD, which has $E_{m.7} = -200$ mV for MD/MD$^{\cdot-}$, can accept an electron from both flavins, each fully reduced flavin having a high activity for this molecule. In the presence of MD, the formation of di-semiquinone species (FADH$^{\cdot}$/FMNH$^{\cdot}$) might be expected from its reactivity with both fully reduced flavins. However, the air-stable semiquinone species, FAD/FMNH$^{\cdot}$, is observed as a major intermediate in the reductase domain of both CPR and iNOS. These data indicate that the interflavin electron transfer from FADH$^{\cdot}$ to FMNH$^{\cdot}$ is relatively fast and the resulting fully reduced FMN($FMNH_2$) rapidly reacts with MD. In both enzymes, the air-stable semiquinone form is a major common intermediate in the catalytic cycle.

On the basis of these data, we have proposed a common model for the catalytic cycle of CPR and NOS reductase domains, in which NOSs are all in the CaM-bound state (Fig. 3). In this model, the internal electron transfer between the flavin redox centers occurs in two steps, 3 and 5. In CPR [15], the different redox potentials, 95 mV (difference between FADH$^{\cdot}$/$FADH_2 = -365$ mV and FMNH$^{\cdot}$/$FMNH_2 = -270$ mV) for the first step is larger than that of 20 mV (difference between FAD/FADH$^{\cdot} = -290$ mV and FMNH$^{\cdot}$/$FMNH_2 = -270$ mV) for the second step (Fig. 3). These values strongly suggest that the reaction of the first step (step 3) is thermodynamically more feasible than that of the second step (step 5). Therefore, in the presence of MD the air-stable neutral semiquinone species could be observed in CPR, and also the iNOS reductase domain and full-length enzyme [58].

Regulation of CPR and NOS reductase domain

In perfused rat liver, the P450 system is mainly controlled by a change of spin state, as described in the previous chapter, whereas the CPR is not strictly regulated. However, nNOS and eNOS reductase domains are regulated by the binding of CaM [32]. The overall rate-limiting step in the NOS system is the reductase domain and heme center, but an increase in flavin domain activity increases the NO synthase activity, indicating the different regulation by CaM of the reductase domain in NOS isoenzymes [59]. These observations suggest that either the reduction of FAD or interflavin electron transfer between FAD and FMN is activated by binding of CaM. Another possibility is that the conversion from the CaM-free closed form to the CaM-bound open form is differentially controlled by the binding of CaM (see later discussion and Fig. 3).

Interestingly, the FMN domain of the nNOS and eNOS reductase domains include an autoinhibitory motif (AI) that controls electron transfer, which is not present in iNOS and CPR. The A1 motif is located between the FMN- and NADPH-binding domains [34]. Two mechanisms have been proposed to explain how A1 controls electron transfer: (i) AI can bind CaM and thereby act as a "sponge" by inhibiting CaM binding to the CaM-binding linker; (ii) interactions between AI and the FMN- and NADPH-binding domains contribute to the "locked" electron-accepting position of the FMN domain. On the basis of structural data, it has been proposed that the binding of CaM induces a large-scale swinging motion of the entire FMN domain

to deliver electrons to the heme domain. However, the interflavin electron transfer in rat and human nNOS reductase domain is accelerated by CaM binding [54–56]. Therefore, the A1 motif may play an additional role. For example, the AI segment might participate in increasing the efficiency of electron transfer by adjusting the distance and orientation between FAD and FMN upon CaM binding [35].

Electron transfer from the FAD–FMN pair to P450

The three-dimensional structure of CPR [24] indicates that the dimethylbenzene edge of the isoalloxazine ring of FMN, which is exposed in the FMN domain, is covered by FAD. The pyrimidine ring of the FMN isoalloxazine is slightly buried by two loops, while the dimethylbenzene edge is exposed to solvent. There are several possible mechanisms for electron transfer from the FAD–FMN pair to the electron acceptor, including P450 and cytochrome *c*. Electron transfer from the FMN to the electron acceptor could occur without an associated conformational change or with a large-scale movement of the FMN domain (Fig. 3). The former model infers that electron transfer involves the pyrimidine ring. In this case, an electron tunneling pathway is presumably involved because the pyrimidine ring is buried in the protein. The aromatic amino acids, which sandwich the isoalloxazine ring of the FMN moiety, may participate in electron tunneling during transfer to the electron acceptor. In this case, domain movement is not necessary for electron transfer.

Alternatively, the fully reduced FMN may transfer electrons to P450 by interaction between the cytochrome P450 and the FMN-binding domain. This interaction occurs at the same structural region of the FAD-binding domain. Therefore, domain movement may be needed to facilitate relative domain–domain reorientation during the electron transfer process. Intramolecular one-electron transfer from the reduced FAD to FMN occurs directly at the closest distance. The dimethylbenzene ring of the FMN moiety must be positioned adjacent to the P450, which could be facilitated by a flexible linker between the FMN domain and the remainder of the molecule. Intriguingly, the isolated FAD domains of CPR and NOSs do not significantly reduce the isolated CaM-bound FMN domains (Iyanagi et al., unpublished data). This observation suggests that the electrostatic interaction between the FAD and FMN domains is less specific, indicating flexibility in the relative orientation between electron donors and electron acceptors during the catalytic cycle. Furthermore, crystal structures of wild type and various mutant CPRs strongly suggest that the relative orientation and position of the two flavin domains with respect to each other are flexible [60].

In the bacterial system, the flavodoxins can shuttle between electron donors and electron acceptors during the catalytic cycle. In CPR, the fully reduced FMN states (IV and VI) may be the open form in which they can transfer electrons to an electron acceptor. The states I, II, III, and V are closed forms in which interflavin electron transfer occurs. However, the P450 and cytochrome *c* cannot access the active site of these states. The model assumes that interflavin electron transfer is linked to a significant movement of the FAD domain (Fig. 3). This could explain why the reactivity of cytochrome *c* with air-stable semiquinone (FAD/FMNH$^{\bullet}$) is very slow, despite the reaction between air-stable semiquinone ($E_{m.1} = -110$ mV) and cytochrome *c* ($E_m = +220$ mV) being thermodynamically favorable. Craig et al. [61] have proposed a model to explain how the movement of the nNOS reductase domain is controlled by conformational changes during catalysis. The model proposes that the CaM-free reductase domain is a closed form whereas the CaM-bound form is an open form, which can donate an electron to an electron acceptor. The catalytic cycle of CPR and CaM bound NOS reductase domain is shown in Fig. 3. The both enzymes are in redox-linked dynamic equilibrium between closed and open forms.

The electron acceptors accept an electron from the $FMNH_2$, while the FMNH$^{\bullet}$ is an inactive intermediate observed during the catalytic cycle. Therefore, the initial reaction in the catalytic cycle starts from the air-stable semiquinone species I, and the IV and VI states are active species for electron acceptors. For cytochrome *c*, a different molecule accepts electrons independently from these states, but a P450 requires two electrons delivered in two one-electron transfer steps, the same P450 accepting two electrons from these states. The P450, which accepts one electron from state IV, has to either move away from the reductase and accept an electron from a different reductase molecule or interact with a different site of the reductase (state V), followed by the redox-dependent conformational change, where a second electron is accepted from state VI. Finally, the two electrons are sequentially transferred to the P450s. In the ER membrane, the FMN domain is fixed by the membrane-binding anchor domain, and an electron transfer between the two proteins, CPR/P450s, occurs in the reactions in near random collision on the plane of the membrane [62].

Acknowledgments

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References

[1] H.S. Mason, W.L. Fowlks, E.W. Peterson, Oxygen transfer and electron transport by the phenolase complex, J. Am. Chem. Soc. 77 (1955) 2914–2915.

[2] O. Hayaishi, M. Katagiri, S. Rothberg, Mechanism of the pyrocatechase reaction, J. Am. Chem. Soc. 77 (1955) 5450–5451.

[3] M. Hayano, M.C. Lindberg, R.I. Dorfman, J.E. Hancock, W.E. Doering, On the mechanism of the C-11β-hydroxylation of steroids; a study with H_2O^{18} and O_2^{18}, Arch. Biochem. Biophys. 59 (1955) 529–532.

[4] H.S. Mason, Mechanisms of oxygen metabolism, Science 125 (1957) 1185–1188.

[5] J. Axelrod, The enzymatic deamination of amphetamine, J. Biol. Chem. 214 (1955) 753–763.

[6] H.S. Mason, J.C. North, M. Vanneste, Microsomal mixed-function oxidations: the mechanism of xenobiotics, Fed. Proc. 24 (1965) 1172–1180.

[7] L.M. Mirica, M. Vance, D.J. Rudd, B. Hedman, K.O. Hodgson, E.I. Solomon, T.D. Stack, Tyrosinase reactivity in a model complex: an alternative hydroxylation mechanism, Science 308 (2005) 1890–1892.

[8] P. Hlavica, Models and mechanisms of O–O bond activation by cytochrome P450. A critical assessment of the potential role of multiple active intermediates in oxidative catalysis, Eur. J. Biochem. 271 (2004) 4335–4360.

[9] I.G. Denisov, T.M. Makris, S.G. Sligar, I. Schlichting, Structure and chemistry of cytochrome P450, Chem. Rev. 105 (2005) 2253–2277.

[10] Y. Hashimoto, T. Yamano, H.S. Mason, An electron spin resonance study of microsomal electron transport, J. Biol. Chem. 237 (1962) 3843–3844.

[11] Y. Miyake, J.L. Gaylor, H.S. Mason, Properties of a submicrosomal particle containing P-450 and flavoprotein, J. Biol. Chem. 243 (1968) 5788–5797.

[12] K. Murakami, H.S. Mason, An electron spin resonance study of microsomal Fe_x, J. Biol. Chem. 242 (1967) 1102–1110.

[13] A.Y. Lu, M.J. Coon, Role of hemoprotein P-450 in fatty acid omega-hydroxylation in a soluble enzyme system from liver microsomes, J. Biol. Chem. 243 (1968) 1331–1332.

[14] T. Iyanagi, H.S. Mason, Some properties of hepatic reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase, Biochemistry 12 (1973) 2297–2308.

[15] T. Iyanagi, N. Makino, H.S. Mason, Redox properties of the reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 and reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductases, Biochemistry 13 (1974) 1701–1710.

[16] B.L. Horecker, Triphosphopyridine nucleotide-cytochrome c reductase in liver, J. Biol. Chem. 183 (1950) 593–605.

[17] Y. Miyake, H.S. Mason, W. Langraf, An electron spin resonance study of liver and adrenal mixed function oxidases near the temperature of liquid helium, J. Biol. Chem. 242 (1967) 393–397.

[18] A.L. Shen, K.A. O'Leary, C.B. Kasper, Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase, J. Biol. Chem. 277 (2002) 6536–6541.

[19] D.M. Otto, C.J. Henderson, D. Carrie, M. Davey, T.E. Gundersen, R. Blomhoff, R.H. Adams, C. Tickle, C.R. Wolf, Identification of novel roles of the cytochrome P450 system in early embryogenesis: effects on vasculogenesis and retinoic acid Homeostasis, Mol. Cell. Biol. 23 (2003) 6103–6116.

[20] N. Huang, A.V. Pandey, V. Agrawal, W. Reardon, P.D. Lapunzina, D. Mowat, E.W. Jabs, G. Van Vliet, J. Sack, C.E. Fluck, W.L. Miller, Diversity and function of mutations in P450 oxidoreductase in patients with Antley–Bixler Syndrome and disordered steroidogenesis, Am. J. Hum. Genet. 76 (2005) 729–749.

[21] W.L. Miller, Regulation of steroidogenesis by electron transfer, Endocrinology 146 (2005) 2544–2550.

[22] J.L. Vermilion, M.J. Coon, Identification of the high and low potential flavins of liver microsomal NADPH-cytochrome P-450 reductase, J. Biol. Chem. 253 (1978) 8812–8819.

[23] T.D. Porter, C.B. Kasper, Coding nucleotide sequence of rat NADPH-cytochrome P-450 oxidoreductase cDNA and identification of flavin-binding domains, Proc. Natl. Acad. Sci. USA 82 (1985) 973–977.

[24] M. Wang, D.L. Roberts, R. Paschke, T.M. Shea, B.S.S. Masters, J.-J.P. Kim, Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes, Proc. Natl. Acad. Sci. USA 94 (1997) 8411–8416.

[25] D.S. Bred, P.M. Hwang, C.E. Glatt, C. Lowenstein, R.R. Reed, S.H. Snyder, Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase, Nature 351 (1991) 714–718.

[26] T. Omura, E. Sanders, R.W. Estabrook, D.Y. Cooper, O. Rosenthal, Isolation from adrenal cortex of a nonheme iron protein and a flavoprotein functional as a reduced triphosphopyridine nucleotide-cytochrome P450 reductase, Arch. Biochem. Biophys. 117 (1966) 660–673.

[27] T. Kimura, K. Suzuki, Components of the electron transport system in adrenal steroid hydroxylase. Isolation and properties of non-heme iron protein (adrenodoxin), J. Biol. Chem. 242 (1967) 419–485.

[28] K. Yamaguchi, Y. Watanabe, I. Morishima, Push effect on the heterolytic cleavage of peroxoiron (III) porphyrin adducts, Inorg. Chem. 31 (1992) 156–157.

[29] S. Nagano, J.R. Cupp-Vickery, T.L. Poulos, Crystal structure of the ferrous dioxygen complex of wild-type cytochrome P450eryF and mutants, A245S and A245T, J. Biol. Chem. 280 (2005) 22102–22107.

[30] S. Nagano, T.L. Poulos, Crystallographic study on the dioxygen complex of wild-type and mutant cytochrome $P450_{CAM}$: implications for the dioxygen activation mechanism, J. Biol. Chem. 280 (2005) 31659–31663.

[31] K. Yamaguchi, Y. Watanabe, I. Morishima, Direct observation of the push effect on the O–O bond cleavage of acylperoxoiron (III) porphyrin complexes, J. Am. Chem. Soc. 115 (1993) 4058–4065.

[32] L.J. Roman, P. Martasek, B.S.S. Masters, Intrinsic and extrinsic modulation of nitric oxide synthase activity, Chem. Rev. 102 (2002) 1179–1190.

[33] R.A. Marcus, N. Sutin, Electron transfer in chemistry and biology, Biochim. Biophys. Acta 811 (1985) 265–322.

[34] E.D. Garcin, C.M. Bruns, S.J. Lloyd, D.J. Hosfield, M. Tiso, R. Gachhui, D.J. Stuehr, J.A. Tainer, E.D. Getzoff, Structural basis for isozyme-specific regulation of electron transfer in nitric-oxide synthase, J. Biol. Chem. 279 (2004) 37918–37927.

[35] T. Iyanagi, Interflavin one-electron transfer in the NADPH-cytochrome P450 reductase and nitric oxide synthase reductase domain, in: T. Nishino, R. Miura, M. Tanokura, K. Fukui (Eds.), In Flavins and Flavoproteins, ArchiTeck Inc., Tokyo, 2005, pp. 415–429.

[36] Y.T. Gao, S.M.E. Smith, J.B. Weinberg, H.J. Montgomery, E. Newman, J.G. Guillemette, D.K. Ghosh, L.J. Roman, P. Martasek, J.C. Salerno, Thermodynamics of oxidation–reduction reactions in mammalian nitric-oxide synthase isoforms, J. Biol. Chem. 279 (2004) 18759–18766.

[37] H. Sies, B. Brauser, Interaction of mixed function oxidase with its substrates and associated redox transitions of cytochrome P-450 and pyridine nucleotides in perfused rat liver, Eur. J. Biochem. 15 (1970) 531–540.
[38] L.A. Reinke, R.G. Thurman, F.C. Kauffman, Oxidation–reduction state of free $NADP^+$ during mixed-function oxidation in perfused rat livers—evaluation of the assumptions of near equilibrium by comparisons of surface fluorescence changes and calculated $NADP^+$: NADPH ratios, Biochem. Pharmacol. 28 (1979) 2381–2387.
[39] T. Iyanagi, T. Suzaki, S. Kobayashi, Oxidation–reduction states of pyridine nucleotide and cytochrome P450 during mixed-function oxidation in perfused rat liver, J. Biol. Chem. 256 (1981) 12933–12939.
[40] T. Suzaki, T. Iyanagi, S. Kobayashi, Effect of 2,4-dinitrophenol on the oxidative metabolism of hexobarbital by cytochrome P-450 in perfused rat liver, J. Biochem. (Tokyo) 94 (1983) 361–371.
[41] S.G. Sligar, D.L. Cinti, G.G. Gibson, J.B. Schenkman, Spin state control of the hepatic cytochrome P450 redox potential, Biochem. Biophys. Res. Commun. 90 (1979) 925–932.
[42] Y. Imai, R. Sato, T. Iyanagi, Rate-limiting step in the reconstituted microsomal drug hydroxylase system, J. Biochem. 82 (1977) 1237–1246.
[43] T. Iyanagi, F.K. Anan, Y. Imai, H.S. Mason, Studies on the microsomal mixed function oxidase system: redox properties of detergent-solubilized NADPH-cytochrome P-450 reductase, Biochemistry 17 (1978) 2224–2230.
[44] T. Iyanagi, I. Yamazaki, One-electron-transfer reactions in biochemical systems. III. One-electron reduction of quinones by microsomal flavin enzymes, Biochim. Biophys. Acta 172 (1969) 370–381.
[45] T. Iyanagi, I. Yamazaki, One-electron-transfer reactions in biochemical systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase), Biochim. Biophys. Acta 216 (1970) 282–294.
[46] T. Iyanagi, On the mechanisms of one- and two-electron transfer by flavin enzymes, Chem. Scr. 27A (1987) 31–36.
[47] N. Cenas, Z. Anusevicius, H. Nivinskas, L. Misevicinen, J. Sarlauskas, Structure–activity relationships in two-electron reduction of quinones, Methods Enzymol. 382 (2004) 258–277.
[48] H. Matsuda, S. Kimura, T. Iyanagi, One-electron reduction of quinones by the neuronal nitric-oxide synthase reductase domain, Biochim. Biophys. Acta 1459 (2000) 106–116.
[49] J. Fu, K. Yamamoto, Z.-W. Guan, S. Kimura, T. Iyanagi, Human neuronal nitric oxide synthase can catalyze one-electron reduction of adriamycin: role of flavin domain, Arch. Biochem. Biophys. 427 (2004) 180–187.
[50] T. Iyanagi, R. Makino, F.K. Anan, Studies on the microsomal mixed-function oxidase system: mechanism of action of hepatic NADPH-cytochrome P-450 reductase, Biochemistry 20 (1981) 1722–1730.
[51] J.L. Vermilion, D.P. Ballou, V. Massey, M.J. Coon, Separate roles for FMN and FAD in catalysis by liver microsomal NADPH-cytochrome P-450 reductase, J. Biol. Chem. 256 (1981) 266–277.
[52] D.D. Oprian, M.J. Coon, Oxidation–reduction states of FMN and FAD in NADPH-cytochrome P-450 reductase during reduction by NADPH, J. Biol. Chem. 257 (1982) 8935–8944.
[53] A. Gutierrez, L.Y. Lian, C.R. Wolf, N.S. Scrutton, G.C.K. Roberts, Stopped-flow kinetic studies of flavin reduction in human cytochrome P450 reductase and its component domains, Biochemistry 40 (2001) 1964–1975.
[54] H. Matsuda, T. Iyanagi, Calmodulin activates intramolecular electron transfer between the two flavins of neuronal nitric oxide synthase flavin domain, Biochim. Biophys. Acta 1473 (1999) 345–355.
[55] Z-W. Guan, T. Iyanagi, Electron transfer is activated by calmodulin in the flavin domain of human neuronal nitric oxide synthase, Arch. Biochem. Biophys. 412 (2003) 65–76.
[56] Z.-W. Guan, D. Kamatani, S. Kimura, T. Iyanagi, Mechanistic studies on the intramolecular one-electron transfer between the two flavins in the human neuronal nitric-oxide synthase and inducible nitric-oxide synthase flavin domains, J. Biol. Chem. 278 (2003) 30859–30868.
[57] A. Gutierrez, A.W. Munro, A. Grunau, C.R. Wolf, N.S. Scrutton, G.C.K. Roberts, Interflavin electron transfer in human cytochrome P450 reductase is enhanced by coenzyme binding, Eur. J. Biochem. 270 (2003) 2612–2621.
[58] K. Yamamoto, S. Kimura, Y. Shiro, T. Iyanagi, Interflavin one-electron transfer in the inducible nitric oxide synthase reductase domain and NADPH-cytochrome P450 reductase, Arch. Biochem. Biophys. 440 (2005) 65–78.
[59] C.R. Nishida, P.R. Ortiz de Montellano, Electron transfer and catalytic activity of nitric oxide synthases. Chimeric constructs of the neuronal, inducible, and endothelial isoforms, J. Biol. Chem. 273 (1998) 5566–5571.
[60] P.A. Hubbard, A.L. Shen, R. Paschke, C.B. Kasper, J.-J.P. Kim, NADPH-cytochrome P450 oxidoreductase. Structural basis for hydride and electron transfer, J. Biol. Chem. 276 (2001) 29163–29170.
[61] D.H. Craig, S.K. Chapman, S. Daff, Calmodulin activates electron transfer through neuronal nitric-oxide synthase reductase domain by releasing an NADPH-dependent conformational lock, J. Biol. Chem. 277 (2002) 33987–33994.
[62] H. Taniguchi, Y. Imai, T. Iyanagi, R. Sato, Interaction between NADPH-cytochrome P-450 reductase and cytochrome P-450 in the membrane of phosphatidylcholine vesicles, Biochim. Biophys. Acta 550 (1979) 341–356.