Distinct Conformational Behaviors of Four Mammalian Dual-Flavin Reductases (Cytochrome P450 Reductase, Methionine Synthase Reductase, Neuronal Nitric Oxide Synthase, Endothelial Nitric Oxide Synthase) Determine Their Unique Catalytic Profiles

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Distinct conformational behaviors of four mammalian dual-flavin reductases (cytochrome P450 reductase, methionine synthase reductase, neuronal nitric oxide synthase, endothelial nitric oxide synthase) determine their unique catalytic profiles

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Keywords
conformational equilibrium; electron transfer; flavoprotein; kinetic model; nitric oxide

Multidomain enzymes often rely on large conformational motions to function. However, the conformational setpoints, rates of domain motions and relationships between these parameters and catalytic activity are not well understood. To address this, we determined and compared the conformational setpoints and the rates of conformational switching between closed unreactive and open reactive states in four mammalian diflavin NADPH oxidoreductases that catalyze important biological electron transfer reactions: cytochrome P450 reductase, methionine synthase reductase and endothelial and neuronal nitric oxide synthase. We used stopped-flow spectroscopy, single turnover methods and a kinetic model that relates electron flux through each enzyme to its conformational setpoint and its rates of conformational switching. The results show that the four flavoproteins, when fully-reduced, have a broad range of conformational setpoints (from 12% to 72% open state) and also vary 100-fold with respect to their rates of conformational switching between unreactive closed and reactive open states (cytochrome P450 reductase > neuronal nitric oxide synthase > methionine synthase reductase > endothelial nitric oxide synthase). Furthermore, simulations of the kinetic model could explain how each flavoprotein can support its given rate of electron flux (cytochrome c reductase activity) based on its unique conformational setpoint and switching rates. The present study is the first to quantify these conformational parameters among the diflavin enzymes and suggests how the parameters might be manipulated to speed or slow biological electron flux.
Introduction

The diflavin reductases transfer electrons from NADPH to a family of one-electron acceptors [1, 4]. They consist of attached NADPH, FAD and FMN binding domains [1, 5] that are analogous to existing proteins: the NADPH and FAD binding domains [NADPH/FAD or ferredoxin-NADP⁺-reductase-like domain (FNR)] resemble NAD⁺-ferredoxin reductases, whereas the FMN domains are analogous to flavodoxins [1, 6-8]. Either flavin cofactor can exist in three different redox states: oxidized, semiquinone (sq) and hydroquinone (hq). The electron flux through these enzymes follows a linear pathway: NADPH → FAD → FMN → final acceptors [9]. Each flavin has a unique function: the FAD accepts a hydride from NADPH and then transfers reducing equivalents to FMN one at a time [10, 11]. Because the mammalian diflavin reductases stabilize their one-electron reduced (semiquinone) FMN (FMNsq), only the two-electron reduced (hydroquinone) FMN (FMNhq) acts as an electron donor to external acceptors and then cycles between the FMNsq and FMNhq forms. The diflavin reductase family includes mammalian members NADPH-cytochrome P450 reductase (CPR) [6, 12], the three nitric oxide synthases (NOS) [13 15], methionine synthase reductase (MSR) [16 18] and novel reductase 1 [19], and includes bacterial members sulfite reductase [20] and flavocytochrome P450 BM3 [21, 22].

Electron transfer (ET) through diflavin enzymes involves specific domain domain interactions. An interface that forms between the NADPH/FAD (FNR) and FMN domains allows the interflavin ET that forms FMNhq. Crystal structures of rat and human CPRs [23, 24] and the reductase domain of rat neuronal nitric oxide synthase (nNOSred) [25] show the isoalloxazine rings of the bound FAD and FMN cofactors are closely positioned for rapid ET. However, in this conformation, the FMN cofactor is buried from solvent and is incapable of performing ET with protein redox partners or artificial acceptors such as cytochrome c. Transition of the conformationally-closed structures to more open forms is thought to be required, as was confirmed recently for CPR [26]. Electron flux through all diflavin enzymes is thought to involve conformational shuttling between closed and open states [27-31]. Crystal structures and biophysical studies have increased our understanding of the conformationally-closed and -open states of diflavin reductases and how they may participate in catalyzing electron flux to their natural acceptor proteins or to cytochrome c [15, 23, 25, 27, 30, 32-39]. However, it is not clear how the conformational equilibria and rates of conformational switching relate to electron flux, or how these parameters compare among the diflavin reductases. To address this, we proposed a simple four-state kinetic model (Fig. 1) [15, 34, 35] that relies on cytochrome c reductase activity to assess electron flux through the diflavin enzymes. Under typical experimental conditions with excess NADPH and cytochrome c, the rate of flavin reduction or cytochrome c reduction (k4) is fast and not rate-limiting [30, 36, 37, 40, 41] and so the electron flux through the diflavin enzyme is only dependent on the rates of FMN domain conformational switching (k1, k − 1, k3, k − 3) and the rate of interflavin electron transfer between the FAD and FMN cofactors (k2). The model incorporates the one-electron redox cycling of the FMNhq and FMNsq, which occurs naturally during turnover, and therefore includes two temporally-separate equilibrium terms, Ksq and Khq, which differ according to the reduction state of the FMN (FMNsq versus FMNhq). We define Ksq = k1/k−1 and Ksq = k3/k−3 (Fig. 1), such that higher K values indicate a greater abundance of open conformations. The equilibrium described by Khq involves a conformational opening step that allows the FMNhq to reduce an acceptor (in this case cytochrome c) and consequently become oxidized to FMNsq. The equilibrium described by Ksq involves a conformational closing step that allows the FMNhq to receive another electron from the FNR domain. For simplicity, the model assumes: (a) the interflavin electron transfer step (k2) is irreversible; (b) the reduction of FAD by NADPH and the reduction of cytochrome c by FMNhq (k4 step) are rapid and irreversible steps relative to all the other kinetic steps; and (c) the conformational equilibrium setpoint is unaffected by the redox state of the FMN (i.e. Ksq = Khq). We previously utilized this approach to determine estimates for the Khq setpoint and rates of

\[ \text{Cyt c$_{red}$} \rightarrow \text{Cyt c$_{ox}$} \rightarrow \text{Cyt c$_{red}$} \]

Fig. 1. Kinetic model for electron flux through a dual flavin enzyme. The model uses four kinetic rates: association (k1 or k−1) and dissociation (k−1 or k−3) of the FMN and FNR domains; the FMNH⁺ reduction rate (k3) and the cytochrome c reduction rate (k4). The fully reduced enzyme in the open conformation (species a) reduces cytochrome c and generates species b, which then undergoes successive conformational closing, interflavin electron transfer, and conformational opening steps to complete the cycle.
conformational switching and interflavin ET in the flavoprotein domains of endothelial and neuronal NOS [reductase domain of endothelial NOS (eNOSred), reductase domain of nNOS (nNOSred)] [34,36] and in site-specific variants of nNOSred [35].

In the present study, we expanded our studies to investigate CPR and MSR and we re-analyzed nNOSred and eNOSred in parallel, aiming to understand the range of conformational behaviors displayed across the mammalian dual-flavin reductase family, and how their conformational parameters may determine their different electron flux profiles. Simulations of the kinetic model show how each enzyme’s unique blend of conformational equilibrium and kinetic parameters help determine its steady-state species distribution and catalytic activity. The present study provides the first measures of conformational setpoints and switching rates, which in turn have protein structural, mechanistic and functional implications.

Results

Steady-state electron flux varies widely among family members

We compared the NADPH-dependent cytochrome c reductase activities of the four flavoproteins at 10 and 25 °C (Table 1). Steady-state cytochrome c reductase activity indicates the maximal electron flux that can be achieved through the four flavoproteins because cytochrome c reacts quickly and irreversibly to accept an electron from their reduced FMN domains [1,15,17,27,35,36]. The reductase activities that we obtained matched with earlier reports [17,27,35,36] and, at either temperature, gave a rank order of CPR > nNOSred > eNOSred = MSR that spanned almost two orders of magnitude. This difference became the premise for the present study.

Table 1. Steady state cytochrome c reductase activities of dual flavin reductases. The cytochrome c reductase activities were determined at 25 and 10 °C as described in the Experimental procedures. The values are the mean ± SD of three measurements carried out under identical conditions, using two different protein preparations for each enzyme. Activities of nNOSred and eNOSred were measured under CaM-free conditions.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>10 °C (min⁻¹)</th>
<th>25 °C (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOSred</td>
<td>27 ± 2.3</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>nNOSred</td>
<td>474 ± 29</td>
<td>752 ± 42</td>
</tr>
<tr>
<td>CPR</td>
<td>1680 ± 160</td>
<td>3629 ± 290</td>
</tr>
<tr>
<td>MSR</td>
<td>37 ± 3</td>
<td>56 ± 4.2</td>
</tr>
</tbody>
</table>

Conformational $K_{eq}$ setpoints vary among the fully-reduced flavoproteins

We estimated the conformational $K_{eq}$ setpoints ([open-reactive]/[closed-unreactive]) of each fully-reduced flavoprotein by monitoring its reaction with an excess of cytochrome c in a stopped-flow spectrophotometer, as done previously [35,36]. Under our particular experimental conditions (final cytochrome c concentration was 50 μM after mixing, second-order rate constant of $4.5 \times 10^6$ M⁻¹s⁻¹ at 10 °C), the portion of reduced enzyme molecules that are in a cytochrome c reactive conformation (which we define as an open conformation) will transfer an electron from FMNₐ to cytochrome c at approximately 225 s⁻¹, and thus this reaction will occur almost within the instrument mixing dead time (approximately 4 ms). In contrast, the portion of enzyme molecules that exist in a cytochrome c unreactive conformation (which we define as closed conformation) will only reduce cytochrome c gradually and in relation to the rate of conformational opening. Thus, the extent to which reduction of the first molar equivalent of cytochrome c exhibits a fast reduction (approximately within the mixing dead-time) versus a slower reduction indicates what proportion of the fully-reduced enzyme molecules were in an open versus closed conformational state at the time of mixing, and gives an estimate for the conformational $K_{eq}$ of the fully-reduced enzyme [34 36].

We rapid-mixed the reduced, NADPH-bound forms of each flavoprotein with an excess (100 μM) of cytochrome c in the stopped-flow spectrometer under anaerobic conditions at 10 °C, and monitored the increase in $A_{550}$ during the first few electron transfers to cytochrome c (i.e. the first few turnovers). Traces that were averaged from six to eight consecutive mixing reactions are shown in Fig. 2. Initial absorbance readings for $t = 0$ were determined by rapid-mixing the enzyme-free reaction buffer with the cytochrome c reaction solution. The experimental data are plotted in terms of the increase in absorption from the $t = 0$ point (a value designated as 0 on the left y-axis in Fig. 2, all panels). The right y-axis of each panel in Fig. 2 indicates the moles of cytochrome c reduced per mole of total flavoprotein that was present in each reaction. The blue dotted box indicates the absorbance change that occurred and the time that elapsed during reduction of the first molar equivalent of cytochrome c.

In all four enzyme reactions, we observed an initial burst phase that was followed by a slower and almost linear rate of cytochrome c reduction, as we had observed previously for nNOSred and eNOSred under...
similar reaction conditions [34, 36]. This behavior suggests the transition to steady-state cycling already occurred within the first few turnovers in all four flavoprotein reactions. This concept was supported by our finding that the enzyme activity calculated from a tangent line drawn onto each kinetic trace within the time taken to reduce the first two equivalents of cytochrome c (tangents are indicated as red dashed lines in Fig. 2) essentially matched the steady-state activity that was determined for each flavoprotein in a standard steady-state assay run in a cuvette for 3 min at 10 °C (Tables 2 and 3).

The data shown in Fig. 2 suggest that the reduced diflavin enzymes have different conformational equilibrium setpoints ($K_{hq}$). For example, in Fig. 2 (upper left), the $t = 0$ value of the nNOSred absorbance trace, relative to the $y$-axis intercept of the blue dashed line, shows that approximately 50% of the first equivalent of cytochrome c became reduced within the mixing dead time and the remaining 50% of the first equivalent became reduced during the subsequent observable period (within the dotted blue box). This implies the NADPH-bound, fully-reduced nNOSred molecules equally populated the open reactive and closed unreactive conformations at the time of mixing (species a and d in Fig. 1, respectively), giving an estimated $K_{hq} = 1$ for the calmodulin (CaM)-free, NADPH-bound, fully-reduced nNOSred, which matches with our previous $K_{hq}$ estimate for nNOSred [35,36]. The situation drastically differed for eNOSred (Fig. 2, upper right). In this case, only approximately 11% of the absorbance change occurred in the instrument dead time during reduction of the first equivalent of cytochrome c, leaving approximately 89% of the absorbance change to be observable. This indicates the CaM-free, NADPH-bound, fully-reduced eNOSred predominantly populates the closed unreactive conformation, according to estimated $K_{hq}$ of approximately 0.12, again similar to our earlier study [34,36].

For the NADPH-bound, fully-reduced CPR (Fig. 2, lower left), the trace showed that 33% of the first equivalent of cytochrome c was reduced within the instrument dead time, indicating that CPR also favors the closed unreactive conformation according to $K_{hq} = 0.5$. In contrast, the NADPH-bound, fully-reduced MSR (Fig. 2, lower right) completed most of its first turnover within the mixing dead time of the instrument, leaving only approximately 28% of the first turnover observable, and giving an estimated $K_{hq} = 2.6$. Thus, the four flavoproteins displayed a

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**Table 2.** Summary of results from reactions mixing dual flavin reductases with excess cytochrome c. An excess of cytochrome c was mixed with fully reduced proteins in a stopped flow instrument at 10 °C as described in the Experimental procedures.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Time (first turnover)</th>
<th>Fraction open</th>
<th>Fraction closed</th>
<th>$K_{hq}$</th>
<th>Steady state electron flux (min $^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOSred</td>
<td>1.8 s</td>
<td>0.11</td>
<td>0.89</td>
<td>0.125</td>
<td>29.5</td>
</tr>
<tr>
<td>nNOSred</td>
<td>40 ms</td>
<td>0.51</td>
<td>0.49</td>
<td>1.04</td>
<td>486</td>
</tr>
<tr>
<td>CPR</td>
<td>9 ms</td>
<td>0.33</td>
<td>0.67</td>
<td>0.5</td>
<td>1620</td>
</tr>
<tr>
<td>MSR</td>
<td>117 ms</td>
<td>0.72</td>
<td>0.28</td>
<td>2.57</td>
<td>42</td>
</tr>
</tbody>
</table>
Table 3. Kinetic parameters derived from fitting the experimental traces according to the four-state kinetic model. For details, see text. Data are representative of two or three trials with each protein. Values in parentheses are experimental values.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Measured $k_\text{A}$</th>
<th>Best fit $k_1$, $k_3$ [s$^{-1}$]</th>
<th>Best fit $k_1$, $k_3$ [s$^{-1}$]</th>
<th>Best fit $k_3$ [s$^{-1}$]</th>
<th>Fitted steady state electron flux [s$^{-1}$] (experimental value)</th>
<th>Fitted time required for first turnover (experimental value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOSred</td>
<td>0.125</td>
<td>5</td>
<td>0.63</td>
<td>5</td>
<td>0.49 (0.45)</td>
<td>1.76 (1.8) ms</td>
</tr>
<tr>
<td>nNOSred</td>
<td>1.0</td>
<td>53</td>
<td>53</td>
<td>22</td>
<td>8.1 (7.9)</td>
<td>38 (40) ms</td>
</tr>
<tr>
<td>CPR</td>
<td>0.5</td>
<td>460</td>
<td>230</td>
<td>52</td>
<td>27 (28)</td>
<td>9 (9) ms</td>
</tr>
<tr>
<td>MSR</td>
<td>2.57</td>
<td>8</td>
<td>20.5</td>
<td>2.8</td>
<td>0.7 (0.62)</td>
<td>114 (117) ms</td>
</tr>
</tbody>
</table>

range of conformational $K_{\text{hq}}$ setpoints in their NADPH-bound, fully-reduced states, according to the rank order: MSR > nNOSred > CPR > eNOSred (Table 2). MSR predominantly populated a reactive open conformation, nNOS was poised equally between the reactive open and unreactive closed conformations, and CPR and eNOS predominantly populated an unreactive closed conformation.

Rates of conformational switching and interflavin ET differ among the four flavoproteins

To derive rate estimates for the conformational transitions between the open reactive and closed unreactive states, and to derive an estimated rate of interflavin ET in each flavoprotein, we used computer simulations to fit the observed kinetic traces of cytochrome $c$ reduction in Fig. 2 according to the four-state kinetic model in Fig. 1 that links the rates of conformational motions and interflavin ET to the electron flux to cytochrome $c$ [34]. We previously used the same approach to derive rate estimates for nNOSred, nNOSred mutants and eNOSred [34,35].

In the present study, we incorporated the estimated $K_{\text{hq}}$ values derived above into the simulations and then used an iterative method to find rate pairs for interflavin ET ($k_2$) and FMN domain conformational transitions ($k_1$, $k_{1\rightarrow}$, $k_3$, $k_{3\rightarrow}$) that support the experimentally-observed rate of electron flux to cytochrome $c$ for each flavoprotein [34,35]. This analysis generated combinations of rate pairs that can each support the observed steady-state electron flux rate for each enzyme, with the general relationship being that faster rates of interflavin ET can pair with slower rates of FMN conformational switching and vice versa. The traces in the graphs of Fig. 3 indicate the allowable rate pairs for each enzyme that we derived from this fitting process. This analysis is instructive because it indicates the lower rate boundaries for the interflavin ET and the conformational opening and closing steps in each enzyme [34,35]. For example, the horizontal blue tangent line drawn in the nNOSred panel of Fig. 3 (upper left) indicates the lowest boundary rate of conformational switching (17 s$^{-1}$) that would be able to maintain the observed electron flux to cytochrome $c$ (approximately 8 s$^{-1}$ for nNOSred at 10 °C), when paired with a comparatively fast rate of interflavin ET. Similarly, a vertical blue tangent line indicates the lowest boundary rate for interflavin ET ($k_2$ approximately 20 s$^{-1}$) that would still be able to maintain the experimentally observed electron flux, when paired with a very fast rate of conformational switching. Because the conformational $K_{\text{hq}}$ is 1 in nNOSred, $k_1 = k_{1\rightarrow}$, $k_3 = k_{3\rightarrow}$, and so only one lower boundary rate value is needed to describe all four of the conformational opening and closing steps in the kinetic model. However, for the three other flavoproteins, their conformational $K_{\text{hq}}$ values are either greater or < 1, and so stipulate that $k_1 \neq k_{1\rightarrow}$ and $k_3 \neq k_{3\rightarrow}$. Thus, two distinct lower boundary values are derived in this case that describe the conformational opening and closing rates (indicated by the blue lines in the main panels and in the insets in Fig. 3).

To obtain the best-fit rate pair values for each flavoprotein, we incorporated as an additional fitting parameter the elapsed time that was needed by each enzyme to reduce the first equivalent of cytochrome $c$ [34,35] (obtained from the data in Fig. 2 as discussed above). These simulations provided a best-fit rate pair for each enzyme, whose values are marked by a blue or black box on each trace in Fig. 3, and they are listed in Table 3. The simulated kinetic traces of cytochrome $c$ reduction for each enzyme, using these best-fit rate values, are overlaid with the actual experimental traces in Fig. 4, and show that a reasonable fit was obtained in each case. Together, the analysis suggests that the rates of conformational switching differ by over two orders of magnitude among the four diflavin reductases, according to rank order: CPR > nNOSred > MSR > eNOSred, whereas their corresponding rates of interflavin ET range over one order of magnitude according to the same rank order.
The approach to and the final steady-state distributions of enzyme species differ among the flavoproteins

The simulations can also predict how the four enzyme conformational/redox species noted in the kinetic model (species a d in Fig. 1) populate as each fully-reduced flavoprotein (species d a) reacts with excess cytochrome c as depicted in the Fig. 2 kinetic traces. Figure 5 shows how the species distribution changes with time in all four enzymes. The kinetics of redistribution differ broadly among the flavoproteins as a direct consequence of their different rates of conformational transitions and interflavin ET. Also, only the eNOSred achieves a steady-state species distribution before the first equivalent of cytochrome c is reduced (time point indicated by a dotted vertical line in Fig. 5), whereas the three other flavoproteins only approach their steady-state distributions within the time needed to reduce the first equivalent. All four enzymes also have unique steady-state species distributions, as indicated by the pie graphs in Fig. 5, as well as in Fig. 6. None of the four flavoproteins build up as the conformationally-open FMN\textsubscript{sq} species (species a) during catalysis because this species reacts so quickly with the cytochrome c, although there are significant differences in the build up of species b, c, and d. For example, the fast conformational transitions in CPR cause its closed FMN\textsubscript{sq} species (species c) to build up because the subsequent interflavin ET step is relatively slow. In contrast, eNOSred predominantly builds up as its closed FMN\textsubscript{sq} species d because it opens relatively slowly to react with cytochrome c. The MSR and nNOSred both build up mainly as their open FMN\textsubscript{sq} species b, primarily because their conformational \( K_{sq} \) values provide a relatively poor driving force for the open form to transition to the closed form, as is required for the FMN\textsubscript{sq} to be reduced. These properties also determine what percentage of FMN\textsubscript{sq} versus FMN\textsubscript{bq} should be present in each flavoprotein during its steady-state reaction with cytochrome c (Fig. 7). The eNOSred is predicted
to contain 80% FMNhq as a result of its build up primarily as the closed reduced species d during catalysis. In marked contrast, the CPR, MSR and nNOSred are predicted to contain between 80 and 95% FMNsq during their steady-state reactions. The difference between eNOSred and MSR is particularly striking because they have nearly equivalent slow cytochrome c reductase activities (Table 1). Thus, their electron flux rates are similarly limited but for opposite reasons: eNOS has slow opening when it is in the reduced (FMNhq) closed form, whereas MSR has slow closing when it is in the oxidized (FMNsq) open form.

**Discussion**

Translating the cytochrome c reduction data to enzyme conformational parameters

It is important to note that our stopped-flow experiments measured the reactivity of the reduced FMN domains (FMNhq) toward cytochrome c, and did not directly measure specific changes in the protein conformation. Thus, linking our data to flavoprotein structural changes depends on two assumptions. First, that the FMNhq is unreactive toward cytochrome c when the enzyme is in a conformationally-closed form. A recent study showed that a covalently-cross-linked closed form of CPR (whose crystal structure was also reported) only supported 5% electron flux to cytochrome c relative to the unlinked CPR [26]. We have obtained the same result for a similarly cross-linked nNOSred (Y. Dai and D. J. Stuehr, unpublished observations). Thus, the assumption holds for CPR and nNOSred and we assume that it also holds for the other two flavoproteins, given their structural and mechanistic similarities. Secondly, we assume that the reduced FMN domain is very reactive toward cytochrome c once it becomes sufficiently unencumbered enough for its bound FMNhq to come within a distance that allows fast ET to...

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**Fig. 4.** Simulated reactions of fully reduced dual flavin enzymes with excess cytochrome c. The traces were obtained by simulating the kinetic model in Fig. 1, using different rates of conformational motion and interflavin electron transfer ($k_2$) to match experimental traces for each of the four dual flavins under study. Total protein concentration is 1.0 and the concentration of enzyme species $d + a$ was set equal to 1.0 at $t = 0$ in the simulations. For nNOSred, conformational motion rates were set so that $K_{hq} = K_{sq} = k_3$, $k_1 = k_3$ and $k_3 = 53$ and $k_2 = 22$. For eNOSred, conformational motion rates were set so that $K_{hq} = K_{sq} = k_1 = k_2 = k_3 = k_3 = 0.125 \times k_1$. The best fit of experimental traces was found when the kinetic settings (s$^{-1}$) were $k_1 = 5.0$, $k_1 = 0.3$, $k_3 = 230$, $k_2 = 52$. For MSR, conformational motion rates were set so that $K_{hq} = K_{sq} = k_1 = k_3 = 0.5$, $k_1 = k_3 = 2.57$, $k_1 = k_3 = 2.57 \times k_1$. The best fit was found when the kinetic settings (s$^{-1}$) were $k_1 = 8$, $k_1 = 20.5$, $k_2 = 2.8$. Simulated traces (black lines) are overlayed with experimental traces (red lines).
The cytochrome c reactivity of the reduced, isolated FMN domains of CPR and nNOS [37,42] are sufficiently reactive to support this assumption, and we assume that this is also the case for MSR and eNOS.

At equilibrium, the four reduced flavoproteins likely populate a myriad of closed and open forms that distribute across a conformational and energetic landscape, as described, for example, by Hay et al. [43]. Crystal structures of nNOSred and CPR illustrate their tightly closed forms [23,25]. More recent crystallographic, cryogenic, small-angle X-ray (SAXS) or neutron scattering (SANS) and solution NMR or EPR studies of CPR and its variants or of nNOS have identified conformationally-open forms and shown that multiple states are indeed populated at equilibrium [27,32,44 46]. The FMN domain in each of three open conformer crystal structures of CPR is sufficiently unencumbered to engage in fast ET with cytochrome c. However, it is unclear which of these open conformations may actually form during catalysis, or how well they each might cycle to participate in steady-state electron flux to cytochrome c. Obviously, minimal conformational changes that would still allow ET from FMN$^{\text{Hq}}$ to cytochrome c would be likely to enable the fast conformational cycling and fast electron flux that is seen in CPR. In contrast, the more extended open conformers may participate poorly, given that these conformers were observed in the structure of CPR variants that display very low steady-state cytochrome c reductase activity [27]. Populating extended conformations or undergoing conformational relaxations back to closed conformations have been suggested to be gradual processes [47,48] and possibly artifacts of dilution [30,49] or of the long equilibration times that enzyme molecules experience when being readied for laboratory studies. We do not know whether such relaxations redistributed the conformationally-open or closed forms in the fully-reduced flavoproteins prior to our stopped-flow mixing experiments. However, the traces in Fig. 2 may suggest that this did not happen to any significant extent. The cytochrome c reductase activities calculated from tangents drawn to the stopped-flow traces in Fig. 2 matched fairly well with the steady-state activities determined for MSR, CPR, eNOSred and nNOSred in standard activity assays (Table 3) that were run over several minutes and did not involve a pre-reduction of the

![Figure 5: Patterns of enzyme distribution versus reaction time in dual flavin enzymes. Rate pairs of conformational motion and interflavin electron transfer (obtained from the simulated best fit values) (Fig. 4) were used in each case to simulate and achieve experimentally obtained cytochrome c reduction rates. Lines indicate the relative concentrations of each enzyme species a-d (Fig. 1), with the total enzyme concentration being 1.0 and the concentration of enzyme species d set equal to 1.0 at t = 0 in the simulations. The blue dotted line marks the time required for the enzyme to reduce one equivalent of cytochrome c. Kinetic settings were: For nNOSred: $k_1 = k_3 = 53$, $k_2 = 22$; for eNOSred: $k_1 = k_3 = 5.0$, $k_1 = k_3 = 0.63$, $k_2 = 5$; for CPR: $k_1 = k_3 = 460$, $k_1 = k_3 = 230$, $k_2 = 52$; for MSR $k_1 = k_3 = 8$, $k_1 = k_3 = 20.5$, $k_2 = 2.8$. Pie graphs show different species distributed at steady state. Color scheme for different species: black, species a; red, species b; green, species c; blue, species d.](image)
enzymes before their activity was measured. Indeed, the kinetic traces in Fig. 2 imply that each fully-reduced flavoprotein quickly achieved the conformational and redox cycling that normally occurs during its steady-state reaction with cytochrome $c$. If instead, a significant relaxation to poorly-cycling open conformers or a relaxation to closed conformers had occurred during the time required to prepare the enzymes for the stopped-flow experiments, then we would expect a mismatch between the tangent-based activity derived from the traces in Fig. 2 and the steady state activity measured by the standard method.

**Possible relationships between the enzyme redox state and conformational behaviors**

Studies with CPR have suggested that the flavin reduction may impact the conformational setpoint by shifting CPR to favor more open forms [44,45,50]. NADPH binding site occupancy can also impact conformational behaviors of CPR and nNOS [13,15,30,35,41,50,51]. Moreover, the interflavin ET step that generates FMN$_{hq}$ ($k_2$ in Fig. 1) has been suggested to gate a subsequent conformational opening step that frees the reduced FMN$_{hq}$ domain in CPR ($k_3$ in Fig. 1) [41,43,52]. These concepts are fundamentally important but are not directly addressed by our results because we only investigated the conformational behaviors of the fully-reduced flavoproteins. However, some of our observations may shed some light. First, we found that none of the four flavoproteins existed in a completely open conformation when they were fully-reduced (i.e. when they contained two-electron reduced (hydroquinone) FAD, FMN$_{hq}$ and bound NADPH). Indeed, our evidence suggests that two of the four (eNOSred and CPR) exist predominantly in their closed conformations even when fully reduced. This implies that reduction to create FMN$_{hq}$ has at most a partial and variable capacity to trigger conformational opening among the four flavoproteins. This can be viewed within the context of recent SAXS and SANS studies on CPR, which reported that a two-electron reduction of the fully-oxidized enzyme caused some opening and stabilized approximately 50% of the enzyme in an open conformation [45]. Note that CPR in the two-electron reduced state would predominantly contain FAD semiquinone (FAD$_{sq}$) and FMN$_{sq}$, and so the conformational change that was reported in their study was predominantly associated with the transition of FMN$_{ox}$ to FMN$_{sq}$ in CPR. The study did not address what effect, if any, that further reduction of FMN$_{sq}$ to FMN$_{hq}$ might cause on the CPR conformation. Given that our study estimated that fully-reduced CPR is one-third open and two-thirds closed, our results imply there may be no further opening upon reduction of its FMN$_{sq}$ to FMN$_{hq}$. Second, a comparison of the best-fit rate estimate that we derived for the interflavin ET step ($k_2$) and the rate for the conformational opening step of each reduced flavoprotein ($k_3$) shows that no correlation exists between these two rates (Table 3). For example, the estimated rate of interflavin ET in e-NOSred is eight-fold faster than its estimated rate of
conformational opening. For MSR and CPR, the situation is reversed, and their estimated rates of conformational opening are seven and four times faster than their estimated rates of interflavin ET, respectively. In contrast, the estimated conformational closing rates of the FMN$_{sq}$-containing species ($k_1$ in Fig. 1), which is the step that precedes the interflavin ET are more similar to the estimated rates of interflavin ET in eNOSred and MSR (Table 3). Further investigation is clearly warranted but, in any case, the present study suggests that one need not invoke a conformational sensitivity toward the FMN$_{sq}$ and FMN$_{hq}$ redox states, or a gating of the FMN domain motion to the interflavin ET step, in order to model electron flux through these flavoproteins.

**Structural considerations and possible basis for different electron flux among the diflavin reductases**

Perhaps the most remarkable findings suggested by the present study is that the four diflavin reductases differ so widely in their conformational equilibrium setpoints and also exhibit a two-order of magnitude range in their rates of FMN domain conformational switching. We found that MSR and eNOSred have conformational equilibrium setpoints that lie on either side of the optimum setting ($K_{hq} = 1$ being approximately optimal in this model) [15,34] and their $K_{sq}$ and $K_{hq}$ setpoints therefore help to limit their electron flux as a result of an imbalance between their conformational closing and opening rates. However, on balance, it is their slow rates of conformational switching that present the biggest barrier to their electron flux. Consider that the $K_{hq}$ setpoint of CPR is also suboptimal but its much faster rates of conformational switching supersede this limitation. Interestingly, our results predict that slightly shifting the $K_{hq}$ of CPR toward its open reactive form would increase its activity above wild-type enzyme, and we suspect that this effect might explain a recently-characterized CPR point mutant with a higher than normal activity [45].

The best-fit rates we derived for CPR conformational switching (230 and 460 $s^{-1}$) are in the middle to high range of similar switching rates reported in other multidomain redox enzymes [28,41,51,53], whereas the rates we estimated for nNOSred, MSR and eNOSred are in the lower to extreme lower end of the range. This suggests that ‘suppressive’ features exist in these three flavoproteins that slow down their rates of conformational cycling relative to CPR or other multidomain redox enzymes and, for MSR and eNOSred, may also give them suboptimal conformational $K_{hq}$ settings, thus preventing them from achieving a faster electron flux as is seen for CPR. What might these features be? We know that calmodulin binding to nNOSred boosts its cytochrome $c$ reductase activity to the level of CPR [15,36,40,54,58] and thus calmodulin likely relieves the suppressive features that are present in the nNOSred [15,40,56,59].

Regarding the protein structural features held in common among diflavin reductases, we may consider the FNR FMN domain linker that is directly involved in conformational switching and differs considerably with regard to its length and amino acid sequence [18,19,25,27,60] and also consider the domain domain interface. We have examined the available structural information on dual-flavin reductases. The structure of the diflavin module has been elucidated for nNOS and CPR. We used the Protein Data Bank (PDB) files 1TLL [25] for nNOS and 1AMO [23] for CPR. Because the structures of the complete diflavin module are not available for eNOS and MSR, we have built structural models using available modeling servers (See Experimental procedures).

The analysis of the FNR/FMN domain interfaces in the structures and models is shown in Table 4. In summary, it appears that the interface area between FNR and FMN domains is very similar for nNOS and CPR. We used the Protein Data Bank (PDB) files 1TLL [25] for nNOS and 1AMO [23] for CPR. Because the structures of the complete diflavin module are not available for eNOS and MSR, we have built structural models using available modeling servers (See Experimental procedures).

The analysis of the FNR/FMN domain interfaces in the structures and models is shown in Table 4. In summary, it appears that the interface area between FNR and FMN domains is very similar for nNOS, eNOS and CPR (approximately 2500 Å$^2$), whereas the interface is smaller in the case of MSR (approximately 2000 Å$^2$). The arrangement of the FNR and FMN domains appears to be very similar in all cases (Fig. 8) and the changes in surface area can be ascribed to the

<table>
<thead>
<tr>
<th>Protein</th>
<th>nNOS</th>
<th>eNOS</th>
<th>CPR</th>
<th>MSR</th>
<th>MSR</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB file</td>
<td>1TLL</td>
<td>Model (template: 1TLL)</td>
<td>1AMO</td>
<td>Model (template: 1AMO)</td>
<td>Model (template: 1TLL)</td>
</tr>
<tr>
<td>Total interface area (Å$^2$)</td>
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<td>2502.2</td>
<td>2501.5</td>
<td>1970.4</td>
<td>1980.8</td>
</tr>
<tr>
<td>% Charged residues</td>
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<td>47.4</td>
<td>57.1</td>
<td>52.9</td>
<td>31.2</td>
</tr>
<tr>
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<tr>
<td>Salt bridges</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
presence of 'extra' elements in NOS and CPR compared to MSR. NOS proteins include an additional autoinhibitory helix element in their FMN domain that is known to modulate the interaction with CaM (amino acids 836-849 in nNOS and 609-621 in eNOS) [25,61] and also contain a C-terminal extension in their FNR domain that modulates the FMN FNR domain interaction and CaM response [39]. Both elements contribute to their FMN FNR interface. In the case of CPR, the FMN domain possesses an extra helical segment (amino acids 67-78) that interacts with the FNR domain, including the formation of a salt bridge between amino acids Arg78 of the FMN domain and Asp352 of the FNR domain. The MSR sequence does not incorporate any of these elements, and thus its resulting interaction area is approximately 500 Å² smaller than for NOS or CPR (Table 4).

MSR has a long hinge region (approximately 80 amino acids) between the FMN and FNR domains, different from the shorter hinges (14-25 amino acids) in NOS or CPR. Due to its location in the primary structure, it is reasonable to think that this hinge region could mediate additional FMN/FNR domain interactions in MSR. However, some secondary evidence suggests that this may not be the case. First, crystallization of the hinge and FNR portion of MSR (residues 166-698) was only able to determine the structure of the portion of the hinge between amino acids 217 and 247 [62]. This section stretches away from the putative location of the FMN module and is unlikely to contribute to FNR-FMN binding; the region between residues 166 and 217 was too flexible for the structure to be determined. Secondly, difficulty in obtaining a crystal structure of the whole MSR protein also points towards a transient, unstable FNR FMN complex in MSR. Thirdly, our experimental data indicate that an open conformation is prevalent in MSR (Table 2). It is possible that the presence of the FMN module helps to arrange the hinge region in a more stable structure that provides additional stability to the FMN FNR module, although the evidence discussed above appears to argue against this hypothesis. In the absence of more structural data, the contribution of this sequence to interdomain binding remains an open question.

As noted previously, NOS and CPR include a shorter (approximately 14 amino acids in CPR and 25 amino acids in NOS), flexible hinge region between the FMN and FNR domains. The composition of the hinge modulates the equilibrium between open and closed conformations, as shown by studies on CPR [27,63] and NOS [60,64]. Due to the flexibility of these regions, they do not appear in the crystal structures available, and we excluded the sequences from our analysis in the modeled structures. As discussed in the case of MSR, the hinge can contribute to the energy of the closed conformation. However, we expect that contribution to be minor in terms of complex stabilization because these fragments do not form a fixed, 'clamp' structure between the FNR and FMN domains, as confirmed by the crystallography experiments. In turn, they appear to operate on the conformational equilibrium by limiting the degrees of freedom of the open conformations [15,27,60,63,64]. In this way, the effect of the hinges will be more related to the kinetics of the conformational changes between...
open and closed conformations, whereas the equilib-
rium setpoint between both conformations will be
more related to the strength of the interaction between
the FNR and FMN domains in the closed conforma-
tion. Thus, we consider that the omission of the hinge
fragments from the interface analysis will not modify
the conclusions of the present study.

The comparison of the binding interactions for the
structures and models indicates that the number of
hydrogen bonds plus salt bridges is higher for eNOS
(n = 4) and CPR (n = 9) than for nNOS (n = 3)
(Table 4). Qualitatively, and considering that the sta-
bility of the complex will be mainly determined by the
interface area, the stability of the ‘closed’ confor-
mation will be mainly determined by the
(ET, conformational
these and other structural features influence interflavin
ET, conformational
remains a topic of ongoing study.

In sum, the present study provides the first compar-
estimates of the conformational equilibria set-
points, interflavin ET rates and conformational
switching rates in four mammalian dual-flavin reducta-
ases, and shows how differences in these parameters can
explain their widely different capacities to support
electron flux from NADPH to cytochrome c.

Experimental procedures

Steady-state cytochrome c reduction assays

Cytochrome c reductase activity was determined at 25 and
10 °C by monitoring A550 and using an extinction coeffi-
cient of ε550 = 21 mm−1cm−1 as described previously
[17,27,35,36].

Reaction of fully reduced proteins with excess
cytochrome c

The rate of reduction of excess cytochrome c by fully
reduced proteins was measured in the stepped flow instru-
ment under anaerobic conditions at 10 °C as described pre-
viously [35,36]. The nNOSred or eNOSred (10 12 μm)
proteins in 40 m m 4 (2 hydroxyethyl) 1 piperazinepropane
sulfonic acid buffer (pH 7.6) with 10% glycerol and
150 m m NaCl containing EDTA (2 m m) were fully reduced
by titrating them with anaerobic sodium dithionite solu-
tion. We used 0.1 m potassium phosphate buffer (pH 7.4)
with 10% glycerol for CPR and MSR proteins, otherwise
keeping the rest of the procedure the same as that used for
the two NOSred proteins. An anaerobic solution of each
fully reduced protein containing NADPH (200 μm) was
mixed with an anaerobic solution of cytochrome c (100 μm)
while monitoring the changes in A550. Initially, the solution
of cytochrome c was mixed with anaerobic buffer alone to
obtain the initial A550 reading at t = 0. All mixing reactions
were repeated consecutively six to eight times, and then the
individual kinetic traces were averaged. The entire analysis
was then repeated using a separately purified batch of each
enzyme. In the reactions of reduced enzyme with cyto-
chrome c, a linear regression analysis of late points in the
traces and/or numeric derivatives of the traces were run to
determine the deflection points separating the early burst of
fast reacting and late slow reacting phases (see Results).

Simulation of the kinetic traces of fully reduced
flavoproteins with excess cytochrome c

We used the computer software GEPASI, version 3.30 [65] to
simulate the experimental electron flux to cytochrome c
using the kinetic model outlined in Fig. 1. Details of this
type of simulations have been reported previously [34,35].
Here, we set the reaction rate with cytochrome c (k4 in the
kinetic model) at 225 s−1 in all cases, based on a reported
second order rate constant (4.5 × 106 m−1s−1) for the reac-
tion of cytochrome c with the reduced conformationally
open nNOSred, or with its reduced isolated FMN domain,
at 10 °C [36,37,40]. Values for each of the four conforma-
tional rates (k1, k 1, k3 and k 3) and the interflavin electron
transfer rate (k2) were input into the software. Each simu-
lated reaction began with 100% of the enzyme in the fully
reduced state (represented by species d and a in Fig. 1). The
initial concentrations of species d and a were deter-
mined based on the experimentally obtained Keq values.

Structure-based analysis of the domain interfaces

Existing PDB structures were used when available. We used
1TLL [25] for rat nNOS and 1AMO [23] for rat CPR. In
the case of bovine eNOS and human MSR, structures were
modeled using the Swiss Model Server [66]. The available
structure of rat nNOS (1TLL) was used as template for the
bovine eNOS model, being by far the template with the
highest sequence identity. In the case of human MSR, a
structure for the FNR domain is available [62], although
the structure of the FMN domain has not been determined.
Because both nNOS and CPR have significant sequence
identity (approximately 33%) to MSR, we built two differ-
ent models for MSR using either nNOS or CPR structures
as templates.
The interface area and the atom interactions for each structure were calculated using the 2P2I server [67]. The output of the 2P2I server was also used to compile the tables of atom and residue contacts (Supplemental Table S1).

The definition of the FNR like and FMN domains for each protein was: rat nNOS, FMN domain amino acids 750 942, FNR domain amino acids 968 1413; CPR, FMN domain amino acids 64 235, FNR domain amino acids 243 678; eNOS, FMN domain amino acids 519 721, FNR domain amino acids 728 1180 (segment 722 727 removed for calculations); MSR/1TLL model, FMN domain amino acids 6 162, FNR domain amino acids 210 697 (segment 163 209 removed for calculations); MSR/1AMO model, FMN domain amino acids 2 162, FNR domain amino acids 210 698 (segment 163 209 removed for calculations). In the available structures of CPR and nNOS, the hinge fragments between the FNR and FMN domains are too flexible and not visible in the crystal structure (236 242 in CPR; 952 958 in nNOS). We considered that the structure of the homologous fragment in the eNOS model (722 727) was not reliable and was removed before analysis. In the case of MSR, the polypeptide including the FNR domain and the hinge domain (amino acids 166 698) has been analyzed but only the fragment between amino acids 217 and 698 yield a crystal structure [62]. We therefore excluded the fragment from our study. Comparison of the MSR models with the available MSR structure [62] indicates that the estimated location of the segments between amino acids 210 and 270 is not consistent with the reported structure. Nevertheless, we did not remove these fragments because they are far from the FNR/FMN domain interface and do not modify our calculations. The PDB files used for the analysis are available in the supplementary materials.

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Author contribution

MMH and MB designed and performed the experiments and analyzed the data. JT did the structural analyses of proteins. CTK performed the steady state assays and helped in preparing first draft of the manuscript. NMP and SCI provided the purified CPR proteins. MMH, MB and JT drafted the paper. LW analyzed the data. DJS designed the research, analyzed the data and wrote the final paper.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web site:
Table S1. Residue contacts in different dual-flavin enzymes. Models and PDB files used for the structural analyses.