Conformation Dependent Hydride Transfer in Neuronal 
NO Synthase Reductase Domain**

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Abbreviations:
nNOSrd, recombinant neuronal nitric oxide synthase reductase domain; nNOS, rat neuronal nitric oxide synthase; CPR, mammalian cytochrome P450 reductase; CaM, calmodulin.

Supporting information:
The following supplementary material is available:

**Fig. S1.** Steady-state ferricyanide reduction.

**Fig. S2.** Reaction of nNOS FAD domain with NADPH.

**Fig. S3.** Reduction of nNOSrd (one-electron reduced) by excess NADPH at different ionic strengths. This supplementary material can be found in the online version of this article.

Keywords:
Nitric Oxide; Electron Transfer; Kinetics; Isotope Effect; NOS
Abstract

Calmodulin (CaM) activates the constitutive isoforms of mammalian NO synthase by triggering electron transfer from the reductase domain FMN to the heme. This enables the enzymes to be regulated by Ca\(^{2+}\) concentration. CaM exerts most of its effects on the reductase domain, which include activation of electron transfer to electron acceptors and an increase in the apparent rate of flavin reduction by substrate NADPH. While it has been shown that the former is caused by a transition from a conformationally locked form of the enzyme to an open form as a result of CaM binding, improving FMN accessibility, the latter effect has not been explained satisfactorily. Here we report the effect of ionic strength and isotopic substitution on flavin reduction. We find a remarkable correlation between the rate of steady-state turnover of the reductase domain and the rate of flavin reduction over a range of different ionic strengths. The reduction of the enzyme by NADPH is biphasic, and the amplitudes of the phases determined through global analysis of stopped-flow data correlate with the proportion of enzyme known to exist in open and closed conformations. The different conformations of the enzyme molecule appear to have different rates of reaction with NADPH. Thus, proximity of the FMN inhibits hydride transfer to the FAD. In the CaM-free enzyme, slow conformational motion (opening and closing) limits turnover. It is now clear that this motion also controls hydride transfer during steady-state turnover, by limiting the rate at which NADPH can access the FAD.

Introduction

In mammals, the production of nitric oxide has been linked to a range of cell signaling events and physiological processes \[1, 2\]. The nitric oxide synthases (NOS) \[3, 4, 5\] responsible have evolved a series of post-translational regulation mechanisms based on the control of the enzymes’ catalytic activity. A molecular-level understanding of these is now developing.

NOS is a dimeric enzyme composed of an oxygenase domain, which is the site for NO synthesis, and a reductase domain, which is related by sequence and structure to cytochrome P450 reductase (CPR). X-ray crystal structures of both the isolated domains have been determined along with CaM-bound FMN domain \[6-9\], but as yet it is unclear how the holoenzyme is assembled. NO synthesis from L-arginine follows a unique mechanism involving consecutive mono-oxygenation reactions, leading to the formation of citrulline via the intermediate N-hydroxy-arginine \[3, 10\].

The oxygenase and reductase domains are connected by a 20 amino-acid linker containing a calmodulin (CaM) binding motif. In the constitutive NOS isoforms (neuronal and endothelial), elevated levels of free Ca\(^{2+}\) cause CaM to bind and activate electron transfer from the reductase domain to the heme of the oxygenase domain \[11\]. This is a primary mechanism of enzyme regulation, enabling NO synthesis activity to be increased.
from a negligible level. However, activity can also be modulated up or down by kinase-dependent phosphorylation of the reductase domain and association with inhibitor proteins [12].

The reductase domain of NOS is itself composed of two domains, one of which binds NADPH and FAD, and the other FMN [13]. The FAD domain is related to the ferredoxin reductase family of enzymes and catalyses NADPH dehydrogenation [14]. It passes the two electron equivalents one at a time to the FMN domain, which is related to the flavodoxin family of electron carriers [15]. Typical of these, the FMN oscillates between a stable one-electron reduced neutral semiquinone form and a two-electron reduced hydroquinone form, shuttling single electrons between the FAD and the heme [16, 20]. For the constitutive enzyme forms, at low Ca\(^{2+}\) concentrations or in the absence of CaM, the enzymes enter a catalytically repressed state. This is characterized by the rate of NO synthesis falling to zero, the rate of steady-state cytochrome c reduction decreasing by 10-fold, the sensitivity of the enzyme to oxygen decreasing and the rate of reduction by NADPH decreasing [17]. These effects are also manifested in the isolated reductase domain (which retains the CaM binding site at the N-terminus) and are similarly relieved by CaM binding [18]. A series of unusual protein inserts mediate the effects of CaM binding and appear to stabilize the repressed form of the reductase domain. These include an approximately 40 amino-acid insert in the FMN domain (the autoinhibitory loop) [19, 20], a 30 amino-acid extension to the C-terminus [21, 22], an insert in the hinge between the FMN and FAD domains [23], a protruding section of the FAD domain [24] and the CaM-binding site itself. Repression of cytochrome c reduction is also induced by NADPH binding [25]. Many of these elements form an extended contact area between the FAD and FMN domains, as compared to CPR [26], and may serve to stabilize the conformation of the protein reported in the respective X-ray crystal structures [27]. Both have the cofactors in close proximity, apparently in an ideal position for FAD to FMN electron transfer. In both structures NADP\(^+\) is bound in a passive conformation and substantial rearrangement is anticipated before either hydride transfer from NADPH to FAD or electron transfer from FMN to acceptor could take place. A recent structure of a mutant CPR shows the enzyme in an open conformation indicating the degree of motion possible [28]. It is likely that the conformational changes required during catalytic turnover of both enzymes have been restricted in NOS causing repression in the absence of CaM. Beyond this, our understanding of the CaM-dependent activation mechanism is limited by the absence of an available structure of the CaM-bound enzyme, although a structure of the CaM-bound FMN domain has recently been reported [8], and by the lack of a coherent model to explain the many reported effects of CaM binding on the enzyme.

One of the least well understood effects of CaM on nNOS reductase domain (nNOSrd) is the increase in the rate of flavin reduction observed on direct reaction of the enzyme with NADPH. This should be a simple process largely involving hydride transfer from NADPH to FAD. However, previous studies show multiple reduction phases with intermediate states that are difficult to assign to distinct species. Thus, it is unclear whether CaM activates the hydride transfer event itself, formation of a precursor complex, product dissociation, electron transfer or a rebalances series of equilibria. Here we investigate the influence of ionic
strength and isotopic substitution on this reaction in order to elucidate which steps are activated by CaM and whether reduction of the FAD is rate-determining in the catalytic action of the reductase domain.

Results and discussion

Steady state turnover: The surrogate electron acceptors, cytochrome c and ferricyanide, are known to short-circuit the turnover of nNOS by accepting electrons from the reductase domain of the enzyme before they can be transferred to the oxygenase domain \[^{[17]}\]. The isolated reductase domain (nNOSrd) has similar rates of turnover to the full-length enzyme and is activated by CaM binding to the same extent \[^{[18]}\]. Cytochrome c accepts electrons exclusively from the FMN of nNOS, whereas ferricyanide can access the FAD also, although not as readily \[^{[16]}\]. The cytochrome c dependence of nNOSrd therefore follows Michaelis Menten kinetics, with the \(k_{\text{cat}}\) increased by 10-fold on CaM binding (Table 1). Data for the ferricyanide dependence of nNOSrd are shown in Supplementary Fig S1. These show that the rate of turnover increases linearly with the concentration of ferricyanide over the range shown, intercepting the y-axis. It is logical to assume that the intercept corresponds to the rate of electron transfer from the FMN, whereas the electron transfer from FAD to ferricyanide causes the linear increase \[^{[20]}\]. This has been confirmed by studies on the isolated domains and FMN-deficient nNOS \[^{[20, 34]}\]. Furthermore, the intercept values, with and without bound CaM, correspond approximately to the rates of steady-state cytochrome c reduction. From the ferricyanide data, it appears that the rate of hydride transfer from NADPH to FAD is fast enough to support ferricyanide reduction at rates in excess of 100 s\(^{-1}\) regardless of whether or not CaM is bound. This suggests that electron transfer from FAD to FMN is slow and limits the turnover of nNOS with cytochrome c. It is logical to suppose that this step is the event activated by CaM \[^{[35, 42]}\]. However, this is not the whole story, in the presence of a large excess of ferricyanide it is likely that the stable FMN semiquinone is oxidized. This seemingly innocuous change in redox state has a profound effect on the behaviour of the enzyme, as demonstrated by the pre-steady-state data (below).

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Ca(^{2+})/CaM</th>
<th>(k_{\text{cat}})</th>
<th>(K_m) (cytc)</th>
<th>(D_V)</th>
<th>(D_{V/K})</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mM NaCl</td>
<td>-</td>
<td>10.4 ± 0.4</td>
<td>3 ± 1</td>
<td>1.9</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>104 ± 6</td>
<td>8 ± 2</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>150 mM NaCl</td>
<td>-</td>
<td>15 ± 1</td>
<td>8 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>105 ± 3</td>
<td>10 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250 mM NaCl</td>
<td>-</td>
<td>23 ± 1</td>
<td>12 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>95 ± 6</td>
<td>12 ± 3</td>
<td></td>
<td></td>
</tr>
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</table>

Parameters were derived by fitting initial rate data to the Michaelis Menten Equation by non-linear regression analysis. Kinetic isotope effects \(D_V\) and \(D_{V/K}\) were determined as described in the text.

**Table 1.** Kinetic parameters for the steady-state reduction of cytochrome c by nNOSrd in the presence or absence of bound CaM, with different salt concentrations.
Pre-steady-state flavin reduction: Reduction of nNOSrd by NADPH has been the subject of several previous studies, many of which reach different conclusions [25, 31, 35, 36]. It has proved difficult to reconcile the rate-constants and amplitudes observed with steady-state observations, to such an extent that it is unclear which steps are rate-determining in the steady-state reactions and which are activated by CaM. Fig. 1 shows the reduction of nNOSrd by an excess of NADPH on rapid mixing in the absence and presence of bound CaM. Two enzyme forms are compared here, fully oxidized (by addition and subsequent removal of ferricyanide) and one-electron reduced (titrated with dithionite until the FMN semiquinone is fully formed) both prepared anaerobically. Both have been studied previously in isolation, but have not been previously been compared. The oxidized form is not a natural component of the catalytic cycle of NOS, which utilizes only the FMN semiquinone/hydroquinone redox couple to pass electrons to the heme, but may be formed in the steady-state reaction with ferricyanide (above). The one-electron reduced form is a natural component of both the NO synthesis and cytochrome c reduction catalytic cycles; the FMN semiquinone cannot be oxidized by either of the ferric heme species involved in these reactions. The most obvious thing to note from Fig. 1 is that CaM appears to have a much greater effect on the one-electron reduced form of the enzyme. Essentially, the CaM-free one-electron reduced form is reduced more slowly by NADPH than the fully oxidized version. Unfortunately the reduction kinetics of the fully oxidized version are highly complicated and difficult to interpret. This form of the enzyme reacts sequentially with 2 equivalents of NADPH and has many possible intermediates (see Scheme 1 and [31]). However, it is clear that the CaM-free oxidized form reacts more quickly with NADPH, and this is likely to influence the behaviour of the enzyme during steady-state turnover with ferricyanide (see above). The one-electron reduced form is the catalytically relevant form and its kinetics should be easier to interpret, this will therefore be our focus.

Figure 1. Stopped-flow reduction of nNOSrd by excess NADPH. A, diode array spectra showing reduction of the 1-electron reduced enzyme in the absence of CaM and, inset in the presence of bound CaM; C, the
corresponding normalized reaction timecourses measured at 456 nm. B, diode array spectra showing reduction of the fully oxidized enzyme in the absence of CaM and, inset in the presence of bound CaM; D, the corresponding normalized reaction timecourses measured at 456 nm.

![Scheme 1](image)

**Scheme 1.** Sequential reduction of the one-electron reduced form of nNOSrd by excess NADPH.

According to Scheme 1, the one-electron reduced enzyme should primarily react with a single equivalent of NADPH before undergoing slower inter-molecular electron transfer reactions. Thus, the absorbance change at 456 nm should involve a single hydride-transfer phase constituting the majority of the amplitude, during which the FAD is reduced to the hydroquinone form. Transfer of an electron to the FMN should have minimal effect on the absorbance. Fig. 1A and C show the nNOSrd spectra and timecourses respectively during reduction. However, as reported previously, the timecourses in the presence and absence of CaM are not monophasic single step reactions and were both fitted to double exponential functions (Table 2). For the CaM-bound enzyme, as expected, reduction occurs with a large fast phase constituting 80% of the absorbance change, whereas the CaM-free enzyme is reduced with a small fast phase constituting 20% of the absorbance change and a large slow phase with rate constant $6.5 \text{ s}^{-1}$. This has proved difficult to interpret. The various possibilities for the biphasic nature of this reaction include the presence of a metastable intermediate state, such as a charge-transfer complex, slow product dissociation, heterogeneity within the enzyme sample or conformational heterogeneity with slow interconversion between states. Global analysis of the reaction (Fig 2 and Fig 3C) enables the spectrum of the intermediate state to be calculated. It is essentially identical to the starting spectrum, but with a lower band at 457 nm. The transition from here to the final species occurs via an identical spectral change. Formation of charge-transfer complexes between NADPH and FAD were not observed to any great extent indicating that stacking between NADPH and FAD is transient under these conditions.
conditions. For comparison, figures showing the reduction of the isolated FAD domain by NADPH are in the supplementary material (Supplementary Fig S2). These show clear formation of a [FADH+/NADP+] charge transfer complex after a very rapid reaction. An equivalent long-wavelength band is not evident in Figs 1 or 3. Experiments performed in the presence of NADP+ do not show strong inhibition, indicating that product release is not slow. That leaves some form of heterogeneity within the sample. Apparently, 20% of the enzyme molecules react with NADPH at a faster rate than the remainder. The slow phase is close to the steady-state catalytic rate constant for cytochrome c reduction per electron transferred and is a candidate for limiting the overall rate. However, the reason for the apparent heterogeneity in the reaction of NADPH with CaM-free nNOSrd must be addressed, before this can be assigned. No evidence of heterogeneity could be found in the samples of nNOSrd purified, but it is possible for proteolytic truncation of a short section of the C-terminus to cause an increase in the activity of the CaM-free enzyme and be difficult to detect [21]. This question can be resolved by looking at the effect of ionic strength on the reaction.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>substrate</th>
<th>Ca2+/CaM</th>
<th>$k_1 (s^{-1})$ (Amplitude)</th>
<th>$k_2 (s^{-1})$ (Amplitude)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-electron reduced nNOSrd</td>
<td>NADPH</td>
<td>-</td>
<td>$35 \pm 1$ (20%)</td>
<td>$6.5 \pm 0.1$ (80%)</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>+</td>
<td>$93 \pm 2$ (80%)</td>
<td>$5.1 \pm 0.1$ (20%)</td>
</tr>
<tr>
<td></td>
<td>NADPD</td>
<td>-</td>
<td>$24 \pm 1$ (30%)</td>
<td>$4.3 \pm 0.1$ (70%)</td>
</tr>
<tr>
<td></td>
<td>NADPD</td>
<td>+</td>
<td>$60 \pm 1$ (80%)</td>
<td>$4.0 \pm 0.1$ (20%)</td>
</tr>
</tbody>
</table>

*Rate constants $k_1$ and $k_2 (s^{-1})$ were derived in each case by fitting to a double exponential function with the percentage amplitude for each phase given. *For the ionic strength data.

Table 2. Kinetic parameters for the stopped-flow reduction of nNOSrd by excess NADPH or NADPD in the presence or absence of bound CaM.

← Figure 2. Stopped-flow reduction of nNOSrd by excess NADPH, dependence on ionic strength. Normalised reaction timecourses measured at 455 nm in the absence of CaM, in the presence of increasing concentrations of NaCl. Fit lines determined by global analysis of data using a consecutive 2-step kinetic model, fit parameters are presented in Table 2, simulated spectra are presented in Fig. 4.
**Figure 3.** Calculated spectra resulting from global analysis of the pre-steady-state reduction of wt nNOSrd (1-electron reduced) by excess NADPH under different ionic strength conditions. Conditions used were as in Fig. 3 with 100 mM KCl, 150 mM KCl and 250 mM KCl for Panels A, B and C. In each case the three spectra represent the three species observed in a consecutive 2-step reaction (i.e. A to B to C) Kinetic fit parameters are listed in Table 2.

**Ionic Strength Effects:** Modulating the ionic strength of the buffer has previously been shown to affect the steady-state rate of cytochrome c reduction by nNOS \[^{37}\], with higher ionic strength correlating with a decrease in affinity for the electron acceptor. Under our conditions (Table 1), the results show a significant effect on the CaM-free enzyme, with 2-fold faster turnover in the presence of 250 mM NaCl and a lesser effect on the CaM-bound enzyme. Thus, there is a decrease in the CaM-dependence of the enzyme at higher ionic strength. This result suggests that the rate-determining step for steady-state cytochrome c reduction in the absence of CaM is ionic-strength dependent. More comprehensive earlier studies of the effect of different salts and protein denaturing agents on the steady-state activity of NOS by Nishimura and Narayanasami *et al.*, \[^{38, 39}\] also show this effect. In fact it was shown that the effect of CaM on cytochrome c reduction can be completely eliminated in the presence of guanidinium thiocyanate, with the CaM-free activity being elevated to that of CaM bound. Although at the extreme, catalytic turnover was not sustained for long. These studies are consistent with the idea that interactions between the FAD and FMN domain inhibit catalytic cytochrome c reduction, and that its disruption through addition of denaturing agents produces a similar effect to CaM-binding.

The pre-steady-state reduction of CaM-free nNOSrd by excess NADPH was also found to be ionic-strength dependent (Fig. 2), with faster reduction occurring at higher ionic strength. Again, the traces collected for the one-electron reduced enzyme were found to be biphasic, with faster slow phases with greater ionic strength (Table 3), and with different amplitudes for the fast and slow phases. The proportion of fast phase increases from 36 % to 53 % on inclusion of 0.25M NaCl in the buffer. The global analysis data indicate that similar
spectral changes occur at each different ionic strength. This indicates that the heterogeneity observed during reduction of CaM-free nNOSrd by NADPH is ionic strength dependent. It cannot therefore be caused by partial degradation or re-oxidation of the sample. The most likely cause is conformational heterogeneity; different conformations of the same protein co-existing in solution, with a slow inter-conversion rate. One form of the protein is highly susceptible to reduction by NADPH, the other either reacts slowly or is unreactive and must undergo a slow conformational rearrangement to convert to the active form. The equilibrium balance between the two forms is dependent on the ionic strength of the buffer. The slow reduction phase is also faster at higher ionic strength, indicating that the conversion rate is ionic strength dependent as well as the equilibrium position. The accessibility of the FMN to cytochrome c in reduced nNOSrd, as estimated from pre-steady-state experiments is around 40% in the absence of CaM and NADPH compared to that in the presence of both [25]. A similar distribution of open/closed conformers in the one-electron reduced enzyme is likely to lead to the 36%:64% distribution of amplitudes observed in the pre-steady-state reduction by NADPH at low ionic strength. A further important observation is that the rate constants for the slow phases are close to the rates of steady-state turnover at the corresponding ionic strength. Thus the rate of reduction of FAD by NADPH appears to limit the rate of cytochrome c reduction, but is itself limited by the rate of conformational motion.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[NaCl]</th>
<th>Ca\textsuperscript{2+}/CaM</th>
<th>(k_1) (s\textsuperscript{-1}) (Amplitude)</th>
<th>(k_2) (s\textsuperscript{-1}) (Amplitude)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-electron reduced nNOSrd</td>
<td>100 mM</td>
<td>-</td>
<td>48 ± 1 (36%)</td>
<td>5.6 ± 0.1 (64%)</td>
</tr>
<tr>
<td></td>
<td>150 mM</td>
<td>-</td>
<td>41 ± 1 (41%)</td>
<td>6.5 ± 0.1 (59%)</td>
</tr>
<tr>
<td></td>
<td>250 mM</td>
<td>-</td>
<td>44 ± 1 (53%)</td>
<td>9.1 ± 0.1 (47%)</td>
</tr>
</tbody>
</table>

**Table 3.** Kinetic parameters for the stopped-flow reduction of nNOSrd by excess NADPH in the presence of different concentrations of NaCl. Rate constants were derived using global analysis to fit to a biphasic kinetic model. In this case amplitudes refer to absorbance changes at 455 nm.

The effect of increasing the salt concentration on the pre-steady state reduction kinetics of CaM-free nNOSrd is similar to the effect of binding CaM. It is possible, therefore, that the active conformational form of CaM-free nNOSrd is related to the CaM-bound form and that CaM-binding acts on a similar conformational equilibrium. This model is shown in Fig. 4, with the conformational change being represented by opening and closing of the hinge connecting the FAD and FMN domains of nNOSrd, to form hinged “open” and “closed” conformations. If the hinged open form only is able to undergo rapid NADPH to FAD hydride transfer, then the pre-steady-state reduction kinetics would be biphasic, with the amplitudes of the two phases corresponding to the position of the conformational equilibrium, provided that the hinge-opening process A is also slow. It
has also been established through mutation studies that the FAD:FMN domain interface is a strong determinant of the activity of the enzyme and particularly its CaM-dependence. Mutations to the interface usually affect steady-state turnover, FMN accessibility to cytochrome c and flavin reduction kinetics similarly and simultaneously. However, we do not yet have data on a fully locked (hinged closed) form of the enzyme to determine whether or not hydride transfer occurs in this conformation. Thus information relating to step C in the model is currently lacking.

Figure 4. Proposed model for the action of nNOSrd. Calmodulin alters the position of equilibrium between the open and closed conformations of nNOSrd (A). NADPH rapidly forms a charge transfer (CT) complex with the FAD in the open form (B), but not in the closed form (C).

Fluorescence Spectroscopy: Fluorescence emission spectra of 2 µM nNOSrd collected in 50 mM Tris/HCl buffer pH7.5 with variable amounts of KCl are shown in Fig. 5. Addition of salt to the enzyme sample caused the fluorescence intensity to increase by 50%. A larger increase is observed on addition of Ca and CaM, but the effect is similar. These results are consistent with the theory that higher ionic strength induces a more open enzyme conformation in which the flavin is more fluorescent, in a similar manner to the structural change induced by CaM binding. It should be noted that the enzyme used in this study had been oxidized by
addition of ferricyanide prior to the experiment and is therefore different to that used in the other studies, in which the stable FMN semiquinone is retained.

**Figure 5.** Fluorescence emission spectra of nNOSrd with excitation at 450 nm, recorded in 50 mM Tris HCl pH 7.5 and with addition of 0.1 M, 0.22 M and 0.5 M KCl.

**Kinetic Isotope Effects:** It has been shown that the A-side hydrogen atom of NADPH is exclusively transferred as a hydride to the FAD of the family of enzymes to which NOS belongs \(^\text{[41]}\). Substitution of this hydrogen by deuterium to form NADP\(^2\)H (NADPD) enables kinetic isotope effects to be determined for reactions involving the transfer of this hydride. Table 1 shows the steady-state kinetic isotope effects for nNOSrd. The isotope effects determined for V/K (i.e. the second-order rate constant for reaction of NADPH with enzyme under steady-state conditions) were low, at 1.1 and 1.4 in the presence and absence of CaM respectively. This indicates that the rate of hydride transfer from NADPH to FAD is slowed slightly when the hydride is replaced by deuteride, due to the larger mass of deuterium. Kinetic isotope effects on hydride transfer can be as high as 8, or beyond if quantum-mechanical tunneling effects are important. Therefore, it is unlikely in this case that the hydride transfer step itself is entirely rate-determining. The results are consistent with those of Wolthers and Schimerlik \(^\text{[41]}\) who draw similar conclusions.

The pre-steady-state flavin reduction experiments enable the hydride transfer step to be observed more directly, by mixing NADPD with oxidized or one-electron reduced enzyme. Traces collected in the presence and absence of CaM are shown in Fig. 6, in comparison to equivalent NADPH reduction traces. It is apparent that isotopic substitution affects the rate of FAD reduction through slowing the rate of hydride transfer. The
traces were analyzed by fitting to double exponential functions as described above, and parameters listed in Table 2. Reduction of nNOSrd by NADPD proceeded in a similar way to the reaction with NADPH, resulting in similar spectral changes and similar amplitudes for fast and slow phases. However, the rate-constants for the fast and slow phases were lower in each case. Comparison of individual values for NADPD versus NADPH under similar conditions provides a kinetic isotope effect for that particular reaction. For the one-electron reduced enzyme, isotope effects on the fast and slow phases \( (k_1 \text{ and } k_2) \) were approximately 1.5 for both, in the presence and absence of CaM. Those for the fully oxidized enzyme were slightly larger, and consistent with those of Knight and Scrutton \([31]\), who reached similar conclusions. The lack of a CaM effect on the kinetic isotope effect indicates that the transition state for hydride transfer is similar in both cases. In other words, CaM does not increase the rate of FAD reduction by altering the affinity of FAD for the hydride, or the orientation of nicotinamide stacking above the FAD. The logical conclusion is that CaM increases the rate at which NADPH can adopt the appropriate orientation for hydride transfer, i.e., the rate of conformational change. In this case the conformational change is the displacement of the FAD stacking residue, F1395, the motion of which is likely to be linked to the overall enzyme conformation.

**Figure 6.** Stopped-flow reduction of nNOSrd by excess NADPH or NADPD. Normalised reaction timecourses measured at 456 nm in the presence (a, b) or absence (c, d) of bound CaM, for reaction with NADPH (a, c) or NADPD (b, d).

**How does the enzyme conformation control the rate of flavin reduction?:** The only structure of nNOSrd available \([7]\) appears to show the enzyme in a hinged-closed conformation with the FMN 5 Å from the FAD and inaccessible to electron acceptors. Therefore, this is a useful structure on which to base mechanistic
hypotheses. In comparison to the structure of CPR [26], nNOSrd has an extended interface between the FAD and FMN domains, brought about by the “autoinhibitory loop” in the FMN domain and the C-terminal extension to the FAD domain. Unfortunately both of these features are only partially resolved in the X-ray structure and it is difficult to speculate on their interactions beyond this. Presumably the extra contact area stabilizes the closed conformation and inhibits hinge-opening. The two antoinhibitory modules may also help to direct the path of the FMN domain as it moves to contact the oxygenase domain in the CaM-bound state. Interestingly, the C-terminal extension (CTX) to nNOSrd begins where a series of catalytically important amino-acid residues lie close to the FAD. The most obvious of these is F1395, which stacks with the FAD isalloxazine ring. It has been well documented that this residue must move considerably during catalysis in order for the NADPH nicotinamide substituent to transfer a hydride to the FAD (in NOS and related enzymes such as ferredoxin reductase and CPR) [7, 14, 26, 30, 34, 43, 44]. It has been proposed therefore that motion of F1395 is dependent on the position/motion of the C-terminal extension [43, 44]. One function of F1395 is to destabilize the stacking interaction between nicotinamide and FAD, enabling NADP+ to dissociate from the charge transfer complex [44]. During catalysis, its rate of movement away from the FAD is likely to control the rate of formation of this charge transfer complex and therefore of hydride transfer. Thus, there is a direct link between the hydride transfer step and position of the CTX. The CTX extends across the surface of the FMN domain, its orientation, movement and perhaps also its structure are likely to be dependent on the enzyme conformation. Even partial truncation of the CTX removes its inhibitory properties [50]. It is also interesting that nNOSrd enters a “conformationally locked” state in the reduced state with NADPH bound, in which the FMN is shielded from external electron acceptors presumably through being buried in the domain-domain interface. The FAD hydroquinone and NADPH are unlikely to be able to form a stable charge-transfer complex and may be unable to stack together. With no driving-force for dislocation of the F1395 residue, both it and the CTX may be locked in place restricting motion of the FMN domain. With the FAD in the oxidized state awaiting hydride transfer, the FMN domain appears to be inhibitory [16]. Its proximity to the FAD may stabilize the position of the CTX and inhibit movement of F1395. Consequently, destabilisation of the domain-domain interface through mutagenesis of the CTX, autoinhibitory loop, or individual amino acids inevitably leads to activation of the CaM free enzyme with respect to flavin reduction and electron transfer to external acceptors [12, 16, 20-24, 27, 36, 40, 43, 44, 46].

The unique autoinhibition mechanism found in the constitutive NO synthases is likely to have evolved to prevent electron transfer to the heme in the absence of CaM and to limit the futile consumption of NADPH by the reductase domain in the presence of oxygen, also preventing superoxide generation [50]. The inhibition of flavin reduction is unlikely to have been an evolutionary priority, but is a consequence of restricting the motion of the FMN domain by extension of the C-terminus. The full importance of domain-domain motion in the catalytic action of this enzyme remains to be seen.
Conclusions

Through the work of many groups it has been established that both NO synthase reductase domain and CPR undergo large-scale conformational movement during catalysis \[3, 7, 12, 25, 26, 27, 36, 40, 44, 46-49\]. Evidence also indicates that this motion, the transition between open and closed enzyme forms, limits the rate of enzyme turnover under some conditions. For nNOSrd, it has been proposed that electron transfer from the FMN to electron acceptors is inhibited by these slow conformational changes, limiting the overall rate of turnover \[46-48\]. In this paper we show that reduction of the FAD by NADPH in nNOSrd is equally affected by the conformation of the enzyme. This is most apparent in the ionic strength studies where both the proportion of enzyme in the most active form and the rate of transition to this form appear to be increased by adding salt. The steady-state rate constants and the amplitudes and rate constants of the slow phases observed in the pre-steady-state show that this is the case and are consistent with each other. Both sets of data point to the active form of the enzyme being in an open conformation and support the role of electrostatic attraction in stabilizing the FAD-FMN domain-domain interface \[46\].

The control of individual kinetic steps by the sampling of different enzyme conformations is a feature of this family of enzymes. In the CaM-free form of nNOSrd, motion appears to be slowed down enough to limit the rate of enzyme turnover. However, it is likely that the same mechanical motion is required in the other members of the family, although it can occur at much greater speeds.

Experimental procedures

**Materials:** All reagents were supplied by Sigma-Aldrich Ltd unless stated otherwise.

**Preparation of nNOSrd and Calmodulin:** The reductase domain of nNOS (nNOSrd) was expressed in *E. coli* BL21(DE3) cells using plasmid pCRNNR \[29\], and purified on 2’,-5-ADP-agarose (1 x 20 cm) and CaM-agarose (1 x 10 cm) as previously described \[25\]. Protease Inhibitor Cocktail (Sigma-Aldrich) was additionally used at the cell lysis stage and all buffers were degassed prior to use, to ensure that the bound FMN retained its semiquinone form. Enzyme concentration was determined by UV-visible spectrometry (Cary 50) with an extinction coefficient of 20940 M$^{-1}$cm$^{-1}$ \[30\]. Bovine brain CaM, co-expressed with nNOSrd, was purified using the previously established protocol \[25\].

**Preparation of NADPD:** Deuterated A-side NADPH (NADPD) was prepared enzymatically using alcohol dehydrogenase from *T. brockii* \[31, 32\], using a preparation in Tris-HCl pH 9 containing 2mM NADP$^+$ and 1 M ethanol-d$_6$. Incubation took place at 42°C for 45 minutes and was monitored by the appearance of a UV-Vis absorption peak at 340 nm. The alcohol dehydrogenase was removed by centrifugation and the resulting mixture of NADP$^+$ and NADPD was purified using a 1 ml Q-sepharose resource-Q column on an FPLC system (Akta). Binding of the nucleotides took place in 10 mM NH$_4$HCO$_3$ and elution was achieved using a
50 ml gradient of 500 mM NH₄HCO₃. Collected fractions were assessed for purity by comparison of the UV-Vis spectra, with peaks at 260 nm (non-deuterated) and 340 nm (deuterated). Fractions with a ratio of 260:340 less than 3 were pooled together and lyophilised.

**Steady-state turnover:** The activity of nNOSrd was characterised by the reduction of electron acceptors cytochrome c (from Horse Heart) and potassium ferricyanide (FSA Lab supplies) using a Cary 50 UV/Vis or Shimadzu UV-1601 spectrophotometer. Assays were carried out at 25°C and initiated by the addition of enzyme (10-40 nm), NADPH (1 mM) and either EGTA (1 mM) or Ca²⁺/CaM (1 mM/100 µM). All experiments were performed in 50 mM Tris-HCl, pH 7.5, with the addition of 100 mM, 150 mM or 250 mM NaCl.

Turnover experiments with NADPD were carried out using a stopped-flow (Applied Photophysics SX.18MV) mixing method, where a catalytic amount of enzyme (0.1 µM) was pre-incubated with NADPH or NADPD (2-10 µM) and reacted with an excess (100 µM) of ferric cytochrome c. The resultant traces were analysed (Origin 7.5, Microcal) to give $k_{cat}$ and $K_m$ values according to the method of Gibson [33].

**Pre-steady-state flavin reduction:** Pre steady-state reduction of the bound flavins of nNOSrd was carried out by stopped-flow mixing at 25°C of 10 µM enzyme with 100 µM NADPH or NADPD, in the presence or absence of Ca²⁺/CaM. The normal buffer used was 50 mM Tris-HCl, pH 7.5 plus 100 mM NaCl, with the addition of extra salt in the ionic strength experiments, up to 150 mM or 250 mM. The change in absorbance at 457 nm was monitored and full spectra were recorded using a diode-array detector at 2.5 ms intervals. The enzyme was either one-electron reduced by titration against dithionite in an anaerobic glove box (Belle Technology), or fully oxidised by addition of excess potassium ferricyanide followed by removal on size exclusion chromatography (Sephadex G-25). Data were analysed by fitting the decay in absorbance at 457 nm to double exponential functions using Origin 7.0 (Microcal). For the salt-dependent studies diode array datasets were analysed using Applied Photophysics proprietary ProK global analysis software by fitting to a 2-step sequential kinetic model (A to B to C).

**Fluorescence Spectroscopy:** Fluorescence emission spectra of 2 µM nNOSrd were collected in 50 mM Tris/HCl buffer pH7.5 using a Shimadzu RF5301 spectrofluorimeter at an excitation wavelength of 450 nm. In order to increase the salt concentration of the samples, the required amount of solid KCl was added to a 3 ml enzyme sample and dissolved. Spectra were recorded after stabilization of the signal (approximately 10 min). This method ensures direct comparability between measurements. The salt did not affect the stability of the protein.
References


