



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

## Conformation-dependent hydride transfer in neuronal nitric oxide synthase reductase domain

**Citation for published version:**

Welland, A & Daff, S 2010, 'Conformation-dependent hydride transfer in neuronal nitric oxide synthase reductase domain', *The FEBS Journal*, vol. 277, no. 18, pp. 3833-3843. <https://doi.org/10.1111/j.1742-4658.2010.07787.x>

**Digital Object Identifier (DOI):**

[10.1111/j.1742-4658.2010.07787.x](https://doi.org/10.1111/j.1742-4658.2010.07787.x)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

The FEBS Journal

**Publisher Rights Statement:**

Copyright © 2010 by The Authors. Journal compilation copyright © 2010 by the Federation of European Biochemical Societies. All rights reserved.

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



This is the peer-reviewed version of the following article:

Welland, A., & Daff, S. (2010). Conformation-dependent hydride transfer in neuronal nitric oxide synthase reductase domain. *FEBS Journal*, 277(18), 3833-3843.

which has been published in final form at

<http://dx.doi.org/10.1111/j.1742-4658.2010.07787.x> This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for self-archiving (<http://olabout.wiley.com/WileyCDA/Section/id-817011.html>).

Manuscript received: 04/06/2010; Accepted: 22/07/2010; Article published: 16/08/2010

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

Andrew Welland and Simon Daff\*

EaStCHEM, School of Chemistry, Joseph Black Building, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, UK.

[\*]Corresponding author; e-mail: [Simon.Daff@ed.ac.uk](mailto:Simon.Daff@ed.ac.uk)

[\*\*]This work was supported by a University Royal Society Fellowship to SD and a BBSRC Ph.D. studentship to AW.

### 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  $\text{Ca}^{2+}$  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<sup>[1, 2]</sup>. The nitric oxide synthases (NOS)<sup>[3, 4, 5]</sup> 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<sup>[6-9]</sup>, 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<sup>[3, 10]</sup>.

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  $\text{Ca}^{2+}$  cause CaM to bind and activate electron transfer from the reductase domain to the heme of the oxygenase domain<sup>[11]</sup>. 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 <sup>[12]</sup>.

The reductase domain of NOS is itself composed of two domains, one of which binds NADPH and FAD, and the other FMN <sup>[13]</sup>. The FAD domain is related to the ferredoxin reductase family of enzymes and catalyses NADPH dehydrogenation <sup>[14]</sup>. It passes the two electron equivalents one at a time to the FMN domain, which is related to the flavodoxin family of electron carriers <sup>[15]</sup>. 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 <sup>[16,20]</sup>. For the constitutive enzyme forms, at low  $\text{Ca}^{2+}$  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 <sup>[17]</sup>. 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 <sup>[18]</sup>. 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) <sup>[19,20]</sup>, a 30 amino-acid extension to the C-terminus <sup>[21,22]</sup>, an insert in the hinge between the FMN and FAD domains <sup>[23]</sup>, a protruding section of the FAD domain <sup>[24]</sup> and the CaM-binding site itself. Repression of cytochrome *c* reduction is also induced by NADPH binding <sup>[25]</sup>. Many of these elements form an extended contact area between the FAD and FMN domains, as compared to CPR <sup>[26]</sup>, and may serve to stabilize the conformation of the protein reported in the respective X-ray crystal structures <sup>[27]</sup>. Both have the cofactors in close proximity, apparently in an ideal position for FAD to FMN electron transfer. In both structures NADP<sup>+</sup> 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 <sup>[28]</sup>. 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 <sup>[8]</sup>, 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

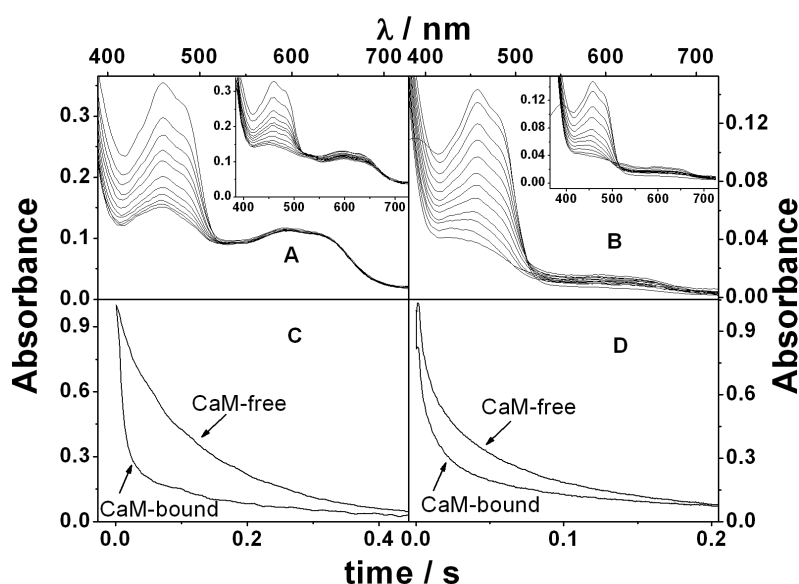
**Steady state turnover:** The surrogate electron acceptors, cytochrome *c* and ferricyanide, are known to short-circuit the turnover of nNOS by accepting electrons from the reductase domain of the enzyme before they can be transferred to the oxygenase domain [17]. The isolated reductase domain (nNOSrd) 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  $k_{cat}$  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<sup>-1</sup> 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).

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

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.

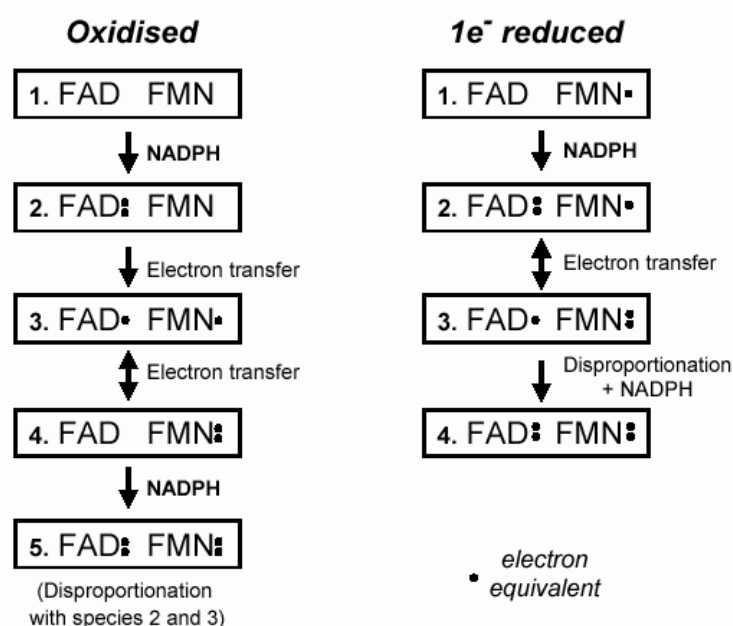
**Table 1.** Kinetic parameters for the steady-state reduction of cytochrome *c* by nNOSrd in the presence or absence of bound CaM, with different salt concentrations.

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

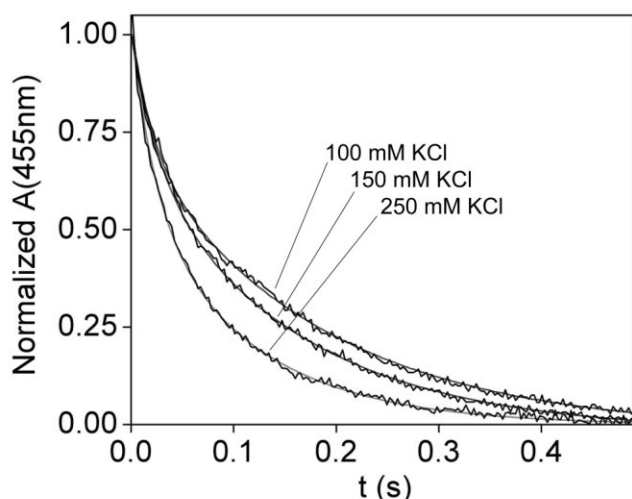
According to Scheme 1, the one-electron reduced enzyme should primarily react with a single equivalent of NADPH before undergoing slower inter-molecular electron transfer reactions. Thus, the absorbance change at 456 nm should involve a single hydride-transfer phase constituting the majority of the amplitude, 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. 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<sup>-</sup>/NADP<sup>+</sup>] 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<sup>+</sup> 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<sup>[21]</sup>. This question can be resolved by looking at the effect of ionic strength on the reaction.

Enzyme	substrate	Ca <sup>2+</sup> /CaM	$k_1$ (s <sup>-1</sup> ) (Amplitude)	$k_2$ (s <sup>-1</sup> ) (Amplitude)
1-electron reduced nNOSrd	NADPH	-	35 ± 1 (20%)	6.5 ± 0.1 (80%)
	NADPH	+	93 ± 2 (80%)	5.1 ± 0.1 (20%)
	NADPD	-	24 ± 1 (30%)	4.3 ± 0.1 (70%)
	NADPD	+	60 ± 1 (80%)	4.0 ± 0.1 (20%)

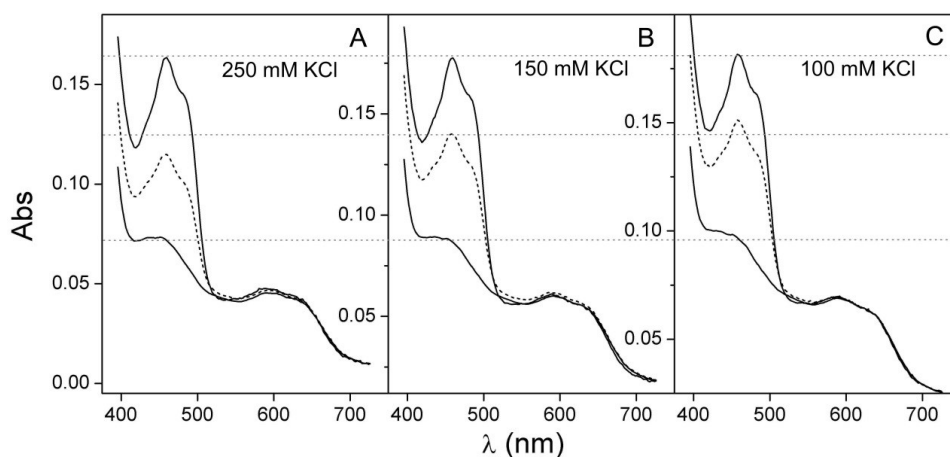
<sup>a</sup>Rate constants  $k_1$  and  $k_2$  (s<sup>-1</sup>) were derived in each case by fitting to a double exponential function with the percentage amplitude for each phase given. <sup>b</sup>For the ionic strength data.

**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 100mM 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<sup>[37]</sup>, 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.*,<sup>[38, 39]</sup> 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

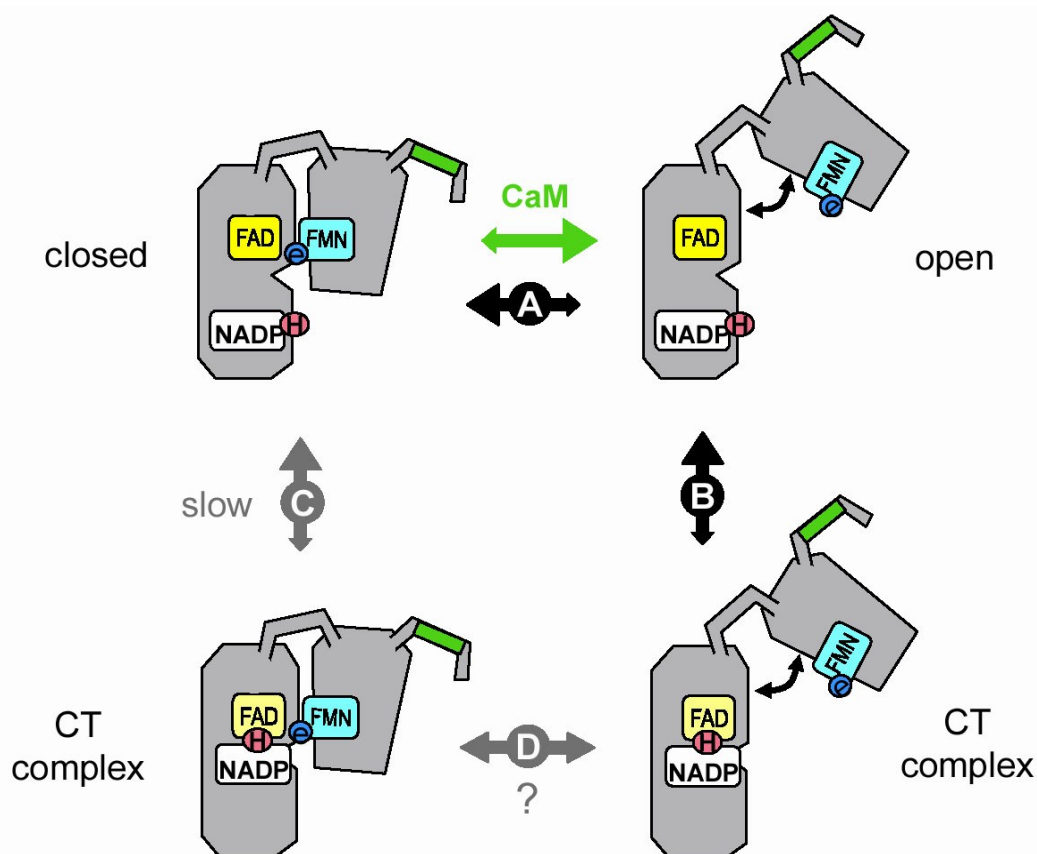
spectral changes occur at each different ionic strength. This indicates that the heterogeneity observed during reduction of CaM-free nNOSrd by NADPH is ionic strength dependent. It cannot therefore be caused by partial degradation or re-oxidation of the sample. The most likely cause is conformational 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 <sup>[25]</sup>. 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.

Enzyme	[NaCl]	Ca <sup>2+</sup> /CaM	$k_1$ (s <sup>-1</sup> ) (Amplitude)	$k_2$ (s <sup>-1</sup> ) (Amplitude)
1-electron reduced nNOSrd	100 mM	-	48 ± 1 (36%)	5.6 ± 0.1 (64%)
	150 mM	-	41 ± 1 (41%)	6.5 ± 0.1 (59%)
	250 mM	-	44 ± 1 (53%)	9.1 ± 0.1 (47%)

**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

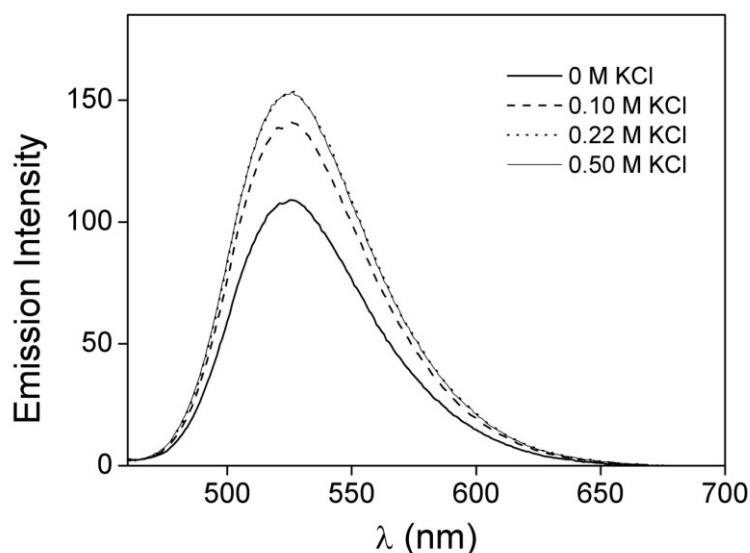
has also been established through mutation studies that the FAD:FMN domain interface is a strong determinant of the activity of the enzyme and particularly its CaM-dependence. Mutations to the interface usually affect steady-state turnover, FMN accessibility to cytochrome c and flavin reduction kinetics similarly and simultaneously<sup>[40, 45-47]</sup>. 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  $\mu\text{M}$  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<sup>[18]</sup>. 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.

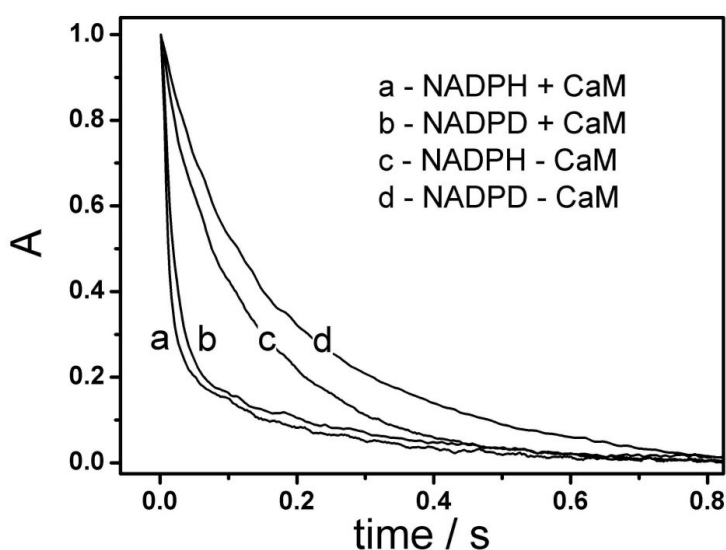


**Figure 5.** Fluorescence emission spectra of nNOSrd with excitation at 450 nm, recorded in 50 mM Tris HCl pH 7.5 and with addition of 0.1 M, 0.22 M and 0.5 M KCl.

**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<sup>[41]</sup>. Substitution of this hydrogen by deuterium to form NADP<sup>2</sup>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<sup>[41]</sup> 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 ( $k_1$  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<sup>[31]</sup>, 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<sup>[7]</sup> 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 <sup>[26]</sup>, 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 autoinhibitory 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 isoalloxazine 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) <sup>[7, 14, 26, 30, 34, 43, 44]</sup>. It has been proposed therefore that motion of F1395 is dependent on the position/motion of the C-terminal extension <sup>[43, 44]</sup>. One function of F1395 is to destabilize the stacking interaction between nicotinamide and FAD, enabling NADP<sup>+</sup> to dissociate from the charge transfer complex <sup>[44]</sup>. 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 <sup>[50]</sup>. 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 <sup>[16]</sup>. 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 <sup>[12, 16, 20-24, 27, 36, 40, 43, 44, 46]</sup>.

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 <sup>[50]</sup>. 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<sup>-1</sup>cm<sup>-1</sup> [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<sup>+</sup> and 1 M ethanol-d<sub>6</sub>. 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<sup>+</sup> 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<sub>4</sub>HCO<sub>3</sub> and elution was achieved using a

50 ml gradient of 500 mM  $\text{NH}_4\text{HCO}_3$ . 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 (10-40nM), NADPH (1mM) and either EGTA (1mM) or  $\text{Ca}^{2+}$ /CaM (1mM/100µM). All experiments were performed in 50mM Tris-HCl, pH 7.5, with the addition of 100mM, 150mM or 250mM 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  $k_{\text{cat}}$  and  $K_{\text{m}}$  values according to the method of Gibson<sup>[33]</sup>.

**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 10µM enzyme with 100µM NADPH or NADPD, in the presence or absence of  $\text{Ca}^{2+}$ /CaM. The normal buffer used was 50mM Tris-HCl, pH 7.5 plus 100mM NaCl, with the addition of extra salt in the ionic strength experiments, up to 150mM or 250mM. The change in absorbance at 457nm was monitored and full spectra were recorded using a diode-array detector at 2.5ms 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

- [1] Schmidt H & Walter U (1994) NO at work. *Cell* **78**, 919-925.
- [2] Masters BSS (2000) in *Nitric Oxide, Biology and Pathobiology* (Ignarro L.J. Ed) pp91-104 Academic Press, New York.
- [3] Daff S (2010) NO Synthase: Structures and Mechanisms, *Nitric Oxide* **23**, 1-11.
- [4] Alderton WK, Cooper CE & Knowles RG (2001) Nitric Oxide Synthases: structure, function and inhibition. *Biochem J* **357**, 593-615.
- [5] Stuehr DJ (1999) Mammalian nitric oxide synthases. *Biochim Biophys Acta* **1411**, 217-230.
- [6] Crane BR, Arvai AS, Ghosh DK, Wu C, Getzoff ED, Stuehr DJ & Tainer JA (1998) Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science* **279**, 2121-2126.
- [7] Garcin ED, Bruns CM, Lloyd SJ, Hosfield DJ, Tiso M, Gacchui R, Stuehr DJ, Tainer JA & Getzoff, ED (2004) Structural basis for isozyme-specific regulation of electron transfer in Nitric Oxide synthase. *J Biol Chem* **279**, 37918-37927.
- [8] Xia C, Misra I, Iyanagi T & Kim J-JP (2009) Regulation of Interdomain Interactions by Calmodulin in Inducible Nitric-oxide Synthase. *J Biol Chem* **284**, 30708-30717.
- [9] Raman CS, Li HY, Martasek P, Kral V, Masters BSS & Poulos TL (1998) Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pterin function involving a novel metal center. *Cell* **95**, 939-950.
- [10] Stuehr DJ, Santolini J, Wang Z, Wei C & Adak S (2004) Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem* **279**, 36167-36170.
- [11] Abu-Soud HM & Stuehr DJ (1993) Nitric-oxide synthases reveal a role for calmodulin in controlling electron transfer. *Proc Natl Acad Sci USA*. **90**, 10769-10772.
- [12] Roman LJ, Martasek P & Masters BSS (2002) Intrinsic and Extrinsic Modulation of Nitric Oxide Synthase Activity. *Chem Rev* **102**, 1179-1189.
- [13] Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR & Snyder SH (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351**, 714-718.
- [14] Karplus PA, Daniels MJ & Herriott JR (1991) Atomic structure of ferredoxin-NADP<sup>+</sup> reductase : prototype for a structurally novel flavoenzyme family. *Science* **251**, 60-66.

- [15] Watenpaugh DK, Sieker LC & Jensen LH (1973) The binding of riboflavin-5'-phosphate in a flavoprotein: flavodoxin at 2.0Å resolution. *Proc Natl Acad Sci USA*. **70**, 3857-3860.
- [16] Adak S, Ghosh S, Abu-Soud HM & Stuehr DJ (1999) Role of reductase domain cluster 1 acidic residues in neuronal nitric-oxide synthase. *J Biol Chem* **274**, 22313-22320.
- [17] Abu-Soud HM, Yoho LL & Stuehr DJ (1994) Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and inter- domain electron transfer. *J Biol Chem* **269**, 32047-32050.
- [18] Gacchui R, Presta A, Bentley DF, Abu-Soud HM, McArthur R, Brudvig G, Ghosh DK & Stuehr DJ (1996) Characterisation of the reductase domain of rat neuronal nitric oxide synthase generated in the methylophilic yeast *Pichia pastoris*. *J Biol Chem* **271**, 20594-20602.
- [19] Salerno JC, Harris DE, Irizarry K, Patel B, Morales AJ, Smith SME, Martasek P, Roman LJ, Masters BSS, Jones CL, Weismann BA, Lane P, Liu Q & Gross SS (1997) An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase. *J. Biol. Chem.* **272**, 29769-29777.
- [20] Daff S, Sagami I & Shimizu T (1999) The 42-Amino Acid Insert in the FMN domain of Neuronal Nitric Oxide Synthase exerts control over Ca<sup>2+</sup>/Calmodulin electron transfer. *J Biol Chem* **274**, 30589-30595.
- [21] Roman LJ, Martasek P, Miller RT, Harris DE, de la Garza MA, Shea TM, Kim J-JP & Masters BSS (2000) The C termini of constitutive nitric oxide synthases control electron flow through the flavin and heme domains and affect modulation by Calmodulin. *J Biol Chem* **275**, 29225-29232.
- [22] Jachymova M, Martasek P, Panda S, Roman LJ, Panda M, Shea TM, Ishimura Y, Kim J-JP & Masters BSS (2005) Recruitment of governing elements for electron transfer in the nitric oxide synthase family. *Proc Natl Acad Sci USA* **102**, 15833-15838.
- [23] Haque MM, Panda K, Tejero J, Aulak KS, Fadlalla MA, Mustovich AT & Stuehr DJ (2007) A connecting hinge represses the activity of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA*, **104**, 9254-9259.
- [24] Jones RJ, Smith SME, Gao YT, DeMay BS, Mann KJ, Salerno KM & Salerno JC (2004) The function of the small insertion in the hinge subdomain in the control of constitutive mammalian nitric-oxide synthases. *J Biol Chem* **279**, 36876-36883.
- [25] Craig DH, Chapman SK & Daff S (2002) Calmodulin activates electron transfer through neuronal Nitric Oxide Synthase Reductase Domain by releasing an NADPH-dependent conformational lock. *J Biol Chem* **277**, 33987-33994.

- [26] Wang M, Roberts DL, Paschke R, Shea TM, Masters BSS & Kim J-JP (1997) Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD- containing enzymes, *Proc Natl Acad Sci USA*. **94**, 8411-8416.
- [27] Roman LJ & Masters BSS (2006) Electron transfer by Neuronal Nitric Oxide Synthase is regulated by concerted interaction of Calmodulin and two Intrinsic Regulatory Elements. *J Biol Chem* **281**, 23111-23118.
- [28] Hamdane D, Xia C, Im S-C, Zhang H, Kim J-JP & Waskell L (2009) Structure and Function of an NADPH-Cytochrome P450 Oxidoreductase in an Open Conformation Capable of Reducing Cytochrome P450. *J Biol Chem* **284**, 11374-11384.
- [29] Newton DC, Montgomery HJ & Guillemette JG (1998) The reductase domain of the human inducible nitric oxide synthase is fully active in the absence of bound calmodulin. *Arch Biochem Biophys* **359**, 249-257.
- [30] Garnaud PE, Koetsier M, Ost TWB & Daff S. (2004) Redox Properties of the Isolated Flavin Mononucleotide- and Flavin Adenine Dinucleotide-Binding Domains of Neuronal Nitric Oxide Synthase. *Biochemistry* **43**, 11035-11044.
- [31] Knight K. & Scrutton NS (2002) Stopped-flow kinetic studies of electron transfer in the reductase domain of neuronal nitric oxide synthase: re-evaluation of the kinetic mechanism reveals new enzyme intermediates and variation with cytochrome P450 reductase. *Biochem J* **367**, 19-30.
- [32] Pollock VV & Barber MJ (2001) Kinetic and mechanistic properties of biotin sulfoxide reductase. *Biochemistry* **40**, 1430-1440.
- [33] Gibson QH (1969) Rapid mixing: stopped flow. *Meth. Enzymol* **16**, 187-228.
- [34] Dunford AJ, Marshall KR, Munro AW & Scrutton NS (2004) Thermodynamic and kinetic analysis of the isolated FAD domain of rat neuronal nitric oxide synthase altered in the region of the FAD shielding residue Phe1395. *Eur J Biochem* **271**, 2548-2560.
- [35] Guan Z-W, Kamatani D, Kimura S & Iyanagi T (2003), 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**, 30859-30868.
- [36] Tiso M, Konas DW, Panda K, Garcin ED, Sharma M, Getzoff ED & Stuehr DJ (2005) C-terminal residue Arg1400 Enables NADPH to Regulate Electron Transfer in Neuronal Nitric Oxide Synthase. *J Biol Chem* **280**, 39208-39219.

- [37] Schrammel A, Gorren ACF, Stuehr DJ, Schmidt K & Mayer B (1998) Isoform-specific effects of salts on nitric oxide synthase activity. *Biochem Biophys Acta* **1387**, 257-263.
- [38] Nishimura JS, Narayanasami R, Miller RT, Roman LJ, Panda S & Masters BSS (1999) The stimulatory effects of Hofmeister ions on the activities of neuronal nitric-oxide synthase - Apparent substrate inhibition by L-arginine is overcome in the presence of protein-destabilizing agents. *J. Biol. Chem.* **274**, 5399-5466.
- [39] Narayanasami R, Nishimura JS, McMillan K, Roman LJ, Shea TM, Robida AM, Horowitz PM & Masters BSS (1997) The influence of chaotropic reagents on neuronal nitric oxide synthase and its flavoprotein module. Urea and guanidine hydrochloride stimulate NADPH-cytochrome c reductase activity of both proteins. *Nitric Oxide* **1**, 39-49.
- [40] Panda K, Hague MM, Garcin-Hosfield ED, Durra D, Getzoff ED & Stuehr DJ (2006) Surface charge interactions of the FMN module govern catalysis by nitric-oxide synthase. *J Biol Chem* **281**, 36819-36827.
- [41] Wolthers KR & Schimerlik MI (2002) Neuronal nitric oxide synthase: substrate and solvent kinetic isotope effects on the steady-state kinetic parameters for the reduction of 2,6-dichloroindophenol and cytochrome  $c^{3+}$ . *Biochemistry* **41**, 196-204.
- [42] Wolthers KR & Schimerlik MI (2001), Reaction of neuronal nitric-oxide synthase with 2,6-dichloroindophenol and cytochrome  $c^{3+}$ : influence of the electron acceptor and binding of  $Ca^{2+}$ -activated calmodulin on the kinetic mechanism. *Biochemistry* **40**, 4722-4737.
- [43] Adak S, Sharma M, Meade AL & Stuehr DJ (2002), A conserved flavin-shielding residue regulates NO synthase electron transfer and nicotinamide coenzyme specificity. *Proc. Natl. Acad. Sci.* **99**, 13516-13521.
- [44] Konas DW, Zhu K, Sharma M, Aulak KS, Brudvig GW & Stuehr DJ (2004) The FAD-shielding residue Phe1395 regulates neuronal nitric-oxide synthase catalysis by controlling  $NADP^+$  affinity and a conformational equilibrium within the flavoprotein domain. *J Biol Chem* **279**, 35412-35425.
- [45] Noble MA, Munro AW, Rivers SL, Robledo L, Daff SN, Yellowlees LJ, Shimizu T, Sagami I, Guillemette JG & Chapman SK (1999) Potentiometric Analysis of the Flavin Cofactors of Neuronal Nitric Oxide Synthase. *Biochemistry* **38**, 16413-16418
- [46] Welland A, Garnaud PE, Kitamura M, Miles CS & Daff S (2008) Importance of the Domain-Domain Interface to the Catalytic Action of NO Synthase Reductase Domain. *Biochemistry* **47**, 9771-9780.

- [47] 47. Ilagan RP, Tejero J, Aulak KS, Sougata SR, Hemann C, Wang Z-Q, Gangoda M, Zweier JL & Stuehr DJ (2009) Regulation of FMN Subdomain Interactions and Function in Neuronal Nitric Oxide Synthase. *Biochemistry* **48**, 3864-3876.
- [48] 48. Stuehr DJ, Tejero J & Haque MM (2009) Structural and mechanistic aspects of flavoproteins: electron transfer through the nitric oxide synthase flavoprotein domain. *FEBS J* **276**, 3959–3974.
- [49] 49. Ellis J, Gutierrez A, Barsukov IL, Huang WC, Grossmann JG & Roberts GCK (2009) Domain Motion in Cytochrome P450 Reductase CONFORMATIONAL EQUILIBRIA REVEALED BY NMR AND SMALL-ANGLE X-RAY SCATTERING. *J Biol Chem* **284**, 36628-36637.
- [50] 50 Tiso M, Tejero J, Panda K, Aulak KS & Stuehr DJ (2007) Versatile regulation of neuronal nitric oxide synthase by specific regions of its C-terminal tail. *Biochemistry* **46**, 14418-14428.