Published in final edited form as:

J Inorg Biochem. 2013 July ; 124: 1–10. doi:10.1016/j.jinorgbio.2013.03.008.

Dissecting the Kinetics of the NADP⁺ - FADH₂ Charge Transfer Complex and Flavin Semiquinones in Neuronal Nitric Oxide Synthase

Huiying Li^{*}, Joumana Jamal, Georges Chreifi, Vikram Venkatesh, Hoda Abou-Ziab, and Thomas L. Poulos

Departments of Molecular Biology and Biochemistry, Pharmaceutical Sciences, and Chemistry, University of California, Irvine, California 92697-3900

Abstract

Electron flow within the neuronal nitric oxide synthase reductase domain (nNOSrd) includes hydride transfer from NADPH to FAD followed by two one-electron transfer reactions from FAD to FMN. We have used stopped flow spectrometry to closely monitor these electron transfer steps for both the wild type and the ΔG810 mutant of nNOSrd using a protocol involving both global analyses of the photodiode array spectral scans and curve fittings of single wavelength kinetic traces. The charge transfer complex and interflavin electron transfer events recorded at 750 nm and 600 nm, respectively, show the kinetics in different time frames. All electron transfer events are slow enough at 4 °C to enable measurements of rate constants even for the fast charge transfer event. To our knowledge this is the first time the rate constants for the charge transfer between NADP⁺ and FADH₂ have been determined for NOS. These procedures allow us to conclude that (1) binding of the second NADPH is necessary to drive the full reduction of FMN and; (2) charge transfer and the subsequent interflavin electron transfer have distinct spectral features that can be monitored separately with stopped flow spectroscopy. These studies also enable us to conclude that interflavin electron transfer reported at 600 nm is not limiting in NOS catalysis.

Keywords

nitric oxide synthase; diflavin reductase; electron transfer; charge transfer

1. Introduction

Nitric oxide synthases (NOS) are present in mammals as three different isoforms, neuronal nNOS, endothelial eNOS, and inducible iNOS. All isoforms catalyze the same two-step reaction requiring O₂ and NADPH that first oxidizes the substrate L-arginine to N^ω-hydroxy-L-arginine and then to citrulline and nitric oxide (NO) [1]. NO is vital in basic physiological functions either as a signaling molecule in the nervous and cardiovascular systems [2] or as a cytotoxic agent in the immune response [3]. All three NOS isoforms share the same domain architecture with an N-terminal domain harboring the heme catalytic

© 2013 Elsevier Inc. All rights reserved.

^{*}To whom correspondence should be addressed: Huiying Li, Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92697, USA, Tel: 949-824-1953; Fax: 949-824-3280; hli@uci.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

site and a nearby cofactor site for tetrahydrobiopterin (H₄B), and a C-terminal reductase domain that provides the binding sites for FMN, FAD, and NADPH [4]. This domain arrangement is similar to that of bacterial cytochrome P450BM3 where the heme and flavin domains are fused in a single polypeptide [5] while in the mammalian microsomal P450 system, the heme and flavins reside in two separate partner proteins, P450 and P450 reductase [6,7]. NOS was first identified as a flavoprotein [8] before being recognized as a heme enzyme [9,10]. Electron flow in NOS is similar to that observed in a P450 system, from NADPH to FAD then to FMN, and finally reaching the heme catalytic center. This electron transfer (ET) pathway is not only thermodynamically controlled by the relative redox potentials of each redox center [11] but also greatly influenced by the conformational changes that define the dynamics between functional domains. One of the major differences between NOS and P450, in addition to the involvement of a redox-active H₄B in the heme catalytic chemistry, is the binding of calmodulin (CaM) in NOS to a helical binding motif between the heme and reductase domains. CaM binding to nNOS and eNOS *in vivo* is regulated by cellular Ca²⁺ levels [12] while CaM tightly binds to iNOS as a subunit even at the basal intracellular Ca²⁺ concentration [13].

The pathway of ET within the diflavin reductase has been well studied and widely accepted as illustrated in Scheme 1 (CT = charge transfer) [14,15]. NADPH is a 2-electron donor and delivers its electrons to FAD through a hydride transfer event while the flavins transfer electrons one at a time which means that one-electron reduced semiquinones (sq) are intermediates. The fully reduced FMNH₂ hydroquinone (hq) is the low potential, active species that delivers electrons to heme to support catalysis [16].

The kinetics of ET from NADPH to flavins within nNOS reductase domain (nNOSrd) can be followed by spectral changes that reflect various oxidation states of each redox center utilizing stopped flow spectrometry [17–20]. One problem is that in the long wavelength region around 600 nm, where the one-electron reduced FAD (or FMN) blue, neutral sq absorbs, the CT complex between the oxidized NADP⁺ and the reduced FADH₂ will also cause subtle absorbance changes [21,22]. The blue flavin sq formation reported at 600 nm is thus biased by CT complex formation. This causes some confusion in the interpretation of kinetic data at this wavelength [14,18,20,23]. Another problem encountered for the NOS reductase is the almost identical spectral features for the semiquinoid form of FAD and FMN, both of which are the neutral, blue sq with a protonated N5 atom in the isoalloxazine ring, making the timing and sequence of ET between FAD and FMN difficult to dissect.

We recently reported a mutagenesis study in which we were able to change the redox behavior of FMN in nNOS by deletion of Gly810 in the FMN binding loop [24]. The one-electron reduced FMN in the ΔG810 mutant is now an anionic, red sq. This work was inspired by the FMN binding environment in P450BM3. The N5 atom of FMN in P450BM3 is an H-bond acceptor from the backbone amide nitrogen of Asn537 [25] whereas the N5 atom of FMN in nNOS is an H-bond donor to the carbonyl oxygen atom of Gly810 [26]. The deletion of Gly810 results in a shorter binding loop that closely resembles P450BM3, which presumably enables the N5 of FMN to accept an H-bond from the amide nitrogen of Asn811 thus stabilizing an anionic sq, FMN^{•−}, as in the case of P450BM3. As a result, the FMN and FAD semiquinones in ΔG810 exhibit spectrally distinct features. However, the full length ΔG810 nNOS showed only 5% of the wild type NO synthesis activity but catalyzed the cytochrome *c* reduction as efficiently as the wild type enzyme [24]. More interestingly, the cyt. *c* reduction activity is independent of CaM binding. The distinct behaviors of the ΔG810 enzyme compared to the wild type in both enzyme catalysis and flavin spectral features prompted us to further investigate the ET kinetics within nNOSrd. In the following study we apply dual detection modes on samples and then combine information from global analyses of the photodiode array spectral scans and curve fittings of

single wavelength kinetic traces that enable us to dissect formation of the CT complex from interflavin ET.

2. Experimental Section

2.1. Protein cloning, expression, and purification

This section is in Supporting Information.

2.2. Setup for stopped flow spectrometry

All stopped flow kinetic measurements were performed under anaerobic conditions using an SX.18MV stopped flow spectrometer (Applied Photophysics) which has a dead time of ~ 1.0 ms. The nNOSrd samples, both wild type and Δ G810, were the reductases copurified with CaM. The buffer used in the assays was 25 mM potassium phosphate, pH 7.5, 100 mM KCl, including 0.5 mM CaCl_2 . The buffer was degassed extensively by alternately purging with ultrapure argon gas and then evacuating with a vacuum pump. Buffer solutions were then brought in to a glovebox (Coy Laboratory Products) and used for preparing the running buffer that contained 5 units/mL catalase, 5 units/mL glucose oxidase, and 5 mM glucose as an oxygen scrubbing system. Two 5 mL gas tight glass syringes were filled with the running buffer. Each of the 5 mL buffer syringe was connected to a 3-way stopcock that was in turn connected to a 2.5 mL syringe containing one of the two reactants in the same running buffer: 1) nNOSrd (either wild type or Δ G810) at 15 μM ; 2) NADPH at various concentrations (at an NADPH to protein molar ratio from 1:1 up to 10:1. NADPH concentration was determined at 340 nm with an extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). To avoid errors caused by volume shrinkage during sample degassing the concentrations of both nNOSrd and NADPH were checked with the degassed samples right before preparing sample solutions for syringes. The two sets of syringes were brought out of the glovebox and mounted on the two drive syringes of the stopped flow spectrometer. The stopped flow system was made anaerobic by washing with the degassed running buffer from the 5 mL syringes before the contents of 2.5 mL reactant syringes were loaded into the drive syringes for data acquisition.

2.3. Photodiode array detection and global analysis

For the photodiode array detection at 25 °C the reference (baseline) was carefully set with the optical cell filled with the NADPH solution from the drive syringe. This is important since the 340 nm maximum for NADPH can interfere with the 390 nm maximum for the flavins. The monochromator entrance and exit slit widths were adjusted to obtain the optimal signal level in the photodiode array mode but 0.5 mm slit width was typical for the photomultiplier. An integration time 2.56 ms was set for the photodiode array detector. To gauge the extent of flavin reduction a control spectrum was taken by stopped flow mixing the same amount of nNOSrd (wild type or mutant) with the plain buffer without NADPH. Global analyses of the photodiode array spectra were performed with the Pro-K software provided with the instrument. For the Δ G810 nNOSrd a reversible 2-step reaction model, $A \rightleftharpoons B \rightleftharpoons C$, was used to obtain the deconvoluted spectra and concentration distribution for the spectral species. For the wild type nNOSrd the 2-step model worked fine when using equimolar NADPH, but a 3-step model, $A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D$, was needed when the excess NADPH drove the reduction further. The resulting simulated spectra were agreeable with the experimental ones with residuals less than $\pm 4 \text{ mAU}$.

2.4. Single wavelength kinetic traces and curve fitting

Wavelengths for the single wavelength kinetic traces were chosen based on the spectral features observed in the photodiode array scans. For wild type nNOSrd, 457 and 600 nm were used for reporting the overall flavin reduction and formation of flavin blue sq,

respectively. For $\Delta G810$ nNOSrd, 470 nm was for overall flavin reduction because of the peak position shift in the mutant; also 395 and 600 nm were for the red and blue flavin sq, respectively. For both reductase samples, 750 nm was chosen to monitor the CT event based on some trial runs in the longer wavelength with the photomultiplier. The small absorbance changes at 520 nm characteristic to sq FADH[•] were also observed if the single wavelength data being plotted from the diode array data, which were similar to the features reported in the literature [19]. However, the small signals rendered quantification difficult. The 520 nm was thus not used in our analysis.

To precisely monitor the fast kinetic events occurring within the first 100 ms, single wavelength traces were measured using a split timebase of 100 + 500 ms with 500 data points in each base. Acquisitions were carried out typically with an NADPH to protein ratio of 10:1 at either 25 °C or 4 °C (controlled by a cooler circulating water to the sample handling unit). An average of 3 repetitive shots was taken for each trace. The 3-fold averaged traces at each wavelength were taken multiple times (n value) and with different batches of protein samples to afford the error estimation for the derived rate constants. When the single wavelength kinetic traces were measured in the range of 700–800 nm a 645 nm cutoff filter was mounted in front of the photomultiplier in order to remove the stray light bias to the outcome of the measurements.

Curve fittings for the kinetic traces were performed with either Agor Pro (WaveMetrics) or the software provided with the stopped flow instrument (Applied Photophysics).

$$\frac{k_1}{k_2 - k_1} c_1 (e^{-k_1 t} - e^{-k_2 t}) + b \quad (1)$$

$$\frac{k_1}{k_2 - k_1} c_1 (e^{-k_1 t} - e^{-k_2 t}) + c_2 e^{-k_3 t} + b \quad (2)$$

The kinetic traces at 395, 600, and 750 nm showing up-and-down features posed challenge in curve fittings. User defined equation (1) shown above was used to handle the 395 nm traces where either up- or down-phase was monophasic whereas equation (2) was needed to better fit the 600 nm traces that has a biphasic nature in its down-phase. However, for 750 nm traces that had a sharp peak, fitting each phase individually with a single-exponential function, rather than the user defined equations above, provided more accurate rate constants (Fig. S1). The amplitude changes at 457 nm (470 nm for the mutant) traces were fit by a standard double-exponential or triple-exponential function. While kinetic traces were measured and compared at both 25 °C and 4 °C the traces in the range of 600–750 nm exhibited small and complex amplitude changes at 25 °C, which made curve fitting difficult. Therefore, curve fittings were only feasible for the 4 °C traces and the results are compiled in Table 1.

3. Results and Discussion

3.1. Full spectral scans and global analyses

There are two detection modes with the SX.18MV stopped flow instrument: single wavelength traces with a photomultiplier and full spectral scans with a photodiode array detector. Although diode array detection provides a full picture of the spectral changes during the course of reactions, it takes at least 1.3 ms before the detector can record the first spectral scan. To capture the early phase of a fast reaction, single wavelength detection is a more appropriate choice since finer data points with a fraction of ms interval can be

achieved to provide better sensitivity. We first characterize nNOSrd, both in wild type and Δ G810 mutant, with diode array detection.

ET from NADPH to flavins highlighted in Scheme 1 was monitored for 1 s with photodiode array detection for the CaM-bound wild type nNOSrd at various NADPH to protein ratios. Interpretation of the photodiode array data requires knowledge of the spectral features of individual flavin cofactor of nNOSrd in various redox states that have been well documented in the literature [19,24]. The oxidized flavin (FAD or FMN) has a major absorption peak at 457 nm, while fully reduced flavin (FADH_2 or FMNH_2) bleaches at this wavelength but with no absorption in the longer wavelength range (Fig. 4 in [19] for FADH_2 and Fig. 3 in [24] for FMNH_2). The one-electron reduced form of either FAD or FMN ($\text{FADH}^\bullet$ or $\text{FMNH}^\bullet$) in wild type nNOS is a blue, neutral sq that exhibits a double-humped peak in 600–650 nm region while $\text{FADH}^\bullet$ shows an additional bump at 520 nm (Fig. 4 in [19]). As shown in Fig. 1A, when a stoichiometric amount of NADPH is used reductions of the fully oxidized FAD – FMN are restricted to the following 2-electron reduced species: FADH_2 – FMN and $\text{FADH}^\bullet$ – $\text{FMNH}^\bullet$. Compared to the control spectrum recorded with the oxidized nNOSrd alone (gray curve in Fig. 1) the first spectral scan at 1.3 ms reflects little reduction of flavin. As reduction proceeds there is an apparent isosbestic point at around 510 nm, indicating that there are only two major species in the system. Although the charge transfer between NADPH and FAD will generate the hq species FADH_2 – FMN, this species shows almost the same spectral features as FAD – FMN with the only exception of a decreased amplitude at the major flavin absorption band of 457 nm. Moreover, the FADH_2 – FMN species will not accumulate owing to the large difference between the redox potentials of $\text{FADH}^\bullet/\text{FADH}_2$ and $\text{FMN}/\text{FMNH}^\bullet$ couples [11]. Instead it quickly proceeds through interflavin ET to form the disemiquinone $\text{FADH}^\bullet$ – $\text{FMNH}^\bullet$, leading to a pronounced 600–650 nm peak and a drop at 457 nm. Therefore, the system is dominated by only two major species: FAD-FMN and $\text{FADH}^\bullet$ – $\text{FMNH}^\bullet$ with an apparent isosbestic point. The transient formation of hq FADH_2 (or the charge transfer complex FADH_2 – NADP^+) and its immediate conversion to the sq are not clearly shown due to the limit of the photodiode array detection but is evidenced by a sharp peak (2 – 3 ms at 25 °C) and its fast decay in the kinetic traces in the 700–800 nm range that will be fully discussed in section 3.2. A global analysis of the spectral scans shown in Fig. 1A, using a 2-step reversible sequential reaction model, $A \rightleftharpoons B \rightleftharpoons C$, results in a deconvoluted spectrum shown in Fig. 2A. Although the spectral species in the global analysis are not necessarily the enzymatic intermediates, the spectral features for species A and B resemble that of the oxidized FAD – FMN and disemiquinone $\text{FADH}^\bullet$ – $\text{FMNH}^\bullet$, respectively. Spectral species C has an elevated 600 nm peak thus reflecting further accumulation of $\text{FADH}^\bullet$ – $\text{FMNH}^\bullet$.

When twice as much NADPH as protein is used flavin reduction proceeds further as shown in Fig. 1B (the global fitting in Fig. S2A) as evidenced by the 457 nm peak decreasing almost twice as much over 1 s compared to that in Fig. 1A. The lack of a clear isosbestic point at 510 nm indicates that there are more than two species. In addition to $\text{FADH}^\bullet$ – $\text{FMNH}^\bullet$, another 2-electron reduced species FAD – FMNH_2 forms but FMNH_2 is a low potential, unstable species if it cannot deliver its electron to an acceptor (such as cytochrome c or heme that are absent in this assay). A loss of the 600 nm sq signature along with a larger decrease in absorbance at 457 nm is indicative of even further reduction of flavins to FADH_2 – FMNH_2 . However, this 4-electron reduced species also is not accumulating due to the almost equipotential of $\text{NADPH}/\text{NADP}^+$ and $\text{FADH}_2/\text{FADH}^\bullet$ redox couples. Instead, FADH_2 – FMNH_2 will disproportionate with the FAD – FMNH_2 species to form the 3-electron reduced species, $\text{FADH}^\bullet$ – FMNH_2 , the final products observed under our conditions (Scheme 1). The fact that excess NADPH is necessary to drive flavin reduction to FAD – FMNH_2 , as indicated by comparison of Fig. 1A and 1B, implies that binding of the second NADPH is needed to drive the full reduction of FMN.

We have used the same protocols to examine flavin reduction at various NADPH concentrations for the $\Delta G810$ reductase. Similar to the wild type protein there is an apparent isosbestic point in diode array spectra in the first 100 ms with equimolar NADPH indicating only two major species at the moment, the oxidized FAD – FMN and $FADH^{\bullet} - FMN^{\bullet-}$ (Fig. 3A). That means in the early stage of flavin reduction, similar to the case of wild type nNOSrd, the hq $FADH_2 - FMN$ quickly converts to the disemiquinone and does not accumulate. However, since more $FADH_2 - FMN$ is generated and not consumed quickly enough, the decreased flavin absorption band of $FADH_2 - FMN$ will blur the isosbestic point after 100 ms (Fig. 3A). This behavior is most likely due to a slower two-electron reduction of FMN. In the mutant the one-electron reduction of FMN gives the anionic red sq (Scheme 2), $FMN^{\bullet-}$, which does not have absorption in the 600 nm region [24] so the increase at 600 nm reflects only formation of the blue $FADH^{\bullet}$ sq owing to ET from $FADH_2$ to FMN. Species B in the global analysis (Fig. 2C) gives the typical spectral features for $FADH^{\bullet} - FMN^{\bullet-}$. Formation of the FMN red sq is characterized by a strong absorption at 395 nm. Equimolar NADPH cannot drive reduction much beyond the disemiquinone, $FADH^{\bullet} - FMN^{\bullet-}$ and, therefore, species C in the global analysis (Fig. 2C) shares similar spectral features with species B and both are dominated by the disemiquinone, $FADH^{\bullet} - FMN^{\bullet-}$. Interestingly, although the accumulation level is about the same (see insets of each panel) for species C of wild type nNOSrd, a disemiquinone $FADH^{\bullet} - FMNH^{\bullet}$, in Fig. 2A and for species C of $\Delta G810$ nNOSrd, a disemiquinone $FADH^{\bullet} - FMN^{\bullet-}$, in Fig. 2C, the 600 nm blue sq peak in Fig. 2A is more pronounced because both $FADH^{\bullet}$ and $FMNH^{\bullet}$ are blue sq in the wild type case.

When the NADPH concentration is raised to twice as much as protein (Fig. 3B and global fitting in Fig. S2B), flavin reduction proceeds further with a larger decrease at 470 nm. The 600 nm peak becomes more pronounced, indicating a higher accumulation of the disemiquinone, $FADH^{\bullet} - FMN^{\bullet-}$. The anionic sq $FMN^{\bullet-}$ is a low potential, less stable, electron donating species [24]. In the absence of an electron acceptor as in our experiment the $FMN^{\bullet-}$ is further reduced to the more stable hq $FMNH_2$. The 395 nm peak drops more significantly along with the decrease in absorbance at 470 nm (Fig. 3B). However, the reduction of FMN in $\Delta G810$ nNOSrd experiences a significant delay. By analogy to P450BM3 reductase [27], the protonation of the anionic sq, $FMN^{\bullet-}$, forming the neutral sq, $FMNH^{\bullet}$, before it can be reduced to hq (Scheme 2), may represent a kinetic trap that slows down the full reduction of FMN. However, we observed no blue FMN semiquinone species when monitoring the dithionite-driven reduction of FMN domain [24]. The neutral blue sq, $FMNH^{\bullet}$, in this case might be only a transient species that is not experimentally detectable (represented in parentheses in Scheme 2). Therefore, the population of the disemiquinoid species, $FADH^{\bullet} - FMN^{\bullet-}$, in the mutant remains high within 1 s resulting in a high absorption at 600 nm (Fig. 3B).

We also carried out experiments for the wild type nNOSrd where the NADPH to protein ratio increased to 10:1 (Fig. 1C). Note the significant difference when NADPH is in much higher excess. Formation of the CT complex is almost complete within the dead time and conversion of flavins to disemiquinone has started as evidenced by the substantial decrease at 457 nm and the appearance of 600–650 nm peak even in the first spectrum at 1.3 ms (black curve) compared to the control spectrum of the oxidized enzyme (gray). The quicker disemiquinone formation and its further reduction with 10-fold excess of NADPH is also demonstrated by the early up-and-down phases in the 600 nm kinetic trace shown in section 3.2. This quick formation and further reduction of the disemiquinone species, showing a peak profile in the 600 nm trace within 50 ms time frame with a high excess of NADPH, is typical for a diflavin reductase such as the wild type nNOS [18,19] and cytochrome P450 reductase [23]. Comparison of the global analysis species A in Fig. 2B with that in Fig. 2A also illustrates the much faster disemiquinone formation when NADPH is in excess. While

the species A in Fig. 2A is flat in the 600 nm region the species A in Fig. 2B has significant features of the blue flavin sq (peak at 600 nm) which results from the decay of FADH₂ in CT2 complex through interflavin ET that happens within the dead time. The smaller amplitude changes at 600 nm for species B, C, and D in Fig 2B compared to the pronounced 600 nm peak for species C in Fig. 2A indicates that with excess NADPH the disemiquinone, FADH[•] – FMNH[•], does not build up. This is consistent with reports from other labs that the disemiquinoid species does not accumulate in the presence of excess NADPH [18,20]. The disemiquinone rapidly proceeds to further reduction to form FADH₂ – FMNH₂ (the major component in species C in global fitting shown in Fig. 2B), which does not absorb at 600 nm. However, this 4-electron reduced species does not accumulate owing to its low potential compared to that of NADPH. Instead, as mentioned above, there is a disproportionation between FADH₂ – FMNH₂ and FAD – FMNH₂ to form more stable 3-electron reduced species, FADH[•] – FMNH₂, which is dominant in the final species D in global fitting (Fig. 2B, Scheme 1)

For the ΔG810 nNOSrd a further increase in NADPH concentration to 10-fold excess (Fig. 3C and Fig. 2D) also results in an increase in flavin reduction represented by large bleaching at 470 nm even in the first spectrum recorded at 1.3 ms. However, the peak at 600 nm remains strong because the disemiquinones, FADH[•] – FMN^{•–}, accumulates for a long time due to a kinetic trap (protonation of FMN^{•–}) to the full reduction of FMN to hq. Species B in the global fitting (Fig. 2D) with a 2-step model for the spectra in Fig. 3C represents a typical disemiquinone, FADH[•] – FMN^{•–}, with a strong 600 nm peak for the blue sq FADH[•] and a strong 395 nm peak for the red sq FMN^{•–}. A persistently strong 600 nm peak within 1 s (Fig. 3C) indicates that the further reduction to give FAD – FMNH₂ has not yet been completed. In global fitting subtraction of the species C spectrum from that of species B gives a strong 395 nm peak with weak 600 nm representing a typical red sq FMN^{•–}. This means FMN^{•–} has been consumed by protonation and further reduction in going from species B to C (Fig. 2D). Species C has strong features for the blue sq, which might be a mixture of two species: FAD – FMNH₂ and FADH[•] – FMN^{•–}. By the end of 1 s the ΔG810 nNOSrd still has a significant contribution from species B (inset in Fig. 2D) reflecting incomplete FMN reduction. This process cannot be fit with a 3-step global fitting model. This contrasts to much more flavin reduction in wild type nNOSrd (inset in Fig. 2B). For either wild type or ΔG810 nNOSrd NADPH cannot drive the flavins to a full 4 electron reduction because the redox potential of the FAD_{sq/hq} couple is almost as low as that of NADPH/NADP⁺ [11], which is consistent with previous work [28].

The slow reduction of the red sq FMN^{•–} in ΔG810 nNOSrd is an interesting observation, which indicates that protonation of N5 on FMN is slow because a backbone amide (Asn811) stabilizes the deprotonated N5. Fortunately, FMN^{•–} itself is the low potential, electron donating species in ΔG810 nNOS, similar to P450BM3, thus slow reduction of red sq would not limit catalysis since FMN can cycle through oxidized and sq forms during turnover [27]. For wild type nNOSrd, on the other hand, the deprotonation of N5 in the blue sq FMNH[•] must be slow because the proton on the N5 atom is stabilized by an H-bond acceptor, the Gly810 backbone carbonyl. This might be why the blue sq FMNH[•] is air stable. This would not affect catalysis either because the wild type nNOSrd can cycle between the 1-electron and 3-electron reduced forms to support the turnover [19]. The kinetic trap of protonation/deprotonation happens to be at the step that is not crucial to the enzyme function in both cases.

3.2. Single wavelength kinetic traces

The photodiode array data have provided a full picture of various ET events in nNOSrd. It is clear that the CT between NADPH and FAD as well as the following interflavin ET occur within the first 50 ms at 25 °C. In order to better monitor these fast events we next turned to

single wavelength kinetic traces. Moreover, the wavelength range (~350 – 725 nm) of the diode array detector limits analyzing the long wavelength region (> 700 nm) that is crucial to the CT event. Two major technical challenges are 1) how to dissect the signals of CT from that of interflavin ET; and 2) how to reliably derive the rate constants for these fast events.

The CT complex between NADPH and FAD was first detected from FAD containing flavoproteins [21] with the characteristic broad band with small extinction in the long wavelength range (600–800 nm). More thorough studies have been reported for p-hydroxybenzoate hydroxylase [29] and recently for ferredoxin-NADP⁺ reductase [30] where the spectral features for the CT1 complex of FAD and NADPH (peak maximum ~ 610 nm) were distinguished from that of the CT2 complex between reduced FADH₂ and oxidized NADP⁺ (peak maximum beyond 730 nm). However, for a FAD and FMN containing diflavoprotein the CT complex is only formed transiently because the reduced FADH₂ has a strong tendency to transfer an electron to FMN, the flavin with a higher redox potential [11]. This ET results in the disemiquinoid species, FADH[•] – FMNH[•], which has a stronger absorption band in the 600–650 nm range with two humps. Therefore, observing the CT complex in a diflavin reductase is more difficult owing to overlap of its absorption band with that of the disemiquinone [23,31,32]. Nevertheless, differentiation of the two events can still be achieved since: 1) the CT complex must form prior to interflavin ET and; 2) the two-hump absorption peak of the blue, neutral flavin sq is quite distinct from the broad absorption band of a CT complex. Therefore, a careful analysis of the single wavelength kinetic traces, especially the low temperature traces, will enable resolution of the two events.

We first examined the kinetic traces measured at 600, 650, 700, 750 and 800 nm (Fig. 4, 800 nm trace was not shown for reasons explained in Fig. S3) using the CaM-bound wild type nNOSrd with 10-fold excess of NADPH in order to examine the kinetics in the pseudo-first order regime. The impact of stray light (at a one-half of the applied wavelength) from the monochromator was most obvious at 800 nm but less significant at wavelengths shorter than 750 nm (Fig. S3). The event recorded at 700, 750, and 800 nm has a sharp peak at ~15 ms at 4 °C that is apparently different from the later event recorded at 600 and 650 nm (Fig. 4). We assign the early change recorded at > 700 nm as CT complex formation (it is most likely the CT2 complex since CT1 complex formation is too fast to be observed by stopped flow). Since the absorption band of the CT complex is broad and extends into the 600–650 nm region, kinetic traces collected at these wavelengths must be “contaminated” by the CT complex, at least in the early fast phase of the flavin blue sq formation. This is the most likely reason why there are some contradicting interpretations for the kinetic traces measured at 600 nm [14,18,20,23].

It is clear from the photodiode array data that hydride transfer between NADPH and FAD as well as the early phase of interflavin ET are over within 50 ms at 25 °C. In order to better resolve the fast events and derive rate constants we had to slow down the reaction by carrying out experiments at 4 °C (Fig. 5). Formation of the CT2 complex monitored at 750 nm has been slowed down from 2–3 ms at 25 °C to 13–15 ms at 4 °C (Figs. 5B and 5D). The flavin blue sq formation in wild type nNOSrd reported at 600 nm starts within the dead time of the instrument and peaks at 12 ms at 25 °C, but at 4 °C the trace is a broader peak at around 100 ms with a resolved shoulder in the up-phase that most likely reflects the contribution from the CT2 complex (Fig. 5A). Nevertheless, the absorbance change at 600 nm should still be dominated by the flavin sq because the rise of 600 nm peak is concomitant with the decay of the 750 nm peak (Fig. 5B) and maximizes in a later time frame (Fig. 5A). In other words, the up phase at 600 nm reports mainly on the transition from FADH₂ – FMN to FADH[•] – FMNH[•] (Scheme 1).

The assignment of 750 nm peak as a CT event is further supported by the kinetic data with the $\Delta G810$ nNOSrd that has a red sq FMN^{•-} (Scheme 2) showing a strong 395 nm absorption peak (see photodiode array data in Fig. 3 in section 3.1). The first trough in the 395 nm trace (Fig. 5C) is likely to report on the fast formation of hq FADH₂ due to formation of the CT2 complex, because the formation of FADH₂ shows absorbance loss at both 470 and 395 nm, and the timing of the trough coincides with the CT2 peak at 750 nm (Fig. 5D). This is the case at both 25 °C and 4 °C but at low temperature the trough at 395 nm is much better resolved from the following rise of the absorption and its further decay. This up-and-down phase of the 395 nm trace represents the formation of a red sq FMN^{•-} and its further reduction, respectively. Time course analyses of the formation of either a blue sq FMNH[•] in wild type nNOSrd or a red sq FMN^{•-} in $\Delta G810$ nNOSrd add an additional level of confidence that 750 nm traces report on formation of the CT2 complex.

The kinetic traces measured at 4 °C show little loss in the dead time and a well resolved peak at 750 nm so that we have attempted, for the first time to our knowledge, to derive the observed rate constants for the CT event in the pseudo first order regime (10-fold excess of NADPH) and to correlate the rate of CT to the rate constants for the following interflavin ET. Again, the interpretation for the $\Delta G810$ nNOSrd is the easiest because of the additional 395 nm trace that provided clear boundary between different kinetic phases. The rates for the up- and down-phase of 750 nm peak are 122 and 38.9 s⁻¹ (Table 1 and Fig. 6D), which represent the formation of CT complex and its decay upon electron flow from FADH₂ to FMN forming FADH[•] – FMN^{•-}, respectively. The early sharp decay of 395 nm trace (Fig. 6B) with a rate of 129 s⁻¹ also reports on formation of the CT complex (or reduction of FAD), which is followed by a slower rise (35.0 s⁻¹) representing the formation of disemiquinone FADH[•] – FMN^{•-}. Therefore, this provides clear evidence that the decay of the 750 nm peak (38.9 s⁻¹) directly relates to the interflavin ET leading to the formation of disemiquinone. The same event is also reflected in the 600 nm trace (Fig. 6C). The slower rate, 40.9 s⁻¹, represents disemiquinone formation, while the faster rate, 191 s⁻¹, accounting for only 23% of the overall absorbance change reflects the “contamination” of the CT event at this wavelength. Consistently, the fast k_1 constant (97.5 s⁻¹) in the 470 nm trace (Fig. 6A) should mainly report on the CT event while the interflavin ET events are reflected in the following two phases.

The interpretation for the wild type nNOSrd is not as straightforward (Fig. 7). The 750 nm trace exhibits similar features with a fast up rate (122 s⁻¹) and a slow down rate (39 s⁻¹) derived from the sharp peak. The fast phase corresponds to the fast phase (120 s⁻¹) at 457 nm (Fig. 7A). Upon interflavin ET the CT complex, FADH₂ – FMN, should convert to disemiquinone, FADH[•] – FMNH[•], which leads to the absorbance increase at 600 nm. The catalytic function of the wild type nNOSrd requires a quick further reduction of FADH[•] – FMNH[•] to form hq, FAD – FMNH₂, an electron donating species to the electron acceptors. This formation and further reduction of the disemiquinone is recorded as a peak in the 600 nm traces (Fig. 7B). The influence of the CT event at this wavelength is shown as a pronounced shoulder in the up-phase of the 600 nm peak. This posed difficulties in deriving rate constants from the curve fitting at 600 nm. The best one can do is to skip the shoulder and fit with Equation (2) in the range of 25 to 600 ms to give an up rate of 13.4 s⁻¹ and biphasic down rates of 13.1 s⁻¹ and 3.4 s⁻¹, representing the formation of disemiquinone and its further reduction to hq, FAD – FMNH₂. It is not clear why the hq formation shows a biphasic nature. A possible explanation is the impact from the second hydride transfer that causes a gentle absorbance increase in the long wavelength region, showing as a slow rising phase (6.7 s⁻¹) beyond 100 ms in 750 nm traces (Fig. 7C). The change in CT complex in this phase is concomitant with the reduction of disemiquinone to hq, FAD – FMNH₂, reported in 600 nm trace (Fig. 7B). Interpretation of the slow kinetic phase in 750 nm traces as the second hydride transfer in nNOSrd is consistent with the observation in the

photodiode array data that excess of NADPH is necessary to drive the full reduction of FMN.

One remaining question is the rate of disemiquinone formation. The 13.4 s^{-1} rate is likely underestimated compared to 13.1 s^{-1} for the following hq formation. The underestimation is caused by the truncation of the shoulder in the 600 nm trace (within 25 ms). A reasonable way around this problem is to use the decay rate of CT (39 s^{-1}) derived from the 750 nm trace as the upper limit for disemiquinone formation. The rates of disemiquinone formation and its further reduction to the redox active hq species would thus be fast enough to sustain the NOS turnover. We have demonstrated that the CT event can be explicitly monitored at wavelengths higher than 700 nm. While CT will inevitably contaminate the kinetic traces at 600 nm because of its broad absorption band, the absorbance changes originated from the flavin blue sq have much large amplitude than that of CT (Figs. 6 and 7). Therefore, the 600 nm traces should still reflect formation and the further reduction of FMN blue sq, if the contamination from CT can be taken in to consideration. However, if the peak in the 600 nm traces is interpreted as the formation and decay of the charge transfer complex as reported in the literature [18], only could the later phases be assigned to the formation of FMN sq and its reduction to hq, resulting in much slower rates for these catalytically important events which could have been too slow to support the NO formation at the heme active site.

3.5. Conclusions

In summary, our work helps to resolve some discrepancies in the literature concerning the kinetic events reported at 600 nm. Owing to the quite different spectral features of the red anionic sq, $\text{FMN}^{\bullet-}$, the ΔG810 mutant has proven useful in providing additional spectral windows into following the various intermediates thus enabling a better dissection of the hydride transfer from the interflavin ET. The CT event is better monitored at 750 nm which correlates well with the following interflavin ET events. The rate constants for the CT event are, for the first time, derived from the 4 °C kinetic traces. Formation of FMNH_2 , the direct electron donor to the heme for wild type nNOS, is at least at 13 s^{-1} at 4 °C (Fig. 7B) and much faster at 25 °C (Fig. 5A) which should be fast enough to not be limiting in NO synthesis. In addition, we demonstrate that the formation of hq FMNH_2 crucial to the NOS catalysis is accelerated by the second hydride transfer from molar excess of NADPH (Fig. 1). The slow protonation of N5 atom of red sq $\text{FMN}^{\bullet-}$, a kinetic trap in ΔG810 nNOSrd that slows down the full reduction of FMN, is in sharp contrast to the otherwise fast first interflavin electron transfer in the mutant (Fig. 6B).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant GM57353.

We thank Dr. Irina Sevrioukova for her critical reading of the manuscript and Stephanie Hai Pineda for her technical assistance.

4. Abbreviations

NOS	nitric oxide synthase
nNOS	neuronal NOS
eNOS	endothelial NOS

iNOS	inducible NOS
nNOSrd	rat nNOS reductase domain
NO	nitric oxide
ET	electron transfer
CT	charge transfer
sq	semiquinone
hq	hydroquinone
H₄B	5,6,7,8-tetrahydrobiopterin
CaM	calmodulin
PMSF	phenylmethanesulfonyl fluoride
IPTG	Isopropyl b-D-thiogalactopyranoside

References

1. Griffith OW, Stuehr DJ. *Annu Rev Physiol.* 1995; 57:707–736. [PubMed: 7539994]
2. Moncada S, Palmer RM, Higgs EA. *Pharmacol Rev.* 1991; 43:109–142. [PubMed: 1852778]
3. Nathan CF, Hibbs JB Jr. *Curr Opin Immunol.* 1991; 3:65–70. [PubMed: 1711326]
4. Alderton WK, Cooper CE, Knowles RG. *Biochem J.* 2001; 357:593–615. [PubMed: 11463332]
5. Narhi LO, Fulco AJ. *J Biol Chem.* 1987; 262:6683–6690. [PubMed: 3106360]
6. Peterson, JA.; Prough, RA. Cytochrome P-450 reductase and cytochrome b5 in cytochrome P-450 catalysis. In: Ortiz de Montellano, PR., editor. *Cytochrome P-450 Structure, Mechanism, and Biochemistry.* Plenum Press; New York and London: 1986. p. 89-118.
7. Murataliev MB, Feyereisen R, Walker FA. *Biochim Biophys Acta.* 2004; 1698:1–26. [PubMed: 15063311]
8. Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. *Nature.* 1991; 351:714–718. [PubMed: 1712077]
9. McMillan K, Bredt DS, Hirsch DJ, Snyder SH, Clark JE, Masters BS. *Proc Natl Acad Sci USA.* 1992; 89:11141–11145. [PubMed: 1280819]
10. White KA, Marletta MA. *Biochemistry.* 1992; 31:6627–6631. [PubMed: 1379068]
11. Noble MA, Munro AW, Rivers SL, Robledo L, Daff SN, Yellowlees LJ, Shimizu T, Sagami I, Guillemette JG, Chapman SK. *Biochemistry.* 1999; 38:16413–16418. [PubMed: 10600101]
12. Vogel HJ. *Biochem Cell Biol.* 1994; 72:357–376. [PubMed: 7605608]
13. Cho HJ, Xie QW, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Nathan C. *J Exp Med.* 1992; 176:599–604. [PubMed: 1380065]
14. Guan ZW, Iyanagi T. *Arch Biochem Biophys.* 2003; 412:65–76. [PubMed: 12646269]
15. Gutierrez A, Grunau A, Paine M, Munro AW, Wolf CR, Roberts GC, Scrutton NS. *Biochem Soc Trans.* 2003; 31:497–501. [PubMed: 12773143]
16. Iyanagi T, Makino N, Mason HS. *Biochemistry.* 1974; 13:1701–1710. [PubMed: 4151581]
17. Matsuda H, Iyanagi T. *Biochim Biophys Acta.* 1999; 1473:345–355. [PubMed: 10594372]
18. Knight K, Scrutton NS. *Biochem J.* 2002; 367:19–30. [PubMed: 12079493]
19. Guan ZW, Kamatani D, Kimura S, Iyanagi T. *J Biol Chem.* 2003; 278:30859–30868. [PubMed: 12777376]
20. Konas DW, Zhu K, Sharma M, Aulak KS, Brudvig GW, Stuehr DJ. *J Biol Chem.* 2004; 279:35412–35425. [PubMed: 15180983]
21. Massey V, Palmer G. *J Biol Chem.* 1962; 237:2347–2358. [PubMed: 14470919]
22. Massey V, Ghisla S. *Ann NY Acad Sci.* 1974; 227:446–465.

23. Gutierrez A, Lian LY, Wolf CR, Scrutton NS, Roberts GC. *Biochemistry*. 2001; 40:1964–1975. [PubMed: 11329263]
24. Li H, Das A, Sibhatu H, Jamal J, Sligar SG, Poulos TL. *J Biol Chem*. 2008; 283:34762–34772. [PubMed: 18852262]
25. Sevrioukova IF, Li H, Zhang H, Peterson JA, Poulos TL. *Proc Natl Acad Sci USA*. 1999; 96:1863–1868. [PubMed: 10051560]
26. Garcin ED, Bruns CM, Lloyd SJ, Hosfield DJ, Tiso M, Gachhui R, Stuehr DJ, Tainer JA, Getzoff ED. *J Biol Chem*. 2004; 279:37918–37927. [PubMed: 15208315]
27. Sevrioukova I, Shaffer C, Ballou DP, Peterson JA. *Biochemistry*. 1996; 35:7058–7068. [PubMed: 8679531]
28. Gachhui R, Presta A, Bentley DF, Abu-Soud HM, McArthur R, Brudvig G, Ghosh DK, Stuehr DJ. *J Biol Chem*. 1996; 271:20594–20602. [PubMed: 8702805]
29. Ortiz-Maldonado M, Entsch B, Ballou DP. *Biochemistry*. 2003; 42:11234–11242. [PubMed: 14503873]
30. Tejero J, Peregrina JR, Martinez-Julvez M, Gutierrez A, Gomez-Moreno C, Scrutton NS, Medina M. *Arch Biochem Biophys*. 2007; 459:79–90. [PubMed: 17224127]
31. Roitel O, Scrutton NS, Munro AW. *Biochemistry*. 2003; 42:10809–10821. [PubMed: 12962506]
32. Welland A, Daff S. *Febs J*. 2010; 277:3833–3843. [PubMed: 20718865]

Highlights

- We present the full spectral scans with photodiode array detection of the NADPH-driven flavin reductions in both the wild type and the $\Delta G810$ mutant of nNOSrd.
- We have identified 750 nm as the wavelength to monitor the charge transfer between NADP^+ and FADH_2 .
- We directly derived, for the first time, the rate constants for the charge transfer event at 4 °C.

FIGURE 1.

Photodiode array detection of the NADPH-driven flavin reduction in the wild type CaM-bound nNOSrd at 25 °C with an NADPH to protein ratio of (A) 1:1, (B) 2:1, and (C) 10:1. The final protein concentration was 7.5 μ M. Four hundred scans were taken in the timebase of 1.02 s with the first scan recorded 1.3 ms after the stopped flow mixing. Before acquisition the baseline was set up with the optical cell filled with the solution from the drive syringe containing known amount of NADPH. For comparison the spectrum of nNOSrd in plain buffer is overlaid by normalizing to the absorbance of the last scan (1.02 s) at 725 nm in each panel.

FIGURE 2.

Deconvoluted spectra from the global fitting with a reversible 2-step model (for panels A, C, and D), $A \leftrightarrow B \leftrightarrow C$, or a 3-step model (panel B), $A \leftrightarrow B \leftrightarrow C \leftrightarrow D$. Panels A and B correspond to the spectral scans shown in Fig. 1A and 1C for wild type nNOSrd; Panels C and D for spectra in Fig. 3A and 3C for $\Delta G810$ nNOSrd, respectively.

Concentration distributions of spectral species are shown in insets. The rate constants (s^{-1}) derived from global fitting are: for wild type nNOSrd, panel A: $k_{+1} = 101$; $k_{-1} = 0.14$; $k_{+2} = 1.1$; $k_{-2} = 0.0019$; and panel B: $k_{+1} = 102$; $k_{-1} = 2.8$; $k_{+2} = 43$; $k_{-2} = 0.71$; $k_{+3} = 2.3$; $k_{-3} = 0.058$; for $\Delta G810$ nNOSrd, panel C: $k_{+1} = 97$; $k_{-1} = 0.0019$; $k_{+2} = 1.3$; $k_{-2} = 0.19$; and panel D: $k_{+1} = 159$; $k_{-1} = 0.0040$; $k_{+2} = 1.4$; $k_{-2} = 0.069$.

FIGURE 3.

Photodiode array detection of the NADPH-driven flavin reduction in the CaM-bound $\Delta G810$ nNOSrd at 25 °C with an NADPH to protein ratio of (A) 1:1, (B) 2:1, and (C) 10:1. The experimental conditions are similar to that for Fig. 1. The spectrum of $\Delta G810$ nNOSrd in plain buffer is also overlaid by normalizing to the absorbance of the last scan (1.02 s) at 725 nm in each panel.

FIGURE 4.

Kinetic traces at 4 °C for the NADPH-driven flavin reduction within the CaM-bound nNOSrd in various long wavelength (A) 600 nm; (B) 650 nm; (C) 700 nm (without filter); (D) 750 nm (without filter). The NADPH to protein ratio is 10:1 with protein concentration in the final mix at 7.5 μ M. Each trace in the plot is an average of 2 repetitive shots recorded with a split timebase of 0.1 + 0.5 s with 500 data points in each base. All panels are drawn in different absorbance scales.

FIGURE 5.

Comparison of the kinetic traces at 25 °C and 4 °C for wild type nNOSrd at (A) 600 nm, (B) 750 nm (without filter); and for $\Delta G810$ nNOSrd at (C) 395 nm, (D) 750 nm (without filter). Kinetic traces in each panel has been offset in order to plot the pair closer. Four panels use different absorbance scales. Each trace is an average of 3 repetitive shots and data acquisition conditions are otherwise similar to that in Fig. 4.

FIGURE 6.

Representative kinetic traces at 4 °C for the CaM-bound Δ G810 nNOSrd at (A) 470 nm, (B) 395 nm, (C) 600 nm, and (D) 750 nm (with 645 nm cutoff filter). The averaged observed rate constants derived from curve fittings (Table 1 and Fig. S1) are labeled for each kinetic phase.

FIGURE 7.

Representative kinetic traces at 4 °C for the CaM-bound wild type nNOSrd at (A) 457 nm, (B) 600 nm, and (C) 750 nm (with 645 nm cutoff filter). The averaged observed rate constants derived from curve fittings (Table 1 and Fig. S1) are labeled for each kinetic phase.

SCHEME 1.

The electron flow pathway in the wild type nNOSrd.

SCHEME 2.

The electron flow pathway in the $\Delta G810$ nNOSrd. The species in parenthesis are transient and experimentally unobserved.

Table 1
Observed rate constants derived from the stopped flow kinetic traces with 10-fold excess of NADPH at 4 °C

Each trace was measured as an average of three 100+500 ms shots with a 10-fold molar excess of NADPH; each averaged trace was fit by either single, double or triple exponential function or the user defined equations (see Experimental Section) to obtain the observed rate constants. Amplitude of each phase in double or triple exponential fitting are given in percentage. The constants derived from multiple averaged traces (n value) were used to calculate the mean and standard deviation. ND, not determined.

WT nNOSrd CaM-bound		
Wavelength	Model for fitting	Observed rate s ⁻¹ Phase Amplitude %
457 nm	Triple-exp	
	k1 down	120 ± 3.3
	A1 (n)	53 ± 2 (6)
	k2 down	13.4 ± 0.6
	A2 (n)	19 ± 1 (6)
	k3 down	8.7 ± 0.3
600 nm	A3 (n)	28 ± 2 (6)
	Equation (2)	
	k1 up (n)	ND (7)
	k2 up (n)	13.4 ± 0.3 (7)
	k3 down (n)	13.1 ± 0.4 (7)
750 nm	k4 down (n)	3.4 ± 0.8 (7)
	Single-exp	
	k1 up (n)	123 ± 7.6 (6)
	k2 down (n)	39.1 ± 1.3 (6)
	k3 up (n)	6.7 ± 1.8 (6)
ΔG810 nNOSrd CaM-bound		
Wavelength	Model for fitting	Observed rate s ⁻¹ Phase Amplitude %
470 nm	Triple-exp	
	k1 down	98 ± 5.9
	A1 (n)	60 ± 7 (9)
	k2 down	33 ± 4.5
	A2 (n)	26 ± 8 (9)
	k3 down	1.6 ± 0.1
395 nm	A3 (n)	14 ± 3 (9)
	Single-exp & Equation (1)	
	k1 down (n)	129 ± 7.5 (7)
	k2 up (n)	35 ± 2.8 (7)
	k2 down (n)	0.6 ± 0.2 (7)
600 nm	Double-exp	
	k1 up (n)	191 ± 55 (4)
	k2 up (n)	41 ± 3.6 (4)

ΔG810 nNOSrd CaM-bound		
Wavelength	Model for fitting	Observed rate s⁻¹ Phase Amplitude %
750 nm	Single-exp	
	k1 up (n)	122 ± 23 (8)
	k2 down (n)	39 ± 3.2 (8)