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# Effect of solution viscosity on intraprotein electron transfer between the FMN and heme domains in inducible nitric oxide synthase

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ABSTRACT

**The FMN–heme intraprotein electron transfer (IET) kinetics in a human inducible NOS (iNOS) oxygenase/FMN construct were determined by laser flash photolysis as a function of solution viscosity (1.0–3.0 cP). In the presence of ethylene glycol or sucrose, an appreciable decrease in the IET rate constant value was observed with an increase in the solution viscosity. The IET rate constant is inversely proportional to the viscosity for both viscosogens. This demonstrates that viscosity, and not other properties of the added viscosogens, causes the dependence of IET rates on the solvent concentration. The IET kinetics results indicate that the FMN–heme IET in iNOS is gated by a large conformational change of the FMN domain. The kinetics and NOS flavin fluorescence results together indicate that the docked FMN/heme state is populated transiently.**


## 1. Introduction

Nitric oxide (NO) is a ubiquitous signaling molecule for vasodilation and neurotransmission and a cytotoxin [1,2]. NO's bio-availability is tightly regulated at the synthesis level by the NO synthase (NOS) enzyme. There are three mammalian NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Mammalian NOS is a homodimeric flavo-hemoprotein that catalyzes the oxidation of L-arginine (Arg) to NO and citrulline with NADPH and $O_2$ as co-substrates [3]. Each NOS subunit comprises of an N-terminal oxygenase domain (containing a catalytic heme active site) and a C-terminal reductase domain (containing the FAD and FMN cofactors), with a calmodulin (CaM) binding region between the two domains [4,5]. The substrate, L-Arg, and a cofactor, (6R)-5,6,7,8-tetrahydrobiopterin ($H_4B$), both bind near the heme center in the oxygenase domain [6]. The iNOS isoform binds CaM irreversibly while nNOS and eNOS bind CaM reversibly in response to intracellular $Ca^{2+}$ concentration [3,5].

The intraprotein electron transfer (IET) processes are key steps in NO synthesis [3,5,7]. Specifically, the CaM-controlled intersubunit FMN–heme IET is essential in coupling electron transfer in the reductase domain with NO synthesis in the heme domain [8]. It is generally accepted that CaM-binding has little or no effect on the thermodynamics of redox processes in NOS [9–12], indicating that the regulation of the IET processes within the enzyme by CaM binding is accomplished dynamically through controlling conformational changes required for effective IET. Because of the biomedical significance of the NOS isoforms, it is of current interest to study the CaM-modulated FMN–heme IET [7,13,14]. A laser flash photolysis approach, recently developed in our laboratory [15], has been used for *direct* determination of kinetics of the FMN–heme IET in the bi-domain oxygenase/FMN (oxyFMN) constructs [15–18] and in the full-length nNOS and iNOS enzymes [17,19].

An "FMN-domain tethered shuttle" model was originally proposed by Ghosh and Salerno [16,20], and supported by kinetics [15–17,19,21–26] and thermodynamic [27] studies, in which CaM activates NO synthesis in eNOS and nNOS through a conformational change of the FMN domain from its shielded electron-accepting (input) state to a new electron-donating (output) state. Our recent IET kinetics results indicate that iNOS holoenzyme remains mainly in the input state [17] and CaM is also required for proper alignment of the FMN and heme domains in iNOS [18]. The structure of the functional output state has not yet been

*Abbreviations:* NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; eNOS, endothelial NOS; CaM, calmodulin; oxyFMN, bi-domain NOS construct in which only the heme-containing oxygenase and FMN domains along with the CaM binding region are present; $FMN_{hq}$, FMN hydroquinone; IET, intraprotein electron transfer; ET, electron transfer; $k_{et}$, rate constant for electron transfer; $\eta$, absolute viscosity; dRF, 5-deazariboflavin; $H_4B$, (6R)-5,6,7,8-tetrahydrobiopterin

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determined. Bi-domain NOS oxyFMN constructs, in which only the oxygenase and FMN domains, along with the CaM binding region, are expressed, were recently designed and constructed [22,28,29]. Biochemical, kinetics and spectroscopic results have shown that these homologous dimeric oxyFMN constructs are validated models of the NOS output state for NO production [15,16,28,30,31].

Davidson has proposed three types of electron transfer (ET) mechanisms between proteins [32]. One is "true" ET. When a fast inter-conversion equilibrium precedes the ET step, the overall reaction is "coupled". When the ET step is combined with a slower and thus rate-limiting structural rearrangement, the overall reaction is "gated" [32,33]. In the cases of true and coupled ET, the observed ET rates are expected to be independent of viscosity [34]. Viscosity effects on electron transfer in protein-protein complexes have been used as evidence for ET gated by dynamic fluctuations in inter-protein orientation in the bound complex [33,35,36]. A few studies of the effect of viscosity on intraprotein electron transfer have also been reported [37,38].

The present study of effect of solution viscosity on the NOS FMN–heme IET kinetics is designed to directly test the hypothesis that a significant conformational change is required for efficient IET between the FMN and heme domains (Fig. 1). We utilized a human iNOS oxyFMN construct [17], a validated model of the NOS output state for NO production (see above). The viscosity of solutions was adjusted by adding an appropriate amount of buffered solutions of ethylene glycol or sucrose. The IET rate constant is inversely proportional to the viscosity for both viscosogens. The IET kinetics results demonstrate that the FMN–heme IET in NOS is gated by a large conformational change. The kinetics and NOS flavin fluorescence results together indicate that the docked FMN/heme state is populated transiently.

## 2. Materials and methods

### 2.1. Expression and purification of human iNOS oxyFMN

The human iNOS oxyFMN vector was a generous gift from Dr. Guy Guillemette (University of Waterloo, Canada). Purification of the protein was performed as previously described [17].

### 2.2. Laser flash photolysis

CO photolysis experiments were conducted using an Edinburgh LP920 laser flash photolysis spectrometer, in combination with a Q-switched Continuum Surelite I-10 Nd:YAG laser and Continuum Surelite OPO. A 446 nm laser pulse out of the OPO was focused onto the sample cuvette to trigger the IET reactions. The CO photolysis experiments were performed at room temperature, as previously described [15,16,19]. All the experiments were repeated at least twice. The transient absorbance changes were analyzed using OriginPro 8.5 (OriginLab).

### 2.3. Fluorescence spectra of NOS flavin

The NOS flavin fluorescence spectra were measured by a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at room temperature. 1–6 μM of the iNOS protein in degassed buffer (40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, ethylene glycol or sucrose at the selected concentration, pH 7.6) was filtered by a 0.2 μM membrane filter. Excitation wavelength was set at 446 nm. The flavin fluorescence intensities at 525 nm were plotted versus protein concentrations. Linear regression analysis of the data gives slope of the line for each sample (i.e., NOS flavin fluorescence per μM).

## 3. Results and discussion

### 3.1. The FMN–heme IET kinetics in iNOS oxyFMN construct as a function of solution viscosity

As expected, upon a 446 nm laser excitation, the absorption at 580 nm of the partially reduced iNOS oxyFMN in the presence of 40% (v/v) ethylene glycol decays below the pre-flash baseline (Fig. 2), which is due to the FMN–heme IET (Eq. 1, where $FMN_{hq}$ stands for FMN hydroquinone), resulting in $FMNH^{\cdot}$ depletion [17], with a rate constant of 169.0 ± 1.1 $s^{-1}$.

$$[Fe^{II}][FMNH^{\cdot}] \rightleftharpoons [Fe^{III}][FMN_{hq}] \quad (1)$$

This is followed by a much slower recovery toward baseline (apparent rate constant = 2.32 ± 0.04 $s^{-1}$; Fig. S1 in the Supporting Information), which is due to CO re-binding to Fe(II) [17]. Note the spectral "transition" (i.e. a reversal in direction of absorption changes over time) in the 580 nm traces. Importantly, the rate constant of the rapid decay $k_{et}$ (Fig. 2) is independent of the signal amplitude (data not shown), i.e., reduced protein concentration, confirming an intra-protein process. Additionally, the IET kinetics at other solvent concentrations is independent of the signal amplitude within the range of viscosity studied (1–3 cP), indicating that the decay is still due to a first-order intramolecular electron transfer process at these concentrations of ethylene glycol and sucrose. Table S1 in the Supporting Information lists the IET rate constants in the presence of ethylene glycol and sucrose.

The IET rate constant with added 40% ethylene glycol (169.0 ± 1.1 $s^{-1}$) is significantly decreased by ~55%, compared to that with 10% ethylene glycol (373.8 ± 14.7 $s^{-1}$, Table S1). Fig. 3 shows that the $k_{et}$ values decrease progressively with increase of viscosity. Note that all the normalized values of the IET rate constants, determined in buffered mixtures of water with the two different viscosogens, ethylene glycol and sucrose, fall on the same line when plotted versus the viscosity. These results together show that it is solution viscosity, and not other properties of the added viscosogens, that is responsible for the remarkable dependence of IET rates on the solvent concentration.

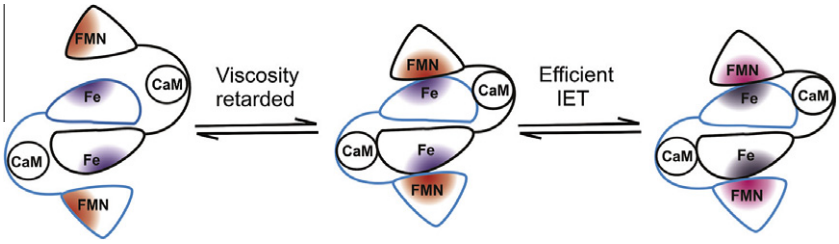


**Fig. 1.** Proposed mechanism of the IET between the FMN and heme domains in iNOS. A large conformational change of the FMN domain moves it from open conformation to closed conformation (i.e., the FMN and Fe centers in close proximity), resulting in efficient FMN–heme IET. CaM binds to iNOS irreversibly. The NOS FAD and NADPH binding domains are omitted for clarity.

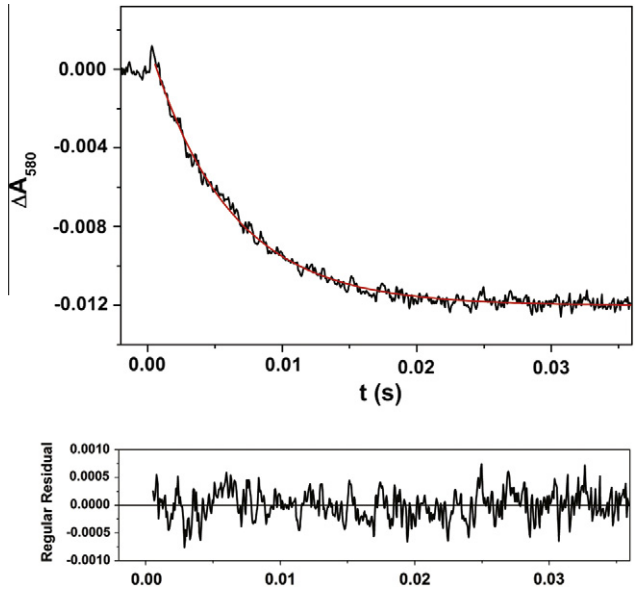


**Fig. 2.** Transient trace at 580 nm at 0–0.04 s obtained for the [Fe(II)–CO][FMNH·] form of human iNOS oxyFMN flashed by 446 nm laser excitation; 40% (v/v) ethylene glycol was present. The trace can be best fitted by a single exponential model (see the residual plot). Anaerobic solutions contained 10 μM iNOS, ~20 μM 5-deazariboflavin and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM Bis–Tris propane, 400 mM NaCl, 2 mM L-Arg, and 1 mM $Ca^{2+}$).

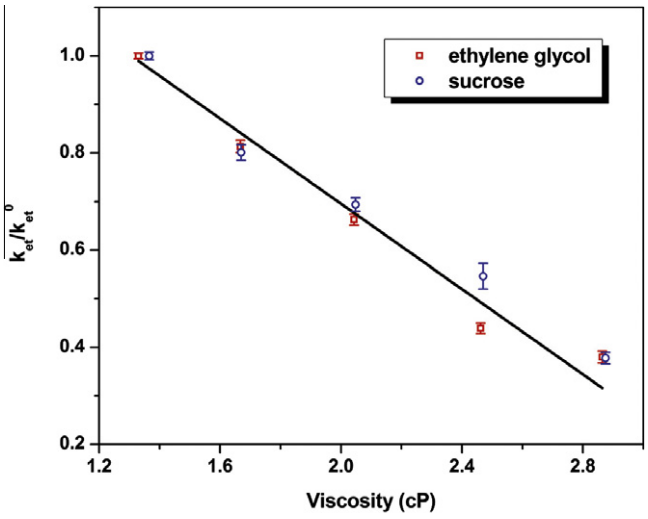


**Fig. 3.** Plot of normalized values ($k_{et}/k^0_{et}$) versus viscosity in ethylene glycol (square) and sucrose (circle). $k^0_{et}$ denotes the IET rate constant at the lowest viscosogen concentration used in this study (10 v/v % and 10 w/w % for ethylene glycol and sucrose, respectively). As is evident, the two data sets can be fit by the same line, showing that $\alpha$ in Eq. (2) equals to 1.

The role of solvent viscosity in controlling chemical reaction kinetics was originally proposed by Kramers [39]. Studies of solvent viscosity effects on protein ET kinetic processes have been recently carried out [40], and in most cases the observed rate constant $k_{obs}$ is inversely proportional to the fractional power ($\alpha$, normally but not always $\leqslant 1$) of the viscosity $\eta$ (Eq. 2) [33,35,37].

$$k_{obs} \propto \eta^{-\alpha} \quad (0 < \alpha \leqslant 1) \tag{2}$$

The viscosity dependence of $k_{et}$ was fitted to the modified Kramers' theory (Eq. 2), giving $\alpha = 1$ (Fig. 3), i.e., the IET rate constant in the iNOS oxyFMN construct is inversely proportional to the viscosity in the presence of either viscosogen.

The dependence of IET can be explained by the following mechanism (also see Fig. 1),

$$[Fe^{II}][FMNH^{\cdot}]_{NR} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [Fe^{II}][FMNH^{\cdot}]_{R}$$

$$[Fe^{II}][FMNH^{\cdot}]_{R} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} [Fe^{III}][FMN_{hq}]$$

which shows two conformationally different precursors, being designated NR (for electron transfer non-reactive) and R (for electron transfer reactive), respectively. Based on steady-state approximation, one can derive Eq. 3 (assuming $FMN_{hq}$ is $FMNH^-$) or Eq. 4 (assuming $FMN_{hq}$ is $FMNH_2$) below for the above mechanism.

$$k_{et(obsd)} = (k_1k_2 + k_1k_{-2} + k_{-1}k_{-2})/(k_{-1} + k_2 + k_{-2}) \tag{3}$$

$$k_{et(obsd)} = (k_1k_2[H^+] + k_1k_{-2} + k_{-1}k_{-2})/(k_{-1} + k_2[H^+] + k_{-2}) \tag{4}$$

In either case, of the four microscopic rate constants, only $k_1$ and $k_{-1}$ are expected to depend on viscosity $\eta$, giving $1/k_{et(obsd)} \propto \eta$. Thus the mechanism is in agreement with the experimental results (Fig. 3).

It is important to note that there is no rapid IET component in the kinetic traces obtained at higher viscosity (i.e., all the IET traces within the viscosity range can be best fitted with a single exponential model, Fig. 2). This is expected because we are in fact measuring the rate of movement of the FMN domain towards the heme domain (which is dependent on viscosity, see above). Another possible interpretation is that the solvent simply alters the FMN domain conformational equilibrium position (open/closed conformations, Fig. 1) and the observed IET rate would depend on the proportion in the closed (i.e., docked) conformation. As such, one would expect to see biphasic kinetics under any solvent conditions, with the ratio of the fast to slow processes being dependent on the equilibrium position. If a viscosity change alters this ratio, it would only alter the ratio of fast to slow processes in the biphasic kinetics, not the observed rate constant values. This is not what is observed. Thus, the latter model of conformational equilibrium is not the case here.

Because the rate constant for IET between the Fe and FMN centers in iNOS is independent of protein concentration and is thus due to an intramolecular process, a plausible explanation for the significant viscosity dependence behavior is that a slower (and thus rate-limiting) conformational change precedes the intramolecular electron transfer in iNOS (Fig. 1). This model is further supported by the fluorescence results below. Thus, these IET kinetics and spectroscopic results can be considered as new evidence that the observed IET rate is not due to the intrinsic electron transfer process, but rather is gated by a large conformational change, presumably mediated by the CaM-binding linker (in between the FMN and heme domains). Indeed, four conformations in the crystal structure of CaM-bound iNOS FMN domain indicate the FMN domain movements in electron transfer (to fine tune the distance/orientation between the redox centers) [41]. Based on the crystal structure of an intact reductase domain, Garcin et al. proposed a similar FMN domain docking model for the IET in NOS enzyme [42].

### *3.2. Fluorescence spectra of iNOS oxyFMN in the presence of ethylene glycol and sucrose*

The characteristic fluorescence of NOS flavin at 525 nm is a sensitive probe for the local environment of the FMN cofactor [24,43]. Additionally, the association between the NOS heme and FMN domains triggered by CaM binding can place the FMN molecule close enough to the paramagnetic heme for the FMN fluorescence being quenched [44]. Indeed, addition of EDTA, a calcium chelator, into the human iNOS oxyFMN sample resulted in a correlated increase in the NOS FMN fluorescence (Fig. S2). This indicates that EDTA

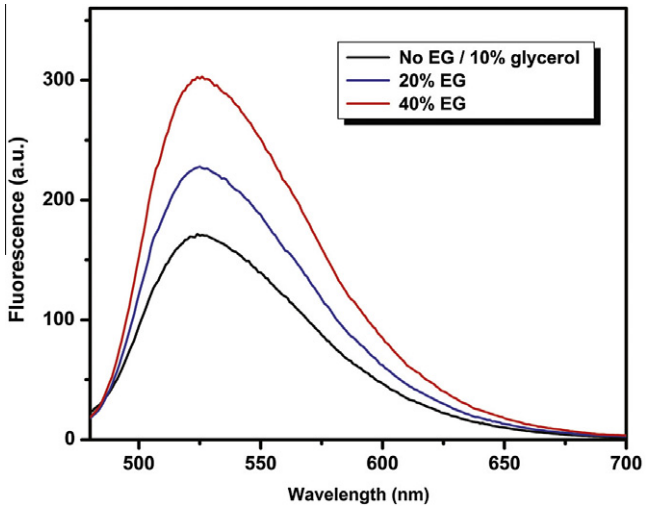


**Fig. 4.** Flavin fluorescence spectra of human iNOS oxyFMN samples with added ethylene glycol (EG). The protein concentrations were 2 μM, and all the measurements were performed under the same conditions. The black trace is of sample without added EG, while 10% glycerol was still present (to stabilize the NOS protein). The samples with added sucrose behaved similarly.

deactivation of CaM causes the FMN domain to move away from heme, resulting in a smaller proportion of the iNOS oxyFMN ensemble existed in the docked state.

We measured the fluorescence spectra of the iNOS oxyFMN protein with or without the solvents (ethylene glycol or sucrose) under identical conditions (i.e., protein concentration, buffer, and fluorometer settings) (Fig. 4). A significant increase in the fluorescence intensities with the added solvents can be noted from the observed spectra. This indicates that the protein moves into a more open conformation upon addition of ethylene glycol/sucrose, i.e., FMN further from the heme. In supportive of this interpretation, it was shown that the fluorescence of isolated nNOS FMN domain is stronger than that of nNOS oxyFMN construct [22].

Additionally, the fluorescence intensities at 525 nm (per μM) of the proteins were determined (Fig. S3). The NOS flavin fluorescence per μM with the added solvents increases progressively with the solvent concentration (Table S2, Fig. S4). Also note that the fluorescence per μM values, determined in the buffer with ethylene glycol and sucrose, overlap well when plotted versus the viscosity (Fig. S4). These fluorescence results together indicate that the FMN domain conformation is altered upon addition of the solvent, and the effect appears to be due to solution viscosity. In combination with the EDTA results (Fig. S2), it is very likely that the increased fluorescence upon addition of either ethylene glycol or sucrose is due to the fact that the FMN domain stays in the docked FMN/heme state transiently (see below), and the interdomain FMN/heme docking is retarded by increased viscosity (Fig. 1), resulting in less percentage of the docked FMN domain conformation in the sample (i.e., less FMN fluorescence quenched by the proximal paramagnetic heme center).

In line with the model in Fig. 1, Daff recently proposed several mechanisms [45] that can limit the FMN–heme IET: (i) the conformational change is slow and thus gates the IET; (ii) the enzyme may spend only a small percentage of time in the docked state; and (iii) the intrinsic rate constant may be slow in the docked output state. The present work clearly demonstrates that factor (i) is responsible for the viscosity-retarded IET kinetics. Additionally, it is likely that the docked state is populated only transiently [14,22], and the fluorescence results appear to support such mechanism in which increased viscosity lowers the percentage of the docked FMN/heme conformation. To date, it is difficult to determine the conformational equilibrium in Fig. 1. This work suggests the need for new efforts to carry out detailed studies of the relative alignment of the NOS FMN and heme domains (including the mean FMN···Fe distance, the distance distribution, and the relative orientation of the FMN and heme centers) [13].

In conclusion, an appreciable decrease in the FMN–heme IET rate constant value of a human iNOS construct was observed with an increase in the solution viscosity. The IET kinetics and NOS flavin fluorescence results indicate that the FMN–heme IET is gated by a large conformational change of the FMN domain, and that the docked FMN/heme state in iNOS is populated transiently. The correlation between fluorescence effect and IET kinetics is interesting and should be explored further.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.07.022.

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