Supplementary material

A BRIDGING INTERACTION ALLOWS CALMODULIN TO ACTIVATE NO SYNTHASE THROUGH A BI-MODAL MECHANISM *

Jesús Tejero[‡, 1, 3], Mohammad Mahfuzul Haque[‡, 1], Deborah Durra[‡], and Dennis J. Stuehr[‡, 2]

From the Departments of [‡]Pathobiology, Lerner Research Institute, The Cleveland Clinic, Cleveland, Ohio 44195, USA

[1] These authors contributed equally to this work.

[2]To whom correspondence should be addressed: Dennis J. Stuehr, Department of Pathobiology, NC-22, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH, 44195; Phone: (216)-445-6950; Fax: (216)-636-0104; Email: stuehrd@ccf.org.

[3] Present address: Vascular Medicine Institute of the University of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, PA 15260, USA.

Supplemental Table I. Primers used for site-directed mutagenesis.

Supplemental Table II. Steady-state NO synthesis activities of wild-type nNOS *versus* different concentrations of wild-type or mutant CaM proteins.

Supplemental Table III. Steady-state cytochrome *c* reductase activities of wild-type and mutant nNOS in the absence of CaM or in the presence of wild-type or mutant CaM proteins.

Supplemental Table IV. Heme reduction rates of wild-type and mutant nNOS in the presence of wild-type or mutant CaM proteins.

Supplemental Table V. Steady-state NO synthesis activities of wild-type and mutant nNOS in the presence of wild-type or mutant CaM proteins.

Supplemental Table VI. Steady-state NADPH oxidase activities of wild-type and mutant nNOS in the absence of CaM or in the presence of wild-type or mutant CaM proteins.

Supplemental Table VII. Calculated Rates used for simulation.

Supplemental Figure S1. UV-visible spectra of purified wild-type nNOS and R752 nNOS mutants.

Supplemental Figure S2. Modeled interactions of NOS/CaM combinations.

Supplemental Table I. Primers used for site-directed mutagenesis. Mutated bases are shown in bold.

PRIMER	SEQUENCE
R752E nNOS sense	5′ GGCCATGGCCAAG**GAG**GTCAAGGCGACC 3′
R752E nNOS antisense	5′ GGTCGCCTTGAC**CTC**CTTGGCCATGGCC 3′
R752Q nNOS sense	5′ GGCCATGGCCAAG**CAG**GTCAAGGCGACC 3′
R752Q nNOS antisense	5′ GGTCGCCTTGAC**CTG**CTTGGCCATGGCC 3′
E47R CaM sense	5′ CCCCACAGAAGCA**AGG**CTGCAGGACATGATCAATG 3′
E47R CaM antisense	5′ CATTGATCATGTCCTGCAG**CCT**TGCTTCTGTGGGG 3′
E47Q CaM sense	5′ CCCACAGAAGCA**CAG**CTGCAGGACATGATC 3′
E47Q CaM antisense	5′ GATCATGTCCTGCAG**CTG**TGCTTCTGTGGG 3′
E47A CaM sense	5′ CCACAGAAGCA**GCG**CTGCAGGACATGATC 3′
E47A CaM antisense	5′ GATCATGTCCTGCAG**CGC**TGCTTCTGTGG 3′

Supplemental Table II. Steady-state NO synthesis activities of wild-type nNOS in the presence of wild-type or mutant CaM proteins. Measurements were done at 25^{o}C at different concentrations of wild-type and mutant CaMs. Values are in min^{-1}. Assay conditions are described under 'Material and Methods'. Values represent the average and standard deviations of at least five or more independent measurements.

nNOS-Fl	CaM Concentration [μM]	With WT-CaM	With E47R-CaM	With E47A-CaM	With E47Q-CaM
0.1 μM	0.1	38 ± 4	6.3 ± 0.8	25.3 ± 1.3	23 ± 2.1
	0.15	40 ± 3.1	7.2 ± 0.5	29.2 ± 2.1	23.5 ± 2.4
	0.2	42 ± 2.8	8.0 ± 1.0	30.5 ± 1.8	22.8 ± 1.47
	0.3	39 ± 4.1	7.6 ± 0.2	30 ± 3.1	22.2 ± 0.8
	0.5	41 ± 2.6	7.8 ± 0.8	29 ± 2.75	24 ± 1.9
	0.8	41 ± 3.9	8.2 ± 0.6	28.5 ± 1.65	23.5 ± 2.5
	1.0	42 ± 1.9	8.1 ± 0.3	30 ± 1.96	24 ± 0.8
	2.0	41 ± 1.6	8.2 ± 0.95	30 ± 1.1	23.8 ± 1.1

Supplemental Table III. Steady-state cytochrome *c* reductase activities of wild-type and mutant nNOS in the absence of CaM or in the presence of wild-type or mutant CaM proteins. Activities were measured at 25°C in the presence of SOD and either in the absence or presence of CaM. Values are in min^{-1} and are representative of independent measurements (n=5) done under identical conditions, using two different protein preparations for each mutant.

		CaM				
		WT	**E47R**	**E47A**	**E47Q**	**---**
nNOS	WT	7700±900	760±40	630±30	730±15	480±50
	R752E	860±130	870±20	850±20	910±20	630±90
	R752Q	740±140	840±30	890±30	980±20	610±40
	tr1401	7800±500	5600±150	6200±200	6100±200	4600±200
	tr1401 R752E	6900±150	7200±200	7100±150	7300±200	6300±200

Supplemental Table IV. Heme reduction rates of wild-type and mutant nNOS in the presence of wild-type or mutant CaM proteins. All reactions were carried out at 10 °C in a stopped-flow spectrophotometer as described under "Materials and Methods". Values are in s^{-1}. The values for heme reduction are the means ± standard deviation of 7–10 individual reactions and are representative of experiments done with two enzyme preparations. The numbers in parentheses indicate the percentage of heme reduction considering wild type as 100%.

		CaM			
		WT	**E47R**	**E47A**	**E47Q**
nNOS	WT	7.1 ± 0.28 (100%)	0.7 ± 0.066 (23%)	2.6 ± 0.105 (52%)	2.5 ± 0.15 (43%)
	R752E	0.8 ± 0.06 (48%)	1.8 ± 0.108 (29%)	1.8 ± 0.105 (52%)	1.3 ± 0.08 (52%)
	R752Q	0.95 ± 0.042 (22%)	0.85 ± 0.055 (34%)	1.8 ± 0.081 (46%)	1.2 ± 0.09 (33%)
	tr1401	6.7 ± 0.26 (97%)	3.8 ± 0.114 (77%)	4.3 ± 0.38 (75%)	4.7 ± 0.16 (74%)
	tr1401 R752E	0.7 ± 0.066 (29%)	0.8 ± 0.084 (22%)	0.9 ± 0.068 (32%)	0.6 ± 0.035 (30%)

Supplemental Table V. Steady-state NO synthesis activities of wild-type and mutant nNOS in the presence of wild-type or mutant CaM proteins. Measurements were done at 25°C. Values are in min^{-1}. Assay conditions are described under 'Material and Methods'. Values represent the mean and standard deviations (n=5) of independent measurements.

		CaM			
		WT	**E47R**	**E47A**	**E47Q**
nNOS	WT	38±3	7.7±0.5	32±1	25±1
	R752E	9.5±1.2	27±4	17±2	12±1
	R752Q	11±2	9.6±0.8	16±1	16±2
	tr1401	37±6	90±7	87±6	94±8
	tr1401 R752E	10±2	27±1	15±1	11±1

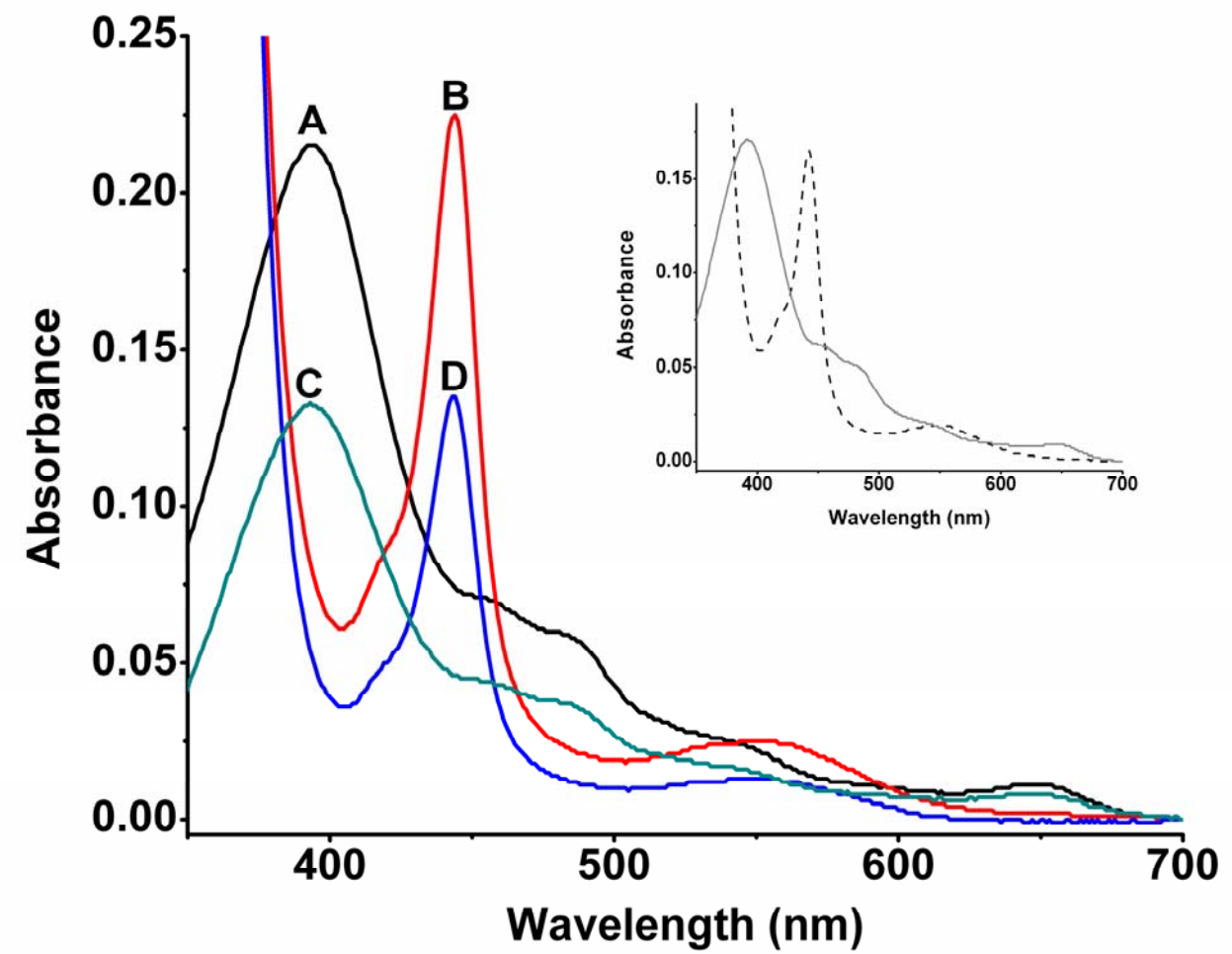
Supplemental Table VI. Steady-state NADPH oxidase activities of wild-type and mutant nNOS in the absence of CaM or in the presence of wild-type or mutant CaM proteins. Measurements were done at 25°C. Values are in min^{-1}. Assay conditions are described under 'Materials and Methods'. Values represent the mean and standard deviations of at least five (or at least three for –CaM assays) independent measurements. Values in parenthesis are ratio of number of moles of NADPH oxidized for the synthesis of one mole of NO.

		CaM				
		WT	**E47R**	**E47A**	**E47Q**	**---**
nNOS	WT	88±7 (2.31)	19±5 (2.47)	52±5 (1.62)	39±2 (1.56)	7.0±4.2
	R752E	23±3 (2.42)	44±2 (1.63)	34±2 (2.0)	26±8 (2.17)	4.5±0.9
	R752Q	21±4 (1.91)	21±1 (2.19)	31±8 (1.94)	29±5 (1.81)	4.1±0.9
	tr1401	96±3 (2.59)	151±14 (1.68)	141±11 (1.62)	174±16 (1.85)	26±3
	tr1401 R752E	50±6 (5.0)	71±12 (2.63)	59±5 (3.93)	46±4 (4.18)	41±6

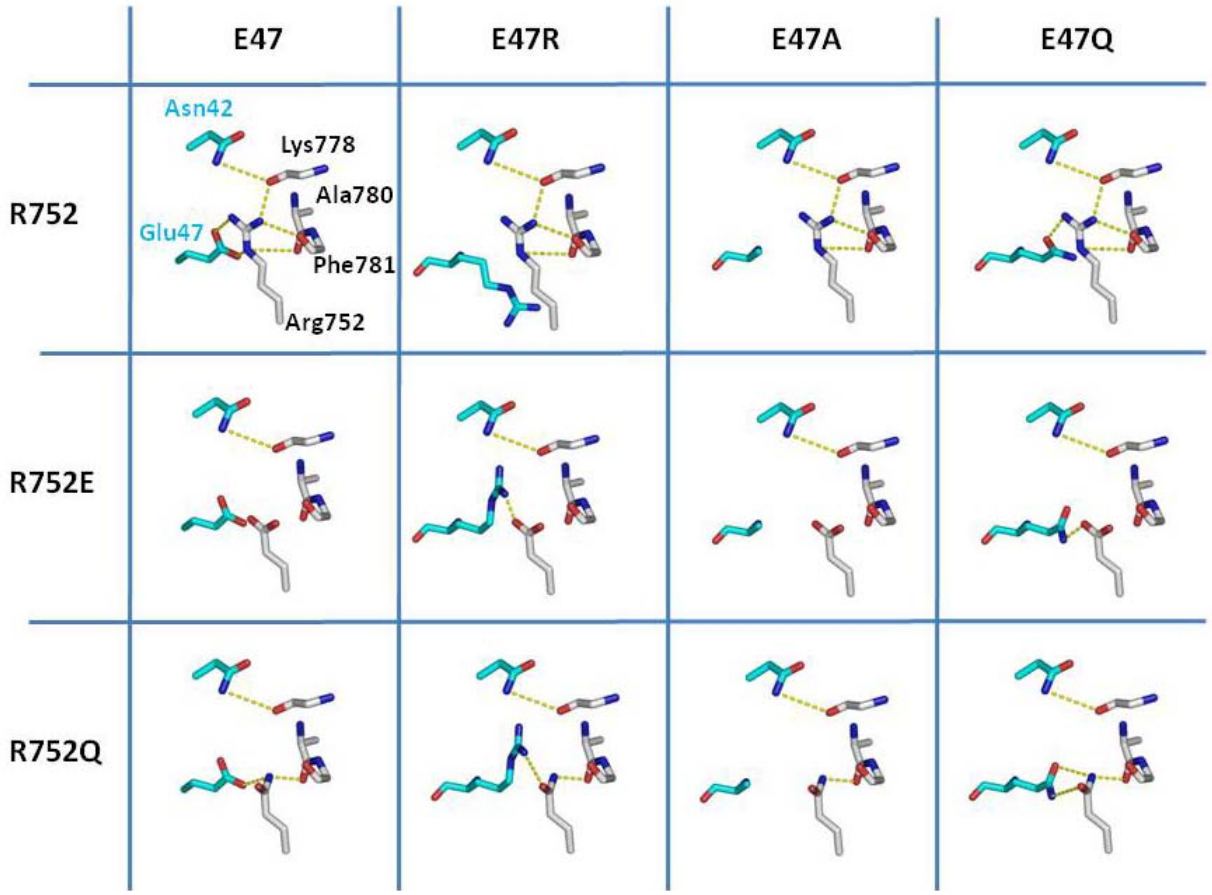
Supplemental Table VII. Calculated Rates used for simulation

			Values at 10 °C
k1	Fe^{III}(a) --> Fe^{II}(a)	k_r	5.26
k2	Fe^{II}(a) + O_2 --> $Fe^{II}O_2$(a)		57.5
k3	$Fe^{II}O_2$(a) --> Fe^{III}(b)	k_{cat1}	13.5
k4	Fe^{III}(b) --> Fe^{III}(c)	$k_{r'}$	5.26
k5	Fe^{III}(c) --> Fe^{II}(c)	$k_{r''}$	5.26
k6	Fe^{II}(c) + O_2 --> $Fe^{II}O_2$(c)		57.5
k7	$Fe^{II}O_2$(c) --> Fe^{III}NO	k_{cat2}	28.7
k8	Fe^{III}NO --> Fe^{III}(a) + NO	k_d	3.26
k9	Fe^{III}NO --> Fe^{II}NO	$k_{r'''}$	5.26
k10	Fe^{II}NO --> Fe^{II}(a) + NO		0.000096
k11	Fe^{II}NO --> Fe^{III}(a) + NO_x	k_{ox}	0.161

(a) denotes L-Arg-bound enzyme with H_4B.
(b) denotes NOHA-bound enzyme with $H_4B^{\bullet+}$
(c) denotes NOHA-bound enzyme with H_4B.
k10, Calculated from Salerno JC, FEBS Lett. 2008; 582(10):1395-9 data.
k11, determined kox value x 2.



Supplemental Figure S1. UV−visible spectra of purified nNOS full-length proteins. Scan A is ferric R752E nNOS in the presence of L-Arg and H_4B, while scan B is the ferrous heme-CO complex of R752E nNOS. Scan C is ferric R752Q nNOS in the presence of L-Arg and H_4B, and scan D is the ferrous heme-CO complex of R752Q nNOS. Spectra of wt-nNOS in the presence of L-Arg and H_4B (solid gray line) and ferrous heme-CO complex (dashed line) are shown as an *Inset*.



Supplemental Figure S2. Tentative interaction network for NOS/CaM combinations. The models were made on the basis of the homology model of the Arg752-Glu47 interaction network shown in Figure 2 (see methods for details) and depict the maximum number of interactions that can be maintained by any nNOS/CaM combination assuming the overall structure is unaltered from the initial, homology-modeled structure. Note that after the homology modeling the conformer of Asn42 (with the nitrogen atom closer to the Lys778 oxygen) is different from that shown in the iNOS FMNCaM-oxy structure (with the oxygen occupying the nitrogen position and forming an interaction with Arg752). Both conformations may coexist. Arg752 and/or Glu47 were replaced (keeping the rest of the structure unchanged) by the corresponding residues using the mutagenesis function on PyMol, version 0.99rc6 (www.pymol.org) and the conformer was selected by eye from the provided conformers trying to avoid steric clashes and to maximize possible interactions. For each structure possible salt bridges and hydrogen bonds (less than 4 angstroms distance) between FMN domain and CaM residues or the 752 residue and any other residues are shown as yellow dashes.