Supporting Information

Pulsed Electron Paramagnetic Resonance Study of Domain Docking in Neuronal Nitric Oxide Synthase: The Calmodulin and Output State Perspective

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Construction of CaM T110C mutant plasmid. Site-directed mutagenesis was performed on the kanamycin resistant pET9dCaM plasmid using a QuikChange mutagenesis kit (Agilent Technologies–Stratagene). The forward and reverse primers used for the CaM T110C mutant were: 5′ GCTTCGCCACGTGATGTGCAACCTTGGAGAG 3′, and 5′ CTCTCCAAGGTTGCACATCACGTGGCGAAGC 3′. The mutated plasmid was confirmed by DNA sequencing.

Expression and purification of CaM T110C mutant. The mutant plasmid was transform into *Escherichia coli* BL21(DE3) cells by electroporation (MicroPulser, Bio-Rad). The transformed cells were grown at 37 °C in LB in the presence of 30 mg/L kanamycin. The protein expression was induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 0.8. After further incubation at 37 °C for 3 h, the cells were harvested.

The cells were resuspended in pH 7.5 buffer containing 50 mM MOPS, 100 mM KCl, 1 mM EDTA, and 1 mM DTT. The cells were lysed using a Branson digital sonicator 250. The lysate was centrifuged in a type 70Ti rotor using a Beckman ultracentrifuge L7 at 30,000 rpm for 40 min at 4 °C. $CaCl_2$ was added into the supernatant to a final concentration of 5 mM. The Ca^{2+} saturated supernatant was then loaded onto a phenyl sepharose fast flow high sub column (GE Healthcare) pre-equilibrated with Buffer A (50 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.5). The column was washed with five-bed volumes of Buffer A, and was further washed with five-bed volumes of Buffer B (50 mM Tris-HCl, 500 mM NaCl, 1 mM $CaCl_2$, pH 7.5) to remove non-specific bound proteins. The column was subsequently washed with five-bed volumes of Buffer A to remove NaCl. The bound CaM protein was finally eluted with two-bed volumes of elution buffer (10 mM Tris–HCl, 10 mM EDTA, pH 7.5). Fractions displaying the characteristic absorbance peaks of CaM at 277 nm (for tyrosine residues) and 269, 265, 259, and 253 nm (for phenylalanine residues) were pooled, concentrated and dialyzed into Buffer A containing 1 mM DTT. The CaM concentration was determined using the extinction coefficient of 3029 M^{-1} cm^{-1} at 277 nm.[24] The protein was aliquoted and stored at -80 °C.

Labeling of the CaM mutant. The CaM T110C mutant was dialyzed into labeling buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH 7.2) to remove DTT in the storage buffer. (1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTSSL) spin label (Enzo Life

Sciences) was dissolved in 100% acetonitrile to a stock concentration of 200 mM. To avoid protein precipitation, 5 μL of the MTSSL stock solution was added into 100 μL labeling buffer, and the diluted MTSSL solution was mixed with 1 mL of 100 μM CaM mutant. The reaction mixture was gently shaken overnight at room temperature. Unreacted spin label was removed by dialysis against the labeling buffer. The spin labeling efficiency and selectivity has been confirmed by tryptic digestion and mass spectrometry (see below).

Mass spectrometry analysis. The spin labeling efficiency was determined by mass spectrometry. Unlabeled and MTSSL-labeled CaM protein samples were digested overnight at 37 °C by trypsin (Thermo Fisher Scientific Inc.) at a final protease to protein ratio of 1:50 (w/w) following the manufacturer's instruction. The samples were then mixed with the same volume of 10 mg/mL α-cyano-4-hydroxycinnamic acid (Thermo Fisher Scientific Inc.) in a 70:30 (v/v) acetonitrile/water solution containing 0.1 % trifluoroacetic acid. 1 μL of each mixture was deposited in triplicate on the MALDI plate, and allowed to dry at room temperature. MALDI-TOF-MS analyses were performed on an Applied Biosystems 4700 Proteomics Analyzer (TOF/TOF) as described.[25] Mass spectra were acquired with a total of 2000 laser pulses over a mass range of m/z from 500 to 2000 Da using a focus mass of 1000 Da. Final mass spectra were the summation of 16 subspectra, each acquired with 125 laser pulses.

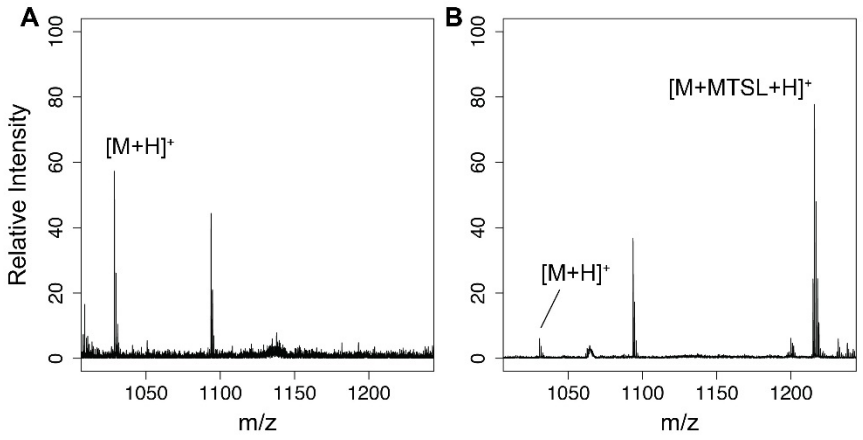

Figure S1. Mass spectrometric analyses of a tryptic digest of unlabeled (A) and MTSL-labeled (B) T110C CaM proteins. The protein samples were digested by trypsin overnight, and analyzed by MALDI-TOF MS. Other regions of the MS spectra are identical in the two samples. The m/z 1030.5 peak denoted $[M+H]^+$ in both panels belongs to the unlabeled peptide HVMCNLGEK (positions 107-115; C110 underlined) in the tryptic digest. Note that the MTSL labeling at Cys110 introduces a +184.1 m/z shift, and the intensity of the labeled peptide signal $[M+MTSL+H]^+$ is over 10-fold of that of the unlabeled peptide $[M+H]^+$ (panel B). These results clearly illustrate that the MTSL label has been effectively and selectively attached to the Cys110 site in T110C CaM protein.

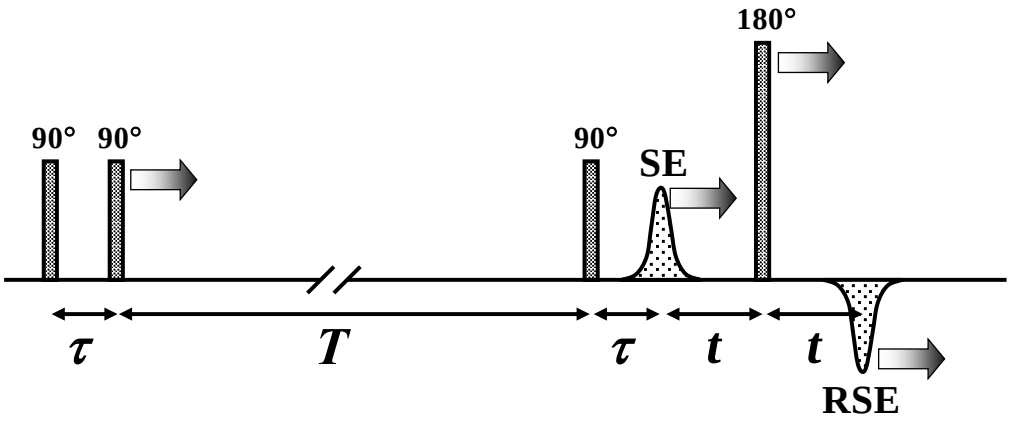

Figure S2. Refocused stimulated ESE pulse sequence used for RIDME measurements. The optimal nominal flip angles of the mw pulses are indicated. The labels "SE" and "RSE" indicate the stimulated ESE and refocused stimulated ESE signals, respectively. The arrows show the mw pulses and ESE signals whose positions are varied during the experiment. In RIDME experiment, only time interval τ is varied, while T and t are kept constant. The echo signal of interest is the refocused stimulated ESE ("RSE"). Adapted from ref. 33.

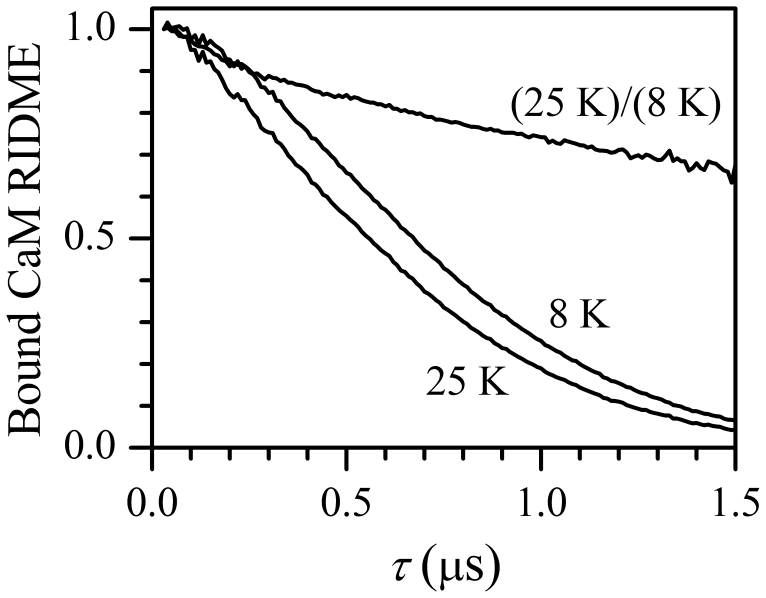

Figure S3. Representative RIDME traces for a sample of 55 μM nNOS oxyFMN + 110 μM SL CaM at [Ca^{2+}] = 1 mM. Experimental conditions: mw frequency, 30.276 GHz; B_o = 1077.2 mT (maximum of the SL signal); mw pulses: 20, 20, 20, and 30 ns, time interval between the second and third mw pulses, T = 20 μs.

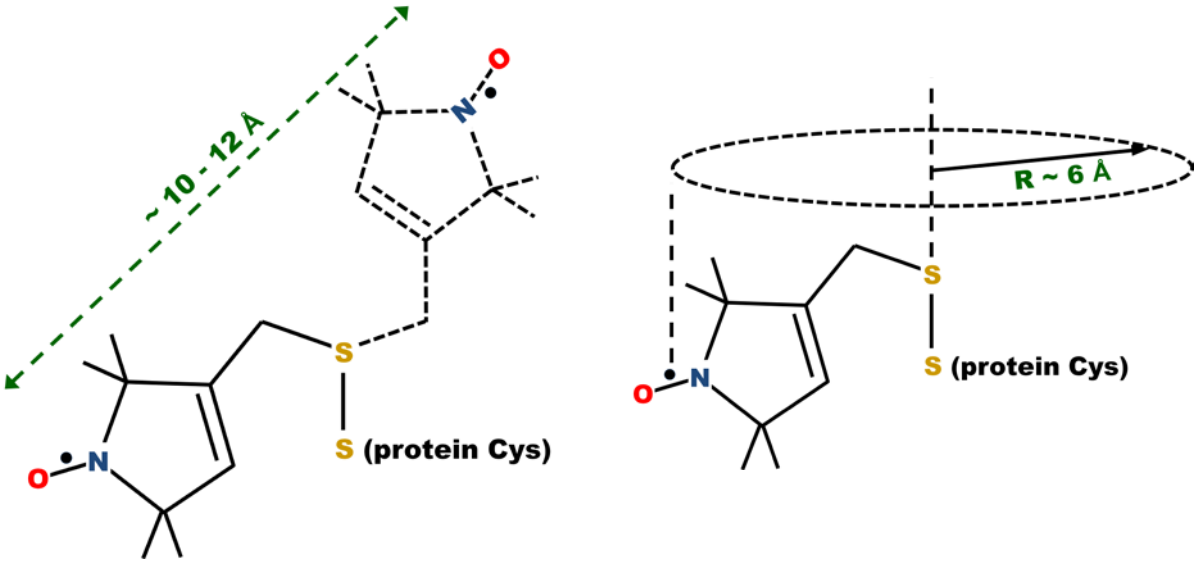

Figure S4. Uncertainty in the SL position arising from the rotational degrees of freedom of single bonds between the S–S bridge and the nitrogen atom of the radical >N–O• fragment.

Simulations of the RIDME trace corresponding to the docked conformation. The RIDME trace calculated for the open conformation (Fig. 4b in main text) was multiplied by the scaling factor C_{open} (< 1) to approximately match the decay of the experimental RIDME trace (see Fig. S5 below), from which it was then subtracted. The resulting difference trace corresponds to the docked conformations of nNOS oxyFMN. Numerical simulations of the difference trace were then performed for various SL positions. The SL position in these simulations was characterized by the distance R_{SL} from the midpoint between the Fe(III) ions of the heme centers (that are 34 Å apart) and by the angle θ_{SL} the radius-vector $\mathbf{R_{SL}}$ makes with the line joining the heme centers (the definitions are shown in the graphical form in Fig. S6). In order to fit the amplitude of the difference RIDME trace, the simulated traces were multiplied by the scaling factor C_{dock} (< 1).

The simulations have shown that only the difference traces obtained with C_{open} = 0.45 – 0.52 could be reproduced by numerical simulations subject to the restricting condition that the SL has to be located outside the oxygenase domain. Examples of simulations for the limiting values of C_{open} are shown in Figs. S7 and S8.

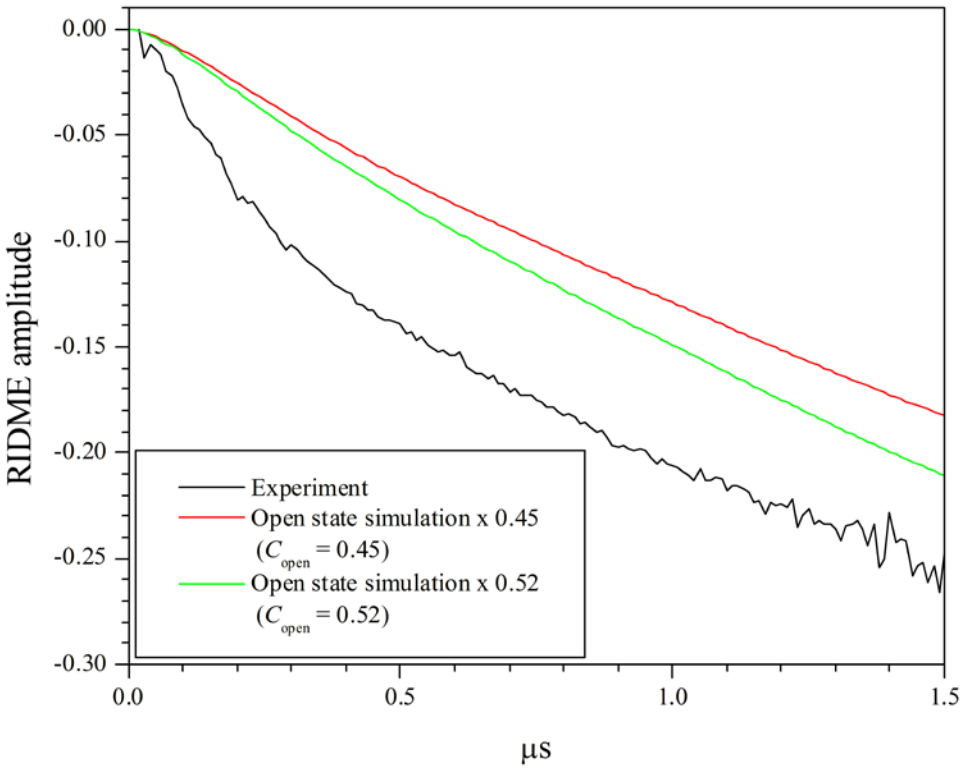

Figure S5. Black trace, experimental RIDME trace corresponding to the interaction of SL of the bound CaM with the heme centers. Color traces correspond to the simulated RIDME trace for open conformations of nNOS oxyFMN (long-dashed trace in Fig. 4b of the main text) multiplied by scaling factors C_{open} = 0.45 (red) and 0.52 (green).

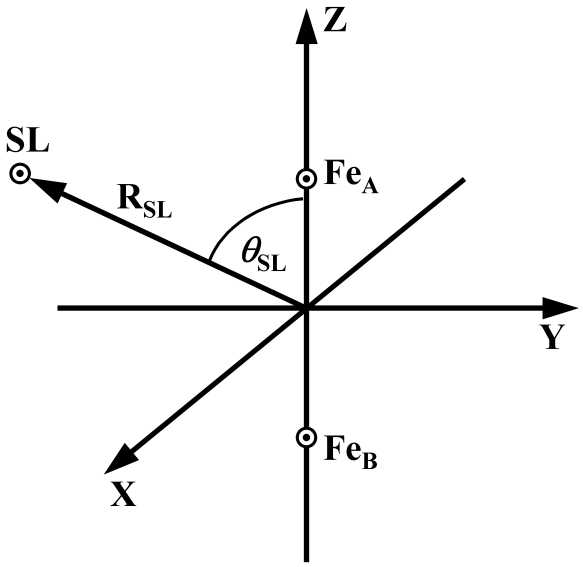

Figure S6. Structural model used in simulation of the RIDME effect corresponding to the docked state. The SL CaM interacts with two ferric heme centers of the oxygenase domain. The line joining the heme centers is taken as axis Z. The coordinate origin is halfway between the two heme centers. Due to the axial symmetry, axes X and Y are arbitrary, but are chosen in such a way that the SL lies in the XZ plane.

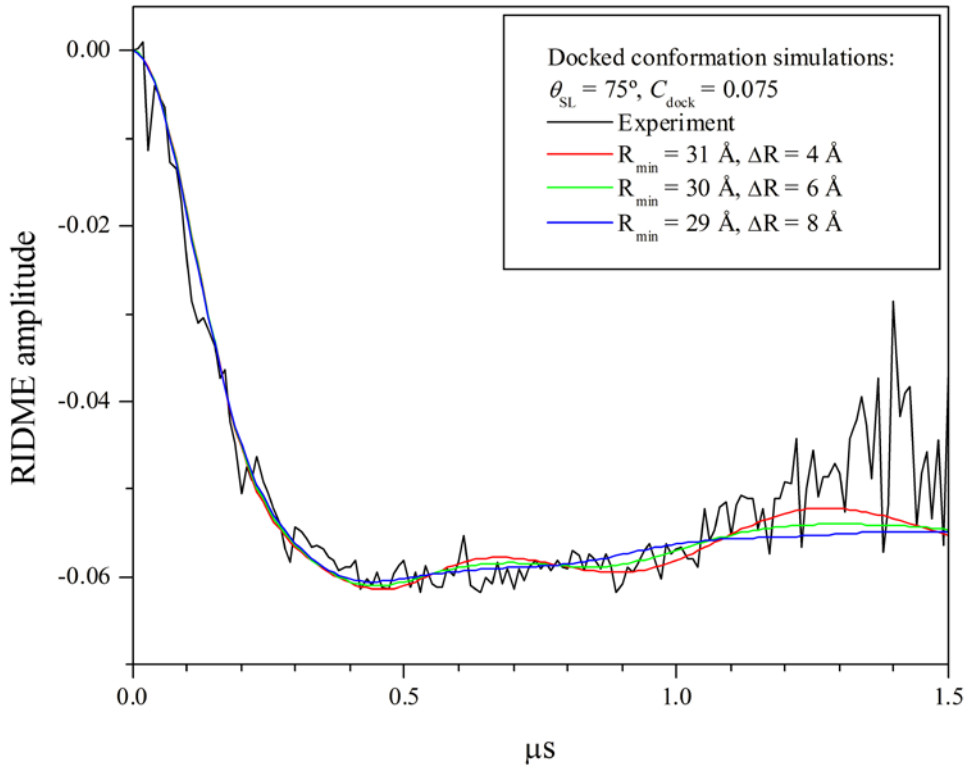

Figure S7. Black trace, the difference experimental RIDME trace obtained using C_{open} = 0.52. Color traces are simulated for θ_{SL} = 75° and using uniform distributions of R_{SL} of different widths. The amplitude scaling factor is C_{dock} = 0.075 for all simulated traces. The optimal distribution width, ΔR_{SL}, is about 6 Å.

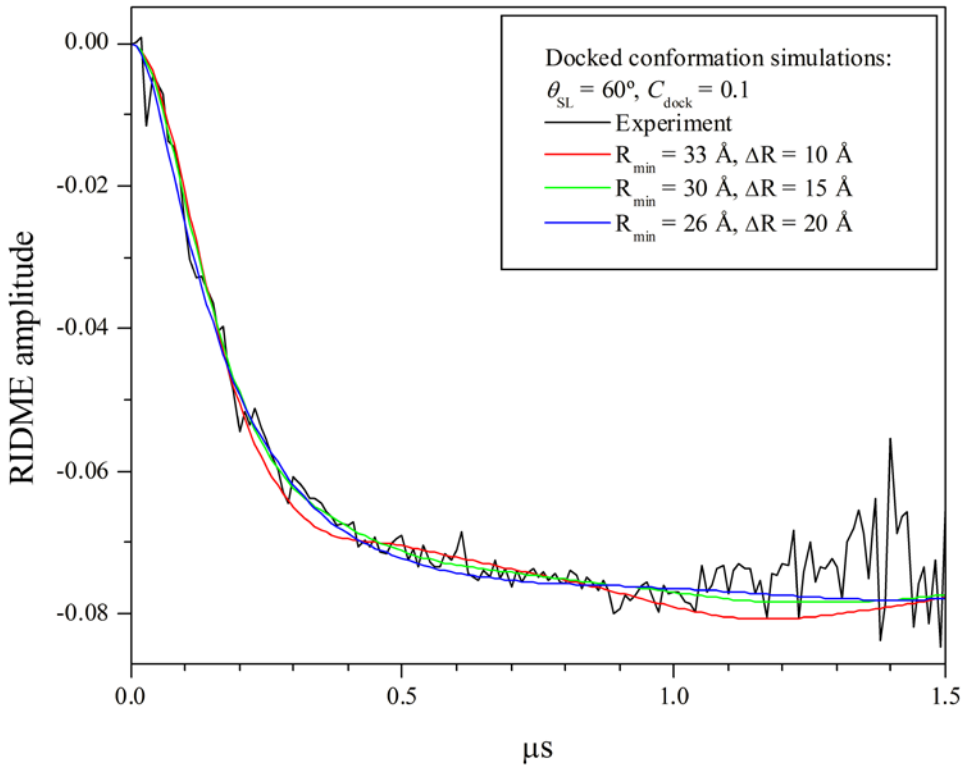

Figure S8. Black trace, the difference experimental RIDME trace obtained using C_{open} = 0.45. Color traces are simulated for θ_{SL} = 60° and using uniform distributions of R_{SL} of different widths. The amplitude scaling factor is C_{dock} = 0.1 for all simulated traces. The optimal distribution width appears to be about 15 Å. For larger ΔR_{SL} (e.g. ΔR_{SL} = 20 Å, blue trace) the formal fits can be obtained, but they are meaningless because the SL at shorter distances is inside the oxygenase domain (at θ_{SL} = 60°, the minimum possible R_{SL} is about 28 Å), which is impossible. For still larger ΔR_{SL} the shape of the initial decay (resembling Gaussian in experiment) cannot be reproduced.