Supporting Information

L337H Mutant of Rat Neuronal Nitric Oxide Synthase Resembles Human Neuronal Nitric Oxide Synthase Toward Inhibitors

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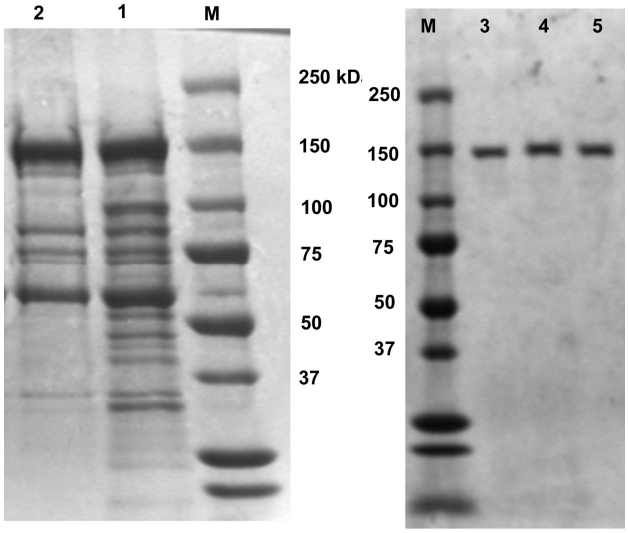
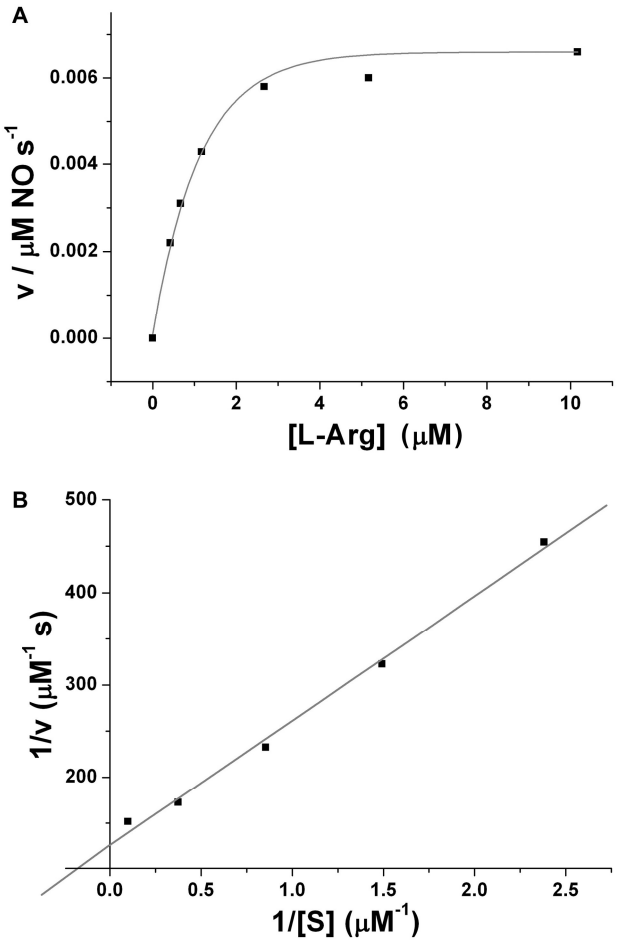
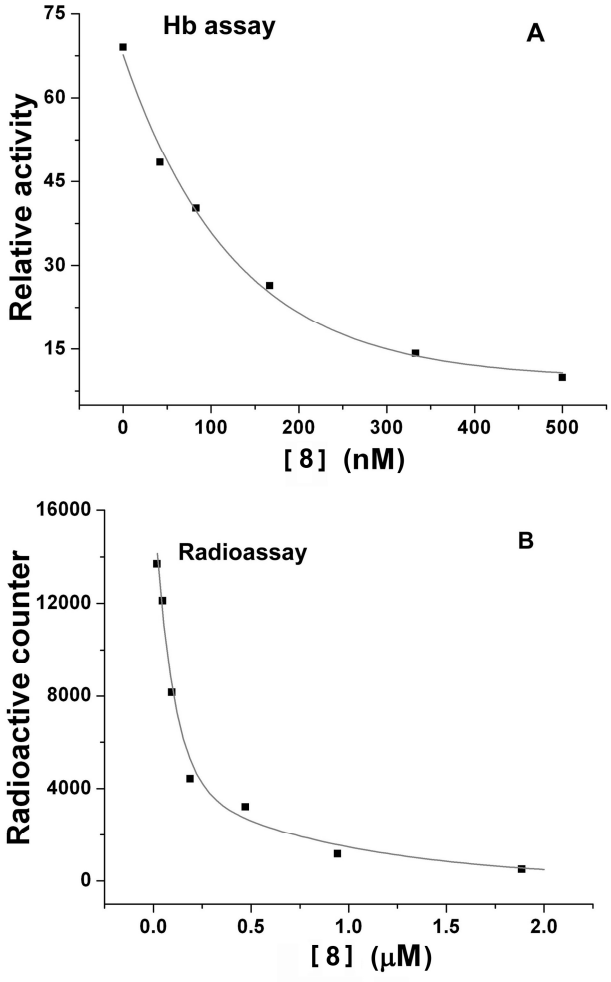
Supporting Information Figure 1. Sequence alignment for the oxygenase domains of rat nNOS and human nNOS. The amino acid sequences of NOS were retrieved from the PIR protein sequence database, and the sequences are human nNOS (entry P29475) and rat nNOS (entry P29476). Blue letters show the same amino acid residues. Black letters show different amino acid residues. Red letters show residues involved in substrate/inhibitor binding. There is only one amino acid difference in red letters, *i. e.,* L337 in rat nNOS and H342 in human nNOS.



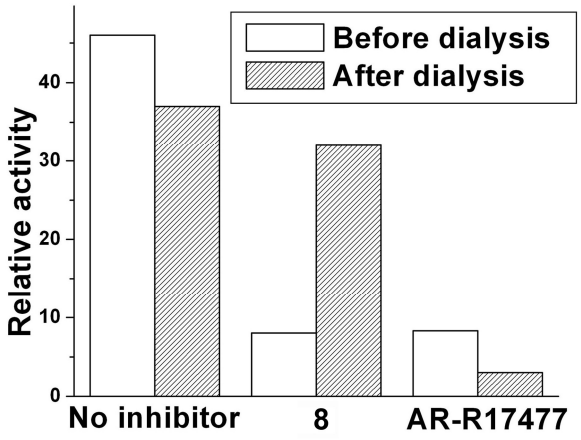
Supporting Information Figure 2. SDS-PAGE analysis of nNOS during purification. Lane 1: crude *E. coli* cell lysate after 2', 5'-ADP-Sepharose column purification; Lane 2: sample from lane 1 after CaM-Sepharose column purification; Lane M: prestained protein molecular weight markers; Lanes 3-5: human nNOS, L337H rat nNOS, and WT rat nNOS after the third Sephacryl S-300 column purification. Protein electrophoresis was carried in a 4-15% polyacrylamide gel, and separated proteins were stained with Coomassie G-250.



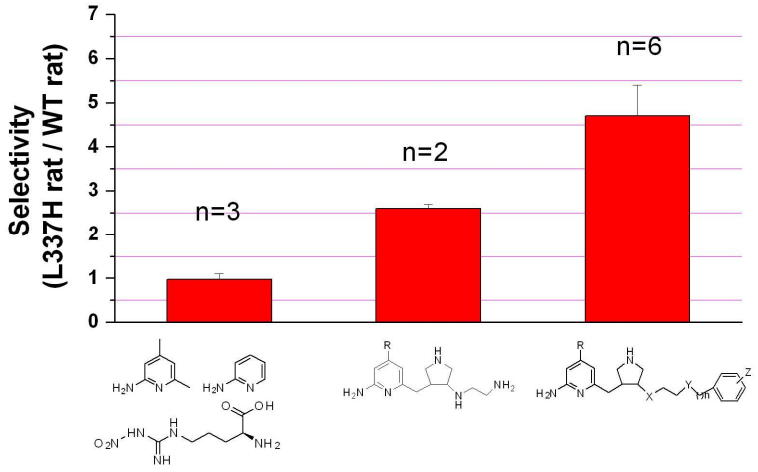
Supporting Information Figure 3. Determination of K_m and V_{max} values for the three enzymes. A. WT rat nNOS (1 μg) was incubated with different concentrations of its substrate and other cofactors. The rate of NO production was monitored by the Hb assay at room temperature. B. K_m and V_{max} values were obtained by a Lineweaver-Burk plot. Figures for human nNOS and L337H nNOS are not shown but all data are listed in Table 1 in the text.



Supporting Information Figure 4. Determination of inhibition efficiency of inhibitors. Different concentrations of compound **8** were introduced into the enzyme reaction mixture before addition of WT rat nNOS to initiate the enzymatic reaction, and the enzyme activity was monitored by (A) the Hb assay or (B) radioassay. IC_{50} values were derived by exponential decay fitting. Other figures are not shown but all data are summarized in Table 2 in the text.



Supporting Information Figure 5. Comparison of rat nNOS activity before and after removal of inhibitors by dialysis. Ten μg of WT rat nNOS was incubated with 1.4 μM of compound **8** or 1.4 μM of AR-R17477 in Hepes buffer (100 mM, pH 7.4) in a final volume of 1100 μL. Six hundred μL of the above mixture was transferred into a dialysis bag (10,000 molecular weight cutoff), and the dialysis bag was placed into 500 mL of dialysis buffer (1 mM DTT, 5 μM BH_4, 5% glycerol, and 1 μM L-arginine in 50 mM Hepes buffer, pH 7.4) on ice with occasional agitation. The remaining enzyme and inhibitor mixture (500 μL) was kept on ice. After 3 h, 300 μL of the reaction mixture was removed to assay the enzyme activity by the Hb assay. To assess the stability of rat nNOS during dialysis, the enzyme was treated with the same conditions as above but without inhibitor.



Supporting Information Figure 6. Trend of selectivity in relation to the tail length of inhibitors. Data are expressed as mean ± SEM (Standard Error of the Mean).

Overexpression and purification of rat nNOS, human nNOS, and L337H rat nNOS

L337H rat nNOSpCW was cotransformed with pGroELS into the protease-deficient competent *E. coli* strain BL21 cells. Transformants were plated on LB agar containing ampicillin at 50 μg/mL and chloramphenicol at 35 μg/mL. A flask containing 1 L of modified Terrific Broth (20 g of yeast extract, 10 g of tryptone, 2.65 g of KH_2PO_4, 4.33g of Na_2HPO_4, and 4 mL of glycerol) and ampicillin (50 μg/mL) and chloramphenicol (35 μg/mL) were inoculated with 4 mL of overnight culture cells and shaken at 240 rpm at 37 ^oC. Protein expression was induced at OD_{600} around 1.2 with the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.5 mM. The flask was kept at 25 ^oC and shaken at 240 rpm in the dark. The cells were harvested at about 40 h after induction and lysed in 30 mL of lysis buffer (50 mM Tris, pH 8.0/ 50 mM NaCl/ 1 mM

EDTA/ 0.5 mM L-arginine/ complete protease inhibitors and 1 mg/mL lysozyme). After 1 h on ice, DTT, BH_4, and glycerol were added to reach final concentrations of 1 mM, 10 μM, and 10% (v/v), respectively. Then the lysis solution was further treated by pulsed sonication. Cell debris was removed by centrifugation at 150,000 x g for 1 h at 4 ^{o}C. The enzyme activity in the supernatant was measured by the Hb assay. The supernatant was applied to a 2',5'–ADP-Sepharose column first, and the bound enzyme was eluted with $NADP^+$. After addition of $CaCl_2$, the eluate was directly loaded onto a CaM-Sepharose column, and the bound enzyme was eluted with EGTA. Finally, the eluted enzyme was passed through a Sephacryl S-300 column. The eluted enzyme activity was measured by the Hb assay, and fractions with high activity were combined. The enzyme was aliquoted and kept at –80 ^{o}C. The entire purification process was performed at 4 ^{o}C. Using the same procedure, WT rat nNOS and human nNOS were also purified. The purity of enzymes was analyzed by SDS-PAGE, and the protein concentration was measured by SDS-PAGE using BSA as a standard.