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Identification of a Flavin Mononucleotide Module Residue Critical for Activity of Inducible Nitrite Oxide Synthase

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Inducible NO synthase (iNOS) contains an amino-terminal oxygenase domain, a carboxy-terminal reductase domain, and an intervening calmodulin-binding domain. For the synthesis of NO, iNOS is active as a homodimer formed by oxygenase domains, while the reductase domain is required to transfer electrons from NADPH. In this study, we identify glutamate 658 in the FMN domain of human iNOS to be a critical residue for iNOS activity and we explore the underlying mechanism for such role. Mutation of glutamate to aspartate almost abolished iNOS activity and reduced dimer formation. Substitution of this residue with non-charged alanine and glutamine, or positively charged lysine did not affect dimer formation and maintained around 60% of iNOS activity. These results suggest that the negative charge specific to glutamate plays an important role in iNOS activity. The Journal of Immunology, 2009, 183: 5977–5982.

N O is an important signaling and cytotoxic molecule that is synthesized from L-arginine by isozymes of NO synthase (NOS),1 the constitutive endothelial and neuronal and the high-output inducible enzymes (1–5). The latter form, termed inducible NOS (iNOS), is widely expressed in diverse cell types under transcriptional regulation by inflammatory mediators (2, 6). iNOS has been implicated in the pathogenesis of many diseases, some of which include Alzheimer’s disease, tuberculosis, asthma, glaucoma, inflammatory bowel disease, stroke, and septic shock (7, 8).

Like all NOSs, iNOS has three domains: 1) an amino-terminal oxygenase domain (residues 1–504) that binds heme, tetrahydrobuioterin (HbB), and L-arginine; 2) a carboxy-terminal reductase domain (residues 537–1153) that binds flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and NADPH; and 3) an intervening calmodulin binding domain (residues 505–536) that regulates electron transfer between the oxygenase and reductase domains (9–12).

The oxygenase domains are critical for dimer formation (3–5, 10). The iNOS reductase domain belongs to dual-flavin reductase that contains an N-terminal FMN containing module and a carboxy-terminal NADPH- and FAD-binding module (13). The FMN module swings back and forth to transfer electrons from FAD-binding domain to heme (13–15). In addition to electron transport, this structural flexibility of FMN module is supposed to play a role in triggering protein conformational change. In this study, we identify residue E658 in the FMN domain of iNOS to be a critical residue for iNOS activity and we explore the underlying mechanisms for such role.

Materials and Methods
iNOS induction and immunoprecipitation

The murine macrophage-like cell line RAW 264.7 was cultured at 37°C in 5% CO2 in DMEM (Mediatech) supplemented with 10% heat-inactivated FBS (16, 17). iNOS expression was induced by incubating cells in the presence of LPS (100 ng/ml) and murine IFN-γ (10 U/ml) for 16 h. iNOS was immunoprecipitated from cell lysates with anti-iNOS Ab.

Mass spectrometry

Immunoprecipitated iNOS was subjected to SDS/PAGE. In-gel digestion and nano-HPLC/MS/MS for methylation site mapping was conducted. Gels were stained with GelCode Blue Stain Reagent (Pierce). The iNOS protein bands were excised, destained with 50 mM ammonium bicarbonate solution in 50% methanol, and washed in HPLC-grade water overnight. Gel pieces were then digested with 100 ng of trypsin in 50 mM NH4HCO3 (pH 8.5) for 4 h in a volume of 15 μl. After digestion, peptides were extracted by the addition of 200 μl of acetonitrile. The supernatants were dried in a Speed-Vac dryer. Each dried sample was dissolved in 20 μl of 5% methanol/95% water/0.1% formic acid solution and injected into Surveyor HPLC system (ThermoFinnigan) using autosampler. An 100 mm × 75 μm, C18 column (5 μm, 300 Å pore diameter, PicoFrit, New Objective) with mobile phases of A (0.1% formic acid in water) and B (0.1% formic acid in methanol) was used with a gradient of 5–95% of mobile phase B over 15 min followed by 95% B for 5 min at a flow rate of 200 nl/min. Peptides were directly elutrospayed into mass spectrometer (Finnigan LTQ, ThermoFinnigan) using nano-spray source. LTQ were operated in the data-dependant mode acquiring fragmentation spectra of the top 20 strongest ions. Obtained MS/MS spectra were analyzed against modified NCBI-ref protein sequence database. Each database modified with mass increase of methylation of glutamate methylation using BioWorks database search engine (BioWorksBrowser ver 3.2, Thermo Electron). All methylated peptide identification with stringent BioWorksBrowser filtering criteria, peptide probability >5 × 10−5 and Xcorr score >4.0, was manually examined and all methylation sites have to be identified by consecutive b- or y-ions so that false identifications were eliminated (18).

Stable isotope labeling by amino acids in cell culture (SILAC) in vivo determination of methylation

SILAC was conducted according to One et al. (19). SILAC labeling medium is DMEM (Invitrogen) lacking methionine reconstituted with [13C6]methionine (Sigma-Aldrich) and supplemented with 10% dialyzed...
FBS (Invitrogen). RAW 264.7 cells were cultured in SILAC labeling medium for 5–7 days to allow adaptation and full incorporation of the stable isotope-containing amino acids.

**Histone extraction**

Histone extraction was done as previously described (20). The cells growing in SILAC medium were collected and lysed on ice for 30 min in PBS containing 1% Triton-X 100 in the presence of protease inhibitors mixture (BD Biosciences). Lysates were centrifuged at 2000 rpm for 10 min at 4°C. The pellet was washed with the same buffer and resuspended in 0.2% HCl to acid extract the histones overnight at 4°C. The sample was centrifuged at 2000 rpm for 10 min at 4°C. The supernatant containing histone proteins was used to analysis histone methylation with mass spectrometry to confirm the effective SILAC procedure.

**Mutagenesis and transfection**

cDNA of human iNOS was inserted into the expression vector pcDNA 3.1 Directional TOPO (Invitrogen). Site-directed mutagenesis was performed by using the QuikChange site-directed mutagenesis kit (Stratagene) (16, 17). All mutations were confirmed by DNA sequence analysis. C-terminal truncation mutations of human iNOS or E658D mutant iNOS were generated by PCR. Human embryonic kidney (HEK)-293 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Cell lysis and Western analysis**

Cells were lysed on ice for 30 min in 40 mM Bis-Tris propane buffer (pH 7.7), 150 mM NaCl, 10% glycerol, 3 mM DTT, and 1% Triton X-100, in the presence of protease inhibitors. Lysates were centrifuged (5 min, 4°C), and supernatants were used for Western analysis (21).

**Partial purification of iNOS**

All purification steps were conducted at 4°C. HEK-293 cells, transfected with wild-type or mutant iNOS, were resuspended in buffer A (40 mM Bis-Tris propane (pH, 7.7), 150 mM NaCl, 10% glycerol, 3 mM DTT, 1 mM t-arginine, 4 mM BH4, 4 mM FAD, and 0.1 mM PMSF), and then disrupted with sonication for 10 s, 10 times. Cell lysates were centrifuged at 10,000 × g for 15 min. The supernatant was applied to a 2.5’-Sepharose 4B column (Amersham Pharmacia) equilibrated with buffer A. The column was successively washed with buffer A and buffer A containing 500 mM NaCl. iNOS was eluted with buffer A containing 4 mM NADPH (17, 22). iNOS-containing fractions were pooled and concentrated using Centricon YM-30 (Millipore).

**Gel-permeation chromatography**

Size-exclusion chromatography was done at 4°C using a Pharmacia Superdex 200 HR column and a fast performance liquid chromatography system (Amersham Pharmacia) (16, 17, 21). The column was equilibrated with buffer A. Partially purified iNOS (30 μg) was injected in 200 μl sample volume, and proteins in the column effluent were monitored at 280 nm (16, 17, 21). Collected fractions were mixed with laemmi sample buffer immediately or stored at −80°C for Western analysis. The column was calibrated with the following Mₙ standards (Pharmacia): thyroglobulin (669,000), ferritin (440,000), catalase (232,000), aldolase (158,000), albumin (67,000), OVA (43,000), chymotrypsinogen A (25,000), and RNase A (13,700).

**Immunofluorescence**

Cells were grown on poly (D-lysine)-coated 22-mm glass coverslips to 70% confluence in six-well clusters. Cells were washed with PBS containing 1.2% sucrose and fixed with 4% formaldehyde at room temperature for 10 min. iNOS was immunolabeled by Anti-iNOS mAb 5D5-H7 (Research and Diagnostic Antibody) and a goat anti-mouse conjugated to Alexa Fluor 488 (green). Coverslips were mounted by using the SlowFade Gold anti-fade reagent with DAPI (Invitrogen) and viewed by a Zeiss Axioblock microscope. Imaging was performed by using a Zeiss 100 (1.4 numerical aperture) oil immersion lens, and Z sections were collected at an optical depth of 0.2 μm. Images were optimized by deconvolution software (23).

**Results**

Our initial experimental approach was directed toward characterization of novel posttranslational modifications in iNOS. Specifically, we studied potential methylation sites in iNOS. Protein methylation is an important posttranslational modification. Most protein methylation reported to involve lysine, arginine, and histidine, whereas glutamate methylation is only found in membrane receptor proteins in bacteria (24). We used iNOS, immunoprecipitated from lysates of mouse macrophage RAW 264.7 cells following their stimulation with LPS and IFN-γ, iNOS was subjected to HPLC/MS/MS analysis (18). We detected an unmodified form of a murine iNOS tryptic peptide (m/z 896.7 with sequence 589LSHL GASQLAPTGEDELGSGQEDAFR664) at the retention time of 23.99 min (supplementary Fig. S1A). Interestingly, we also found two modified counterparts (m/z 901.3 and 901.2) at the same peptide as the retention of 34.06 and 23.44 min, respectively (supplementary Fig. S1, B and C). The major difference of these two different kinds of spectra is +14 mass shifts suggesting methylation as a potential modification. A comparison of the three MS/MS spectra conclusively localized the modified sites to E652 and E660 in murine iNOS. Murine residue E652 corresponds to human iNOS E658 and is conserved in both iNOS nNOS (Fig. 1). Murine E660 (human E666) is conserved in all NOSs. We then tried to confirm the methylation of the above two sites in live cells. We used SILAC for in vivo incorporation of “heavy” methyl group donated by [13CD₃]S-adenosyl methionine, which is metabolically converted from supplemented [13CD₃]methionine in culture medium (19). However, multiple experimental attempts failed to detect heavy methyl incorporation in cultured cells subjected to SILAC. Effective SILAC procedure was confirmed by mass spectrometry analysis demonstrating concomitant heavy methylation of histones (data not shown). Therefore, we concluded two possibilities. The first one is that detection of methylation on immunoprecipitated iNOS was merely an artifact induced during sample processing. The second possibility is that methylation of those residues occur nonenzymatically and thus independent of the donor [13CD₃]S-adenosyl methionine (18). Nevertheless, although we could not confirm or exclude the possibility of glutamate methylation of iNOS, functional characterization of these residues provided previously unexpected critical role of human iNOS residue E658 in NO production.

**Identification of residue E658 as critical for human iNOS activity**

Human iNOS residues E658 and E666 are both in the FMN module of iNOS reductase domain (9–11, 14). Crystal structures of nNOS reductase domain suggest that the FMN module is electronegative, implying charge-enabling function (13–15). Methylation is known to exert regulatory effects by neutralizing negative charges (24). We therefore, substituted the negatively charged glutamate residues E658 and E666 in human iNOS with the negatively charged amino acid, aspartate (D). The glutamate to aspartate mutation was chosen because it preserves both the structure and the negative charge but it eliminates the possibility of charge silencing by a potential glutamate-specific methylation. The iNOS

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**FIGURE 1.** Sequence alignment of iNOS residues. * and # denote studied glutamate residues E658 and E666 of human iNOS, respectively.

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*The online version of this article contains supplemental material.*
mutants were characterized after their expression in HEK293 cells, which do not express any endogenous NOS genes. Whereas no significant change in NO production was observed in cells transfected with iNOS mutant E666D, compared with wild-type iNOS, cells transfected with iNOS mutant E658D showed a dramatic decrease by around 90% in NO accumulation in the culture medium (Fig. 2).

**iNOS mutant E658D does not form dimers on SDS/PAGE**

For NO synthesis, NOS is active only as a homodimer (10, 17). Previously, we have reported that iNOS can form “undisruptable” dimers on SDS/PAGE and that those dimers could not be observed in dimerization-defective iNOS mutants (21). Because iNOS E658 mutant was in essence inactive in producing NO, we evaluated its ability to form dimers on SDS/PAGE. In contrast to wild-type iNOS and to iNOS mutant E666D, no dimers were detected in cell lysates transfected with iNOS mutant E658D (Fig. 3). These data indicate that iNOS E658D inability to produce NO is associated with a defect in dimer formation.

**Detailed mutational analysis of E658 residue reveals striking effect of E to D mutation**

To further study the mechanism of charge-enabling function E658, we mutated that residue to either neutral alanine (A) or glutamine (Q), or to a positively charged lysine (K). Interestingly, HEK293 cells transfected with any of the three iNOS mutants E658A, E658K, or E658Q exhibited around 60% NO production compared with that transfected with wild-type iNOS, but significantly higher than cells transfected with iNOS mutant E658D (Fig. 4A). Consistent with the NO production data, and in contrast to iNOS E658D mutant, all three iNOS mutants E658 to A, K or Q exhibited dimers on SDS/PAGE detected by Western analysis (Fig. 4B). These results suggest that the negative charge on E658 may function as a reversible negative regulator in iNOS activity. Silencing of this negative charge, e.g., by methylation would be required for iNOS activity.

Regarding the general importance of this residue, we further studied the sensitivity of iNOS mutants to BBS-2, a selective iNOS dimerization inhibitor (25, 26). We observed that 2 μM BBS-2 decreased NO production around 75% by wild-type iNOS or iNOS mutants E658A, E658K, and E658Q (supplementary Fig. S2). This result indicated that the dimers formed by iNOS mutants were similar to those formed by wild-type iNOS in terms of antagonist sensitivity.

**Gel-permeation chromatography confirms dimerization defect of iNOS E658D**

To further study dimer formation by iNOS mutants, we partially purified iNOS or iNOS mutants from HEK293-transfected cells and subjected the purified proteins to size analysis by size exclusion chromatography under conditions that maximize iNOS dimer formation (10, 17). As expected, wild-type iNOS eluted mostly as a dimer with molecular mass between 232 and 440 kDa (Fig. 5). Similar results were obtained with iNOS mutants E658A, E658K, and E658Q. In contrast, most E658D iNOS mutant eluted at a molecular mass bigger than 440 kDa or lower than 232 kDa, which indicated a shift to multimer and monomer, respectively, as previously shown in iNOS dimerization defective mutants (16). These data confirm that the substitution of Glu-658 with aspartate affected iNOS dimerization.

**Cellular augmentation of tetrahydrobiopterin does not rescue iNOS E658 activity**

To further characterize the defect in iNOS activity and dimerization, we evaluated its response to increased levels of cellular H4B.
Tetrahydrobiopterin is an important cofactor for all NOSs (24, 27). It is generally accepted that H4B binding plays a role in stability of NOS dimers (5, 22, 28). Augmentation of H4B cellular level was done by the addition of sepiapterin, a stable precursor of H4B (29, 30). Although the levels of cellular H4B in HEK293 are not known to be limiting factors in NO synthesis by iNOS, sepiapterin addition significantly increased NO production of cells transfected with wild-type iNOS but not in those transfected with iNOS mutant E658D (Fig. 6). These results suggest that increased levels of H4B did not have significant effect on iNOS E658D activity.

Discussion

In this study, we show that the substitution of the iNOS E-658 with negatively charged aspartate dramatically affected NO synthesis mediated via exaggeration of C terminus-flavins interaction. To test this hypothesis, we removed the 23-amino acids C-terminal tail of human iNOS as previously done by Roman et al. for murine iNOS (31) and determined whether this truncation could restore the activity of iNOS E658D mutant. However, this truncation did not rescue the activity of E658D or its dimerization when tested by transfecting the truncated mutant in HEK293 cells and analyzing nitrite accumulation and dimer formation by Western blot. However, truncated E658D iNOS mutant remained inactive and could not form dimers (Fig. 7, A and B, respectively). These results suggest that the C terminus of iNOS is not likely to be involved in the loss of activity of E658D iNOS mutant. Interestingly, C-terminal truncation of wild-type iNOS significantly reduced NO synthesis in transfected cultured cells. These results are at variance with data previously obtained for recombinant murine iNOS tested in vitro (31). This variance might represent difference between conditions in vitro and cultured cells.

E658 mutation does not affect subcellular localization of iNOS

We have previously shown that iNOS is regulated by its subcellular sequestration forming an aggresome in perinculear region (23). We tested whether loss of activity of iNOS E658D mutant was caused by altered subcellular localization. We then use immunofluorescence to examine subcellular localization of iNOS and iNOS mutants E658D, E658A, E658K, or E658Q in HEK 293T cells. There were no detected differences in subcellular localization between wild-type iNOS and any of iNOS mutants tested (supplementary Fig. S3).

FIGURE 5. Evaluation of effect of various mutations of E658 on iNOS dimerization analyzed by gel permeation chromatography. HEK 293T cells were transfected with plasmid containing LacZ, wild-type iNOS (iNOS wt), or one of the iNOS mutants E658D, E658A, E658K, or E666Q. Sixteen hours following transfection, iNOS or iNOS mutants were purified from cell lysates using 2’5’ ADP-Sepharose 4B column and subjected to a fast performance liquid chromatography system using a Superdex 200 HR column in the presence of 3 mM DTT, 1 mM L-arginine, 4 mM BH4, and 4 μM FAD. Fractions eluted between 8 and 14.5 ml were evaluated by Western blot analysis with iNOS Ab. Arrows indicate molecular masse of corresponding fractions according to Mr standards.

FIGURE 6. Effect of BH4 precursor sepiapterin on iNOS or iNOS E658D mutant activity. HEK 293T cells were transfected with plasmid containing LacZ, wild-type iNOS (iNOS wt), or iNOS mutant E658D and incubated in the presence or absence of 10 μM sepiapterin. Nitrite accumulation in the culture medium was assessed 16 h after transfection. Data represent means ± SD; n = 3; *, p < 0.05; †, p > 0.05 for sepiapterin treatment compared with control conditions, respectively.

FIGURE 7. Effect of C terminus truncation on NO production and dimerization of iNOS and iNOS mutant E658D. HEK 293T cells were transfected with plasmid containing LacZ, wild-type iNOS (iNOS wt), and iNOS mutant E658D. Parallel experiments were done using iNOS or iNOS E658D with deletion of the last 23 amino acid (iNOS wt-tr or E658D-tr, respectively). A, Nitrite accumulation in the culture medium was assessed 16 h after transfection. Data are means ± SD, n = 3; **, p < 0.001; †, p > 0.05 compared with nontruncated controls, respectively. B, Cell lysates (50 μg) were evaluated by Western blot analysis with Abs against iNOS or GAPDH.
activity and reduced iNOS dimerization. However, the replacement of this residue with noncharged alanine, glutamine, or positively charged lysine did not have such dramatic effect on iNOS activity. These results identify E658 in iNOS as a previously unsuspected critical residue for iNOS activity. They also propose an intriguing regulatory mechanism for NO synthesis by suggesting that the negative charge in E658 serves as a “silencer” for iNOS activity and/or dimerization. For iNOS to produce NO, the “silencer” will have to be neutralized, e.g., by methylation or other yet to be revealed mechanism. In iNOS E658D mutant, although aspartate is negatively charged, the resulting loss of iNOS activity suggest that the mechanism of slicing glutamate negative charge is residue-specific. Nevertheless, the structural change from glutamate to alanine, glutamine, or lysine still reduced NO synthesis by ~40%, suggesting that E658 is critical for iNOS active conformation. Taken together, our results indicate that E658 is a critical residue for iNOS activity and its negative charge might be an important negative regulator for NO synthesis. In this context, Panda et al. (15) recently reported that the substitution of E762 (E546 in human iNOS) of rat nNOS on noncharged asparagin led to a two fold increase in NO synthesis, suggesting that the negative charge of that glutamate residue might be a negative regulator for NOS activity.

In the FMN domain of the NOS reductase crystallographic structure for rat nNOS, E-916 (equivalent to human iNOS E-658) is located in the edge strand of the central β-sheet (14). The negatively charged E-916 side chain forms a salt bridge with H-897 (equivalent to human iNOS 639) in the previous α-helix (corresponding to iNOS AFAHIDDQLSH-645 of Fig. 1), presumably helping to anchor and align this helix with the underlying β-sheet framework. The α-helix containing H-897 begins at the buried end of the FMN cofactor, where residues of the previous loop (corresponding to human iNOS SSMPQFC) bind and tune FMN. The first and last main-chain nitrogen atoms of this loop hydrogen bond with FMN O₂, whereas their conserved Ser and Cys side chains contribute a hydrogen bond and hydrophobic packing, respectively, to the loop conformation. Cys and preceding conserved Phe are in van der Waals contact with the E-916 side chain. Conserved Tyr π-stacks with the isoalloxazine ring and, together with the subsequent conserved Pro and their flanking residues, participates in the interface between the FMN- and FAD-binding domains.

Why does the Glu-to-Asp mutation nearly abolish iNOS activity, whereas substitution with Ala, Gln, or Lys leads to less reduction in activity? How could Asp at this position be so detrimental, while seemingly less conservative mutations (like Ala) have much milder effects? There are several possibilities. Asp still forms a salt bridge with His, but the shorter side chain leads to distortions that impact orientation of the His-containing helix with respect to the underlying β-sheet, or disturb the FMN binding loop described above. The Asp side chain might fail to make the salt bridge with His, but instead make an alternative interaction that distorts the FMN binding site or the FMN-binding domain fold, leading to similar consequences. Could mutant Asp side chain cause FMN redox changes via 885 Gly-Ser 886 and/or Phc-Cys peptide flips? If Gly-Ser peptide flips, the hydrogen bond from Ser NH to FMN O₂ could be swapped with hydrogen bond from redox active FMN N1 to Gly C = O.

The Glu mutations could affect the interface between the FMN and FAD domains and/or the interaction of the FMN domain with the oxygenase module, when delivering electrons to the heme (through the “back face” of the heme, opposite from the Arg binding pocket, in our model). If so, the mutations could shift the equilibrium for the “swinging” FMN domain. Changing this equilibrium might in itself destabilize the dimer. Each FMN domain participates in some interactions across the interface of the reductase dimer. Because the reductase module operates in trans, when delivering electrons to the oxygenase module, so direct interactions across the dimer interface are expected there as well.

Previous studies have revealed that iNOS oxygenase domain is responsible for forming the dimer interface (32–34). Based on the crystal structure available (32–34), E658D is not part of the dimer interface but rather it is on the solvent-exposed part of NOS. It is not clear why E658D mutant would not form dimers. The presence of E658 on the surface of iNOS suggests a possible role in protein-protein interaction. Recent studies suggested mobility of the FMN region during NO synthesis, which entails interaction between the FMN module and the oxygenase domain (15). The exact residues involved in this interaction are not yet determined. Our study suggests that E658 might be a candidate for such role. Finally, this study reveals a previously unsuspected critical residue for activity of iNOS and shed further light on the importance of the FMN module for such activity.

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Disclosures

The authors have no financial conflict of interest.

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