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Complementation analysis of mutants of nitric oxide synthase reveals that the active site requires two hemes

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ABSTRACT **For catalytic activity, nitric oxide synthases (NOSs) must be dimeric. Previous work revealed that the requirements for stable dimerization included binding of tetrahydrobiopterin (BH_4), arginine, and heme. Here we asked what function is served by dimerization. We assessed the ability of individually inactive mutants of mouse inducible NOS (iNOS; NOS2), each deficient in binding a particular cofactor or cosubstrate, to complement each other by generating NO upon cotransfection into human epithelial cells. The ability of the mutants to homodimerize was gauged by gel filtration and/or PAGE under partially denaturing conditions, both followed by immunoblot. Their ability to heterodimerize was assessed by coimmunoprecipitation. Heterodimers that contained only one COOH-terminal hemimer and only one BH_4-binding site could both form and function, even though the NADPH-, FAD-, and FMN-binding domains (in the COOH-terminal hemimer) and the BH_4-binding sites (in the NH_2-terminal hemimer) were contributed by opposite chains. Heterodimers that contained only one heme-binding site (Cys-194) could also form, either in cis or in trans to the nucleotide-binding domains. However, for NO production, both chains had to bind heme. Thus, NO production by iNOS requires dimerization because the active site requires two hemes.**

Nitric oxide synthases (NOSs) (EC 1.14.13.39) are heme-containing (1–3), dimeric (4–7) flavoproteins that catalyze the oxidation of L-arginine to form L-citrulline and nitric oxide (NO), a radical with diverse physiological roles (7–10). Catalysis involves sequential electron transfer from NADPH to FAD, FMN, heme, and oxygen (11). Tetrahydrobiopterin (BH_4) is also required (7, 9, 11) and may serve to protect the heme from NO (12). Three isoforms–NOS1, NOS2 or iNOS, and NOS3–have been cloned from mammals (13), and there are avian (14) and insect (15) homologs. The basic organization of the NOS structure appears to be conserved: the COOH-terminal hemimer binds the three nucleotides; the basic, hydrophobic central peptide is postulated to attach calmodulin (7, 11); and the NH_2-terminal hemimer binds BH_4, heme, and L-arginine (16–19) and by itself can homodimerize (16, 17).

Deletion and mutation analyses have begun to delineate the individual residues required for binding of cosubstrates and prosthetic groups. Residues 1121–1144 of mouse iNOS, comprising its extreme COOH terminus, are essential for the binding of NADPH (20). For example, in a mutant termed LS, replacement of these residues with a different sequence resulted in abolition of enzymatic activity and NADPH binding, without affecting dimerization or the binding of calmodulin or heme (20). In contrast, in the NH_2-terminal hemidomain, mutants G450A and A453I were inactive because they failed to bind BH_4 or dimerize; these mutants, however, still bound NADPH, calmodulin, and heme (21). Finally, mutation of Cys-184 abolished the binding of heme in human NOS3 (18), as did mutation of the corresponding Cys-415 in rat NOS1 (16, 19). In this work, we extended the latter observation to the cognate Cys-194 in mouse iNOS. The aforementioned iNOS mutants and additional constructs described below provided tools with which to map functional interactions between monomers. We asked which mutants could homo- and heterodimerize, and which could complement each other's function upon cotransfection.

EXPERIMENTAL PROCEDURES

Plasmids. Fig. 1 describes the iNOS coding region of the plasmids used and summarizes the properties of the expressed proteins. Wild-type L8 and mutants S2, LS, Lm3, G450A, and A453I were constructed previously (20, 21). The mutant C194A was constructed through PCR mutagenesis, changing the nucleotide sequence *TGC* encoding Cys-194 to *GCC*, encoding Ala. After confirmation by DNA sequencing, the mutated iNOS cDNA was subcloned into the vector pcDNAI/Amp (Invitrogen). Mutant N(1–619) was constructed from Lm4a (20), which was first digested by *Afl*III and then by *Bal*31. The product, after further digestion by *Eco*RV, was self-ligated and transformed into *Escherichia coli* strain XL2-Blue (Stratagene). Plasmids from transformants were analyzed for the 3′ deletion and verified by DNA sequencing. N(1–619) consists of the NH_2-terminal 619 amino acid residues of L8 fused with residue Ile (due to the *Eco*RV site) followed by a stop codon (TGA) and the 3′-untranslated region (20). The double mutant N(1–619)C194A was constructed by introducing the C194A mutation into the COOH-terminal truncation mutant, N(1–619).

Cell Cultures and Transfection. The 293 human embryonic renal epithelial cell line (American Type Culture Collection) was cultured in DMEM (Sigma, D5523) supplemented with 10% horse serum, 200 units/ml penicillin, and 200 μg/ml streptomycin at 37°C in 5% CO_2/95% air. Transient transfection of 293 cells was performed with calcium phosphate (20).

NOS Activity. NOS activity was determined for intact cells and their lysates. For intact cells, 100 μl of culture medium was placed in a well of a 96-well plate and mixed with 100 μl of Griess's reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2.5% H_3PO_4), with dilutions of sodium nitrite as standards. Absorbance at 550 nm was recorded in a microplate reader (Dynatech). For enzyme assays transfected cells were lysed by freeze-thaw in 40 mM Tris (pH 7.9) containing 2 mM arginine, 5 μg/ml each of the protease inhibitors aprotinin, leupeptin, pepstatin, and chymostatin, and 1 mM phenylmethylsulfonyl fluoride. The reaction volume of 100 μl per well contained lysate, 2 mM NADPH, 2 μM FAD, 3 μM BH_4, and 3 mM DTT. After incubation at 37°C for 3 h,

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Abbreviations: NOS, nitric oxide synthase; iNOS, inducible NOS; BH_4, tetrahydrobiopterin.

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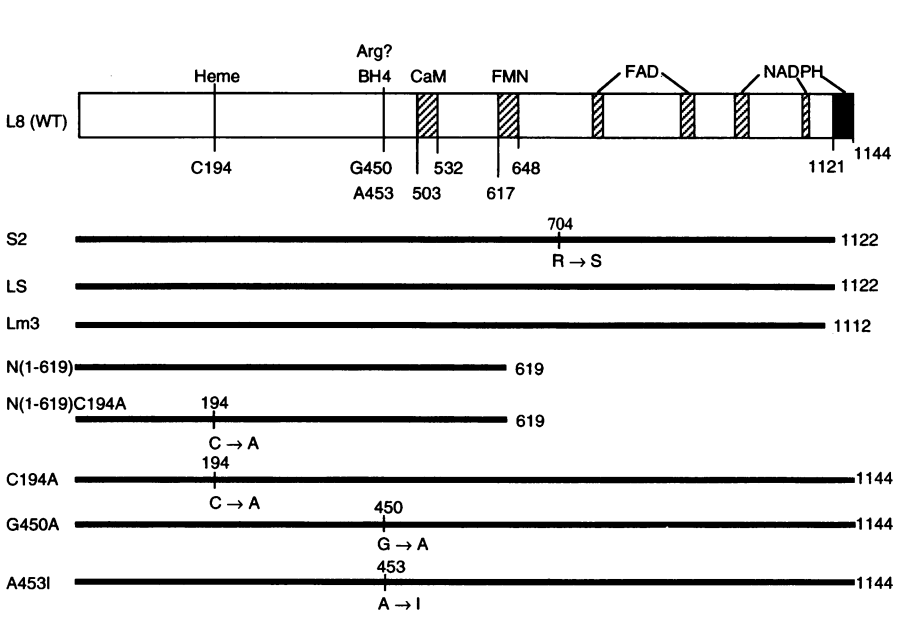

	NOS Activity	Binding of: NADPH	BH4	Heme	Dimer Formation	Ref.
L8 (WT)	+	+	+	+	+	20, 21
S2	-	-	nd[3]	+	+	20
LS	-	-	nd	+	+	20
Lm3	-	(-)[1]	nd	nd	+	20
N(1-619)	-	(-)[1]	nd	+	+	This study
N(1-619)C194A	-	(-)[1]	nd	(-)[4]	-	This study
C194A	-	(+)[2]	nd	-	-	This study
G450A	-	+	-	+	-	21
A453I	-	+	-	+	-	21

FIG. 1. Coding region of iNOS included in the plasmids; properties of the expressed proteins. (*Left*) The full-length (1144 amino acid), wild-type (WT) coding region in plasmid L8 is shown by the bar at top. Hatched boxes indicate putative binding domains for calmodulin (CaM), FMN, FAD, and NADPH as inferred by sequence analysis (22). Experimental evidence supports designation of the the filled box at the COOH terminus (1121–1144) as a component of the NADPH binding site (20), G450 and A453 as components of the BH_4-binding site (21), and C194 as a heme-binding residue (this study) (solid lines). Coding regions in mutant plasmids are shown by bold lines below L8, with numbers on the right indicating length. Point mutations are indicated. (*Right*) 1, NADPH binding was not determined. It is predicted that these mutants do not bind NADPH because the C′-terminal hemidomain that contains the NADPH-binding site was deleted. 2, NADPH binding was not determined. It is predicted that NADPH is bound because there is only one point mutation at Cys-194, a residue that is not involved in binding of NADPH. 3, Not determined. (4), Heme binding was not determined. It is predicted to be deficient based on results for C194A.

10 μl of lactic dehydrogenase (Sigma, L-2625 1:25 dilution in 500 mM sodium pyruvate) was added, followed 15 min later by Griess's reagent. Protein content of lysates was determined by the method of Bradford (23).

Antibodies. Antisera were raised in rabbits. Ab(iNOS) was raised against intact iNOS purified from RAW 264.7 cells (22). Ab(NO16) was raised against a thyroglobulin conjugate of the synthetic peptide (C-Nle-EEPKATRL) corresponding to the COOH terminus of wild- type iNOS (20).

Heme Assay. As described (21), cell lysate (1–2 mg protein) from transfected 293 cells was immunoprecipitated with Ab(iNOS) together with protein A-Sepharose (Sigma). An aliquot of the immunoprecipitate was reserved for immunoblot. The remainder was dried and boiled for 30 min in 0.5 ml of 2 M oxalic acid and cooled. Porphyrin fluorescence was analyzed in a Hitachi/Perkin–Elmer MPF2A fluorescence spectrophotometer (excitation, 400 nm; emission, 540–710 nm) in conjunction with heme standards.

Detection of Homodimer Formation. Proteins were separated by SDS/PAGE on 7.5% or gradient gels (as indicated) by a standard procedure (20) with modifications. A stock of 4× sample buffer (250 mM Tris, pH 6.8/8% SDS/4% 2-mercaptoethanol/0.004% bromphenol blue/40% glycerol) was heated at 100°C for 5 min, aliquoted, and stored at -70°C until use. Lysates were mixed with one-third vol of 4× sample buffer and the mixtures were either boiled for 3–5 min (fully denaturing conditions) or not boiled (partially denaturing conditions), as indicated, before being loaded on the gel. In both cases, prestained molecular weight standards (GIBCO/BRL) were boiled with sample buffer according to the manufacturer's instructions. After SDS/PAGE, proteins were electroblotted to 0.2 μm pore size nitrocellulose membranes (Schleicher & Schuell). The membrane was blocked in 5% nonfat milk in TBST (25 mM Tris, pH 7.5/150 mM NaCl/0.1% Tween 20) and incubated with the indicated antibody followed by washing with TBST. Detection was by means of an enhanced chemiluminescence system (Amersham).

Detection of Heterodimer Formation. Lysate (0.5–1 mg protein) from transfected cells was immunoprecipitated with Ab(NO16), which is directed against an epitope present in G450A and C194A but not N(1–619). The immunoprecipitate and an aliquot of the original lysate (60 μg protein) were subjected to SDS/PAGE and immunoblot with Ab(iNOS), which reacts with wild-type iNOS and all mutants used.

RESULTS

Experimental Strategy. To develop a panel of mutants for complementation analysis, we first added 3 mutants, C194A, N(1–619), and N(1–619)C194A, to those described in previous reports (20, 21). We then tested pairs of individually inactive mutants for their ability to produce NO when cotransfected, and for their ability to homo- and heterodimerize. Fig. 1 summarizes the characteristics of the panel when transfected individually.

Mutant C194A Lacks Heme and Fails to Homodimerize. Mutation of Cys-194 to Ala inactivated mouse iNOS: in six experiments, 293 cells transfected with wild-type iNOS produced 0.38 ± 0.06 nmol NO_2^- per min per mg lysate protein (mean ± SD), whereas those transfected with C194A produced 0.01 ± 0.00 nmol NO_2^-/ per min per mg lysate protein. The latter value corresponded to the background signal observed with cells transfected with vector pcDNAI alone. At the protein level, expression of C194A was not less than that of wild type, judging from immunoblots of the lysates (not shown) and the immunoprecipitates (Fig. 2*A*). Lack of activity of C194A most likely resulted from its failure to bind heme, as reflected in the absence of the porphyrin signal in the immunoprecipitate (Fig. 2*B*).

As expected, only monomers of iNOS (≈130 kDa) were detected on SDS/PAGE followed by immunoblot, when transfected cell lysate was mixed with sample buffer containing SDS and 2-mercaptoethanol and boiled before loading on the gel (fully denaturing conditions; Fig. 3*A*). However, when boiling

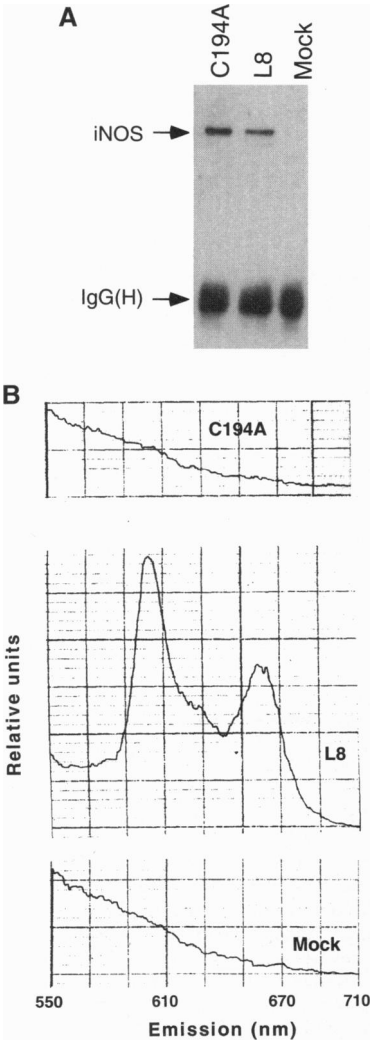

FIG. 2. Lack of heme in C194A. Lysates (1–2 mg) from 293 cells transfected with mutant C194A, wild-type piNOS-L8 (L8), or vector pcDNAI/Amp alone (mock) were immunoprecipitated with Ab (iNOS). (*A*) Five percent of the immunoprecipitate was analyzed by immunoblot after SDS/PAGE on a 7.5% gel under fully denaturing conditions. iNOS and the heavy chain of IgG (H) are indicated. (*B*) Ninety percent of the immunoprecipitate was used to detect porphyrin by fluorescence.

of the sample was omitted, an additional, more slowly migrating band (marked "dimer") was seen for the iNOS proteins from wild-type (L8) and short-form iNOS (S2) (20). Both have been shown by size-exclusion chromatography to elute as a mixture of dimers and monomers (20, 21). In contrast, only monomers were detected for the mutants G450A and A453I, which were previously characterized by chromatography as being dimer-free in conjunction with their inability to bind BH_4 (21) (Fig. 3*B*). The same partially denaturing SDS/PAGE analysis indicated that C194A failed to form dimers (Fig. 3*B*). The inability of C194A to homodimerize was also confirmed in a detergent-free setting by gel filtration chromatography on a Superdex 200 HR 10/30 column (Pharmacia) followed by immunoblot (21) (not shown).

COOH-Terminal Truncation Mutant N(1–619) Binds Heme and Homodimerizes. N(1–619) lacks the COOH-terminal 524 residues of iNOS, including all known binding sites for FAD, FMN, and NADPH, but it retains the wild-type NH_2-terminal 619 residues, including normal binding sites for heme and BH_4. Indeed, N(1–619) bound heme (Fig. 4*A*). Moreover, N(1–619) homodimerized, as judged by SDS/PAGE under partially denaturing conditions followed by immunoblot (Fig. 4*B*).

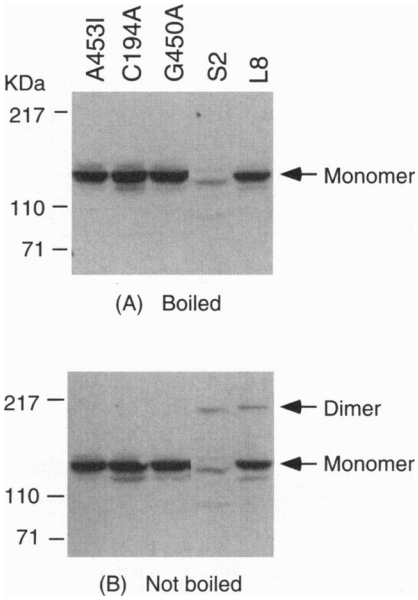

FIG. 3. Inability of C194A to homodimerize. Lysates (60 μg) from 293 cells transfected with wild type (L8), or the mutants S2, C194A, G450A, or A453I were subjected to SDS/PAGE in a gradient gel (6–15%) followed by immunoblot with Ab(iNOS). The lysates were mixed with sample buffer and loaded on the gel after boiling for 3–5 min (*A*) or without boiling (*B*). Positions of monomer and dimer are indicated.

Partial Functional Complementation Between the NADPH-Deficient, COOH-Terminal Mutant LS and the BH_4-Deficient, NH_2-Terminal Mutant G450A. The COOH-terminal mutant LS and the NH_2-terminal mutant G450A were each expressed as proteins of expected apparent molecular weight in 293 cells, whether transfected alone or together (not shown). No nitrite accumulated in the medium of cells transfected with either mutant alone. However, cotransfectants produced nitrite to a level 24% as much as wild type (Fig. 5*A*), and their lysates displayed NOS activity to a level 7.4% of wild type (Fig. 5*B*). Lm3, a mutant with deletion of the COOH-terminal 32 amino acids (20), also partially complemented G450A: NO_2^- accumulation in the medium was 24.3% of wild-type and NOS activity in the lysates was 9.3%. Results were similar with both LS and Lm3 when the partner G450A was replaced by another BH_4-deficient mutant, A453I (not shown).

Partial Functional Complementation Between the Nucleotide-Deficient, COOH-Terminal Truncation Mutant N(1–619) and the BH_4-Deficient, NH_2-Terminal Mutant G450A. The COOH-terminal mutant N(1–619) expressed a truncated iNOS protein that was well separated, by virtue of its lower molecular weight, from full-length iNOS protein such as L8 (Fig. 4*B*), as revealed by immunoblot with Ab(iNOS). N(1–619) was used to substitute for LS in the next set of experiments. Like LS, N(1–619) functionally complemented G450A. Cotransfected cells produced 41% as much nitrite as those transfected with wild-type iNOS (Fig. 6*A*), and their lysates displayed 14% as much NOS activity as with wild type (Fig. 6*B*).

Lack of Functional Complementation Between the Nucleotide-Deficient, COOH-Terminal Truncation Mutant N(1–619) and the Heme-Deficient, NH_2-Terminal Mutant C194A. In contrast, cotransfection of N(1–619) with C194A led to a level of apparent nitrite accumulation (Griess reactivity) that nearly matched that of cells transfected with vector alone (Fig. 6*A*) and was not affected by the presence of the NOS inhibitor N^{ω}-monomethyl-L-arginine. Moreover, the minimal Griess reactivity

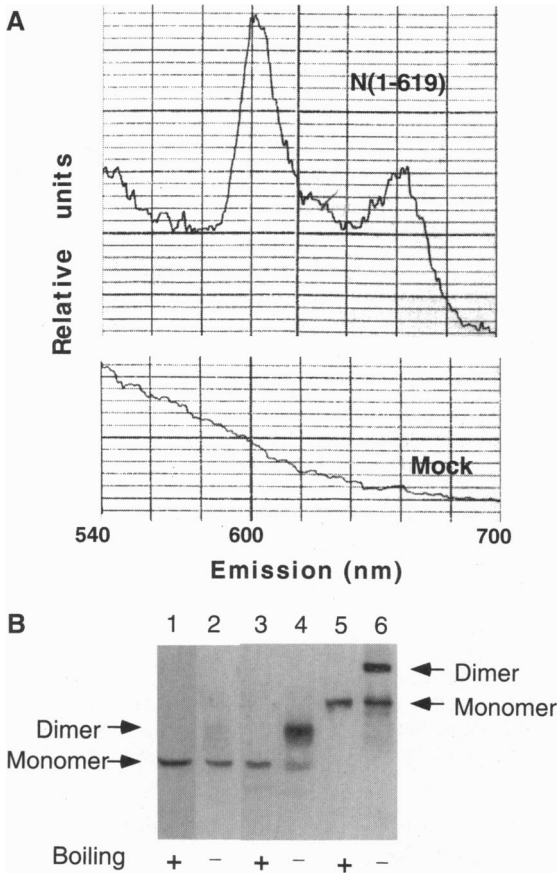

FIG. 4. Characterization of mutants N(1–619) and N(1–619)C194A. (*A*) Binding of heme in N(1–619). Cell lysates (1–2 mg) from 293 transfectant with mutant N(1–619) or vector alone (mock) were immunoprecipitated with Ab(iNOS). Ninety percent of the immunoprecipitates were used to detect porphyrin by fluorescence. (*B*) Homodimerization. Lysates from 293 cells transfected with N(1–619)C194A (lanes 1 and 2), N(1–619) (lanes 3 and 4), or L8 (lanes 5 and 6) were mixed with sample buffer, with (+; lanes 1, 3, and 5) or without (−; lanes 2, 4, and 6) boiling, and then subjected to SDS/PAGE followed by immunoblot with Ab(iNOS). Arrows indicate positions of monomers and dimers.

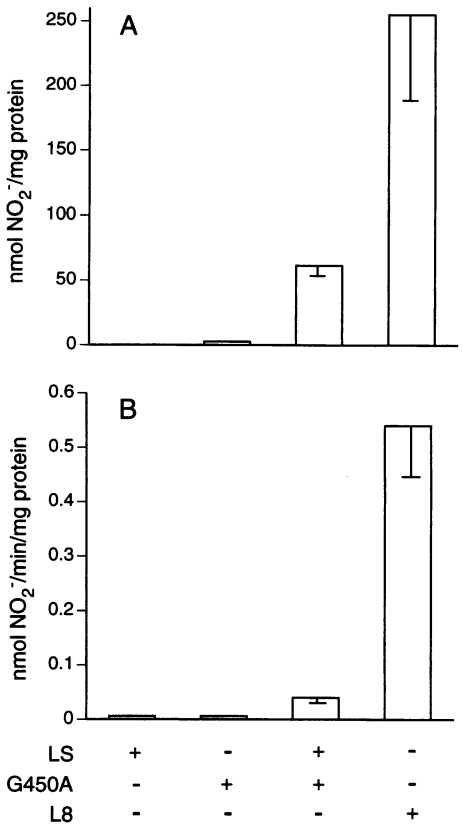

FIG. 5. Complementation between COOH-terminal mutant LS and NH_2-terminal mutant G450A. NOS activity is assessed by (*A*) nitrite accumulation in cell culture medium, and (*B*) NOS assay. Mutants LS alone, G450A alone, or the combination of LS and G450A were transfected into 293 cells. Wild-type L8 served as positive control. Nitrite was quantified in culture medium and results expressed as nmol per mg of cell protein, mean − SD for the number of experiments indicated in parentheses: LS (10), G450A (3), LS plus G450A (12), L8 (7). The cell lysates were used for enzyme assay in the same experiments and expressed in the same terms except on a per minute basis. Where error bars are not visible, they are smaller than the thickness of the line.

generated by lysates of these cotransfectants (Fig. 6*B*) did not increase with time of incubation (not shown).

Lack of Functional Complementation Between the Double Mutant N(1–619)C194A and the BH_4-Deficient, NH_2-Mutant G450A. In the preceding section, lack of complementation may have resulted from the inability of electrons to flow in trans from the nucleotide binding domains of C194A to the heme of N(1–619). Accordingly, we introduced the mutation Cys-194 → Ala into N(1–619) and asked if the resulting construct, N(1–619)C194A, acting as a source of BH_4, could complement G450A, acting as a source of both electrons and heme in cis. As shown in Fig. 6, cotransfection of N(1–619)C194A and G450A did not result in functional complementation.

COOH-Terminal Truncation Mutant N(1–619) Forms Heterodimers with Both G450A and C194A, and Double Mutant N(1–619)C194A also Heterodimerizes with G450. To evaluate the aforementioned results, it was necessary to investigate whether N(1–619) heterodimerized with G450A or C194A, and whether N(1–619)C194A heterodimerized with G450A. Lysates of 293 cells individually transfected with N(1–619), G450A, or C194A, or cotransfected with the pairs N(1–619) plus G450A or N(1–619) plus C194A, all contained the respective species of NOS proteins, as revealed by immunoblot with Ab(iNOS) (Fig. 7*A*, lanes 1–5). Aliquots of the same lysates were then immunoprecipitated with Ab(NO16), which only reacts with the wild-type COOH terminus. The latter is present in G450A and C194A, but not in N(1–619). When the immunoprecipitated products were subjected to SDS/PAGE followed by immunoblot with Ab(iNOS), N(1–619) did not appear in the lysates of 293 cells transfected with N(1–619), G450A, or C194A alone, but did appear in the lysates of cells cotransfected with N(1–619) plus G450A or N(1–619) plus C194A (Fig. 7*A*, lanes 6–10).

The same analysis was applied to cells transfected with the double mutant N(1–619)C194A. N(1–619)C194A appeared in the whole cell immunoblot, but did not appear in the Ab(NO16) immunoprecipitate unless cotransfected with G450A (Fig. 7*B*).

DISCUSSION

Conclusions with New Mutants Studied Individually. C194A lacked heme, strongly suggesting that the presumed thiolate ligand for heme in mouse iNOS is normally provided by this residue, consistent with mutation analysis of the cognate residues in NOS3 (18) and NOS1 (16, 19). The ability of C194A to heterodimerize militated against the alternative explanation—its structure was so grossly perturbed that heme was lost despite its covalent attachment to a different cysteine. Conversely, the inability of C194A to homodimerize in the intracellular milieu extends the previous observation that heme was necessary for dimerization by purified monomers in a nonphysiologic buffer (5).

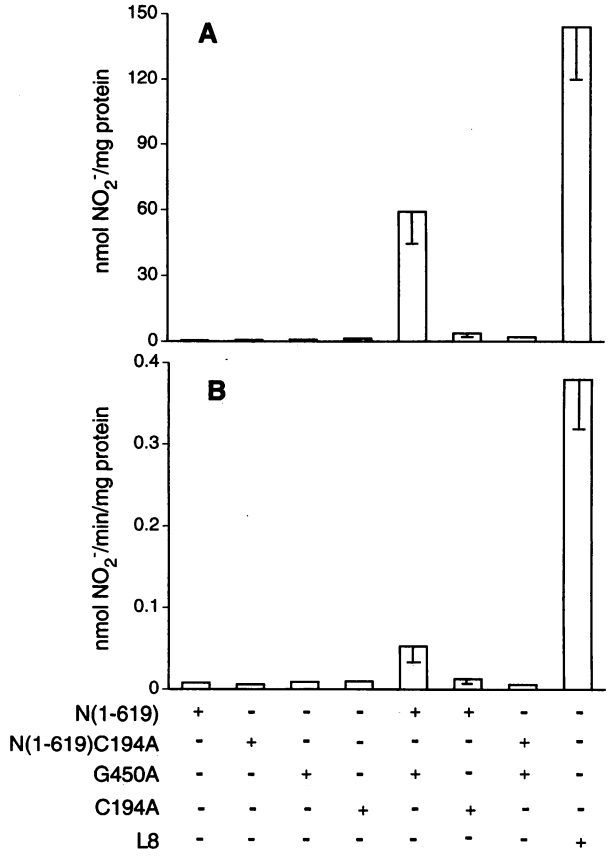

FIG. 6. Complementation of COOH-terminal truncation mutant N(1–619) by G450A but not by C194A; lack of complementation of N(1–619)(C194) by G450A. NOS activity is assessed by (*A*) nitrite accumulation in cell culture medium and (*B*) NOS assay of the cell lysates as in Fig. 5. The plasmids used for transfection are indicated. Results are means − SD for the number of experiments indicated in parentheses: N(1–619) (5), G450A (4), C194A (6), N(1–619)C194A (3), N(1–619) plus G450A (9), N(1–619) plus C194A (12), N(1–619)C194A plus G450A (3), L8 (10). Where error bars are not visible, they are smaller than the thickness of the line.

The ability of the COOH-terminal truncation mutant N(1–619) to homodimerize establishes that the NH_2-terminal hemimer is sufficient to sustain dimerization of iNOS under conditions pertaining within the cell, confirming observations made with isolated, proteolytically-generated NH_2-terminal hemimers of NOS1 (16) and iNOS (17). We have not tested whether COOH-terminal hemimers may also dimerize, as described for NOS3 (24). N(1–619)C194A lost the ability to homodimerize (Fig. 4*B*) in conjunction with the mutation of its heme binding Cys-194 to Ala.

Klatt *et al.* (25) recently reported that the capacity of NOS to dimerize can be monitored by SDS/PAGE conducted without boiling of the samples. We have independently reached the same conclusion (Fig. 3), as validated by gel chromatography (refs. 20 and 21, and results not shown).

Conclusions from Mutants Studied in Pairs. Results are schematized in Fig. 8. Partial functional complementation occurred between the NADPH-deficient, COOH-terminal mutants LS, Lm3, and N(1–619) on the one hand and the BH_4-deficient, NH_2-terminal mutants G450A and A453I (not shown) on the other hand. A partial degree of complementation was all that could be expected, because the formation of heterodimers must have occurred in competition with the formation of inactive homodimers of the COOH-terminal mutants. By the criteria available to us, the stability of homodimers of the COOH-terminal mutants was indistinguishable from that of wild type. In contrast, the heterodimers of

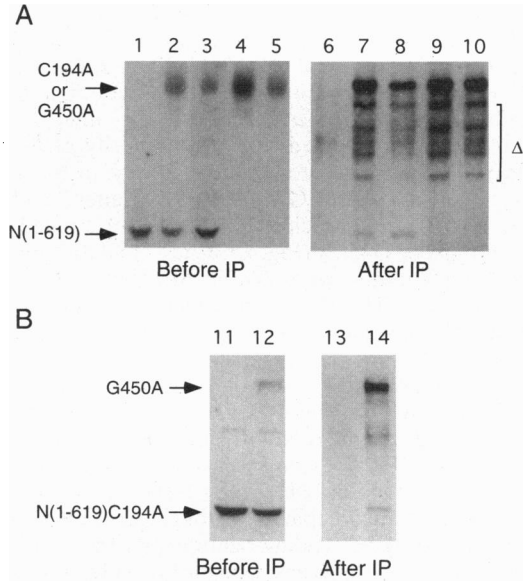

FIG. 7. Heterodimer formation between N(1–619) and either G450A or C194A and between N(1–619)C194A and G450A. (*A*) Lysates of 293 cells transfected with N(1–619) (lanes 1 and 6), C194A (lanes 4 and 9), or G450A (lanes 5 and 10) alone, or lysates of 293 cells cotransfected with N(1–619) plus C194A (lanes 2 and 7) or N(1–619) plus G450A (lanes 3 and 8) were subjected to immunoblot with Ab(iNOS) before (lanes 1–5, 60 μg/sample) or after (lanes 6–10, 600 μg/sample) immunoprecipitation (IP) with Ab(NO16), which is directed against iNOS residues 1137–1144; these are lacking in N(1–619). The area marked by Δ indicates degradation products of iNOS arising during immunoprecipitation. (*B*) As in *A*, except cells transfected with N(1–619)C194A alone (lanes 11 and 13) or N(1–619)C194A plus G450A (lanes 12 and 14).

mutant pairs were expected to be less stable, because one set of the partners (G450A and A453I) could not form homodimers (Fig. 3 and ref. 21). Compared to wild-type homodimers, the mutant heterodimers displayed relatively lower NOS activity in cell lysates than they did in intact cells; this may

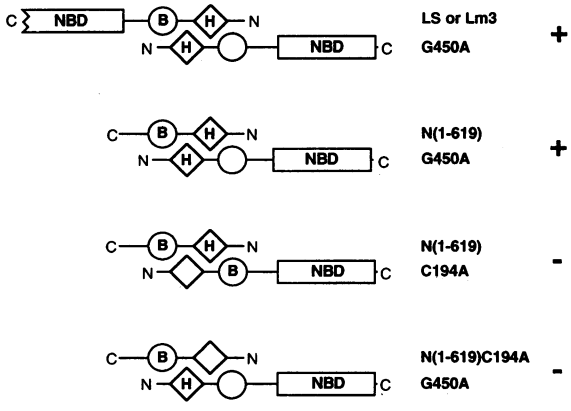

FIG. 8. Schematic summary: pairs of mutant monomers form heterodimers with (+) or without (−) capacity to produce NO. Nucleotide- (NADPH-, FAD-, FMN-) binding domains are indicated by box labeled NBD; jagged edge indicates truncation within NBD that prevents NADPH binding. Diamonds labeled H indicate heme-binding domain. Circles labeled B indicate BH_4 binding domain. H and B are arbitrarily centered on individual residues currently known to make an essential contribution to the binding of heme or BH_4, respectively. Open circles in the corresponding positions denote mutants deficient in binding heme or BH_4. N and C denote NH_2 and COOH termini. Calmodulin is not depicted. N(1–619)C194A is predicted to bind BH_4 because it sustains heterodimerization with the BH_4-deficient mutant, G450A. Therefore, C194A, which is less disrupted than N(1–619)C194A, is also predicted to bind BH_4.

reflect greater instability of the mutant heterodimers under nonphysiologic conditions, such as dilution. The link between functional complementation and heterodimerization was confirmed by coimmunoprecipitation of one mutant monomer with antibody capable of recognizing only the other. In whole lysates of doubly transfected cells, the intensity of immunostaining of N(1–619) was comparable to that of its cotransfection partners, C194A or G450A (Fig. 7*A*, lanes 2 and 3). In contrast, in coimmunoprecipitates, N(1–619) stained much less than C194A or G450A (Fig. 7*A*, lanes 7 and 8), supporting the conclusion that there were significantly fewer heterodimers than homodimers. The degree to which heterodimerization normalized NO production in cotransfected cells is all the more notable.

Although homodimers did not form and NO was not produced when BH_4 binding sites were altered on both chains, heterodimers did form and NO was produced when only one chain could bind BH_4. We cannot exclude that the mutant that was incapable of binding BH_4 when tested alone may have acquired BH_4-binding capacity through a conformational change imparted by heterodimerization. Barring such an unlikely circumstance, electrons flowing initially through the monomer with intact nucleotide binding domains must have been able to proceed to substrate through the aid of BH_4 provided by the other monomer.

Because only one BH_4 binding site was required for dimerization and catalysis, we asked the same question for heme. The answer was distinctly different. A single heme binding site could sustain heterodimerization, but catalysis required two binding sites. No functional complementation was seen when a lone heme was bound either in cis or in trans to the electron flow from the COOH-terminal hemimer, as tested in the cotransfected pairs N(1–619)C194A plus G450A and N(1–619) plus C194A, respectively (Figs. 6 and 8).

Thus, NO production by iNOS requires two heme binding sites and, presumably, two hemes. Each heme may help maintain the structure of the other chain's independently functioning active site. Alternatively, there may be a single active site, to which each heme makes an essential contribution.

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