Neurogranin (Ng) is a neuron-specific protein kinase C-selective substrate, which binds calmodulin (CaM) in the dephosphorylated form at low levels of Ca\(^{2+}\). This protein contains redox active Cys residues that are readily oxidized by several nitric oxide (NO) donors and other oxidants to form intramolecular disulfide. Identification of the Cys residues of rat brain Ng, Cys\(_3\), Cys\(_4\), Cys\(_9\), and Cys\(_{51}\), involved in NO-mediated intramolecular disulfide bridge formation was examined by site-directed mutagenesis. Mutation of all four Cys residues or single mutation of Cys\(_{51}\) blocked the oxidant-mediated intramolecular disulfide formation as monitored by the downward mobility shift under nonreducing SDS-polyacrylamide gel electrophoresis. Single mutation of Cys\(_3\), Cys\(_4\), or Cys\(_9\) or double mutation of any pair of these three Cys residues did not block such intramolecular disulfide formation, although the rates of oxidation of these mutant proteins were different. Thus, Cys\(_{51}\) is an essential pairing partner in NO-mediated intramolecular disulfide formation in Ng. Cys\(_3\), Cys\(_4\), and Cys\(_9\) individually could pair with Cys\(_{51}\), and the order of reactivity was Cys\(_9\) > Cys\(_4\) > Cys\(_3\), suggesting that Cys\(_3\) and Cys\(_{51}\) form the preferential disulfide bridge. In all cases tested, the intramolecularly disulfide bridged Ng proteins displayed dramatically attenuated CaM-binding affinity and —2–3-fold weaker protein kinase C substrate phosphorylation activity. The data indicate that the N-terminal Cys\(_3\), Cys\(_4\), and Cys\(_9\) are in close proximity to the C-terminal Cys\(_{51}\) in solution. The disulfide bridge between the N- and C-terminal domains of Ng renders the central CaM-binding and phosphorylation site domain in a fixed conformation unfavorable for binding to CaM and as a substrate of protein kinase C.

Nitric oxide (NO)\(^1\) has been established as a messenger molecule in physiological processes as diverse as host defense, vascular regulation, and neuronal communication (1–4). NO is synthesized by a family of nitric oxide synthases (NOS) which utilize arginine as their substrate in the 5-electron oxidation of the guanidino nitrogen. NO is a free radical gas that readily diffuses into cells where it reacts with molecular targets. NO is extremely susceptible to both oxidation and reduction resulting in the formation of NO surrogates such as nitrosamine (NO(\(~\)\)) and nitroso anion (NO(\(~\)\)) (5). In addition, NO free radical reacts readily with other free radical such as superoxide anion (O\(_2^-\)) to form peroxynitrite (ONO\(_2^-\)) (6), with O\(_2\) to form NO\(_2\), and with transition metal ions to form adducts (5). These secondary reaction products and products of NO oxidation and reduction are capable of reaction with metals, thiols, and additional targets to give further products with biological activities (5, 7). The signal transduction pathways of NO can be broadly classified as cGMP-dependent and -independent; the former pathway involves NO binding at the heme of soluble guanylyl cyclase leading to stimulation of cGMP formation and the latter pathway involves reactions with other heme and nonheme iron, N-nitrosoation of nucleic acids, and modifications of target proteins by S-nitrosoation, ADP-ribosylation, and tyrosine nitration (2, 3). S-Nitrosation of thiols is a common occurrence in biological systems; NS-NO serves as a bioactive reservoir of NO that targets reactive sulphydryl groups in S-nitrosothiol-thiol exchange reactions and accelerates intramolecular disulfide formation from vicinal thiol groups (8). Reversible formation of disulfide bonds is utilized biologically in enzyme catalysis, transport of reducing equivalents, metabolic regulation, cellular defense, and provision of structural stability (9).

Rat brain neurogranin (Ng), also known as RC3 or BICKS, is a 78-amino acid PKC-selective substrate which binds calmodulin (CaM) at low levels of Ca\(^{2+}\) (10–15). This protein and another PKC substrate neuromodulin (also known as GAP-43, F1, or B-50) contain a conserved 19-amino acid sequence region, where the CaM-binding and phosphorylation site domain is located (11, 16, 17). Phosphorylation of these two proteins by PKC weakens their binding affinities for CaM. It has been proposed that the PKC-catalyzed phosphorylation of these two proteins frees the CaM for other CaM-dependent enzymes (18, 19). The signal transduction pathway involving activation of PKC and phosphorylation of Ng and neuromodulin has been linked to the modulation of ion channel and neurotransmitter release and the induction and maintenance of long term potentiation (20–25). Recently, neuromodulin has been shown to be a target of nitric oxide, which inhibits thioester-linked longchain fatty acylation possibly through a direct modification of Cys thiois (26). Both Ng and neuromodulin contain two N-terminal Cys residues at position 3 and 4; the former contains two additional Cys residues at position 9 and 51. So far, there is no evidence for the long-chain fatty acylation of any of the Ng Cys residues (27). Treatment of rat brain Ng with NO donors or

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\(^{1}\) The abbreviations used are: NO, nitric oxide; Ng, neurogranin; PKC, protein kinase C; PKM, PKM, protein kinase M; PS, phosphatidylserine; DG, diacylglycerol; CaM, calmodulin; DEANO, diethyl amine nitric oxide; SNP, sodium nitroprusside; SIN-1, 1-morpholinosydnonimine; SNOG, S-nitrosoglutathione; SNAP, S-nitroso-N-acetylpenicillamine; IBZ, o-iodosobenzoic acid; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; WT, wild type.
Nitric Oxide Modification of Neurogranin

Table 1 Summary of the various Ng mutant proteins

| Protein        | Mutation(s) |
|----------------|-------------|
| WT             | None        |
| Single Cys mutants |            |
| C3S            | Cys3 → Ser  |
| C4G            | Cys4 → Gly  |
| C9S            | Cys9 → Ser  |
| C51G           | Cys51 → Gly |
| Double Cys mutants |         |
| C3SC/4S        | Cys3 → Ser, Cys4 → Ser |
| C3SC/9S        | Cys3 → Ser, Cys9 → Ser |
| C4GC/9S        | Cys4 → Ser, Cys9 → Ser |
| Tetra Cys mutant |            |
| Tetra          | Cys3 → Ser, Cys4 → Gly, Cys9 → Ser, Cys51 → Gly |

Nitric Oxide Modification of Neurogranin

other oxidants, such as H2O2 or α-iodosobenzoic acid (IBZ), results in intramolecular disulfide(s) formation with resulting attenuation of this protein’s binding to CaM and the ability to serve as a substrate of PKC (28). Oxidation of Ng was also seen in the NO donor-treated rat brain synaptosomes when analyzed by immunoblot with an antibody against Ng. Here, we examine the role of the four Cys residues of rat brain Ng, Cys3, Cys4, Cys9, and Cys51, in NO modification of its function by intramolecular disulfide bridge formation. Cys3, Cys4, and Cys9 individually can form a disulfide bridge with Cys51 and the order of reactivity of the former three Cys residues with Cys51 is Cys3 > Cys9 > Cys4, indicating that Cys3-Cys51 is the preferential intramolecular disulfide bridge formed by NO modification.

Experimental Procedures

Materials—Commercial products utilized were: γ-32P]ATP (28 Ci/ mmol) from DuPont NEN; α-32P]-dATP (1,000 Ci/mmol) from Amersham Corp.; oligodeoxynucleotides from Curachem; PCR reagent system and 100-base pair ladder from Life Technologies, Inc.; Wizard miniprep kits, competent HB101 and JM109 cells from Promega; pRSET expression vector, cloning vector (pCRII). Subcloning into the pRSET expression vector and the HI site linker. Full-length WT and mutant Ng proteins were expressed and purified in E. coli from Novagen; all restriction endonucleases, T4 ligase, plasmid and TA cloning kit from Invitrogen; competent BL21(DE3) cells—expression cells from Novagen; all other chemicals were reagent grade or of higher purity. The following oligodeoxynucleotide primers were utilized in the PCR reactions:
P1, 5′-ACCAGATATGAGCTCTGGCAGCAGGCC-3′; P2, 5′-GCCGCGATCCAATCTGCCGGCCGGCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGCACGCAGGACCC-3′; C3S, 5′-AACCCGATATGGAGAC...
frozen and lyophilized, and the dried fractions were resuspended in 100 μl of distilled water. An aliquot of each fraction was analyzed by 10–20% SDS-PAGE, and those fractions containing >95% Ng or mutant proteins were pooled. Ng was additionally purified from frozen rat brain as described previously (15).

Oxidation of Ng Proteins by Nitric Oxide Donors and Other Agents—WT and Cys mutant Ng proteins were reduced in the presence of 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT (37 °C, 30 min) and then oxidized by addition of 2.5–10 mM H2O2, DEANO, SNP, SIN-1, SNOG, SNAP, or IBZ (20 °C, 1–15 min). Reactions were terminated by addition of 20 mM ascorbic acid and diluted with SDS-sample buffer lacking β-mercaptoethanol and bromphenol blue, unless otherwise noted. Analysis was carried out by electrophoresis on 10–20% SDS-polyacrylamide gels that had been prerun at 40 mA/slab gel for 15 min. Gels were stained with 0.05% Coomassie Brilliant Blue R250.

WT and Cys Mutant Ng Binding to CaM-affinity Column—The ability of Ng to bind CaM in the absence of Ca2+ was determined using a CaM-Sepharose column. Ng (20 μg) was reduced in the presence of 20 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT (37 °C, 30 min) and was then oxidized or not by addition of 10 mM DEANO (20 °C, 15 min); the oxidation was terminated by addition of 20 mM ascorbic acid. Reaction mixtures were diluted 4-fold with 50 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA and applied to a 1-ml CaM-Sepharose column equilibrated in 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.1 mM NaCl, and 2 mM ascorbic acid. The column was washed with 2.2 ml of the same buffer and then eluted with 2.4 ml of 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 6 mM CaCl2, 0.5 mM NaCl, and 2 mM ascorbic acid. Fractions of elution drops were collected and reduced by addition of 25 mM DTT (37 °C, 30 min) and then assayed for the presence of Ng by phosphorylation with either PKC or PKM.

PKC, PKM, and Protein Quantitation Assays—Rat brain PKC and PKM (30) were purified to near homogeneity by the previously described methods. PKC activity was determined using a lipid vesicle assay containing 30 mM Tris-Cl (pH 7.5), 6 mM Mg-acetate, 60 μM UDP-[γ-32P]ATP (1,000–5,000 cpm/pmol), 100 μg/ml PS, 20 μg/ml DG, variable substrate, 400 μM CaCl2, and 0.5–1.0 μg/ml PKC, unless otherwise noted. PKM activity was determined under similar conditions in the presence of 2 mM EGTA but without PS, DG, and CaCl2. Reactions were carried out at 37 °C in a final volume of 25 μl and [32P]ATP incorporation was determined using P81 phosphocellulose paper (31) or a Dowex 1/DEAE cellulose mini column (15). Stocks of phospholipid/DG were prepared by drying mixtures in CHCl3 under a N2 stream; lipids were resuspended in 20 mM Tris-Cl (pH 7.5), vortexed, and sonicated (2 min, bath sonicator). The concentration of WT and integrin Ng proteins was estimated by either quantitative phosphorylation (37 °C, 1 h) or by amino acid analysis.

RESULTS

The four Cys residues, Cys3, Cys4, Cys9, Cys51, of rat brain Ng were examined for their role in NO modification of this protein by site-directed mutagenesis. WT, four single, C3S, C4G, C9S, C51G, three double, C3S/C4S, C3S/C9S, C4S/C9S, and a Tetra Cys mutant Ng proteins were constructed by recombinant DNA methods and expressed in and purified from Escherichia coli. The purified WT and eight Cys mutant Ng proteins were constructed by recombinant DNA methods and expressed in and purified from E. coli. The purified WT and eight Cys mutant Ng proteins were constructed by recombinant DNA methods and expressed in and purified from E. coli. The purified WT and eight Cys mutant Ng proteins were constructed by recombinant DNA methods and expressed in and purified from E. coli. The purified WT and eight Cys mutant Ng proteins were constructed by recombinant DNA methods and expressed in and purified from E. coli.

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various NO donors, DEANO, SNP, SIN-1, SNOG, and SNAP, as well as the general oxidant IBZ were examined for their ability to form intramolecular disulfide bridging in the WT and Cys mutant Ng proteins (Fig. 3). The NO donors, DEANO, SNAP, and IBZ were variably effective in causing intramolecular disulfide bridging in WT, single Cys mutants, C3S, C4G, and C9S, and double Cys mutants, C3S/C4S, C3S/C9S, and C4S/C9S to form intramolecular disulfide bridge indicates that Cys3, Cys4, and Cys51 individually can pair with Cys51 (Fig. 2).

Various NO donors, DEANO, SNP, SIN-1, SNOG, and SNAP, as well as the general oxidant IBZ were examined for their ability to form intramolecular disulfide bridging in the WT and Cys mutant Ng proteins (Fig. 3). The NO donors, DEANO, SNP, and SNAP, and IBZ were variably effective in causing intramolecular disulfide bridging in WT, single Cys mutants, C3S, C4G, and C9S, and double Cys mutants, C3S/C4S, C3S/C9S, and C4S/C9S. In contrast, SIN-1 and SNOG were ineffective (Fig. 3, all panels, lanes 5 and 6). SIN-1 simultaneously generates 1 mol of NO and 1 mol of O2/mol of SIN-1, and reaction of these products forms peroxynitrite (32). Peroxynitrite, a strong oxidant, oxidizes the SH group to sulfenic and sulfonic acids (32, 33) which likely explains why SIN-1 does not induce disulfide bridging in WT or any of the Cys mutant Ng proteins (Fig. 3, all panels, lane 5). SNOG treat-
FIG. 3. Oxidation of WT and mutant Ng proteins by NO donors and IBZ. WT and mutant Ng proteins were reduced in the presence of 40 mM Tris-Cl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT (37 °C, 30 min) and then oxidized with 5 mM each of H2O2, DEANO, SNP, SIN-1, SNOG, SNAP, or IBZ for 10 min (30 °C). Reactions were terminated by addition of 20 mM ascorbic acid, diluted with SDS sample buffer lacking the sulfhydryl agent, and electrophoresed on 10–20% SDS-PAGE. Coomassie Brilliant Blue R250-stained proteins are as indicated and only the region of the reduced and oxidized Ng proteins is shown. H2O2 oxidized Ng proteins are shown for reference.

To determine the only other NO agent tested that did not cause disulfide bridging in the WT or Cys mutant Ng proteins, resulted in an upward shift on SDS-PAGE, or a somewhat higher Mr (Fig. 3, all panels except E, lane 6). We interpret this result as SNOG forming stable disulfide adducts (1–4 mol of SNOG/mol of protein, depending on the Ng protein) without concomitant intramolecular disulfide bridge formation. SNOG treatment of the Tetra Cys mutant Ng, as expected, did not result in a shift to the higher Mr species (Fig. 3E, lane 6), whereas all other Ng proteins tested did. DEANO and SNP were similarly effective in the extent of disulfide bridging induced in WT and Cys mutant Ng proteins, with the exception of Tetra and C51G proteins which were not reactive. In contrast, SNAP was less effective in the extent of disulfide bridging induced for virtually all of the reactive Ng proteins (Fig. 3, all panels, lane 7). As expected, the Tetra Cys mutant Ng lacking all four Cys residues, showed no intramolecular disulfide bridging with all oxidants tested as demonstrated by a lack of a mobility shift on SDS-PAGE (Fig. 3E). Similar to the results with H2O2, C51G was the only other mutant Ng which could not be intramolecularly disulfide bridged by any of the oxidants tested with resulting increase in electrophoretic mobility, indicating that Cys51 is a critical pairing partner in NO-mediated disulfide bridging in Ng. Also similar to the H2O2 results, the ability of single Cys mutants, C3S, C4G, C9S, and double Cys mutants, C3S/C4S, C3S/C9S, and C4S/C9S to form intramolecular disulfide bridging with NO donors, DEANO, SNP, and SNAP, indicates that Cys3, Cys4, and Cys9 individually can pair with Cys51 in NO-mediated disulfide bridging (Fig. 3).

Among the three double mutants, the extent of disulfide bridge formation after 10 min of reaction was Cys3/Cys51 (C3S/C4S/C9S), Cys3/Cys51 (C3S/C9S), and Cys51/Cys9 (C3S/C4S) by the gel mobility shift assay (Fig. 4). DEANO-induced intramolecular disulfide bridge formation in WT and C3S/C4S double mutant are faster and to a greater extent than those of C3S/C9S and C4S/C9S mutants. Among the three double mutants, the extent of disulfide bridge formation after 10 min of reaction was Cys3/Cys51 (C3S/C4S/C9S) pairing > Cys3/Cys51 (C3S/C9S) pairing > Cys3/Cys9 (C4S/C9S) pairing, indicating that Cys3–Cys51 is the preferential disulfide bridge of the three (Fig. 4).

To determine the role of disulfide bridge formation in Ng’s ability to bind CaM in the absence of Ca2+, WT, and the eight Cys mutant Ng proteins were examined for their ability to bind CaM under reducing and DEANO-oxidized conditions using a CaM-affinity column assay. Reduced or DEANO-oxidized WT or the eight Cys mutant Ng proteins were applied to a CaM-Sepharose column in the presence of EGTA and after washing in the presence of EGTA (fractions 1–13), the proteins were eluted in the presence of Ca2+ (fractions 14–28) (Fig. 5). Reduced WT Ng bound to CaM-Sepharose in the presence of EGTA and was eluted with Ca2+, as expected, whereas the majority of DEANO-oxidized WT Ng did not bind and was excluded from the column, indicating that the NO-mediated intramolecularly disulfide bridged WT Ng was unable to bind CaM (Fig. 5, WT panel). A small fraction of DEANO-oxidized WT Ng did bind to the column and was eluted with Ca2+, this is attributed to the minor unoxidized fraction of WT Ng. Similar to WT Ng all of the eight Cys mutant Ng proteins in the reduced state totally bound to the CaM-affinity resin in the presence of EGTA and were eluted with Ca2+, indicating that single, double, or tetra mutation of the various four Cys residues of Ng did not affect the ability of Ng to bind to CaM (Fig. 5). The single Cys mutant Ng proteins, C3S, C4G, C9S, similar to WT Ng, when DEANO-oxidized, the majority of each of these
proteins did not bind and were excluded from the column (Fig. 5). Since the Tetra Cys mutant Ng lacks all Cys residues, it is expected that DEANO oxidation will not affect its binding to CaM. This is in fact what was observed. The majority of the DEANO-treated mutant protein bound to the CaM-affinity resin in the presence of EGTA and was eluted with Ca\(^{2+}\), and a minor fraction was eluted at the tail part of the EGTA washing. The nature of this material remains unknown (Fig. 5, panel Tetra, fractions 7–15). The C51G mutant when DEANO-oxidized, similar to the Tetra Cys mutant, totally bound the CaM-affinity resin in the presence of EGTA and was eluted with Ca\(^{2+}\) (Fig. 5, panel C51G). This result again indicates that Cys\(^{51}\) is a critical pairing partner in DEANO-mediated disulfide bridge formation in Ng. The ability of the three double Cys mutants, C3S/C4S, C3S/C9S, and C4S/C9S, when DEANO-oxidized, to bind CaM was also determined. For the DEANO-oxidized C3S/C4S mutant (Cys\(^3\)-Cys\(^{51}\) pairing), C3S/C9S mutant (Cys\(^3\)-Cys\(^{51}\) pairing), and C4S/C9S mutant (Cys\(^4\)-Cys\(^{51}\) pairing) the relative CaM-affinity ratios of not bound to bound protein in the presence of EGTA were \(-90\%/10\%\), \(-70\%/30\%\), and \(-50\%/50\%\), respectively (Fig. 5). These results indicate that the order of reactivity for Cys\(^3\), Cys\(^4\), and Cys\(^8\) pairing with Cys\(^{51}\) is Cys\(^8\) > Cys\(^4\) > Cys\(^3\), which is consistent with the kinetic results (Fig. 4).
C3S, C4G, and C9S, when H2O2-oxidized were also poorer substrate for PKC (Fig. 6,
kinase M.

Cys residues and the single mutation at Cys51 to other amino
located at positions 3, 4, 9, and 51. Both the mutation of all four
residues, was, as expected, not affected by H2O2 oxidation. The
lack of an effect on substrate ability for H2O2-oxidized C51G
indicates that Cys51 is a critical pairing partner for disulfide
bridging; this is consistent with the above data supporting an
essential role of Cys51 in NO-mediated disulfide bridging of Ng.

Rat brain Ng when disulfide bridged by oxidants is a poorer substrate for PKC (28). It was of interest to know how the oxidized Cys mutant Ng proteins behave as substrates for PKC. WT Ng when oxidized with H2O2 was a 2-fold poorer substrate for PKC (Fig. 6, WT). The single Cys mutant Ng proteins, C3S, C4G, and C9S, when H2O2-oxidized were also poorer substrates for PKC, indicating that the disulfide bridging in these proteins occurs as in WT and results in a poorer substrate for PKC. In contrast, the Tetra Cys and C51G mutant Ng proteins when H2O2-oxidized, displayed normal activity as a substrate for PKC. The Tetra Cys mutant, lacking all four Cys residues, was, as expected, not affected by H2O2 oxidation. The lack of an effect on substrate ability for H2O2-oxidized C51G indicates that Cys51 is a critical pairing partner for disulfide bridging; this is consistent with the above data supporting an essential role of Cys51 in NO-mediated disulfide bridging of Ng and the subsequent effects on CaM binding and PKC substrate ability.

**DISCUSSION**

The two known functions of rat brain Ng, namely, binding of CaM and as a substrate of PKC, are simultaneously attenuated following oxidation by several NO donors and other oxidants, such as H2O2 and IBZ, to form intramolecular disulfide. The rate of oxidation of Ng by NO is considerably faster than that of serum albumin, glutathione, or DTT (28), an indication that Ng is a highly favorable acceptor of NO. This previous study did not define the pairing among the four Cys residues of Ng located at positions 3, 4, 9, and 51. Both the mutation of all four Cys residues and the single mutation at Cys51 to other amino acids prevent the intramolecular disulfide cross-linking that converts the M₉ = 17,000 to the 10,000 species under non-reducing SDS-PAGE. Single mutation at Cys³, Cys⁴, or Cys⁹ does not prevent the oxidation. Thus, Cys⁵¹ must form disulfide bond with either Cys³, Cys⁴, or Cys⁹, that results in bridging the N- and C-terminal ends of the Ng molecule. This conclusion is supported by the observations that double mutants involving any two of the Cys³, Cys⁴, and Cys⁹ still can be oxidized. Since the intramolecular disulfide cross-linking is the predominant reaction products of the wild type and the various mutants, this indicates that Cys⁵¹ must be in close proximity to Cys³, Cys⁴, and Cys⁹ so that the high local concentration favors intramolecular disulfide. Comparison of the rates of oxidation of the three double mutants involving Cys³, Cys⁴, and Cys⁹ indicates that Cys³¹ pairs with Cys³ most favorably among the three.

The current study defines only the oxidant-induced intramolecular disulfide bridge formation involving Cys³¹ and any one of the three N-terminal Cys residues that induces mobility shift upon SDS-PAGE. It is conceivable that other disulfide bonds among the N-terminal Cys residues are likely to form upon oxidation, especially for the C51G mutant in which Cys³ or Cys⁹ could form disulfide with Cys⁵¹.

Reversible oxidation/reduction of Ng affects both the CaM-binding affinity and substrate phosphorylation by PKC. The wild type and all the Cys mutants of Ng are phosphorylated by PKC, and they all bind to the CaM-affinity column under reducing condition. Thus, all four Cys residues are not directly involved in either the CaM binding or substrate recognition by PKC. This is perhaps anticipated since the PKC phosphorylation site and the CaM-binding domain is located within a 19-amino acid Ng/neuromodulin homology (11) situated at the middle of the Ng molecule. Synthetic peptides corresponding to this domain bind CaM and serve as an effective substrate of PKC (15). Intramolecular disulfide cross-linking between the N- and C-terminal ends of Ng results in a gross conformational change that renders the central domain unable to bind CaM and also results in a poorer substrate of PKC. It has been suggested that the Ng central domain can be induced to form α-helical structure upon binding to CaM (34). Disulfide cross-linking between the two ends apparently disrupts the “induced-fit” between Ng and CaM. Similar structural constraint probably also plays a role in converting Ng to a weaker PKC substrate following oxidation.

The physiological relevance of Ng oxidation by NO in controlling its binding to CaM and as a substrate of PKC has not been established. Toward this goal, we have shown that Ng associated with rat brain synaptosomes can be oxidized by DEANO when analyzed by immunoblot analysis (28). In addition, we noticed that the oxidized form of Ng was present in the rat brain extracts prepared in buffer without reducing agent, and this oxidized form can be reduced by NADPH.² It appears that there are enzymes catalyzing the redox reactions of Ng in the brain. In this respect, Ng is similar to thioredoxins and glutaredoxins (35); all three are small molecular mass proteins (7.5–12 kDa) containing redox active thiois. A major function of thioredoxin and glutaredoxin is as a cofactor for the enzyme ribonucleotide reductase, which catalyzes the conversion of ribonucleotides to deoxyribonucleotides (36). In the case of thioredoxin, the reduced cofactor is regenerated by specific NADPH-dependent thioredoxin reductase (37). However, oxidized glutaredoxin is reduced directly by cellular glutathione, and the resulting GSSG, in turn, is reduced by NADPH via the glutathione reductase (38). Ng does not exhibit any sequence homology with these two proteins, including the short sequence regions containing redox active Cys residues for thioredoxin (-Cys-Gly-Pro-Cys-) (39) and glutaredoxin (-Cys-Pro-Tyr-Cys-) (40). The redox-active thios of Ng are far apart in its primary structure but are in close proximity in solution. Binding of Ng

² J. H. Pak, C. W. Mahoney, and K.-P. Huang, unpublished results.
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to CaM in the absence of Ca\(^{2+}\) blocks the oxidation of Ng (28), indicating that these active thiols are no longer available for redox reactions. Thus, Ng can be considered as a Ca\(^{2+}\)-sensitive redox protein as a rise in [Ca\(^{2+}\)] favors its dissociation from CaM and subsequent oxidation by NO due to activation of Ca\(^{2+}\)/CaM-dependent NO synthase or other oxidants.

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