# Calcium-Sensitive Interaction between Calmodulin and Modified Forms of Rat Brain Neurogranin/RC3

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ABSTRACT: Neurogranin (NG) binding of calmodulin (CaM) at its IQ domain is sensitive to $Ca^{2+}$ concentration and to modifications by protein kinase C (PKC) and oxidants. The PKC phosphorylation site of NG is within the IQ domain whereas the four oxidant-sensitive Cys residues are outside this region. These Cys residues were oxidized forming two pairs of intramolecular disulfides, and could also be glutathiolated by *S*-nitrosoglutathione resulting in the incorporation of four glutathiones per NG. Circular dichroism (CD) showed that modification of NG by phosphorylation, oxidation forming intramolecular disulfides, or glutathiolation did not affect the α-helical content of this protein. Mutation of the four Cys residues [Cys(−)-NG] to Gly and Ser did not affect the α-helical content either. Interaction of CaM with the reduced (red)-, glutathiolated (GS)-, or Cys(−)-NG in the $Ca^{2+}$-free solution resulted in an increase in the α-helicity determined by their CD spectra, but relatively little change was seen with the oxidized NG (ox-NG) or phosphorylated NG ($PO_4$-NG). The binding affinities between the various modified forms of NG and CaM were determined by CD spectrometry and sedimentation equilibrium: their affinities were Cys(−)-NG > red-NG, GS-NG > ox-NG > $PO_4$-NG. Unlike Cys(−)-, red-, and GS-NG, neither ox- nor $PO_4$-NG bound to a CaM-affinity column. Thus, both oxidation of NG to form intramolecular disulfides and phosphorylation of NG by PKC are effective in modulating the intracellular level of CaM. These results indicate that modification of NG to form intramolecular disulfides outside the IQ domain provides an alternative mechanism for regulation of its binding affinity to CaM.

Neurogranin/RC3 (NG)[1] is a postsynaptically located protein kinase C (PKC) substrate expressed in selective neurons within the cerebral cortex, hippocampus, and striatum of the adult brain. This protein accumulates in the dendritic spines and shafts of these brain regions and has been implicated in the postsynaptic second messenger cascade of long-term potentiation and depression (for review, see *1* and *2*). Rat brain NG is a 78-amino acid protein that binds calmodulin (CaM) in a $Ca^{2+}$-sensitive manner; namely, NG forms a complex with CaM in the absence of $Ca^{2+}$, and the complex dissociates in the presence of $Ca^{2+}$ (*3*−*6*). The CaM-binding domain of NG has been mapped to a region containing basic amino acids and an abundance of hydrophobic residues with a high propensity to form an amphiphilic α-helix (*3*−*6*). This region shares sequence homology with several other IQ domain (IQXXXRGXXXR)-containing proteins (*7, 8*), whose binding to CaM does not require $Ca^{2+}$. Among them, NG resembles most closely the growth cone membrane-associated protein neuromodulin/GAP-43. Both proteins contain a PKC phosphorylation site within this region (*3, 4, 9, 10*), which is also referred to as phosphorylation site/CaM-binding domain (PSD). Phosphorylations of both NG and neuromodulin by PKC attenuate their binding affinities for CaM (*3, 4, 9, 10*). As the concentrations of each of these two proteins, NG in the postsynaptic and neuromodulin in the presynaptic compartments, account for nearly 0.5% of the total brain protein in cell body-rich regions (*11*−*13*), it has been proposed that the PKC-mediated phosphorylation of these two proteins results in an increase in the free CaM level for the activation of many $Ca^{2+}$/CaM-dependent enzymes in the neurons (*1, 2*).

Rat and mouse brain NGs have identical amino acid sequences within which there are four Cys residues located at positions 3, 4, 9, and 51 (*4, 14, 15*). Unlike the rodent protein, other species contain three Cys residues with rodent Cys51 being replaced by Arg in the human (*16, 17*), bovine (*3*), and ovine proteins (*18*). Purified rat brain NG is

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[1] Abbreviations: NG, neurogranin; ox-NG, oxidized neurogranin; red-NG, reduced neurogranin; GS-NG, glutathiolated neurogranin; Cys(−)-NG, C3S/C4G/C9S/C51G mutant neurogranin; $PO_4$-NG, phosphorylated neurogranin; PSD, phosphorylation site/calmodulin-binding domain; CaM, calmodulin; PKC, protein kinase C; GSNO, *S*-nitrosoglutathione; TFA, trifluoroacetic acid; TFE, trifluoroethanol; ES-MS, electrospray ionization mass spectrometry.

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susceptible to oxidation by air and by several chemical oxidants in a reaction that resulted in the formation of intramolecular disulfide bonds. This modification can be detected by a downward mobility shift of the oxidized NG upon nonreducing SDS−PAGE (*19*, *20*). NG in the rat brain synaptosomal preparations was shown to be oxidized by nitric oxide donors (*19*). More recently, we have demonstrated that oxidation of this protein in rat brain slices was stimulated by the neurotransmitter *N*-methyl-D-aspartate and inhibited by the nitric oxide synthase inhibitors $N^G$-nitro-L-arginine methyl ester and $N^G$-monomethyl-L-arginine (*21*). These findings point to the possibility that oxidation of rodent NG in the brain is mediated by nitric oxide generated in situ. However, the physiological significance of this modification in controlling the activities of CaM-dependent enzymes is unknown. Site-directed mutagenesis of each of these four NG Cys residues indicates that Cys51 is essential for forming the intramolecular disulfide bond with either Cys3, -4, or -9, that bridges the amino- and carboxyl-terminal ends of the molecule (*20*). The oxidized NG (ox-NG) is a poorer substrate for PKC and has a lower affinity for CaM when compared with the reduced NG (red-NG). Based on studies with circular dichroism (CD) spectrometry, Gerendasy et al. (*6*) demonstrated that binding of CaM to NG in the absence of $Ca^{2+}$ specifically induced α-helix formation within the PSD of NG. However, the structural changes associated with the phosphorylation and oxidation of rat brain NG have not been investigated.

Previously, we found that treatment of NG with *S*-nitrosoglutathione (GSNO) resulted in a retarded mobility upon nonreducing SDS−PAGE as compared to that of the reduced form (*20*). The GSNO-mediated modification of NG has been characterized as glutathiolation in this study. Modification of the Cys residue of a protein by *S*-glutathiolation is known to regulate the activity of several enzymes including human immunodeficiency virus type I protease (*22*), glutathione transferase (*23*), carbonic anhydrase III (*24*), creatine kinase (*25*, *26*), glyceraldehyde-3-phosphate dehydrogenase (*27*−*29*), glycogen phosphorylase *b* (*30*), and aryl sulfotransferase IV (*31*). The effect of glutathiolation on the function of rat brain NG is unknown.

In this study, we employed CD spectrometry to investigate the effects of oxidation, phosphorylation, and glutathiolation on the structure of NG and on its interaction with CaM. We found that modification of NG by oxidation to form two pairs of intramolecular disulfide bonds, by phosphorylation with PKC at a single site, or by glutathiolation of all four Cys residues did not change the potential α-helical content of the protein. In the presence of EGTA, interactions of the red-NG, glutathiolated NG (GS-NG), and Cys(−)-NG (a mutant protein in which all of its Cys residues have been replaced by Ser and Gly) with CaM all resulted in increases in their α-helical contents, whereas relatively little change was seen with the ox-NG or phosphorylated NG ($PO_4$-NG). The affinities of the various modified forms of NG for CaM were determined by CD spectrometry and sedimentation equilibrium. Our results suggest that (1) both oxidation of NG-forming intramolecular disulfides and phosphorylation of NG by PKC are effective in modulating the intracellular level of free CaM, and (2) modification of NG by oxidation outside the IQ domain can also affect its binding affinity for CaM.

## EXPERIMENTAL PROCEDURES

*Preparation of Rat Brain, Recombinant WT, Cys(−) Mutant, and Glutathiolated NG*. Rat brain (*4*) and recombinant WT, and Cys(−) mutant (C3S/C4G/C9S/C51G) NG (*20*) were purified to homogeneity as previously described. Rat brain and recombinant WT NG undergo spontaneous oxidation in the air and can be reduced by DTT (*19*). The GS-NG was prepared by incubation of the reduced WT NG (in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) with 5 mM GSNO (Calbiochem) at 30 °C for 30 min. The reaction mixture was applied to a C4 reverse phase HPLC column (Vydac 214TP54, 0.46 × 25 cm) and eluted with 0.1% TFA (Solvent A) and 0.1% TFA + acetonitrile (Solvent B) gradient at 0.5 mL/min as follows: 0−10 min, Solvent B at 10%; 10−15 min, Solvent B increased to 15%; 15−55 min, Solvent B increased to 80%; and 55−60 min, Solvent B increased to 100%. The GS-NG was eluted at 50% Solvent B. The concentrations of NG were determined by amino acid analysis or by the BCA protein assay reagent (Pierce).

*Chromatography of ox-, red-, and GS-NG on a CaM-Affinity Column*. Purified ox-NG was reduced by incubation in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT at 30 °C for 30 min followed by the addition of 20 mM ascorbic acid. The ox- or GS-NG (10−30 μg) was applied to a CaM−Sepharose (Pharmacia Biotech) column (1 mL bed volume packed in a Pasteur pipet) equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 0.1 M NaCl, and 2 mM ascorbic acid. The column was washed with 2.5 mL of the same buffer and eluted with 2.5 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 0.5 M NaCl, 6 mM $CaCl_2$, and 2 mM ascorbic acid. The DTT-reduced NG was analyzed under the same conditions except that both the washing and elution buffers also contained 2 mM DTT. Fractions (8 drops, 0.16 mL) were collected for assay of NG by exhaustive phosphorylation with PKC or PKM.

*Phosphorylation of NG by PKC*. NG was phosphorylated by PKC in 30 mM Tris-HCl buffer, pH 7.5, containing 6 mM $MgCl_2$, 0.12 mM [$\gamma$-$^{32}$P]ATP, 50 μg/mL PS, 10 μg/mL DG, 0.4 mM $CaCl_2$, 5 mM DTT, and NG or by PKM under the same conditions without PS/DG/$Ca^{2+}$ but containing 2 mM EGTA. $^{32}$P incorporation into NG was measured by the Dowex AG1x8/DEAE-cellulose mini-column method (*4*). Phosphorylated NG used for mass spectrometry was prepared in the presence of nonradioactive ATP. The phosphorylated protein was purified by HPLC on an analytical C4 reverse phase column (Vydac 214TP54, 4.6 × 250 mm) using a 10−60% acetonitrile gradient in 0.1% TFA. PKC and PKM were purified as previously described (*32*).

*Electrospray Ionization Mass Spectrometry (ES-MS)*. ES-MS was performed using M-Scan's Quattro II upgraded Bio-Q instrument with quadrupole analyzer. Myoglobin was used to calibrate the instrument. Sample aliquots of 10 μL were injected into the instrument, and elution was carried out using a mixture of acetonitrile, 0.1% TFA, methoxyethanol, and 2-propanol (1:1:1:1) at a flow rate of 5 μL/min. The spectra were deconvoluted and the masses expressed as daltons.

*Circular Dichroism Spectrometry*. CD spectra (195−260 nm) were recorded in a Jasco J-500C spectropolarimeter as

described elsewhere (*33*). Spectra were measured in a quartz cuvette with a light path of 0.1 cm. For the measurement of the CD spectra of the oxidized NG, the buffer (0.2 mL) contained 5 mM phosphate buffer, pH 7.2, and 100 mM NaCl (Buffer A), and for that of the reduced NG, the same buffer contained 2 mM DTT. For determining the interaction of NG with rat brain CaM, Buffer A also contained 2 mM EGTA, 2.1 mM $CaCl_2$, and 2 mM DTT when added. Mean residue ellipticity $[\theta]$ was determined from the equation: $[\theta] = (\theta \times 100 \times M_r)/(c \times d \times N_a)$ where $\theta$ is the measured ellipticity in degrees, $c$ is the protein concentration in milligrams per milliliter, $d$ is the light path in centimeters, $M_r$ is the molecular weight of the protein, and $N_a$ is the number of the amino acid residues. The spectra were analyzed in terms of secondary structures using the CONTIN program (*34*).

*Analytical Ultracentrifugation.* Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge using an 8-hole An 50Ti rotor. Two-channel Epon centerpieces were filled with 0.14 mL of solutions containing NG, neuromodulin, and CaM at concentrations of approximately 5−10 $\mu$M in Buffer A containing 0.1 mM EGTA. Sedimentation equilibrium was attained consecutively at rotor speeds of 30 000 and 40 000 rpm at 4 °C, and the absorbance distributions were recorded at 230 nm. Extinction coefficients for all samples were determined spectrophotometrically. The buoyant molar masses of NG, neuromodulin, and CaM were measured by global analysis of multiple equilibrium profiles. Mixtures were analyzed by nonlinear regression with the well-known expressions for the radial distribution of reversibly interacting macromolecules in the centrifugal field (*35*). Global analysis was applied to several equilibrium profiles obtained from experiments at different ratios of loading concentrations and different rotor speeds, treating the binding constant as a global parameter and the loading concentrations as local parameters. Calculations were performed with the software MLAB (Civilized Software, Inc., Bethesda, MD). All sedimentation profiles were well-described by the best-fit distributions of a model allowing for a complex formation with 1:1 stoichiometry. Confidence intervals for the binding constants were estimated using projections of the objective function (*36*) and *F*-statistics (*37*). For fundamental reasons, and indicated by the statistical analysis, the similarity of the molar masses of neuromodulin and CaM allows only one limit for the binding constant to be determined ($K_d < 10$ $\mu$M). The more favorable molar mass ratio of NG with CaM, however, allowed well-defined binding constants to be obtained.

## RESULTS

*Characterization of ox-, red-, $PO_4$-, and GS-NG by Mass Spectrometry*. Purified rat brain NG in solution without reducing agent is spontaneously oxidized to form intramolecular disulfide bonds (*19*). ES-MS analysis of the oxidized protein showed a mass of 7534.14 ± 0.18 Da (Figure 1A), and upon reduction with 1 mM DTT, the mass was increased to 7538.88 ± 0.90 Da (Figure 1B), suggesting that two pairs of disulfide bonds are formed in the ox-NG. This conclusion was further confirmed by the observation that incubation of the oxidized rat brain NG with iodoacetamide did not increase the mass of NG (data not shown). Phosphorylation of rat brain NG with PKC or PKM, the catalytic fragment of PKC, resulted in the incorporation of 1 mol of phosphate/mol of NG at Ser36 with an increase of 81 Da over the oxidized NG to 7615.43 ± 0.81 Da (Figure 1C). Incubation of the red-NG with iodoacetamide resulted in the incorporation of 4 mol of acetamide/mol of NG and an increase in mass to 7766.49 ± 0.16 Da (Figure 1D). The recombinant WT NG in the oxidized form had a mass of 7493.79 ± 0.31 Da (Figure 1E), which is 41 Da less than the protein purified from rat brain. This difference is likely due to acetylation of the amino-terminal methionine of the rat brain protein. Glutathiolation of the recombinant WT NG with GSNO resulted in the incorporation of 4 mol of glutathione/mol of NG with a resulting increase in mass to 8719.85 ± 0.19 Da (Figure 1F), indicating that all four Cys residues are modified. We noticed that glutathiolation of NG was more effective with a partially decomposed GSNO preparation (aqueous solution kept at room temperature overnight) as compared to a freshly prepared one. It should be noted that all the purified NG preparations contain a minor component having a mass 16−17 Da greater than the major component. We speculate that this minor NG component contains a methionine sulfoxide in this protein.

*CD Spectrometry of ox-, red-, $PO_4$-, and Cys(−)-NG*. The CD spectra of the recombinant WT ox- and red-NG showed marginal negative ellipticities at 220 nm (Figure 2A), suggesting that both forms are largely in the random-coil conformation. The CD spectra of the reduced and oxidized native rat brain NG, which are acetylated at the amino terminus, also showed marginal ellipticities at 220 nm (Figure 2B). Although oxidations of both the recombinant and native rat brain NG increase their electrophoretic mobilities in a nonreducing SDS−polyacrylamide gel, reduction of NG with DTT has no effect on the α-helical content of these proteins. In 90% trifluoroethanol (TFE), two minima at 205 and 220 nm were seen in the recombinant (Figure 2A) and native rat brain (Figure 2B) NG both in the oxidized and in the reduced forms. The estimated secondary structures of these proteins were 36−73% α-helix and 0−21% $\beta$-sheet.

The CD spectra of $PO_4$-NG (Figure 2C) were similar to those of the nonphosphorylated NG either in the presence or in the absence of DTT, suggesting that phosphorylation does not change the secondary structure of the protein either. The Cys(−)-NG does not contain any Cys residue, and its CD spectra in the presence and absence of DTT were unchanged (Figure 2D). In 90% TFE, both $PO_4$- and Cys(−)-NG showed similar CD spectra as the ox- and red-NG. A similar result was obtained with GS-NG in the absence of DTT (data not shown).

*Interaction of Cys(−)-, red-, ox-, and $PO_4$-NG with CaM.* Since the Cys(−)-NG has Cys4 and -51 replaced by Gly and Cys3 and -9 by Ser, this mutant protein cannot form any disulfide bond. Mixing of Cys(−)-NG (10 $\mu$M) with CaM (5.2 $\mu$M) in the absence of DTT resulted in a greater than additive increase in the signal at 220 nm (Figure 3A), indicating an increase in the α-helical content due to interaction of NG with CaM. Under the same conditions, the CD spectra of ox-NG + CaM (Figure 3C) exhibited a slight increase in the signal at 220 nm over the theoretical noninteracting sum of the individual spectra, suggesting that the ox-NG interacts poorly with CaM. Interaction of the red-NG with CaM (Figure 3B) resulted in a similar degree of

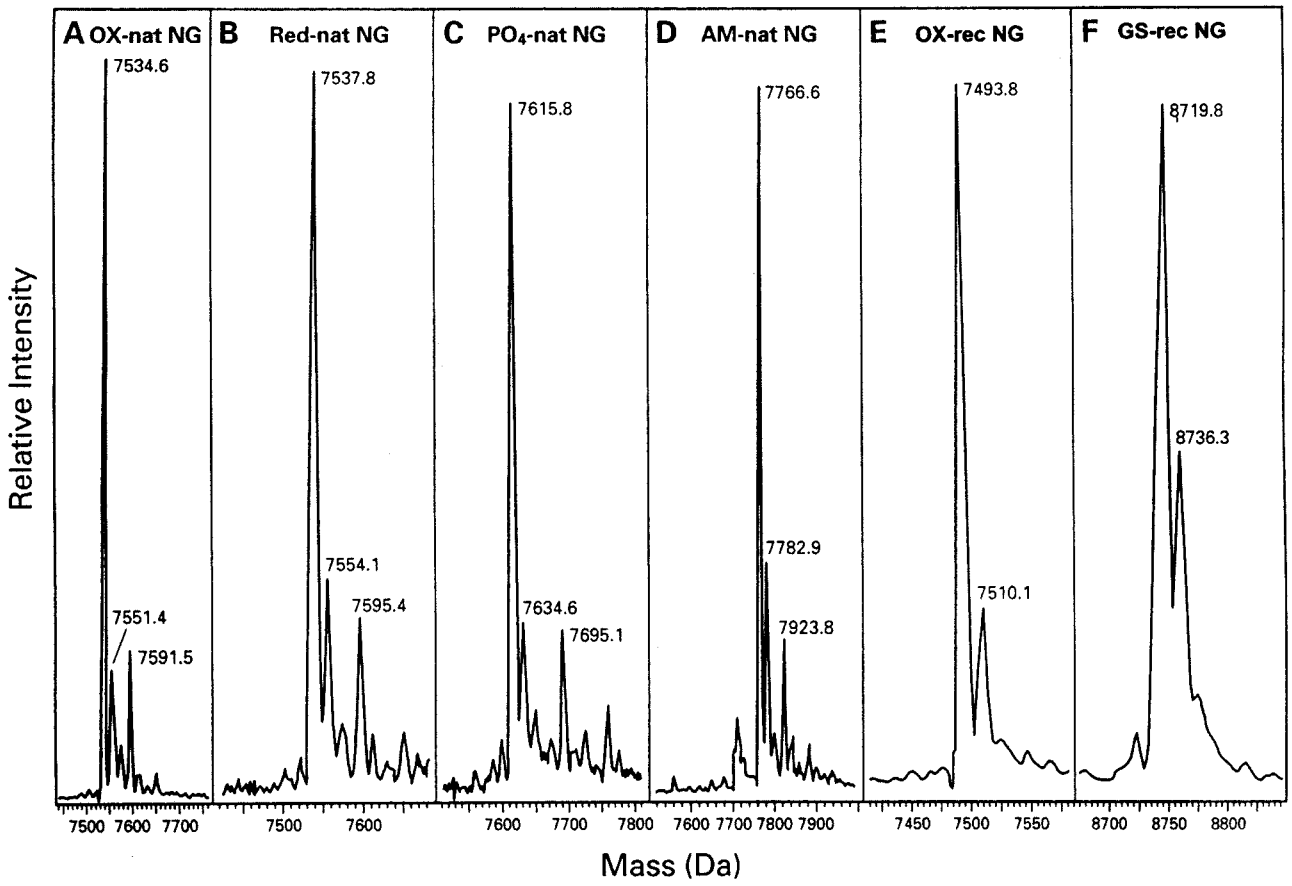


FIGURE 1: Mass spectrometric analysis of ox-, red-, $PO_4$-, and GS-NG. The purified native rat brain (nat NG) and recombinant NG (rec NG) were already in the oxidized form. The iodoacetamide-treated, $PO_4$-, and GS-NG were purified by C4 reverse phase HPLC and lyophilized. The ox-, $PO_4$-, and GS-NG were analyzed directly, and the red-NG was prepared by incubation of the ox-NG with 1 mM DTT immediately before analysis. The estimated mass of the ox-nat NG is 7534.14 ± 0.18 Da (panel A), red-nat NG 7538.88 ± 0.90 Da (panel B), $PO_4$-nat NG 7615.43 ± 0.81 Da (panel C), iodoacetamide-treated reduced rat brain NG (AM-nat NG) 7766.49 ± 0.16 Da (panel D), ox-rec NG 7493.79 ± 0.31 Da (panel E), and GS-rec NG 8719.85 ± 0.19 Da (panel F).

enhancement in the CD spectra as those seen with the Cys-(−)-NG. The enhanced signal at 220 nm is an indication of the increase in the α-helical content primarily due to interaction of the NG PSD with CaM as predicted by Gerendasy et al. (*6*). Due to the time required for measuring the individual spectra and the interaction of red-NG with CaM, the concentration of DTT was kept at 2 mM to ensure that the NG was in the reduced form throughout the experiment. The $PO_4$-NG + CaM under the reducing condition exhibited only a marginal increase in the signal at 220 nm over the sum of these two components (Figure 3D), an indication that the PSD of $PO_4$-NG interacts poorly with CaM. These results confirm the previous finding that the PKC-phosphorylated NG does not bind to the CaM-affinity column (*3*). Titration of CaM with increasing concentrations of Cys(−)- and red-NG gave apparent $K_d$ values of 2.9 ± 0.5 and 5.4 ± 0.48 μM, respectively (Figure 4). However, the $K_d$ values of $PO_4$- and ox-NG were, however, too large to be determined by CD spectrometry.

*Interaction of GS-NG with CaM.* The ox-NG does not contain any free sulfhydryl group and bound poorly to the CaM-affinity column (Figure 5B). Thus, we sought to determine if oxidation of the four sulfhydryl groups of NG by another mechanism, namely, glutathiolation, would also affect its binding to CaM. Glutathiolation is one of the mechanisms frequently involved in the modification of the protein sulfhydryl group in vivo. Rat brain NG could be modified by glutathiolation at all four Cys residues by treatment with partially decomposed GSNO. The GS-NG, like red-NG (Figure 5A), bound to the CaM-affinity column in the presence of EGTA and could be eluted with buffer containing $Ca^{2+}$ (Figure 5C). Thus, oxidation of the four sulfhydryl groups of NG by glutathiolation without forming an intramolecular disulfide bond does not affect its binding to the CaM-affinity column. The GS-NG, however, was found to be a poorer substrate of PKC than red-NG; the $V_{max}$ of the former was approximately half of the latter, and the $K_m$ values of both were comparable. The CD spectra of GS-NG + CaM were greater than the sum of the two individual components at 220 nm (Figure 6A). This increment in ellipiticity at 220 nm was greatly reduced in the presence of $Ca^{2+}$ (Figure 6B), indicating that the interaction between GS-NG and CaM is sensitive to $Ca^{2+}$. Titration of CaM with increasing concentrations of GS-NG gave an apparent $K_d$ of 5.6 ± 0.63 μM (Figure 4), which is similar to that of the red-NG. It seems that formation of mixed disulfides with the four sulfhydryl groups is not the major cause for the observed reduction in binding affinity of ox-NG. The formation of intramolecular disulfides that results in a restricted movement of the amino- and carboxyl-terminus may impede the interaction between the ox-NG and CaM.

*Determination of the $K_d$ of Interaction between CaM and the Various Forms of NG by Sedimentation Equilibrium.* To verify the observed differences in binding affinities of the

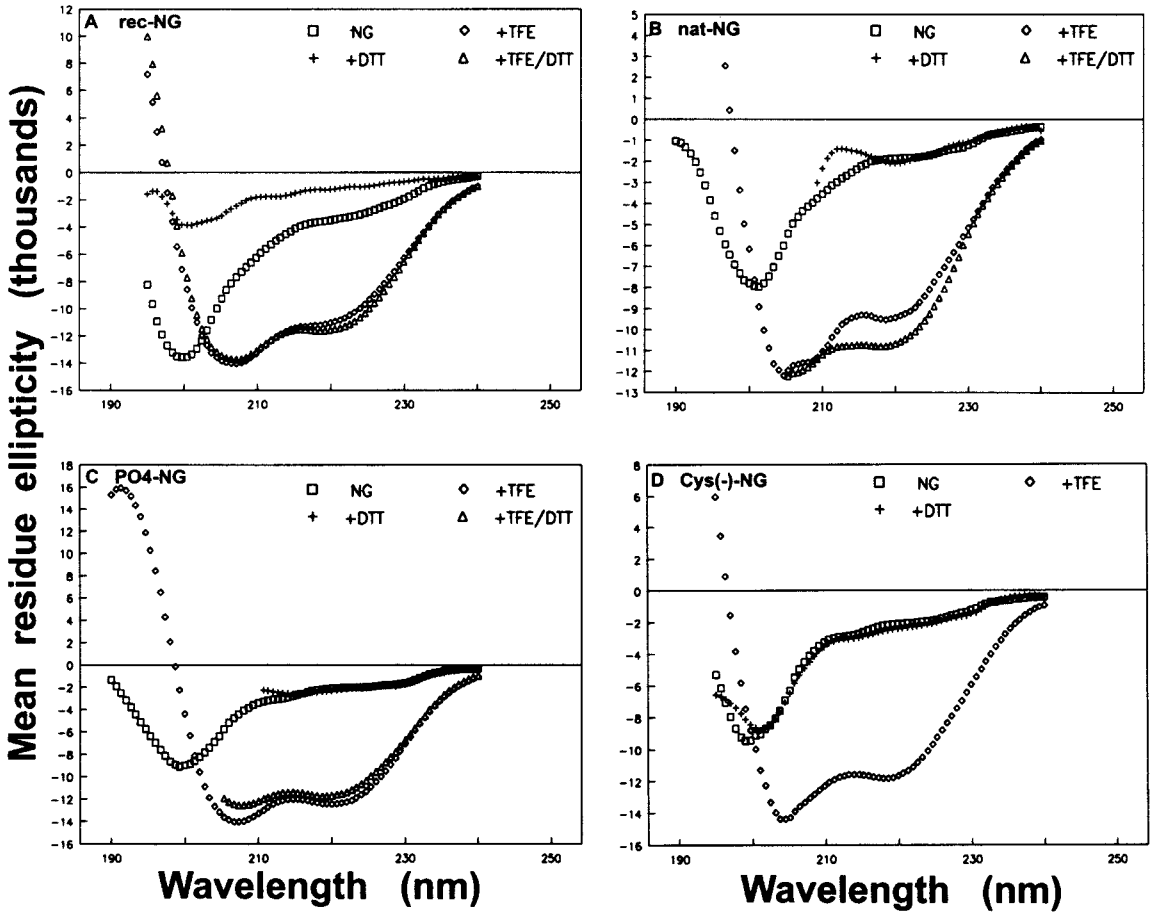


FIGURE 2: CD spectra of the WT, phosphorylated WT, Cys(−) mutant, and native rat brain NG ± DTT. The CD spectra of the purified recombinant WT NG (rec-NG) (panel A), native rat brain NG (nat-NG) (panel B), phosphorylated recombinant NG ($PO_4$-NG) (panel C), and Cys(−)-NG (panel D) were measured in 5 mM phosphate buffer, pH 7.2, containing 100 mM NaCl (□) or in 90% TFE (◇). To measure the effects of DTT, these proteins at 10 times concentration were preincubated with 1 mM DTT in 0.1 M phosphate buffer, pH 7.6, at room temperature for 10 min and then diluted 10-fold into 5 mM phosphate buffer, pH 7.2, containing 100 mM NaCl (+). For measuring the CD spectra of the DTT-treated samples in 90% TFE, the samples were centrifuged at 14 000 rpm in an Eppendorf centrifuge for 1 min, and the supernatant was used for analysis (△) (panels A−C). Mean residue ellipticities were plotted as a function of wavelength. The spectra were smoothed for clarity in presentation.

various forms of NG and CaM by CD spectrometry, we also employed sedimentation equilibrium experiments to determine the $K_d$ of these interactions. This approach, however, was not suitable for the red-NG as this protein became oxidized during several days of centrifugation in the presence of 50 $\mu$M DTT, an upper limit that would not interfere with optical detection of NG at 230 nm. The $K_d$ values of interactions of the various forms of NG and CaM determined by sedimentation equilibrium and CD spectrometry are summarized in Table 1. By sedimentation equilibrium, the interaction of Cys(−)-NG and CaM had a lower $K_d$ than those of GS- and ox-NG, whereas no interaction was detectable between CaM and $PO_4$-NG. By CD spectrometry, Cys(−)-NG also exhibited a lower $K_d$ than those of the red- and GS-NG. Based on these measurements, it appears that $PO_4$-NG has the lowest affinity for CaM. It should be noted that the $K_d$ values determined by sedimentation equilibrium were much higher than those determined by CD spectrometry perhaps due to the differences in the temperatures of the experiments. The CD spectrometry experiments were done at room temperature, and those of sedimentation equilibrium were at 4 °C. The differences in the $K_d$ values between ox-NG and those of Cys(−)-, red-, and GS-NG determined by CD spectrometry and sedimentation equilibrium were consistent with the experiments using CaM-affinity chromatography, in which the ox-NG did not bind to the affinity column. It has been shown previously that $PO_4$-NG does not bind to the CaM-affinity column (*3*). Based on the results of three separate analyses, it is apparent that phosphorylation of the PSD exerts the most pronounced effect on the binding affinity of NG to CaM.

## DISCUSSION

Rat brain NG contains four Cys residues that are readily oxidized by many oxidants in vitro (*19*) as well as by the neurotransmitter *N*-methyl-D-aspartate in brain slices in situ (*21*). Previously, we have characterized the oxidation of NG based on its shift in the electrophoretic mobility in nonreducing SDS−PAGE (*19*, *20*). This assay detects the gross changes in the molecular shape of NG in the presence of SDS. The exact number of disulfide bonds formed was not determined. In this study, by using ES-MS we have demonstrated that there are two pairs of disulfide bonds formed in the ox-NG. The reduced rat brain NG has a mass of 7538 Da and that of the oxidized one 7534 Da. The mass of the recombinant ox-NG is 7493 Da, which is that expected from the cloned cDNA sequence (7496 Da for the reduced and 7492 Da for the oxidized form) (*14*, *15*). Thus, the

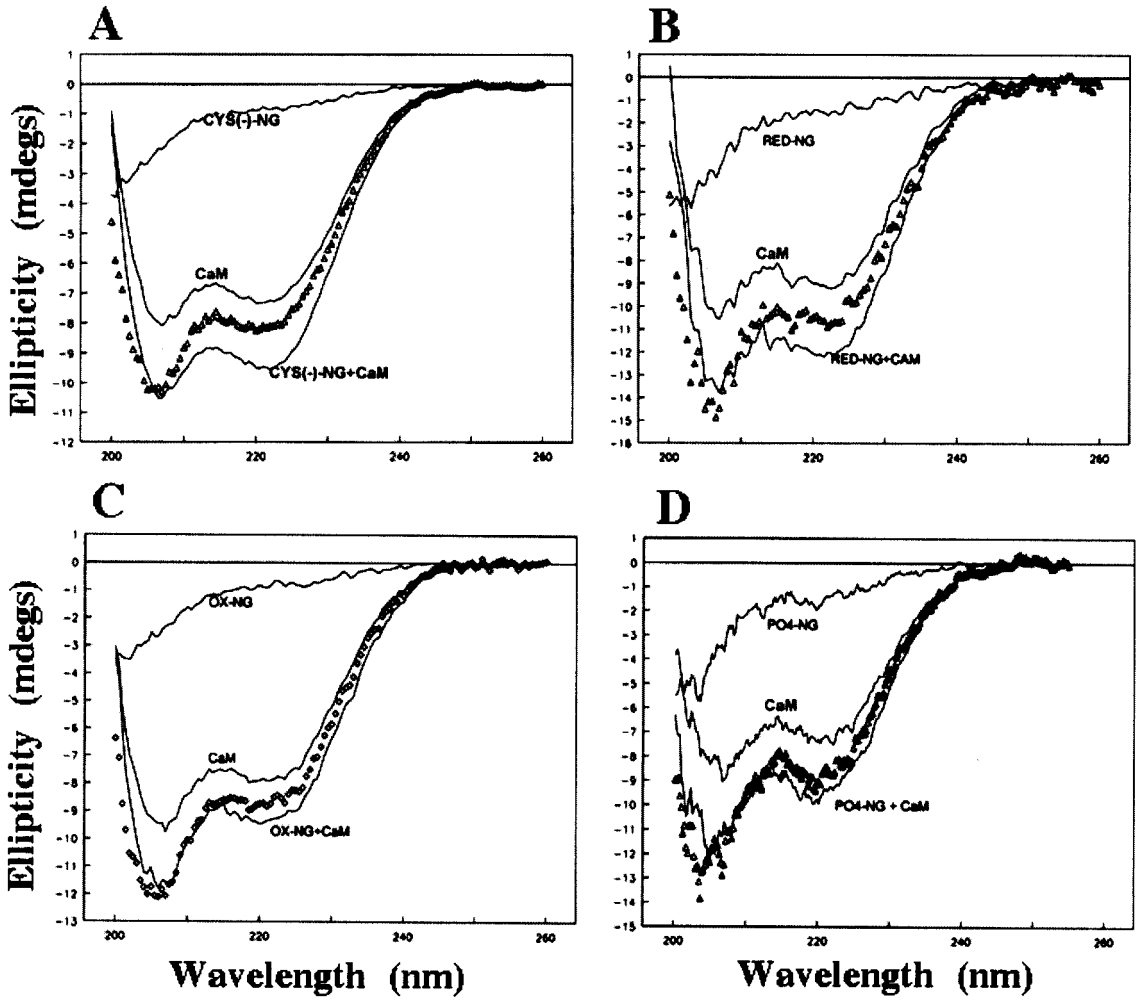


FIGURE 3: Interaction of Cys(−)-, red-, ox-, and $PO_4$-NG with CaM in the presence of EGTA. CD spectra of Cys(−)-NG (panel A), red-NG (panel B), ox-NG (panel C), or $PO_4$-NG (panel D), each at 10 $\mu$M, CaM (5.2 $\mu$M), and a mixture of each of the various forms of Ng and CaM were measured in 5 mM phosphate buffer, pH 7.2, containing 100 mM NaCl and 2 mM EGTA (panels A and C) or the same buffer containing 2 mM DTT (panels B and D). The spectra of each individual component, their sum (△ or ◊), and those after mixing are shown. Each data point represents the average of three measurements.

difference between the rat brain and recombinant NG is 41 Da, which may be assigned as an acetyl group that modifies the amino-terminal methionine. The amino-terminus of rat brain NG has been shown to be blocked (*4*), and now we can assign the modification as *N*-acetylation. The PKC-phosphorylated rat brain ox-NG has a mass of 7615 Da, which is 81 Da higher than the nonphosphorylated form. The major site of phosphorylation by PKC has been determined as Ser36 (*4*). A recent report by Di Luca et al. (*38*), also using ES-MS, assigned the mass of rat brain NG as 7450 Da and its phosphorylated form 7534 Da. These authors apparently did not take into consideration that the amino-terminus of rat brain NG is modified by acetylation, and thus their assignment of the mass of rat brain NG was underestimated. Their assigned mass of phosphorylated NG of 7534 Da is, in fact, that of the oxidized form as we have shown in this study, and the 7450 Da species has not been detected in our purified rat brain NG preparations. Thus, the observations made by Di Luca et al. (*38*) on the high level of NG phosphorylation in vivo may be questionable.

The oxidized NG contains two pairs of intramolecular disulfide bonds bridging the carboxyl-terminal Cys51 and the amino-terminal Cys3, -4, or -9. The exact pairing between these Cys residues has not been determined; however, our previous mutagenesis study indicates that Cys51 can pair with any one of the amino-terminal Cys residues (*20*). As Cys3 and -4, two neighboring Cys residues, are not likely to form a disulfide due to structural constraint imposed by the peptide bond, we predict that the two pairs of disulfide bonds are formed between Cys51 and Cys3 or Cys4 and between Cys9 and either of the latter two Cys residues. Upon oxidation, rat brain NG preferentially forms intramolecular and rarely intermolecular disulfide bonds. Thus, we predict that Cys51 must be in close proximity to the amino-terminal Cys residues. Cys51 is located in a region rich in basic amino acids (RKKIKSGEC$^{51}$GRK), whereas the amino-terminal 25 amino acids of NG contain 8 acidic and only 1 basic amino acid. It seems that the charge interaction may stabilize these two regions and thus favors the intramolecular disulfide bond formation. The carboxyl-terminal end (residues 55−78) of the NG molecule contains 13 Gly and 4 Pro residues with a sequence similar to collagen and likely forms a random coil. The disulfide bridge formation between Cys51 and any one of the amino-terminal Cys residue fixes NG in such a shape that it migrates as a smaller molecular weight species than the red-NG on nonreducing SDS−PAGE. In addition, we

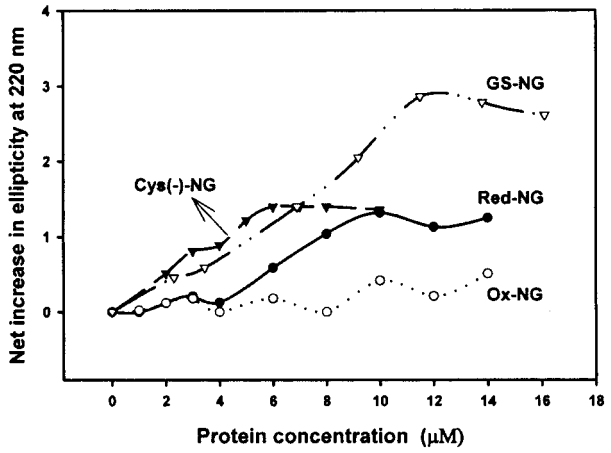


FIGURE 4: Changes in the ellipticity as a function of increasing NG concentrations. Net increases in ellipticity at 220 nm following interactions of the various forms of NG and CaM (5.2 $\mu$M) over the sum of individual spectra were plotted as a function of increasing concentrations of Cys(−)- (▼), red- (●), ox- (○), and GS-NG (▽). Each data point represents the average of three measurements. The apparent $K_d$ values determined from these data were as follows: Cys(−)-NG, 2.9 ± 0.5 $\mu$M; red-NG, 5.4 ± 0.48 $\mu$M; and GS-NG, 5.6 ± 0.63 $\mu$M. The apparent $K_d$ values of ox- and $PO_4$-NG were too large to be determined by this method.

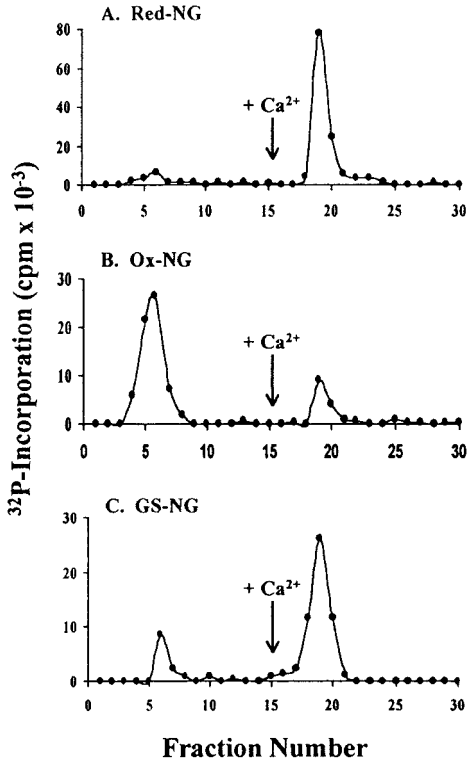


FIGURE 5: Binding of GS-NG to the CaM-affinity column. Purified red-NG (panel A), ox-NG (panel B), and GS-NG (panel C), 20 $\mu$g of each, were applied to a CaM-affinity column equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 2 mM ascorbic acid ± 2 mM DTT (see Experimental Procedures for details) and eluted with the buffer containing 6 mM $CaCl_2$ (indicated by the arrow). Effluent fractions were exhaustively phosphorylated by PKM for the detection of NG.

also noticed that the ox-NG could be separated from the red-NG by Mono Q ion-exchange chromatography at pH 7.5, in which the reduced form was eluted at a higher salt concentration than that of the oxidized form. The reduced

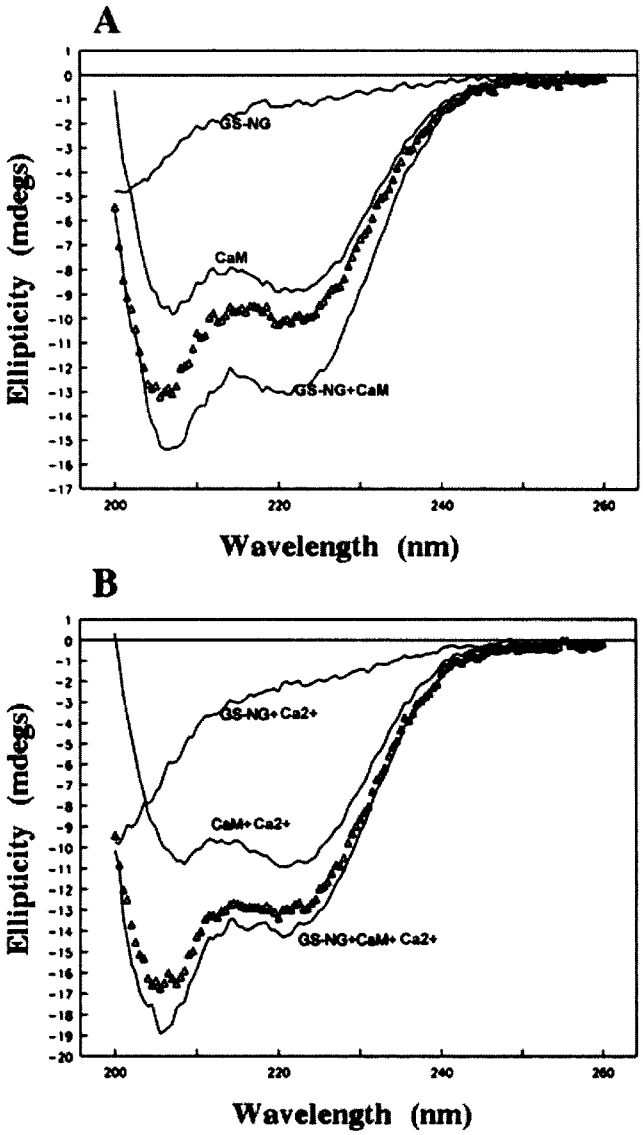


FIGURE 6: Interaction of GS-NG with CaM. CD spectra of GS-NG (11.5 $\mu$M) were measured in 5 mM phosphate buffer, pH 7.2, 100 mM NaCl, and 2 mM EGTA ± CaM (5.2 $\mu$M) (panel A) and under the same conditions + 2.15 mM $CaCl_2$ (panel B). The spectra of each individual component, their sum (△), and those after mixing are shown.

Table 1: $K_d$ of the Interactions between the Various Forms of NG and CaM Determined by Sedimentation Equilibrium and CD Spectroscopy

| | $K_d$ ($\mu$M) | | |
|---|---|---|---|
| protein | sedimentation equilibrium | CD spectrometry | binding to CaM-affinity column |
| red-NG | ND[a] | 5.4 ± 0.48 | + |
| ox-NG | 40 ± 10 | ND | − |
| $PO_4$-NG | no binding | ND | − |
| GS-NG | 20 ± 9 | 5.6 ± 0.63 | + |
| Cys(−)-NG | 14 ± 9 | 2.9 ± 0.5 | + |

[a] Not determined.

form apparently has more exposed negative charges, including the ionization of -SH groups, than those of the oxidized form for interaction with the anion-exchange matrix. Despite these differences between ox- and red-NG, both forms show random-coil conformation. Similarly, $PO_4$-, Cys(−)-, and GS-NG also assume a random-coil conformation, suggesting that

oxidation, phosphorylation, and replacement of the four Cys residues with Ser and Gly do not induce any significant change in the backbone structure of this protein.

NG is among a few IQ motif-containing proteins, including neuromodulin (GAP-43) (*39*), unconventional myosins (*40*), PEP-19 (*41*), and the Igloo protein of *Drosophila* neurons (*42*), that bind CaM preferentially in the absence of $Ca^{2+}$. The primary amino acid sequence of this domain in NG ($A^{29}$AAKIQASFRGHMARKKIKS$^{48}$), also called PSD, has a marked propensity to form α-helix. Synthetic peptides corresponding to this domain (NG29−48) interact with CaM in a fashion similar to the intact protein (*4*−*6*). These previous results suggested that this domain contributed to the observed changes in the α-helical content resulting from the NG/CaM interaction. NG both in the oxidized and in the reduced forms exhibit relatively low α-helical content; their CD spectra are similar despite the structural constraint imposed on ox-NG by the two pairs of disulfide bridges. Oxidation of NG to form intramolecular disulfides causes a reduction in the binding affinity for CaM without directly modifying the PSD. Thus, the region outside the PSD may also contribute to the stabilization of the NG/CaM complex. Phosphorylation of Ser36 within the PSD by PKC greatly attenuates the binding affinity of NG for CaM. Introduction of negative charges within the PSD [AAAKIQAS$^{36}$($PO_4$)-FRGHMARKKIKS] disrupts the interaction of this basic amphiphilic domain with CaM.

Both phosphorylation and oxidation of NG are physiological events; the former is catalyzed by PKC $\gamma$ (*43*) and the latter by nitric oxide upon stimulation of neurons with neurotransmitter (*21*). Treatment of rat brain cortical slices with NMDA caused a transient oxidation of over 25% of the endogenous NG, an indication that this protein is a major target of nitric oxide. The extent of phosphorylation of NG under the same conditions has not been determined. However, it is predictable that stimulation of neurons by NMDA will activate PKC and cause phosphorylation of NG. Thus, the combination of phosphorylation and oxidation of NG can convert a substantial amount of NG to forms with low affinities for binding to CaM. Under such conditions, the intracellular level of free CaM will rise and becomes available for many CaM-dependent enzymes. Recently, we have shown that stimulation of NG phosphorylation and oxidation by treatments of mouse hippocampal slices with okadaic acid (0.5 $\mu$M) and sodium nitroprusside (0.5 mM), respectively, caused an increase in the autophosphorylation of calcium/calmodulin-dependent kinase II. The net increase in the autophosphorylated kinase was much greater in the wild type than that of the NG-negative mutant mice (*44*). These findings further support the contention that both phosphorylation and oxidation of NG contribute to the regulation of calcium/calmodulin-dependent enzymes. The physiological role of NG glutathiolation is not clear at present; this modification as well as oxidation to form intramolecular disulfides did occur upon treatment of rat cortical slices with a combination of sodium nitroprusside and xanthine/xanthine oxidase.

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