S-Nitrosation of Ca^{2+}-Loaded and Ca^{2+}-Free Recombinant Calbindin D_{28K} from Human Brain†

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ABSTRACT: Calbindin D_{28K} is noted for its abundance and specific distribution in mammalian brain and sensory neurons. It can bind three to five Ca^{2+} ions and may act as a Ca^{2+} buffer to maintain intracellular Ca^{2+} homeostasis, but its exact role is still unknown. In the present study, mass spectrometric analysis reveals that the five cysteine residues in recombinant human brain calbindin D_{28K} (rHCaBP) are derivatized with *N*-ethylmaleimide, consistent with the determination of 5.3 ± 0.4 and 4.7 ± 0.4 free thiols in the protein using the thiol-specific reagents 5,5′-dithiobis(2-nitrobenzoic acid) and 5-(octyldithio)-2-nitrobenzoic acid, respectively. The results of UV−vis and circular dichroism absorption, intrinsic fluorescence, and mass spectrometry measurements indicate that both Ca^{2+}-loaded (*holo*) and Ca^{2+}-free (*apo*) rHCaBP are S-nitrosated by *S*-nitrosocysteine (CysNO). The number of cysteine residues S-nitrosated in *holo*rHCaBP and *apo*rHCaBP are 2.6 ± 0.05 and 3.4 ± 0.09, respectively, as determined by the Saville assay. *Holo*rHCaBP also undergoes S-nitrosation at one to three cysteine residues when exposed to *S*-nitrosoglutathione (GSNO), and Cys100 was found to be an S-nitrosation site by peptide mass mapping. Treatment of *holo*rHCaBP with free NO resulted in a mass increase of 59 ± 2 Da, corresponding to two NO adducts. Since up to four cysteine residues can be S-nitrosated in rHCaBP, it is proposed that the protein may act as a NO buffer or reservoir in the brain in a manner similar to serum albumin in blood. It is significant in this context that rHCaBP is found coexistent with nitric oxide synthase in cerebellum and that S-nitrosation varies with Ca^{2+} binding, with S-nitrosation occurring to a greater extent in *apo*rHCaBP than in the holoprotein. Furthermore, exposure of rHCaBP to either CysNO or GSNO also leads to rapid S-thiolation of Cys187. We demonstrate here for the first time that intrinsic protein fluorescence is a sensitive probe of protein S-nitrosation. This is due to efficient Förster energy transfer (R_0 ∼17 Å) between tryptophan donors and S-nitrosothiol acceptors.

Calbindin D_{28K}, also known as Ca^{2+}-binding protein (CaBP),[1] is a member of a large family of intracellular Ca^{2+}-binding proteins (*1*). The sequence of CaBP, which possesses six EF hands and binds three to five Ca^{2+} ions (*2*), is highly conserved in the human, bovine, rat, and chick protein. Sequence conservation is not just in the Ca^{2+}-binding domains, indicating that CaBP may be involved in other biologically important functions in addition to the proposed role of Ca^{2+} buffer (*3*, *4*). CaBP is also noted for its abundance and specific distribution in mammalian brain and sensory neurons (*5*). It is reported that neurodegenerative diseases such as Alzheimer's and Parkinson's are linked to deficiencies in CaBP, which exhibits diminished expression with age (*6*). Human CaBP (HCaBP) seems to be also linked to epilepsy, amyotrophic lateral sclerosis, and Huntington's disease (*7*−*9*).

NO is a molecule that has, in recent years, been shown to play many roles in bioregulation. It is known to be involved in vasodilation, platelet aggregation, inflammation, and neuronal communication (*10*). In addition, NO has been implicated in many neurodegenerative diseases such as AIDS dementia and Huntington's and Parkinson's diseases (*11*). Since NO is a labile free radical, how it exerts its biological effects has become the focus of intense research. Recently, it has been discovered that NO can react with the thiol groups of small molecules such as glutathione or cysteine to form *S*-nitroso compounds, which in turn can donate NO to proteins in a process called *trans*-S-nitrosation (*12*). These *S*-nitroso compounds are believed to be prevalent in the body (*13*) and are thought to facilitate NO transport, to prolong

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[1] Abbreviations: ANS, 8-anilinonaphthalenesulfonic acid; *apo*rHCaBP, Ca^{2+}-free rHCaBP; $Ca(OAc)_2$, calcium acetate; CD, circular dichroism; CysNO, *S*-nitrosocysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, DL-dithiothreitol; EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; GSNO, *S*-nitroso-L-glutathione; rHCaBP, human brain calbindin D_{28K}; *holo*rHCaBP, Ca^{2+}-loaded rHCaBP; IPTG, isopropyl β-D-thiogalactoside; MALDI, matrix-assisted laser desorption ion source; NEM, *N*-ethylmaleimide; NO, nitric oxide; ODNB, 5-(octyldithio)-2-nitrobenzoic acid; TFA, trifluoroacetic acid; TNB^{2-}, 2-nitro-5-thiobenzoate anion.

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the life of NO in blood and tissues, to target its delivery, and to mitigate its cytotoxic potential (*14*). Stamler and coworkers have proposed that proteins with the consensus sequence C (D, E) are likely candidates for S-nitrosation on their cysteine residues (*15*). The crystal structure of human CaBP is not yet known, but the primary sequence reveals that the protein possesses five cysteine residues, two of which (Cys100 and Cys119) have the putative *trans*-S-nitrosation consensus sequence C (E).

In the brain, NO influences synaptic plasticity, apoptosis, neuronal development, and even complex behavioral responses (*16*). CaBP is especially abundant in the brain, making up 0.1−1.5% of the total soluble protein (*17*). It is known that Ca^{2+}-loaded calmodulin, another well-known Ca^{2+}-binding protein, is required to activate NO synthase (*16*), and association of calmodulin with the constitutive NO synthase isozymes is essential for NO production (*18*). Hence it is reasonable to propose that CaBP may also play a role in NO biochemistry in brain. Moreover, there is evidence showing that CaBP may interact with as yet unknown target molecules (*1*); could perhaps one of these be NO synthase? Interestingly, CaBP is found coexistent with NO synthase in the cerebellum (*19*). Also, intercellular Ca^{2+} waves in rat hippocampal slices and dissociated glial neuron cultures are mediated by NO (*20*), and Ca^{2+} regulates S-nitrosation and denitrosation of tissue transglutaminase (*21*).

Given the possible cross talk between Ca^{2+} and NO signaling (*20*, *21*), it is of interest to determine whether Ca^{2+}-binding proteins also react with free NO or NO donors. In this work, a number of spectroscopic methods and mass spectrometry are used to directly probe S-nitrosation of the cysteine residues in recombinant human CaBP (rHCaBP).

MATERIALS AND METHODS

Materials. NO was purchased from Praxair. Isopropyl β-D-thiogalactoside (IPTG), DL-dithiothreitol (DTT), L-cysteine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), mercuric chloride, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, ammonium sulfamate, and *N*-ethylmaleimide (NEM) were purchased from Sigma. 5-(Octyldithio)-2-nitrobenzoic acid (ODNB) was purchased from Fluka, sodium nitrite was from Anachemia Chemicals, *S*-nitroso-L-glutathione (GSNO) was from Cayman, calcium acetate [$Ca(OAc)_2$] and acetonitrile (HPLC grade) were from Fisher, and trifluoroacetic acid (TFA, HPLC grade) was from Aldrich. Nanopure water from a Barnstead or Millipore system was used to prepare all solutions.

Preparation of rHCaBP. A cDNA clone of rHCaBP (*22*) was inserted into the *Nhe*I and *Xba*I sites of the expression vector pGYMX (*23*) to produce an amino-terminal His-tagged fusion protein. Expression from the pGYMX-HCaBP vector was not genetically stable, and the rHCaBP gene along with a DNA fragment encoding the His tag was subcloned into pTrc99A (Amersham Pharmacia Biotech) using the *Nco*I and *Xba*I restriction sites. *Escherichia coli* cells transformed by pTrc99A-rHCaBP were grown in LB media and induced with 1 mM IPTG overnight. Harvested cells were passed through a French press, and the centrifuged cell lysate was applied to a nickel chelate affinity column (Invitrogen) and eluted using increasing concentrations of imidazole. Fractions found to contain rHCaBP at >95% purity, as judged by Coomassie-stained SDS−PAGE, were pooled and dialyzed against 20 mM Tris-HCl (pH 7.4) and 1 mM DTT. The concentration of the protein was determined spectrophotometrically (ϵ_{280} = 28037 M^{-1} cm^{-1} for *holo*rHCaBP and 26000 M^{-1} cm^{-1} for *apo*rHCaBP) (*1*). The protein was stored as a stock solution (6 mg/mL) in 20 mM Tris-HCl (pH 7.4), 0.1 mM $Ca(OAc)_2$, and 1 mM DTT at −80 °C. DTT was removed immediately prior to use by gel filtration on a G25 NAP-10 1.3 × 2.6 cm column (Amersham Pharmacia Biotech) equilibrated with 5 mM Tris-HCl (pH 7.4) and concentrated by ultrafiltration (Amicon Centricon unit with a YM-10 filter; Millipore). Given the nature of the experiments carried out here, it was not possible to use working solutions containing DTT as recommended for HCaBP due to its propensity to form disulfide cross-links (*24*). Care was taken to minimize handling of the protein at room temperature and to work with dilute solutions to avoid cross-linking. No evidence of covalent dimer formation was obtained by mass spectrometry. A 10−20-fold excess of EGTA was added to generate the Ca^{2+}-free protein (*apo*rHCaBP), which was purified on the NAP-10 column and concentrated as before.

Preparation of S-Nitrosocysteine. CysNO was synthesized by combining equimolar amounts of cysteine in 250 mM HCl containing 0.1 mM Na_2EDTA with sodium nitrite in water (*25*), and the pH was adjusted to 7.4 by adding 1 M NaOH dropwise. This stock solution of CysNO was diluted to the required concentration using 5 mM Tris-HCl buffer (pH 7.4). CysNO was always prepared freshly before use, shielded from light, and kept on ice.

Preparation of NO Solutions. MilliQ water in a rubber-stoppered vial was sparged for 30 min with N_2 and 30 min with NO gas. Higher nitrogen oxides were removed by passage of the gas through a 10% KOH trap before the collection vial. This resulted in a saturated solution of NO (∼1.5 mM) as measured by a NO meter (ISO-NO, World Precision Instruments, Sarasota, FL).

S-Nitrosation of rHCaBP. The protein was treated with a 10-fold molar excess of CysNO or GSNO in 5 mM Tris-HCl (pH 7.4) at room temperature for 30 min and gel-filtered on a NAP-10 column to remove excess CysNO. The protein was also exposed to an ∼20-fold molar excess of free NO in 10 mM ammonium acetate buffer at pH 4.0 for 30−40 min at room temperature.

Determination of Free Cysteines in rHCaBP with DTNB and ODNB. The number of free cysteine residues in rHCaBP was determined by monitoring the absorbance at 412 nm (ϵ = 14.15 mM^{-1} cm^{-1}) on a Cary Varian spectrophotometer of the 2-nitro-5-thiobenzoate anion (TNB^{2-}) generated by 2−4 μM rHCaBP in 100 mM potassium phosphate buffer (pH 7.27) containing 1 mM EDTA. The TNB^{2-} anion was produced by the reaction of DTNB or ODNB with the free SH groups of rHCaBP (*26*). To further determine the status of the five cysteine residues in rHCaBP, the protein was incubated with a 33-fold molar excess of NEM at room temperature in 5 mM Tris-HCl (pH 7.4) for 5 min and frozen at −80 °C to quench the reaction. The NEM-modified protein was analyzed by mass spectrometry as outlined below.

Spectrophotometric Analyses. UV−vis and CD absorption and fluorescence measurements were used to investigate the S-nitrosation of rHCaBP. Spectra were recorded at 25 °C

on a Beckman DU Series 650 spectrophotometer, a Jasco J-710 spectropolarimeter purged with N_2 at a flow rate of 5 L/min, and an AMINCO-Bowman Series 2 luminescence spectrophotometer. All samples were in 5 mM Tris-HCl buffer (pH 7.4) in 1 cm cuvettes. Appropriate blanks, run under the same conditions, were subtracted from the sample spectra.

S-Nitrosothiols in rHCaBP. The Saville method was used to determine the number of *S*-nitrosothiols (*27*). Briefly, 1 mL of ammonium sulfamate (0.5% w/v in water) was added to 200 μL of ~60 μM CysNO-exposed rHCaBP in 5.8 mL of 0.25 M sulfuric acid. Then 10 mL of $HgCl_2$/sulfanilamide solution (prepared from 1 volume of 1% w/v $HgCl_2$ in water and 4 volumes of 3.4% w/v sulfanilamide in 0.4 M HCl) was added, 0.1% w/v of *N*-(1-naphthyl)ethylenediamine in 0.4 M HCl was added to the mark in a 25 mL volumetric flask, and the absorbance at 540 nm was read after 10 min. A standard curve was prepared by the same procedure but substituting rHCaBP with CysNO.

Calculation of R_0. The Förster distance, R_0, is the donor−acceptor separation at which the donor fluorescence is quenched by 50% and is defined by (*28*)

$$R_0 = (9.79 \times 10^3)(k^2 n^{-4} Q_a J)^{1/6} \quad (1)$$

k^2 was assumed to be 0.67 for random orientation between the donor and acceptor transition dipoles (*29*); n, the refractive index of the protein matrix between the donor and acceptor, was given a value of 1.4 (*30*); and Q_a, the quantum yield for tryptophan fluorescence in the absence of acceptors, was taken as 0.2 (*31*). The spectral overlap J (cm^3 M^{-1}) between the donor emission (tryptophan residues in rHCaBP) and acceptor absorption [free GSNO, which is more stable than free CysNO at pH 7.4 (*32*), was used to estimate CysNO absorption in rHCaBP because of interfering protein absorption at ~300 nm] was calculated from (*28*)

$$J = \frac{\int F_\lambda \epsilon_\lambda \lambda^4 \, d\lambda}{\int F_\lambda \, d_\lambda} \quad (2)$$

where F_λ is the emission intensity of the donor at wavelength λ (nm) and ϵ_λ is the extinction coefficient of the acceptor at λ (nm). The integrals in eq 2 were calculated numerically between 300 and 400 nm using a step of 1 nm.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) was carried out on a ThermoFinnigan SSQ7000. Samples in 5 mM Tris-HCl buffer (pH 7.4) were mixed (1:20 v/v) with 75% acetonitrile/0.05% TFA and injected into the ESI source at a flow rate of 5 μL/min. The ESI source temperature was maintained at 70 °C. Spectra were scanned from m/z 500−2200 at a rate of 3 s/scan. Mass-scale calibration was carried out using myoglobin and L-methionylarginylphenylalanylalanine acetate (MRFA) as reference compounds. Protein mass spectra were deconvoluted using BioWorks software (ThermoFinnigan).

Peptide Mass Mapping. Endoproteinase Glu-C digestion was carried out in 0.4% ammonium acetate (pH 4.0) at 40 °C for 2 h at a rHCaBP/Glu-C ratio of 15/1 (w/w). The reaction was stopped by addition of methanol to a final concentration of 5%. The peptides were desalted using C18 tips ($ZipTip_{C18}$; Millpore) and eluted from the tips with 60% acetonitrile/0.1% TFA. The eluate (1.5 μL) was mixed with 1.5 μL of matrix solution [100 μL of α-cyano-4-hydroxycinnamic acid (40 mg/mL in acetone), 10 μL of nitrocellulose (20 mg/mL in acetone), 40 μL of acetone, and 50 μL of 2-propanol], and 1.5 μL of the peptide−matrix mixture was spotted onto a 100-well, gold-plated MALDI plate and air-dried. Samples were analyzed using an Applied Biosystems Voyager-DE MALDI-TOF mass spectrometer, equipped with a 337 nm N_2 laser. Parent ion masses were measured by the time-of-flight (TOF) analyzer with an accelerating voltage of 20 kV. The mass spectrometer was calibrated externally using calibration mixture no. 1 from Applied Biosystems.

Table 1: Determination of Free Thiols in rHCaBP

reagent	free thiols/protein (mol/mol)[a]
DTNB	5.3 ± 0.4
ODNB	4.7 ± 0.4

[a] The molar ratios given represent the mean ± RMSD (root mean square deviation) of three to four determinations.

RESULTS

Determination of Free Cysteines in rHCaBP with DTNB and ODNB. As the crystal structure for rHCaBP has not yet been determined, reactions with excess DTNB and ODNB were performed in 100 mM phosphate buffer (pH 7.27) to determine how many free cysteines are present in the protein and thus the number of potential S-nitrosation sites. The determination of free thiols with DTNB and ODNB is quite sensitive due to the strong absorption (ϵ_{412} = 14.15 mM^{-1} cm^{-1}) of the product, TNB (*26*). ODNB reacts faster with free thiols in proteins than DTNB because of the absence of one negative charge and the presence of a lipophilic hydrocarbon chain in the former (*33*, *34*). Sequence information shows that HCaBP has five cysteine residues (*3*), and the DTNB and ODNB assays determined five free thiols (Table 1), indicating that rHCaBP has no disulfide bonds.

Spectroscopic Characterization of S-Nitrosated rHCaBP. *(1) UV−Vis Absorption*. This is a commonly used method to determine the presence of a S-nitrosated product because the N−π* electronic transition between nitrogen and sulfur in S-nitroso compounds gives rise to absorption at ~335 and 550 nm (*35*). The 550 nm band is nearly electric dipole forbidden, so its extinction coefficient is much less than that at 335 nm. After the reaction of CysNO with rHCaBP, the solution was passed through a NAP-10 column to remove excess CysNO, and the UV−vis spectrum was recorded. Figure 1 shows that the rHCaBP product exhibits an absorption maximum at 333 nm, consistent with S-nitrosation of cysteine residues in the protein. Furthermore, the S-nitrosated apoprotein exhibits stronger absorption than the holoprotein.

(2) Circular Dichroism (CD). Although ~335 nm absorption is a convenient method to detect S-nitrosation, other nitrosated derivatives such as nitrosated tryptophan (*36*) as well as NO_2^-, a byproduct of CysNO decomposition, absorb at 330−350 nm. To confirm the S-nitrosation of rHCaBP and to investigate the microenvironment of the S−NO bonds in the protein, CD spectra were recorded. *S*-Nitroso compounds give characteristic CD bands at 335 and 550 nm. In contrast to the absorption of unpolarized light, CD absorption is stronger at 550 nm than at 335 nm, and the visible absorption is well separated from protein CD signals,

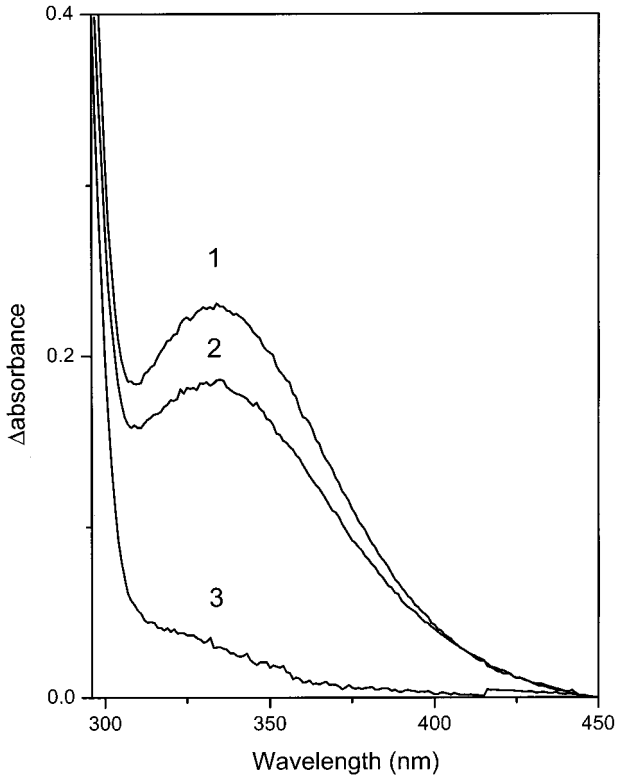

FIGURE 1: UV−vis absorption of S-nitrosated rHCaBP. UV−vis spectra of (1) 66 μM S-nitrosated *apo*rHCaBP, (2) 67 μM S-nitrosated *holo*rHCaBP, and (3) 69 μM untreated *holo*rHCaBP. Spectra were recorded in a 1 cm cuvette at 25 °C in 5 mM Tris-HCl buffer (pH 7.4). Excess CysNO was removed from the S-nitrosated samples by gel filtration on a NAP-10 column (see text).

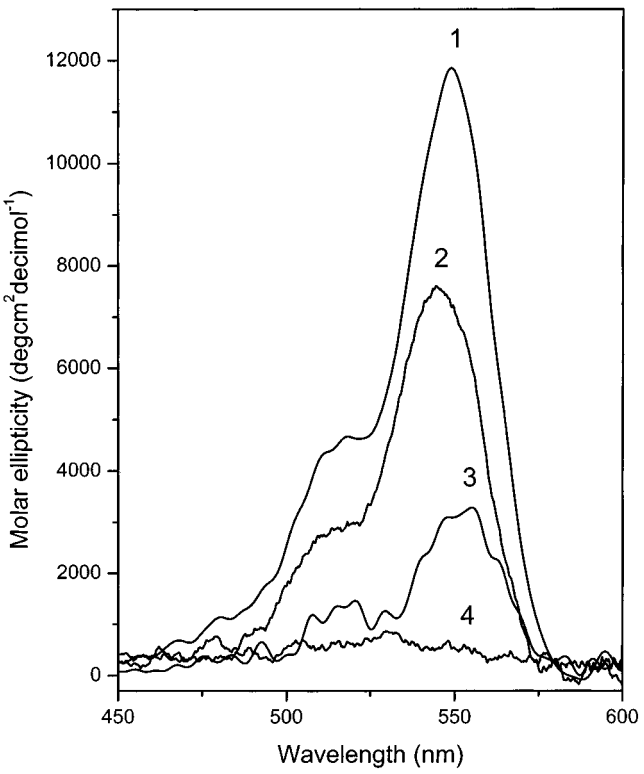

FIGURE 2: Visible CD spectra of S-nitrosated rHCaBP: (1) 66 μM S-nitrosated *apo*rHCaBP, (2) 58 μM S-nitrosated *holo*rHCaBP at pH 7.4, (3) 69 μM S-nitrosated *holo*rHCaBP at pH 3, and (4) 59 μM *holo*rHCaBP at pH 7.4. See Figure 1 for experimental conditions. The pH was adjusted to 3 in sample 3 by the addition of 1 M HCl.

including other NO-modified species (*35*). The visible−CD spectra of rHCaBP taken before and after incubation with CysNO are shown in Figure 2. S-Nitrosated *apo*rHCaBP exhibits a stronger CD signal than the S-nitrosated holoprotein. Red shifting of the CD band reveals that the SNO moiety is more exposed in the absence of Ca^{2+} (549 nm) than in the Ca^{2+}-loaded protein (545 nm) given that the CD maximum for free CysNO is at 551 nm (data not shown). Finally, the relative intensities of the CD (Figure 2) and unpolarized absorption (Figure 1) indicate greater S-nitrosation of Ca^{2+}-free vs Ca^{2+}-loaded rHCaBP.

(3) Intrinsic Fluorescence. Because of the weak intensity of SNO bands, UV−vis and CD absorption methods consume a significant amount of sample. Since NO donors also absorb at the same wavelengths as the S-nitrosated protein products, excess CysNO must be removed before the protein spectra are recorded. To find a more sensitive method, which would also be useful in kinetic analyses of the CysNO/rHCaBP reaction, intrinsic fluorescence of the rHCaBP reactants and products was examined. rHCaBP has 14 Phe, 8 Tyr, and 2 Trp residues. When excited at 295 nm, *holo*rHCaBP emits at 328 ± 1 nm (Figure 3A). Thus, compared to free tryptophan (350 nm), the environment around the tryptophan residues in *holo*rHCaBP is quite hydrophobic. When Ca^{2+} is removed from the protein, the tryptophan environment does not appear to be perturbed, as the intrinsic fluorescence of the apo- and holoprotein is very similar (Figure 3A). In contrast, Berggard and co-workers reported significantly higher intrinsic fluorescence for their Ca^{2+}-loaded rHCaBP compared to the apo form, but surprisingly the wavelength maximum and shape of the emission bands were exactly the same. Since fluorescence intensities are error prone due to the many factors that can lead to quenching, we also recorded the emission of our rHCaBP at high concentration (77 μM), similar to that used by Berggard et al. (68 μM) (Figure 5 in ref *1*). We obtained the same results (data not shown) as those shown in Figure 3 using 8 μM protein. Second, their rHCaBP displayed an emission maximum at 335 nm whereas ours fluoresces maximally at 328 nm. Third, they reported that ANS bound to *apo*rHCaBP gave a higher intensity and blue-shifted emission compared to ANS bound to the Ca^{2+}-loaded form. We obtained opposite results with our rHCaBP (data not shown); the fluorescence of ANS bound to *apo*rHCaBP increased and blue shifted when Ca^{2+} was added to the protein solution, just as observed for chicken calbindin D_{28K} and calmodulin (*1*).

Spectral overlap between tryptophan emission at 320−340 nm and S−NO absorption at 330−340 nm should give rise to efficient energy transfer between tryptophan and CysNO residues. As excepted, the fluorescence intensity of both *holo*- and *apo*rHCaBP decreases dramatically following S-nitrosation, with greater quenching in the apoprotein (Figure 3A). To confirm that the quenching is due only to tryptophan → CysNO energy transfer, additional experiments were carried out. Figure 3B shows that incubation of free tryptophan with CysNO at room temperature for 30 min has a negligible effect on its fluorescence, establishing that the quenching in Figure 3A is not due to freely diffusing CysNO or its dissociation products.

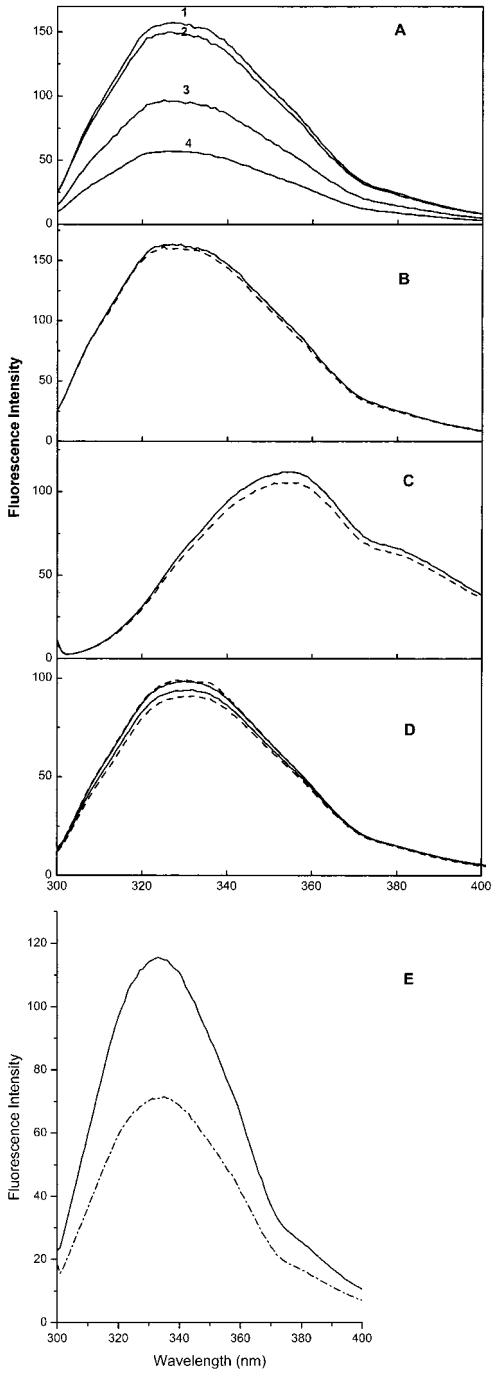

FIGURE 3: Effect of S-nitrosation on the intrinsic fluorescence of rHCaBP. (A) spectrum 1, 8 μM *holo*rHCaBP; spectrum 2, 8 μM *apo*rHCaBP; spectrum 3, 8 μM S-nitrosated *holo*rHCaBP; and spectrum 4, 8 μM S-nitrosated *apo*rHCaBP. (B) 8 μM *holo*rHCaBP in the absence (solid line) and presence of 160 μM $NaNO_2$ (dashed line). (C) 10 μM free tryptophan before (solid line) and after incubation with 160 μM CysNO for 30 min (dashed line). (D) 6.8 μM *holo*rHCaBP incubated with 240 μM NEM for 30 min (upper solid line) and then 160 μM CysNO for 30 min (upper dashed line); 7.0 μM *apo*rHCaBP incubated with 240 μM NEM for 30 min (lower solid line) and then 160 μM CysNO for 30 min (lower dashed line). (A−D) Spectra were recorded in 5 mM Tris-HCl buffer (pH 7.4) with 80 μM $Ca(OAc)_2$ for *holo*rHCaBP and 200 μM EGTA for *apo*rHCaBP samples. S-Nitrosation of rHCaBP was carried out in a 30 min incubation with CysNO in 5 mM Tris-HCl (pH 7.4). (E) rHCaBP (5 μM) before (solid line) and after (dashed line) incubation with an NO-saturated solution (100 μM) in 10 mM ammonium acetate buffer with 1 mM EDTA at pH 4.0 for 40 min at room temperature. All of the spectra were recorded in a 1 cm cuvette at 25 °C.

Since it is not stable at pH 7, excess CysNO in the reaction solution will form NO_2^- as proposed by Pietraforte and coworkers (*12*):

$$2RSNO \rightarrow RSSR + 2NO^{\bullet}$$

$$2NO^{\bullet} + O_2 \rightarrow 2NO_2^{\bullet}$$

$$NO_2^{\bullet} + NO^{\bullet} \rightarrow N_2O_3$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$$

Free NO_2^- has been shown to be an efficient quencher of horse liver alcohol dehydrogenase, alkaline phosphatase, and *N*-acetyltryptophanamide fluorescence (*37*) at high concentration (~50 mM). Figure 3B shows that micromolar NO_2^- does not quench rHCaBP fluorescence under our experimental conditions. To investigate the possible quenching effect of other NO-modified species, such as nitrosotryptophan and tyrosine-NO_2, both *holo*- and *apo*rHCaBP were incubated with NEM for 30 min before incubation with CysNO to block the cysteine residues in rHCaBP. The effect of CysNO on the intrinsic fluorescence of NEM-treated rHCaBP is negligible (Figure 3D).

The mass spectra discussed later revealed the presence of one mixed disulfide in S-nitrosated rHCaBP. Since tryptophan fluorescence can be quenched by nearby disulfides (*38*), we sought to distinguish between S−NO and S−S quenching. Exposure to the oxidized form of glutathione (GSSG) resulted in a single disulfide bond formation between glutathione and a protein cysteine residue, but this S−S bond formation did not quench the intrinsic fluorescence of rHCaBP (data not shown). The mass spectrum (Figure 5B) of rHCaBP mixed with a NO-saturated solution revealed that two S−NO bonds formed in the protein at pH 4.0. The corresponding fluorescence spectra, also recorded at pH 4.0 (Figure 3E), revealed extensive fluorescence quenching and a red shift of the emission maximum from 329 to 333 nm, which indicated that the tryptophan residues become more exposed at low pH. The extent of rHCaBP S-nitrosation and protein quenching is less in the NO-treated sample compared to the CysNO-treated samples. Taken together, these results indicate that efficient Trp → CysNO energy transfer occurs in S-nitrosated rHCaBP due to the spectral overlap between tryptophan emission at 329 nm and CysNO absorption at 334 nm. The strong quenching of fluorescence in S-nitrosated rHCaBP is consistent with efficient Trp → GSNO Förster energy transfer, which exhibits a J of 8.82×10^{-16} (cm^3 M^{-1}) and a R_0 of 17 Å. Also, Trp → CysNO energy transfer is more efficient in *apo*- than in *holo*rHCaBP (Figure 3A), indicating greater S-nitrosation of cysteine residues within quenching distance of the two tryptophan residues in *apo*rHCaBP (Trp20 and Trp107).

Mass Spectrometry. ESI mass spectra were obtained to determine if incubation with the *S*-nitrosothiols or NO caused any spectroscopically silent alterations in rHCaBP. The deconvoluted ESI mass spectrum of our rHCaBP exhibits a single peak with M_r 31722 ± 2 Da (Figure 4A). This corresponds to the average mass predicted for rHCaBP from the native HCaBP sequence (*3*) plus the His tag ($MATSH_6$-IEGRAS) minus the initial methionine (M). As expected, Ca^{2+} binding was not detected under the experimental

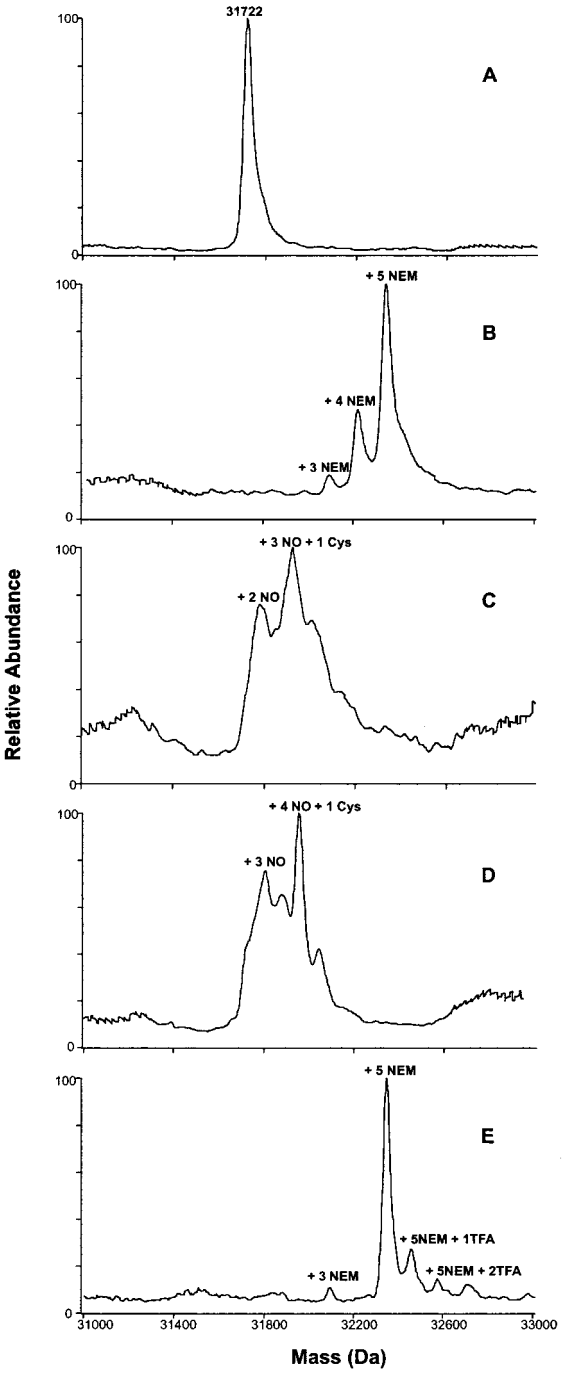

FIGURE 4: ESI-MS analysis of rHCaBP S-nitrosation: (A) 5 μM *holo*rHCaBP; (B) 1 μM NEM-labeled *holo*rHCaBP; (C) 1 μM S-nitrosated *holo*rHCaBP; (D) 1.5 μM S-nitrosated *apo*rHCaBP; (E) 1 μM NEM-labeled *apo*rHCaBP after a 30 min incubation with 10-fold molar excess CysNO. Experimental conditions: All samples were in 5 mM Tris-HCl (pH 7.4), and the S-nitrosated samples contained 0.5% TFA to stabilize the S−NO bond. NEM-labeled *holo*rHCaBP (B) and *apo*rHCaBP (E) were prepared by incubating the protein with a 33-fold molar excess of NEM for 5 and 30 min, respectively, at 25 °C, and the samples were frozen at −80 °C until used. The S-nitrosated proteins were prepared as described in Figure 1. Samples for MS analysis were diluted 20-fold into 75% acetonitrile/0.05% TFA and directly infused at a flow rate of 5 μL/min into the ESI source of the mass spectrometer with capillary temperatures of 250 °C (A, B) or 180 °C (C−E), a spray voltage of 4.5 kV, and a sheath gas pressure of 30 psi.

conditions used to record the mass spectrum (75% acetonitrile/0.05% TFA, positive ion mode). Following incubation with a 33-fold molar excess of NEM, labeling of rHCaBP with 3−5 NEM is seen in Figure 4B, confirming the results in Table 1 that five free cysteine residues are present in rHCaBP.

Mass spectrometry was also used to confirm the chemical nature of the species formed during the incubation of rHCaBP with CysNO. Since the S−NO bond is thermally labile, altering the temperature of the metal capillary transfer tube can alter the intensity of S-nitrosated species in the mass spectrum (*39*). At a capillary temperature of 180 °C, the spectrum of *holo*rHCaBP is dominated by peaks with M_r values that are 59 ± 3 and 206 ± 3 Da higher than those of untreated rHCaBP (Figure 4C). These mass shifts (ΔM_r) are assigned to rHCaBP modified with two NO groups and three NO plus one Cys, respectively. For *apo*rHCaBP, two dominant species were also detected with ΔM_r of 87 ± 3 and 235 ± 3 Da, which corresponds to protein derivatized with three NO groups and protein derivatized with four NO plus one Cys (Figure 4D).

To determine whether CysNO incubation resulted in modification of only cysteine residues, the protein was first treated with a 33-fold molar excess of NEM for 30 min. A 30 min incubation of NEM-treated rHCaBP with a 10-fold excess of CysNO was then carried out before the mass spectra were recorded. Figure 4F shows a single dominant peak with a ΔM_r of 625 Da higher than untreated rHCaBP, which reveals labeling with five NEM groups. There are also minor peaks corresponding to protein labeled with three NEM and TFA adducts of the dominant (rHCaBP−NEM_5) species. These results indicate that a 33-fold excess of NEM blocks all five cysteine residues in rHCaBP and that CysNO does not modify the protein at other residues such as tryptophan or tyrosine under the experimental conditions used in this work.

Because the GSNO is likely the dominant intracellular *S*-nitrosothiol, *holo*rHCaBP was incubated with a 10-fold excess of GSNO. The mass spectrum (Figure 5A) revealed that, despite the larger size of GSNO, up to three cysteine residues were S-nitrosated and a glutathione adduct was also formed. The mass increase of 306 Da for the glutathione adduct resulted in much better peak separation compared to that in the CysNO-modified protein (Figure 5A vs Figure 4C). A comparison of the peptide mass maps obtained by MALDI-TOF for modified and unmodified rHCaBP indicated that Cys187 is the S-thiolation site and Cys100 is one of the S-nitrosation sites (data not shown). Peptides containing the other cysteine residues in rHCaBP (Cys94, Cys219, and Cys257) were found as unmodified peptides in the MALDI mass spectra.

Determination of S-Nitrosothiols in rHCaBP by the Saville Assay. The spectroscopic and mass spectral results all show that *apo*rHCaBP undergoes greater S-nitrosation than the holoprotein on treatment with CysNO. To confirm this important conclusion, the Saville assay for nitrosothiol quantitation was selected as the method of choice. The numbers of *S*-nitrosothiols per protein molecule determined by the Saville assay (Table 2) show that in *holo*rHCaBP and *apo*rHCaBP about two to four cysteine residues are S-nitrosated, with a greater extent of S-nitrosation in *apo*rHCaBP.

DISCUSSION

The reaction with the thiol-specific reagents, DTNB and ODNB, shows that rHCaBP possesses five free cysteines

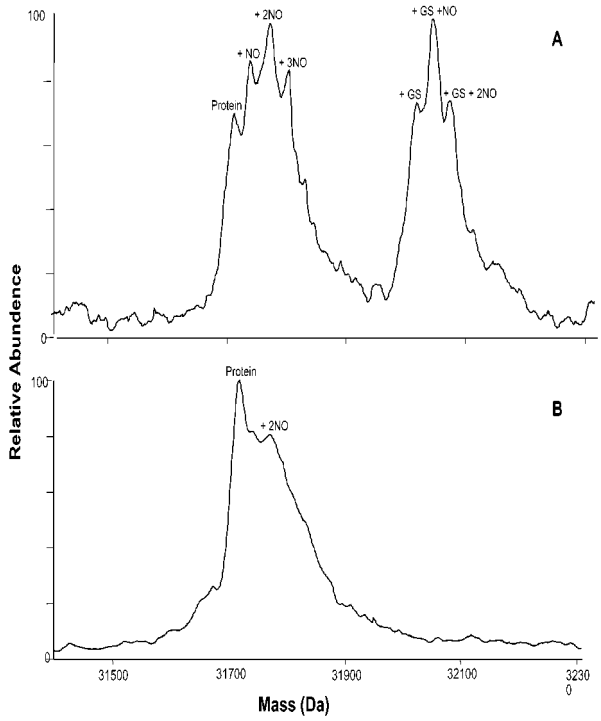

FIGURE 5: ESI-MS of GSNO- and NO-treated rHCaBP. (A) 2.3 μM *holo*rHCaBP treated with a 10-fold molar excess of GSNO for 30 min in 5 mM Tris-HCl (pH 7.4) at room temperature. (B) 2.2 μM *holo*rHCaBP treated with a 20-fold molar excess of NO for 30 min in 10 mM ammonium acetate buffer (pH 4.0) at room temperature. The mass spectrometry conditions were the same as in Figure 4.

Table 2: Determination of CysNO in rHCaBP by the Saville Assay[a]

protein	CysNO/protein (mol/mol)[b]
*holo*rHCaBP	2.6 ± 0.05
*apo*rHCaBP	3.4 ± 0.09

[a] See Materials and Methods. [b] The molar ratios given represent the mean ± RMSD (root mean square deviation) of three to four determinations.

(Table 1). This is consistent with the fact that most members of the troponin C superfamily of proteins do not contain disulfide bonds (*40*). The cytosol provides a reducing environment that prevents the formation of disulfide linkages by maintaining cysteine residues in their reduced form (*24*). Mass spectrometry revealed that five NEM adducts are formed (Figure 4E), confirming that rHCaBP has five reactive cysteines. Since the rHCaBP species labeled with three and four NEM groups also show quite strong signals (Figure 4B), the mass spectral data additionally indicate that one cysteine residue is likely buried in the protein and a second semiburied. The Saville assay demonstrates that 2.6 ± 0.05 S−NO groups are present in *holo*rHCaBP and 3.4 ± 0.09 in *apo*rHCaBP after incubation with CysNO (Table 2).

Modified rHCaBP exhibited UV absorbance at ~333 nm, which confirms the presence of the S−NO group. S-nitrosated *holo*rHCaBP and *apo*rHCaBP possess ϵ_{333} values of 2.7 ± 0.3 and 3.3 ± 0.2 mM^{-1} cm^{-1}, respectively. From the published values of 0.87 and 0.76 mM^{-1} cm^{-1} for BSA−SNO (*41*) and GSNO (*42*), respectively, which each possess only one free thiol, the average ϵ_{333} per S−NO group is 0.82 mM^{-1} cm^{-1}. On the basis of this number, S-nitrosated *holo*rHCaBP possesses 3.3 SNO groups and S-nitrosated *apo*rHCaBP 4.0. These estimates of SNO groups per rHCaBP are in good agreement with the results from the Saville assay (Table 2) and mass spectrometry (Figure 4C,D).

Mohney and Walker (*35*) demonstrated that changes in the Cys34-NO CD signals of BSA can be correlated with conformational change in the protein. The SNO CD absorption band at 545 nm of S-nitrosated *holo*rHCaBP decreases in intensity and red shifts by 9 nm when the pH is dropped from 7.4 to 3 (Figure 2). In contrast, the CD signal of Cys34-NO in BSA is stronger under acidic conditions. Interpretation of the visible CD spectra of S-nitrosated rHCaBP is more complicated since it has about three to four SNO groups compared to the single SNO group in S-nitrosated BSA. Nonetheless, the 4 nm red shift for *apo*rHCaBP compared to *holo*rHCaBP at pH 7.4, as well as the 9 nm red shift in *holo*rHCaBP on lowering the pH to 3, indicates that the CysNO residues are more buried on average in the holoprotein at pH 7.4 than in the apo form at pH 7.4 or the holo form at pH 3. Thus, removal of Ca^{2+} or decreasing the pH to 3 (which may also result in loss of Ca^{2+}) leads to more exposed CysNO groups in rHCaBP.

ESI-MS is a useful method for the study of S-nitrosation. In addition to accurate mass measurements, the ESI-source conditions can be varied to cleave the labile S−NO bond during the analysis (*39*). Thus, S−NO bonds formed in reactions between NO donors and proteins or peptides can be distinguished from other bonds such as disulfides formed on S-thiolation (*43*). In this study, we varied the temperature of the metal capillary transfer tube to distinguish between S−NO bond formation, which is observed only at low capillary temperatures due to its lability, and other modifications. The mass spectra reveal that, in addition to S-nitrosation, a single cysteine residue of rHCaBP is S-thiolated on incubation with CysNO (Figure 4C,D) or GSNO (Figure 5A). With *S*-nitrosothiols as NO donors, the formation of mixed disulfides has been reported in a number of other studies (*34*, *43*, *44*). NO or NO donors can also modify tyrosine or tryptophan residues in proteins (*36*, *39*, *45*). However, the combined mass spectrometry (Figure 4) and fluorescence (Figure 3) data on NEM-labeled rHCaBP confirm that, under the present experimental conditions, CysNO targets only the protein's cysteine residues. NEM-blocked rHCaBP exhibits no peaks due to additional adducts in its mass spectrum and negligible quenching of intrinsic fluorescence following incubation with CysNO.

Because of spectral overlap between tryptophan emission at 320−340 nm and SNO absorption at 330−340 nm, efficient energy transfer is expected. Our experimental data (Figure 3A) confirm this, and a R_0 value of ~17 Å was estimated for S-nitrosated rHCaBP on the basis of Trp/GSNO spectral overlap (eq 2). Efficient intramolecular Förster energy transfer is also seen in other small *S*-nitrosothiols, such as *N*-dansyl-*S*-nitrosohomocysteine (*46*) and other fluorophore-labeled *S*-nitrosothiols (*47*). For proteins that contain tryptophan residues, quenching of intrinsic fluorescence provides a rapid and sensitive method to detect S-nitrosation of cysteine residues.

Two cysteine residues were S-nitrosated in the reaction of rHCaBP with NO (Figure 5B). At low GSNO to rHCaBP molar ratios (1−5:1), about one to two cysteine residues were also the targets of S-nitrosation (data not shown). Interestingly, two of the five cysteine residues (Cys100 and Cys119)

of rHCaBB fall within putative S-nitrosation consensus motifs (*15*). Peptide mass mapping of modified rHCaBP revealed that Cys100 is S-nitrosated and Cys187 S-thiolated. Extensive attempts at peptide mass mapping using Glu-C, pepsin, and cyanogen bromide digests and both MALDI and ESI sources failed to identify other S-nitrosation sites in rHCaBP. The reasons for the instability of the other S-nitrosated peptides under the peptide mass mapping conditions are not clear. Further analysis using Cys → Ser mutants of rHCaBP is needed to investigate the other site(s) of S-nitrosation as well as mechanisms of S-nitrosation and S-thiolation of the protein.

In conclusion, this study reports that CaBP can be easily S-nitrosated. Hence rHCaBP may act not only as a Ca^{2+} buffer but also as a NO buffer or reservoir in mammalian brain and sensory neurons. Furthermore, since Ca^{2+} binding affects the extent of S-nitrosation of rHCaBP, there is likely cross talk between these two buffering capacities of the protein. Accessibility of cysteine residues may be different in the apo- and holoprotein, as suggested by the CD results, and/or ligation of carboxylate residues by Ca^{2+} may affect the reactivity of cysteine residues toward S-nitrosation. This posttranslational modification is reportedly identified by an "acid−base consensus motif" where cysteine is flanked by acidic and/or basic residues (*15*, *48*). We are currently studying the relationships between Ca^{2+} binding, S-nitrosation, and S-thiolation in rHCaBP and its mutants.

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