Mechanism of S-Nitrosation of Recombinant Human Brain Calbindin D_{28K}[†]

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ABSTRACT: Mass spectrometry and UV−vis absorption results support a mechanism for NO donation by *S*-nitrosoglutathione (GSNO) to recombinant human brain calbindin D_{28K} (rHCaBP) that requires the presence of trace copper, added as either Cu,Zn-superoxide dismutase (CuZnSOD) or $CuSO_4$. The extent of copper-catalyzed rHCaBP S-nitrosation depends on the ratio of protein to GSNO and on the reaction time, and NO-transfer is prevented when copper chelators are present. CuZnSOD is an efficient catalyst of rHCaBP S-nitrosation, and the mechanism of CuZnSOD-catalyzed S-nitrosation involves reduction of the active-site Cu^{II} by a number of the five free thiols in rHCaBP, giving rise to thiyl radicals. The Cu^{I}-ZnSOD formed catalyzes the reductive cleavage of GSNO present in solution to give GSH and release NO. rHCaBP thiyl radicals react with NO to yield the *S*-nitrosoprotein. Cu^{II}ZnSOD is also reduced by GSH in a concentration-dependent manner up to 5 mM but not at higher GSH concentrations. However, unlike the rHCaBP thiyl radicals, $GS^{\bullet}$ radicals dimerize to GSSG faster than their reaction with NO. The data presented here provide a biologically relevant mechanism for protein S-nitrosation by small *S*-nitrosothiols. S-nitrosation is rapidly gaining recognition as a major form of protein posttranslational modification, and the efficient S-nitrosation of CaBP by CuZnSOD/GSNO is speculated to be of neurochemical importance given that CaBP and CuZnSOD are abundant in neurons.

Nitric oxide (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 (*1*). In addition, NO has been implicated in many neurodegenerative diseases such as AIDS dementia and Huntington's and Parkinson's diseases (*2*). Since NO is a labile free radical, the way it exerts its biological effects has become the focus of intense research. Recently, it has been reported 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 protein thiols (*3*). *S*-Nitrosothiols are believed to be prevalent in the body (*4*), having been detected in the human airway, plasma, platelets, and neutrophils (*5*, *6*). They can confer NO-like biological activities (*7*) and are thought to facilitate NO transport, to prolong its life in blood and tissues, to target its delivery, and to mitigate its cytotoxic potential (*8*). *S*-Nitrosothiols have also been shown to regulate protein function in numerous systems (*9*, *10*). For example, sulfhydryl S-nitrosation (*11*) has been reported to affect the function of serum albumin (*8*), tissue-type plasminogen activator (*12*), gyceraldehyde-3-phosphate dehydrogenase (*13*), and protein-phosphotyrosine phosphatase (*14*). The latter two enzymes are inactivated by S-nitrosation of their active-site cysteine residues.

The mechanisms of formation of low and high molecular weight *S*-nitroso compounds in vivo are still incompletely understood. Attention has focused to date on the reactivity of NO with molecules such as glutathione, cysteine, and BSA[1]. It has been reported that *S*-nitrosothiols are formed by the autoxidation of NO to higher oxides of nitrogen (NOx) (*15*) by metal catalysis (*16*), by the action of dinitrosyl−iron complexes (*17*), or by direct reaction between NO and thiols in the presence of electron acceptors (*18*). Low molecular weight compounds are generally thought to undergo instantaneous trans-S-nitrosation or SNO−SH exchange reactions (*19*, *20*), but the mechanism of NO donation from small *S*-nitrosothiols to protein thiols has received little attention. Previous results from our lab (*21*) have shown that S-nitrosation of hemoglobin by GSNO, unlike trans-S-nitrosation (*22*), requires the presence of redox-active copper:

$$GSNO + Cu^{I} + H^{+} \rightleftharpoons GSH + NO + Cu^{II} \quad (1)$$

$$protein{-}SH + NO + Cu^{II} \rightleftharpoons protein{-}SNO + Cu^{I} + H^{+} \quad (2)$$

Questions arise as to whether copper catalysis is a general mechanism of protein S-nitrosation and if it can occur in vivo, where there is less than one free copper ion per

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[1] Abbreviations: BF_4NO, nitrosonium tetrafluoroborate; BSA, bovine serum albumin; CuZnSOD, Cu,Zn-superoxide dismutase; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; ESI−MS, electrospray ionization mass spectrometry; GSH, reduced glutathione; GSNO, *S*-nitrosoglutathione; ICP−MS, inductively coupled plasma mass spectrometry; Mb, myoglobin; neocuproine, 2,9-dimethyl-1,10-phenanthroline; rHCaBP, recombinant human brain calbindin D_{28K}.

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cell (*23*). Interestingly, it has been reported that Cu,Zn-superoxide dismutase (CuZnSOD) catalyzes the decomposition of *S*-nitrosothiols in the presence of a reductant such as GSH (*24*).

In the present study, the mechanism of rHCaBP S-nitrosation by GSNO is investigated in detail. CaBP is a Ca^{2+}-binding protein noted for its abundance and specific distribution in mammalian brain and sensory neurons. Previously, we reported that rHCaBP has five free thiols and is readily S-nitrosated and S-thiolated (*25*). GSNO, which has been identified as a low molecular weight NO carrier in vivo (*26*, *27*), was used as an NO donor. We now report that the redox turnover of trace copper is required for S-nitrosation of rHCaBP by GSNO and that CuZnSOD is a likely catalyst in vivo. Cu^{II}ZnSOD is reduced first by free thiols to give thiyl radicals and Cu^{I}ZnSOD. The latter catalyzes the reductive cleavage of GSNO to release NO, and rHCaBP-based thiyl radicals react with the nascent NO to give the S-nitrosated protein.

MATERIALS AND METHODS

Materials. Recombinant human brain calbindin D_{28K} (rHCaBP) was prepared as described previously (*25*). Glycine *N*-(*N*-L-γ-glutamyl-*S*-nitroso-L-cysteinyl) (GSNO) was obtained from Cayman, diethylenetriaminepentaacetic acid (DTPA) was obtained from ICN Pharmaceuticals, and $CuSO_4 \cdot 5H_2O$ was from Anachemia. 2,9-Dimethyl-1,10-phenanthroline hydrochloride (neocuproine) and nitrosonium tetrafluoroborate (BF_4NO) were purchased from Fluka; copper,zinc-superoxide dismutase (CuZnSOD) and bovine serum albumin (BSA) were from Roche Molecular Biochemicals; and reduced glutathione (GSH) and horse heart myoglobin were from Sigma. Nanopure water (MilliQ) from a Millipore system was used to prepare all solutions.

Aerobic Modification of rHCaBP by GSNO. rHCaBP (172 μM) was incubated with different molar ratios of GSNO in 1 mM Tris-HCl buffer (pH 7.4) for 5−180 min at room temperature in the presence or absence of chelators (200 μM DTPA and 1 mM neocuproine), 50 μM $CuSO_4$, or 40 μM CuZnSOD.

Anaerobic Modification of rHCaBP by GSNO. Anaerobic manipulations were performed under a nitrogen atmosphere (<2 ppm O_2) in a glovebox (Mbraun UniLab Workstation) at 25 °C. rHCaBP (20 μM) was incubated with 2.5-fold molar excess GSNO in 1 mM Tris-HCl buffer (pH 7.4) for 20 min in the presence of 40 μM CuZnSOD.

Modification of rHCaBP by BF_4NO. A stock solution of BF_4NO (15 mM) was prepared just prior to use in 0.2 M HCl since NO^+ is stable under acidic conditions. An aliquot (0.5 μL) of the BF_4NO stock was added to 19 μM rHCaBP in 10 mM Tris-HCl buffer (pH 7.4) to give to a final molar ratio of 1:20 (rHCaBP/BF_4NO). Addition of this quantity of BF_4NO did not alter the pH of the buffer. The reactions were carried out for 30 min at 30 °C in the presence or absence of chelators.

Myoglobin Assay for Free NO. A solution of horse heart oxymyoglobin (~1.6 mM) in 100 mM potassium phosphate buffer (pH 7.4) was prepared by dithionite reduction of metmyoglobin followed by desalting in air on a G25 NAP-10 1.3 X 2.6-cm column (Amersham Pharmacia Biotech) equilibrated with 100 mM phosphate buffer (pH 7.4). The oxymyoglobin solution was aliquoted and stored at −80 °C until needed (*26*). Release of NO from 250 μM GSNO was monitored by following the conversion of oxymyoglobin to metmyoglobin spectrophotometrically in solutions containing 10 μM myoglobin and 50 μM CuZnSOD in 10 mM PBS/1 mM EDTA with or without free thiols. Spectra were recorded in a 1-cm path length cuvette at 37 °C.

Reduction of CuZnSOD. Conversion of active-site Cu^{II} to Cu^{I} was monitored using the characteristic visible absorption peak of Cu^{II}ZnSOD at 680 nm (*28*). Cu^{II}ZnSOD (0.93 mM) was incubated with or without free thiols (2.0 mM GSH, 2.0 mM Cys, 2.0 mM BSA, or 0.5 mM rHCaBP) in 50 mM phosphate buffer/0.2 mM EDTA (pH 7.4) over 30 min at 37 °C. Spectra were recorded immediately in a 1-cm path length cuvette and again every 3 min over ~ 35 min. The time course of 1 mM CuZnSOD reduction by different concentrations of GSH was also examined. For each concentration of GSH used, spectra were recorded every 0.5 min over 30 min. The data presented are the average of triplicate measurements.

Mass Spectrometry. Electrospray ionization mass spectrometry (ESI−MS) was carried out on a ThermoFinnigan SSQ7000 mass spectrometer. Samples for protein mass spectra (10 μL) in Tris-HCl buffer (pH 7.4) were desalted using C18 pipet tips ($ZipTip_{C18}$; Millpore) and eluted from the tips with 50% acetonitrile/0.05% TFA. The eluate (10 μL) was mixed with 200 μL of 75% acetonitrile/0.2% formic acid and directly infused at a flow rate of 3 μL/min into the ESI source of the mass spectrometer with source and capillary temperatures of 70 and 185 °C, respectively, a spray voltage of 4.0 kV, and a sheath-gas pressure of 35 psi. Spectra were scanned over an *m*/*z* range of 700−1800 at a rate of 5 s/scan. The low-mass range (*m*/*z* 300−700) of the spectra was examined following sample (100 μL) infusion into the ESI source of the mass spectrometer by flow injection from a 100-μL loop (but no column) attached to the HPLC (Agilent 1090) at 50 μL/min with 75% acetonitrile/0.05% trifluoroacetic acid as a mobile phase. The mass-scale calibration was carried out using myoglobin and L-methionyl-arginyl-phenylalanyl-alanine acetate (MRFA) as reference compounds. Protein mass spectra were deconvoluted using BioWorks software (ThermoFinnigan).

ICP−MS Analysis. The copper content in 5 mM rHCaBP and 25 mM Tris-HCl was determined using previously published procedures (*29*).

RESULTS

The number of cysteine residues in a protein that are S-nitrosated or converted to protein-mixed disulfides can be readily established from accurate mass measurements. To determine the time course and concentration dependence of S-nitrosation and S-glutathiolation of rHCaBP, the protein was incubated with GSNO at different molar ratios for 5−180 min. The mass spectra in Figure 1A−C show that at a molar ratio of 1:10 rHCaBP/GSNO, the extent of S-nitrosation is incubation-time dependent. When the incubation time was increased from 5 min (Figure 1A) to 180 min (Figure 1C), the major species in solution changed from native protein to doubly NO-labeled protein. Figure 1A−C

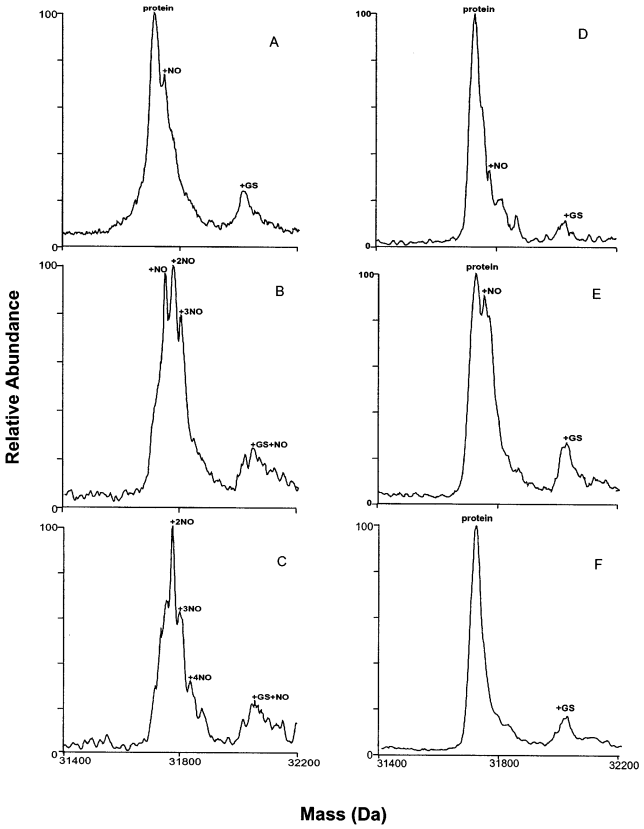

FIGURE 1: Effect of incubation time, GSNO/rHCaBP molar ratio, and metal chelators on the S-nitrosation of rHCaBP. Mass spectra of ~1 μM rHCaBP treated with 10-fold molar excess of GSNO for (A) 5 min, (B) 20 min, and (C) 180 min, and ~1 μM rHCaBP treated with (D) 2.5-fold and (E) 5-fold molar excess of GSNO. (F) Same as sample E but with 200 μM DTPA and 1 mM neocuproine added. rHCaBP (172 μM) was incubated with GSNO in 1 mM Tris-HCl buffer (pH 7.4) at room temperature. Prior to MS analysis, the samples were desalted and diluted into 50% acetonitrile/0.2% formic acid and directly infused at a flow rate of 3 μL/min into the ESI source of the mass spectrometer with a capillary temperature of 185 °C, a spray voltage of 4.0 kV, and a sheath-gas pressure of 30 psi.

also reveals that, although GSNO is in 10-fold excess, after 180 min some of the thiols in the protein are not modified, which may be due to their lower accessibility or reactivity. When the incubation time was fixed at 20 min, the extent of rHCaBP S-nitrosation increased with the molar ratio of rHCaBP to GSNO (Figure 1B,D−E).

Copper is known to catalyze the breakdown of GSNO with NO release (*30*). The metal (1.5 μM) was detected in 5 mM rHCaBP in 25 mM Tris-HCl by ICP−MS (data not shown). Thus, the effects of copper chelators on rHCaBP modification were examined. In the presence of both DTPA (a Cu^{II}-specific chelator) and neocuproine (a Cu^{I}-specific chelator), only the GS adduct of rHCaBP was detected in the mass spectrum after 20 min incubation of the protein with 5-fold excess GSNO (Figure 1F), suggesting that S-nitrosation but not S-glutathiolation is a metal-catalyzed process. However, DTPA or neocuproine alone did not completely prevent S-nitrosation (data not shown), revealing that both chelators are necessary to fully inhibit turnover of the trace copper present in solution. This would explain why *S*-nitrosothiol formation is observed in the presence of the commonly used Cu^{II}-chelators, DTPA and EDTA (*11*, *16*). Examination of the low *m*/*z* range of the mass spectra showed that incubation with GSNO did not modify the chelators (data not shown).

Could Cu-catalyzed rHCaBP S-nitrosation (eqs 1 and 2) occur in vivo? The catalyst would likely have to be protein-bound copper since there is essentially no free aqueous copper in cells (e.g., yeast contain ≤1 copper ion per cell) (*23*, *31*). The dominant copper-containing enzyme in cells is CuZnSOD with reported concentrations of 10 μM in yeast and 10−30 μM in erythrocytes and hepatocytes, and immunostaining revealed exceptionally high concentrations in motor neurons (*31*). Thus, the effect of bovine CuZnSOD on rHCaBP modification by GSNO was examined. After 20-min incubation of rHCaBP with 2.5-molar excess GSNO, the protein was extensively NO-labeled in the presence but not in the absence of CuZnSOD (Figure 2A vs Figure 1D). In contrast, CuZnSOD had little effect on the extent of rHCaBP S-glutathiolation (Figure 2A vs Figure 1D). Interestingly, when copper was added as $CuSO_4$, the yields of rHCaBP S-nitrosation and S-glutathiolation are clearly lower and higher, respectively, than in the presence of CuZnSOD (Figure 2B vs Figure 2A). This reveals that free copper is a less selective catalyst than CuZnSOD; thus, copper released from the enzyme is unlikely a cocatalyst in CuZnSOD-catalyzed rHCaBP S-nitrosation (Figure 2A).

It has been proposed that *S*-nitrosothiols such as GSNO act as NO^+ donors and undergo direct trans-S-nitrosation with thiols such as cysteine (CysH) (*32*, *33*):

$$\mathrm{GSNO + CysH \rightleftharpoons GSH + CysNO} \qquad (3)$$

BF_4NO is exclusively an NO^+ donor that can force *S*-nitrosoprotein generation by direct attack of NO^+ on free thiols. Thus, to distinguish between NO^+ and NO release from GSNO, BF_4NO-treated rHCaBP was examined by ESI−MS in the presence and absence of copper chelators. Although the mass spectra are noisy because of salt effects and not all peaks can be identified, the results reveal that S-nitrosation of rHCaBP by BF_4NO is actually more extensive in the presence of the chelators (Figure 3B) than in their absence (Figure 3A). Since copper chelation prevents protein S-nitrosation by GSNO (Figure 1F) but not by NO^+ (Figure 3B), this eliminates direct NO^+ transfer from GSNO to rHCaBP as shown for free CysH in reaction 3.

What is the mechanism of CuZnSOD-catalyzed rHCaBP S-nitrosation? Incubation of rHCaBP/GSNO/CuZnSOD under anaerobic conditions gives rise to a protein product with a mass spectrum very similar to that in Figure 2A (data not shown), revealing that O_2 does not play a role in the S-nitrosation process. Does CuZnSOD act as an NO transferase? Oxymyoglobin ($MbFe^{II}O_2$) was used as a scavenger to detect if any NO was released during the incubation. NO is quickly scavenged [$k = (43.6 \pm 0.5) \times 10^6$ M^{-1} s^{-1} at pH 7.0] (*34*) by $MbFe^{II}O_2$ to give metmyoglobin ($MbFe^{III}$):

$$\mathrm{MbFe^{II}O_2 + NO \rightleftharpoons MbFe^{III} + NO_3^-} \qquad (4)$$

Reaction 4 can be readily monitored spectrophotometrically since a decrease in absorbance at 542 and 580 nm ($MbFe^{II}O_2$ decay) is accompanied by increased absorbance at 632 and 502 nm ($MbFe^{III}$ growth), and the Soret band at 416 nm

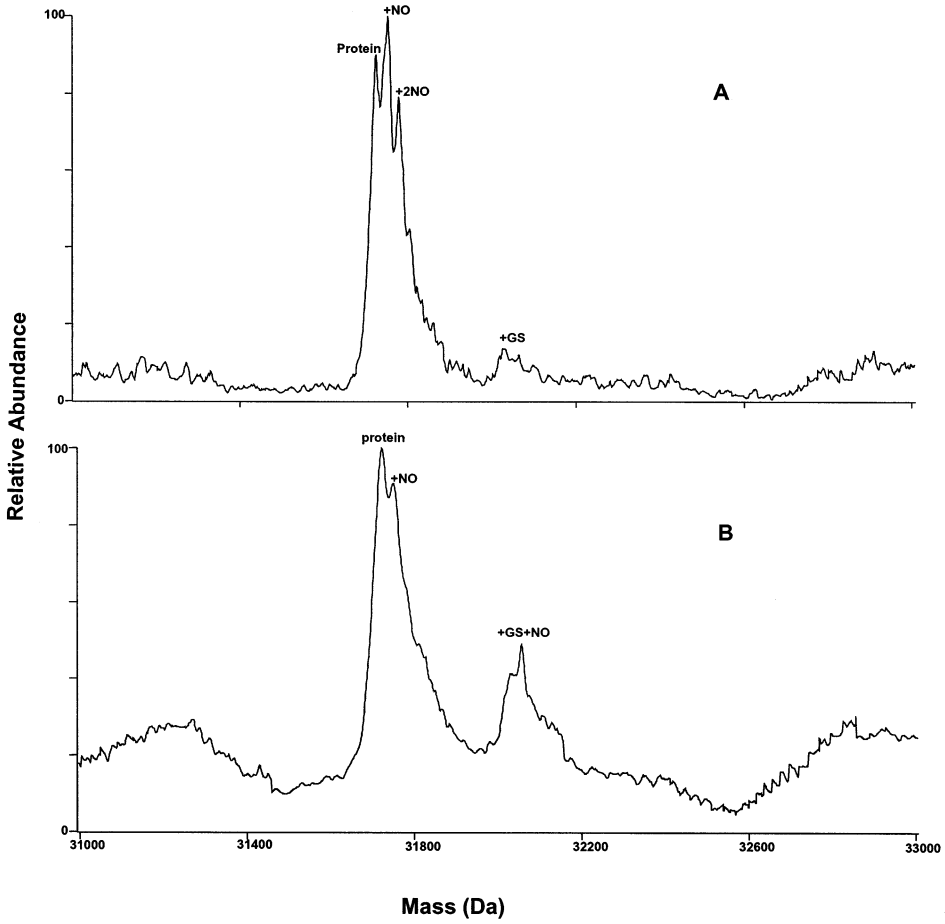

FIGURE 2: Effect of CuZnSOD and $CuSO_4$ on the S-nitrosation of rHCaBP. Mass spectra of ~1 μM rHCaBP treated with (A) 2.5-fold molar excess of GSNO with 40 μM CuZnSOD and (B) 2.5-fold molar excess of GSNO with 50 μM $CuSO_4$ for 20 min in 1 mM Tris-HCl buffer (pH 7.4) at room temperature. The mass spectral conditions are given in Figure 1.

($MbFe^{II}O_2$) shifts to 408 nm ($MbFe^{III}$). Figure 4A shows that CuZnSOD and GSNO together have negligible effect on the Mb absorption spectra, but when a free thiol, such as GSH (Figure 4B) or rHCaBP (Figure 4C) is added, absorbance changes consistent with $MbFe^{II}O_2$ decay and $MbFe^{III}$ formation are observed, indicating that NO was released from GSNO and scavenged by $MbFe^{II}O_2$ (eq 4).

Reduction of Cu^{II}ZnSOD to Cu^{I}ZnSOD by the free thiols was detected by directly monitoring the visible spectrum of the protein. A band centered at 680 nm has been assigned to the d−d absorption of Cu^{II} at the active site of CuZnSOD (*28*). When Cu^{II} is reduced to Cu^{I}, the 680-nm band disappears. The inset to Figure 5 reveals that in the absence of free thiols, the Cu^{II}ZnSOD absorbance at 680 nm was essentially constant over 30 min at 37 °C but decreased on a similar time scale when free thiols, such as GSH (Figure 5A) or rHCaBP (Figure 5B), were added. Since rHCaBP has five free thiols (*25*), four of which are quite exposed based on DTNB titrations (data not shown), a 4-fold molar excess of GSH over rHCaBP was used. Since the time scale of NO scavenging (Figure 4) is similar to that of active-site Cu^{II} reduction (Figure 5), the latter must be the rate-limiting step in Cu^{I}ZnSOD-catalyzed reductive cleavage of GSNO.

The reduction of Cu^{II}ZnSOD at higher concentrations of GSH was investigated since GSH levels are usually 2−10 mM in vivo (*35*). At ≤5 mM GSH, the reaction showed a free-thiol concentration dependence, which disappeared above 5 mM, and higher GSH concentrations were inhibiting (Figure 6A). Moreover, Cu^{II}ZnSOD was never reduced fully by physiological concentrations of GSH even after 3 h of incubation (data not shown).

Free cysteine and BSA were used to further probe the efficiency of various free thiols as donors to Cu^{II}ZnSOD. On addition of cysteine, the absorbance at 680 nm decreased to almost zero within 10 min as reported (*36*), followed by a slow increase, indicating reoxidation of the active-site copper in air (Figure 6B, trace 5). No absorbance loss at 680 nm was observed in the presence of BSA (Figure 6B, trace 2), although titration with DTNB revealed that ~ 40% of BSA possessed a free thiol (data not shown).

To fully investigate the mechanism of CuZnSOD-catalyzed rHCaBP S-nitrosation, it is necessary to also know the species produced from GSNO. After a 20-min incubation of rHCaBP with 2.5-fold molar excess of GSNO in the presence of CuZnSOD, only GSH was detected by mass spectrometry (Figure 7A).

DISCUSSION

The extent of rHCaBP S-nitrosation by GSNO depends on the incubation time and rHCaBP/GSNO ratio (Figure 1). After 20 min, ~1−2 cysteine residues were S-nitrosated (rHCaBP/GSNO = 1:10), which increased to ~3−4 after 180 min. These results are consistent with a number of

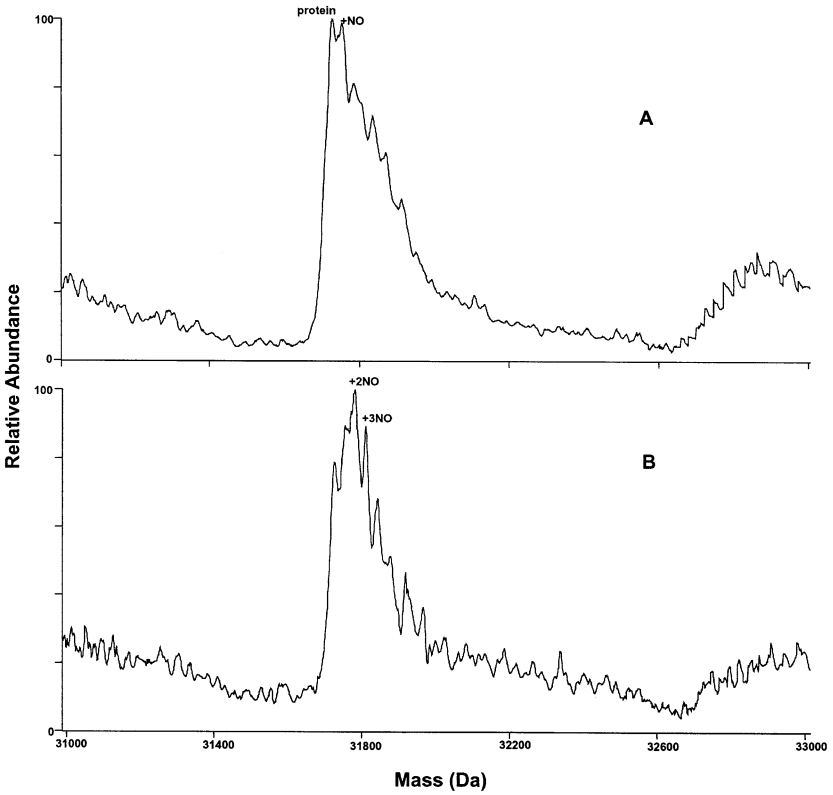

FIGURE 3: Effect of chelators on the S-nitrosation of rHCaBP by BF_4NO. Mass spectra of ~1 μM BF_4NO-treated rHCaBP in the (A) absence and (B) presence of chelators. rHCaBP was incubated with 20-fold molar excess of BF_4NO for 30 min in 10 mM Tris-HCl buffer (pH 7.4) at 30 °C with or without 200 μM DTPA and 1 mM neocuproine. The mass spectral conditions are given in Figure 1.

studies that demonstrated that enzymes with active-site cysteines are inhibited by NO donors is a time- and concentration-dependent manner (*37*). rHCaBP S-nitrosation occurs only in the presence of redox-active copper (e.g., Figure 1E vs Figure 1F). Turnover of copper by redox cycling is required (eqs 1 and 2), and the present results suggest that both Cu^{I} and Cu^{II} chelators are necessary to fully inhibit this turnover. Previous results from our lab (*22*) indicated that S-nitrosation of oxyhemoglobin at Cysβ93 by GSNO also requires redox-active copper. In contrast to S-nitrosation, S-thiolation of rHCaBP by GSNO occurred within 5 min and only slightly increased after 25 min. This mirrors the reported time course of S-glutathiolation of glyceraldehyde-3-phosphate dehydrogenase (*38*).

The presence of CuZnSOD dramatically increases rHCaBP S-nitrosation (Figure 2A). Because free copper also catalyzes rHCaBP S-nitrosation (Figure 2B), it is important to establish whether CuZnSOD-catalyzed S-nitrosation is due to its active-site copper or to copper nonspecifically associated with the enzyme. Since copper added as $CuSO_4$ is a less selective catalyst than CuZnSOD (Figure 2B vs Figure 2A), the S-nitrosation activity of the enzyme must be associated with its active-site copper. This was confirmed by pretreating CuZnSOD with DTPA to remove free copper (*36*), which did not diminish the ability of the enzyme to promote NO release from GSNO as detected by the Mb assay (eq 4) (data not shown). Furthermore, Figure 5A,B shows that both GSH and rHCaBP reduce the active-site copper, thus allowing its redox turnover. Combined, these data establish that S-nitrosation of rHCaBP is mediated by the active-site copper in CuZnSOD rather than free copper. Nonetheless, the possibility exists that free copper may be a much more rapid catalyst of both *S*-nitrosothiol formation and breakdown (i.e., eq 2) (*31*), thereby decreasing the yield of protein S-nitrosation as compared to that in the CuZnSOD-catalyzed reaction. Chelation of free Cu^{II} by GSSG (*30*) or rHCaBP would also explain the lower yield of S-nitrosation in incubates containing $CuSO_4$ (Figure 2B).

Using $MbFe^{II}O_2$ as an NO scavenger (eq 4), it was found that CuZnSOD catalyzes NO release from GSNO in the presence of GSH or rHCaBP (Figure 4). Since electron donors such as thiols are required for the reductive cleavage of GSNO, formation of Cu^{I}ZnSOD in the presence of various free thiols was investigated. By monitoring the time course of decay of the Cu^{II} d−d absorption at 680 mm, the ability of free thiols to reduce Cu^{II}ZnSOD was found to be different (Figures 5 and 6). Cysteine, the smallest thiol used, was the fastest reductant, whereas no reduction was observed with BSA (Figure 6B). Thus, steric control of access to the catalytic site may be a critical factor in CuZnSOD-mediated S-nitrosation. For example, the active-site Cu^{II} appears to be accessible to 3−4 of the five thiols in rHCaBP (*25*) and to Cysβ93 of oxyhemoglobin (A. Romeo, personal communication) but not to the single free thiol (Cys34) of BSA. The intrinsic reactivity of a protein thiol depends on its pK_a value since the thiolate anion is much more reactive toward S-nitrosation than the thiol (*39*). Cys34, which contains the single sulfhydryl group of BSA, has a low pK_a (4.5) as compared to GSH (8.75) (*26*, *40*), and the X-ray structure of human serum albumin (*41*) showed that Cys34 is partially solvent exposed. BSA is readily S-nitrosated at Cys34 by free copper and NO (*16*), so its lack of reactivity in CuZnSOD-catalyzed S-nitrosation indicates

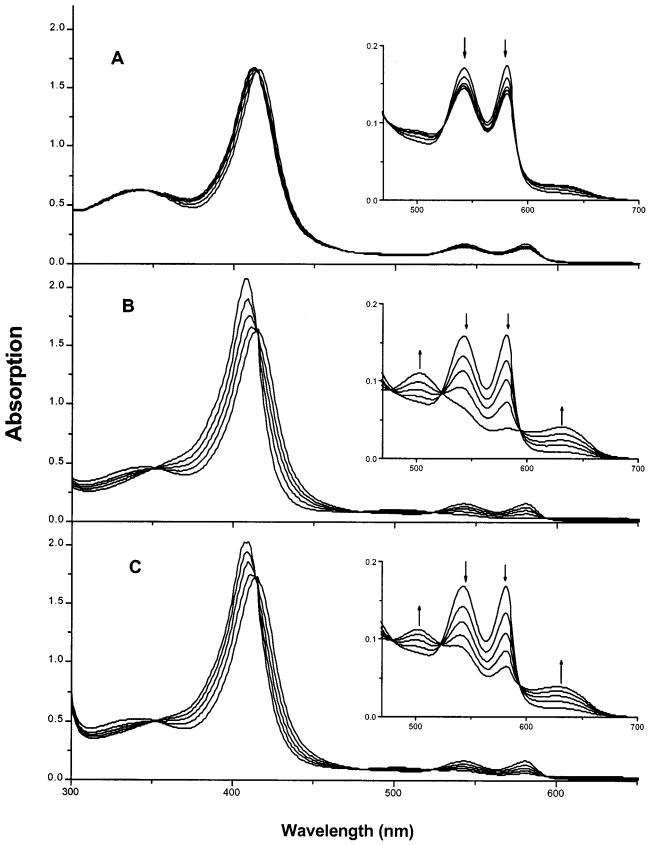

FIGURE 4: Time-dependent spectral changes in $MbFe^{II}O_2$ because of NO scavenging. Absorbance decreases at 416, 542, and 580 nm ($MbFe^{II}O_2$ decay) are accompanied by increases at 408, 502, and 632 nm ($MbFe^{III}$ growth). (A) Mb + CuZnSOD + 250 μM GSNO; (B) Mb + CuZnSOD + 125 μM GSNO + 0.5 mM GSH; and (C) Mb + CuZnSOD + 125 μM GSNO + 0.1 mM rHCaBP. All solutions contained 10 μM $MbFe^{II}O_2$ and 50 μM CuZnSOD in 10 mM PBS with 1 mM EDTA at 37 °C. Spectra were recorded at 0, 5, 10, 15, and 20 min in a 1-cm path length cuvette.

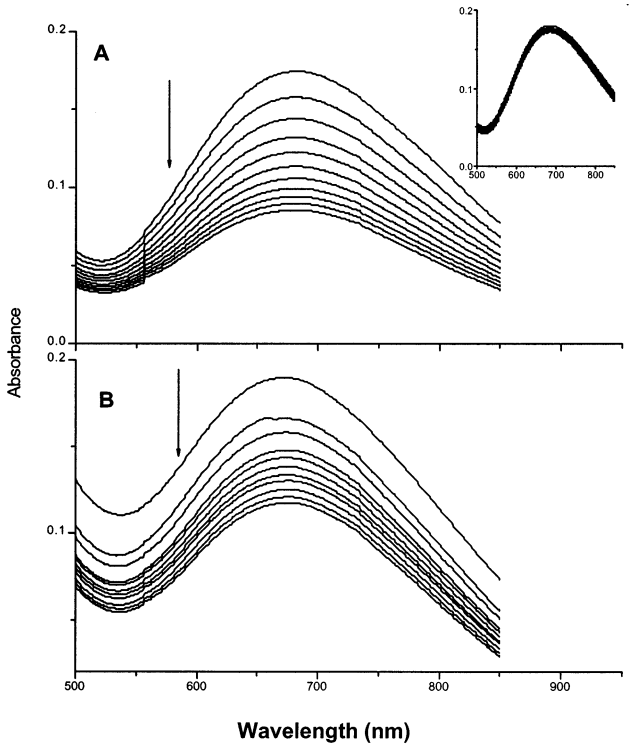

FIGURE 5: Effects of GSH and rHCaBP on the visible absorption spectrum of 0.93 mM Cu^{II}ZnSOD. (A) CuZnSOD with 2 mM GSH and (B) CuZnSOD with 0.5 mM rHCaBP and CuZnSOD alone (inset, panel A). Incubations were in 50 mM phosphate buffer/0.2 mM EDTA (pH 7.4) over 30 min at 37 °C. Spectra were recorded at 0, 3, 9, 15, 18, 21, 24, 27, 30, 33, and 36 min in a 1-cm path length cuvette.

that selectivity is dictated by specific protein−protein interactions. Thus, sites of protein S-nitrosation in vivo will likely depend not only on concentration but also on access to catalysts such as CuZnSOD, giving rise to specificity. Unfortunately, the crystal structure of rHCaBP is not yet known, so modeling its interaction with CuZnSOD is not now possible.

The rate of Cu^{II}ZnSOD reduction by GSH (Figure 6A) was found to be concentration dependent up to 5 mM. Negligible increase in reduction was observed above 5 mM GSH, which suggests product inhibition. Figure 7C shows that GSSG is present in the products, and more GSSG was formed at higher concentrations of GSH (Figure 7C vs Figure 7B). GSSG chelates free Cu^{II} (*30*), but Figure 6A shows that 10 mM GSSG causes negligible change in the 680-nm absorbance of Cu^{II}ZnSOD, indicating that it does not remove Cu^{II} from the enzyme. GSSG may block the active-site crevice and inhibit further reduction of the Cu^{II} center by GSH. This would allow CuZnSOD to function as a superoxide dismutase in the presence of high cytosolic GSH. Because of the activity of glutathione reductase (*42*), GSSG levels in vivo are < 5% those of GSH, but they may be sufficient to inhibit reduction of Cu^{II}ZnSOD by GSH.

Taking all the present results into consideration, the following mechanism for CuZnSOD-catalyzed protein S-nitrosation is proposed:

$$Cu^{II}ZnSOD + CaBP\text{-}SH \rightleftharpoons Cu^{I}ZnSOD + CaBP\text{-}S^{\bullet} + H^{+} \quad (5)$$

$$Cu^{I}ZnSOD + GSNO + H^{+} \rightleftharpoons GSH + NO^{\bullet} + Cu^{II}ZnSOD \quad (6)$$

$$NO^{\bullet} + CaBP\text{-}S^{\bullet} \rightleftharpoons CaBP\text{-}SNO \quad (7)$$

The active-site Cu^{II} in CuZnSOD is first (partially) reduced to Cu^{I} by free protein thiols, such as those of rHCaBP (eq 5). Then Cu^{I} catalyzes the reductive cleavage of GSNO (eq 6), and the nascent NO radical is scavenged by a protein-based thiyl radical such as CaBP-S$^{\bullet}$ yielding the S-nitrosated protein (eq 7). A role for any oxygen-derived species in CuZnSOD-mediated S-nitrosation is excluded since the yields of S-nitroso-rHCaBP were the same in the absence or presence of O_2. Furthermore, NO gas added to an anaerobic rHCaBP/CuZnSOD incubation did not lead to detectable nitrosation of rHCaBP (data not shown). This supports the mechanism given in eqs 5−7 since the thiyl radical yield would be low in the absence of a reaction that regenerates Cu^{II}ZnSOD (eq 6).

Cu-catalyzed dimerization of low molecular weight thiyl radicals such as GS$^{\bullet}$ is efficient and competes with their reaction with NO (*16*). However, no CuZnSOD-catalyzed dimerization of rHCaBP was detected by SDS−PAGE (data not shown), presumably because steric hindrance prevents rHCaBP cross-linking via the thiyl radicals formed. Thus, protein thiyl radicals undergo reaction 7, whereas GS$^{\bullet}$

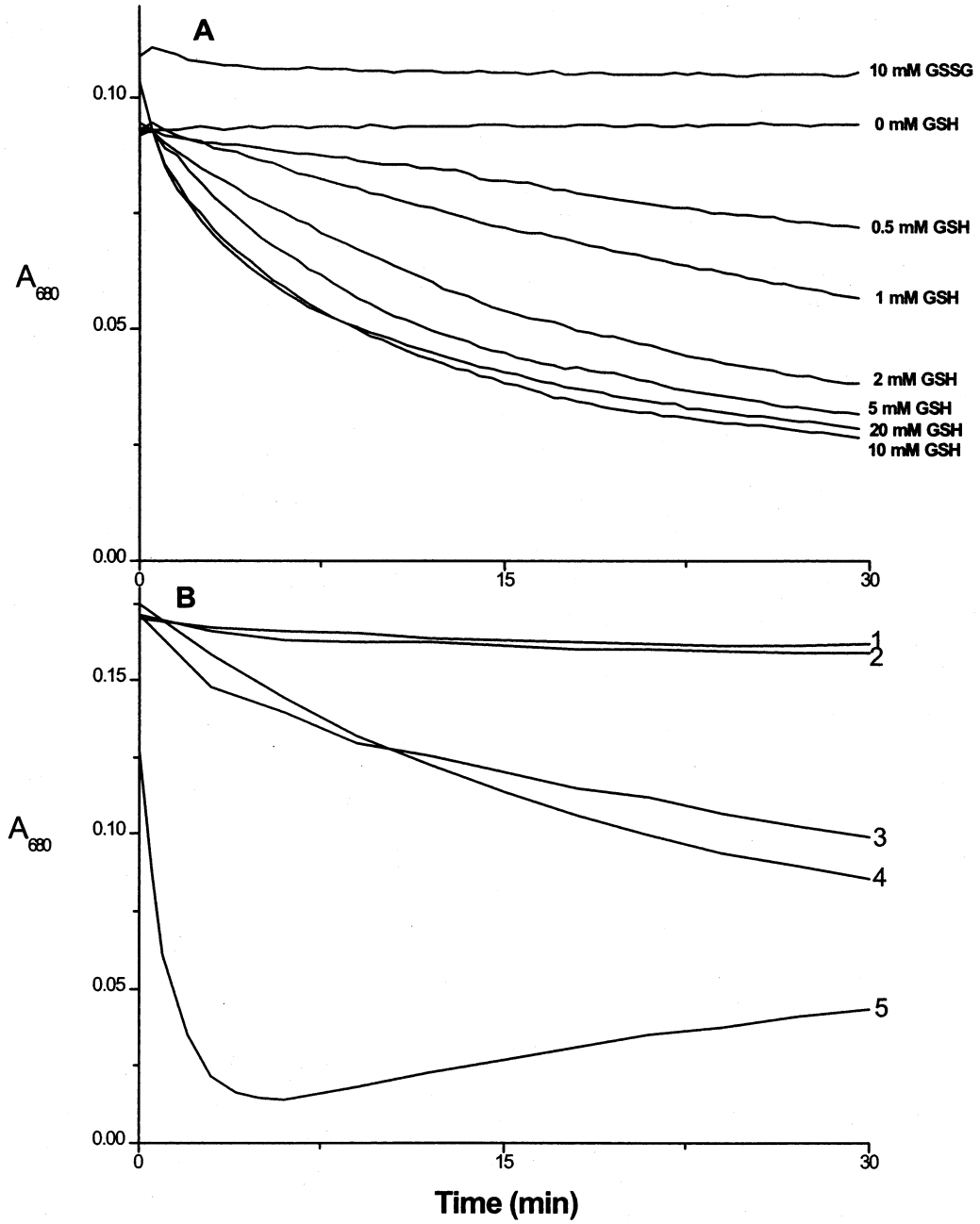

FIGURE 6: Plot of Cu^{II} d−d absorption at 680 nm vs time on incubation of Cu^{II}ZnSOD with thiols. (A) 0.58 mM Cu^{II}ZnSOD with 10 mM GSSG or 0.5 mM Cu^{II}ZnSOD with the GSH concentrations indicated on the traces. The data are the averages of triplicate experiments. (B) 0.93 mM Cu^{II}ZnSOD only (trace 1) and Cu^{II}ZnSOD with 2 mM BSA (trace 2), 0.5 mM rHCaBP (trace 3), 2 mM GSH (trace 4), and 2 mM cysteine (trace 5). Samples were in 50 mM phosphate buffer/0.2 mM EDTA (pH 7.4) at 37 °C. Absorbances at 680 nm were recorded immediately after thiol addition to Cu^{II}ZnSOD and repeated every 0.5 min over 30 min.

radicals dimerize to GSSG (Figure 7C) following their production when GSH is a donor as shown for CaBP in reaction 5.

Since CaBP, Ca^{2+}, NO, and CuZnSOD are implicated in neurodegenerative disorders such as Parkinson's and Huntington's diseases (*2*, *43*), a full understanding of their chemistry in vitro is crucial to understanding their possible interactions in vivo. For example, as shown here and elsewhere (*31*), CuZnSOD catalyzes GSNO breakdown (reaction 6), but we have not yet established if and under what conditions CuZnSOD will catalyze *S*-nitroso-rHCaBP breakdown and NO release.

We speculate that CuZnSOD is an efficient catalyst of CaBP S-nitrosation in vivo. CaBP levels in auditory neurons are estimated to reach 2 mM (*44*). Muller and co-workers (*45*) report that granule cells most likely contain several hundred micromolar CaBP, which controls calcium microdomains in hippocampal neurons and acts as a freely diffusible intracellular calcium buffer. Thus, high local concentrations of CaBP would compete with millimolar GSH (*3*) as a reductant of Cu^{II}ZnSOD. The thiyl radicals formed would efficiently trap any NO released from GSNO or other nitrosothiol, allowing CaBP to serve as an NO buffer in addition to its function as a calcium buffer (*46*). In this context, it is of note that the prototypical calcium-sensor protein, calmodulin, does not possess a single cysteine residue (*47*) in contrast to the five free cysteine residues present in CaBP (*25*).

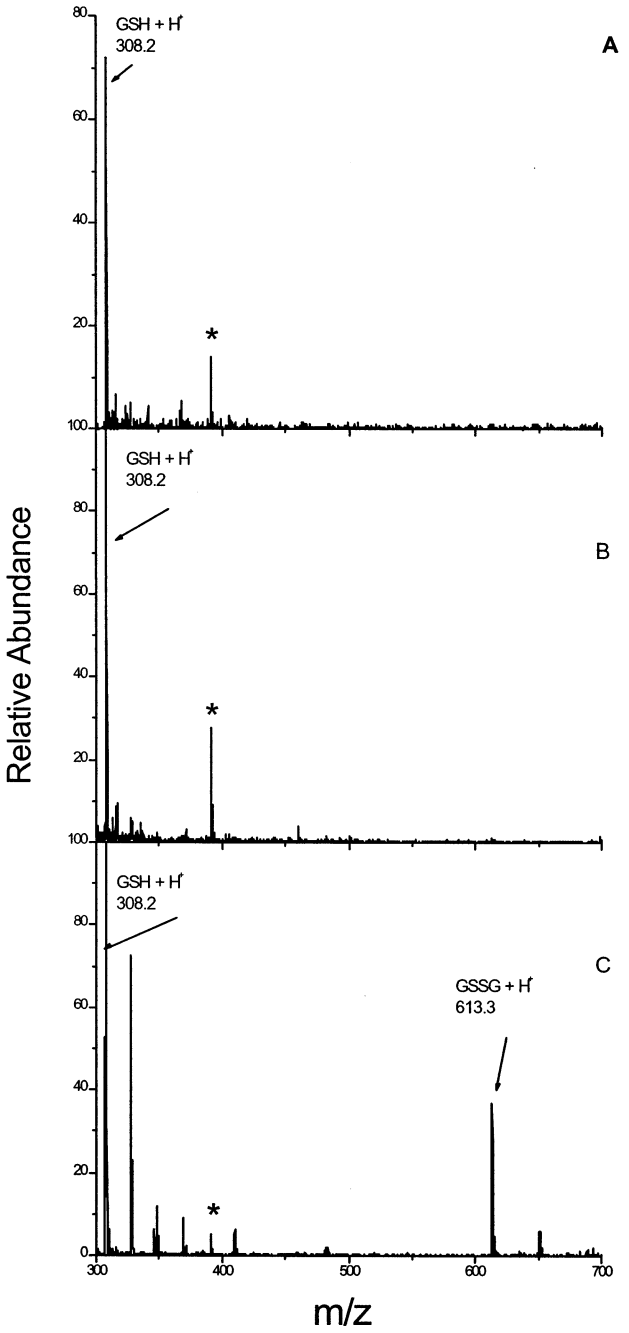

FIGURE 7: Mass spectra in the low *m*/*z* range. (A) rHCaBP (~15 μM) was incubated with 2.5-fold molar excess of GSNO in the presence of 40 μM CuZnSOD for 20 min in 1 mM Tris-HCl buffer (pH 7.4) at room temperature. (B) 0.5 mM or (C) 20 mM GSH was incubated with 1 mM CuZnSOD in the absence of GSNO for 2 h in 50 mM phosphate buffer/0.2 mM EDTA (pH 7.4) at 37 °C. Samples (100 μL) were infused into the electrospray source of the mass spectrometer by flow injection at 50 μL/min with 75% acetonitrile/0.05% trifluoroacetic acid as a mobile phase. The capillary temperature was 180 °C, and the spray voltage was 4.0 kV. The mass spectrum of the mobile phase was recorded and subtracted from the spectra of the samples. The peak at *m*/*z* 391 marked with an asterisk is due to a bis(2-ethyl-hexyl) phthalate impurity in the system (*48*).

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