The endogenous nitric oxide synthase inhibitors l-Nω-methylarginine and l-Nω,Nω-dimethylarginine are catalyzed by the enzyme dimethylargininase. Dimethylargininase-1 from bovine brain contains one tightly bound Zn(II) coordinated by two cysteine sulfur and two lighter ligands. Activity measurements showed that only the apo-enzyme is active and that the holo-enzyme is activated by zinc removal. In this work, the effect of NO on dimethylargininase-1 structure and its activity was investigated using 2-(N,N-dimethylamino)diazeneolate-2-oxide as an NO source. The results showed that whereas the holo-form was resistant to S-nitrosylation, the apo-form could be modified. The results of absorption spectroscopy, mass spectrometry, and fluorometric S-NO quantification revealed that two of five cysteine residues reacted with NO yielding cysteine-S-NO. The modification reaction is specific, because by liquid chromatography/mass spectrometry experiments of digested S-NO-dimethylargininase-1, cysteines 221 and 273 could be identified as cysteine-NO. Because Zn(II) protects the enzyme against nitrosation, it is suggested that both cysteines are involved in metal binding. However, specific cysteine-S-NO formation occurred in the absence of a characteristic sequence motif. Based on a structural model of dimethylargininase-1, the activation of both cysteines may be accomplished by the close proximity of charged residues in the tertiary structure of the enzyme.

The free radical NO acts as a signaling molecule in various tissues. In neuronal tissue it functions as a neurotransmitter that, as a gas molecule, is able to freely permeate through cell membranes. NO binding to the heme moiety of soluble guanylate cyclase activates this enzyme, leading to the generation of the second messenger, cyclic GMP (1). Apart from this signaling pathway, NO plays an important role in a number of biological processes, including the regulation of protein function through the nitrosation of sulfur atoms of cysteine residues (2). Well-characterized examples are those of caspase-3 (3) or the N-methyl-ω-aspartate (NMDA) receptor (4). In most examples of Cys-S-nitrosylation, the target protein is inhibited. NO is produced by at least three isozymes of nitric oxide synthase (NOS) of which nNOS occurs predominantly in neuronal tissues (5). The activity of this isozyme is mainly regulated by Ca2⁺ (6). Several other regulatory mechanisms for nNOS, including its inhibition by phosphorylation, have also been described (6). It is well established, moreover, that the two l-Arg derivatives, l-Nω-methylarginine (MMA) and l-Nω,Nω-dimethylarginine (ADMA), act as endogenous competitive inhibitors of NOS (7–10). Their cytosolic concentrations are regulated by the enzyme dimethylargininase (l-Nω,Nω-dimethylarginine dimethylaminohydrolase, DDAH, EC 3.5.3.18). This cysteine hydrolase catalyzes MMA and ADMA to l-citrulline (l-Cit) and CH₃NH₂ or (CH₃)₂NH, respectively (11). Increased levels of MMA and ADMA in plasma and urine were found in neuronal diseases characterized by low NO levels such as schizophrenia (12) as well as diseases linked to vascular dysfunction, e.g. uremia, atherosclerosis, hypercholesterolemia, diabetes mellitus, hypertension, and homocysteinemia (13). Conversely, NO overproduction is apparently responsible for migraine (14). In this case, the observed increase in expression of various enzymes including DDAH has been linked to an imbalance in NOS inhibitors (15).

In mammals, two isozymes of DDAH, DDAH-1 and DDAH-2, exist (16). Whereas DDAH-2 is mainly expressed in the heart, kidney, and placenta, DDAH-1 is predominantly expressed in the brain (16). The specific role for DDAH-1 in the regulation of nNOS has been suggested based on recent studies showing that both nNOS and DDAH-1 are up-regulated in injured neurons (17). The characterization of DDAH-1 from bovine brain revealed a monomeric 31.2-kDa protein containing one non-catalytic Zn(II) (18–20). Activity measurements of native and metal-free DDAH-1 showed that the endogenously bound Zn(II) inhibits the enzyme. Extended x-ray absorption fine structure (EXAFS) studies suggested that Zn(II) is tetrahedrally coordinated through two sulfur (Cys) ligands and two nitrogen (or oxygen) ligands (20). However, Zn(II)-containing DDAH-1 could be fully or partially activated by various concentrations of phosphate, imidazole, histidine, and histamine, a process accompanied by the release of Zn(II). Based on this and the apparent Zn(II) dissociation constant in the nanomolar

**Zn(II)-free Dimethylargininase-1 (DDAH-1) Is Inhibited upon Specific Cys-S-Nitrosylation**

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1 The abbreviations used are: NMDA, N-methyl-D-aspartate; ADMA, l-Nω,Nω-dimethylarginine (symmetric dimethylated l-Arg); Cit, citrulline; DDAH, l-Nω,Nω-dimethylarginine dimethylaminohydrolase (dimethylargininase); DEA NONOate, 2-(N,N-dimethylamino)diazeneolate-2-oxide; DTT, 1,4-dithio-D,L-threitol; ESI-MS, electrospray ionization mass spectrometry; EXAFS, extended x-ray fine structure; HPLC, high pressure liquid chromatography; LC-MS, liquid chromatography/mass spectrometry; MEK, 2-morpholino-ethanesulfonic acid; MMA, l-Nω-methylarginine (monomethylated l-Arg); NO, nitric oxide synthase; TCEP-HCl, tris(2-carboxyethyl)-phosphine hydrochloride; TEA, triethanolamine; TFA, trifluoroacetic acid.
range, a regulatory role for Zn(II) has been suggested (19, 20). Increased levels of intracellular Zn(II) under oxidative or nitrosative stress conditions are well documented (21–23). This may account for the reduction of DDAH-1 activity and the presence of elevated ADMA levels observed in cell culture studies under these conditions (10). Alternatively, because DDAH is a cysteine hydrolase, the enzyme inhibition through its nitrosation is also likely. Upon submission of the present work, the in vitro inhibition of prokaryotic DDAH by NO donors has been reported. Moreover, in endocytic cells heterologously expressed DDAH-2 was found S-nitrosylated following the cytokine induced expression of inducible NOS. In both instances, DDAH nitrosation was detected by an anti-S-nitrosocysteine antibody (24).

The present work was conducted with the aim of gaining insights into the effect of NO on the structure and activity of mammalian DDAH-1 in vitro. The nitrosation reaction was studied with the Zn(II)-containing and Zn(II)-free enzymes. DDAH-1 nitrosation was followed by electrospray ionization mass spectrometry (ESI-MS), electronic absorption spectroscopy, fluorometric SNO quantification, and measurements of enzymatic activity. The results showed that whereas the holo-form is resistant to nitrosation, two of five Cys residues of the apo-form were modified. To examine the specificity of the reaction, the amino acid sequence of DDAH-1 was determined, and the tryptic peptides of the NO modified enzyme were analyzed by ESI-MS. The results allowed the conclusion that the formation of Cys-S-NO is specific and that Zn(II) protects the enzyme against NO modification. Homology modeling revealed that the close proximity of charged residues in the tertiary structure of DDAH-1 might be responsible for the specific Cys-S-nitrosylation.

**MATERIALS AND METHODS**

MMAHOAc and sodium 2-(N,N-dimethylamino)-diazenolate-2-oxide (DEA NONOate) were purchased from Alexis, and Tris-(2-carboxy-ethyl)-phosphine hydrochloride (TCEP-HCl) was purchased from Pierce. Trypsin from bovine pancreas (sequencing grade) was obtained from Roche Molecular Biochemicals, and the endopeptidase Glu-C from Staphylococcus aureus strain V8 was purchased from Sigma. Dialysis tubing (cut off, 12–14 kDa; Spectrum) was rendered metal-free as described previously (25). DDAH-1 was purified from bovine brain using the previously reported procedure (20). The protein homogeneity was checked by SDS-PAGE and ESI-MS.

**Metal and Protein Quantification**—The zinc concentration was determined by atomic absorption spectroscopy (IL Video 12), and that of DDAH-1 was determined by absorption spectroscopy (Cary 3, Varian) using the molar extinction coefficient ε210 nm = 14420 M−1 cm−1 (19).

**Activity Measurements**—The product of the enzymatic reaction (t-Cit) was determined by our previously reported colorimetric method in reduction. Briefly, to 10 ml of freshly isolated DDAH-1 (5 μg/ml) from bovine brain contained between 0.95 and 0.85 mol equiv-}

containing fractions were combined and concentrated using Centricon-10 (Millipore).

**Generation of Zn(II)-free DDAH-1**—The preparation of the apo-enzyme was performed by dialysis. All dialysis steps were carried out at 4 °C, and the outer solutions were steadily bubbled with argon. Buffers were rendered metal-free as described previously (19). To remove Zn(II) from native DDAH-1, 200 μl of protein sample (~50 μM) were dialyzed against 100 ml of metal-free 100 mM imidazole/HCl and 10 mM DTT, pH 6.8. The dialysis buffer was changed four times (every 2 h), and the dialysis was completed overnight. To remove imidazole and DTT, the protein was dialyzed against two changes of 100 ml of metal-free 10 mM MES/NaOH and 100 mM KCl, pH 6.2.

**Inactivation of DDAH-1 through NO**—To monitor DDAH-1 activity as a function of the DEA NONOate concentration in the presence and absence of Zn(II), both holo- and apo-DDAH-1 (8.7 μM) were tested either in 250 mM MES/NaOH, 20% (v/v) glycerol, pH 6.4, or 250 mM imidazole/HCl, 20% (v/v) glycerol, pH 6.4. To the protein samples, DEA NONOate was added to a final concentration of 0, 0.2, 0.5, 1, 2, 5, or 10 mM, and the solution mixture was incubated for 20 min at room temperature. Subsequently, samples of 5 μl were removed and added to 57 μl of 250 mM imidazole/HCl and 7 mM MMA, pH 7.4. Finally, the mixtures were incubated for 30 min at 37 °C, and their t-Cit concentrations were determined.

To monitor the time dependence of DDAH-1 activity in the presence of constant NO donor concentration, two samples of holo-DDAH-1 were incubated in 250 mM imidazole/HCl, pH 6.4, containing either 1 mM or 5 mM DEA NONOate. Aliquots were taken after 0, 10, 20, 40, or 90 min, and their residual enzymatic activities were determined.

**Correlation of Cys-S-Nitrosylation with DDAH-1 Activity**—Six samples of 10 μM apo-DDAH-1 in 250 mM imidazole/HCl, pH 6.4, were incubated with 0, 0.2, 0.7, 5, 10, or 20 mM DEA NONOate for 30 min at 37 °C. Aliquots of 2 μl of each sample were placed into a polystyrene 96-well microtiter plate and diluted with 50 μl of 250 mM imidazole/HCl, pH 6.4. Upon the addition of 5 μl of 80 mM MMA, the plate was incubated for 30 min at 37 °C. Afterward, the t-Cit concentration was determined.

In parallel to the activity measurements, the number of Cys-S-NOs was quantified. For this purpose the fluorometric method of Park and Kostka (27) was adapted for use in 96-well microtiter plates. Briefly, another 2-μl aliquot from the initial DEA NONOate incubation was added to 98 μl of 1% (w/v) ammonium sulfamate in 0.25 mM HCl placed in a white 96-well microtiter plate. The probes were incubated for 10 min at room temperature. In the next step, 10 μl of 0.05 mg/ml 2,3-diaminonaphthalene in 0.25 mM HCl was added to each well. The probes were incubated for 10 min at room temperature. The fluorescence at 430 nm (excitation: 360 nm) was determined using a microtiter plate reader (HTS 7000 Plate Reader/Kimber Life Sciences). A stock solution of NaN2O5 was calibrated photometrically at 210 nm employing the molar extinction coefficient ε210 nm = 5380 M−1 cm−1 (28). The standard NaN2O5 curve was obtained by adding 2 μl of 0, 5, 10, 20, 50, 100, 200, or 500 μM NaN2O5 into 98 μl of 0.25 mM HCl, and the samples were treated as described for the blanks. All measurements were performed in triplicate.

**Spectroscopic Characterization of Nitrosated DDAH-1**—For the spectroscopic characterization of nitrosated DDAH-1, 450 μl of 26 μM DDAH-1 in 250 mM imidazole/HCl, pH 6.4, were titrated with DEA NONOate. The titration was performed with 1-μl aliquots of 100 mM DEA NONOate in 10 mM NaOH. Spectra were recorded between 300 and 800 nm (Cary 3, Varian). After 10 min after NO donor addition, the sample was titrated until a maximum was reached (about 10 mM DEA NONOate added in total).

**Mass Spectrometric Analysis of Nitrosated DDAH-1**—20 μl of 12 μM apo-DDAH-1 in 20 mM NH4OAc/HOAc, pH 6.4, were incubated with 0, 5, or 20 mM DEA NONOate for 20 min at 37 °C. Before injection, the samples were desalted using C8-ZenTips (Millipore) from which proteins were eluted with 10 μl of 78:21 (v/v) CH3OH/H2O/HCOOH. ESI mass spectra of 5 μl of this solution were acquired on an API III* triple quadrupole instrument (AB/MDS Scie, Toronto, Canada). The following set of parameters was applied: ionization voltage, 4800 V; orifice voltage, 80 V; mass range, m/z 700–1700; step size, 0.15 Da; scan duration, 5 s.

**Amino Acid Sequence Determination of DDAH-1**—We have already determined 20% of the amino acid sequence of bovine brain DDAH-1 using peptides of a tryptic digest (19). This approach was expanded in the present studies using peptides from digests with trypsin and endo-
proteinase Glu-C. The resulting peptides were separated by HPLC and analyzed using a combination of MS/MS and Edman peptide sequencing. The tryptic digest was performed as described (19). The second digest was done with one-twentieth of endoproteinase Glu-C in 25 mM NH4HCO3/NH4OH, 0.5 mM guanidinium chloride, pH 7.8, for 2 h at 25 °C. The sequenced peptides were aligned according to the mammalian DDAH-1 sequences available to date. The sequenced peptides were aligned according to the mammalian DDAH-1 sequences available to date. The sequenced peptides were aligned according to the mammalian DDAH-1 sequences available to date.

**Position of Cys-S-NO in DDAH-1 Amino Acid Sequence—10 μM DDAH-1 was incubated in 280 μl of 250 mM imidazole/HCl, pH 6.4, with 20 μM DEA NONOate for 30 min at 37 °C.** The sample was washed extensively with 300 μl of 0.1% (v/v) TFA in a Microcon-10 (Millipore). After a final washing step with 100 μl of Tris/HCl and 10% (v/v) CH3CN (pH 8.0) to a final volume of 150 μl, 0.2 mol equivalents of trypsin were added, and the sample was incubated for 2 h at 37 °C. 100 μl of the digest was mixed with 1 μl of TFA and applied to a reversed phase HPLC (Vydac MS-C18, 1 × 254 mm). The flow rate was 50 μl min⁻¹, and the absorption was monitored at 215 nm. Eluent A consisted of 0.2% (v/v) TFA, and eluent B was 80% (v/v) CH3CN and 0.2% (v/v) TFA. The following gradient program was applied: 0 min 5% B; 5 min 5% B; 65 min 50% B; 75 min 60% B; 80 min 100% B. The eluate was split to allow 10% to enter the electrospray source (5 μl min⁻¹), the remainder being diverted for manual fraction collection. ESI mass spectra were acquired from m/z 450 to 1700 with a step size of 0.25 Da and a scan duration of 5 s. MS/MS spectra of the doubly charged S-nitrosylated peptides were recorded in the collision energy range of 32–42 eV using argon (2.5 × 10⁻¹⁰ molecules cm⁻³) as collision gas.

**Determination of a Model Structure for Bovine DDAH-1**—the model structure of the bovine DDAH-1 was calculated using the program 3D-PSSM V2.60 (www.sbg.bio.ic.ac.uk/~3dpsmm/) (29). The bovine DDAH-1 sequence aligned best against Pseudomonas aeruginosa DDAH (PDB 3H70). A further refinement was performed using the program Swiss-PdbViewer V3.7 (30), placing the amino acid sequence of bovine DDAH-1 (this work) and the x-ray diffraction coordinates of P. aeruginosa DDAH (31). A final energy minimization was done with the GROMOS96 implementation of the Swiss-PdbViewer using 50 steps of steepest descent and 100 steps of conjugated gradients.

**RESULTS**

Bovine brain DDAH-1 used in this study contained ~0.95 mol equivalents of Zn(II). Previously, we could show that the bound metal inhibits the enzyme and that only the apo-enzyme is active. Zn(II) is bound with an apparent dissociation constant of 4.2 mM (20). Metal removal by strong and rather bulky metal chelators like EDTA or 1,10-phenanthroline takes place very slowly. However, Zn(II) can be dissociated from the protein rather easily in the presence of high concentrations of imidazole/HCl or phosphate buffer (100–500 mM), suggesting that the metal ion is buried in the protein structure. In other common buffers such as MES/NaOH, HEPES/NaOH, Tris/HCl, and TEA/HCl, no metal release occurs (20).

**Inhibition of Apo-DDAH-1 with DEA NONOate—**The influence of varying concentrations of the NO donor DEA NONOate on the activity of holo-DDAH-1 was examined in either 250 mM MES/NaOH or 250 mM imidazole/HCl at pH 6.4. Upon enzyme incubation in both buffers with DEA NONOate, the protein was transferred to 250 mM imidazole/HCl, pH 7.4. Note that in the latter buffer the activity of the in situ generated apo-form was measured (20) (see above). It appeared that the enzyme pre-incubated in imidazole/HCl was dramatically inhibited by NO, whereas its inhibition in MES/NaOH was only minor (Fig. 1A). In addition, apo-DDAH-1 was also generated through dialysis of the holo-enzyme as described (19) and incubated with DEA NONOate both in 250 mM MES/NaOH and 250 mM imidazole/HCl. In both buffers, the effect of NO on the enzymatic activity was similar to that observed with holo-DDAH-1 in 250 mM imidazole/HCl (data not shown). These results allow the conclusion that whereas apo-DDAH-1 is inhibited by NO, the presence of Zn(II) in the protein structure protects the enzyme against nitrosation. To further characterize the nitrosated DDAH-1, the experimental conditions under which the major part of the protein is nitrosated were sought. For this purpose, in situ generated apo-DDAH-1 was incubated with 1 or 5 μM DEA NONOate, and the changes in enzymatic activity followed as a function of time (Fig. 1B). In both cases, a hyperbolic decrease of activity was discerned. The -75% enzyme inhibition seen after 20 min of sample incubation with 5 mM NO donor (Fig. 1B) is very similar to that reached with 10 mM NO donor in the same time (Fig. 1A).

**Characterization of the Product of DDAH-1 Nitrosation—**Protein nitrosation gives rise to various protein modifications. Besides the formation of Cys-S-NO, which has been described for many proteins (2, 32), the formation of Tyr-NO2 (33), Cys-S-O2H, and Cys-S-O3H were also reported (35–37). To characterize the species formed in the case of nitrosated DDAH-1, its absorption spectrum between 300–500 nm was recorded (Fig. 2A). The spectrum reveals a maximum at 341 nm being characteristic for the n0→π∗ transition of the SNO group (38, 39). Independent evidence for the presence of Cys-S-NO was provided by ESI-MS spectra recorded upon the addition of 0, 5, or 20 mM DEA NONOate to apo-DDAH-1 (Fig. 2, B–D). Besides the major peak of apo-DDAH-1 at 31199 Da (arrow), up to three additional species with masses of 31228 (a), 31256 (b), and 31285 Da (c) (Fig. 2, C and D) were observed. The additional peaks (a–c) with mass differences of a multiple of 29 Da compared with the unmodified protein suggest the presence of DDAH-1 species containing one, two, and three NO-modified Cys, i.e. Cys-S-NO. Based on ESI-MS spectra of SNO-DDAH-1 recorded after up to 5 days, the relatively unstable Cys-S-NO presumably formed various new oxidation products like Cys-S-O2H, Cys-S-O3H, and others (data not shown). However, compared with the half-life of small S-nitroso thios (from minutes to hours), the half-life of SNO-DDAH-1 in the range of 1 or 2 days is rather high, resembling that of other S-nitrosylated proteins, e.g. SNO-BSA (39).

**Quantification of NO-modified Cys Residues in DDAH-1—**Using electronic absorption spectroscopy and ESI-MS, the nitrosation product of the reaction of DEA NONOate with DDAH-1 could be unambiguously characterized (see above).
Specific Cys-S-Nitrosylation of DDAH-1

level. The sequence determination was performed by Edman degradation and/or MS/MS analysis of peptides from cleavage with trypsin and endoproteinase Glu-C separated on reversed phase HPLC, and their molecular masses were determined by ESI-MS. The herein reported amino acid sequence of bovine brain DDAH-1 (Fig. 4) has been deposited into the Swiss-Prot Protein Data Bank and is available under the accession number P56965. Considering that the N terminus of DDAH-1 is acetylated (19), the calculated molecular mass of 31157 Da for the apo-protein agrees well with that determined previously by ESI-MS (18). Thus, similar to DDAH-1 isolated from rat kidney (40) and human liver (41), no further permanent post-translational modification is present in the bovine brain protein. Fig. 4 shows the alignment of the bovine DDAH-1 sequence with the mammalian sequences known to date. The sequence of DDAH from *P. aeruginosa*, for which the crystal structure has recently been determined, is also included (31). The sequence identity of the mammalian species is 91%; the overall identity of all species present in Fig. 4 amounts to 28%. In the structure of the procaryotic DDAH, the residues of the catalytic triad have been identified as Cys-249, His-162, and Glu-114 (31). By analogy, the corresponding residues in the mammalian species are Cys-273, His-172, and Asp-126 (Fig. 4).

Localization of Cys-S-NO in Bovine DDAH-1—Cys-S-NO derivatives are rather unstable at alkaline pH values (39). Therefore, the tryptic digest for the detection of the Cys-S-NO within the DDAH-1 sequence was performed at pH 8.0 using a high trypsin to DDAH ratio and a short incubation time (2 h) (42). A comparison of the peptide maps of the untreated and nitrosated DDAH-1 revealed two Cys residues containing peptides Leu-57 to Arg-97 and Leu-211 to Lys-229. In a third peptide (Leu-57 to Arg-97) containing two Cys residues, the corresponding residues in the mammalian species are Cys-273, His-172, and Asp-126 (Fig. 4). Double nitrosation of this peptide was detected.

The assignment of the peptides containing S-nitrosylated Cys-221 and Cys-273 was confirmed by MS/MS analysis. Even at relatively low collision energies, an almost complete homolytic cleavage of the S-NO bond occurred in agreement with previous studies (43). At higher collision energies, the peptide ions resulting from a loss of the NO radical further fragmented, giving rise to sequence-dependent fragment ions of the denitrosated peptides. The NH₂- and COOH-terminal ions of the a, b, and y series were accompanied by relatively abundant, uncommon b-1 and y-1 ions (a discussion of the nomenclature used in identifying fragment ions can be found in Ref. 44). The

However, neither of these two methods provides quantitative results. Thus, depending on the protein, the molar absorption coefficients per Cys-S-NO substantially differ (39). In addition, the ordinary ESI-MS spectra allow only a qualitative analysis. For the quantification of Cys-S-NO we have employed the fluorescence detection of 1-[H]–naphthotriazol, generated in the reaction of 2,3-diaminonaphthaline with Hg(II)-released NO. The method described by Park and Kostka (27) was modified for the use in 96-well microtiter plates. The degree of Cys-S-NO modification as a function of increasing DEA NONOate concentrations (between 0–20 mM) was examined. The results were correlated with the residual enzymatic activity. Fig. 3 reveals that the absence of enzymatic activity above 10 mM DEA NONOate were correlated with the residual enzymatic activity. Fig. 3 reveals a mass of 31199 Da (concentrations, species with mass differences of 29 (a), 58 (b), and 87 Da (c), respectively, were formed.

A comparison of the peptide maps of the untreated and nitrosated DDAH-1 revealed two Cys residues containing peptides Leu-211 to Lys-229 and Val-266 to Lys-280 in which a mass difference of 29 Da was found (Table I). The amount of S-nitrosylated species was estimated from the ratio of the counted ions in the LC-MS experiment of the nitrosated protein (Table I). In a third peptide (Leu-57 to Arg-97) containing two Cys residues (Fig. 4), only a minor nitrosation of each single Cys was found. The two mono-nitrosated peptides eluted in two different chromatographic peaks (Table I). No double nitrosation of this peptide was detected.

The assignment of the peptides containing S-nitrosylated Cys-221 and Cys-273 was confirmed by MS/MS analysis. Even at relatively low collision energies, an almost complete homolytic cleavage of the S-NO bond occurred in agreement with previous studies (43). At higher collision energies, the peptide ions resulting from a loss of the NO radical further fragmented, giving rise to sequence-dependent fragment ions of the denitrosated peptides. The NH₂- and COOH-terminal ions of the a, b, and y series were accompanied by relatively abundant, uncommon b-1 and y-1 ions (a discussion of the nomenclature used in identifying fragment ions can be found in Ref. 44). The

\[ \text{DDAH-1} \]

**Fig. 2.** Characterization of nitrosated bovine DDAH-1 by absorption spectroscopy and ESI-MS. A, absorption spectrum of SNO-DDAH-1. B–D, deconvoluted ESI-MS of DDAH-1 upon incubation with 0 (B), 5 (C), and 20 mM (D) DEA NONOate. Non-nitrosated DDAH-1 reveals a mass of 31199 Da (arrow). With the raising of DEA NONOate concentrations, species with mass differences of 29 (a), 58 (b), and 87 Da (c), respectively, were formed.

\[ \text{DDAH-1} \]

**Fig. 3.** Correlation of the degree of Cys-S-NO formation with the enzymatic activity of apo-DDAH-1. DDAH-1 activity was determined in 250 mM imidazole/HCl, pH 7.4, upon pre-incubation with various concentrations of DEA NONOate (■). The number of Cys-S-NOs was determined by fluorometric S-NO quantification (○).

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\[ ^2 \] M. Knipp and S. Kozlov, unpublished results.
Specific Cys-S-Nitrosylation of DDAH-1

b-1 and y-1 ions appear to contain free radicals, which originate from cleavage of the S-NO bond.

Homology Model of DDAH-1—The crystal structure of P. aeruginosa DDAH has recently been determined (31). Because bovine DDAH-1 and the P. aeruginosa DDAH share an amino acid sequence identity of 39%, it is possible to obtain a model structure for bovine DDAH-1 using the crystallographic data available for the P. aeruginosa protein. The model building was based on the sequence alignment shown in Fig. 4, which was obtained from the secondary structure prediction by PSI-Pred (www.globin.bio.warwick.ac.uk/psipred/) included in the program 3D-PSSM (29). Fig. 5A shows the superposition of the prokaryotic DDAH crystal structure (white) with the DDAH-1 model structure (blue). The root mean square deviation of 1.71 Å between both structures indicates a high significance of the model. The fold of the prokaryotic DDAH consists of five modules of more or less degenerated βαβ-modules arranged in a 5-fold pseudo-symmetry. A similar fold was also found for L-arginine:glycine amidinotransferase and L-arginine:inosamine-phosphate amidinotransferase (31, 45). The corresponding modules in the model structure of the mammalian DDAH-1 are shown in Fig. 5B.

DISCUSSION

In the present work we have demonstrated that only apo-DDAH-1 reacts with NO, leading to enzyme inhibition (Fig. 1). The reaction product could be identified as SNO-DDAH-1. A fluorometric quantification of Cys-S-NO revealed the nitrosation of two of five Cys residues (Fig. 3). To answer the question as to the specificity of the Cys-S-NO formation, the amino acid sequence of bovine brain DDAH-1 was determined, and the nitrosated apo-enzyme was subjected to a tryptic digest. From the ratio of the counted ions of the nitrosated and the non-nitrosated peptides in an LC-MS experiment, semi-quantitative results were obtained. The product analysis showed the presence of two major Cys-S-NO-containing peptides (Table I), with Cys-221 and Cys-273 being the major S-nitrosylated residues. A slight nitrosation of the third tryptic peptide of DDAH-1 (see Table I) presumably represents a side product of the reaction or a product of NO interchange with Cys-S-NO-containing peptides upon the tryptic digest. Thus, the results suggest that the enzyme nitrosation is specific. Because Cys-273 is the active site residue of DDAH-1 (Fig. 4), the inhibition of the enzymatic activity is due to its S-nitrosylation. However, both the correlation between the number of Cys-S-NO residues and the enzymatic activity (Fig. 5) and the LC-MS-experiments (Table I) suggest that Cys-221 is S-nitrosylated to the same degree as Cys-273.

The specific Cys-S-nitrosylation is surprising, because in the primary structure of bovine DDAH-1 the sequence motif XC/D/E for Cys-S-nitrosylation (2, 32), where X usually stands for basic or acidic amino acids, is absent. A growing number of proteins have been recognized as being regulated through the S-nitrosylation of specific Cys residues (2). In many examples, including caspase-3 and methionine adenosyl transferase, the preference of a specific Cys for S-nitrosylation seems to be achieved through a close spatial contact resembling the continuous sequence motif mentioned above (46, 47). To gain an insight into the structural features of bovine DDAH-1 responsible for the specific Cys-S-nitrosylation, a homology model of this enzyme was generated (Fig. 5, A and B). Modeling studies based on the structure of P. aeruginosa DDAH have suggested the presence of five similar βαβ-modules also in DDAH-1. The large loop between residues 14 and 25 in module I of P. aeruginosa DDAH (yellow) closes over the active site when a substrate or a product is bound (31). In contrast, module I of the mammalian DDAH-1 consists of a αααβ-fold, resulting in a small helix-turn-helix motif (light blue). This structural feature is more similar to module I of L-arginine:glycine amidinotransferase (45), used as a template for the solution of the P. aeruginosa DDAH structure (31). These differences would represent major structural differences between the structure of mammalian and the prokaryotic DDAH.

To evaluate whether structural properties of bovine DDAH-1 are also important for the activation of both Cys-221 and Cys-273 for Cys-S-nitrosylation, the structural model was examined further. Its closer inspection revealed that the S° of Cys-273 is surrounded by the carboxylic groups of Glu-77 and Asp-78 at the distance of 3.4 and 5.0 Å, respectively. His-172, located at the distance of 6.2 Å, seems also to influence the S° of Cys-221 (Fig. 5D). These structural features compare well with the spatial arrangement of the catalytic Cys present in the structural model of methionine adenosyl transferase (46) and in the crystal structure of caspase-3 (protein data base number 1CP3). Taken together, it would appear that the spatial proximity of charged residues in DDAH-1 might be responsible for the activation of Cys-221 and Cys-273 in the nitrosation reaction. However, it should be emphasized that this interpretation is based on a structural model and that more studies are needed to support this conclusion.

The Cys-S-nitrosylation of enzymes is often accompanied by a reversible loss of activity (2). The enzyme activity of DDAH-1 is inhibited with IC50 ~ 1 mM of DEA NONOate (Fig. 1A). Although this donor concentration appears to be a rather high, because of a short half-life of NO in solution (minutes) (48) and...
its slow release with time, the actual concentration of free NO in solution is much lower. In cellular systems intracellular NO concentrations can be relatively high. For instance, in neurons the concentration ranges between 2–4 μM (49), and in activated neutrophils and macrophages it is below 10 μM (50). Therefore, it can be assumed that the free NO concentrations used in this study are of physiological relevance.

The reversibility of this reaction was tested by incubation of SNO-DDAH-1 with 5 mM 2-mercaptoethanol, DTT, or glutathione. However, in contrast to a number of reports on other nitrosated proteins/enzymes where such a treatment restored the enzymatic activity, our attempts to restore the enzymatic activity of nitrosated DDAH-1 remained unsuccessful (data not shown). Because the removal of Cys-S-bound NO from proteins is likely to occur through trans-nitrosylation, we ascribe this effect to the inaccessibility of the nitrosylated Cys-273 and Cys-221 residues to these reagents. In the model of DDAH-1, both residues are buried in the protein structure (Fig. 5B). It should be noted that also in the case of caspase-3 the reversibility of the Cys-S-nitrosylation is partial and requires a rather high DTT concentration (47).

Apart from the enzyme nitrosation, DDAH-1 is also inhibited by Zn(II) ions with an apparent binding constant lying in the range of intracellular “free” Zn(II) concentrations (20). Based on activity measurements of DDAH-1 in brain tissue homogenate, it would appear that ~30% of the enzyme is present in the Zn(II)-bound form (20). In many Zn(II)-containing proteins such as metallothioneins, zinc finger proteins, and liver alcohol dehydrogenase, NO binds preferentially to Cys-S ligands, thereby displacing bound Zn(II) (51–54). However, our data demonstrate that for DDAH-1 this is not the case. Because the bound Zn(II) on the one hand inhibits the enzyme and on the other hand protects the two Cys residues against S-nitrosylation, we propose that for DDAH-1 this is not the case. Because the bound Zn(II) on the one hand inhibits the enzyme and on the other hand protects the two Cys residues against S-nitrosylation. We propose that for DDAH-1 this is not the case.
between the S atoms should be substantially lower (−3.7 Å). Consequently, the mutual approach of both residues would require changes in the protein structure. Indeed, based on the circular dichroism studies, a structural alteration upon Zn(II) binding to apo-DDAH-1 takes place (19).

The reduction of DDAH-1 activity and the presence of elevated ADMA levels were observed in cell culture studies under oxidative stress conditions (10). This observation was paralleled by a decrease in NO production (55, 56), suggesting that under these conditions intracellular DDAH activity would be substantially inhibited. In very recent endothelial cell culture studies, heterologously expressed DDAH-2 was found S-nitrosylated following the cytokine-induced expression of inducible NOS. The enzyme nitrosation was established by means of an anti-S-nitrosocysteine antibody (24). Independently, in vitro S-nitrosylation of one (Cys-249) of a total of five Cys residues of structurally well characterized bacterial DDAH has also been shown. In the present work, S-nitrosylation of two Cys residues of DDAH-1 (Cys-221 and Cys-273) has been demonstrated. The differences in the primary structures between bovine DDAH-1 and P. aeruginosa DDAH (30% identity) are most likely the reason for this effect. An inhibition of protein function by both Zn(II) and NO has already been reported, e.g., in the case of caspase-3 (47) and the NMDA receptor (57). Whereas for the NMDA receptor the residues involved in Zn(II) binding are to date not known, for caspase-3 the active site Cys is clearly the date not known, for caspase-3 the active site Cys is clearly the

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