Nitrosation of Tryptophan Residue(s) in Serum Albumin and Model Dipeptides

BIOCHEMICAL CHARACTERIZATION AND BIOACTIVITY*

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Nitrosation of bovine serum albumin with acidified NaNO₂ was compared to that of carboxymethyl-bovine serum albumin in which the thiol group is covalently blocked. Differential ultraviolet-visible (UV-Vis) spectroscopy and a modified Saville assay indicated that a non-cysteine residue(s) in carboxymethyl-bovine serum albumin was nitrosated. The nitrosated carboxymethyl-bovine serum albumin exhibited similar vasorelaxation activity as that observed with nitrosated bovine serum albumin. Identification of the nitrosated non-cysteine residue(s) was studied using 16 model dipeptides, each of which contained a glycyl residue and a variable residue. Using photolysis-chemiluminescence analysis, modified Saville assay, differential UV-Vis spectroscopy, and bioassays, L-glycyl-L-tryptophan (Gly-Trp) was modified. Using photolysis-chemiluminescence analysis, indicated that a non-cysteine residue(s) in carboxymethyl-bovine serum albumin was nitrosated. The nitrosated carboxymethyl-bovine serum albumin exhibited similar vasorelaxation activity as that observed with nitrosated bovine serum albumin. Identification of the nitrosated non-cysteine residue(s) was studied using 16 model dipeptides, each of which contained a glycyl residue and a variable residue. Using photolysis-chemiluminescence analysis, modified Saville assay, differential UV-Vis spectroscopy, and bioassays, L-glycyl-L-tryptophan (Gly-Trp) was found to be the only dipeptide that underwent significant nitrosation under these conditions. Liquid chromatography-UV-Vis spectroscopy-mass spectrometry showed that the NO group was attached to the indole nitrogen of tryptophan. Nitrosated Gly-Trp exhibited dose-dependent vasorelaxation and platelet inhibiting activity as that observed with nitrosated bovine serum albumin; HPLC, high performance liquid chromatography.

*This work was supported in part by National Institutes of Health Grants HL 48743, HL 53919, and P50 HL 55993, a Merit Review Award from the U.S. Veterans Administration, and by a grant from NitroMed, Inc. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: -NO, nitroso group; NO₂, nitric oxide; GSNO, S-nitrosoglutathione; Cys-NO, S-nitroso-L-cysteine; BSA, bovine serum albumin; CM-BSA, carboxymethyl bovine serum albumin; CD, circular dichroism; GSH, glutathione; Gly-Trp, L-glycyl-L-tryptophan; HPLC, high performance liquid chromatography.

The nitrosation reaction introduces the nitroso group (-NO) into an organic molecule, leading to the formation of C-nitroso, N-nitroso, O-nitroso, or S-nitroso derivatives of the parent molecule. Commonly used nitrosating agents include HNO₂, N₂O₃, and N₂O₄ (for review, see Ref. 1), but NO₂ has also been suggested to be effective in this regard (2).

Nitrosation reactions may be relevant to the metabolism and actions of nitric oxide (NO), a gaseous molecule that is generated by various mammalian cells and is involved in a variety of physiological and pathophysiological processes (3). Oxidation of NO生成 N₂O₃ and N₂O₅, which can, in turn, cause nitrosation. By contrast, S-nitrosation products, such as S-nitroso-N-acetylpenicilliamine, S-nitrosoglutathione (GSNO), and S-nitroso-L-cysteine (Cys-NO), have been shown to induce similar biologic effects as that observed with NO (4, 5). Several studies have also demonstrated that NO⁺ or S-nitroso compounds induce DNA mutations and changes in enzyme function by nitrosation (6-10).

It has been assumed that the S-nitroso compounds induce NO⁺-like biological activity by releasing NO through homolytic fission of the S-NO bond. Although such cleavage does occur under photolytic (11) or Cu²⁺ (12) catalysis, a recent kinetic study demonstrated that the rates of formation of NO⁺ from S-nitroso-N-acetylpenicilliamine and Cys-NO are orders of magnitude smaller than the rates of transfer of -NO from these compounds to other thiols (13). Thus, -NO groups may reach their biological target via a transnitrosation pathway rather than by spontaneous release followed by nitrosation.

To explore the possible involvement of other amino acid residues in nitrosation reactions, we studied nitrosation of serum albumin and model dipeptides. S-Nitrosation of cysteine or cysteine-containing peptides (e.g. glutathione) has been previously studied in detail, and the resulting S-nitrosated derivatives produce vasorelaxation and platelet inhibition (4, 14-16). In this study, we examined whether or not other amino acid residue(s), in addition to cysteine, can be nitrosated and whether or not these nitrosated derivatives can manifest NO⁺-like activity. BSA was used as a model protein for this purpose and the nitrosation of carboxymethyl-BSA (CM-BSA), a modified derivative in which the thiol group is covalently blocked, was carried out. The identification and activity of the nitrosated non-cysteine residues were compared with those of 16 model dipeptides.

EXPERIMENTAL PROCEDURES

Sulfanilamide, ammonium sulfamate, and N-(1-naphthyl)ethylenediamine dihydrochloride were obtained from Aldrich Chemical Co. The following reagents were purchased from Sigma: BSA (bovine albumin, essentially fatty acid free, catalog No. A-6003), CM-BSA (S-carboxymethyl albumin, bovine, catalog No. A-6285), glutathione (GSH), all dipeptides, and S,5-dithiodisulfo-2-nitrobenzoic acid.

Nitrosation—NaNO₂ plus HCl were used as a nitrosating agent in this study. The combination of these two compounds will principally produce HNO₃; however, small amounts of N₂O₃ and NOCl may also form in the reaction solution, and these species are stronger nitrosating agents than HNO₃. For this reason, we use the denotation, NaNO₂/HCl, throughout this study to describe the nitrosating species used.
With the exception of the time course experiments conducted with UV-Vis spectroscopic analysis, nitrosation was produced by first dissolving serum albumin (BSA and CM-BSA (200 mg/ml for ~3 mm)) or peptides (10 mm) in water; Gly-Trp was dissolved in 0.14 M HCl. Immediately before nitrosation, NaNO2 (prepared in H2O at 50 mm) was diluted in 0.94 or 0.8 M HCl and mixed with an equal volume of protein (or peptide) dissolved in 0.1 M EDTA at pH 7.3. The reaction was carried out at room temperature for 30–40 min and terminated by neutralizing the solution to pH 7.5 with 5 N NaOH in 0.5 mm Tris buffer. The control samples (acid-treated) were prepared following the same procedure except that NaNO2 was omitted from the reaction solution.

UV Visible Spectroscopy—Spectra were recorded at room temperature on a Cary 4E UV Visible spectrophotometer (Varian, Inc., Australia Pty., Ltd.). Protein or peptide was diluted in 0.5 M HCl and placed in both reference and sample cuvette. After correcting the baseline, NaNO2 was added to the sample and an equivalent volume of H2O to the reference cuvette. Scanning was initiated immediately and 13 scans were performed at 5-mm intervals. Computer-stored data (light transmission) were converted to absorbance and plotted using a MicroCal Origin program (MicroCal Software, Inc., Northampton, MA).

Circular Dichroism—Circular dichroic (CD) spectra were recorded on an Aviv 62DS CD Spectropolarimeter (Aviv Associates, Lakewood, NJ) calibrated from 500 to 190 nm with a 10-camphorsulfonic acid (1 M mg/ml in ethan). Spectra were recorded at 25 °C in 0.05-cm quartz cells from 250 to 190 nm. Protein concentrations of 0.3 mg/ml. Multiple spectra (5–10) were recorded for duplicate samples prepared on different days. These spectra were averaged and corrected for baseline contribution from the buffer. Molar ellipticity values, [θ], were calculated according to the equation: [θ] (deg cm2/dmole) = D × MRW × l/c, where D is the displacement from the baseline value × the full range in degree, MRW is the mean residue weight of the amino acids, l is the pathlength of the cuvette in cm, and c is the concentration of the protein in g/ml.

Data points were analyzed at 1-nm intervals between 250 and 190 nm by non-linear, constrained least-squares curve-fitting procedures to obtain estimates of each type of secondary structure. CD spectra were analyzed with Chemical Sheet, β-turn, and the LINEQ program of Cynthia Teeters as described by Mao and Wallace (20) utilizing the reference data sets of Greenfield and Fasman (18), Chang and colleagues (19), and Brahms and Brahms (20).

Thiol Content—Thiol content was measured according to a modified (21) Ellman assay (22). Briefly, protein or peptide was diluted in 0.1 M sodium phosphate containing 6 M guanidine HCl and 1 mM EDTA at pH 7.3, 5.5, 4.0. Sodium cyanoborohydride was added to a final concentration of 0.15 M. The increase of absorbance at 412 nm was followed until maximal absorption was achieved (typically less than 5 min). The concentration of free thiol was calculated from the molar extinction coefficient of the nitrosothiolate ion in 6 M guanidine HCl (ε = 13,700 M−1 cm−1).

Nitroso Content—Nitroso content was measured by two methods: Saville assay and photoinduction-chromiunmescence analysis. In the Saville assay (23), the sample containing 5-nitroso-dinitrophenyl was first mixed with 0.1% ammonium sulfate in 0.4 M HCl (total volume = 0.5 ml) for 1 min to remove NO2 or HNO2, from the sample. A solution (0.5 ml) containing 3% sulfanilamide and 0.25% HgCl2 in 0.4 M HCl was then added, followed by 0.5 ml of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 0.4 M HCl. The mixture was incubated at room temperature for 10 min and the absorbance was read at 540 nm. The nitroso content was calculated according to a standard curve constructed with 2.5–20 μM NaNO2. (The ammonium sulfate step was omitted in the standard curve measurements.)

Photoinduction-chromiunmescence analysis was performed using a thermal energy analyzer (TEA model 543 analyzer, Thermedics Detection, Inc., Chelmsford, MA) equipped with a photolytic interface (Nitrolyte™, Thermedics Detection Inc., Chelmsford, MA). The design of the instrument has been previously described (24–26). Sample (100 μl, 0.5–1 μM peptide) was introduced into the system via an HPLC pump with H2O as the mobile phase (flow rate = 1 ml/min). NO liberated from the sample by photolysis was carried by argon, separated from the liquid path by condensation, passed into the thermal energy analyzer under vacuum, and mixed with ozone. Chemiunmescence resulting from the transition of NO2+ to its ground state was detected and recorded, and the nitroso content calculated using a standard curve constructed using 100–1,500 mm 5-nitrosothiolate as a standard synthesized by reacting glutathione with acidified nitrite, as described above.

Liquid Chromatography/Mass Spectrometry—Experiments were carried out using an atmospheric pressure ionization-electrospray liquid chromatograph/mass spectrometer (API-Electrospray LC/MS) system (Hewlett Packard Co., Palo Alto, CA), which consists of a HP-1090 liquid chromatograph, a HP 59987 API-Electrospray LC/MS interface, a HP 5998B MS engine, and a HP ChemStation data system with HP G1047A LC/MS software. A 2.1 × 150-mm high surface area silica column (Vydac 150H52521, Vydac Co., Hesperia, CA) was used for the HPLC separation. The samples were injected into the column equilibrated with 10% acetonitrile, 90% H2O, and 0.1% trifluoroacetic acid (solvent A) at a flow rate of 0.3 ml/min. A linear gradient was started 5 min after injection to reach 90% acetonitrile, 10% H2O, and 0.1% trifluoroacetic acid (solvent B) in 40 min and then held at 100% solvent B for 5 min. The eluent was monitored at 275 and 335 nm by a diode array detector, which also recorded simultaneously the full UV-Vis spectra of the elution peaks. The eluent then entered a spray chamber with drying nitrogen gas at 245 °C and passed through the capillary at an exit voltage of 72 or 150 V as indicated. Analyte ions were scanned over a range of 50–800 m/z and the background was subtracted. Filtered raw data were presented without further refinement.

Vasoconstriction Assay—New Zealand White male rabbits (2.4–3.6 kg) were sacrificed with pentobarbital (120 mg/kg) injected via a marginal ear vein. The aorta was excised and placed in ice-cold Krebs’s buffer. Extrinsic tissue and the vascular endothelium were carefully removed. The aorta was cut into 5-mm rings and suspended in an organ chamber (Radnotli Glass Co., Inc., Monrovia, CA) containing 10 or 20 ml of Krebs’s buffer (37 °C, pH 7.4) aerated with 5% O2/95% CO2. Strips of Aorta. The control samples (acid-treated) were prepared immediately before use.

Platelet Aggregation—Freshly obtained human platelets were kindly provided by the Naval Blood Research Laboratory (Boston, MA) and collected by mechanical apheresis, using the Haemonetics Mobile Collection System (Haemontics, Inc., Braintree, MA) from donors who had not ingested any platelet inhibitor for at least 2 weeks. The blood was collected in acid-citrate-dextrose (ACS, NIH formula A) anticoagulant. The platelet-rich plasma was isolated by discontinuous-flow centrifugation with the Mobile Collection System. Platelets were counted with a Coulter Counter, model ZM (Coulter Electronics, Hialeah, FL), and diluted with platelet-poor plasma to a concentration of 1.7–2.0 × 10^10/ml. Aggregation was carried out on a PAP-4 aggregometer (Biodata, Hatboro, PA). Platelets were incubated with nitrosated or acid-treated (control) peptides/proteins for 15 min at 37 °C. ADP (5–20 μM, concentration required to achieve 80% aggregation response of control platelets) was added, and the platelets were stirred (1,000 rpm, 37 °C) while aggregation was recorded. The extent of aggregation described in this report was recorded as the maximal extent of change in light transmission.

RESULTS

Thiol Content and Effects of Nitrosation—We initially studied the nitrosation of three molecules, BSA, CM-BSA, and glutathione. Glutathione was used as a reference known to undergo stoichiometric nitrosation under these conditions with NaNO2/HCl (27).

The thiol (SH) content of these molecules was first examined. BSA had 0.41 ± 0.01 mol of SH/mol of protein, while CM-BSA and glutathione had 0.02 ± 0.004 and 0.98 ± 0.08 mol/mol, respectively. The SH content in BSA was consistent with what has been published previously; i.e., the free cysteine residue in purified BSA is involved, in part, in mixed disulfide bond formation, and the most carefully prepared albumin contains at most 0.65–0.7 mol of SH/mol of protein (28).

Owing to the possibility that acid could cause hydrolysis of disulfide bonds in proteins, the SH content of HCl-treated BSA was also determined. There was no significant change in SH content when the proteins were incubated with 0.5 M HCl for 40 min and then neutralized to pH 7.5, the conditions used for nitrosation.

BSA, CM-BSA, and glutathione were then nitrosated with equimolar NaNO2/HCl. Thiols of the nitrosated molecules were completely undetectable after the reaction.

UV Visible Spectroscopy—Figs. 1 and 2 show the time-de-
ependent UV-Vis absorption spectra of a newly generated chromophore in glutathione, BSA, and CM-BSA during the course of reaction with NaNO2/HCl. After addition of NaNO2 to the solution, spectra were recorded immediately and repeated every 5 min for a total of 13 cycles. Final concentration of glutathione was 0.3 mM in panels A-C, and the final concentration of NaNO2 was 0.3, 1.2, 4.8, and 4.8 mM in panels A-D, respectively. Spectra were recorded against a blank containing glutathione in 0.5 N HCl (panels A-C).

**Fig. 1.** UV-Vis absorption spectra of chromophore generation during the reaction of glutathione and NaNO2/HCl. After addition of NaNO2 to the solution, spectra were recorded immediately and repeated every 5 min for a total of 13 cycles. Final concentration of glutathione was 0.3 mM in panels A-C, and the final concentration of NaNO2 was 0.3, 1.2, 4.8, and 4.8 mM in panels A-D, respectively. Spectra were recorded against a blank containing glutathione in 0.5 N HCl (panels A-C).

Increasing the NaNO2 concentration 4- and 16-fold did not enhance the GSNO chromophore (Fig. 1, B and C), but at these molar excesses of NaNO2, the absorption peak of HNO2 became apparent. HNO2 absorption is illustrated by the tetrad of peaks between 300–400 nm with \( \lambda_{\text{max}} \) values of 347, 358, 371, and 386 nm (Fig. 1D); a small shoulder is also visible at 337–339 nm. The gradual decrease in chromophore intensity indicated that the initially formed HNO2 underwent decomposition over the time course of these experiments (3HNO2 → 2NO2⁻ + HNO3 + H2O).

The SH group of BSA's Cys34 reacted promptly to produce a chromophore spectrum reminiscent of glutathione (Fig. 2A);
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TABLE I

| Sample                  | λ\(_{\text{max}}\) (nm) | Apparent molar absorbance | λ\(_{\text{max}}\) (nm) | Apparent molar absorbance |
|-------------------------|--------------------------|---------------------------|--------------------------|---------------------------|
| BSA/NaNO\(_2\) (1:1)    | 334                      | 467                       | 333                      | 1303                      |
| BSA/NaNO\(_2\) (1:4)    | 335                      | 835                       | 335                      | 3862                      |
| BSA/NaNO\(_2\) (1:16)   | 337                      | 1724                      | 335                      | 8073                      |
| CMBSA/NaNO\(_2\) (1:1)  | 301                      | 1332                      | 314                      | 1354                      |
| CMBSA/NaNO\(_2\) (1:4)  | 305                      | 1264                      | 334                      | 3540                      |
| CMBSA/NaNO\(_2\) (1:16) | 325                      | 1744                      | 336                      | 7515                      |
| GSH/NaNO\(_2\) (1:1)    | 335                      | 879                       | 335                      | 879                       |
| GSH/NaNO\(_2\) (1:4)    | 336                      | 957                       | 336                      | 808                       |
| GSH/NaNO\(_2\) (1:16)   | 337\(^a\)               | 1240                      | 337\(^a\)               | 995                       |

\(^a\) The λ\(_{\text{max}}\) before HNO\(_2\) absorption tetrad.

TABLE II

| Sample                  | Nitroso/peptide Without Hg\(_{2}^{2+}\) | Nitroso/peptide With Hg\(_{2}^{2+}\) | S-Nitroso peptide |
|-------------------------|----------------------------------------|-------------------------------------|-------------------|
|                         | S-Nitroso content measured | Nitroso content | measured | Nitroso content | measured |
| BSA/NaNO\(_2\) (1:0)    | 0.00                     | 0.00                     | 0.00 | 1.43 | 0.43 |
| BSA/NaNO\(_2\) (1:1)    | 0.00                     | 0.37                     | 0.37 | 0.00 | 0.00 |
| BSA/NaNO\(_2\) (1:4)    | 0.04                     | 0.37                     | 0.33 | 0.00 | 0.00 |
| BSA/NaNO\(_2\) (1:16)   | 0.11                     | 0.38                     | 0.27 | 0.00 | 0.00 |
| CMBSA/NaNO\(_2\) (1:0)  | 0.00                     | 0.00                     | 0.00 | 0.00 | 0.00 |
| CMBSA/NaNO\(_2\) (1:1)  | 0.01                     | 0.01                     | 0.00 | 0.00 | 0.00 |
| CMBSA/NaNO\(_2\) (1:4)  | 0.04                     | 0.05                     | 0.01 | 0.00 | 0.00 |
| CMBSA/NaNO\(_2\) (1:16) | 0.11                     | 0.14                     | 0.03 | 0.00 | 0.00 |
| GSH/NaNO\(_2\) (1:0)    | 0.00                     | 0.00                     | 0.00 | 0.00 | 0.00 |
| GSH/NaNO\(_2\) (1:1)    | 0.00                     | 0.87                     | 0.87 | 0.00 | 0.00 |
| GSH/NaNO\(_2\) (1:4)    | 0.00                     | 0.75                     | 0.75 | 0.00 | 0.00 |
| GSH/NaNO\(_2\) (1:16)   | 0.00                     | 0.63                     | 0.63 | 0.00 | 0.00 |

\(^a\) Calculated from the nitroso content measured in the presence and absence of added Hg\(_{2}^{2+}\).

As shown in Table II, the S-nitroso content of BSA was 0.37 mol/mol. The S-nitroso content decreased with increasing NaNO\(_2\) in the nitrosation reaction. A similar decrease was also observed with glutathione. However, in contrast to glutathione, the decrease in S-nitroso content was offset by an equivalent increase in non-S-NO content. Note that without neutralization, the S-nitroso content in nitrosated GSH was 0.97 (data not shown). CM-BSA did not contain detectable amounts of S-nitroso groups, but did have a measurable nitroso group at high NaNO\(_2\)/HCl concentrations that could be detected in the absence of Hg\(_{2}^{2+}\). These data indicated that the nitrosated group in CM-BSA is a non-thiol group.

CD Spectroscopy—To examine the secondary structure of the serum albumin species after their nitrosation and neutralization, far UV CD spectra of non-treated, HCl-treated and nitrosated BSA and CM-BSA were recorded and analyzed for specific elements of secondary structure. All spectra (data not shown) are characterized by negative minima at 222 and 208 nm and a positive maximum at ~190 nm, which are similar to data reported by others (29) and characteristic of a protein that possesses a predominantly \(\alpha\)-helical conformation. All samples exhibit ~60% \(\alpha\)-helix, ~40% \(\beta\)-sheet, ~4–6% \(\beta\)-turn, and the remainder as random coil. Thus, neither nitrosation, HCl treatment, nor carboxymethylation alters the conformation or secondary structure of BSA.

Nitrosation of Model Peptides—In order to clarify which...
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functional group of BSA was nitrosated, studies on model peptides were carried out. Sixteen different dipeptides (Gly-Gly, Gly-Glu, Gly-Gln, Gly-Asp, Gly-Asn, Gly-Ser, Gly-Thr, Gly-Met, Gly-His, Gly-Pro, Pro-Gly, Arg-Gly, Gly-Lys, Gly-Phe, Gly-Tyr, and Gly-Trp) were examined. Among these peptides, the glycine residue was invariably included for the reasons of solubility and blocking of the \( \alpha \)-amino group of the variable residue. The variable residues in the dipeptides included all the amino acids whose side chains contain a functional group in addition to hydrocarbon. Arg-Gly was used because of its commercial availability, and Pro-Gly was tested owing to the presence of a secondary amine group in the proline residue. Nitrosation of the peptides was carried out under the same conditions as those used for BSA, but the molar ratio between peptide and \( \text{NaNO}_2 \) was kept at 1:1. The degree of nitrosation of the dipeptides was estimated by three different methods: photolysis chemiluminescence analysis, Saville assay, and spectroscopic analysis.

Among the 16 dipeptides tested, only nitrosated Gly-Trp released significant amounts (25%) of NO\(^{-} \) after photolysis. Nitrosated Gly-Lys, Gly-Phe, and Gly-Tyr released 4, 5, and 3%, respectively. The remaining dipeptides released 1–3% NO\(^{-} \), similar to that of control, Gly-Gly, which was released significant amounts (25%) of NO\(^{-} \) from a nitrophenol, rather than a nitrosophenol, structure. Arg-Gly was used because of its commercial availability, and Pro-Gly was tested owing to the presence of a secondary amine group in the proline residue. Nitrosation of the peptides was carried out under the same conditions as those used for BSA, but the molar ratio between peptide and \( \text{NaNO}_2 \) was kept at 1:1. The degree of nitrosation of the dipeptides was estimated by three different methods: photolysis chemiluminescence analysis, Saville assay, and spectroscopic analysis.

UV visible spectroscopy was used to monitor the nitrosation process of the dipeptides. Three types of spectra (Fig. 3) were observed among the reactions of the 16 dipeptides. Nitrosation of Gly-Trp produced a chromophore with an absorption \( \lambda_{\text{max}} \) of 335 nm at the onset of the reaction and a \( \lambda_{\text{max}} \) of 316 nm at 60 min. Neutralization of the solution at the end of the reaction led to a reversion of \( \lambda_{\text{max}} \) to 335 nm. The apparent molar absorption of the chromophore was 1684 M\(^{-1} \) cm\(^{-1} \). The reaction of Gly-Tyr generated a wide absorbance band at 258–318 nm with an apparent molar absorption of 107 M\(^{-1} \) cm\(^{-1} \) (at 60 min) at 287 nm. The reactions of the other dipeptides showed similar spectra as that found for Gly-Phe. These spectra were equivalent to the \( \text{HNO}_2 \) spectra shown in Fig. 1D. From these spectra, it was concluded that the chromophore produced in the nitrosated CM-BSA was derived from a tryptophan residue in the protein. BSA is known to contain two tryptophan residues with one of them located in an aqueous solvent-exposed environment (30).

Reaction Products in Nitrosated Gly-Trp—In the peptide study described above and the biological activity study shown below, the reaction solutions of nitrosated peptide were used without separation of reaction products. To identify the reaction products in the solution, we analyzed the nitrosated Gly-Trp solution on a HPLC-coupled mass spectrometry system (API-electrospray LC/MS, see “Experimental Procedures”).

As shown in Fig. 4A, the HPLC separation of nitrosated Gly-Trp revealed two major peaks with retention times of 8.7 (I) and 16.4 (II) min. Peak I exhibited identical UV-Vis spectrum and mass spectrum as that found in the control sample (acid-treated Gly-Trp, Fig. 4B). The mass ion at 262.6 m/z indicated that the molecule was a protonated form of Gly-Trp.

Peak III was identified as N-nitroso-Gly-Trp (N-NO-Gly-Trp) with the -NO group attached to the indole-nitrogen of tryptophan. This peak had UV-Vis absorption maxima at 267 and 335 nm and a shoulder at 273–274 nm, similar to that reported for N-acetyl-N\(^1\)-nitrosotryptophan (267, 274, and 335 nm (31)). The two major ion species (261.3 and 187.4 m/z) in the mass spectrum were identified as the fragments of nitroso-Gly-Trp with cleavages at the N-NO bond (261.3 m/z) and the N-C bond between tryptophan's \( \alpha \)-carbon and \( \alpha \)-amino residue (187.4 m/z), and the ion at 291.7 m/z was identified as the molecular ion. The low abundance of the molecular ion indicated that the homolytic N-NO bond fission had readily occurred during the electrospray process, which is consistent with a recent report that denitrosation occurs readily at high temperatures during electrospray-mass spectrometric analysis (32).

As we also observed that N-NO-Gly-Trp had less structural stability than Gly-Trp. The fragmentation at the C-N bond (to generate the 187.4 m/z ion) occurred at a capillary exit voltage of 72 V in the N-NO-Gly-Trp sample but did not appear in
Fig. 4. Separation and identification of nitrosation products of Gly-Trp by API-electrospray LC/MS. The nitrosation reaction solution of Gly-Trp (A) and the reaction control (acid-treated Gly-Trp) (B) were loaded onto a C18 HPLC column and eluted according to the gradient
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**DISCUSSION**

In this study, a non-cysteine residue(s) in CM-BSA was found to be nitrosated by NaNO2/HCl. Using 16 model dipeptides, the non-cysteine residue was characterized and the structural properties indicated that the residue was tryptophan. Nitrosated CM-BSA and Gly-Trp exhibited NO-like vasorelaxation and antiplatelet activity, similar to that of S-nitroso-Gly-Trp.

**Effect of nitrosated BSA and CM-BSA on vascular tone.** Endothelium-denuded rabbit aortic rings were contracted with 1 μM phenylephrine and relaxed with 1.5 or 15 μM protein prepared as described under “Experimental Procedures.” Relaxation is expressed as percent decrease in tension. All values are expressed as the mean ± S.D. with n = 10 animals in each group.

Gly-Trp until 150 V were applied (Fig. 4B). When 150 V were applied during the N-NO-Gly-Trp analysis, the 261.3 and 291.7 m/z ions completely disappeared, leaving ions at 187.4 and 130.7 m/z as equally dominant (data not shown).

Several other small peaks were also found in the HPLC elution profile (Fig. 4A). Peak II (14.8 min) was identified as N-nitro-Gly-Trp (N-NO2-Gly-Trp), which exhibited a major molecular ion peak at 307.4 m/z. The structure of two other minor peaks at retention times of 3.0 and 5.8 min were not identified. Both of the peaks had UV-Vis absorption maxima at 275–277 nm and no absorption at higher wavelengths. By mass spectral analysis, the 3-min peak showed a single ion species at 279.4 m/z and the 5.8-min peak showed two ion species at the 280.2 and 313.4 m/z.

Vasorelaxation Activity—Vasorelaxation by nitrosated CM-BSA was examined and compared to that of S-nitrosated BSA. In this experiment, the proteins were reacted with equimolar NaNO2 in 0.47 N HCl for 40 min and the pH neutralized to 7.5 immediately before the bioassay. As shown in Fig. 5, S-nitrosated BSA caused relaxation of aortic vessel rings by 13 ± 14 and 71 ± 22% at protein concentrations of 1.5 and 15 μM, respectively. Nitrosated CM-BSA exerted less, but significant, relaxation: 5 ± 7 and 63 ± 21% at 1.5 and 15 μM, respectively.

Dose-dependent Bioactivity of Nitrosated Gly-Trp—Vasorelaxation activity of all the NaNO2/HCl-treated dipeptides was examined. Nitrosated Gly-Trp was the only dipeptide that produced significant vasorelaxation, leading to 60.7, 81.5, and 90.5% relaxation at 1.5, 5, and 15 μM, respectively. The other dipeptides produced relaxation in the range of 0–3% at 1.5 μM, 0–8.5% at 5 μM, and 7.2–18.8% at 15 μM. The modest degree of nitrosation at the higher concentrations was likely a consequence of unreacted HNO2 in the reaction mixture (see below).

To determine if the vessel relaxation caused by nitrosated Gly-Trp was exerted through a specific pharmacologic mechanism, the dose-dependence of relaxation was next examined. Nitrosated Gly-Trp was compared with three other compounds: acid-treated Gly-Trp, NaNO2, and S-nitrosoglutathione. As shown in Fig. 6, acid-treated Gly-Trp had no effect on vascular tone whereas nitrosated Gly-Trp exhibited a clear dose-dependent relaxation effect. The EC50 for relaxation was calculated as 1.13 ± 0.25 μM, which was 2.5-fold less potent than that of GSNO (0.43 ± 0.33 μM). Because neither of the peptides were purified to homogeneity and the nitroso content of Gly-Trp and glutathione was approximately 22 and 89%, respectively, the actual potency of N-nitroso-Gly-Trp may be equal to or slightly greater than that of GSNO. NaNO2 was significantly less potent than nitrosated Gly-Trp and GSNO; it produced 5, 17, and 41% relaxation at concentrations of 5, 15, and 50 μM, respectively. The relaxations caused by all of the above compounds were reversible, indicating that no significant vascular toxicity occurred in the assay to account for the reduction in tone.

Inhibition of Platelet Aggregation—The effect of nitrosated Gly-Trp on platelet aggregation was also studied and compared to that of three other nitrosated molecules: glutathione, BSA, and CM-BSA. As shown in Fig. 7, all of these molecules produced dose-dependent inhibition of platelet aggregation. The IC50 values for the nitrosated derivatives of glutathione, Gly-Trp, BSA, and CM-BSA were 0.7 ± 0.3, 3.5 ± 0.9, 1.3 ± 0.3, and 17.3 ± 7.9 μM, respectively. Again, these values represent lower limits of the actual IC50 values owing to the use of peptide or protein molarity rather than nitroso content.
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Fig. 7. Inhibition of platelet aggregation. Platelets (1.7–2.0 × 10^8/ml) were incubated with nitrosated GSNO (squares), nitrosated BSA (diamonds), nitrosated CM-BSA (circles). Aggregation was carried out as described under “Experimental Procedures.” Inhibition was calculated as the percent decrease of the maximal extent of aggregation produced by a given nitrosated species compared with control.

>pete unfavorably with cysteine. In addition, tryptophan nitrosation has other characteristics different from cysteine nitrosation. For example, nitrosation of tryptophan is a completely reversible reaction, but nitrosation of cysteine is rarely reversible because denitrosation of S-NO-cysteine usually produces an oxidized form of cysteine (cystine). If both cysteine and tryptophan residues are involved in a repeated transnitrosation process, the former will require a reduction system to recycle the cysteine, but the latter will not.

The biological effects of N-nitrosotryptophan have been reported in terms of its mutagenicity. Using synthetic N-acetyl-N^1-nitrosotryptophan, Venitt and colleagues (38) showed that the compound causes mutation in a series of E. coli WP2 strains (trp^- to trp^+) and several Salmonella typhimurium strains (his^- to his^+). Since a similar effect has been observed with NO^- gas (39, 40), these data observations demonstrate that N-nitrosotryptophan, in addition to mediating vasorelaxation and antiplatelet aggregation, can produce other NO^-like biological effects.

How extracellular N-nitrosotryptophan causes intracellular biological effects has not been investigated. It is obvious that this molecule cannot freely diffuse through cellular membranes and does not spontaneously release NO^- radical via homolytic N-NO bond fission. Therefore, to mediate its NO^-like effects, the -NO group on the N-nitrosotryptophan (either N-NO-Gly-Trp or nitrosated CM-BSA) must be transferred to guanylyl cyclase (for vasorelaxation) or DNA (for mutation) via intermediate -NO carrier(s), and one of these must be located on or associated with the plasma membrane. Interestingly, such a carrier does not seem to be strictly selective regarding the type of nitroso group because the potency of N-NO-GlyTrp was similar to that of S-nitroso glutathione. There is little current knowledge about the biochemical mechanism(s) of transnitrosation, although such a mechanism appears to be relevant for the biologic actions of nitroso compounds.

Acknowledgment—We thank Teresa Wright, John K. Snyder, Carol Ng, and Mats Hamberg for their stimulating scientific discussion; and Stephanie Tribuna for expert secretarial assistance.

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J. Biol. Chem. 1996, 271:14271-14279.
doi: 10.1074/jbc.271.24.14271

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