Corrections

BIOCHEMISTRY. For the article "Roles of the anaphase-promoting complex/cyclosome and of its activator Cdc20 in functional substrate binding," by Esther Eytan, Yakir Moshe, Ilana Braunstein, and Avram Hershko, which appeared in issue 7, February 14, 2006, of *Proc. Natl. Acad. Sci. USA* (**103,** 2081–2086; first published February 2, 2006; 10.1073/pnas.0510695103), the last two lines on page 2083 were omitted from the print journal, due to a printer's error. The sentence "As may be expected, the affinity of the binding of very high concentrations (1,800 nM) of the mutant substrate (Fig. 2*B*)" should read: "As may be expected, the affinity of the binding of the DM substrate to Cdc20 is even lower, as indicated by the observation that Cdc20 binding continued to increase even at very high concentrations (1,800 nM) of the mutant substrate (Fig. 2*B*)." This error does not affect the conclusions of the article. The online version is correct.

www.pnas.org/cgi/doi/10.1073/pnas.0601553103

IMMUNOLOGY. For the article "Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome," by Jacques-Eric Gottenberg, Nicolas Cagnard, Carlo Lucchesi, Franck Letourneur, Sylvie Mistou, Thierry Lazure, Sebastien Jacques, Nathalie Ba, Marc Ittah, Christine Lepajolec, Marc Labetoulle, Marc Ardizzone, Jean Sibilia, Catherine Fournier, Gilles Chiocchia, and Xavier Mariette, which appeared in issue 8, February 21, 2006, of *Proc. Natl. Acad. Sci. USA* (**103,** 2770–2775; first published February 13, 2006; 10.1073/pnas.0510837103), the authors would like to acknowledge a grant from Réseau de Recherche Clinique, Institut National de la Santé et de la Recherche Médicale (INSERM), on Sjögren's syndrome. In addition, J.-E.G. received a grant from INSERM and performed his work thanks to a "Poste d'Accueil INSERM."

www.pnas.org/cgi/doi/10.1073/pnas.0601677103

MEDICAL SCIENCES. For the article "SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures," by Gang Hao, Behrad Derakhshan, Lei Shi, Fabien Campagne, and Steven S. Gross, which appeared in issue 4, January 24, 2006, of *Proc. Natl. Acad. Sci. USA* (**103,** 1012–1017; first published January 17, 2006; 10.1073/pnas.0508412103), the authors note the omission of an important step of the SNOSID method for SNO peptide isolation. On page 1016, last paragraph, right column, "Proteins were precipitated by addition of 2 volumes of iced acetone" should be inserted after: "Some lysates were subjected to S-nitrosylation by pretreatment with GSNO at 37°C for 30 min; control samples were either untreated or incubated under identical conditions with 5 mM DTT." In addition, on page 1017, the second full sentence of the left column, "Protein pellets were solubilized and biotinylated by 1 h incubation at room temperature in 5 ml of resuspension buffer (20 mM Tris, pH 7.6/4 mM ascorbate/0.2 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide) (Pierce, Rockford, IL)" should read: "Protein pellets were solubilized and biotinylated by 1 h of incubation at room temperature in 5 ml of resuspension buffer (20 mM Tris, pH 7.6/1.0% SDS/1 mM EDTA/0.1 mM neocuproine/4 mM ascorbate/0.2 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide) (Pierce, Rockford, IL)." These errors do not affect the conclusions of the article.

www.pnas.org/cgi/doi/10.1073/pnas.0601557103

MEDICAL SCIENCES. For the article "Defective cerebellar response to mitogenic Hedgehog signaling in Down's syndrome mice," by Randall J. Roper, Laura L. Baxter, Nidhi G. Saran, Donna K. Klinedinst, Philip A. Beachy, and Roger H. Reeves, which appeared in issue 5, January 31, 2006, of *Proc. Natl. Acad. Sci. USA* (**103,** 1452–1456; first published January 23, 2006; 10.1073/pnas.0510750103), it should be noted that all instances of "Down syndrome" incorrectly appeared as "Down's syndrome," due to an editorial error that occurred after author approval of the proofs.

www.pnas.org/cgi/doi/10.1073/pnas.0601630103

SNOSID, a proteomic method for identification of cysteine S-nitrosylation sites in complex protein mixtures

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Reversible addition of NO to Cys-sulfur in proteins, a modification termed S-nitrosylation, has emerged as a ubiquitous signaling mechanism for regulating diverse cellular processes. A key first-step toward elucidating the mechanism by which S-nitrosylation modulates a protein's function is specification of the targeted Cys (SNO-Cys) residue. To date, S-nitrosylation site specification has been laboriously tackled on a protein-by-protein basis. Here we describe a high-throughput proteomic approach that enables simultaneous identification of SNO-Cys sites and their cognate proteins in complex biological mixtures. The approach, termed SNOSID (SNO Site Identification), is a modification of the biotin-swap technique [Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P. & Snyder, S. H. (2001) *Nat. Cell. Biol.* 3, 193–197], comprising biotinylation of protein SNO-Cys residues, trypsinolysis, affinity purification of biotinylated-peptides, and amino acid sequencing by liquid chromatography tandem MS. With this approach, 68 SNO-Cys sites were specified on 56 distinct proteins in *S*-nitrosoglutathione-treated (2–10 μM) rat cerebellum lysates. In addition to enumerating these S-nitrosylation sites, the method revealed endogenous SNO-Cys modification sites on cerebellum proteins, including α-tubulin, β-tubulin, GAPDH, and dihydropyrimidinase-related protein-2. Whereas these endogenous SNO proteins were previously recognized, we extend prior knowledge by specifying the SNO-Cys modification sites. Considering all 68 SNO-Cys sites identified, a machine learning approach failed to reveal a linear Cys-flanking motif that predicts stable transnitrosation by *S*-nitrosoglutathione under test conditions, suggesting that undefined 3D structural features determine S-nitrosylation specificity. SNOSID provides the first effective tool for unbiased elucidation of the SNO proteome, identifying Cys residues that undergo reversible S-nitrosylation.

mass spectrometry | nitric oxide

With the discovery that covalent NO addition to Cys-sulfur is a generic protein modification that regulates diverse protein functions, the term S-nitrosylation has crept into the lexicon of protein biochemists (1). Like O-phosphorylation (OPH), S-nitrosylation is stimulus-evoked (2), precisely targeted (3), spatiotemporally restricted (4), and contributes to a cell's flexibility in responding to a continually changing extracellular milieu (for reviews, see refs. 5–8). Thus, S-nitrosylation fulfills essential criteria for service as a bona fide cell-signaling modality.

Whereas S-nitrosylation has been reported for >100 proteins, contributing to NO-mediated signaling in mammalian cells, <30 protein Cys modification sites have been specified (6). In each case, the specification of a NO-targeted Cys residue has been a laborious task, usually involving an iterative combination of mutagenesis and MS-based approaches. Despite the importance and ubiquity of S-nitrosylation in cell signaling, no method has yet been described for unbiased identification of targeted Cys (SNO-Cys) (where NO is covalently bonded to sulfur) modification sites in proteins. Moreover, the accumulated inventory of SNO-Cys sites in the published literature has not enabled a compelling elucidation of molecular "rules" that provide a basis for preferential addition of NO or NO-derived species to particular protein Cys residues. Given this knowledge gap, we sought to develop a method that enables unbiased, facile, and high-throughput specification of SNO-Cys sites on NO-regulated target proteins in complex biological samples. Here we describe SNOSID (SNO-Site Identification), a method that appears to fulfill this need.

SNOSID is an extension of the biotin-switch method originally described by Jaffrey, Snyder, and colleagues (9). The first step in the original biotin-switch method is methylthiolation of all free Cys-thiols in a protein mixture, followed by selective reduction of S–NO bonds, thereby generating a new unmodified thiol at each former SNO-Cys site. These new thiols are then marked by introduction of a mixed-disulfide bond with a biotin-tagging reagent, captured on immobilized avidin, and selectively released from avidin by reduction of the disulfide linker. Several laboratories have used the biotin-switch method in combination with 1D or 2D PAGE and in-gel trypsinolysis for identification of SNO proteins by mass spectrometry (10–14). To extend the capability of this approach, we introduced a proteolytic digestion step before avidin capture. This added step provides for the selective isolation of peptides that previously contained SNO-Cys residues, rather than intact SNO proteins (as with the original method). We then exploit nanoflow liquid chromatography (nLC) tandem MS (nLC-MS/MS) for high-throughput peptide identification, thereby inventorying the set of SNO-Cys sites on proteins.

Utility of the SNOSID approach is demonstrated by our identification of 68 SNO-Cys peptides from 56 rat cerebellar proteins after treatment with *S*-nitrosoglutathione (GSNO). Additionally we show that at least six of these SNO-Cys peptides (from four proteins) are endogenously S-nitrosylated in brain. This study demonstrates an unbiased and large-scale identification of SNO-Cys residues in proteins. To elucidate the environment of a given Cys that potentially makes it prone to NO modification, we applied a bioinformatic approach that searches for motifs in flanking sequences of identified SNO-Cys sites. With a machine-learning algorithm and training protocol that effectively identified sequence patterns that predict OPH in a control experiment, stable S-nitrosylation by GSNO was not predicted by a linear amino acid consensus motif. Instead, the propensity of a protein thiol to undergo nitrosylation is appar-

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: EIC, extracted ion chromatograph; GSNO, *S*-nitrosoglutathione; nLC, nanoflow liquid chromatography; nLC-MS/MS, nLC tandem MS; NOS, nitric oxide synthase; OPH, O-phosphorylation; SNO, *S*-nitrosothiol; nNOS, neuronal NO synthase.

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www.pnas.org/cgi/doi/10.1073/pnas.0508412103

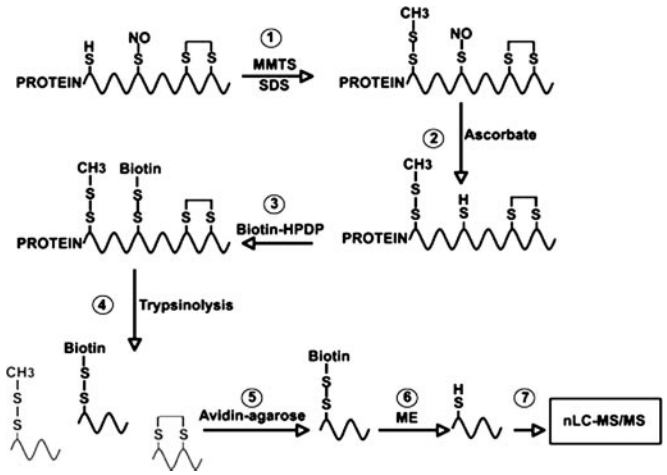

Fig. 1. Schematic of the SNOSID method for identifying protein SNO-Cys modification sites. Steps 1–3 are performed essentially as described previously for affinity capture of SNO proteins (9). Biotinylated proteins are then subjected to trypsinolysis (step 4), and the resulting biotinylated peptides are affinity-captured on streptavidin-agarose (step 5), followed by elution as the unmodified peptide with 2-mercaptoethanol (ME) (step 6). Finally, peptides (formerly containing SNO-Cys) are resolved and analyzed through nLC-MS/MS and database searching (step 7).

ently encoded in its 3D environment by contextual features that await definitive specification.

Results and Discussion

Isolation of SNO-Cys-Containing Peptides by the SNOSID Method. We sought to adapt the previously described biotin-swap method (9) to enable unbiased specification of SNO-Cys residues on proteins. Given that this method had effectively been used for capture and identification of intact SNO proteins, we reasoned that it could similarly be used to capture SNO-Cys containing peptides in tryptic digests of SNO proteins. Furthermore, for peptides with a suitable m/z and possessing only a single Cys residue, it should be possible to use high-throughput nLC-MS/MS to elucidate the peptide sequence and identify the protein of origin and the site of S-nitrosylation. To test this possibility, we applied the scheme depicted in Fig. 1 for isolation of SNO peptides and analysis by nLC-MS/MS.

As for the previously used affinity purification of SNO proteins (9), initial steps used for purification of SNO peptides were (*i*) methylthiolation of free protein –SH groups by reaction with 20 mM *S*-methylmethanethiosulfonic acid; (*ii*) selective reduction of SNO-Cys by using 4 mM ascorbate, thereby generating a new population of free thiols at sites where NO had been; and (*iii*) covalent addition of a thiol-biotin moiety to these newly generated thiols by mixed disulfide linkage using 0.2 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide. At this stage, rather than capturing intact SNO proteins on streptavidin-agarose, proteins were subjected to complete trypsinolysis. Biotinylated-peptides were affinity-captured on streptavidin-agarose, followed by selective cleavage of the disulfide bond linking the peptide Cys and its biotin tag (using 100 mM 2-mercaptoethanol). This tandem purification procedure incorporates highly selective peptide capture and release for efficient recovery of former SNO peptides in unmodified form. Because the elution of peptides is done under exceedingly gentle conditions, one would not predict significant contamination by peptides that bind streptavidin-agarose either nonspecifically or specifically via biotin incorporated at a non-thiol site (as in decarboxylases, where biotin is covalently bound to Lys ε-amino).

We sought to test the efficacy of this SNO-peptide purification procedure with levels of SNO proteins that may approach tissue accumulations. Cerebellum was selected as a model system because it is known to be a major repository of neuronal NO synthase (nNOS) in brain (15), and prior studies confirmed that endogenous SNO proteins are present in brains of wild-type mice but not nNOS-nullozygous mice (9). As an initial test of the potential for SNOSID to specify SNO-Cys residues, experiments were performed with cerebellum lysates in which SNO-protein levels were modestly enriched by treatment with relatively low levels of GSNO. Notably, GSNO has been detected in rat cerebellum at an estimated 6–8 μM concentration (16) and is implicated as an effector of mammalian NO/cGMP signaling (17). A physiological role of GSNO in regulating the extent of protein S-nitrosylation *in vivo* is provided by the observed up-regulation of SNO-protein levels in mice that are nullozygous for an enzyme that selectively denitrosylates GSNO (GSNOR) (18).

To quantify the relationship between GSNO treatment concentration and SNO-protein content, rat cerebellum lysates (1 mg/ml protein) were pretreated at 37°C for 30 min in the dark with various concentrations of GSNO, from 0 to 30 μM. SNO proteins were acetone-precipitated free of unreacted GSNO, and total SNO content was measured by the Saville–Griess assay. As shown in Fig. 2*A*, without GSNO pretreatment, the SNO content of cerebellum proteins was at the limit of detection (0.02–0.04 μM) but increased progressively with GSNO treatment concentration. Notably, with 10 μM GSNO, the SNO content of cerebellum proteins rose to ≈3% of the accessible protein thiol pool [the accessible thiol pool was determined based on the maximal increase in A_{410} after reaction with 5,5′-dithiobis-(2-nitrobenzoic acid)]. All subsequent experiments aimed at specification of protein SNO-Cys modification sites were performed with GSNO treatments ranging from 0 to 10 μM, which gave ≈0.02–3.0% occupancy of protein thiols by NO (Fig. 2*A*).

Fig. 2*B* shows base peak chromatograms and resolution by nLC of peptides captured by SNOSID, before and after 5 μM GSNO treatment of cerebellum lysates. Before GSNO treatment, a discernible but low abundance of peptide ions was detected (Fig. 2*B*, control), consistent with low endogenous expression of SNO proteins in rat cerebellum. After GSNO treatment, peptide ions were found to increase markedly in abundance and distribution over the base peak chromatograms (Fig. 2*B*, GSNO). The substantive increase in detected ions, indicative of enhanced SNO-protein levels, is in accord with the ≥100-fold increase in total SNO-protein content found after treatment with 5 μM GSNO (Fig. 2*A*).

Identification of SNO-Cys by nLC-MS/MS and Database Searching. MS/MS data from experiments such as described in Fig. 2 were analyzed by SPECTRUM MILL (Agilent Technologies, Palo, Alto, CA). Table 1, which is published as supporting information on the PNAS web site, lists 68 putative SNO-Cys-containing tryptic peptides from 56 proteins that were identified with high stringency (SPECTRUM MILL score, >10; SPI% (the percentage of assigned spectrum intensity of total spectrum intensity), >70%) (19) in at least three of six experiments wherein rat cerebellum lysates were treated with 2, 5, or 10 μM GSNO. It is notable that identification of a protein based on the inferred sequence of a single peptide poses a significant methodological challenge. This limitation likely contributes to significant MS/MS data in our experiments that could not be confidently ascribed to a unique peptide sequence in the database.

Notwithstanding predicted errors of omission, the reliability of identified SNO peptides in Table 1 is compelling. First, 29% (16 of 56) of listed proteins were previously reported to either undergo modification or have their activity altered by S-

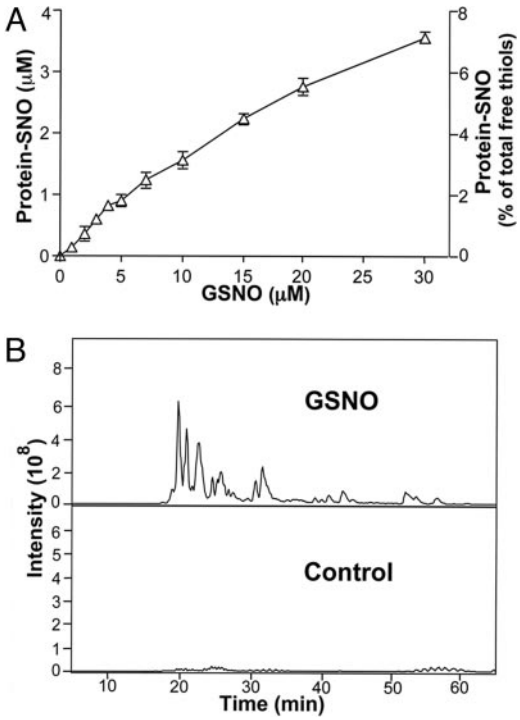

Fig. 2. Application of the SNOSID method for identification of SNO-Cys sites in rat cerebellum, before and after treatment with GSNO. (*A*) Quantification of SNO-Cys in rat cerebellum proteins as a function of GSNO pretreatment concentration. Cerebellum lysates were incubated with the indicated concentrations of GSNO at 37°C for 30 min. Proteins were precipitated in iced acetone, and the SNO-protein content was measured by the Saville–Griess assay. Note that among thiols accessible to modification by 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (49 ± 2 μM thiols per mg of protein), 2% underwent S-nitrosylation after incubation with 5 μM GSNO (the GSNO concentration used in *B*). Points are means ± SEM of triplicate determinations. (*B*) nLC-MS/MS analysis showing base peak chromatograms of eluted peptide ions (MS mode). Results depict SNOSID analysis of a rat cerebellum lysate before (control) and after (GSNO) treatment with 5 μM GSNO. Note the detection of peptide ions at low levels before GSNO treatment and abundant levels after GSNO treatment.

nitrosylation. In 3 of 16 of these proteins, a specific Cys had been implicated as target, and, in each case, our identification by SNOSID concurred (see references in Table 1). Second, although Cys is a relatively rare amino acid (2.2% predicted from the rat genome), Table 1 reveals that 98.6% (68 of 69) of putative SNO peptides contained at least one Cys. It is unclear why one non-thiol-containing peptide (identified as a tryptic peptide from glucosamine-fructose-6-phosphate aminotransferase; GRVNFETNTVLLGGLK, not included in Table 1) copurifies with apparently bona fide SNO peptides. Explanations include that this peptide binds and coelutes with a Cys-containing peptide or that the peptide is actually Cys-containing but is misidentified. The observed enrichment of Cys-containing peptides in putative SNO peptides cannot be explained by chance, which predicts that 26.4% of random tryptic 12-mer peptides contain Cys, but is in accord with the requirement that every SNO peptide contains at least one Cys. Third, the abundance of peptide signals was abolished when ascorbate-mediated reduction of the S–NO bond was omitted from the procedure, ruling out the possibility that incomplete blocking of peptide thiols leads to significant misrepresentation as SNO-Cys-containing peptides. Fourth, detection of peptide ions was suppressed by >95% after denitrosyation of SNO proteins by 5 mM DTT treatment (data not shown).

The 56 identified proteins belong to diverse functional categories, from metabolic enzymes and structural proteins to ion channels, transporters, receptors, and signal transduction molecules. A single Cys is contained in 53 of these peptides, defining the site of S-nitrosylation; for the remaining five peptides, it remains to be shown which of two alternative Cys residues are S-nitrosylation sites. These proteins include those that are involved in glutamate transport and metabolism (sodium-dependent glutamate/aspartate transporters, glutamate decarboxylase, succinate semialdehyde dehydrogenase, and mitochondrial glutamate carrier), proteins involved in calcium transport and sensing (sodium/calcium exchanger 2, plasma membrane calcium-transporting ATPase 2, hippocalcin, and vicinin-like protein-1), and proteins involved in synaptic vesicle trafficking/docking/recycling [clathrin heavy chain, syntaxin binding protein-1, *N*-ethylmaleimide-sensitive factor (NSF) attachment protein β, bassoon, vesicle-fusing ATPase/NSF, ADP-ribosylation factor 1 GTPase-activating protein, and synaptotagmin-2]. In multiple cases, NO was shown to modulate the activity of systems that use these proteins, although S-nitrosylation of a critical protein Cys residue has not yet been examined. One exception is NSF, which was established in brain to undergo physiological S-nitrosylation on a Cys residue that we found herein (Cys-263) and associated with inhibition of exocytosis (20) and up-regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor expression (21). Together, the cerebellar SNO peptides listed in Table 1 are consistent with broad-reaching functions for SNO proteins in neuroregulation and provide a road map for Cys residues that may subserve pivotal roles in transducing NO bioactivities in brain.

Identification of Endogenous SNO-Cys-Containing Peptides. In untreated cerebellum, six endogenous SNO-Cys sites on four distinct proteins were identified: Cys-295 and Cys-376 in α-tubulin, Cys-12 and Cys-239 in β-tubulin, Cys-149 and Cys-153 in GAPDH, and Cys-504 in dihydropyrimidinase-related protein-2/CRMP-2. Table 2, which is published as supporting information on the PNAS web site, contains sequences of these endogenous SNO-Cys-containing peptides. Notably, it had been shown that α- tubulin, β-tubulin, and GAPDH are endogenously S-nitrosylated in brain (9, 20, 22) and that SNO–dihydropyrimidinase-related protein-2/CRMP2 accumulates in brain lysates after incubation with GSNO (9). Of these four proteins, the only ambiguous SNO-Cys site is on GAPDH, wherein the identified SNO-Cys peptide contains two Cys residues, precluding determination of whether Cys-149, Cys-153, or both undergo endogenous S-nitrosylation. However, it is notable that prior mutagenesis studies have revealed Cys-149 as the only endogenous SNO-Cys site on GAPDH. Remarkably, SNO modification of Cys-149 was found to be necessary and sufficient for GAPDH binding to Siah1 (an E3 ubiquitin ligase), resulting in the proapoptotic nuclear translocation of GAPDH (23).

Additional support for peptides listed in Table 2 arising from endogenous SNO proteins is provided by loss of their cognate ion counts when the ascorbate-mediated S–NO reduction step is omitted from the SNOSID procedure (preventing biotinylation at SNO-Cys sites) and their >90% loss after treatment with 5 mM DTT (data not shown). Fig. 3 shows the extracted ion chromatograph (EIC) of m/z equal to 853.4 and 782.9, which are reconciled as the doubly charged peptide ions arising from SNO-GAPDH (IVSNASCTTNCLAPLAK, residues 143–159) (Fig. 3*A*) and dihydropyrimidinase-related protein-2 (GLYDGPVCEVSVTPK, resides 497–511) (Fig. 3*B*), respectively. Note that each EIC appears as a single chromatographic peak at the given m/z (included in dotted boxes in Fig. 3), and peaks are absent when ascorbate reduction is omitted from the SNOSID procedure. Amino acid sequences of these peptide ions were established by MS/MS fragmentation patterns, which are depicted below each EIC in Fig. 3. Sequence assignments were

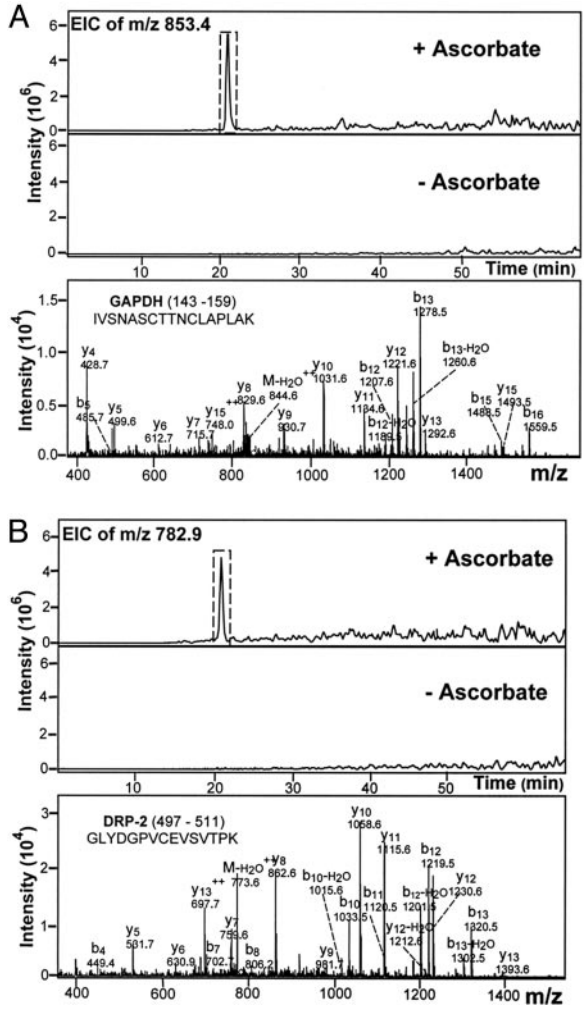

Fig. 3. Identification of endogenous SNO-Cys peptides in rat cerebellum. A single chromatographic peak was detected in each of the EIC at m/z 853.4 and 782.9, which correspond to the expected m/z values for doubly charged SNO peptides IVSNASCTTNCLAPLAK from GAPDH (*A*) and GLYDGPVCEVSVTPK from DPR-2 (*B*). The sequence identities of these two peptides were established by MS/MS, shown below each EIC. The detection of these two peaks was abolished in control experiments wherein the ascorbate reduction step was omitted from the SNOSID procedure.

similarly made for each of the four tubulin-derived tryptic peptides listed in Table 2. When the biotin-swap procedure was performed with intact cerebellar proteins rather than tryptic digests, additional peptides were identified from GAPDH (4), α-tubulin (six peptides) and β-tubulin (six peptides), and DRP-1 (one peptide), providing further confirmation that these are indeed endogenous SNO proteins.

Each of the six endogenous SNO-peptide ions was found to increase progressively in intensity with exposure of cerebellar lysates to increasing concentrations of GSNO (0–10 μM). The extent of increase in peptide ion intensity ranged from 16.9-fold (α-tubulin) to 2,211-fold (β-tubulin). Although endogenous ion signal intensities were only a fraction of maximum observed for each putative SNO peptide, their relative weakness does not preclude significant biological consequences. Indeed, nNOS is discretely distributed in brain (24), making it likely that endogenous SNO proteins concentrate at nNOS-rich brain foci. The further increases in peptide ions elicited by GSNO treatment would predictably extend to nNOS-poor brain regions that may be relatively irrelevant for physiological NO signaling. Future

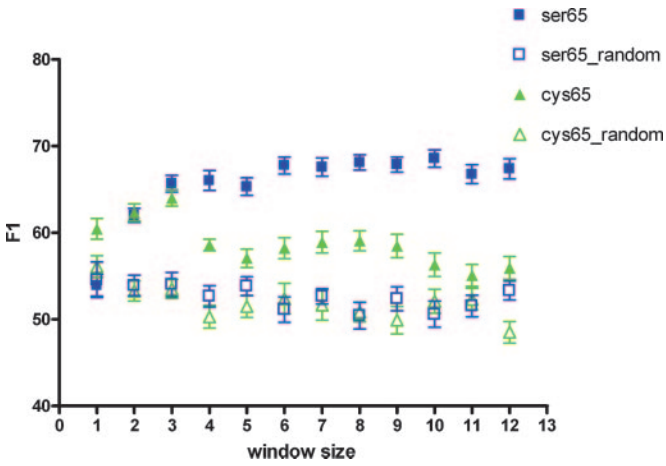

Fig. 4. Evaluation of the primary amino acid sequence of identified SNO peptides for Cys flanking residues. The predictive performance of flanking residues for S-nitrosylation (SNO) and OPH sites are compared. For each window size, 20 random samplings for both Ser-OPH sites and Cys-SNO sites were carried out; for each sampling, 65 positive and 65 negative samples were chosen to obtain a balanced training set of 130 total sites. Training sets were from 57 rat/mouse Ser-OPH protein sequences (140 Ser sites) and 56 SNO rat/mouse protein sequences (65 Cys sites). The negative samples were extracted from the other Ser/Cys residues in the same protein sequences. As controls, the same number of samplings was carried out without differentiating whether the residue is from an OPH/SNO site or not; thus, the 65 positive and 65 negative samples were randomly assigned. Calculations based on these data are denoted as random. The F1 measure, which is the harmonic mean of recall and precision, is plotted against the size of the window of the flanking residues analyzed.

studies will be needed to test whether SNO proteins are indeed differentially distributed in brain and colocalize at sites of nNOS expression.

Primary Sequence Analysis of SNO Proteins. The identification of only 58 SNO peptides in cerebellum after treatment with 2–10 μM GSNO suggests that S-nitrosylation is highly selective, despite the limited current understanding of how selectivity is achieved (6). With a sufficient inventory of protein Cys residues that are most prone to S-nitrosylation, it should be possible to distinguish physicochemical features that explain the selectivity of S-nitrosylation.

As a first attempt toward elucidating the basis for specificity of S-nitrosylation by GSNO, we considered whether Cys-flanking residues in the primary amino acid sequence contain a recognizable S-nitrosylation motif, in accord with a prior suggestion (25). It is well known that the primary sequence of residues flanking OPH sites can effectively predict sites of phosphorylation (26). By analogy, we asked whether primary sequence in Cys-flanking residues is associated with observed S-nitrosylation at this site. To address this question, we used a machine-learning approach to evaluate the presence of primary sequence patterns. Training sets of equal size were used with the same machine-learning protocol for prediction of SNO and OPH from sequence only, with OPH sites serving as a positive control and randomly assigned SNO and OPH sites as negative controls (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). Fig. 4 shows the prediction performance (F1 measure) as a function of the window size (i.e., the number of residues on each side of the acceptor residue). The values of F1 for Ser-OPH (similar results were obtained for Tyr-OPH sites; data not shown) are consistent with the notion that the "kinase physically contacts a stretch of 7–12 residues surrounding the acceptor residue" (26). With the

complete OPH data set, the machine-learning protocol displayed similar predictive OPH performance to that reported (26). For SNO sites, the F1 measure increased slightly with window size to a maximum of 3 and then decreased. Notably, with the current number of available training example for SNO, the support vector machine model barely outperformed random predictions and accordingly, is not useful for predicting SNO sites in new sequences. Despite our inability to define a primary sequence motif that predicts S-nitrosylation by GSNO under the conditions used, it is conceivable that the SNO-Cys residues considered here comprise a subset, selected for susceptibility of S–NO reduction by ascorbate (27); accordingly a primary sequence SNO motif could be predictive for SNO proteins identified by using other methods or NO donors. The predictive ability of a suggested 3D acid–base flanking residue motif that predicts Cys S-nitrosylation (25, 28) awaits rigorous evaluation.

S-Nitrosylation Targets Specific Redox-Sensitive Thiols. Because a basis for S-nitrosylation specificity by GSNO could not be found in the primary sequence of SNO peptides, key determinants of NO-targeting are likely to be encoded in the 3D Cys environment. In this regard, it is noteworthy that among the 20 Cys in tubulin (8 Cys in the α-subunit and 12 Cys in the β-subunit), 4 were identified as sites of endogenous SNO-modification (α-Cys-295, α-Cys-376, β-Cys-12, and β-Cys-239) and 3 additional Cys (α-Cys-347, β-Cys-303 and β-Cys-354) were found to undergo S-nitrosylation after treatment with low GSNO levels. The crystal structure of tubulin (Protein Data Bank ID code 1TUB) reveals that, with the exception of β-Cys-12, none of the NO-targeted Cys reside on the protein surface (29). Moreover, the NO-targeted Cys residues were previously shown to be the most reactive toward thiol-modifying reagents, such as 5,5′-dithiobis-(2-nitrobenzoic acid) (30, 31). Thus, a low pK_a instead of solvent accessibility may be of primary importance for S-nitrosylation to occur.

A basis for reactivity of the Cys that undergo endogenous S-nitrosylation in tubulin is suggested from consideration of the crystal structure. Notably, all identified SNO-Cys are located in an environment of positive charge. Interestingly, α-Cys-295 and α-Cys-347 are positioned within 6 Å of a pair of aromatic residues (F296/Y319 and W346/F343, respectively). Tubulin β-Cys-376 is similarly in proximity with Y272 and hydrogen bonds with a positive residue, R320. Remarkably, electronegative sulfurs of Cys are often observed to engage in favorable electrostatic interactions with electropositive hydrogens on aromatic rings: Such interactions can contribute to protein structure stability (31, 32). Over 50% of protein Cys residues in a database of high-resolution structures were reported to be in proximity to aromatic residues, with Cys-sulfur pointing toward aromatic ring hydrogen atoms and apparently engaged in electrostatic interactions (32). Such sulfur-aromatic electrostatic interactions would provide greatest stability in hydrophobic protein environments, such as that shown for the site of S-nitrosylation on argininosuccinate synthetase (33) and the ryanodine receptor (3). Hence, we propose that Cys-aromatic interactions, in conjunction with hydrogen-bonding to basic residues, provide a general structural motif that renders Cys susceptible to S-nitrosylation.

The importance of thiol reactivity for S-nitrosylation is further supported by consideration of some SNO-Cys residues on proteins listed in Table 1. Cys-160 and Cys-257 in ADP/ATP translocase are known to be primary targets for oxidative stress and undergo disulfide bond formation, resulting in formation of the permeability transition pore in mitochondria, triggering apoptosis (34). Cys-283 of cytosolic creatine kinase and the homologous Cys-317 of sarcomeric mitochondrial isoform are active site thiols at which S-nitrosylation was shown to be inactivating (35). In the case of DJ-1, the identified SNO peptide contained two Cys residues; one of them, Cys-106, was reported to be a primary target for oxidation, leading to mitochondrial relocation and neuroprotectant activity of DJ-1 (36, 37). Cys-299 of protein farnesyltransferase coordinates the zinc finger (38), and zinc finger thiols are well known to be redox-active (39). Cys-228 of calcineurin (protein phosphatase 2B) is recognized as one of two thiols that are most sensitive to oxidation (40). Very recently, Cys-442 of heat-shock protein 60 was reported to be alkylated by a natural electrophilic inhibitor, epolactaene (41). Together, these findings support the general notion that S-nitrosylation selectively targets reactive protein thiols.

In summary, the SNOSID method described here has enabled an unbiased identification of SNO-Cys residues in a biologically complex protein mixture. Results confirm the presence of endogenous SNO proteins in rat brain and define Cys targets therein. With technical advances in nLC-MS/MS-based detection and procedural improvements in SNO-protein recovery and bioinformatics detection, it is likely that the number of endogenous SNO-Cys residues identified in brain and other NOS-containing tissues will expand significantly. Whereas proximity of a protein Cys to NOS may be a determinant of NO-targeting selectivity, as implicated for NMDA receptor subunits (42) and Dexras (4), features of the Cys environment are likely to be predominant. Our findings failed to reveal features in the primary sequences of SNO-Cys residues that could account for selective targeting by GSNO. Instead, the 3D Cys environment is implicated in conferring susceptibility to S-nitrosylation. A large data set of high-resolution structures of SNO-prone protein Cys residues will be invaluable for rigorous definition of physicochemical features that reconcile protein S-nitrosylation specificity.

Materials and Methods

Materials. Rat cerebellum was purchased from Pel-Freez (Rogers, AR), nanospray capillary emitters (15 μm) were from New Objective (Woburn, MA) and capillary LC columns (C18 resin, 75-μm diameter, 150-mm length) were from Agilent Technologies. HPLC grade acetonitrile was from J.T. Baker Chemicals, Phillipsburg, NJ (provided through VWR International, West Chester, PA), and all other chemicals, unless stated otherwise, were purchased from Sigma in the highest available purity.

SNO Quantification by Saville–Griess Assay. SNO-protein levels in cerebellar lysates were quantified by using the Saville-Griess assay, as described in ref. 43. Briefly, untreated and GSNO-treated cerebellar lysates were prepared by precipitation in iced acetone followed by dissolution in incubation buffer, as described above for SNO peptide analysis. Mercury displacement of NO from SNO proteins was elicited by 10 min of incubation with 100 μM $HgCl_2$. Nitrite levels were determined colorimetrically after reaction with 100 μl of working Griess reagent (1:1 mixture of 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 5% *O*-phosphoric acid) and quantified in a microtiter plate spectrophotometer (ThermoMax; Molecular Devices) at A_{540} relative to sodium nitrite standards (44).

The SNOSID Method for SNO Peptide Isolation. Rat cerebellum (1 g) was homogenized in 10 ml of iced lysis buffer (20 mM Tris·HCl/1 mM EDTA/150 mM NaCl/0.4% Triton X-100/0.1 mM neucuproine, pH 7.6) with 10 strokes in a Dounce homogenizer. Some lysates were subjected to S-nitrosylation by pretreatment with GSNO at 37°C for 30 min; control samples were either untreated or incubated under identical conditions with 5 mM DTT. Samples were then centrifuged at 2,000 $\times$ *g* for 15 min, and the supernatant was discarded. Protein pellets were resuspended in blocking buffer (20 mM Tris·HCl, pH 7.7/2.5% SDS/20 mM *S*-methylmethanethiosulfonic acid/1 mM EDTA/0.1 mM neocuproine) to give a final

protein concentration of 0.8–1.0 mg/ml and incubated for 30 min at 50°C. Proteins were precipitated free of *S*-methylmethanethiosulfonic acid by addition of two volumes of iced acetone and pelleted by centrifugation. Protein pellets were solubilized and biotinylated by 1 h incubation at room temperature in 5 ml of resuspension buffer (20 mM Tris, pH 7.6/4 mM ascorbate/0.2 mM *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide) (Pierce, Rockford, IL). Biotinylated proteins were precipitated with iced acetone, pelleted, and solubilized in 5 ml of incubation buffer (20 mM Tris·HCl, pH 7.7/1 mM EDTA/0.4% Triton X-100), followed by digestion with 1:100 diethyl pyrocarbonate-treated trypsin (Sigma) at room temperature for 16 h. Digestion was terminated by addition of 0.5 mM PMSF, and the resulting peptide mixture was incubated with 100 μl of streptavidin-agarose beads (Sigma) at room temperature for 1 h. The streptavidin beads were washed five times in 1 ml of wash buffer (incubation buffer containing 600 mM NaCl), followed by five 1-ml washes in 5 mM ammonium bicarbonate buffer containing 20% acetonitrile. Streptavidin-bound peptides were eluted with 150 μl of 100 mM 2-mercaptoethanol and concentrated in a SpeedVac. Complete drying was avoided to diminish sample loss and thiol oxidation. Trifluoroacetic acid was added to the peptide solution to achieve a final concentration of 0.1%, and samples were analyzed by nLC-MS/MS.

LC-MS/MS Analysis. nLC-MS/MS was used to obtain peptide sequence information and identify cognate proteins of origin. nLC-MS/MS was performed with an 1100 series nLC coupled to an XCT plus ion trap mass spectrometer (Agilent). The avidin-eluted peptide mixture was injected onto a 0.3- × 5-mm Zorbax 300SB-C18 sample-enrichment column (Agilent), at a flow rate of 10 μl/min. Peptides were resolved on a 0.075- × 150-mm Zorbax 300SB-C18 analytical column (Agilent) at a flow rate of 0.3 μl/min with a gradient of 15–40% solvent B for 50 min and 40–85% solvent B for 30 min. Mobile phase solvent A comprised 0.1% formic acid in 3% acetonitrile, and solvent B was 0.1% formic acid in 90% acetonitrile. The LC column eluate was introduced into the ion trap mass spectrometer via electrospray, using a 15-μm diameter silica emitter needle. Mass spectra were acquired in positive-ion mode with automated data-dependent MS/MS on the four most intense ions from precursor MS scans.

Database Search of MS/MS Data for Peptide Sequence Identification. Analysis of MS/MS spectra for peptide identification was performed by protein database searching with SPECTRUM MILL software (Agilent Technologies). Raw MS/MS spectra were first processed to extract MS/MS spectra that could be assigned to at least two y- or b-series ions, and only those spectra were searched against the SwissProt rodent database. Key search parameters were a minimum matched peak intensity of 50%, a precursor mass tolerance of 2.0 Da, and a product mass tolerance of 0.6 Da. The threshold used for peptide identification was a SPECTRUM MILL score of >10.0 and an SPI% (the percentage of assigned spectrum intensity of total spectrum intensity) of >70%. Finally, all MS/MS spectra were validated by manual inspection.

We thank Ye Ingrid Yin (Molecular Pharmacology and Chemistry Program, Sloan-Kettering Institute, New York) for assistance with MS. This work was supported by National Institutes of Health Grants HL46403, HL80702, and RR19355 (to S.S.G.).

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