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Original Contribution

Detection of Protein *S*-Nitrosation using Irreversible Biotinylation Procedures (IBP)

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ABSTRACT

The biotin switch assay for detection of protein *S*-nitrosation has been widely used in the field of nitric oxide and redox signaling. However, here we found that there is experimental and theoretical interference of intermolecular disulfide bonds in S-nitrosated protein identification with avidin purification after biotin switch method: proteins linked to S-nitrosated proteins by intermolecular disulfide bonds can be falsely detected as S-nitrosated targets. Then we developed irreversible biotinylation procedures (IBP) to prevent this interference, in which irreversible biotinylation was used to instead of reversible biotinylation, all the intermolecular disulfide bonds were broken before purification of biotinylated proteins added as a new step, and doing elution by denaturation of avidin after the purification. This strategy enables us to specifically detect protein *S*-nitrosation without the potential interference of intermolecular disulfide bonds. Furthermore, we applied IBP to proteomic approaches and quantitative proteomic approaches for high-throughput studies of protein *S*-nitrosation.

Introduction

Cysteine (Cys, C) is an amino acid with important functions; it can form disulfide bonds (-S-S-) which are important for protein structure stability[1,2], and it is a key amino acid of the catalytic sites of many families of enzymes[2,3]. It is reported that over 95% proteins contain cysteine[4]. Most importantly, as a nucleophile, cysteine can be modified by various electrophiles, thus participating in the regulation of protein function. Therefore, protein post-translational modifications involving cysteine have long been recognized as important regulatory mechanisms for signal transduction[2]. There are different types of redox-related cysteine modifications including protein disulfide bond (-S-S-), S-nitrosothiol (-SNO), sulfenic acid (-SOH), sulfinic acid ($-SO_2H$), and sulfonic acid ($-SO_3H$). Methods for precisely discrimination of these different kinds of modifications are restricted [5] and this limitation has hampered the study of the relationship between cysteine modifications and protein function.

The invention of the biotin switch assay for *S*-nitrosation in 2001 was a significant breakthrough[6]. *S*-Nitrosation (commonly referred to as *S*-nitrosylation) is a post-translational modification of free cysteine residues by a reactive nitrogen species, resulting in the conversion of free cysteines (Cys-SH) to *S*-nitrosothiols (Cys-SNO)[7]. The biotin switch method was designed to detect protein *S*-nitrosation specifically without the interference of other kinds of cysteine modifications, and therefore enabled isolation of the biological effects of *S*-nitrosation from other cysteine modifications. Since the invention of this method, developments in the field of nitric oxide and redox signaling have been dramatically enhanced: hundreds of targets have been identified and the roles of *S*-nitrosation in cellular signaling and diseases have been explored. *S*-nitrosation is regarded as another important cGMP-independent signaling mechanism of NO in a broad range of physiological and pathological processes[8]. The biotin switch method has been combined successfully with proteomic methods and has enabled systematic studies of *S*-nitrosation and various other cysteine modifications. With the increasing application of this method, several modifications have been made to improve its sensitivity, including increasing the ascorbate concentration to enhance the reduction of SNO and therefore increase the signal of *S*-nitrosated proteins[9], and adding copper to increase the biotinylation efficiency via enhancing SNO bond cleavage[10]. Recently, SNO-RAC was developed to enhance the identification of high molecular weight protein targets and dynamic studies of nitrosation/denitrosation in intact cells[11].

Here we found that proteins linked to *S*-nitrosated proteins by intermolecular disulfide bonds can be falsely detected as *S*-nitrosated targets in the original biotin-switch assay after avidin purification. Then we developed irreversible biotinylation procedures (IBP) and IBP-based proteomic approaches which prevent interference from intermolecular disulfide bonds.

Abbreviations: biotin–HPDP, N-[6-(biotinamido)hexyl]-3-(2-pyridyldithio)propionamide; biotin-M, biotin-maleimide; biotin-PEO-M, biotin-PEO-maleimide; DTSP, 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester); GSNO, S-nitrosoglutathione; MS, mass spectrometry; MMTS, methylmethanethiosulfonate; NEM, N-ethylmaleimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ICAT, isotope-coded affinity tag.

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Materials and Methods

Chemicals

S-Nitrosoglutathione (GSNO) was synthesized from glutathione using acidified nitrite. Sodium ascorbate, sodium dodecyl sulfate (SDS), neocuproine, methylmethanethiosulfonate (MMTS), dithiothreitol (DTT), N-ethylmaleimide (NEM), biotin-maleimide (biotin-M), DTSP (Lomant's reagent, 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester)) and streptavidin-agarose were purchased from Sigma (St. Louis, MO). Protease inhibitor cocktail was from Roche. N-[6-(Biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (biotin–HPDP), biotin-PEO2-maleimide (biotin-PEO-M) and monomeric avidin-agarose were purchased from Pierce. Mouse anti-GAPDH monoclonal antibody, rabbit β-actin polyclonal antibody, mouse and rabbit secondary antibodies with HRP were from Santa Cruz Biotech. Rabbit anti-NSF and rabbit anti-PDI polyclonal antibodies were from Abcam. Cleavable ICAT reagent kits were purchased from Applied Biosystems (Foster City, CA, USA). All the other chemicals were local products of analytical grade.

Animals

4-5 week old C57BL/6 mice were sacrificed and mouse brains were harvested for *in vitro* analysis. The Institute of Biophysics Administrative Panel on Laboratory Animal Care approved all procedures involving animals.

Diagonal Electrophoresis

Protein samples were analyzed by non-reducing 12% SDS-PAGE. Lanes were cut out and proteins were reduced using 200 mM DTT in electrophoresis buffer for 15 min at room temperature. The lanes were then rotated 90° and laid horizontally on top of another 12% gel for the 2nd dimension of reducing SDS-PAGE. Silver staining was used after SDS-PAGE to detect protein bands.

Irreversible Biotinylation Procedure (IBP)

Mouse brain was homogenized in 5 ml HEN buffer (250 mM HEPES pH 7.7, 0.1 mM EDTA, 10 mM neocuproine) with 1% NP40 (Nonidet P-40), protease inhibitor cocktail and 1 mM PMSF, sonicated and then centrifuged at 12,000 g for 10 min at 4 °C. The supernatant was treated with 40 μM of a transnitrosation reagent GSNO for 30 min at room temperature or incubated without GSNO. The GSNO was removed by ice-cold acetone precipitation for 20 min followed by centrifugation at 2,000 g for 10 min. The protein pellet was resuspended in blocking buffer (HEN buffer with 2.5% SDS and 20 mM MMTS) to a final concentration of less than 1 mg/ml to ensure full blocking of the free thiols. The free thiols were blocked at 50 °C for 20 min. The excess MMTS was removed by ice-cold acetone precipitation for 20 min followed by centrifugation at 2,000 g for 10 min. This precipitation was repeated several times to remove the residual MMTS. The pellet was recovered in HENS buffer containing 2.5% w/v SDS with 0.2 mM biotin-maleimide and 10 mM ascorbate and incubated for 2 h at room temperature. The excess biotin-maleimide was removed by ice-cold acetone precipitation for 20 min followed by centrifugation at 2,000 g for 10 min. This precipitation was repeated several times. The protein pellet was resuspended in HENS buffer with 200 mM DTT and incubated for 15 min at 100 °C to reduce potential intermolecular disulfide bonds. Two volumes of neutralization buffer (HEN buffer with NaCl) were added and streptavidin-agarose was used to purify the biotinylated proteins. After incubation for 2 hours the agarose was washed 3 times with neutralization buffer containing 0.05% SDS. The biotinylated proteins were eluted by incubating the agarose with 2.5% SDS in HEN at 95 °C for 15 min.

In some experiments, we used 20 mM N-ethylmaleimide (NEM) to replace MMTS or 0.2 mM biotin-PEO-maleimide to replace biotin-maleimide. Similarly, biotin-PEO2-maleimide or any other irreversible thiol reactive biotinylation reagent could be used in place of biotin-maleimide.

In the original biotin switch assay, we used 0.4 mM biotin-HPDP for biotinylation and omitted the reduction step before the purification of biotinylated proteins as described in the original biotin switch method. In some experiments, we used NEM to replace MMTS, or avidin-agarose to replace streptavidin-agarose.

In vitro Cross-linking of Proteins in the Lysates of Mouse Brain

DTSP (Lomant's reagent, 3,3′-Dithiodipropionic acid di(N-hydroxysuccinimide ester)), is a homobifunctional cross-linking reagent containing a cleavable disulfide linkage, typically coupled to molecules containing primary amines by amide bonds buffered at pH 7.5 (6.5-8.5). Here the lysates of mouse brain were treated with DTSP for 30 min to form intermolecular disulfide bonds, Pr-S-S-Pr *in vitro* and was stopped by addition of Tris buffer. The lysates were then analyzed by the original biotin switch method and IBP.

SDS PAGE and Western Blotting

After non-reducing or reducing SDS-PAGE, the gels were western blotted to nitrocellulose (NC) membranes and blocked by 5% non-fat milk with 0.05% tween-20 in Tris buffered saline (TTBS) for 1 hour. Then the membranes were incubated with different primary antibodies with a 1:2000 dilution (0.5% non-fat milk in TTBS) for 1 hour. After washing the primary antibodies with TTBS, the membranes were incubated with the corresponding secondary antibodies with HRP (1:10,000 dilution, 0.5% non-fat milk in TTBS). After washing, the signals were detected by enhanced chemiluminescence detection (Pierce).

Identification of S-Nitrosation by Proteomic IBP (pIBP)

Mouse brain was homogenized in 5 ml HEN buffer with protease inhibitor cocktail and 1 mM PMSF, sonicated and then centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was treated with 40 μM of GSNO for 30 min at room temperature or incubated without GSNO. *S*-nitrosated proteins were purified as above by IBP. Purified proteins were then trypsinized (using a 1:50 ratio of protein: trypsin) in solution for 12-16 h. The peptides were cleaned by SCX ziptip before LC-MS/MS analysis.

For site identification experiments, the purified proteins were trypsinized and biotinylated peptides were subsequently purified with monomeric avidin-agarose. Peptides were eluted and cleaned by SCX ziptip for LC-MS/MS analysis.

Quantitative Irreversible Biotinylation Procedures (qIBP)

Proteins from mouse brain were extracted and analyzed as above in pIBP. We used a urea-based biotin switch method for this purpose. The proteins were blocked with 20 mM MMTS and urea buffer (8 M urea with 250 mM HEPES pH 7.7, 0.1 mM EDTA, and 0.1 mM neocuproine) for 1 hour at room temperature. Acetone precipitation was used to remove excess MMTS. After resuspending the proteins in urea buffer, 10 mM ascorbate and ICAT reagents were added for biotinylation of S-nitrosated proteins. The GSNO treated sample was biotinylated with an ICAT heavy reagent and the control sample was biotinylated with an ICAT light reagent at 37 °C for 2 hours. The two samples were mixed together and trypsinized before purification of

biotinylated peptides. The peptides were then acid-cleaved for LC-MS/MS analysis.

MS/MS analysis

Peptides were separated by reverse-phase capillary liquid chromatography (RP-C18, Column Technology Inc, 0.15 mm × 150 mm) at a flow rate of 200 nl/min. The eluent was directly analyzed by a Thermo LTQ linear trap instrument equipped with a Thermo microelectrospray source, a Thermo Surveyor pump and an autosampler (Thermo Finnigan, San Jose, CA, USA). A survey scan followed by 5 CID events was used. Peptide identification by CID was carried out in automated mode using the 3 min dynamic exclusion option.

MS/MS spectra were searched against NCBI using SEQUEST in Bioworks 3.2 (Thermo Finnigan). The SEQUEST database search criteria for pIBP included a variable modification of 452.54 Da for cysteines when the biotin-maleimide reagent was used. For qIBP, the SEQUEST database search criteria included a static modification of 237.37 and a variable modification of 9 Da for cysteines when ICAT reagents were used. The tolerance of the precursor ion and the fragment ion were 2 and 1 amu, respectively. The SEQUEST filtration criteria were set as follows: XCorr value>1.8 for singly charged, >2.5 for doubly charged and >3.8 for triply charged ions; Delta Cn>0.1; preliminary score (Sp)>500; RSp<5; the P value for identification of false positives was set at <0.001. Peptides were permitted to have up to two miscleavages. Our raw data has been submitted to the PRIDE database.

Results

Intermolecular disulfide bonds are present and interfere with the biotin switch assay followed by avidin-agarose purification

Firstly, we showed that intermolecular disulfide bonds are present in protein samples after the biotin switch assay. Purified biotinylated proteins were obtained from mouse brain lysates treated with 40 μM of *S*-nitrosoglutathione (GSNO) using a biotin switch method in which the final step of eluting biotinylated proteins from streptavidin-agarose was conducted under non-reducing conditions in order to preserve potential intermolecular disulfide bonds. Diagonal electrophoresis was then used to detect proteins containing intermolecular disulfide bonds [12,13] (Fig. 1a). Additional protein bands appeared below the diagonal line after the second dimension of reducing SDS-PAGE, indicating that the presence of intermolecular disulfide bonds does indeed interfere with the biotin switch assay for detecting protein *S*-nitrosation.

We then investigated whether these intermolecular disulfide bonds were formed because of any of the reagents used in the biotin switch assay by replacing reagents involved in the key steps. First, we replaced the reversible thiol blocking reagent MMTS used in the blocking step with an irreversible thiol reactive reagent, N-ethylmaleimide (NEM). However, we still observed the presence of intermolecular disulfide bonds (Supplementary Fig. 1a). Similarly, when we replaced the biotinylation reagent biotin-HPDP with biotin-maleimide or replaced the purification reagent streptavidin-agarose with monomeric avidin-agarose (which has less affinity for the biotin tag compared with streptavidin or avidin), intermolecular disulfide bonds were still apparent (Supplementary Fig. 1b, Supplementary Fig. 1c).

We also analyzed proteins purified after the biotin switch assay using Western blotting with a specific antibody. We found that the widely recognized *S*-nitrosated protein target glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was present after the biotin switch assay as several high molecular weight bands in addition to its monomeric band after lengthening exposure time of Western blotting (Fig. 1b). We analyzed the protein samples before the agarose purification step and found that the high molecular weight bands

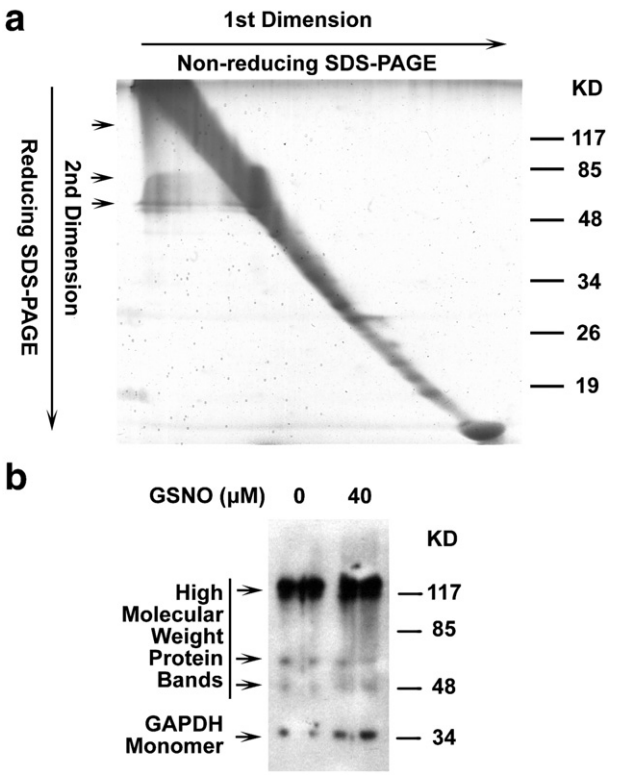

Fig. 1. Intermolecular disulfide bonds are present under non-reducing SDS-PAGE and interfere with the biotin switch assay. a. Diagonal electrophoresis of protein samples purified from 40 μM GSNO-treated mouse brain lysates using the original biotin switch assay. Additional protein bands appear below the diagonal line, as indicated by the arrows. b. Non-reducing SDS-PAGE was used to detect the intermolecular disulfide bonds. *S*-nitrosated proteins were purified by streptavidin-agarose from mouse brain lysates treated with or without 40 μM GSNO by the original biotin switch assay, then analyzed by Western blotting to analyze GAPDH. GAPDH was present as several high molecular weight bands after non-reducing SDS-PAGE, as indicated by arrows, in addition to its monomeric band.

were already present before the purification step, and that the disulfide bonds could be broken by DTT reduction in both the MMTS- (Fig. 2a) and NEM- (Fig. 2b) blocked protein samples. We analyzed two other *S*-nitrosated proteins, parkin and N-ethylmaleimide-sensitive factor (NSF), and also found high molecular weight bands in addition to their monomeric bands (Fig. 2c,d). When we exchanged the biotinylation reagent biotin-HPDP with other reagents such as biotin-maleimide (biotin-M) or biotin-PEO-maleimide (biotin-PEO-M), intermolecular disulfide bonds were still present as shown in Supplementary Fig. 2a. The same results were obtained for NSF (Supplementary Fig. 2b), and NEM blocking did not eliminate these disulfide bonds (Supplementary Fig. 2c).

In order to avoid potential trans-nitrosation of free cysteines and potential oxidation of free thiols to intermolecular disulfide bonds, a thiol blocking reagent such as MMTS should be included in the lysis buffer. We therefore prepared protein samples using lysis buffer containing MMTS and analyzed GAPDH *S*-nitrosation, as shown in Supplementary Fig. 2d, however, high molecular weight protein bands were still present. Our results thus clearly show that proteins with intermolecular disulfide bonds are present and are likely to be falsely identified as *S*-nitrosation targets.

Design of irreversible biotinylation procedures for detection of S-nitrosation

The data presented here prove that intermolecular disulfide bonds interfere with the detection of protein *S*-nitrosation during the

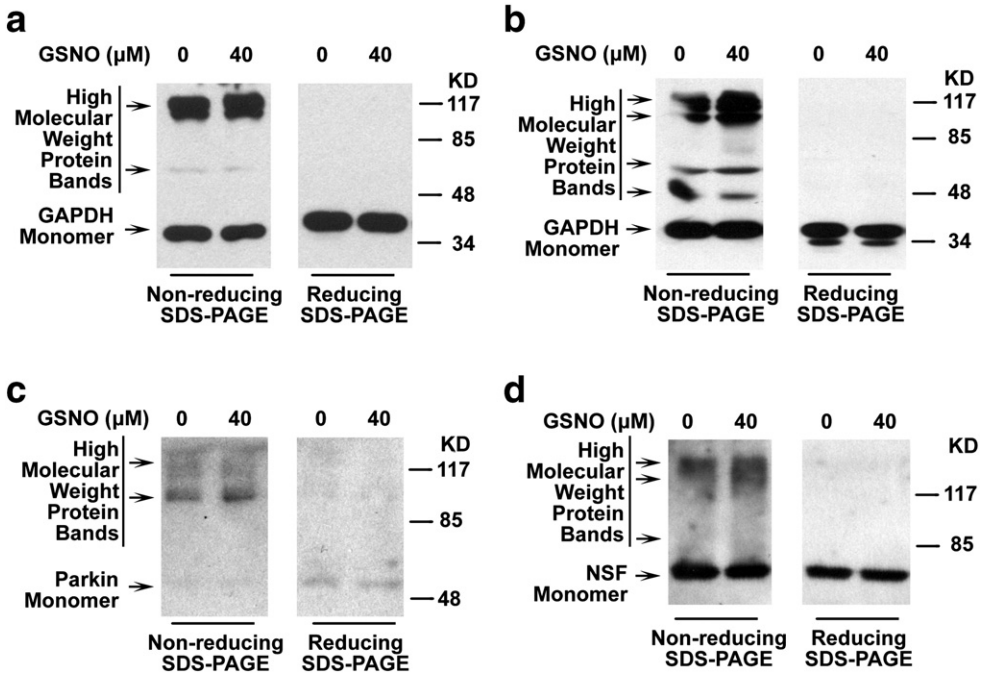

Fig. 2. Single proteins as examples to elucidate the interference of intermolecular disulfide bonds after the biotin switch method without avidin purification. a. Mouse brain lysates were treated with or without 40 μM GSNO, the samples were analyzed by the original biotin switch method.. However, the samples were not treated by streptavidin-agarose purification but analyzed directly by non-reducing or reducing SDS-PAGE in a same gel followed by Western blotting. Some high molecular weight bands were present (indicated by arrows) in addition to the GAPDH monomeric band in non-reducing SDS-PAGE, and disappeared in reducing SDS-PAGE. b. The same initial samples and procedures as in a, with NEM as the blocking reagent instead of MMTS. c. The same initial samples and procedures as in b, high molecular weight bands of Parkin were present after the original biotin switch assay. d. The same initial samples and procedures as in b, high molecular weight bands of NSF were present after the original biotin-switch assay.

biotin switch assay followed by avidin purification. Interference from intermolecular disulfide bonds should not be ignored in protein *S*-nitrosation studies as it may lead to artifactual results concerning the relationship between protein *S*-nitrosation and biological processes. Therefore, a modified method should be developed for future studies of protein *S*-nitrosation to exclude the interference of intermolecular disulfide bonds.

The basic strategy of the biotin switch method involves several critical steps. Firstly, free cysteines are blocked using the free thiol-blocking reagent methyl methane thiosulfonate (MMTS). After excess blocking reagent is removed, the *S*-nitrosated cysteines (Cys-SNO) on the proteins are reduced with ascorbate to give free cysteines which can then be biotinylated using the reversible biotinylation reagent biotin-HPDP (N-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide). Biotinylated proteins are purified using streptavidin-agarose and eluted using disulfide bond reducing reagents such as 2-mercaptoethanol (2-ME) or dithiothreitol (DTT). The eluted proteins are *S*-nitrosated proteins and can be identified by Western blotting or proteomic approaches. This strategy has been slightly modified to suit the detection of S-sulfenation and S-palmitoylation by replacing the *S*-nitrosothiol reducing reagent ascorbate with arsenite[14] or hydroxylamine[15,16], respectively.

We found that interference from the presence of intermolecular disulfide bonds was not considered in the strategy of avidin purification after the original biotin switch assay. From the flow chart of the original biotin switch assay (Fig. 3a), we can see that if there was an intermolecular disulfide bond between proteins A and B, the disulfide bond between these two proteins would be stable throughout the whole process of the assay until the elution of the biotinylated proteins from streptavidin-agarose using disulfide bond reducing reagents. After elution under reducing conditions, the intermolecular disulfide bond would be cleaved and protein B would be detected in the Western blotting step. Protein B would thus be falsely detected as a protein target of *S*-nitrosation. It should be pointed out that Fig. 3a is only a simple example of the S-S bonding relationships that could exist between proteins in a sample; complicated derivatives are likely to be more common (Supplementary Fig. 3).

Removing the interference of intermolecular disulfide bonds is the key to solving these problems. The original biotin switch assay used several special reagents such as MMTS and biotin-HPDP. These reagents all react reversibly with thiols. Reversibility has its advantages for analysis of protein samples, because the proteins maintain their native chemical composition and thus remain suited to biochemical analyses such as Western blotting or mass spectrometry. However, this is the main cause of the interference of intermolecular disulfide bonds. Biotin-HPDP is a reversible biotinylation reagent and after the biotin switch assay, *S*-nitrosated proteins are biotinylated and will be purified by avidin-agarose with biotin affinity. If there are any kinds of disulfide bonds that will interfere, it is not possible to eliminate this interference without loss of the biotinylation signal. If reducing reagents are added to break disulfide bonds, for example, biotinylated proteins will lose their biotin tag and will not be purified by avidin-biotin affinity approaches.

These problems can be resolved by changing the original biotin switch assay into a "irreversible biotinylation procedure" (IBP) as shown in Fig. 3b. We have changed several steps of the original method and added one extra step. In step 2, the reversible biotinylation reagent, biotin-HPDP, is replaced with an irreversible biotinylation reagent biotin-M, i.e., -S-C-linker-biotin will be formed instead of -S-S-linker-biotin. Then in the extra step 3, the samples are reduced with DTT to break any intermolecular disulfide bonds before biotinylated proteins are purified, thus removing any possibility of interference from intermolecular disulfide bonds. Finally in step 4, the purified proteins are eluted from streptavidin. This strategy eliminates the artifactual identification of protein B as a *S*-nitrosation target, and thus enables us to study protein *S*-nitrosation without the potential interference of intermolecular disulfide bonds.

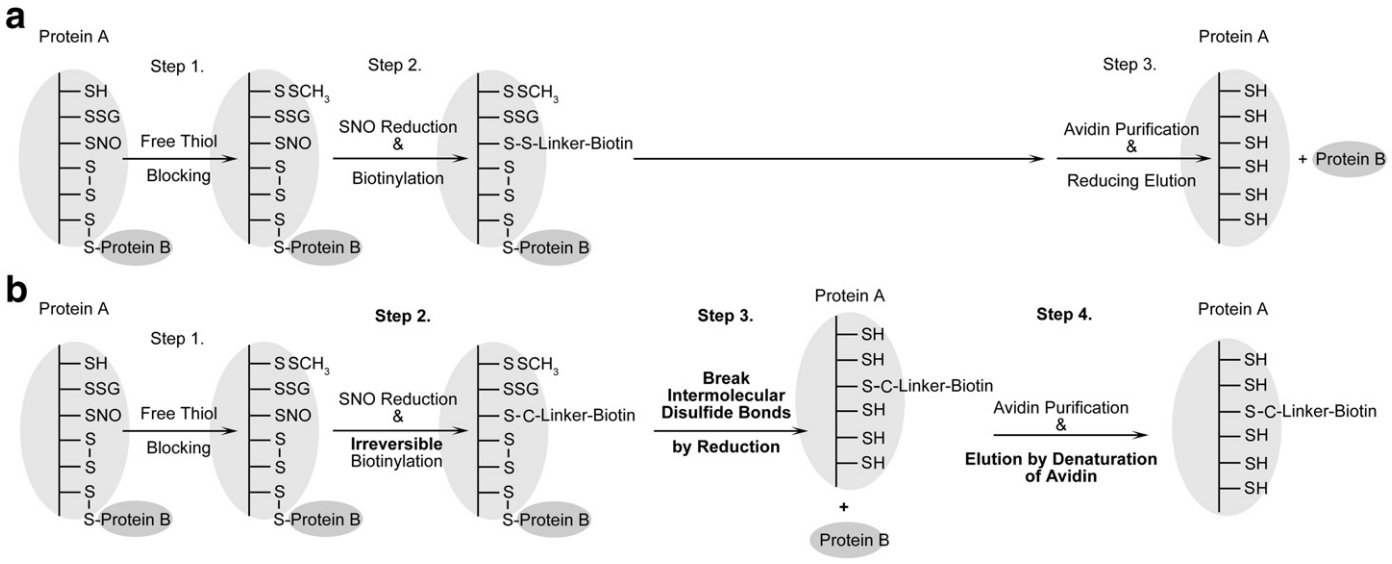

Fig. 3. Theoretical analysis of the original biotin switch assay followed by avidin purification and the construction of the irreversible biotinylation procedure, "IBP". a. Flow chart for the original biotin switch assay[6]. Protein B will be falsely detected as a protein target of *S*-nitrosation because the intermolecular disulfide bond is not broken before purification. b. Flow chart for IBP, which eliminates the interference from protein B by adding a reduction step to release protein B before purification.

Identification of S-nitrosation by IBP

Fig. 4a shows the results of protein samples treated with 40 μM GSNO analyzed using our irreversible biotinylation procedures: no intermolecular disulfide bond interference was detected, proving that this strategy can be applied successfully. The same results were obtained after replacing biotin-M with another irreversible biotinylation reagent, biotin-PEO-M. After treatment of brain lysates with 40 μM GSNO followed by the irreversible biotinylation procedures, using MMTS or NEM as the blocking reagent and biotin-M or biotin-PEO-M as the biotinylation reagent, *S*-nitrosated protein targets of interest were analyzed by Western blotting as shown in Fig. 4b. The results showed that exogenous treatment with GSNO increased protein *S*-nitrosation significantly and that the IBP strategy is efficient for detection of *S*-nitrosation compared with the original biotin switch assay (Fig. 4c). We then analyzed the eluted protein samples and found that the high molecular weight protein bands of GAPDH were no longer present when using the irreversible biotin switch procedure (IBP) (Fig. 4d), indicating that IBP could successfully prevent the interference of intermolecular disulfide bonds.

The interference can be more serious under oxidative stress since non-native disulfide bonds will form. Here we used DTSP to induce Pr-S-S-Pr in the lysates of mouse brain *in vitro* and then analyzed S-nitrosation with the original biotin switch method and IBP, respectively. As indicated in Fig. 5a, there are very strong Pr-S-S-Pr interference signals detected with the original biotin switch assay, however, with the IBP analysis, the Pr-S-S-Pr interference was successfully eliminated because the Pr-S-S-Pr was broken during IBP. The same results were shown in Fig. 5b of GAPDH western blotting analysis. These results again elucidate the influence of intermolecular disulfide bonds when using biotin switch assay and also validate the efficiency of IBP in prevention S-nitrosation detection from the interference of intermolecular disulfide bonds. However, the real situation of redox changes *in vivo* could be much more complicated and needs further studies.

Design of a Proteomic Method for Protein S-Nitrosation and Identification of S-Nitrosation sites by Proteomic IBP (pIBP)

The publication of the biotin switch method resulted in an explosion of interest in the proteomics of *S*-nitrosation[6]. The biotin switch method is particularly well suited to this field as it enables the enrichment of low abundance post-translationally modified proteins, increasing the likelihood of their detection. The original proteomic methods for analyzing biotin switch results were developed bearing in mind the design of the assay. Therefore, just as is the case with the original biotin switch assay, the data generated by these proteomic methods may also be affected by intermolecular disulfide bonds. For example, if a given peptide has more than one cysteine, any non-*S*-nitrosated cysteines in the peptide could form intermolecular disulfide bonds with one or more other peptides, giving rise to false positive identification of these peptides as *S*-nitrosation sites; the higher the number of intermolecular disulfide bonds formed, the greater the possibility that they will be identified as false positive targets (Supplementary Fig. 4a). Whereas there is no such interference with IBP followed by MS identification as shown in Supplementary Fig. 4b.

To identify *S*-nitrosation protein targets, several strategies can be used. The protein targets can be directly identified after the protein purification step of biotin switch, or by identifying *S*-nitrosated peptides after trypsin digestion and subsequent purification. However, these two methods have different advantages and disadvantages. To identify the purified proteins by direct digestion, a protein should be identified by at least two distinct peptides to increase the fidelity of identification. However, information about the sites of *S*-nitrosated cysteines is lost. If *S*-nitrosated peptides are used to identify the *S*-nitrosated proteins, site and protein information can be obtained, but only a limited number of *S*-nitrosated peptides can be identified, and with low fidelity in most cases.

To overcome these disadvantages we developed a combined method, thus both increasing the fidelity of protein identification and obtaining peptide site information, and validated this method by analyzing mouse brain lysates (Fig. 6a). *S*-nitrosated proteins from control and GSNO-treated samples were digested with trypsin and identified directly by LC-MS/MS. Results indicated that significantly more *S*-nitrosated proteins were present after GSNO treatment (Supplementary Table 1, *S*-nitrosated proteins in the sample with GSNO treatment, Supplementary Table 2, *S*-nitrosated proteins in the sample without GSNO treatment). After protein identification, the biotinylated peptides were purified with monomeric avidin and *S*-nitrosation site information was obtained (Supplementary Table 3).

In the first step of pIBP MS (mass spectrometry) experiment, purified S-nitrosated proteins were identified directly by MS with high fidelity (at least 2 distinct peptides to identify a protein in MS) but without site information (Supplemental Table 1). Then in the

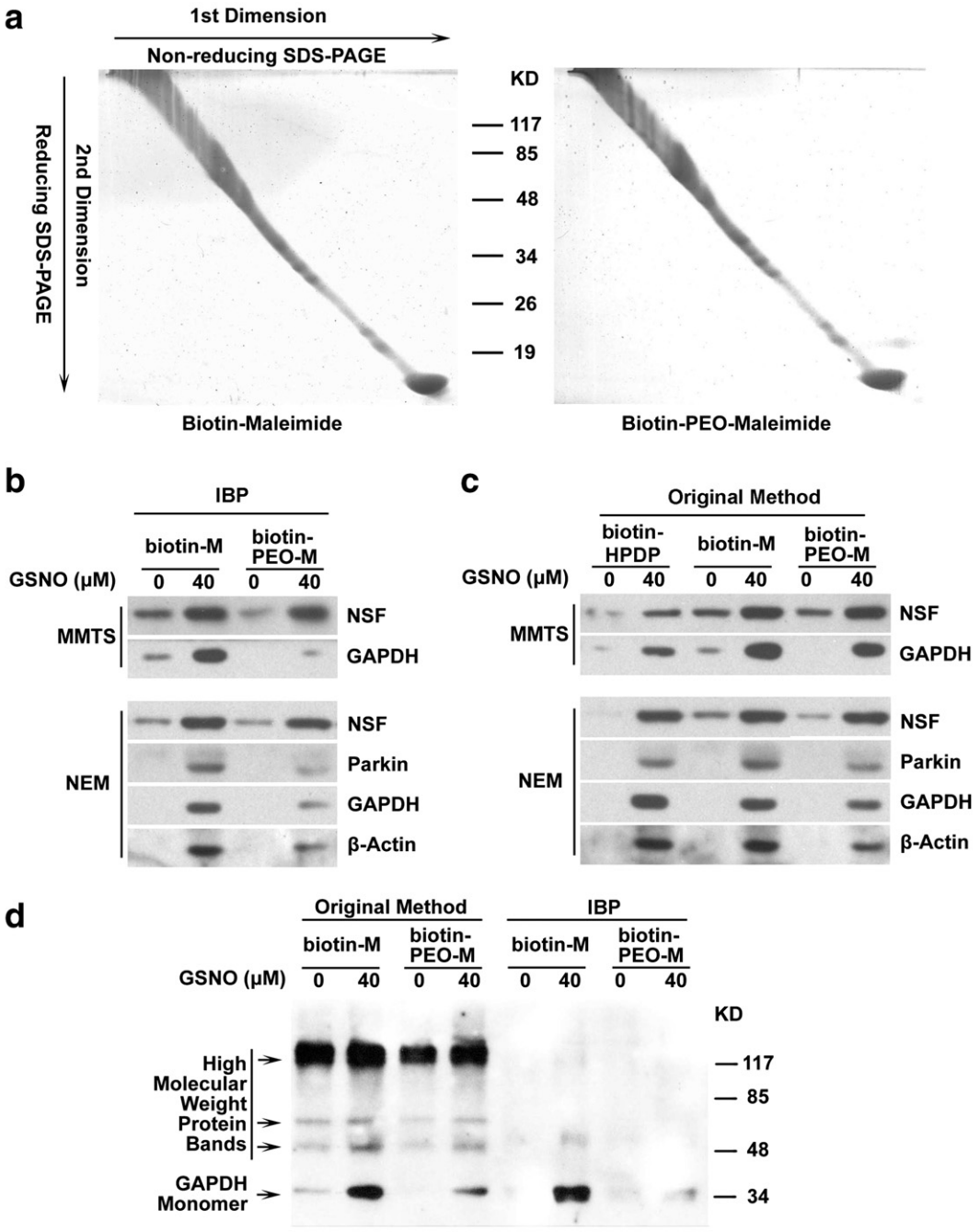

Fig. 4. Validation of the irreversible biotinylation procedure, "IBP". a. Diagonal electrophoresis of protein samples purified from 40 μM GSNO-treated mouse brain lysates using IBP with biotin-maleimide or biotin-PEO-maleimide as the irreversible biotinylation reagent. b. Western blotting of *S*-nitrosated proteins of interest as indicated, *S*-nitrosated proteins were purified after streptavidin-agarose purification from mouse brain lysates treated with or without 40 μM GSNO using IBP followed by reducing SDS-PAGE and Western blotting with interested antibodies. c. The same initial samples and procedures as in b, analyzed by the original biotin switch method followed by reducing SDS-PAGE and Western blotting with interested antibodies. d. Non-reducing SDS-PAGE was used to analyze the presence of intermolecular disulfide bonds after avidin purification combined with IBP or the original biotin switch method. Western blotting analysis of GAPDH purified from mouse brain lysates treated with or without 40 μM GSNO using the original biotin switch assay or IBP. High molecular weight protein bands of GAPDH disappeared after IBP under non-reducing SDS-PAGE, thus successfully avoiding interference by the formation of intermolecular disulfide bonds.

second step of pIBP MS experiment, purified biotinylated peptides were identified (Supplemental Table 3) with low fidelity (only 1 peptide were used for identifying a protein in MS). If a peptide is present in both Supplementary Table 1 and Supplementary Table 3 (marked in bold font), this indicates that the peptide in Supplementary Table 3 belongs to the protein in Supplementary Table 1. If a peptide in Supplementary Table 3 has no corresponding protein in Supplementary Table 1, this peptide is most likely a false positive identification and further experiment is needed to confirm this peptide or protein as a S-nitrosation target.

Quantitative Detection of S-nitrosated Proteins with Quantitative Irreversible Biotinylation Procedures (qIBP)

The pIBP method only provides semi-quantitative information on the difference between two protein samples and is thus not suitable

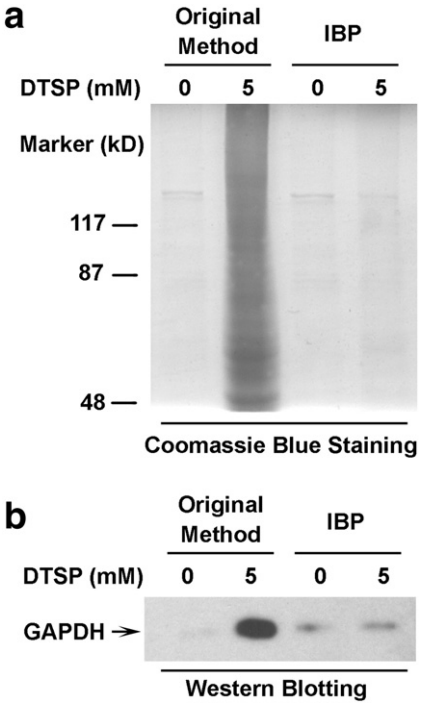

Fig. 5. Analysis of the lysates of mouse brain treated with DTSP *in vitro* with the original biotin switch method and IBP, respectively. a. DTSP was used to induce Pr-S-S-Pr in the lysates of mouse brain *in vitro*, followed by the original biotin switch method or IBP. The purified proteins by streptavidin-agarose were analyzed by reducing SDS-PAGE and stained by coomassie blue. b. The same gel of a was analyzed by GAPDH antibody with Western blotting after reducing SDS-PAGE.

for studying the dynamics of nitrosation/denitrosation. To obtain fully quantitative information, ICAT (isotope-coded affinity tag) irreversible biotinylation reagents were coupled with the peptides for quantitative analysis[4]. Using isotopic tags, a given *S*-nitrosated peptide can easily be distinguished from other peptides which do not have these specific tags but are joined to the *S*-nitrosated peptide by intermolecular disulfide bonds.

ICAT can be used for the quantitative detection of redox sensitive cysteines[4] and for the detection of *S*-nitrosation sites in single proteins. As shown in Fig. 6b, in order to identify the *S*-nitrosation sites here, we used cleavable ICAT reagents to replace biotin-M in the biotinylation step. One sample was labeled with a light ICAT reagent (with nine ^{12}C atoms) and another with a heavy ICAT reagent (with nine ^{13}C atoms), leading to a difference in mass of 9 Daltons. After trypsin digestion of the sample protein mixture, the biotinylated peptides were purified for quantitative analysis. Mouse brain lysates were treated with CysNO and 10 *S*-nitrosated sites were identified by qIBP (Supplementary Table 4). Basically, all positive identifications had heavy/light ratios above 10, indicating that the *S*-nitrosation level of these targets increased significantly after CysNO treatment.

Discussion

In this research, we have described a finding that a significant number of proteins linked to purified *S*-nitrosated proteins via intermolecular disulfide bonds can be detected and possibly wrongly identified as *S*-nitrosated targets. We developed an irreversible biotinylation procedure (IBP) with new strategy to overcome the interference from intermolecular disulfide bonds. Based on IBP, we also developed a proteomic approach (pIBP) and a quantitative approach (qIBP) for high-throughput identification and quantitative study of protein *S*-nitrosation.

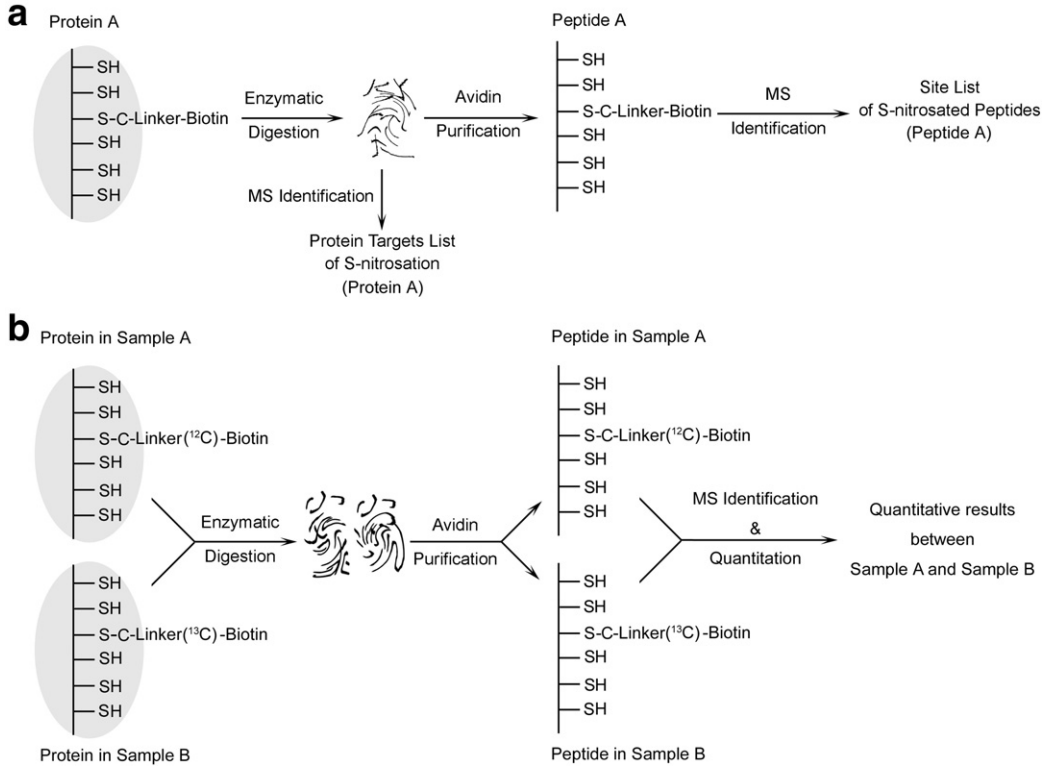

Fig. 6. Design of proteomic IBP (pIBP) and quantitative IBP (qIBP). a. *S*-nitrosated protein samples are biotinylated and purified based on IBP, then enzymatic digested into peptides. Partial peptide sample is used for protein target identification of *S*-nitrosation with MS. The rest part of the peptide sample can be purified with Avidin to get the biotinylated peptides. With MS analysis, the site information of *S*-nitrosation proteins can be obtained. b. One sample was labeled with a light ICAT reagent (with nine ^{12}C atoms) and another with a heavy ICAT reagent (with nine ^{13}C atoms), leading to a difference in mass of 9 Daltons. After trypsin digestion of the sample protein mixture, the biotinylated peptides were purified for MS quantitative analysis.

Table 1
List of proteins linked to S-nitrosated proteins by intermolecular disulfide bonds purified with the original biotin switch method. *S*-Nitrosated proteins in mouse brain lysates treated with 40 μM GSNO were purified by the original biotin switch method and analyzed by diagonal electrophoresis. The proteins below the diagonal line were identified by mass spectrometry. The "Protein" column gives the names of proteins and "Accession" column gives their NCBI Accession numbers. "Table S1" column indicates whether the protein identified here has also been identified as S-nitrosated protein in Supplementary Table 1 (labeled "Yes") or not (labeled "NO"). "Forms" gives the different type of known forms of the "protein" besides monomers: "D" for dimer formation at native condition with or without inter-polypeptide disulfide bonds; "T" for trimer formation with or without covalent disulfide bond, "O" for oligomer formation with or without covalent disulfide bond, "I" for inter-protein disulfide bonds formation (between different proteins); "C" for subunit in native protein complex. "Reference" indicates the reference number in Supplementary data

Protein	Accession	Table S1	Forms	Reference
2',3'-cyclic nucleotide 3' phosphodiesterase	6753476	Yes	D;	[1]
Actin, alpha 2, smooth muscle, aorta	6671507	Yes	D;O	[2–4]
Actin, beta, cytoplasmic	6671509	Yes	D; O	[2–4]
Albumin	163310765	Yes	D	[5]
Aldolase 1, A isoform	6671539	Yes	D	[6,7]
Aldolase 3, C isoform	60687506	Yes	D	[6,7]
ATPase, H+ transporting, lysosomal V1 subunit A	31560731	Yes	C	[8]
Brain glycogen phosphorylase	24418919	Yes	D	[9]
Clathrin, heavy polypeptide (Hc)	51491845	Yes	D	[10]
Creatine kinase, brain	10946574	Yes	D	[4,11]
Enolase 1, alpha non-neuron	158853992	Yes	D	[12,13]
Glutamine synthetase	31982332	Yes	D; O	[14]
Heat shock protein 1, alpha	6754254	Yes	I	[15]
Heat shock protein 1, beta	40556608	Yes	I	[15]
Heat shock protein 8	31981690	Yes	I	[15]
Hemoglobin alpha, adult chain 2	145301549	Yes	D	[16]
Hemoglobin, beta adult major chain	31982300	Yes	D	[16]
Hemoglobin, beta adult minor chain	17647499	Yes	D	[16]
Hypothetical protein	149251837	Yes		
Hypothetical protein	149258064	Yes		
Isocitrate dehydrogenase 3 (NAD+) alpha	18250284	Yes	D	[4,17]
Karyopherin (importin) beta 1	88014720	Yes	I	[13,18,19]
Lactate dehydrogenase A	6754524	Yes	D	[13,20]
Malate dehydrogenase 1, NAD (soluble)	31982178	Yes	D	[4,21]
Malate dehydrogenase 2, NAD (mitochondrial)	31982186	Yes	D	[4,21]
Na+/K+-ATPase alpha 1 subunit	21450277	Yes	C	[8]
Na+/K+-ATPase alpha 3 subunit	21450321	Yes	C	[8]
N-ethylmaleimide sensitive fusion protein	31543349	Yes	T	[22]
Neural cell adhesion molecule 1 isoform 3	164448632	Yes	I	[23]
Neurochondrin	172072590	Yes		
Optic atrophy 1 homolog	19526960	Yes		
Parvalbumin	31980767	Yes	D	[24]
Phosphatidylethanolamine binding protein 1	84794552	Yes	D	[25]
Phosphofructokinase, muscle	31981185	Yes	D	[26]
Phosphoglycerate mutase 1	114326546	Yes	D; O	[27,28]
Protein disulfide isomerase associated 3	112293264	Yes	D,O,I	
Protein kinase C, beta	6679345	Yes	D	[29]
Protein kinase C, gamma	6755080	Yes	D	[29]
Pyruvate kinase, muscle	31981562	Yes	D	[29]
Reticulon 1 isoform RTN1-C	56090141	Yes		[30]
Similar to Cofilin-1	149255823	Yes	O; I	[31]
Similar to GAPDH isoform 4	149265160	Yes	D; O	[4,32]
Similar to Glucose phosphate isomerase 1 isoform 2	94380203	Yes	D	[33]
Similar to valosin isoform 1	94408011	Yes	I	[34]
Similar to Ywhaq protein	149263879	Yes	D	[35]
Solute carrier family 1 (glial high affinity glutamate transporter), member 3	24233554	Yes	D	[36]
Solute carrier family 25 (mitochondrial carrier, Aralar), member 12	27369581	Yes	D	[36]
Solute carrier family 25, member 5	22094075	Yes	D	[36]
Spectrin alpha 2	115496850	Yes	D	[37]
Synapsin II isoform IIb	8567410	Yes	D; I	[38]
Synaptosomal-associated protein 91	7305507	Yes	D; C	[39]
Synaptotagmin I	6678197	Yes	D	[40]
Syntaxin 1B	13259378	Yes	D; I; O; C	[41]
Syntaxin binding protein 1 isoform b	165972305	Yes	D; I; C	[42]
TBP-interacting protein isoform 1	149261084	Yes	C	[43]
Thymus cell antigen 1, theta	6678347	Yes	D; O	[44]
Triosephosphate isomerase 1	6678413	Yes	D	[4,45]
Tubulin, alpha 1B	34740335	Yes	D; O	[13,46]
Tubulin, beta	21746161	Yes	D; O;	[46]
Tubulin, beta 3	12963615	Yes	D; O	[13,46]
Tubulin, beta 4	31981939	Yes	D; O;	[46]
Tubulin, beta 5	7106439	Yes	D; O	[13,46]
Ubiquinol cytochrome c reductase core protein 2	22267442	Yes	D; C	[47]
Ubiquinol-cytochrome c reductase core protein 1	46593021	Yes	D; C	[47]
Vacuolar H+ ATPase B2	19705578	Yes	C	
Voltage-dependent anion channel 1	6755963	Yes	D; O;	[48]
Aconitase 2, mitochondrial	18079339	NO	D	[49]
ADP-ribosylation factor 3	6680718	NO	D	[50]
Alpha isoform of regulatory subunit A, protein phosphatase 2	8394027	NO	D	[51]
ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	31980648	NO	C	[8]
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	31980744	NO	C	[8]
ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	6680748	NO	C	[4,8]
ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	20070412	NO	C	[8]
ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	12025532	NO	C	[8]
ATPase, Na+/K+ transporting, alpha 2 polypeptide	30409956	NO	C	[8]
Calcium/calmodulin-dependent protein kinase II alpha isoform 1	6753250	NO	D	[52]
Calcium/calmodulin-dependent protein kinase II, delta isoform 2	70906477	NO	D	[52]
Calmodulin 1	6753244	NO	D	[53]
Collapsin response mediator protein 1	40068507	NO	D	[54]
Cytochrome c oxidase subunit IV isoform 1	6753498	NO	C	[55]
Dihydropyrimidinase-like 2	40254595	NO	D	[56]
Dynamin	116063570	NO	D	[57]
Dynamin 1-like isoform b	71061455	NO	D	[57]
Dynamin 3	84490431	NO	D	[57]
Enolase 2, gamma neuronal	7305027	NO	D	[58]
Excitatory amino acid transporter 2 isoform 3	7106409	NO	D	[59]
Glucose phosphate isomerase 1	6680067	NO	D	[60]
Glutamate oxaloacetate transaminase 1, soluble	160298209	NO	D	[61]
Glutamate oxaloacetate transaminase 2, mitochondrial	6754036	NO	D	[61]
Glutaminase isoform 2	164607135	NO	D	[62]
Guanine nucleotide binding protein, alpha o isoform B	164607137	NO	I	[63]
Guanosine diphosphate (GDP) dissociation inhibitor 1	33859560	NO	I	[64]
Heat shock protein 2	50345978	NO	I	[15]
Heat shock protein 9	162461907	NO	I	[15]
Hexokinase 1	6754206	NO	D	[65]

The interference of intermolecular disulfide bonds we focused here will only affect the applications combining biotin switch method with avidin purification, not in those only involving the replacement of –SNO with a biotin tag. In fact, this problem is not specific to the pull-down of proteins labeled by biotin switch techniques; the

interference of pulling down proteins with intermolecular disulfide bonds is a common problem for all immuno-precipitation type experiments conducted under non-reducing conditions.

Although non-disulfide-based biotin reagents have been used in the biotin switch method, simply change of reagents could not avoid the interference. The key step to avoid the interference is to break the intermolecular disulfide bonds by reduction before the purification of S-nitrosated proteins as designed in IBP. In our study, we choose non-disulfide-based biotin reagents (biotin-maleimide, biotin-PEO-maleimide, ICAT) by the criterion that they will form thioether (-S-C-) bonds with cysteines, which is not reducible by DTT or 2-ME in the added reduction step in IBP. The "irreversible biotinylation reagents" were chosen to suit the design of IBP, which could exclude the interference of intermolecular disulfide bonds. In fact, if the tag of biotin labeling is not removed during analysis, just as Greco et al did in their proteomic study without loss the biotinylation tag [17], the interference of intermolecular disulfide bonds can be ruled out. However, keeping the intramolecular and intermolecular disulfide bonds in biotinylated proteins will lower the efficiency of trypsin digestion and biotinylated peptides purification, leading to lower signals and fewer number of *S*-nitrosated peptides identified during MS analysis. Proteomic IBP shows advantage in solving this problem.

In fact, specificity of the original biotin switch method has been a concern for some time. The need to rule out the interference of selenocysteine, cysteine sulphate, cysteine sulphinic acid and selenomethionine was borne in mind in the design of the original method. These amino acids were either blocked with MMTS or were unable to react with ascorbate or biotin-HPDP, and thus would not interfere during the biotin switch assay[6]. Interference from cysteine sulphenic acid has also subsequently been excluded[18]. However, not every kind of cysteine-related modification was considered. With respect to the influence of disulfide bonds, intramolecular disulfide bonds and S-thiolation (by cysteine or glutathione) were considered in the original biotin switch method, however, the influence of intermolecular disulfide bonds was not considered.

Results from the analysis of proteins below the diagonal line in Fig. 1a by mass spectrometry (Table 1) showed that nearly 70% of these proteins (66 over 95) were the same as the *S*-nitrosation targets identified with IBP shown in Supplementary Table 1. These results confirmed that intermolecular disulfide bonds and *S*-nitrosation interfere with each other during detection and that proteins prone to form intermolecular disulfide bonds were also prone to *S*-nitrosation, and vice versa. Since intermolecular disulfide bonds commonly exist in proteins and are likely to be regulated by the intracellular redox environment[12,13], interference by intermolecular disulfide bonds has to be considered in protein *S*-nitrosation studies (or studies of other types of cysteine modification). In fact, most of the proteins in Table 1 were found to be able to form dimers, homo-/hetero- oligomers or protein complexes (see the references in Table 1), which will make intermolecular disulfide bonds formation easier during redox regulation.

As shown in Supplementary Fig. 3, when protein A forms homo-oligomers, a single *S*-nitrosated protein A molecule will pull down several non-nitrosated protein A molecules, so that the *S*-nitrosation level detected could be several times greater than the true level. When intermolecular disulfide bonds form with protein B, a single *S*-nitrosated protein A molecule can similarly pull down one (or more) protein B molecules, thus increasing the apparent *S*-nitrosation level of protein B significantly. In this situation, protein B is likely to be identified as a false positive *S*-nitrosation target. If protein B has intermolecular disulfide bonds with different *S*-nitrosated proteins, then protein B will have a higher possibility of being falsely identified as a positive target. When the *S*-nitrosated protein is in a complex, many associated proteins will be identified as false positive *S*-nitrosation targets, especially if the proteins are sensitive to redox modifications. The above mentioned possibilities will increase in models under oxidative stress because non-native disulfide bonds will form[12,13].

The extent of the influence of intermolecular disulfide bonds on results from using the original biotin switch method in previous reports needs to be evaluated case by case in the light of our findings. This is a question that should be addressed in the field of redox signaling. Although the reversible properties of different cysteine modifications may lead to the same regulatory effects, it is still important to distinguish which signaling mechanisms result from which redox modifications. However, our present study indicates that the original biotin switch assay followed by avidin purification actually does not distinguish *S*-nitrosation successfully from intermolecular disulfide bonds. This raises the possibility that specific cellular processes previously shown to be related to *S*-nitrosation can only be said to be redox-related cysteine modifications. IBP and IBP-based proteomic approaches provide specific methods for *S*-nitrosation detection. The strategy in IBP is also a good reference point for developing methods to eliminate the interference of intermolecular disulfide bonds from other cysteine modification detection systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2010.05.001.

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