Mass spectrometry-based analyses for identifying and characterizing *S*-nitrosylation of protein tyrosine phosphatases

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Abstract

All members in the protein tyrosine phosphatase (PTP) family of enzymes contain an invariant Cys residue which is absolutely indispensable for catalysis. Due to the unique microenvironment surrounding the active center of PTPs, this Cys residue exhibits an unusually low pK_a characteristic, thus being highly susceptible to oxidation or *S*-nitrosylation. While oxidation-dependent regulation of PTP activity has been extensively examined, the molecular details and biological consequences of PTP *S*-nitrosylation remain unexplored. We hypothesized that the catalytic Cys residue is targeted by proximal nitric oxide (NO) and its derivatives collectively termed reactive nitrogen species (RNS), leading to nitrosothiol formation concomitant with reversible inactivation of PTPs. To test this hypothesis, we have developed novel strategies to examine the redox status of Cys residues of purified PTP1B that was exposed to NO donor *S*-Nitroso-*N*-penicillamine (SNAP). A gel-based method in conjunction with mass spectrometry (MS) analysis revealed that the catalytic Cys215 of PTP1B was reversibly modified when PTP1B was briefly treated with SNAP. In order to further identify the exact mode of NO-induced modification, we employed an online LC–ESI-MS/MS analysis incorporating a mass difference-based, data-dependent acquisition function that effectively mapped the *S*-nitrosylated Cys residues. Our results demonstrated that treating PTP1B with SNAP led to *S*-nitrosothiol formation of the catalytic Cys215. Interestingly, SNAP-induced modifications were strictly reversible as highly oxidized Cys derivatives (Cys–SO₂H or Cys–SO₃H) were not identified by MS analyses. Thus, the methods introduced in this study provide direct evidence to prove the direct link between *S*-nitrosylation of the catalytic Cys residue and reversible inactivation of PTPs.

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1. Introduction

The superfamily of protein tyrosine phosphatases (PTPs) consists of a large number of enzymes, including classical, phosphotyrosine-specific phosphatases and dual specificity phosphatases (DUSPs) that recognize phosphorylated serine/threonine as well as phosphotyrosine residues in protein substrates. The catalytic activity of PTPs relies completely on the invariant Cys residue located within

the conserved signature motif [I/V]HCxxGxxE[S/T] that defines the PTP superfamily [1,2]. Due to the unique environment surrounding the active site, the catalytic Cys residue displays an unusually low pK_a (between 4.5 and 5.5, compared to pK_a of a typical Cys being ~8.5), allowing its presence as the thiolate anion form at the neutral pH [3,4]. Biochemical analysis has clearly revealed that the nucleophilic attack on the phosphate group of substrates is the first step of reaction catalyzed by PTPs [5]. Therefore, the thiolate anion form of catalytic Cys residue is crucial for enhancing PTP-mediated phosphotyrosyl dephosphorylation of substrates. Interestingly, the low pK_a character also renders the catalytic Cys of PTPs highly susceptible to redox-dependent regulation [6].

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Data accumulated over the last decade have demonstrated that the catalytic Cys residue of cellular PTPs can be easily oxidized by proximal reactive oxygen species (ROS), which are produced by endogenous NADPH oxidases in response to various physiological and pathophysiological stimuli [7]. Interestingly, oxidation of the catalytic Cys residue leads to loss of its nucleophilic characteristic, concomitant with enzymatic inactivation of PTPs. Indeed, numerous reports have shown that the oxidation-mediated inhibition of PTPs plays an essential role in the activation of tyrosine phosphorylation-dependent signaling [8–11]. Biochemical evidence suggests that, upon an increased concentration of environmental ROS, the catalytic Cys is initially converted to the sulfenic acid derivative (Cys–SOH) or the cyclized sulfenyl-amide species [12,13]. It is generally proposed that oxidized PTPs in either sulfenic acid or sulfenyl-amide form are reversible. The endogenous thiol reductants, such as glutathione, can easily react with those oxidized Cys residues to form disulfide intermediates which then would be reduced by enzyme-mediated reactions to complete reactivation of PTPs [12]. In addition to the reversible mode of modification, high concentrations of environmental ROS can promote further oxidation of the catalytic Cys, resulting in the formation of the sulfinic acid (Cys–SO₂H) or the sulfonic acid (Cys–SO₃H) derivative [12–15]. These modified PTPs are permanently inactivated as thiol reductants are unable to reduce the highly oxidized Cys residues.

In addition to ROS, the free radical nitric oxide (NO), which is produced by the nitric oxide synthase (NOS) family of enzymes, and various derivatives of NO collectively termed reactive nitrogen species (RNS), may both function as second messengers that control a broad array of signaling pathways [16]. Although the soluble guanylate cyclase which produces cGMP while its heme-iron group coupled with NO has been viewed as the primary endogenous NO receptor [17], recent investigations suggested that RNS may regulate cell signaling through cGMP-independent mechanisms. Interestingly, among those NO-mediated actions described so far, the *S*-nitrosylation of Cys residue has been regarded as an important post-translational modification that influences various cellular functions [18,19]. It has been proposed that the activity of a number of key enzymes which plays an essential role in the control of cell signaling cascades is regulated by *S*-nitrosylation and denitrosylation. According to the unique low pK_a characteristic of the catalytic Cys residue, it is reasonable to hypothesize that endogenous PTPs are potentially targeted by cellular RNS. Indeed, the enzymatic activity of PTP1B, PTEN, and SHP-2 was suppressed in cells exposed to NO donors, suggesting that the catalytic Cys of these PTPs is susceptible to *S*-nitrosylation [20,21]. Nevertheless, direct evidence for such a RNS-induced post-translational modification of PTPs is not available. Thus, in the current study, we sought to develop novel methods that allow direct measurement of Cys modifications of PTPs in response to stimulation with NO donors. Two strategies were employed in

order to reveal the molecular basis that contributes to NO-mediated inactivation of PTPs.

2. Application of a gel-based method that reveals NO donor-induced reversible inactivation of PTP1B

2.1. Principle

To date, biochemical evidence was not sufficient enough to reveal the underlying mechanism that leads to the inactivation of PTPs in response to NO stimulation. Furthermore, it was not known whether NO induces only the reversible inhibition of PTPs or it can also promote the permanent inactivation of PTPs. To explore the effect of NO on redox regulation of PTP activity, the purified 37 kDa form of PTP1B was reacted with a NO donor *S*-nitroso-*N*-acetylpencillamine (SNAP). A gel-based assay was developed in conjunction with MS analysis to monitor the extent of *in vitro* modifications.

2.2. Procedures

2.2.1. Preparation of *S*-nitrosylated and oxidized PTP1B

The wild type, C-terminally truncated, 37 kDa human PTP1B was purified to homogeneity as described previously [22]. PTP1B was dissolved in the degassed buffer (20 mM Hepes (pH 7.5), 1% NP-40, 150 mM NaCl, 10% Glycerol) at the concentration of 0.7 μM, followed by treatment with various concentration of H₂O₂ (RideldelHaen) for 10 min at 37 °C. For the treatments with *S*-Nitroso-*N*-penicillamine (SNAP, CALBIOCHEM) the reaction time were extended to 20 min at 37 °C. For experiments with SNAP treatment, the catalase and superoxide dismutase (both 100 μg/ml, Calbiochem) were added in order to remove any trace amount of hydrogen peroxide and superoxide radical that might appear in the reaction buffer.

2.2.2. Reaction with iodoacetic acid and iodoacetamide

The SNAP or H₂O₂-reacted PTP1B was subsequently incubated with 50 mM iodoacetic acid (IAA) or iodoacetamide (IAM) for 15 min at 37 °C. This step blocked all unmodified thiol groups, thus preventing further Cys oxidation or nitrosylation. The processed PTP1B was mixed with 2× gel loading buffer containing 100 mM β-mercapto-methanol, boiled for 3 min and then subjected to SDS-PAGE using a 10% SDS-gel. An aliquot of IAA-reacted sample was saved for the in-gel phosphatase activity assay.

2.2.3. In-gel digestion with trypsin and MALDI-MS analysis

The protein bands of interest, which were visualized by Coomassie blue staining of the SDS-gel, was manually excised from the gel and repeatedly destained in 50% acetonitrile until the gel slice became colorless. The gel pieces were dehydrated with acetonitrile for 10 min. In-gel digestion was performed by adding 0.1 μg sequencing-grade trypsin (Promega) in 10 μl of 25 mM ammonium

bicarbonate buffer, followed by incubation at 37 °C for more than 16 h with constant vortexing. The digested peptides were then recovered from the gel by sequential extractions with acetonitrile. All extracts were pooled and dried for MALDI-MS and MS/MS analyses.

2.2.4. In-gel phosphatase activity assay

The in-gel phosphatase activity assay was performed according to the method originally described by Burridge and Nelson [23] and then modified by Meng and Tonks [24]. Briefly, samples were applied to a 10% SDS-gel cast containing ^{32}P -labeled poly (Glu:Tyr) $_{4:1}$ as a substrate (1.5×10^6 cpm/20 ml of gel solution, approximately 2 μM pTyr). Following electrophoresis, the gel was placed sequentially in buffers for fixing, denaturation and renaturation. During the last wash with renaturation buffer for 1 h, 3 mM DTT was included for activating PTP1B, thus leading to the dephosphorylation of substrates immediately surrounding the area of phosphatase in gel. The reaction was terminated by staining solution containing Coomassie blue. After destaining, the gel was dried and then exposed to X-ray film.

2.3. Results

As shown in Figs. 1 and 2, the C-terminally truncated recombinant PTP1B ran normally as a 37 kDa protein band but would shift to a slightly higher apparent molecular weight band if first alkylated with either iodoacetic acid or iodoacetamide. MALDI-MS mapping of the tryptic peptides extracted from in-gel digests of the excised protein

bands revealed that all Cys residues, including the catalytic Cys215, were carboxymethylated or carbamidomethylated respectively (data not shown). However, if prior to alkylation, the purified protein was first treated with H_2O_2 which acted as a representative of ROS, or SNAP as an NO donor, additional bands would appear.

In the case of carboxymethylated PTP1B (Fig. 1a), a lower band became more intense with increasing concentration of applied H_2O_2 . MALDI-MS analysis of the tryptic peptides from this lower band revealed that its catalytic Cys215 retained its free thiol form whereas other Cys residues were carboxymethylated as expected. Indirectly, the data indicates that an increasing fraction of PTP1B was reversibly oxidized. Such modification of the catalytic Cys215 prevented it from being carboxymethylated but would allow it to be reduced back to Cys-SH when treated with β -mercaptoethanol prior to running into the reducing SDS-PAGE. The reversion of the catalytic Cys215 to free thiol form not only resolved this PTP1B variant from the fully carboxymethylated form on gel but also restored its activity, as shown by accompanying in-gel phosphatase activity assay. In contrast, MALDI-MS analysis of the upper band identified that tryptic peptide carried not only carboxymethylated Cys215 but also highly oxidized Cys215 (Cys-SO $_2$ H and Cys-SO $_3$ H). Thus, a significant fraction of irreversibly oxidized PTP1B was also produced as expected which coincidentally ran at the same position as the non-oxidized PTP1B with carboxymethylated Cys215.

In the case of carbamidomethylated PTP1B (Fig. 1b), the irreversibly oxidized PTP1B variants were resolved

Fig. 1. Detection of H_2O_2 -based modifications of recombinant PTP1B in vitro by alkylating reagent labeling method. Recombinant PTP1B was treated with various concentrations of H_2O_2 for 10 min. Following the addition of catalase, samples were incubated with 50 mM iodoacetic acid (IAA) or iodoacetamide (IAM) for 15 min. An aliquot of 2.5 μg PTP1B per lane was subjected to reducing SDS-PAGE (a, upper panel) or in-gel phosphatase activity assay (a, lower panel). As indicated by number 1–4 on the gel image (a-1), PTP1B sample reacted with 10 μM H_2O_2 was resolved into 39 and 37 kDa isoforms. Each band was subjected to in-gel digestion for MALDI-MS mapping of tryptic peptide. The catalytic Cys215 susceptible to oxidation is carried on tryptic peptide T28, giving the $[\text{M}+\text{H}]^+$ signals at m/z 2175, 2207, 2223, 2232, and 2233 (accurate monoisotopic mass), corresponding respectively to the reduced (–SH), sulfinic acid (–SO $_2$ H), sulfonic acid (–SO $_3$ H), carbamidomethylated (–CAM), and carboxymethylated (–CM) forms, as annotated in (c).

Fig. 2. Reversibly inactivated form of PTP1B was generated in response to treatment with NO donor. Recombinant PTP1B was incubated with various concentrations of SNAP prior to treatment with 50 mM iodoacetic acid (IAA) or iodoacetamide (IAM) labeling. An aliquot of sample was subjected to reducing SDS-PAGE (a, upper panel) or in-gel phosphatase activity assay (a, lower panel). *S*-nitrosylation reaction can proceed completely as a dose-dependent manner. Modified in-gel PTP activity assay of *S*-nitrosylated PTP1B was shown (a, lower panel). Interesting bands as indicated by number 5–8 on the gel image (a and b) were subjected to in-gel digestion and analyzed by MALDI-MS analysis. The peak annotation details have described in the legend of Fig. 1.

from the non-oxidized and reversibly oxidized forms as an additional upper band. The gradual increase in intensity of this upper band with increasing concentration of applied H_2O_2 indicates that more and more of the PTP1B was irreversibly oxidized. Thus, both iodoacetic acid and iodoacetamide-based gel assays corroboratively demonstrated that H_2O_2 at $1\text{--}10^3\text{ }\mu\text{M}$ would incompletely oxidize PTP1B in a dose-dependent manner. Importantly, MALDI-MS analysis confirmed that even at $10\text{ }\mu\text{M}$ H_2O_2 , the catalytic Cys215 could be irreversibly oxidized. On a reducing SDS-PAGE, the irreversibly oxidized PTP1B forms exhibited a retarded electrophoretic mobility compared to the reversibly oxidized form which carried a free thiol on the catalytic Cys215. Interestingly, non-oxidized PTP1B with the catalytic Cys215 alkylated with iodoacetic acid or iodoacetamide would co-migrate, respectively, with the irreversibly and reversibly oxidized forms. The complementary use of both gel assays is therefore advantageous in visualizing either of the two oxidized forms.

In contrast to H_2O_2 , it is clear that SNAP induced only a reversible modification on PTP1B (Fig. 2). With the iodoacetic acid-based gel assay (Fig. 2a), treatment with increasing SNAP concentration led to a gradual disappearance of the original protein band concomitant with the appearance and gaining in intensity of a lower band that corresponded to PTP1B with a free thiol on the catalytic Cys215. Thus, inactive PTP1B with carboxymethylated Cys215 was gradually replaced by an active PTP1B form, as supported by the in-gel phosphatase activity assay. With the IAM-based gel assay (Fig. 2b), no additional band was observed. MALDI-MS analysis of the resulting tryptic

peptides nevertheless showed that the catalytic Cys215 existed predominantly in its free thiol form instead of the carbamidomethylated form when SNAP was added. Data from both gel-based assays are therefore consistent with a rather efficient NO-mediated *S*-nitrosylation of the catalytic Cys215 of PTP1B which could be fully reversed by reducing agents such as β -mercaptoethanol. The application of $10^3\text{ }\mu\text{M}$ SNAP, which is equivalent to the production of $\sim 1\text{ }\mu\text{M}$ free NO radical in our measurement (data not shown) and consistent with the previous report [25], was sufficient to completely convert the entire population of PTP1B proteins to the reversibly inactivated form. This is to be contrasted against H_2O_2 -mediated oxidation (Fig. 1), which is not only often incomplete even at an equivalent high dose, but also not fully reversible.

3. Identification of *S*-nitrosylated cysteine residues of PTP1B by mass spectrometry

3.1. Principle

Although MALDI-MS analysis of the tryptic peptides from in-gel digests of the protein bands corresponding to NO-treated PTP1B is indicative of a reversible modification on the catalytic Cys215, it provides no direct evidence for *S*-nitrosylation. To prevent the conversion of an SNO back to SH through the reducing conditions employed for SDS-PAGE, NO-treated PTP1B was directly digested in solution for MS mapping. Based on the unique feature of *S*-nitrosylated peptides in which the characteristic loss of the NO moiety (30 mass units) often occurs under

ESI-MS conditions [26], a mass difference scanning function of the mass spectrometer was employed, as outlined in Scheme 1.

3.2. Procedures

3.2.1. Preparation of *S*-nitrosylated PTP1B for solution-based trypsin digestion

The purified, C-terminally truncated, 37 kDa human PTP1B (0.7 μ M) in 20 mM ammonium bicarbonate buffer (pH 7.5) was incubated with 1 mM of SNAP as an NO donor at 37 °C for 20 min. The *S*-nitrosylated PTP1B was subsequently digested with sequencing-grade trypsin (1:10 protease to protein ratio,) at 37 °C for 3 h.

3.2.2. Application of ESI-MS analysis in conjunction with mass difference scanning function for identifying *S*-nitrosylated Cys residues of PTP1B

For online nanoLC–nanoESI-MS/MS analysis, the peptides in 0.1% formic acid were first loaded at a flow rate of 9 μ l/min onto a 15 mm precolumn (375 μ m o.d./150 μ m i.d.) packed with 5 μ m C18 particles (Nucleosil 120-5 C18, Macherey-Nagel, GmbH & Co. KG). The retained peptides were then eluted and separated on an analytical C18 capillary column (25 cm $\times$ 375 μ m o.d./75 μ m i.d.) packed with the same material, using a 30-min linear gradient from 10 to 80% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min. To facilitate specific detection and automated MS/MS data acquisition of *S*-nitrosylated peptides, a built-in mass difference scanning function of the QSTAR-XL hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) was employed. MS survey data were first acquired in information-dependent acquisition mode, scanning from m/z 400 to 1900. MS to MS/MS switch was set to be dependent upon detecting a mass difference of 29.998 amu

(referred as 30 amu else where in the text) between any two signals, with a narrow mass tolerance 0.03 amu. Up to 2 MS/MS spectra in 6 s would be acquired for any of the implicated signals with ion intensity above 30 counts in a single scan.

3.3. Results

The SNAP-reacted PTP1B was digested with trypsin, and the peptide mixture was then subjected to LC–MS survey scan. The MS/MS analysis was performed upon detection of signal pairs differing in mass by 30 amu during automatic MS scan. Based on the data-dependent MS/MS acquisition, an edited subchromatogram, instead of an overall total ion current chromatogram, was generated. It is important to emphasize that peaks shown in chromatogram were registered only at time points when MS/MS functions were triggered. As shown in Fig. 3a, 5 peptide pairs with 30 mass units difference were highlighted in the subchromatogram. Among which, 4 peptides, designated as T4, T28, T3–4, and T15, contained a Cys residue that was presumably *S*-nitrosylated in response to SNAP stimulation (Fig. 3a). As a representative example, the MS survey scan at a given time point (15.5 min) during LC by which the *S*-nitrosylated peptide T4 was identified is shown in Fig. 3b. The inset highlights the mass regions where the signal pairs of interests were detected (Fig. 3b). In this case, the *S*-nitrosylated T4 peptide (m/z 545.7) and its accompanying thiol radical-carrying peptide (m/z 530.7, the denitrosylated form) were detected as doubly charged forms which were used specifically for subsequent MS/MS analysis. As shown in Fig. 3c, the MS/MS spectrum of the precursor ion at m/z 545.7 identified that Cys32 (T4) of PTP1B was indeed *S*-nitrosylated. Furthermore, the T28 peptide (m/z 735.4) and T15 peptide (m/z 965.1), both of which were detected as triply charged

Scheme 1. The workflow of mass difference scanning function.

Fig. 3. Mass difference-based LC-ESI-MS/MS analysis identified *S*-nitrosylated sites of PTP1B. The experiment is run as an information-dependent acquisition using the mass difference scanning function in the Analyst software. Mass Difference scanning was used to look for pairs of ions in the TOF MS survey scan separated by specific mass differences and this pattern was used as switch criteria for MS/MS. The selected ion chromatograms during the 14–30 min LC-MS/MS run were shown in (a). As an example, the MS survey scan at a given time (15.5 min) during the experiment in which the *S*-nitrosylated peptide T4 was eluted and identified (shown in the inset) represents a magnification of the mass regions where the signal pairs of interests were detected (b). Representative MS/MS spectra for the three *S*-nitrosylated peptides carrying Cys32, Cys215, and Cys92 are shown in (c–e) which allows an unambiguous sequence identification for the respective peptides.

forms, were subjected to MS/MS analyses. Results shown in Fig. 3d and e indicate that Cys215 and Cys92, respectively, were *S*-nitrosylated. An additional pair of signals labeled as T39 (*m/z* 785.4/770.4) in Fig. 3a was assigned as non-Cys containing tryptic peptides of PTP1B. Although the MS/MS spectrum was unable to localize the NO moiety (data not shown), it remains possible that the Trp291 residue in peptide T39 may be targeted by NO, as suggested by a previous report [27,28].

4. Concluding remarks

Employing a gel-based method in conjunction with MS analysis, we showed that modification of the catalytic Cys215 occurred concomitantly with NO donor-induced inactivation of PTP1B. Importantly, under the experimental conditions used in this study, our data demonstrated that the modification of Cys215 of PTP1B was strictly reversible in response to stimulation with SNAP, whereas treating PTP1B with H₂O₂ effectively induced both

reversible and irreversible modifications on Cys215. Furthermore, we developed a direct measurement based on the ESI-MS analysis that ultimately identified the Cys nitrosothiol formation of PTP1B exposed to SNAP. Thus, the underlying mechanism contributing to NO donor-induced reversible inactivation of PTP1B could be assigned to direct *S*-nitrosylation of the catalytic Cys215 residue. Interestingly, in addition to Cys215, the ESI-MS analysis identified that Cys32 and Cys92 of PTP1B were also susceptible to *S*-nitrosylation. The structural study has revealed that both Cys32 and Cys92 are located on the surface-exposed positions [12], suggesting an easy access for NO donors to react with these residues. Further investigations are needed to determine the preferential susceptibility of Cys32, Cys92, and Cys215 to *S*-nitrosylation.

In conclusion, we have introduced novel strategies for revealing the *S*-nitrosylated form of PTP1B. Although data provided by the current study were based on *in vitro* experiments, similar approaches can be applied to examine the redox status of endogenous PTPs in response to stimuli

that induce NO production in cells. For example, once immunoprecipitated by specific antibodies from cell lysates, a particular species of PTP can be eluted for in-solution tryptic digestion, followed by determination of *S*-nitrosylated Cys residues through online nanoLC–nano-ESI-MS/MS analysis. These studies will demonstrate the exact post-translational modification of endogenous PTPs that may account for cellular NO effects on regulation of important signaling pathways.

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