Characterization of Novel Oxidation Products of Cysteine in an Active Site Motif Peptide of PTP1B

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We investigated the formation of hydroxyl radical ($\text{OH}^\cdot$) and H_2O_2 mediated oxidation products of a synthetic peptide, HCSAGIGRS, which is an active site sequence motif of protein tyrosine phosphatase 1B (PTP1B). We determined that a novel cysteine sulfinamide $\text{HC}[\text{S}(\text{O})\text{N}]\text{SAGIGRS}$ is produced in the oxidation reaction by Fenton reagents ($\text{Fe}^{+2}/\text{H}_2\text{O}_2$) as well as by H_2O_2 . These products were characterized by tandem mass spectrometry experiments on both singly and doubly charged precursor ions. MS^3 experiments using an ion trap instrument as well as LC-MS/MS experiments using a quadrupole time-of-flight (Q-TOF) instrument demonstrated that $\text{HC}[\text{S}(\text{O})\text{N}]\text{SAGIGRS}$ is not a water loss product of cysteine sulfinic acid $[\text{HC}(\text{SO}_2\text{H})\text{SAGIGRS}]$. We also obtained data from tandem mass spectrometry experiments that provided evidence for the existence of stable cysteine sulfenic acid $[\text{HC}(\text{SOH})\text{SAGIGRS}]$ in solution. A mechanism for the formation of the cysteine sulfinamide product is proposed based on the above experimental results. The preparation and identification of cysteine sulfinamide in this study may provide insight into the mechanism of both $\text{OH}^\cdot$ and H_2O_2 induced oxidation reactions of protein tyrosine phosphatases. (J Am Soc Mass Spectrom 2009, 20, 1540–1548) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Protein tyrosine phosphatases (PTPs) comprise an important class of enzymes involved in control of the cell cycle and signal transduction by regulating levels of protein tyrosine phosphorylation in response to cellular signals [1–5]. Because of this key function, PTPs have been implicated in the development of cancer, diabetes, rheumatoid arthritis, and hypertension [6, 7]. Cysteine residues in PTPs are highly susceptible to oxidation by reactive oxygen species (ROS)/reactive nitrogen species (RNS), because at neutral pH they exist in thiolate anion (Cys-S^-) form due to their low pK_a (4.7–5.4) [8]. This property directly influences and inhibits PTP activity during signaling processes. Much biochemical evidence suggests that PTP's activity is regulated through reversible oxidation of the catalytic cysteine to cysteine sulfenic acid intermediate by low micromolar concentrations of H_2O_2 [9]. These active sites can be reactivated by thioredoxin electron donors [10] and reduction with glutathione [11]. PTP1B has been studied extensively as it is an important enzyme in the negative regulation of the insulin receptor [12, 13], and a therapeutic target in type II diabetes and obesity [14]. In light of the critical biologic role of PTP1B and its regulation by cysteine

oxidation, Salmeen et al. used X-ray crystallography and mass spectrometry to discover a novel “cysteine sulfenamide” species in the oxidation of PTP1B by H_2O_2 . This species was identified as a reversible intermediate in the oxidative inhibition of PTP1B, which may allow reactivation of PTP1B by biological thiols [15].

Hydroxyl radicals ($\text{OH}^\cdot$) are extremely reactive and can cause irreversible damage to all cellular components [16–18]. They can be produced by the reduction of H_2O_2 in the presence of iron via the “Fenton reaction” [19]. Although this reaction is a major source for $\text{OH}^\cdot$ formation in cells, these radicals often can be produced from other cellular oxidants like ONOOH in their homolytic cleavage reactions [20–22]. Indeed, most of the amino acid residues of proteins are potential targets for oxidation by $\text{OH}^\cdot$ generated by high concentrations of H_2O_2 and $\text{Fe}(\text{II})$ or by ionizing radiation [23–31]. Increasing evidence suggests that $\text{OH}^\cdot$ are also involved in regulating signaling processes of wide variety of proteins including PTPs [32, 33]. Corroborating the role of $\text{OH}^\cdot$ in the control of PTPs, Caselli et al. [34] examined the inactivation mechanism of low molecular weight PTPs by H_2O_2 in vitro and found that addition of metal chelator to the reaction mixtures caused 17% and 28% decrease in the inactivation of IF1 and IF2, respectively. These investigators demonstrated that $\text{OH}^\cdot$ partially contributes to this inactivation. In light of this, we have studied the structures of $\text{OH}^\cdot$ and H_2O_2

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mediated oxidation products of PTP1B *in vitro*. To minimize the complexity of diverse oxidation products, we have selected a short synthetic peptide, HCSAGIGRS, which mimics the active site sequence motif of PTP1B. Here we report the preparation and characterization by tandem mass spectrometry of cyclic cysteine sulfinamide and cysteine sulfenic acid formed in solution by reactive oxygen species ($\text{OH}^\cdot$ and H_2O_2) mediated oxidation reactions of HCSAGIGRS. These novel oxidative species of the catalytic cysteine of PTP1B have been proposed as intermediate steps towards irreversible inactivation of PTP1B [35], but to the best of our knowledge never before directly observed in solution.

Experimental

The synthetic peptide HCSAGIGRS was purchased from GenScript Corp., Scotch Plains, NJ, USA. This peptide was oxidized using Fenton reagents ($\text{Fe}^{+2}/\text{H}_2\text{O}_2$) as reported previously [36]. Briefly, 1 mM peptide solution was incubated at room temperature in the presence of 0.5 mM FeSO_4 and 0.2 mM H_2O_2 for ~30 min. The reaction was terminated by adding aliquots of methionine solution until its concentration reached 1 mM. Similar conditions were used for the oxidation of HCSAGIGRS by H_2O_2 except that the concentration of H_2O_2 was 100 μM . In direct infusion MS and MS/MS experiments on a Q TOF Micro instrument (Micromass, Manchester, United Kingdom), 50% acetonitrile in 0.1% formic acid was used as the carrier and electrospray solvent with a flow rate of 500 nL/min. A 1- μL aliquot of 2- μM sample solution was injected via a divert valve in each run under the following source conditions: spray voltage 1500 to 1800 V, cone voltage 40 V, and source temperature 80 $^\circ\text{C}$. Argon was used as a collision gas for all MS/MS experiments. The low mass and high mass resolution of the quadrupole mass filter before the TOF analyzer were adjusted for transmission of precursor ions through a mass window of only 1.0 Da. Each spectrum was obtained by averaging ~25 scans, and the scan time was 1 s/scan. The Q-TOF Micro instrument was calibrated with [Glu1]-fibrinopeptide B, and the data in both MS and MS/MS modes were acquired at 5000 resolution. Additional direct infusion tandem mass spectrometry (MS^2 and MS^3) experiments were carried out on an LCQ-Deca XP (Thermo Finnigan, San Jose, CA, USA) equipped with a nano-ESI source (Jamie Hill Instrument Services, Arlington, MA, USA). The carrier solution was 50% acetonitrile in 0.1% formic acid with a flow rate of 300–500 nL/min. The oxidation reaction mixture was diluted 5-fold in carrier solution, and infused directly into the mass spectrometer with spray voltage of 2.5 kV and temperature for the heated capillary of 150 $^\circ\text{C}$. The mass width for precursor ion selection in MS/MS was 1 Da, and the normalized collision energy was optimized (18%–25%) so that the precursor ions were clearly present but not the most abundant ions in the product-ion spectra. Each spec-

trum was obtained by averaging ~50 scans, and the time for each scan was 0.1 s.

Data-dependent nanoLC-MS/MS experiments were carried out using a Waters nanoAcquity UPLC and a Waters Q-TOF Premier mass spectrometer (Milford, MA, USA). The analytical column was a Waters Symmetry C18, 100 μm i.d. $\times$ 15 cm, with 3.5 μm particles. Mobile phase A was 2% acetonitrile/0.1% formic acid, while mobile phase B was 90% acetonitrile/0.1% formic acid in water. The gradient was 5%–90% B over 45 min at a flow rate of 200 nL/min. Automatic switching between MS and MS/MS modes was controlled by MassLynx 4.0 (Micromass), dependent on both signal intensity and charge states from MS to MS/MS, and on either signal intensity or time from MS/MS to MS.

Results and Discussion

The peptide HCSAGIGRS was incubated with Fenton reagent to form cysteine oxidation products as described in the Experimental section, and infused directly into the Q-TOF Micro instrument for mass spectrometry analysis. The partial mass spectrum of the above reaction mixture is shown in Figure 1a. This spectrum exhibits singly charged ions at m/z 901.4, 903.4, 919.4 and 935.4, which corresponds to the +14, 16, 32, and 48 Da modifications of HCSAGIGRS (m/z 887.4), respectively. The latter three products at m/z 903.4, 919.4, and 935.4 were characterized as cysteine sulfenic acid (Figure 1b), cysteine sulfinic acid (Figure 1c), and cysteine sulfonic acid (Figure 1d) products based on the characteristic fragment ions formed in their MS/MS spectra. We observed similar MS/MS spectra for the doubly charged ions of the oxidation products (data not shown).

Characterization of Cysteine Sulfinamide (HC[S(O)N]SAGIGRS)

The MS/MS spectra of singly (m/z 901.3) and doubly (m/z 451.2) charged ions of a product formed in the oxidation reaction of HCSAGIGRS by Fenton reagents are shown in Figure 2b and Figure 3b, respectively. Compared with the MS/MS spectrum of unmodified peptide HCSAGIGRS in Figure 2a, a mass shift of 14 Da can be seen in the MS/MS spectrum in Figure 2b for several b ions [b_4 (m/z 413.1), b_5 (m/z 470.1), b_6 (m/z 583.2), $b_8 + \text{H}_2\text{O}$ (m/z 814.3)] as well as for three a ions, [a_4 (m/z 385), a_5 (m/z 442.2), and a_6 (m/z 555.2)]. The formation of a y_6 (m/z 560.3) ion rules out modification of the AGIGRS fragment. The characteristic ion at m/z 853 due to direct loss of a neutral SO from $\text{M} + \text{H}^+$ ions of m/z 901 and an ion at m/z 207 ($b_2\text{-SO}$) localize the site of modification to the cysteine residue. The MS/MS spectrum of the doubly charged ion (m/z 451.2) in Figure 3b displayed an additional characteristic y_8 ion at m/z 764, along with y_3 (m/z 319), y_5 (m/z 489.2), y_6 (m/z 560.3), and $b_2\text{-SO}$ (m/z 207.1) ions, revealing that cys-

Figure 1. (a) Q-TOF partial mass spectrum of $(M + H)^+$ ions of products formed in the oxidation reaction of HCSAGIGRS peptide with Fenton reagent. Q-TOF MS/MS spectra of $(M + H)^+$ ions (b) HC(SOH)SAGIGRS (m/z 903.4); (c) HC(SO₂H)SAGIGRS (m/z 919.4); and (d) HC(SO₃H)SAGIGRS (m/z 935.4).

teine is modified by 14 Da. Thus, the above results suggest that an intramolecular covalent bond is formed between the oxidized cysteine residue and the nitrogen of serine, giving rise to a cysteine sulfinamide product. The structure of this product and its key fragmentation pathways are depicted in Scheme 1. Although the formation of the y_7 ion (m/z 647) in MS/MS spectra of both singly and doubly charged sulfinamide precursor ions (Figures 2b and 3b) would require complex rearrangement reactions in the gas phase, such rearrangements under CID conditions were previously reported for peptides with sequences PFKCG [37] and PRCGVPDVA [38]. For example, a prominent gas-phase rearrangement ion (m/z 373, b_3) was observed in the MS/MS spectra of cyclic cysteine sulfonamide, cysteine sulfinamide, and cysteine sulfenamide products formed in the oxidation of PFKCG by HOCl [37].

With these precedents in mind, we believe the y_7 ion (m/z 647) in MS/MS spectra of both singly and doubly charged precursor ions (Figures 2b and 3b) may be formed by a similarly complex gas-phase rearrangement reaction. We do not believe these spectra provide evidence for a cysteine sulfine because we do not observe the predicted ions of m/z 839.4 (Figure 2b) or m/z 420.4 (Figure 3b) caused by the neutral loss of a stable thio-ketene ($CH_2=S=O$) from a cysteine sulfine. Recently, Gates et al. demonstrated, by using a small organic molecule model of the PTP1B active site, that the sulfenic acid residue possesses sufficient electrophilicity to drive a cyclization reaction with a neighboring amide group, which leads to the formation of a 3-isothiazolidinone analog [39]. In this study, there was no evidence for the formation of cysteine sulfine from the sulfenic acid intermediate. We also do not believe

Figure 2. Q-TOF MS/MS spectra of $(M + H)^+$ ions of (a) HCSAGIGRS (m/z 887.4) and (b) HC[S(O)N]SAGIGRS (m/z 901.4).

our data can be explained by oxidation of histidine because the formation of an abundant histidine immonium ion (Figures 2b and 3b) at m/z 110 and a shift of y_8 ion from m/z 750 (Figure 3a) to m/z 764 (Figure 3b) strongly suggest that histidine is not modified. When we infused the unmodified peptide (m/z 887.4) solution directly in to the MS, we found no oxidation product formed at m/z 901, suggesting oxidation did not occur in

the electrospray source of the mass spectrometer (data not shown).

Interestingly, the MS/MS spectrum shown in Figure 3b is very similar to that of the doubly charged Q-TOF MS/MS spectrum of HC(SO₂H)SAGIGRS (m/z 460.2, +2) (Figure 4a) except for the y_8 fragment ion at m/z 764 and differences in the relative intensities of all other fragment ions. The formation of ions at m/z 273 and

Figure 3. Q-TOF MS/MS spectra of $(M + 2H)^{+2}$ ions of (a) HCSAGIGRS (m/z 444.2) and (b) cyclic cysteine sulfonamide HC[S(O)N]SAGIGRS (m/z 451.1). Ions at m/z 273.0 and 418.1, indicated with an asterisk, are most likely derived from the isobaric contaminant of m/z 451.2 (+2) ions formed by the in-source loss of water from HC(SO₂H)SAGIGRS.

Scheme 1. Fragmentation pathways of doubly charged cysteine sulfinamide HC[S(O)N]SAGIGRS (+2) ions.

418.2 (indicated with asterisks) in Figure 3b raised the possibility of the presence of isobaric impurities at the m/z 451.2 precursor ions as they were found to be present in Figure 4a, although at low abundance. Moreover, an intense peak at m/z 451.2 (+2) was most likely due to the loss of water in the ion trap MS/MS analysis of HC(SO₂H)SAGIGRS, as shown in Figure 4b. These observations led us to carefully investigate the purity as

well as the mechanism of the formation of cysteine sulfinamide m/z 451.2 (+2) ions. To do this, we used an ion trap to perform MS² and MS³ experiments on the m/z 451.2 (+2) parent ions formed in the oxidation reaction of HCSAGIGRS and on 451.2 (+2) product ions formed by the water loss during the MS/MS analysis of HC(SO₂H)SAGIGRS. These spectra are shown in Figure 5. Differences can be seen between the MS/MS spectrum (Figure 5a) and the MS/MS/MS spectrum (Figure 5b). For example, the spectrum in Figure 5b displayed a base peak at m/z 418 (+2) most likely due to a facile loss of water in MS/MS, followed by the neutral loss of H₂SO₂ in MS/MS/MS. Another ion at m/z 273 (b_2 ion of HC(SO₂H)SAGIGRS), which is formed by the cleavage at the C-terminal side of modified cysteine, is absent in Figure 5a. Likewise, the spectrum in Figure 5a contains a base peak at m/z 442 (–H₂O), a series of abundant y ions and a b_6 ion that are of very low intensity or absent in Figure 5b. The y_8 ion, which is more intense in Figure 5a, is especially informative. This suggest that the m/z 451.2 (+2) ions formed by the loss of water from HC(SO₂H)SAGIGRS possess a different structure than the cysteine sulfinamide. Thus, based on the above experimental results, it seems most likely that the majority of the ions at m/z 451.2 (+2) belong to the cysteine sulfinamide product formed directly in the oxidation reaction. However, a minor contribution via the loss of water (most likely in-source) from HC(SO₂H)SAGIGRS under our MS experimental conditions cannot be ruled out. In sum, we conclude that our data are consistent with multiple mechanisms for the formation of the cysteine sulfinamide product.

Figure 4. MS/MS spectra of doubly charged ions of HC(SO₂H)SAGIGRS (m/z 460.2, +2) obtained by (a) Q-TOF and (b) ion trap instruments.

Figure 5. Ion trap spectra: (a) MS/MS spectrum of m/z 451.2 (+2) ions of HC[S(O)N]SAGIGRS and (b) MS/MS/MS spectrum of m/z 451.2 (+2) ions of [HC(SO₂H)SAGIGRS(-H₂O)] produced in the MS/MS of m/z 460.2 (+2).

We have also carried out data-dependent nano LC-MS/MS experiments to separate and characterize the cyclic cysteine sulfinamide oxidation product. We obtained the SICs for m/z 451.2 ions as well as m/z 460.2 ions (Figure 6) from an LC-MS/MS experiment. We found one MS/MS spectrum for a precursor ion of m/z 451.2 (Figure 6d) at 14 min RT (Figure 6c) when the cysteine sulfinic acid oxidation product (m/z 460.2) eluted (Figure 6b). We found another MS/MS spectrum for m/z 451.2 (Figure 6e) acquired at 31 min (Figure 6c). These two MS/MS spectra were recorded with similar S/N ratios, but the spectra differ in several respects. The y_8 ion at m/z 764 is completely absent from the MS/MS spectrum obtained at 14 min (Figure 6d), whereas it is clearly present in the MS/MS spectrum that is obtained at 31 min (Figure 6e). Also, the MS/MS spectrum at 31 min contains a more prominent series of y ions compared with the MS/MS spectrum at 14 min. The formation of a low intensity m/z 418 ion in Figure 6e may be due to poor HPLC separation and contamination by the high concentration of the cysteine sulfinic acid oxidation product. These results suggest that the cysteine sulfinamide product (eluting predominantly at 31 min) is formed directly from the oxidation of HCSAGIGRS peptide, and is not a water loss product of cysteine sulfinic acid (eluting predominantly at 14 min).

These results are complementary to a recent study of PTP1B by Salmeen et al. who discovered a novel intermediate “cysteine sulfinamide” in the hydrogen peroxide mediated inactivation of PTP1B [15]. They

proposed a mechanism for cysteine sulfinamide formation in which active site cysteine thiol undergoes oxidation to give sulfinic acid, followed by cyclization of the amide nitrogen onto the oxidized sulfur residue. These authors reinvestigated cysteine sulfinamide formation in PTP1B and found that the “sulfinamide bond” is stable for more than 5 h [34]. Nevertheless, in the current study, the cysteine sulfinamide structure that we characterized has an additional oxygen atom on the sulfur that could be formed when cysteine sulfinamide undergoes further oxidation. Salmeen et al. proposed an identical cysteine sulfinamide intermediate in the irreversible H₂O₂ oxidation of the cysteine thiol to cysteine sulfinic acid [35]. Based on these previously reported results and our current experimental findings, we propose a mechanism for the formation of a cysteine sulfinamide product starting with an OH[•] attack on the -SH group of cysteine in the HCSAGIGRS peptide as illustrated in Scheme 2.

To investigate the formation of cysteine sulfinamide via H₂O₂ oxidation of the HCSAGIGRS peptide, we incubated the peptide with 100 μ M H₂O₂ at RT for 30 min while minimizing contamination by metal ions. We obtained a Q-TOF LC-MS/MS spectrum virtually identical to that of the cysteine sulfinamide ion at m/z 451.2 (+2) formed in the oxidation reaction of HCSAGIGRS by Fenton reagent. These results suggest that cyclic cysteine sulfinamide may be a key intermediate in the irreversible oxidation by both OH[•] and H₂O₂ oxidants of the active site cysteine in PTP1B.

Figure 6. (a) LC-MS chromatogram of products formed in the oxidation reaction of HCSAGIGRS peptide with Fenton reagent and selected ion chromatograms (SIC) of (b) 460.2 ions and (c) 451.2 ions. LC-MS/MS spectra of m/z 451.2 (+2) ions acquired at RTs (d) at 14 min and (e) at 31 min.

Characterization of Cysteine Sulfenic Acid [HC(SOH)SAGIGRS]

To investigate the formation of a potential cysteine sulfenic acid intermediate, the Fenton reagent oxidation reaction mixture was infused directly into a Q-TOF Micro mass spectrometer immediately after addition of the reagent to HCSAGIGRS peptide. A clear peak at m/z 903.4 can be seen in the mass spectrum (Figure 1a). The MS/MS spectrum of this m/z 903.4 ion (Figure 1b) shows a series of y ions, including y_3 and y_5 – y_7 , which confirmed the amino acid sequence SAGIGRS. Histidine modification is unlikely because of the formation of its abundant immonium ion at m/z 110. These data indirectly suggest that cysteine is modified. In addition, the ion at m/z 853.4 indicating the loss of neutral H_2SO from the parent ion confirmed that cysteine is modified to cysteine sulfenic acid [HC(SOH)SAGIGRS]. A low abundance ion at m/z 853 ion formed by the loss of neutral H_2S is also present in Figure 2a. A similar H_2SO loss was observed in the MS/MS spectrum of another

peptide, PRC(SOH)GVPDVA (m/z 929.4), which we recently characterized as a stable sulfenic acid oxidation product [38]. Furthermore, modification of cysteine to cysteine sulfenic acid induced the preferred formation of abundant y ions over b ions as in the MS/MS spectra of cysteine sulfenic acid [HC(SO₂H)SAGIGRS] and cysteine sulfonic acid [HC(SO₃H)SAGIGRS] (Figure 1c and d). Figure 1b–d show that the y_7 ion formed by the facile cleavage at C-terminal side of the modified cysteine residue is relatively intense while it is almost absent in the MS/MS spectrum of unmodified HCSAGIGRS (Figure 2a). Thus, cysteine sulfenic acid undergoes very similar fragmentation as cysteine sulfenic acid and cysteine sulfonic acid in our MS/MS experiments. It has been proposed that cysteine sulfenic acids in proteins are stabilized by hydrogen bonding with amino or carbonyl groups [38, 40]. We suggest similar hydrogen bonding interactions to stabilize cysteine sulfenic acid in the PTB1B active site sequence HC(SOH)SAGIGRS, perhaps due to strong hydrogen bonding between the SOH group and the imida-

Scheme 2. Proposed mechanism for the formation of cysteine sulfinamide HC[S(O)N]SAGIGRS product and its oxidation to cysteine sulfonic acid.

zole ring of histidine. These results are consistent with evidence for the presence of cysteine sulfinic acid in PTP1B in response to H_2O_2 oxidation obtained by Barrett et al. [41] and Denu et al. [42], using the trapping reagent NBD-Cl. While there are many reports describing the significance of cysteine sulfinic acid intermediates in redox signaling in biologically important proteins (reviewed in [43], our results do not directly demonstrate that cysteine sulfinic acid is an intermediate for the formation of cysteine sulfinamide in PTP1B.

Conclusions

We used mass spectrometry to characterize several oxidation products of a synthetic peptide containing the PTP1B active site sequence motif, HCSAGIGRS. We found a previously unknown oxidation product, cysteine sulfinamide, which may be formed in the irreversible oxidation of catalytic cysteine in PTP1B. The direct identification of cysteine sulfinic acid in our experiments suggests that it might be a key intermediate in the formation of the cysteine sulfinamide product. Our findings suggest plausible $\text{OH}^\bullet$ and H_2O_2 mediated oxidation products of PTP1B that may be formed when the Fe-catalyzed Fenton reaction prevails in cellular responses to high oxidative stress situations, increase in H_2O_2 levels, and/or exposure to high-energy irradiation such as x-rays, γ rays, or UV radiation.

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