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Study of the preferred modification sites of the quinone methide intermediate resulting from the latent trapping device of the activity probes for hydrolases

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Abstract

Use of activity probes has been demonstrated to be a powerful tool in modern chemical proteomic study. Previously we have designed and synthesized a series of mechanism-based activity probes that utilized quinone methide chemistry. Here, we characterized the trend of chemical reactivity for the reactive quinone methide intermediate **3** (QM-**3**) resulting from the latent trapping device. In a competition assay, the labeling of PTP1B by probe **1a** was blocked by externally added cysteine without affecting the catalytic activity of the enzyme. Further sequencing analysis on the trypsin-digested peptides of probe **1a**-labeled PTP1B using tandem mass spectrometry revealed that all six cysteine residues of PTP1B are capable of being modified by probe **1a**. These results indicated that the sulfhydryl group of cysteine residue is the preferred nucleophile for the reactive QM-**3**. Our finding provides the first example in understanding the preferred amino acid residues modified by the reactive QM-**3**, which is also the key structural unit responsible for forming covalent bonds in many biochemical applications.

Keywords: Quinone methide; Hydrolases; Protein tyrosine phosphatase; Glucosidase; Cysteine; Labeling; Proteome; Activity probe; Mechanism-based

In the post-genomic era, the rich information in the genomic databases offers great opportunity in identifying new drug targets and therefore in facilitating the process of drug discovery and development [1–3]. Since proteins are the key biomolecules responsible for most of the cellular functions, uncovering and characterizing all the protein products encoded by the genomes, especially by the human genome, has become a high priority challenge for biochemists and researchers in multidisciplinary fields [4–6]. Although advances in instrumentation including two-dimensional gel electrophoresis and mass spectrometry together with continuously expanding gene/protein databanks have made rapid protein identification possible [7–9], handling the huge number of proteins encoded by the human genome is still a tedious and time-consuming task [10–12]. As an alternative approach, researchers now first focus on certain groups of proteins in the complicated biological systems, expecting to simplify the complexity and to identify targets of interest more efficiently. Chemical probes capable of selectively labeling protein subfamilies have thus been developed to meet the demands [13–18]. Structurally, a typical chemical probe carries two key elements, a reactive unit and a reporter group, in its design. The

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reactive unit of the probe forms covalent linkage with the target proteins. The labeled proteins in turn could be monitored and characterized based on the property of the reporter group.

In our group, we have adopted a mechanism-based approach and developed activity probes for hydrolases such as tyrosine phosphatases and glycosidases [19–21]. These hydrolases were first chosen as they play important roles in post-translational modification of proteins and in many signal transduction systems [22,23]. As shown in Fig. 1, an activity probe carries four major parts in its design, including a recognition head, a latent trapping device, and a reporter group connected through a linker bridge. The approach is unique in that the probes themselves are also the substrates of the corresponding hydrolases, therefore offering an advantage that the target hydrolases could be pre-determined. The recognition head serves to define the specificity for the target hydrolase. Probes **1a** and **1b** share the same structural features, except probe **1a** carries a phenylphosphate head making it selectively target tyrosine phosphatases [19,20], whereas probe **1b** uses a β-glucosyl unit to target β-glucosidase [21]. The design for the linker and reporter moiety could be flexibly tuned to meet demands in different applications. The labeling event is a two-stage process, including activation and alkylation, as shown in Fig. 2. An activity probe **1** is typically stable under incubation conditions in the absence of the target hydrolase. However, when the designated bond between the recognition head and the latent trapping device is selectively cleaved with the assistance of the target hydrolase, it becomes activated by the release of *p*-hydroxybenzylic fluoride intermediate **2**. Intermediate **2** quickly undergoes 1,6-elimination to produce highly reactive quinone methide **3** (QM-**3**), which in turn could alkylate suitable nucleophiles on nearby hydrolases to form labeled adduct **4**. Here, the latent trapping device serves as the core of the probe and the QM-**3** plays a critical role in the covalent labeling of target hydrolase.

The latent trapping device, which exploits the quinone methide chemistry [24], was mainly derived from *p*-hydroxymandelic acid derivatives [25]. This design has also been incorporated in many other applications that required covalent labeling of proteins including probes for hydrolases [19–21,25,26] as well as a hapten design for reactive immunization [27]. However, the preferred labeling sites in these systems have never been studied. In this report, we use PTP1B and probe **1a** as a model and carry out two series of experiments to study the preferred modification sites by the QM-**3**. Knowledge on the preferred labeling sites would certainly help us understand how far we can go with this trapping device and provide opportunities for future applications.

Materials and methods

*Labeling of PTP1B by probe **1a**.* Probe **1a** was prepared according to reported procedures [19,20]. The catalytic domain of human PTP1B (1–321) was expressed in *Escherichia coli* and purified as a 6× His tagged protein using Ni–NTA–agarose (Qiagen) [20]. Two micrograms of purified PTP1B was incubated with 2 mM of probe **1a** in reaction buffer containing 50 mM Tris, pH 8.0, 1 mM EDTA, and 50 mM NaCl at 4 °C for 15 min. For the competition study, two different concentrations of individual amino acid, 0.1 and 1.0 mM, were also included in the labeling mixtures. The reaction products were separated by 10% SDS–polyacrylamide gel electrophoresis. After electrophoresis, the probe **1a**-labeled proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 10% nonfat dried milk, washed with TTBS (0.05% Tween 20, 20 mM Tris, and pH 7.6, 137 mM NaCl), and then treated with a streptavidin–horseradish peroxidase conjugate (Amersham–Pharmacia, 1:2000 dilution) in TTBS with 1% nonfat dried milk for 1 h at 25 °C. Visualization of bound streptavidin–horseradish peroxidase conjugate was achieved by treating the membrane with ECL chemiluminescence reagents

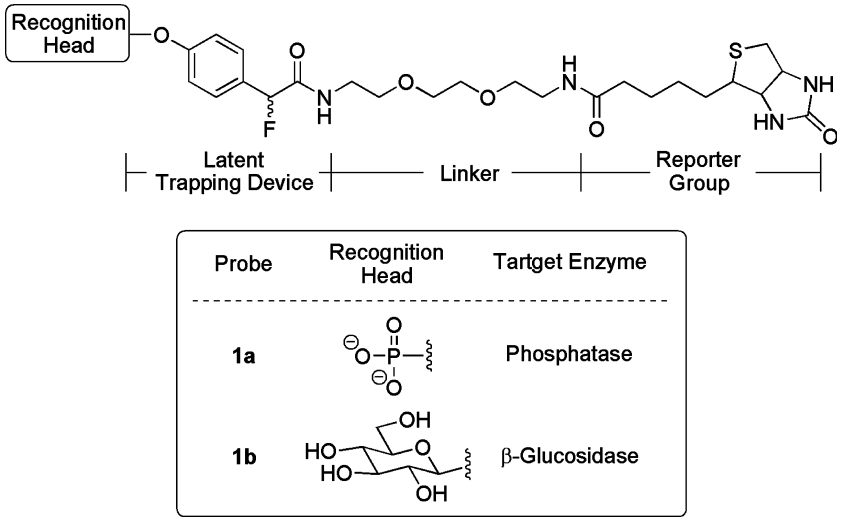

Fig. 1. The structural feature of activity probes **1a** and **1b** that target tyrosine phosphatases and β-glucosidase, respectively.

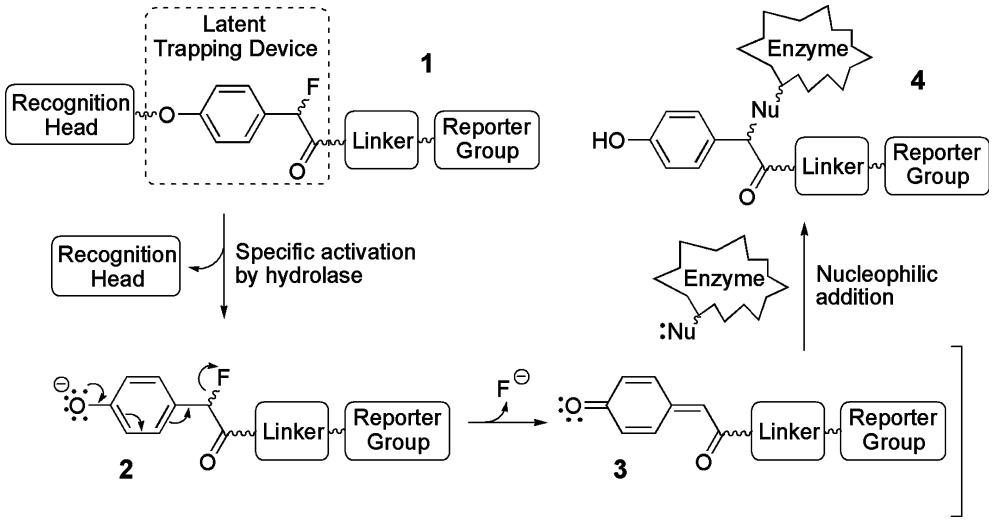

Fig. 2. General design of activity probe **1** and the selective labeling process for the corresponding hydrolase.

(Amersham–Pharmacia), and exposed to film for 0.5–30 min before development.

Activity assay of PTP1B. The PTP1B activity analysis was conducted in acetate buffer (100 mM sodium acetate, pH 7.5, 50 mM NaCl, and 1 mM EDTA) and room temperature using *p*-nitrophenyl phosphate as the substrate. The absorbance at 405 nm was used to determine the rate of *p*-nitrophenyl phosphate hydrolysis. Under our assay condition, 1 μg of the purified PTP-1B hydrolyzed 2.3 μmol of *p*-nitrophenyl phosphate per minute.

MALDI-TOF MS analysis of intact protein molecular weight. The spectra of intact proteins were acquired by using a MALDI-TOF mass spectrometer Voyager DE-PRO (PerSeptive Biosystems, USA) operating in the linear, delayed extraction mode. The instrument was equipped with a 337 nm nitrogen laser at frequency of 3–20 Hz. The spectra were recorded in the linear mode at accelerating voltage 25 kV, 93% grid voltage, 0.15% guide wire voltage, 900 ns delay time, and a low mass gate of 5 kDa. One hundred laser shots were averaged per spectrum. External mass calibration was usually applied, based on a mixture of two reference proteins {cytochrome *c* (MW = 12,361) and myoglobin (MW = 16,952)} covering extended to the *m*/*z* 10–100 K range. All the MALDI-TOF-MS mass spectra were analyzed by using Data-Explorer (PE Biosystems, Foster City, CA, USA).

*LC tandem mass analysis of probe **1a**-labeled peptides.* Protein bands were excised from preparative SDS–PAGE, washed, in-gel reduced by DTT, alkylated by iodoacetamide (IAA), and digested with trypsin (Promega, sequencing grade) in 25 mM NH_4HCO_3, pH 8.0, according to published procedures [28,29]. Nanoflow capillary liquid chromatography–tandem mass spectrometric (LC-MS/MS) analysis of in-gel digests was performed using a capillary LC system (Ultimate, LC Packings, Amsterdam, Netherlands) coupled to a Q-TOF (quadrupole time-of-flight) mass spectrometer (QSTAR Pulsar i, Applied Biosystem/MDS Sciex, Foster City, California). The column of nanoscale capillary liquid chromatography used was a reversed-phase C18 (15 cm × 75 μm i.d., 3 μm) with a flow rate of 200 nL/min. Solvent A was 0.1% formic acid in water/acetonitrile (98/2, v/v) and solvent B was 0.08% formic acid in water/acetonitrile (2/98, v/v). Separation of peptides was performed with a gradient 5–50% B in 30 min. The ES mass spectra were scanned from *m*/*z* 400–2000 at a scan cycle of 1 s/scan. The nanoLC tip of on-line LC/MS used was a New Objective PicoTip (FS360-20-10-D-20) (PicoTip, New Objective, MA, USA). The voltage on the nanoLC tip was 1800–2200 V in positive mode. Tandem mass spectra were automatically collected under the information dependent acquisition (IDA) during the LC-MS/MS run. The ES mass spectra were scanned from *m*/*z* 400 to 2000 at a scan cycle of 1 s/scan, and automated product ion scan from *m*/*z* 70 to 2000 at a scan cycle of 3 s/scan.

Results and discussion

The covalent alkylation of PTP1B occurred at the second stage by reactions of QM-**3** with suitable nucleophiles on yet to be determined amino acid residues. In order to understand the preferential reactivity of the side chain functional groups toward QM-**3**, we first performed a competition study by adding external amino acids to the labeling mixtures. The ones that effectively compete with PTP1B would block the labeling process. Twelve different amino acids which cover all the possible reacting functional groups, including carboxyl (Asp, Glu), amido (Asn, Gln), sulfhydryl (Cys), sulfide (Met), hydroxyl (Ser, Thr), imidazole (His), guanido (Arg), and aromatic (Trp), were selected for the competition experiments. Two concentrations, 0.1 and 1.0 mM, for each of the amino acids were examined. The competed labeling results are shown in Fig. 3. Two sets of gel results are presented. The gels on the left were stained with Coomassie blue that reflected the relative amounts of protein loaded. The gels on the right were visualized with streptavidin-conjugated peroxidase chemiluminescence after transferring the reaction products onto a nitrocellulose membrane. This would allow specific staining for biotinylated proteins. The results indicated that there was no apparent inhibition of labeling at lower concentration (0.1 mM) of added amino acid. However, when the amino acid was used at 1.0 mM, it became clear that cysteine (Cys) was able to block the labeling reaction. Among the rest 11 amino acids, only Ser or Met slightly decreased the labeling reaction. None of the others showed significant inhibitory effect under our assay conditions. In order to rule

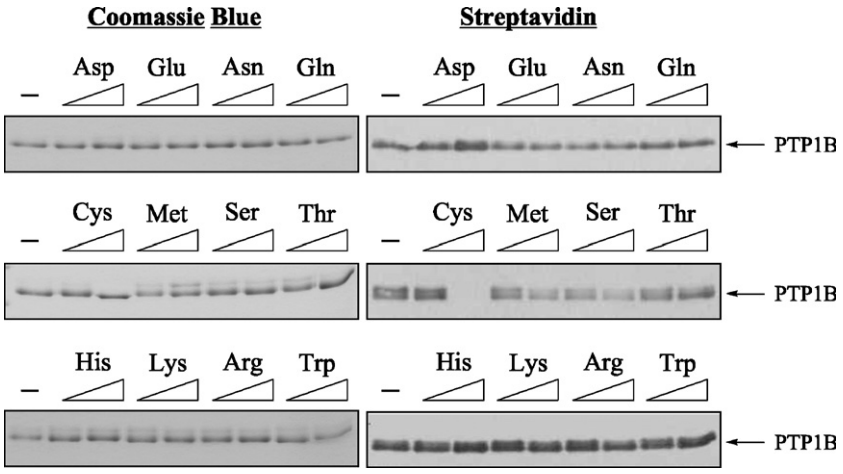

Fig. 3. Inhibition of probe **1a** labeling by Cys residue. Two micrograms each of PTP1B was mixed with 0.1 or 1.0 mM of indicated amino acids and incubated with 2 mM probe **1a** at 4 °C for 15 min. The reaction mixtures were separated by 10% SDS–PAGE and visualized by Coomassie blue stain (left) or Western blotted with streptavidin (right).

out the possibility that the added amino acids might interfere with the first cleavage (activation) stage, namely the hydrolysis of P—O bond by PTP1B, we also examined the catalytic activity of PTP1B in the presence of added amino acids. As shown in Fig. 4, at both concentrations none of the amino acids affected PTP1B activity under our assay conditions. These observations confirmed that the sulfhydryl group of Cys has the highest reactivity over the other amino acids toward QM-**3**. This trend of chemical reactivity is consistent with the results obtained from study with other quinone methides [30]. However, additional factors, including relative concentrations of competing nucleophiles and their accessibility, might also influence the protein modification sites. Other possible labeling sites that have been recorded in peptide and protein models include the N-terminus, as well as the side chains from Lys, His, and Trp residues [30–32]. In our present work, these secondary modification sites were not pursued.

In the second set of experiments, we performed MALDI-TOF MS and LC tandem MS studies on probe **1a**-labeled PTP1B in order to identify the labeled residues. After incubating PTP1B with probe **1a** for 60 min, the MALDI-TOF MS profiles from labeled and intact PTP1B were then compared. The MALDI-TOF mass spectrum shows significant mass shifts compared to those obtained from intact PTP1B, indicating multiple modifications. The phenomenon of multiple modifications has also been observed in the case of phosphotriesterase as characterized by LC-MS with a probe carrying the same trapping device [25]. To further identify the specific labeling sites, both labeled and unlabeled PTP1B samples were digested with trypsin followed by LC-MS/MS analysis. For each modification by QM-**3** there would be an increase in molecular weight of 508 Da. With the aid of BioAnalyst software, we were

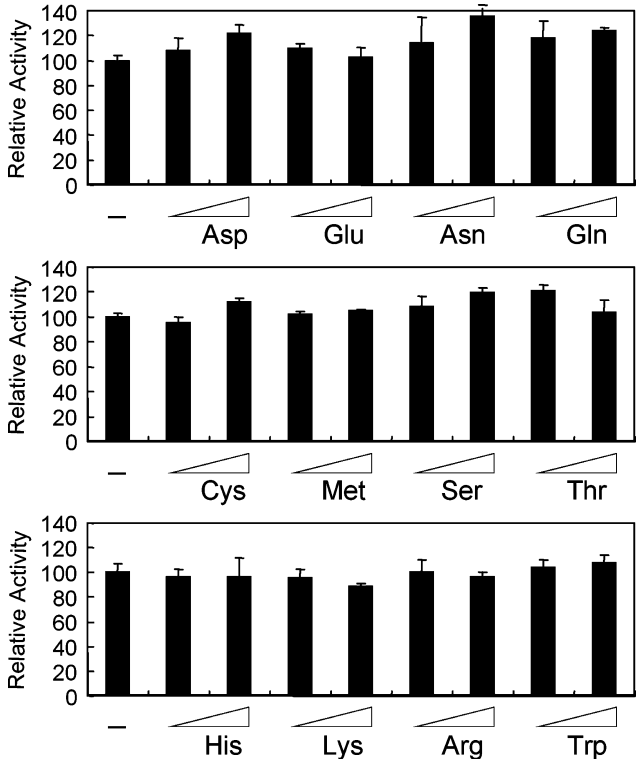

Fig. 4. Presence of amino acids does not affect the activity of PTP1B. Two micrograms of each of PTP1B was mixed with 0.1 or 1 mM of amino acids and incubated with 1 mM *p*-nitrophenyl phosphate in buffer containing 0.1 M sodium acetate, 50 mM NaCl, and 1 mM EDTA. The absorbance at 405 nm was taken at different time intervals. Catalytic rate was determined as the changes of absorption per minute. Here the relative activities are presented using the PTP1B activity in the absence of amino acid as 100%.

able to identify five peptide fragments that were labeled by QM-**3** (Table 1). The observed peptide fragments for the labeled PTP1B are shown in the column of +QM-**3,** while the ones for the unlabeled PTP1B are observed

Table 1
Tryptic peptides observed for labeled and unlabeled PTP1B

Peptide	Molecular weight[a]			Sequence[b]	Fragment[c]
	Calculated	+IAA	+QM-**3**		
1	1061.45	1118.46	1569.44	HEASDFP**C**R	33–41 (C40)
2	2864.35	2920.37	3371.35	SYILTQGPLPNT**C**Q GFWEMVWEQK	88–111 (C100)
3	1409.65	1465.67	1916.47	**C**AQYWPQK	129–136 (C129)
4	2175.06	2231.08	2682.06	ESGSLSPEHGPVVV H**C**SAGIGR	208–229 (C223)
5	1730.82	1843.85	2746.82 (2 QM-**3**), 2295.82 (1 IAA + 1 QM-**3**)	SGTF**C**LADT**C**LLLM DK	230–245 (C234, C239)

[a] Peptide fragments observed for unlabeled (+IAA) and labeled (+QM **3**) PTP1B.
[b] Cysteine residues are shown in bold form.
[c] The position of cysteine residue in each peptide fragment was shown in parentheses.

with modification of IAA and shown under the column of +IAA. By analyzing the amino acid composition of these five peptides, we found only Cys residue was common among them. It is especially worth noting that peptide five carries two Cys residues, leading to two QM-**3** modifications. The results are in consistence with the competition experiments, both supporting that the Cys residues appeared to be the alkylation sites for QM-**3** modification.

To determine the exact sites of QM-**3** modification, tandem mass analysis for these five polypeptides was conducted. The MS/MS spectrum representing the sequence of ^{33}HEASDFPCR^{41} (peptide 1) with addition of one QM-**3** is shown in Fig. 5. In this experiment, the y_n and b_n fragments were assigned based on the model proposed by Roepstorff and Fohlman [33]. Here, a series of y-fragments from y_2 to y_8 were observed, corresponding to the QM-**3** modified fragments, -CR, -PCR, -FPCR, -DFPCR, -SDFPCR, -ASDFPCR, and -EASDFPCR. The y_1 fragment was clearly unlabeled because the mass is identical to the one in free peptide. Similarly, the series of b-fragments were also consistent with this conclusion. Together these results allowed us to unambiguously determine that the QM-**3** molecule was attached to the cysteine residue of the polypeptide fragment. Besides peptide fragment 1, we were able to identify the QM-**3** modified Cys residues in the following peptides, Cys129-Lys136 (peptide 3) and Glu208-Arg229 (peptide 4). Although it is still unclear to us which Cys residues were first modified by QM-**3**, our results clearly indicated that all six Cys residues were labeled by QM-**3**.

Together, in the model system with PTP1B and probe **1a**, we first performed a competed labeling study in the presence of external amino acids to demonstrate that Cys residues have the highest reactivity toward the reactive QM-**3**. MALDI-TOF MS experiments on the five tryptic peptide fragments resultant from QM-**3** labeled

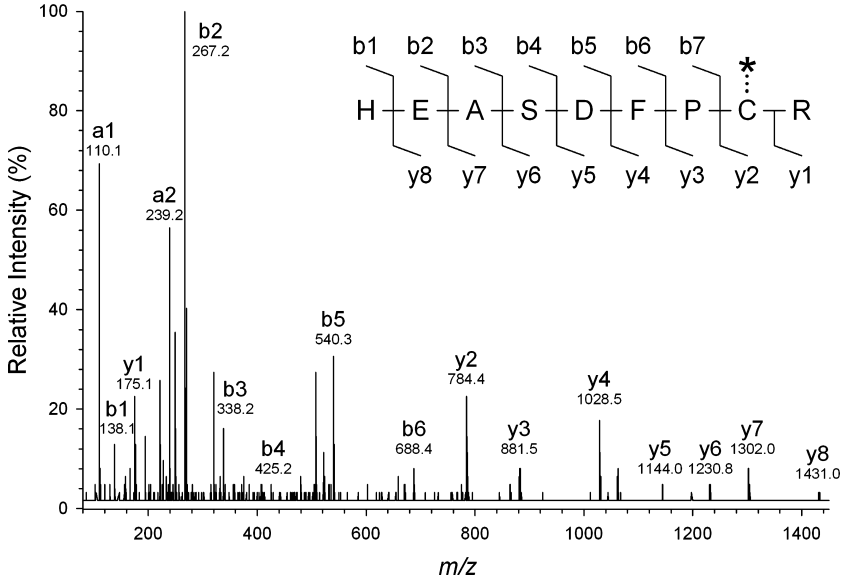

Fig. 5. Tandem mass spectrum of the peptide HEASDFPCR labeled with probe **1a**. Asterisk symbol attached to Cys residue (C) denotes the fragment of QM-**3**.

PTP1B also suggested Cys residues be the possible modification sites. The actual labeling sites were unambiguously confirmed by tandem MS study. In this study the chemical reactivity of QM-**3** has been determined for the first time. All six Cys residues on PTP1B could be labeled by the QM-**3**. The possibility of other amino acids that might serve as the secondary modification sites cannot be completely ruled out [30–32], although they played only a minor role in this system.

Acknowledgments

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