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Using mass spectrometry to study the photo-affinity labeling of protein tyrosine phosphatase 1B

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a potential target for the treatment of Type II diabetes and several companies are developing small molecule inhibitors of this enzyme. Part of the characterization of these compounds as PTP1B inhibitors is the understanding of how they bind in the enzyme active site. The use of photo-activated inhibitors that target the active site can provide such insight. This paper describes the characterization of a photoprobe directed at the active site of PTP1B. Mass spectrometry revealed the specific binding of the probe to the intact protein. Digestion of the labeled protein followed by LC–MS and LC–MS/MS was used to show that the photoprobe binds to a specific active site amino acid. This was confirmed by comparison with the X-ray structure of PTP1B with a PTP1B inhibitor. The probe labels a conserved acidic residue (Asp) that is required for catalytic activity. This photoprobe may prove to be a useful tool for the development of a PTP1B inhibitor or for the study of PTPs in general.

Keywords: Photoprobe; Phosphatase; Photo-affinity

1. Introduction

Protein tyrosine phosphatases (PTPs) are signaling enzymes which govern the dephosphorylation of phosphotyrosine residues in vivo. PTPs have been implicated in the regulation of a number of fundamental cellular processes including cell division, metabolism, gene transcription, and immune response [1]. These enzymes work in an opposing balance with protein tyrosine kinases (PTKs, which phosphorylate tyrosine residues) for the reversible and dynamic phosphorylation of proteins in eukaryotic cells. Protein tyrosine phosphorylation has been shown to play an important role in complex cellular signal transduction pathways. The malfunctioning of this enzymatic balance has been indicated as a possible factor in the development of such diseases as diabetes, cancers, and immune dysfunctions [2].

PTP1B, in particular, is a cytoplasmic PTP that has been implicated through mouse knockout studies to be involved in the development of diabetes and diet-induced obesity [3]. It has been proposed that PTP1B plays a critical role in the dephosphorylation of the insulin receptor. Inhibition of this enzyme, therefore, should stimulate insulin signaling. As a result, PTP1B has become an attractive therapeutic target for the treatment of type II diabetes.

Photo-affinity labeling is a useful tool for studying the interaction of substrates with their receptor or enzyme target. This technique has been used for the isolation and identification of receptors as well as for substrate tracking in cellular systems [4,5]. With this technique, the sample is irradiated with UV light after incubation of the target protein with the photo-activated probe. Photolysis activates the photoprobe to generate a reactive intermediate, which can then go on to form a covalent bond with the recep-

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tor protein. There are several photolabile groups that can be used to generate the highly reactive carbene or nitrene intermediate. Diazirenes, carbene precursors, are advantageous since they are highly chemically and thermally stable. Diazirenes can be activated at wavelengths above the UV range, avoiding UV inactivation of enzymes and protein damage.

In this study, a photoprobe using a labile diazirene moiety was investigated for its ability to modify the active site of the PTP1B. The extent of labeling, the specificity of the substrate, and the amino acid residue to which the photolabel was bound was determined by mass spectral analysis of the intact and digested labeled protein. The probe may prove useful for studying enzyme occupancy of various PTP1B inhibitors.

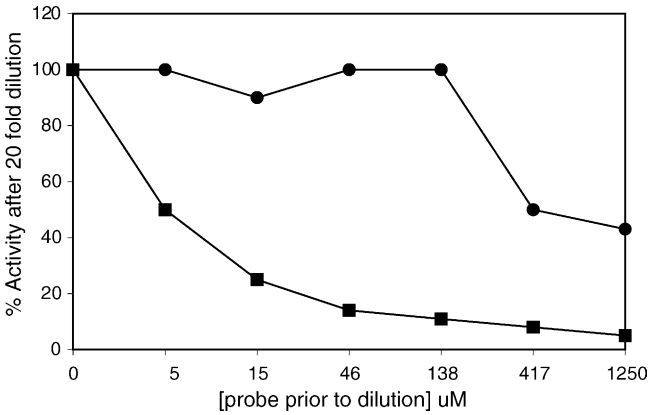

Fig. 2. The percent activity of PTP1B in the presence of photoprobe, with (square symbols) and without (circle symbols) UV irradiation.

2. Experimental

2.1. Materials

Glycerol, BisTris buffer, and EDTA were obtained from Sigma. Triton X-100 was purchased from Pierce. *N*,*N*′-dimethyl-*N*,*N*′-bis(mercaptoacetyl)hydrazine (DMH), a DTT substitute, was synthesized following the procedure outlined by Singh and Whitesides [6]. 3,6-Fluorescein diphosphate (FDP) [7] and *N*-benzoyl-L-glutamyl-[4-phosphono(difluoromethyl)]-L-phenylalanayl-[4-phosphono(difluromethyl)]-L-phenylalanineamide (BzN-EJJ-amide) were synthesized as previously described [8]. The diazirene photoprobe was synthesized at Merck Frosst. All other chemicals were of reagent grade and obtained from either Sigma or Aldrich (St. Louis, MO).

The catalytic domain of PTP1B (residues 1-320, flag fusion protein) was expressed and purified as described by Huyer et al. [9].

2.2. Kinetic parameters for PTPases

Kinetic parameters were determined either by fluorescence detection using a SpectraMax Gemini plate reader (Molecular Devices, Sunnyvale, CA) or by measuring absorbance using a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale CA) in a 96-well plate format. The formation of FMP from the substrate FDP was monitored by absorption at 450 nm ($\varepsilon \sim 34{,}000\,M^{-1}\,cm^{-1}$) or by fluorescence with excitation at 440 nm and emission at 515 nm. IC_{50} values were calculated as previously described using FDP as the substrate, 0.2 μg/ml Flag-PTP1B (1-320), and a threefold serial dilution of the photoprobe starting at 25 mM [10]. The IC_{50} of irradiated probe was determined under the same conditions as above except that the probe (in water) was irradiated on ice with a 40 W lamp (Philips) at a ∼10-cm distance from the lamp for 10 min prior to the inhibition studies. The linear initial rates were fitted to the Michaelis–Menten equation with the aid of the nonlinear regression curve-fitting algorithm provided by Softmax Pro software (version 2.6, Molecular Devices).

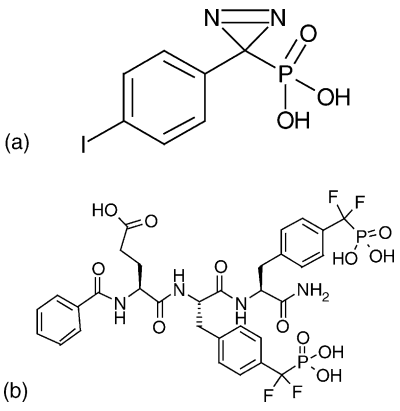

Fig. 1. Structure of (a) photoprobe and (b) BzN-EJJ-amide.

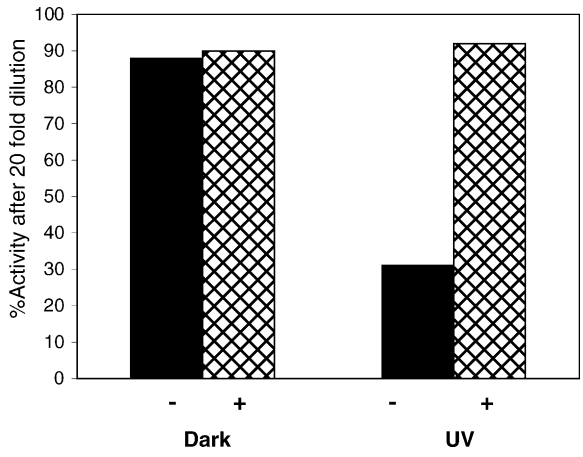

Fig. 3. The percent activity of PTP1B in the presence of 140 μM photoprobe with (+) and without (-) 100 nM BzN-EJJ-amide and with (UV) and without (Dark) irradiation.

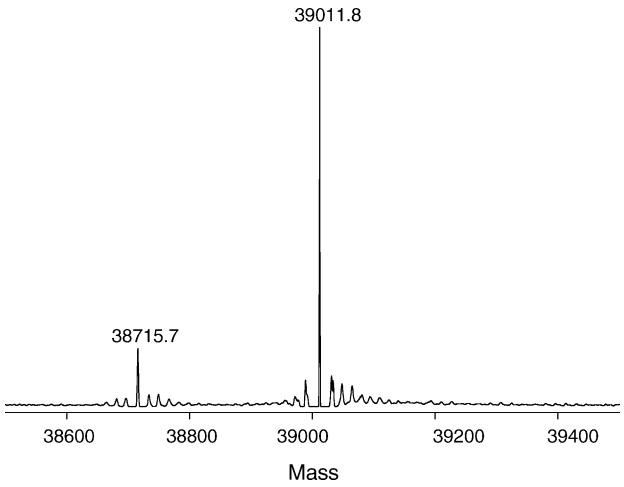

Fig. 4. Deconvoluted LC–MS spectrum of PTP1B modified by photoprobe following UV irradiation.

2.3. Photo-labeling

Flag-PTP1B at 0.2 mg/mL (5 μM) was mixed with 50 μM photoprobe in BisTris buffer (50 mM BisTris, 2 mM EDTA, 2 mM DMH) at room temperature for 5 min. The mixture was then placed on ice and irradiated with a 40 W lamp for 30 min. For competition studies, 50 μM BzN-EJJ-amide was pre-incubated with the enzyme for 1 min prior to the addition of the photoprobe and irradiation treatment. Samples were then submitted for the LC–MS analysis.

2.4. Competition with BzN-EJJ-amide

PTP1B at 0.2 mg/mL (5 μM) was mixed with 50 μM photoprobe and 50 μM BzN-EJJ-amide (10:10:1 probe:inhibitor:enzyme ratio) in BisTris buffer (50 mM BisTris, 2 mM EDTA, 2 mM DMH) at room temperature for 5 min. The mixture was then placed on ice and irradiated with a 40 W lamp for 30 min.

2.5. Reversibility and protection by BzN-EJJ-amide

Reversibility was studied by diluting a premix of Flag-PTP1B at 0.2 ug/mL and photoprobe (threefold serial dilution starting at 25 mM) 20-fold into BisTris buffer (50 mM BisTris, 2 mM EDTA, 2 mM DMH) containing 75 μM FDP after treatment with or without a 10 min irradiation with a 40 W lamp. For protection experiments, the enzyme was pre-incubated with 100 nM BzN-EJJ-amide for 2 min prior to the addition of the photoprobe. The initial rates were measured by absorbance at 450 nm and the percent activity was determined.

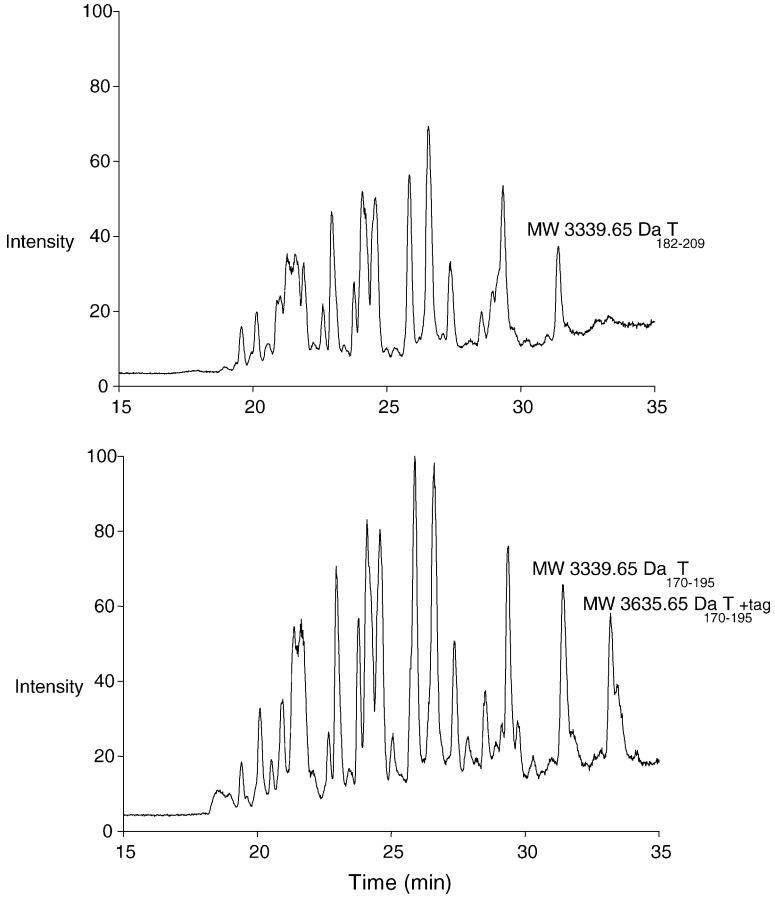

Fig. 5. LC–MS chromatogram of the tryptic digestion of (A) PTP1B native and (B) PTP1B labeled with photoprobe.

2.6. LC–MS analysis

All MS and MS/MS data were acquired using a Micromass Q-ToF Ultima mass spectrometer (Manchester, UK) fitted with a Z-spray electrospray ion source. The mass spectrometer was operated in positive ion mode at a capillary voltage of 3.5 kV with a desolvation gas temperature of 150 °C and a source temperature of 80 °C. The tunnel voltage was maintained at 50 V while the collision voltage was adjusted according to the mass of the parent peptide (25–35 eV).

Samples were delivered to the mass spectrometer using an Agilent 1100 capillary-LC (Palo Alto, CA). Protein analysis was carried out at a flow rate of 50 μl/min using a ThermoHypersil 50 × 1 mm BioBasic-45 μm column. A linear gradient was run from 15% B to 95% B over 20 min. Eluent A was 5% CH_3CN, 0.05% TFA and eluent B was 50% CH_3CN, 50% 2-propanol, 0.05% TFA. All peptide analyses were performed using a Phenomenex 150 × 1 mm C_{18} 3 μm Luna column. Eluent A consisted of 5% CH_3CN, 0.1% formic acid while eluent B was 95% CH_3CN, 0.1% formic acid. The digestion of PTP1B was separated by initially holding the gradient at 0% B for 10 min followed by a linear increase to 50% B over 25 min. To ensure complete elution, the gradient was linearly increased to 80% over 10 min and then to 90% within a minute. A flow rate of 20 μl/min was used.

Tryptic peptides were prepared as follows: protein samples, in BisTris buffer (pH 8.5), were incubated overnight at 37 °C with sequence-grade trypsin (1:20, w/w). Digestion of the resulting tryptic peptides using Glu-C was subsequently carried out by the addition of Glu-C in a 1:20 ratio (w/w). The sample was incubated at 37 °C for 5 h at which time a second aliquot of Glu-C was added, and the incubation was continued for another 6 h. Both the labeled and control (without inhibitor) samples were processed in parallel.

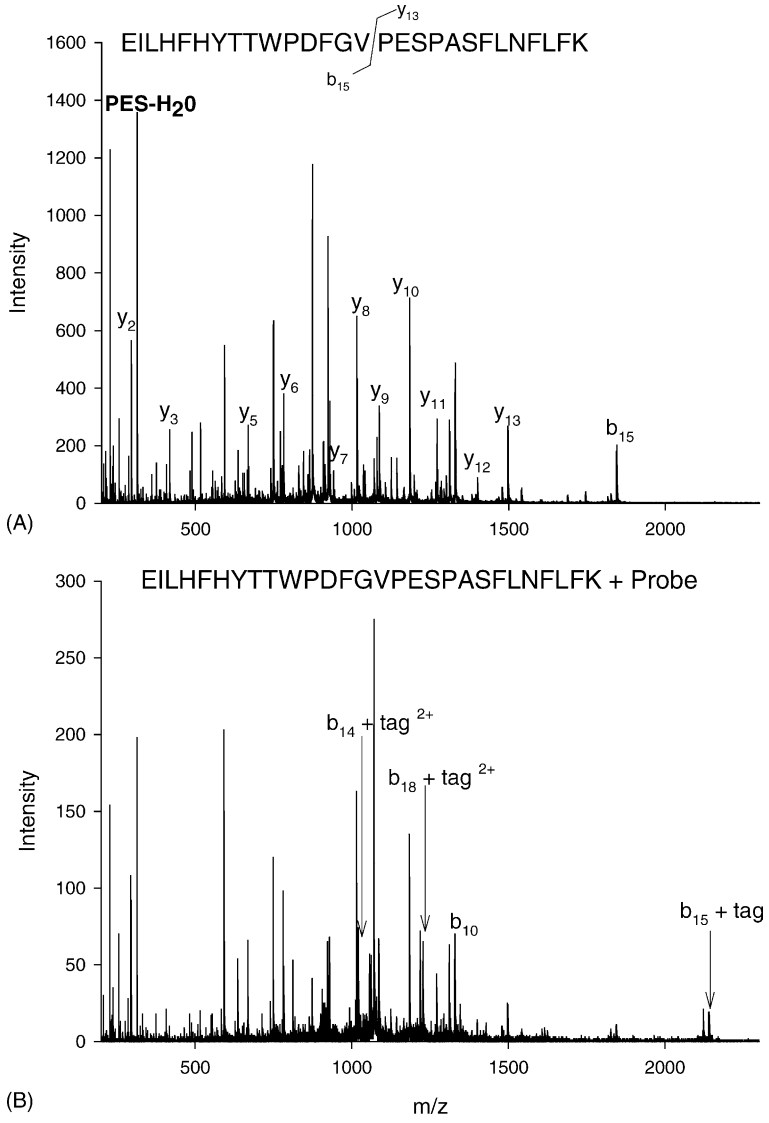

Fig. 6. MS/MS sequencing of the tryptic peptide $T_{170–195}$ (A) unlabeled and (B) labeled with photoprobe.

3. Results and discussion

3.1. Inhibition of PTP1B by the photoprobe

In searching for a suitable active site directed photoprobe for PTP1B, the potency of the photoprobe in question (Fig. 1 a) had to be determined. An IC_{50} value of 120 and 180 μM was determined for the non-irradiated and irradiated probe, respectively. In contrast to the variety of PTP1B inhibitors that have been studied in the literature, this photoprobe has an IC_{50} value that is 10–20 times higher than that which is normally accepted as a potent value. Despite the high IC_{50} values obtained, the utility of this probe as an active site directed inhibitor was investigated further.

The reversibility of the photoprobe inhibition as well as the protection of the enzyme active site was tested in the presence and absence of light using a potent and selective PTP1B active site inhibitor, BzN-EJJ-amide (Fig. 1b). This compound has been shown to reversibly inhibit PTP1B with an IC_{50} of 5 nM [10]. Fig. 2 shows the inhibition of PTP1B in the presence of photoprobe was reversible in the absence of UV radiation, but irreversible after UV treatment. The reversible

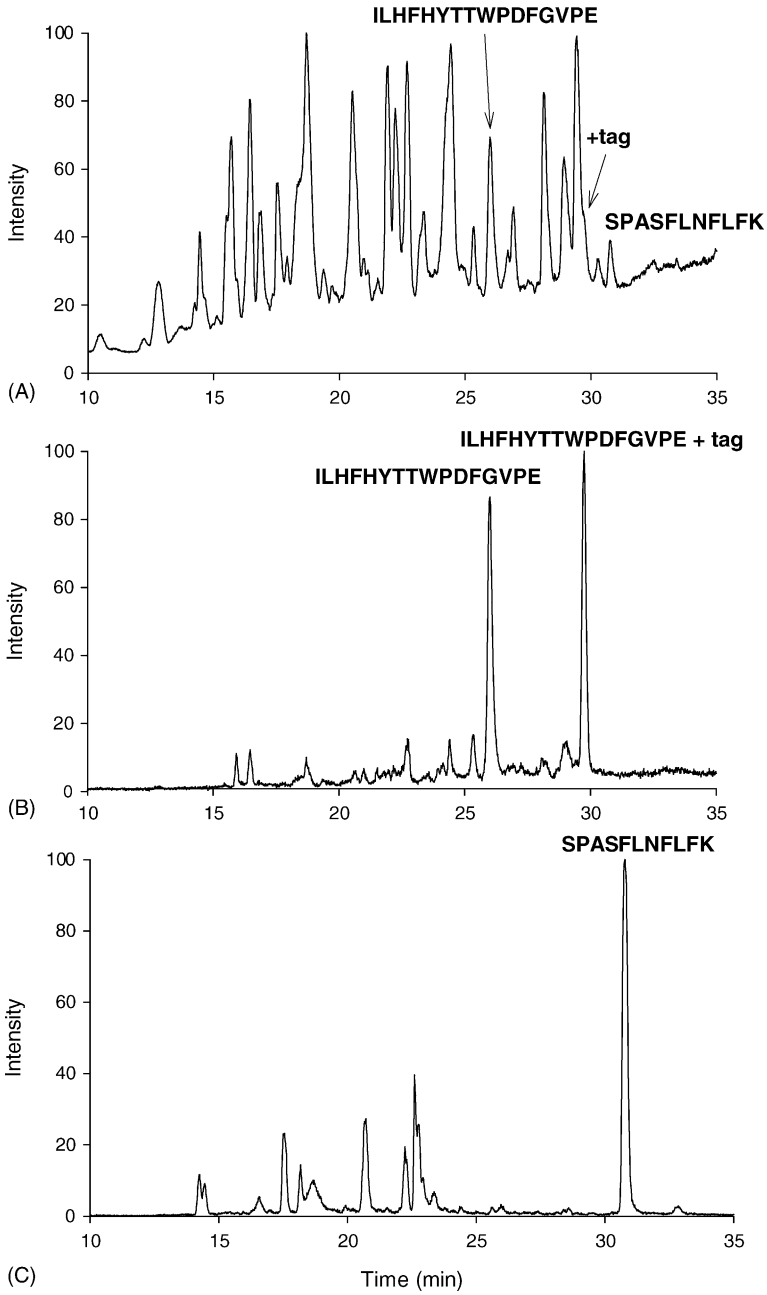

Fig. 7. LC–MS of Glu-C digestion of tryptic peptide $T_{170–195}$ (A) total ion chromatogram, (B) extracted ion chromatogram of photoprobe labeled Glu-C peptide ILHFHYTTWPDFGVPE, and (C) extracted ion chromatogram of unlabeled peptide SPASFLNFLFK.

inhibitor, BzN-EJJ-amide, protected the enzyme from irreversible inactivation by the probe after UV treatment (Fig. 3). The fact that the enzyme is protected by a potent active-site directed inhibitor indicates that the photoprobe is binding either in or near the active site. The control experiment shows that UV irradiation is necessary for inactivation of the protein by the photoprobe; thus, the photo-activation of probe is necessary for inhibition. This data suggests the photoprobe is acting as an active site directed photo-activated probe for PTP1B. Definitive proof was obtained via LC–MS analysis of the intact and digested protein after labeling with the photoprobe.

3.2. Mass spectral analysis of intact PTP1B

LC–MS was used to confirm the covalent labeling of the protein and to estimate the relative amount of protein modified as well as the stoichiometry of the labeling. The deconvoluted spectrum in Fig. 4 shows a small peak with an average mass of 38715.7 Da corresponding to the expected mass of PTP1B (38715.3). The much larger peak at 39011.8 Da is 296.1 mass units higher than the PTP1B protein itself and is equivalent to the addition of the photoprobe after loss of N_2. This spectrum also shows that labeling of the enzyme occurs in a one-to-one stoichiometry with approximately 80% of the total protein having been labeled.

No adduct could be detected by LC–MS when the protein was incubated with probe in the presence of BzN-EJJ-amide. Also no adduct was detected unless there was irradiation of the incubation mixture (data not shown). The mass spectrum of the intact protein indicates that a single copy of the probe labels the protein. However, to determine the site of binding, digestion of the protein followed by MS–MS was undertaken.

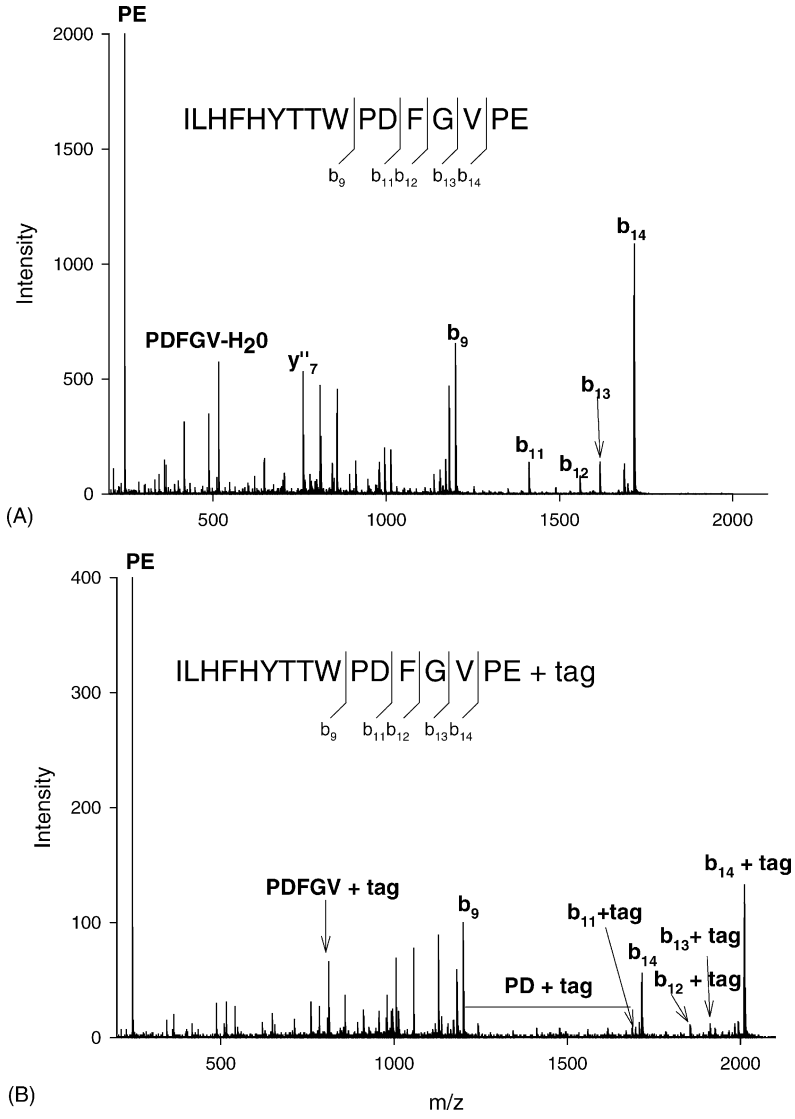

Fig. 8. MS/MS fragmentation of (A) unlabeled and (B) photoprobe labeled Glu-C tryptic peptide ILHFHYTTWPDFGVPE.

3.3. Digestion of labeled PTP1B

The labeled PTP1B sample was digested using trypsin to determine the site of attachment of the photoprobe. Fig. 5 shows the total ion chromatogram of the resulting separation of the tryptic peptides. By comparing the chromatograms of the digested unlabeled PTP1B with that from the labeled sample, only one peptide was identified containing the expected mass shift of the photolabel. The tryptic peptide $T_{170–195}$ with a molecular weight of 3339.65 Da was observed at 31.5 min in both the control and labeled digestions. However, in the tryptic digestion of the labeled PTP1B a new peak was observed at 33.2 min with a molecular weight of 3635.65 Da.

Fragmentation of these peptides confirmed their identity as the tryptic peptide $T_{170–195}$ with the sequence EILHFHYTTWPDFGVPESPASFLNFLFK. The presence of the photoprobe on this peptide was also verified (Fig. 6). Due to the size of the peptide, there was insufficient sequence information to determine the exact amino acid containing the probe. The fragmentation of the labeled peptide, however, showed the b_{14} ion with the tag indicating that the probe was located on the N-terminus of the peptide up to and including the glycine residue (EILHFHYTTWPDFG + tag). The presence of the b_{10} fragment without the tag (EILHFHYTTW) localized the probe to the following residues, PDFG + tag. The presence of glutamic acid residues in this peptide allowed for the subsequent Glu-C digestion resulting in two smaller peptides of a more manageable and potentially informative size (ILHFHYTTWPDFGVPE and SPASFLNFLFK).

Fig. 7A shows the total ion chromatogram of the Glu-C digestion products of the initial trypsin digestion. The N-terminus peptide (ILHFHYTTWPDFGVPE) was found both without and with the photolabel at 25.8 min and 29.7 min, respectively (Fig. 7B). The C-terminus peptide of the original tryptic peptide with the sequence SPASFLNFLFK was found at 30.8 min (Fig. 7C). As expected, this peptide did not contain a photoprobe adduct.

Fragmentation of the labeled peptide and the corresponding unlabeled peptide was used to confirm their identity and identify the site of probe attachment (Fig. 8). Fragmentation of the labeled peptide showed the labeled b_{14} ion with sequential amino acid loss to the labeled b_{11} ion. The presence of the unlabeled b_9 ion indicates a loss of the proline (P180) and aspartic acid (D181) residues as well as the photolabel. The attachment of the label to one of these two amino acids is further supported by the ion at *m*/*z* 812.2, which is equivalent to the PDFGV fragment plus the photolabel. Since fragments for loss of F, G, and V are present without the label attached, the photoprobe must label either the proline or the aspartate residue. The exact residue labeled was elucidated by the use of data obtained from X-ray studies carried out on the PTP1B protein co-crystallized with an active site directed inhibitor.

Fig. 9 shows the X-ray crystal structure of a PTP1B inhibitor in the Cys215-Ser215 PTP1B mutant (numbering based on protein sequence without the Flag) [11]. In this image, however, the residue 215 is shown as a cysteine. The three highlighted amino acids are Pro180, Asp181 (on the right of the image), and the active site residue Cys215 (lower left hand corner of active site). Of the two amino acids identified

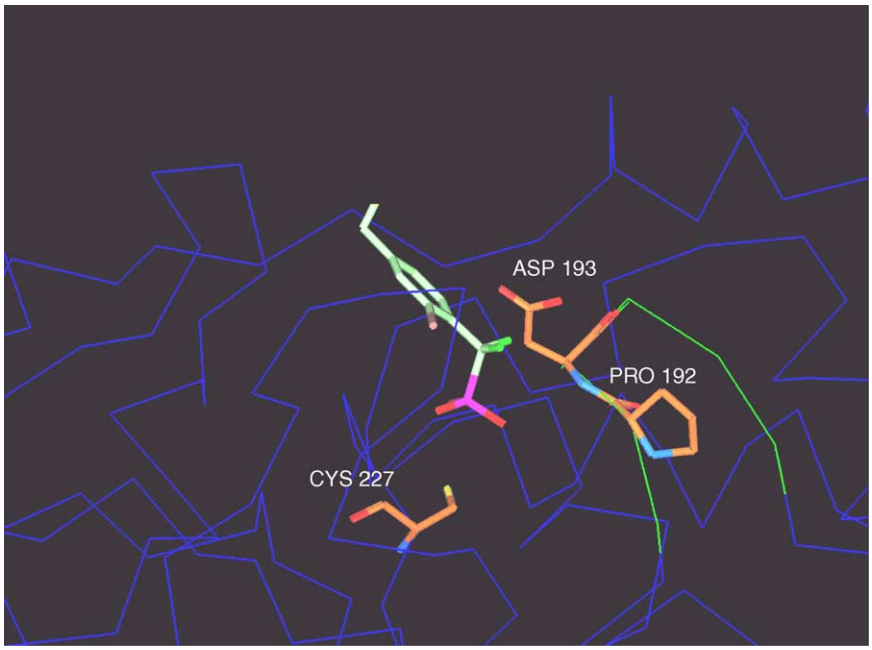

Fig. 9. X-ray crystal structure of PTP1B inhibitor with the Cys215-Ser215 PTP1B mutant showing the active site residues in close proximity with the substrate.

through MS/MS sequencing of the labeled peptide, the label can only be covalently attached to the aspartic acid residue (D181). Pro180 is facing away from the active site while the aspartic acid moiety is in close proximity with where the diazirene of the photoprobe would be expected to lie. The Asp residue is involved in both steps of catalysis and functions as a general acid in the first step and as a general base in the second [12]. The Asp, along with the Pro residue and a Trp residue, comprise the WPD loop, which is involved in substrate binding. The Asp residue is conserved across the PTPs. Given the general phosphotyrosine mimetic structure of the photoprobe, this probe may prove useful in studying other PTP enzymes.

4. Conclusions

Characterization of a new diazirene photoprobe for PTP1B indicates that the photoprobe inactivates the enzyme in a specific and active-site directed manner. The activity of the enzyme is irreversibly inhibited in the presence of the probe and UV light, and the enzyme can be protected by a potent PTP1B inhibitor. It was estimated from the mass spectral analysis of the intact protein that approximately 80% of the total protein was modified with a 1:1 stoichiometry. MS/MS analysis of the intact photo-labeled PTP1B in combination with trypsin and Glu-C digestions confirmed the specific and active site irreversible modification of PTP1B by the photoprobe after UV irradiation. The site of attachment was determined to be located on the tryptic peptide $T_{170–195}$ (EILHFHYTTWPDFGVPESPASFLNFLFK). MS/MS sequencing of the Glu-C digestion of the tryptic digest isolated the label to one of two amino acids—Pro180 and Asp181. To differentiate between the two amino acids and determine the exact site of attachment, the X-ray crystal structure of the Cys215-Ser-215 PTP1B mutant in the presence of an inhibitor was examined. It was found that the Asp181 residue was present in the active site and would be expected to lie in close proximity with the diazirene of the photoprobe. The photoprobe described may prove to be a useful tool for studying the inhibition of PTP1B by active site directed inhibitors.

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