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Synthesis and peptide incorporation of an unnatural amino acid containing activity-based probe for protein tyrosine phosphatases

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ABSTRACT

An unnatural amino acid was synthesized to incorporate a quinone methide-generating activity-based probe for protein tyrosine phosphatases (PTPs) and then integrated into a PTP1B-specific substrate. The resulting probe led to preferential labeling of PTP1B in cell lysates in the presence of PTP4A3.

Protein tyrosine phosphatases (PTPs), just like their counteracting enzymes protein tyrosine kinases (PTKs), mediate protein tyrosine phosphorylation, a central regulatory mechanism for cellular signal transduction.[1] For example, PTP1B functions as a positive regulator of signaling events associated with breast and ovarian tumorigenesis, in addition to playing a role in downregulating insulin and leptin signaling.[1] Another PTP, PTP4A3, is implicated in cell proliferation, migration and cancer metastasis.[1] Since there are more than 100 PTPs in humans, it is desirable to clearly define the partnerships between individual PTPs and phosphoproteins.[1] While substrate-trapping mutants of PTPs can be used to identify their physiologically relevant substrates,[2] no methods are available to allow direct identification of PTPs for phosphopeptide substrates. In this regard, recent progress in the proteomic applications of mechanism-based enzyme inactivators is encouraging.[3] In these applications, activity-based probes have been used to label enzymes including phosphatases.

Among activity-based PTP-targeting probes (Fig. 1), turnover-based suicidal substrates containing fluoromethylphenyl phosphate (e.g., **1** and **2**) have been intensively studied.[4] Hydrolysis of such substrates by PTPs or other phosphatases generates a highly reactive quinone methide, which then reacts with a nucleophile near the phosphatase active site. More recently, the derivatives of α-bromobenzylphosphonate (**3**)[5] and phenyl vinylsulfonate (**4**)[6] have been used as class-specific PTP probes. However, these probes are reactive towards thiols even in the absence of PTPs, especially in neutral or basic aqueous solutions, and thiols are commonly present in the reducing cellular environments. Furthermore, α-bromobenzylphosphonate undergoes solvolysis under similar conditions.[6] It has been hypothesized that the integration of a turnover-based probe into a PTP-specific phosphotyrosyl substrate will avoid the disadvantages of other types of probes as well as improve the specificity in labeling the PTP. To test this hypothesis, we began our investigation by synthesis and peptide incorporation of a quinone methide-based probe-containing amino acid, thus converting a 'miniature' PTP1B-specific substrate into a turnover-based probe (Fig. 1). We report herein our preliminary results of this specific investigation.

First of all, a novel unnatural amino acid **5**, the 3-difluoromethyl analog of phosphotyrosine, was synthesized. While both convergent and divergent routes are possible,[7] we started the synthesis with orthoformylation of the tyrosine phenol moiety,[8] followed by routine functional group transformations[4,9] (Scheme 1).

The 'miniature' substrate **6**, or, when converted into a PTP-resistant form, inhibitor **7**, is highly efficient and selective for PTP1B, and exhibits a PTP1B-interacting pattern that is typical of a physiologically relevant or optimal PTP1B phosphopeptide substrate.[9] Then the presumably PTP1B-specific probe **8** was assembled via solid phase synthesis, that consisted of a PTP-probing component, derived from the 'miniature' PTP1B substrate, and a biotin for Western blot detection by streptavidin-horse radish peroxidase (HRP), with the two separated from each other by a polyamide-polyether linkage. The flexible PEG-like linkage was used to minimize the po-

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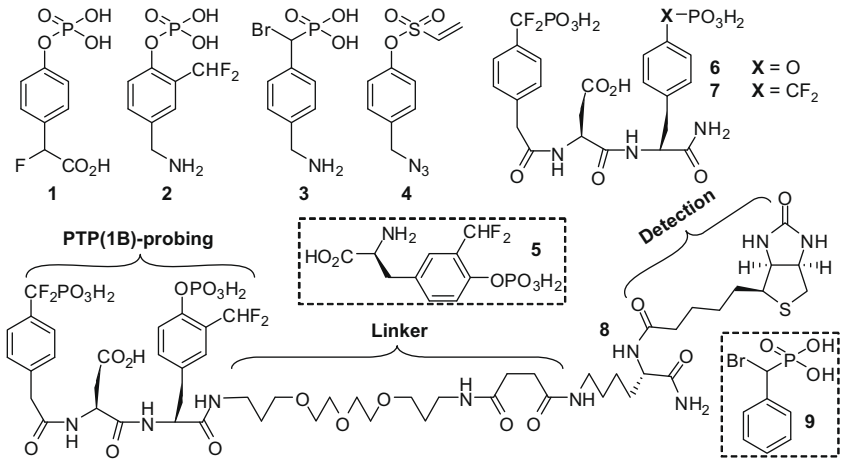
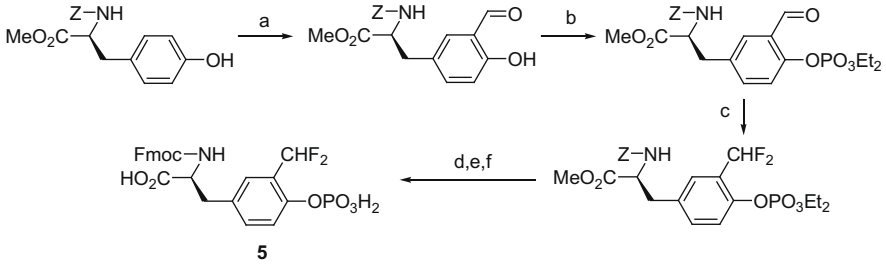


Figure 1. Design of an activity-based probe **8** that is presumably specific for PTP1B: PTP(1B) probing component is linked to biotin via a flexible linker. **1**–**4**, structures of precursors or components of previously studied activity-based probes for PTPs; **5**, the 3-difluoromethyl analog of phosphotyrosine; **6** and **7**, highly efficient and selective PTP1B substrate and inhibitor, respectively; and **9**, a PTP inactivator.



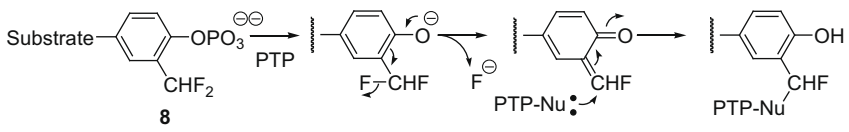
Scheme 1. Synthesis of Fmoc-protected unnatural amino acid **5**. Reagents and conditions: (a) anhydrous $MgCl_2$, Et_3N, $(CHO)_n$, acetonitrile, reflux, 18 h, 43%; (b) Et_3N, $ClPO_3Et_2$, CH_2Cl_2, ice bath to room temperature, overnight, 85%; (c) Et_2NSF_3 (DAST), CH_2Cl_2, ice bath to room temperature, overnight, 64%; (d) TMSBr, CH_2Cl_2, ice bath to room temperature, 16 h; (e) Fmoc-OSu, 1,4-dioxane/aqeous $NaHCO_3$, 3 h; (f) LiOH, THF/water, ice bath, 1.5 h; 61%.

tential interference between biotin and the PTP-probing component.

When **8** interacts with a PTP, it will be dephosphorylated and activated, producing a highly reactive quinone methide, which then reacts with a nearby nucleophile, such as a thiolate. If this nucleophile originates from the PTP, that is indeed in the proximity of the newly-generated quinone methide product, then such a reaction leads to crosslinking of the probe with the PTP (Scheme 2). Since the probe **8** carries specificity for PTP1B, that is, selectively recognized by PTP1B's catalytic machinery, such labeling is expected to recapitulate comparable selectivity for PTP1B over other PTPs. To our knowledge, this is the first example of using an activity-based probe to selectively label an individual PTP in the presence of other PTPs.

To evaluate **8** as an activity-based PTP probe, two purified recombinant PTPs—that are over 100-fold different in k_{cat}/K_m for **6**—PTP1B and PTP4A3, were respectively incubated at neutral to basic pH with the probe (Fig. 2A). As shown in Figure 2B, incubation with the probe at a low concentration led to detectable biotin labeling of PTP1B but not of PTP4A3. However, at a higher concentration of **8**, both PTP1B and PTP4A3 could be labeled. Such labeling is PTP activity-dependent, since incubation of the probe with dPTP1B, the catalytically deficient C215S mutant of PTP1B, did not show any detectable signal. As expected, the probe-PTP covalent complexes run at slightly higher molecular weights than the unmodified PTPs. Multiple bands, that indicated multiple labeling of a single protein molecule, were detected at the higher concentration of **8** in both PTP1B and PTP4A3 labeling experiments. This result is consistent with the previous observations in that the diffusable quinone methide-based labeling is not highly active site-specific and may attack the N-terminal α-amino group as well as non-active-site cysteines.[4] This kind of single-site labeling may not always completely inactivate the PTP although the probe **8** showed time- and dose-dependent inactivation for PTP1B (data not shown).

To assess the selectivity of **8** for PTP detection in a complex proteome, *Escherichia coli* lysates were spiked with purified recombinant PTPs (Fig. 3A). While both PTP4A3 and PTP1B are readily detectable (Fig. 3A, the left lane), diffusable quinone methides did not result in significant nonspecific labeling of other proteins. When PTP4A3 and dPTP1B were spiked into the lysates, only PTP4A3 was detected (Fig. 3A, the right lane). This demonstrates



Scheme 2. Proposed mechanism of PTP labeling by the probe **8** (adapted from Ref. 4).

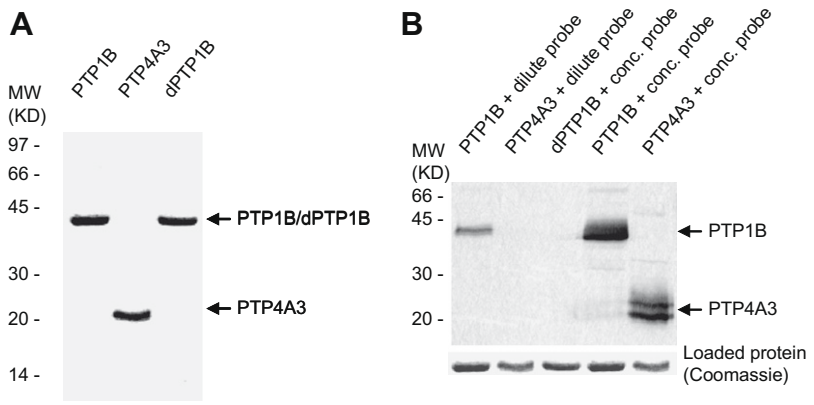


Figure 2. Labeling of purified recombinant PTPs—PTP1B, PTP4A3 and dPTP1B (4 μg each). (A) SDS–PAGE and coomassie staining of purified recombinant PTPs. (B) Western blot using streptavidin-HRP after 1 h incubation with 20 μM (dilute) or 200 μM (conc.) of the probe **8**.

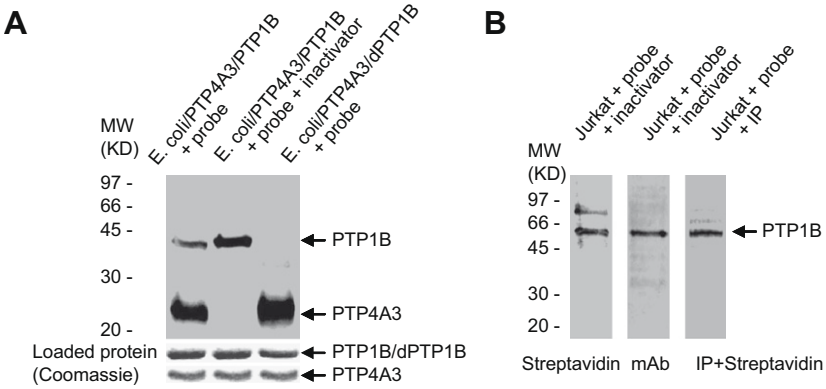


Figure 3. Labeling of PTPs by 0.8 mM of the probe 8 in cell lysates in the absence or presence of 6 mM of the general PTP inactivator 9. (A) *E. coli* lysates (70 μg total proteins) spiked with 4 μg each of PTP4A3 and PTP1B/dPTP1B at 1 h incubation, as detected by Western blot using streptavidin-HRP. (B) Jurkat cell lysates (25 μg total proteins) at 5 h incubation, as detected by Western blot using streptavidin-HRP (left lane), Western blot using monoclonal antibody to PTP1B (mAb) + secondary antibody-HRP (middle lane), and PTP1B immunoprecipitation (IP) + Western blot using streptavidin-HRP (right lane), respectively.

that the labeling by the probe 8 is selective for active PTPs in the presence of the inactive PTP and other endogenous bacterial proteins.

It was of interest to examine whether **8** could distinguish PTP1B from PTP4A3 or other PTPs. Since high doses of the probe **8** would otherwise eventually label any coexisting PTPs, we introduced a nonspecific, competing but non-detecting PTP inactivator **9** (Fig. 1) into our labeling experiments, reasoning that signals resulting from less specific PTPs would be more suppressed.[10] We chose α-bromobenzylphosphonate **9** because of its ready synthetic availability.[11] It was not attached to biotin and hence it is non-detecting in our system. Indeed, with the addition of **9**, PTP1B was readily and preferentially detected in the presence of PTP4A3 in *E. coli* lysates (Fig. 3A, the middle lane). This implies that selective labeling of a PTP in the presence of other PTPs can be facilitated by nonspecific suppression of general PTP activities.

To confirm the above perception, we decided to test **8** against a mammalian proteome that is known to contain PTP1B and multiple other PTPs. To this end, Jurkat cell lysates were studied (Fig. 3B) by the combination of **8** and **9**. Two major bands were observed (Fig. 3B, the left lane), with the stronger signal at the lower molecular weight corresponding to PTP1B, as detected by a monoclonal antibody (Fig. 3B, the middle lane). The identity of PTP1B was further confirmed by PTP1B enrichment from immunoprecipitation using the PTP1B-specific monoclonal antibody and subsequent Western blot using strepavidin-HRP (Fig. 3B, the right lane). At this point, it is not clear whether the other band represents another phosphatase having substrate specificity comparably to PTP1B or indeed a partner protein that is in complex with PTP1B (and hence presumably subject to diffusion-dependent labeling). Nonetheless, further identification is possible with affinity-enrichment, limited proteolysis and LC–MS analysis.

In conclusion, we present here a novel phosphotyrosine analog that incorporates a PTP activity-based probe, which in turn can be incorporated into an individual PTP-specific substrate, leading to the observed specificity in labeling the PTP in the presence of other PTPs. Whereas peptide substrates coupled with small molecule crosslinkers have been used for mechanism-based labeling of specific protein kinases,[12] our results imply that phosphotyrosyl peptide substrates combined with activity-based probes and general suppressors can be used to label specific PTPs responsible for their dephosphorylation. More detailed work on the application of this and other related probes are ongoing and will be reported in due course.

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