Published in final edited form as:

Chembiochem. 2017 October 05; 18(19): 1950–1958. doi:10.1002/cbic.201700253.

Activation of engineered protein tyrosine phosphatases with the biarsenical compound AsCy3-EDT₂

Wai Cheung Chan, Gregory S. Knowlton, and Anthony C. Bishop*

^aAmherst College, Department of Chemistry, Amherst, Massachusetts 01002

Abstract

Methods for activating signaling enzymes hold significant potential for the study of cellular signal transduction. Here we present a strategy for engineering chemically activatable protein tyrosine phosphatases (*act*PTPs). To generate *act*PTP1B, we introduced three cysteine point mutations in the enzyme's WPD loop. Biarsenical compounds were screened for the capability to bind *act*PTP1B's WPD loop and increase its phosphatase activity. We identified AsCy3-EDT₂ as a robust activator that selectively targets *act*PTP1B in proteomic mixtures and intact cells. Introduction of the corresponding mutations in T-cell PTP also generates an enzyme (*act*TCPTP) that is strongly activated by AsCy3-EDT₂. Given the conservation of WPD-loop structure among the classical PTPs, our results potentially provide the groundwork of a widely generalizable approach for generating *act*PTPs as tools for elucidating PTP signaling roles as well as connections between misregulated PTP activity and human disease.

Graphical abstract

Turn it up: Cysteine point mutations on the WPD loop generate protein tyrosine phosphatase (PTP) mutants that can be target-specifically activated with the small molecule AsCy3. Due to the strict conservation of the WPD loop across the classical PTP family, this strategy for achieving direct chemical control over enzyme activity may be applicable to a range of signaling proteins.

Keywords

AsCy3; enzyme activation; enzymes; protein engineering; protein tyrosine phosphatases

Introduction

The protein tyrosine phosphatases (PTPs), which dephosphorylate phosphotyrosine residues in protein substrates, constitute a large family of signaling enzymes whose activity is ubiquitously misregulated in human disease.^[1] Selective modulators of PTP activity are therefore desirable tools for signal-transduction research and are sought after as potential therapeutic candidates.^[2] Unfortunately, PTP-targeted small-molecule discovery has proven notoriously difficult, leading to the designation of PTPs as “undruggable.”^[3]

*Corresponding author. Tel.: +1-413-542-8316; Fax: +1-413-542-2735; acbishop@amherst.edu.

Many PTPs are tumor-suppressor proteins,^[4] making the superfamily particularly attractive for targeted *activation* strategies: compounds that can activate specific PTPs in a cellular context could represent important tools for investigating the mechanisms of tumor suppression. However, the chemical challenges for identifying PTP activators are especially daunting, as there is no established activation site in the PTP domain to target. Although the potential power of PTP activators is clearly recognized,^[4b,5] very little literature on small-molecule PTP activators exists.^[5] One study exemplifies the difficulty in attempting to find such activators: Matilla and co-workers carried out a high-throughput screen of over 64,000 compounds to identify activators of T-cell PTP (TCPTP).^[6] Even from such an exhaustive screen, the TCPTP agonists identified, spermidine and mitoxantrone, are relatively weak activators that have an array of non-TCPTP-specific activities.^[7]

In light of the dearth of effective activators of naturally occurring PTPs, we are attempting to develop methods for the generation of activator-sensitized PTPs: engineered protein phosphatases whose activities can be potently and specifically augmented with small molecules. Most known strategies for engineered-enzyme activation utilize chemical rescue, in which introduction of an enzymatic defect into a target enzyme is coupled with administration of a small molecule that restores activity.^[8] In contrast, we seek to develop activator-sensitized PTPs that retain the basal activity and regulatory-control mechanisms of the corresponding wild-type PTP in the absence of a small-molecule activator. Along these lines, we recently reported that a biarsenical compound (FAsH-EDT₂) can directly activate a mutant of the PTP SHP2 through disruption of autoinhibitory interactions between SHP2's N-terminal Src-homology 2 (SH2) domain and its PTP domain.^[9] Unfortunately, the strategy developed for SHP2 activation is not broadly applicable across the PTP family, as only two of the 37 human classical PTPs utilize SH2-mediated autoinhibition.^[4a]

In the current study, we sought to engineer activatable PTPs that retain wild-type-like basal activity by targeting a structural feature that is conserved across the entire family of classical PTPs—namely, the WPD loop. We show that the biarsenical compound AsCy3-EDT₂^[10] can be used to potently and specifically activate a WPD-loop variant of PTP1B. The activatable PTP1B (*act*PTP1B) contains three cysteine point mutations at non-conserved positions of the WPD loop. Upon administration of AsCy3-EDT₂, *act*PTP1B is rapidly activated through a combination of catalytic rate constant (k_{cat}) augmentation and Michaelis constant (K_M) reduction. Importantly, the highly conserved structure of the WPD loop suggests that other *act*PTPs may be engineered based on the *act*PTP1B prototype, and we show here that the activation approach can be readily applied to a second phosphatase, T-cell PTP (TCPTP).

Results and Discussion

Design of *act*PTP1B

We have previously shown that targeting FAsH-EDT₂ to a two-cysteine insertion in PTP-PEST can be used to rescue activity of the engineered enzyme.^[8f] The PTP-PEST chemical-rescue strategy is, however, limited by a number of factors: for example, the approach cannot be generalized to other PTPs, and the rescue of PTP-PEST's activity is observed only with an artificial small-molecule PTP substrate (data not shown). Moreover, the PTP-PEST

sensitizing motif comprises only two cysteines, as opposed to the tri- or tetra-cysteine motif that would be needed for targeting with specificity in a complex proteome.^[11]

To generate a potentially activatable PTP1B mutant that contains three cysteine point mutations and would potentially retain basal activity in the absence of biarsenicals, we avoided peptide insertions and focused on site-directed mutagenesis at three non-conserved positions in the WPD loop of human PTP1B (encoded by *PTPNI*, amino acid positions 184, 186, and 187). (It is not possible to introduce a complete tetra-cysteine motif within the WPD loop without either using insertions or mutating conserved residues.) We generated the triple cysteine mutant V184C/E186C/S187C PTP1B (*act*PTP1B, Figure 1A; see Figure S1 in the Supporting Information for the complete sequences of all PTPs utilized in this study) and found that *act*PTP1B expressed well from *E. coli*. The mutant enzyme was isolated as a six-histidine-tagged protein at a purity level that is indistinguishable from that of wild-type PTP1B (Figure S2 in the Supporting Information).

Identification of AsCy3-EDT₂ as an activator of *act*PTP1B

To determine if the enzymatic activity of *act*PTP1B could be modulated by binding to biarsenicals, we incubated *act*PTP1B with four commercially available compounds, FAsH-EDT₂, ReAsH-EDT₂, AsCy3-EDT₂, and AsCy5-EDT₂ (Figure 1B).^[10,12] (For brevity, the “-EDT₂” is henceforth omitted from biarsenical names.) Activity assays using the small-molecule PTP substrate *para*-nitrophenylphosphate (*p*NPP) revealed that all four biarsenicals induce measurable increases in activity when compared to a vehicle-only control, with the cyanine-based compounds AsCy3 and AsCy5 producing stronger activation than FAsH or ReAsH (Figure 1C). Pre-incubation with 500 nM AsCy3 yielded the most dramatic increase in *act*PTP1B activity (approximately 600%), while AsCy5, a compound that appears in the patent literature,^[12c] but has not previously been used in published research, also proved to be a robust activator (approximately 500%). By contrast, wild-type PTP1B was not activated by pre-incubation with either AsCy3 or AsCy5 (Figure S3 in Supporting Information). Since AsCy3 was found to be the strongest activator of *act*PTP1B, it was selected for further characterization.

Dose and time dependence of AsCy3-induced activation

Next, we characterized the dose dependence of AsCy3-induced *act*PTP1B activation (Figure 2A). Following incubation of *act*PTP1B with varying concentrations of AsCy3, we observed a robust and potent dose response that plateaued at approximately 1 μ M compound and 10-fold activation. At lower concentrations, relative activity steadily increased with increasing AsCy3; even at the lowest concentration tested (125 nM), a reaction rate more than double that of the DMSO control was observed.

To assess the rate at which AsCy3 binds to *act*PTP1B's engineered WPD loop, we next measured the time dependence of activation. To this end, we incubated *act*PTP1B for various time periods with AsCy3 (2 μ M), then tested for increased activity. As shown in Figure 2B, the AsCy3-induced activation of *act*PTP1B was rapid. Strong activation was observed even after only 5 minutes of pre-incubation and the assay's maximum activation was achieved within 10 minutes.

Michaelis-Menten analysis of *act*PTP1B activation

Having optimized dosage and incubation times for AsCy3-induced activation of *act*PTP1B, we next aimed to compare the kinetic parameters of the activated enzyme with those of unactivated *act*PTP1B (Figure 3A and Table 1). We also directly compared the activity of untreated *act*PTP1B with that of wild-type PTP1B to assess the effect, if any, of the cysteine mutations on PTP1B's inherent enzymatic activity. We found that *act*PTP1B does possess moderately reduced catalytic activity, as compared to the wild-type, in the absence of biarsenical. The k_{cat} value of *act*PTP1B is, however, within a factor of the two of the wild-type value (Table 1), suggesting that the enzyme is capable of carrying out its catalytic functions in the absence of added biarsenicals.

In the presence of AsCy3, *act*PTP1B's catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) increases dramatically (Figure 3A and Table 1). Pre-treatment with 2 μM compound gives rise to both a 4.0-fold increase in k_{cat} and a 5.2-fold decrease in K_{M} —in combination, a 21-fold increase in $k_{\text{cat}}/K_{\text{M}}$. It is also noteworthy that AsCy3-treated *act*PTP1B is strongly activated even when compared to the wild-type enzyme. In the presence of AsCy3, *act*PTP1B displayed a 6.5-fold higher $k_{\text{cat}}/K_{\text{M}}$ than wild-type PTP1B (Table 1), establishing it as truly “activatable.” To our knowledge, this is the first demonstration that PTP1B's catalytic activity can be increased above wild-type levels through application of a small-molecule.

Activity of *act*PTP1B on a phosphopeptide substrate

The previously described experiments were all carried out with *p*NPP as substrate. The cellular substrates of PTPs, however, are phosphorylated proteins; to be useful as a probe of the cellular roles of PTP1B activity, AsCy3 must be capable of increasing *act*PTP1B's catalytic rate regardless of the substrate. To test whether *act*PTP1B's activation is substrate-dependent or independent, we measured the effect of AsCy3 on the enzyme when acting on the phosphopeptide DADE(pY)LIPQQG, which corresponds to an autophosphorylation site on the epidermal growth factor receptor, a physiological substrate of PTP1B.^[13] We found that AsCy3-induced activation of *act*PTP1B is substrate-independent: the presence of AsCy3 induces an increase of approximately 2-fold in *act*PTP1B's activity when it is incubated with 100 μM DADE(pY)LIPQQG (Figure 3B). It should be noted that the level of activation with the phosphopeptide substrate appears to be more modest than measured with *p*NPP (although the activation levels are not directly comparable, as the phosphopeptide assays are not run under Michaelis-Menten conditions). Future experiments will be required to determine whether activation levels for various phosphopeptides are constant or variable. Regardless, our results demonstrate that AsCy3 is capable of directly augmenting the activity of *act*PTP1B with a physiologically relevant phosphopeptide substrate.

Targeting *act*PTP1B with AsCy3 in proteomic mixtures and intact cells

A key criterion for a small-molecule tool to be useful in chemical biology is the ability to effectively engage its biomolecular target in the context of complex proteomes. We therefore investigated AsCy3's efficacy in specifically targeting and activating *act*PTP1B in a crude cell lysate. After incubating lysates from cells that express *act*PTP1B with various concentrations of AsCy3 (or DMSO control), we assayed each mixture for PTP activity with *p*NPP. (Since *E. coli* cells do not naturally express PTPs, there is little to no measurable

background activity from the other proteins in the lysate (data not shown).) We found that AsCy3-induced activation of *actPTP1B* was remarkably robust in the context of a complex proteome (Figure 4A). A dose-dependence experiment revealed strong activation (over 10-fold) that plateaus at 2 μ M AsCy3, a concentration only two times higher than the dosage required to achieve maximum activation with purified protein (Figure 2A).

Although the experiments shown in Figure 4A demonstrate target activation, PTP assays do not directly address the level of *specificity* with which AsCy3 engages *actPTP1B* in a complex proteome. To visualize AsCy3's potential off-target interactions we took advantage of the compound's fluorogenicity.^[10] After incubating crude lysates with varying concentrations of AsCy3, we separated the lysate proteins by SDS-PAGE and visualized AsCy3-bound proteins with UV light. Remarkably, each lane deriving from AsCy3-treated *actPTP1B*-expressing lysates displayed only one band, which appears at a position corresponding to the molecular weight of *actPTP1B* (41 kD, Figure 4B). A single band is observed even after incubation with 26 μ M AsCy3, a concentration an order of magnitude higher than the concentration required to achieve maximal PTP activation (2 μ M, Figure 4A). The absence of off-target bands in the SDS-PAGE experiment suggests that AsCy3 is capable of engaging *actPTP1B* with exquisite specificity, even in the context of a complex proteomic mixture.

The cell permeability of AsCy3 is not well documented, and the compound contains two negatively charged sulfonic-acid moieties, which would presumably hinder its ability to cross cell membranes. Nevertheless, there is some published indication that AsCy3 can penetrate bacterial cells,^[14] leading us to investigate whether the compound could target and activate *actPTP1B* in *E. coli*. We incubated *actPTP1B*-expressing cell suspensions for 30 minutes with varying concentrations of AsCy3, thoroughly washed away free biarsenical from the cells, lysed the cells, and tested the resulting solutions for PTP activity. Consistent with previous results on purified protein and lysates, we found that the presence of AsCy3 robustly augmented *actPTP1B*'s activity in a dose-dependent manner when treatment was performed in intact cells (Figure 4C). Visualization of the AsCy3-bound proteins in these lysates confirmed that AsCy3 had penetrated the cell and engaged *actPTP1B*, without detectably binding any off-target proteins (Figure 4D). A control experiment with cells expressing wild-type PTP1B showed no PTP activation (Figure 4C) or AsCy3-bound proteins (Figure 4D), even after treatment with an AsCy3 concentration (50 μ M) twice that of the highest used to treat *actPTP1B*-expressing cells (25 μ M).

Although the experiments described above show that AsCy3 engages and activates *actPTP1B* in intact cells, the data also suggest room for improvement with regard to the compound's cell permeability. In the cellular dose-dependence experiments, no plateauing of either activation (Figure 4C) or *actPTP1B* binding (Figure 4D) was observed at concentrations as high as 25 μ M. Since an activation plateau was reached at much lower concentrations in lysates (Figure 4A), the cellular results suggest that the bacterial cell membrane and/or wall are significantly hindering the ability of AsCy3 to enter the cytoplasm. Future experiments with AsCy3 derivatives that possess the Cy3 backbone but lack the negatively sulfonic-acid groups^[14] will be necessary to assess whether the cell

permeability of AsCy3 can be boosted without compromising the compound's activating potency.

***ac*fTCPTP: Rationale and design**

As a preliminary attempt to test the generalizability of our PTP-activation approach, we selected human TCPTP (encoded by *PTPN2*). TCPTP is highly homologous to PTP1B (72% catalytic-domain identity),^[15] and the WPD loops of the two enzymes have identical sequences, suggesting that a WPD-loop engineering strategy developed on PTP1B may be readily applied to TCPTP (Figure 5A).

TCPTP is also an important biological target for activation in its own right. The enzyme's PTP activity is implicated in the negative regulation of an array of signaling pathways that lead to oncogenesis when inappropriately stimulated.^[16] Therefore TCPTP activators could be useful, both for probing the role of TCPTP activity in pathogenesis and for validating TCPTP as a potential activation target for therapeutic intervention.^[6,17] Moreover, TCPTP is one of the few PTPs for which an activator of the wild-type enzyme has been reported.^[6] The precedent compound, mitoxantrone, therefore provides a useful comparison point for the evaluation of novel TCPTP activation strategies.^[17]

To engineer a potentially activatable TCPTP mutant, we aligned the WPD loops of PTP1B and TCPTP and generated the triple cysteine TCPTP mutant that corresponds to *ac*fPTP1B: V185C/E187C/S188C TCPTP (*ac*fTCPTP, Figure 5A). As observed with *ac*fPTP1B, *ac*fTCPTP expressed well, and was obtained at a purity level indistinguishable from the wild-type enzyme (Figure S4 in the Supporting Information).

Identification of AsCy3 as an activator of *ac*fTCPTP

To determine if the activity of *ac*fTCPTP could be increased by biarsenicals, we incubated the enzyme with FAsH, ReAsH, AsCy3, and AsCy5. We found, in strong agreement with the *ac*fPTP1B precedent, that pre-incubation with AsCy3 and AsCy5 (1 μ M) gives rise to robust augmentation of the enzyme's activity (approximately 450%), whereas the increases induced by FAsH and ReAsH are relatively small (Figure 5B). Although the Cy3 and Cy5 derivatives gave activation levels that were essentially identical, we selected AsCy3 for further characterization of *ac*fTCPTP to facilitate direct comparisons with our previously obtained results on *ac*fPTP1B.

Characterization of AsCy3-induced activation of *ac*fTCPTP

We next characterized the dose dependence of AsCy3-mediated activation. We also sought to compare the level of *ac*fTCPTP activation with that induced by the previously identified activator of wild-type TCPTP, mitoxantrone.^[17] Under the conditions of our enzymatic assays, neither mitoxantrone (10 μ M) nor AsCy3 (1 μ M) induced any detectable increase in activity of wild-type TCPTP (Figure 6A). By contrast, AsCy3 treatment of *ac*fTCPTP gave rise to a strong and dose-dependent activity increase that plateaued at approximately 500 nM (Figure 6A). Even a concentration as low as 62.5 nM (approximately twice the concentration of enzyme in the assay) yielded robust activation.

Michaelis-Menten analysis of wild-type TCPTP, as well *act*TCPTP in both the absence and presence of AsCy3, revealed that the activated enzyme achieves a kinetic efficiency that is increased approximately 8-fold as compared to non-treated *act*TCPTP and 4-fold as compared to the wild-type enzyme (Figure 6B and Table 2). A time-dependence assay further revealed that the rate at which AsCy3 activates *act*TCPTP is too fast to be measured under our conditions, with the activation being essentially complete after a 1-minute pre-incubation (Figure 6C). Finally, we found that AsCy3 is capable of activating *act*TCPTP with a phosphopeptide substrate (Figure 6D). In sum, the biochemical analyses of AsCy3-induced activation of *act*PTP1B and *act*TCPTP mirror each other very closely, providing evidence in support of the hypothesis that PTP1B would be useful as a prototype for the development of an activatable TCPTP variant. Also, our results suggest that other classical PTPs, with which PTP and TCPTP share similar WPD-loop structure and function,^[18] may be readily engineered to be activatable as well.

Why AsCy3?

In light of the current AsCy3 literature, the *act*PTP peptide sequence targeted by AsCy3 in our experiments can appear perplexing. The originally reported target sequence for AsCy3 (CCKAEAACC) contains five amino-acid residues separating the cysteine pairs,^[10] and a subsequent study showed that the compound can effectively bind to peptides with ten or more residues between the cysteines.^[19] In contrast, the engineered WPD loops of the *act*PTPs reported here contain only one intervening residue between the sets of engineered cysteines (a naturally occurring proline, Figures 1A and 5A). Although we do not currently understand the mechanism of AsCy3-induced activation of *act*PTPs in atomic detail, we hypothesize that the key difference between our “short” target sequence and the previously published “long” AsCy3-binding motifs is that the short sequence lies in the PTP domain’s WPD loop—a structured, extended loop of a folded protein—whereas previous studies on AsCy3-binding motifs were carried out with unstructured peptides.

To better understand the means by which biarsenicals can target the structured WPD loop, we modeled the CPGG sequence of *act*PTP1B in the context of a previously solved PTP1B crystal structure.^[20] The model of the tri-cysteine motif within the extended WPD-loop conformation predicts the distance between the sulfur atom of C184 and those of C186 and C187 to be approximately 10.3 Å and 11.4 Å, respectively (Figure 7). These distances are too large to be effectively spanned by either FAsH or ReAsH (Figure 1B), which have inter-arsenical distances of approximately 5 Å. By contrast, AsCy3 has an inter-arsenical distance of approximately 14 Å in its planar conformation, as well as rotatable bonds between its arsenical units. Since AsCy3 has a longer “wingspan” and is conformationally more flexible than FAsH and ReAsH, it can presumably adopt a conformation that is capable of engaging all three cysteines residues of an *act*PTP’s engineered WPD loop more effectively than the smaller biarsenicals.

More broadly, our results with *act*PTPs suggest that AsCy3 (as well as AsCy5) may possess wide-ranging utility in future applications that utilize biarsenicals to control protein function. As shown by our data, the cyanine-based compounds, which are relatively underexplored as compared to the “default” biarsenicals FAsH and ReAsH,^[21] may be able

to effectively bind a broad range of tri- and tetra-cysteine motifs, due to their size and flexibility. AsCy3 and AsCy5, therefore, may prove to be key facilitators for the development of future biarsenical-sensitization strategies on protein families beyond the PTPs.

Conclusion

Small-molecule activators could potentially prove equally useful as inhibitors for interrogating cell-signaling networks. However, few methods for direct chemical activation of engineered proteins have been described. We have demonstrated that introduction of a tri-cysteine motif into the WPD loop of PTP1B is sufficient to render the enzyme activatable by AsCy3, a biarsenical small molecule. AsCy3-induced activation of the engineered PTP1B is potent, dose-dependent, substrate-independent, and effective in both complex proteomic mixtures and intact cells. Moreover, we show that the activator-sensitizing motif developed on PTP1B can be readily transferred to a homologous phosphatase, TCPTP, to generate a TCPTP variant that is potently activated by AsCy3. Our findings provide a novel and generalizable strategy for the chemical activation of PTPs, affording tools to potentially facilitate the chemical-genetic analysis of PTP signaling roles in cell biology and human pathology.

Experimental Section

General and materials

“PTP Activity (%)” is defined as the initial velocity of a PTP reaction in the presence of a biarsenical divided by the initial velocity of a vehicle-only control, which is defined as 100% activity. Error bars and “±” values represent the standard deviations of at least three independent measurements. Biarsenical compounds were purchased from Toronto Research Chemicals and used without further purification. Mitoxantrone was produced by Selleck Chemical, purchased from Fisher Scientific, and used without further purification.

Cloning and mutagenesis of PTP-encoding genes

The plasmids encoding His₆-tagged PTP1B^[22] and TCPTP^[23] have been previously described. Site-directed mutations were introduced using the Quikchange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Desired mutations were confirmed by sequencing.

Protein expression and purification

PTP1B, *act*PTP1B, TCPTP, and *act*TCPTP were expressed and purified using HisPur Ni-NTA (Thermo Scientific) according to the manufacturer’s instructions and as previously described for other His₆-tagged PTPs.^[24] After purification, proteins were exchanged into storage buffer (3,3-dimethyl glutarate at pH 7.0, 1 mM EDTA, 1 mM TCEP), concentrated, flash-frozen in liquid nitrogen, and stored at –80 °C. Bradford assays were used to measure enzyme concentrations and SDS-PAGE was used to assess purity.

Quenched phosphatase activity assay using *p*NPP

Quenched PTP assays using *p*NPP as substrate were carried out in a total volume of 200 μ L, containing PTP buffer (50 mM 3,3-dimethylglutarate at pH 7.0, 1 mM EDTA, 50 mM NaCl), enzyme (varying concentrations: see figures), 1% DMSO or biarsenical in DMSO solution (varying concentrations), and *p*NPP (added after varying pre-incubation times and at varying concentrations) at 22 °C. PTP reactions were quenched by the addition of 40 μ L of 5 M NaOH, and the absorbances (405 nm) of 200 μ L of the resulting solutions were measured. Kinetic constants were determined by fitting the data to the Michaelis-Menten equation using SigmaPlot 12.3.

Continuous phosphatase activity assay using *p*NPP

After pre-incubations of varying times (see Figure 6C), PTP reactions were started by the addition of *p*NPP (1.0 mM) to solutions containing PTP buffer (see above), *act*TCPTP (64 nM), and AsCy3 (1 μ M) or DMSO (1%). The absorbances (405 nm) of 200 μ L of the resulting solutions were measured continuously for three minutes and the slopes of the lines deriving from AsCy3-treated enzyme were compared to the corresponding DMSO controls.

Continuous phosphatase activity assay with phosphopeptide substrate (DADEpYLIPQQG)

PTP kinetic assays with the phosphopeptide DADEpYLIPQQG were carried out by measuring increasing absorbance at 282 nm, essentially as described.^[25] Assays were performed at 22 °C in a total reaction volume of 180 μ L in peptide buffer (50 mM 3,3-dimethylglutarate at pH 7.0, 50 mM NaCl), with AsCy3 (varying concentrations) or DMSO (1%) and the appropriate PTP (varying concentrations). Reactions were started by the addition of DADEpYLIPQQG (100 μ M, Biomatik).

*act*PTP1B assays with AsCy3-treated crude cell lysates

E. coli overexpressing *act*PTP1B were grown and lysed as described above, with the exception that, prior to lysis, cells were re-suspended in lysis buffer (50 mM 3,3-dimethylglutarate at pH 7.0, 1 mM EDTA, 50 mM NaCl, 1 mM TCEP). Protein concentrations of the clarified lysates were normalized and lysate PTP activities were assessed as described above (quenched PTP assay). Total protein concentration in the PTP assays was 0.025 mg/mL.

*act*PTP1B assays with AsCy3-treated *E. coli* cells

BL21(DE3) *E. coli* cells overexpressing *act*PTP1B were grown and harvested as described above, re-suspended in lysis buffer (see above), and incubated with either AsCy3 (varying concentrations) or DMSO for 30 minutes at 22 °C. After incubation, the cells were pelleted, the supernatant was removed, and the cells were washed twice with lysis buffer. Cells were then lysed by sonication, and PTP activity in the clarified lysates was determined as described above (quenched PTP assay). Reaction rates were normalized according to the concentration of total protein in each sample.

SDS/PAGE and visualization of AsCy3-binding proteins

Cell lysate experiment (Figure 4B): Lysates from *E. coli* overexpressing *actPTP1B* were prepared as described above and AsCy3 (varying concentrations) was added to lysate solutions containing 4 µg total protein in a volume of 14 µL. After a 30-minute incubation lysate proteins were separated by SDS-PAGE gel and visualized under broadband UV light. Novex Benchmark Fluorescent Protein Markers were used for the determination of protein molecular weights. *Intact-cell experiment* (Figure 4D): *E. coli* overexpressing *actPTP1B* were grown, treated with AsCy3, washed, and lysed as described above. Lysate proteins were separated by SDS-PAGE and AsCy3-bound proteins were visualized as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R15GM071388. Funding from Amherst College is also gratefully acknowledged.

References

1. a) Hunter T. Cell. 2000; 100:113–127. [PubMed: 10647936] b) Zhang ZY. Curr Opin Chem Biol. 2001; 5:416–423. [PubMed: 11470605] c) Van Vactor D, O'Reilly AM, Neel BG. Curr Opin Genet Dev. 1998; 8:112–126. [PubMed: 9529614]
2. Blaskovich MA. Curr Med Chem. 2009; 16:2095–2176. [PubMed: 19519384]
3. Zhang ZY. Acc Chem Res. 2017; 50:122–129. [PubMed: 27977138]
4. a) Tonks NK. Nat Rev Mol Cell Biol. 2006; 7:833–846. [PubMed: 17057753] b) Julien SG, Dube N, Hardy S, Tremblay ML. Nat Rev Cancer. 2011; 11:35–49. [PubMed: 21179176]
5. Mattila E, Ivaska J. Anti-Cancer Agents Med Chem. 2011; 11:141–150.
6. Mattila E, Marttila H, Sahlberg N, Kohonen P, Tahtinen S, Halonen P, Perala M, Ivaska J. BMC Cancer. 2010; 10doi: 10.1186/1471-2407-1110-1187
7. a) Otsuka H. J Cell Sci. 1971; 9:71–84. [PubMed: 4327649] b) Evison BJ, Sleebs BE, Watson KG, Phillips DR, Cutts SM. Med Res Rev. 2016; 36:248–299. [PubMed: 26286294]
8. a) Li J, Yu JT, Zhao JY, Wang J, Zheng SQ, Lin SX, Chen L, Yang MY, Jia S, Zhang XY, Chen PR. Nat Chem. 2014; 6:352–361. [PubMed: 24651204] b) Xia Y, DiPrimio N, Keppel TR, Vo B, Fraser K, Battaiei KP, Egan C, Bystroff C, Lovell S, Weis DD, Anderson JC, Karanicolas J. J Am Chem Soc. 2013; 135:18840–18849. [PubMed: 24313858] c) Peracchi A. Curr Chem Biol. 2008; 2:32–49. d) Qiao YF, Molina H, Pandey A, Zhang J, Cole PA. Science. 2006; 311:1293–1297. [PubMed: 16513984] e) Karginov AV, Ding F, Kota P, Dokholyan NV, Hahn KM. Nat Biotechnol. 2010; 28:743–747. [PubMed: 20581846] f) Chen VL, Bishop AC. Chem Comm. 2010; 46:637–639. [PubMed: 20062887] g) Li J, Jia S, Chen PR. Nat Chem Biol. 2014; 10:1003–1005. [PubMed: 25362360] h) Camacho-Soto K, Castillo-Montoya J, Tye B, Ogunleye LO, Ghosh I. J Am Chem Soc. 2014; 136:17078–17086. [PubMed: 25409264]
9. Chio CM, Cheng KW, Bishop AC. ChemBioChem. 2015; 16:1735–1739. [PubMed: 26063205]
10. Cao HS, Xiong YJ, Wang T, Chen BW, Squier TC, Mayer MU. J Am Chem Soc. 2007; 129:8672–8673. [PubMed: 17585763]
11. Chio CM, Yu X, Bishop AC. Bioorg Med Chem. 2015; 23:2828–2838. [PubMed: 25828055]
12. a) Griffin BA, Adams SR, Tsien RY. Science. 1998; 281:269–272. [PubMed: 9657724] b) Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY. J Am Chem

- Soc. 2002; 124:6063–6076. [PubMed: 12022841] c) Mayer-Cumblidge UM, Cao H. PCT [WO 2008127768 A1 20081023]. 2008
13. Zhang ZY, Thieme-Sefler AM, Maclean D, McNamara DJ, Dobrusin EM, Sawyer TK, Dixon JE. Proc Natl Acad Sci USA. 1993; 90:4446–4450. [PubMed: 7685104]
14. Fu N, Xiong YJ, Squier TC. Bioconj Chem. 2013; 24:251–259.
15. Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK, Moller NP. Mol Cell Biol. 2001; 21:7117–7136. [PubMed: 11585896]
16. a) Bourdeau A, Heinonen KM, Brunet DV, Tailor P, Lapp WS, Tremblay ML. Topics Curr Gen. 2004; 5:185–200. b) Lee H, Kim M, Baek M, Morales LD, Jang IS, Slaga TJ, DiGiovanni J, Kim DJ. Sci Rep. 2017; 7doi: 10.1038/srep45077 c) Karlsson E, Veenstra C, Emin S, Dutta C, Perez-Tenorio G, Nordenskjold B, Fornander T, Stal O. Breast Cancer Res Treat. 2015; 153:31–40. [PubMed: 26208487]
17. Ylilauri M, Mattila E, Nurminen EM, Kapyla J, Niinivehmas SP, Maatta JA, Pentikainen U, Ivaska J, Pentikainen OT. Biochim Biophys Acta. 2013; 1834:1988–1997. [PubMed: 23856547]
18. Barr AJ, Ugochukwu E, Lee WH, King ONF, Filippakopoulos P, Alfano I, Savitsky P, Burgess-Brown NA, Muller S, Knapp S. Cell. 2009; 136:352–363. [PubMed: 19167335]
19. Alexander SC, Schepartz A. Org Lett. 2014; 16:3824–3827. [PubMed: 24999741]
20. Jia Z, Barford D, Flint AJ, Tonks NK. Science. 1995; 268:1754–1758. [PubMed: 7540771]
21. a) Pomorski A, Krezel A. ChemBioChem. 2011; 12:1152–1167. [PubMed: 21538762] b) Shen S, Li XF, Cullen WR, Weinfeld M, Le XC. Chem Rev. 2013; 113:7769–7792. [PubMed: 23808632]
22. Davis OB, Bishop AC. Bioconj Chem. 2012; 23:272–278.
23. Hoffman HE, Blair ER, Johndrow JE, Bishop AC. J Am Chem Soc. 2005; 127:2824–2825. [PubMed: 15740097]
24. Chio CM, Lim CS, Bishop AC. Biochemistry. 2015; 54:497–504. [PubMed: 25519989]
25. Zhang ZY, Maclean D, Thieme-Sefler AM, Roeske RW, Dixon JE. Anal Biochem. 1993; 211:7–15. [PubMed: 7686722]

Figure 1.

Design of a chemically activatable PTP1B mutant. (A) Sequence alignment of the WPD loops of wild-type and *act*PTP1B. (B) Chemical structures of the biarsenical compounds used in this study. (C) AsCy3 strongly activates *act*PTP1B. PTP activity of *act*PTP1B (25 nM) was measured with *p*NPP (1.5 mM, quenched assay) in the absence (DMSO) or presence of the indicated biarsenicals (500 nM) after 120-minute pre-incubations.

Figure 2.

AsCy3-induced activation of *actPTP1B* is dose-dependent and rapid. (A) Dose dependence of *actPTP1B* activation. PTP activity of *actPTP1B* (25 nM) was measured with *pNPP* (1.5 mM, quenched assay) in the absence (0) or presence of the indicated concentrations of AsCy3 after 120-minute pre-incubations. (B) Time dependence of *actPTP1B* activation. PTP reaction rates of *actPTP1B* (25 nM) samples were measured with *pNPP* (1.5 mM, quenched assay) in the absence (DMSO) or presence of AsCy3 (2 μM) after pre-incubations of the indicated times.

Figure 3.

Michaelis-Menten and phosphopeptide-dephosphorylation kinetics of AsCy3-treated *act*PTP1B. (A) Michaelis-Menten kinetics. Wild-type (wt) PTP1B (25 nM, filled circles: DMSO) and *act*PTP1B (25 nM, open circles: DMSO; triangles: AsCy3) were assayed for PTP activity with the substrate *p*NPP (indicated concentrations, quenched assay) after a 120-minute preincubation with AsCy3 (2 μM) or DMSO vehicle. The initial rates of the resulting reactions were fit to the Michaelis-Menten equation to derive the kinetic constants shown in Table 1. (B) Phosphopeptide dephosphorylation. The PTP activity of *act*PTP1B (25 nM) was measured with the phosphopeptide DADEpYLIPQQG as substrate after pre-incubation (20 minutes) with AsCy3 (2 μM , open circles) or DMSO (filled circles).

Figure 4.

AsCy3 targets and activates *act*PTP1B in complex proteomes and intact cells. (A) Activation of *act*PTP1B in cell lysates. Clarified lysates from *E. coli* expressing *act*PTP1B were incubated with the indicated concentrations of AsCy3 for 30 minutes, then assayed for PTP activity with *p*NPP (1.5 mM, quenched assay). (B) Visualization of targeted proteins from AsCy3-treated cell lysates. Clarified lysates from *E. coli* expressing *act*PTP1B were incubated with the indicated concentrations of AsCy3 for 30 minutes. The proteins from the resulting solutions were separated by SDS-PAGE and AsCy3-bound proteins were visualized using broadband UV illumination. (C) Activation of *act*PTP1B in intact cells. *E. coli* cells expressing either wild-type (wt) PTP1B or *act*PTP1B were incubated with the indicated concentrations of AsCy3 for 30 minutes, washed thoroughly and lysed. PTP activities of the resulting lysates were measured with *p*NPP (1.5 mM, quenched assay). (D) Visualization of targeted proteins from AsCy3-treated cells. Cells expressing wild-type PTP1B (wt) or *act*PTP1B (*act*) were treated with the indicated concentrations of AsCy3 for

30 minutes and lysates were prepared as described for panel C. AsCy3-bound proteins were visualized as described for panel B.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

Design of a chemically activatable TCPTP mutant. (A) Sequence alignment of the WPD loops of wild-type TCPTP and *act*TCPTP. (B) AsCy3 strongly activates *act*TCPTP. PTP activities of *act*TCPTP (32 nM) samples were measured with *p*NPP (1.5 mM, quenched assay) in the absence (DMSO) or presence of the indicated biarsenicals (1 μ M) after 60-minute pre-incubations.

Figure 6.

Activation of AsCy3-treated *acfTCPTP* is dose-dependent, rapid, and substrate-independent. (A) Dose dependence of *acfTCPTP* activation. The PTP activities of wild-type (wt) TCPTP (32 nM) or *acfTCPTP* (32 nM) were measured with *pNPP* (1.5 mM, quenched assay) in the presence of the indicated concentrations of AsCy3 or mitoxantrone (mito) after 60-minute pre-incubations. (B) Michaelis-Menten analysis of wild-type TCPTP and *acfTCPTP*. Wild-type TCPTP (32 nM, closed circles: DMSO) and *acfTCPTP* (32 nM, open circles: DMSO; triangles: AsCy3) were assayed for PTP activity with the substrate *pNPP* (indicated concentrations, quenched assay) after a 60-minute preincubation with AsCy3 (1 μM) or DMSO vehicle. The initial rates of the resulting reactions were fit to the Michaelis-Menten equation to derive the kinetic constants shown in Table 2. (C) Time dependence *acfTCPTP* activation. PTP activity of *acfTCPTP* (64 nM) was measured with *pNPP* (1.0 mM, continuous assay) in the absence (DMSO) or presence of AsCy3 (1 μM) after pre-incubations of the indicated times. (D) Phosphopeptide dephosphorylation. The PTP activity of *acfTCPTP* (32 nM) was measured with the phosphopeptide DADEpYLIPQQG after pre-incubation (10 minutes) with AsCy3 (1 μM, open circles) or DMSO (filled circles).

Figure 7. Model of *act*PTP1B's WPD loop. Starting with a solved crystal structure of PTP1B in its closed-WPD conformation (PDB: 1PTU^[20]), the WPD-loop model for *act*PTP1B was generated by replacing the side chains of V184, E186, and S187 with cysteine side chains in the UCSF Chimera software package. The indicated distances between sulfur atoms in the highest-probability rotamers were measured using the software's distance function.

Table 1

Kinetic constants of wild-type (wt) PTP1B, *act*PTP1B, and AsCy3-treated (2 μ M) *act*PTP1B assayed with *p*NPP.

enzyme	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{mM}^{-1} \text{s}^{-1}$)
wt PTP1B + DMSO	7.4 ± 2.3	4.3 ± 1.7	1.7
<i>act</i> PTP1B + DMSO	3.9 ± 1.0	7.3 ± 1.5	0.52
<i>act</i> PTP1B + AsCy3	15.7 ± 0.5	1.4 ± 0.1	11

Table 2

Kinetic constants of wild-type (wt) TCPTP, *act*TCPTP, and AsCy3-treated (1 μ M) *act*TCPTP assayed with *p*NPP.

enzyme	k_{cat} (s^{-1})	K_{M} (mM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{mM}^{-1} \text{s}^{-1}$)
wt TCPTP + DMSO	2.9 ± 0.1	3.4 ± 0.1	0.85
<i>act</i> TCPTP + DMSO	2.0 ± 0.2	4.2 ± 1.1	0.48
<i>act</i> TCPTP + AsCy3	7.0 ± 0.2	1.9 ± 0.2	3.7