Engineered Inhibitor Sensitivity in the WPD Loop of a Protein Tyrosine Phosphatase[†]

Xin-Yu Zhang and Anthony C. Bishop*

Department of Chemistry, Amherst College, Amherst, Massachusetts 01002

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ABSTRACT: Small molecules that can be used to turn off the activities of specific cellular proteins are essential tools for chemical biology. Few such compounds are known, however, and they are particularly difficult to identify for members of large protein families. Here, we present a method for insertion of a chemical "off switch" into a catalytically essential loop region (the "WPD loop") of a protein tyrosine phosphatase (PTP). Using a combination of point mutations and amino acid insertions, we have engineered variants of T-cell PTP (TCPTP) that possess cysteine-rich WPD loops. The engineered WPD loops, which contain sequences that appear in no wild-type PTP, confer upon TCPTP the ability to bind a cell-permeable small molecule (the biarsenical fluorescein derivative, FlAsH) that is not an inhibitor of wild-type PTPs. We have identified sites in TCPTP's WPD loop that can be modified to display FlAsH-binding cysteine residues without disrupting TCPTP's inherent PTP activity, as assayed with either small-molecule or phosphorylated-peptide PTP substrates. Upon addition of the FlAsH ligand, however, the activities of the mutants drop dramatically. Inhibition of the FlAsH-sensitized TCPTP mutants is rapid and specific; and strong FlAsH sensitivity was observed in mutants that contain as few as two cysteine point mutations in their engineered WPD loops. Our results show that relatively conservative substitutions can be used to engineer precise small-molecule control of PTP activity. Moreover, since all known classical PTPs utilize the WPD-loop mechanism targeted in this study, it is likely that a substantial fraction of the PTP superfamily can be sensitized through an analogous approach.

The mammalian protein tyrosine phosphatases (PTPs[1]), which catalyze the dephosphorylation of phosphotyrosine residues in protein–substrates, are an attractive family of targets for small-molecule inhibitor discovery (*1, 2*). The PTP family comprises a large and diverse set of signaling enzymes, roughly 100 of which are encoded in the human genome (*3–5*). Protein phosphorylation is, arguably, the most ubiquitous signaling mechanism in mammalian biology, and a complete understanding of signal transduction will require a full accounting of PTP function. Small molecules that specifically inhibit individual PTPs would no doubt expedite the achievement of this far reaching goal.

Introduction of non-natural inhibitor sensitivity through protein engineering can greatly facilitate the targeting of an enzyme or enzyme family with highly specific chemical inhibitors (*6, 7*), and we have made significant progress in the development of a method for engineering novel inhibitor sensitivity into PTP active sites (*8–11*). However, all active-site-directed PTP-inhibitor discovery is limited by the relatively low cellular permeability of known competitive PTP inhibitors, most of which are charged phosphotyrosine mimetics (*2, 12*). In principle, the complications inherent in the engineering of enzyme active sites could be avoided through the introduction of novel allosteric sites. At first glance, however, such an idea may seem fanciful: if novel active-site inhibitor sensitivity is difficult to engineer systematically, how could a *de novo* allosteric site possibly be introduced into a target protein? Recently, two reports have shown that insertion of a small-molecule-binding peptide into a protein domain can indeed be used to generate allosterically controllable enzyme variants (*13, 14*). These studies utilized the cell-permeable small molecule fluorescein arsenical hairpin binder (FlAsH), which is known to bind specifically to peptides containing tetra-cysteine motifs (*15, 16*). When a FlAsH-binding peptide is embedded within an enzyme's catalytic domain, addition of the FlAsH ligand can lead to activity changes that are unique to the mutant/FlAsH pair (Figure 1). Specifically, Liscovitch and co-workers generated FlAsH-sensitive variants of the bacterial antibiotic-resistance enzyme TEM-1 through insertion of an optimized FlAsH-binding peptide Cys-Cys-Pro-Gly-Cys-Cys (CCPGCC) at various regions within the enzyme (*13*). These authors also argued that their approach could constitute a general method for ligand-sensitive protein engineering. Indeed, we have recently shown that insertion of CCPGCC in the catalytic domain of an unrelated enzyme, T-cell PTP (TCPTP), is sufficient for sensitization of the phosphatase to inhibition by FlAsH (*14*).

A significant limitation of the peptide-insertion approach to enzyme sensitization lies in the radical nature of the sensitizing mutation: a six amino acid insertion. The most

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* To whom correspondence should be addressed. Telephone: (413) 542-8316. Fax: (413) 542-2735. E-mail: acbishop@amherst.edu.

[1] Abbreviations: PTP, protein tyrosine phosphatase; TCPTP, T-cell protein tyrosine phosphatase; FlAsH, fluorescein arsenical hairpin binder; *p*NPP, *p*-nitrophenyl phosphate; pY, phosphotyrosine; WT, wild-type.

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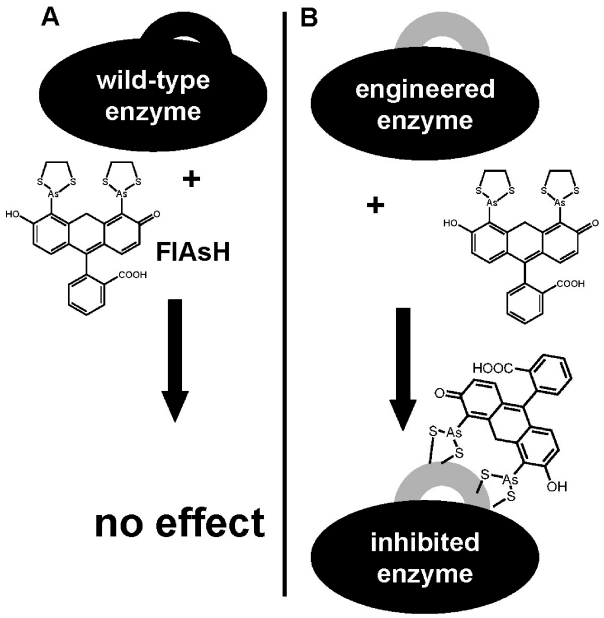


FIGURE 1: Specific enzyme inhibition via mutagenic introduction of FlAsH sensitivity. A loop region of a target enzyme (A, black) is modified to contain a cysteine-rich FlAsH-binding peptide (B, gray). FlAsH does not bind to the wild-type enzyme, whereas it specifically binds to the engineered loop and inhibits the engineered enzyme's activity.

highly FlAsH-sensitive TCPTP mutant identified in our previous work (TCPTP-187) demonstrated significantly reduced activity even in the absence of FlAsH: a 19-fold defect in catalytic-rate constant (k_{cat}) compared to wild-type TCPTP (*14*). While it is not surprising that insertion of a six amino acid peptide can have a deleterious effect on an enzyme's inherent activity, this activity loss limits the prospects of using peptide-insertion mutants as ligand-sensitive protein targets in mammalian cell-culture or *in vivo* studies, as a critical criterion for the biological usefulness of such approaches is that the engineered target can "silently" replace the function of the wild-type (*17*). (Similarly large decreases in activity were observed when CCPGCC insertion was used to generate FlAsH-sensitive variants of the bacterial antibiotic-resistance enzyme TEM-1 (*13*).)

Here we report the discovery of a WPD-loop-targeting approach for the identification of fully active and allosterically controllable TCPTP mutants. In lieu of CCPGCC insertion, we have successfully embedded FlAsH-binding peptides directly into TCPTP's canonical WPD loop (*4*). TCPTP, like all classical PTPs, can exist in either the "open" or "closed" forms, which differ predominantly in the conformation of the WPD loop (*18, 19*). The movement between open and closed WPD-loop conformations is critical for PTP activity, as potential PTP substrates access the active sites of open PTP domains, and a mechanistically critical aspartic-acid residue (the "D" of WPD) can only perform its general-acid/base functions in the closed form (*20*). When FlAsH is specifically targeted to an engineered WPD, the covalent "tethering" of the small molecule to the peptide loop can, in principle, inhibit the open/closed transition and shut down the enzyme's activity (*14*).

Using a limited cysteine-scanning strategy, we found that the PTP WPD loop is remarkably tolerant to cysteine substitutions, even at sites that are strongly conserved throughout the PTP family. Tolerated WPD-loop mutations (and small insertions) were then combined in an iterative strategy that produced a panel of di-, tri-, and tetra-cysteine TCPTP mutants. From the panel, we identified several TCPTP mutants whose activities drop dramatically upon addition of the FlAsH ligand. Importantly, these enzymes demonstrate wild-type-like activity levels in the absence of FlAsH, and FlAsH has no significant effect on the catalytic competency of wild-type TCPTP. Thus we show that highly specific PTP inhibition can be achieved in a rapid and systematic manner via targeting of the PTP WPD loop.

MATERIALS AND METHODS

Materials, Equipment, and General Procedures. The phosphopeptide substrate DADEpYLIPQQG was purchased from EMD. All DNA sequencing was carried out at the Cornell Biotechnology Resource Center. FlAsH was synthesized as described (*14–16*) and dissolved in DMSO. All *p*NPP-based PTP assays were performed in triplicate using Eppendorf Protein LoBind tubes. All error bars and "±" values represent the standard deviations of at least three independent experiments.

Mutagenesis. A plasmid encoding TCPTP-His_6, pHEH042 (~50 ng) (*10*), cloned *Pfu* 10× reaction buffer (5 μL), cloned *Pfu* DNA polymerase (1 μL, 2.5 U, Stratagene), 2 mM dNTP mix (5 μL), and water (36 μL) were combined with appropriate primers (1 μL of each at a concentration of 300 ng/μL) and placed in a temperature cycler. (See Supporting Information for primer sequences.) The reaction mixtures were subjected to one cycle of 95 °C for 30 s, then 18 cycles of 95 °C for 30 s, 45 °C for 1 min, and 68 °C for 16 min. At the end of temperature cycling, 10 U (1 μL) of *Dpn*I restriction enzyme was added, and the reaction mixture was incubated at 37 °C for 1 h. Following *Dpn*I digestion of parental DNA, the mutant DNAs were ethanol-precipitated and redissolved in 3 μL of water. The resulting DNA solutions were used to transform competent DH5α *Escherichia coli*. Plasmid preparations from the resulting ampicillin-resistant colonies were sequenced over the entire coding region of the TCPTP gene. The plasmids encoding P181C/P186C, P181C/E187C, 3C-2, 3C-3, and 3C-5 TCPTP were prepared through two rounds of mutagenesis as described above. In the second round, the P181C mutation was introduced into template plasmids encoding P186C, E187C, 2C-1, P186C/E187C, and 2C-3 TCPTP, respectively.

TCPTP Expression. TCPTP-His_6-encoding plasmids (wild-type and mutants) were transformed into BL21(DE3)-codonPLUS-RIL *E. coli* (Stratagene). Single colonies were picked and used to inoculate 500 mL LB cultures, which were grown to midlog phase at 37 °C, and induced with 0.2 mM IPTG for 16 h at 26 °C. Cells were pelleted and frozen at –80 °C. Cell lysis was achieved by incubation of the cell pellets with 10 mL of BPER (Pierce), and purifications of enzymes were carried out using SwellGel Nickel Chelated Discs (Pierce) according to the manufacturer's instructions. The protein solutions obtained were concentrated with CentriPrep Centrifugal Filter Devices (Millipore) and exchanged into pH 7.0 buffer containing 50 mM 3,3-dimethylglutarate, 1 mM EDTA, 150 mM NaCl, and 1 mM dithiothreitol. The concentrated protein solutions were flash-frozen in liquid nitrogen and stored at –80 °C. Protein

concentrations were determined by Bradford assay, and enzyme purities were estimated by SDS−PAGE.

PTP Activity Assays Using pNPP. The TCPTP activity assays with *p*NPP were carried out at 22 °C in a total reaction volume of 200 μL containing *p*NPP (0.25–10 mM) and the appropriate TCPTP enzyme (50–150 nM) in 1×PTP buffer (50 mM 3,3-dimethylglutarate at pH 7.0, 1 mM EDTA, and 50 mM NaCl). Reactions were quenched after 8 min by the addition of 40 μL of 5 M NaOH. The reaction mixtures (200 μL) were loaded onto a 96-well plate, and the absorbances at 405 nm were measured. Kinetic constants were determined by fitting the data to the Michaelis–Menten equation.

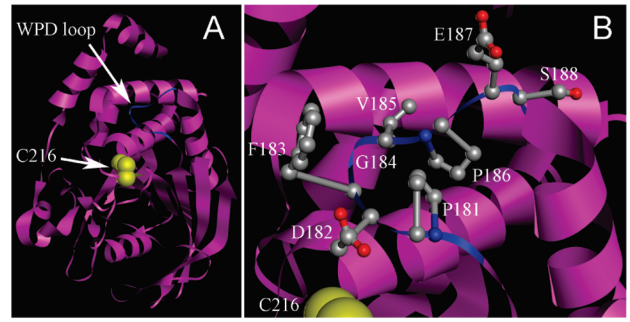
PTP Activity Assays Using DADEpYLIPQQG. Phosphopeptide-dephosphorylation assays were carried out by measuring increasing absorbance at 282 nm essentially as described (*21*). Assays were performed at 22 °C in a total volume of 140 μL and contained the following: 50 mM 3,3-dimethylglutarate pH 7.0, 125 mM NaCl, 1 mM EDTA, 70 μM DADEpYLIPQQG, and the appropriate TCPTP (20–900 nM). Kinetic constants were obtained by nonlinear regression to the integrated Michaelis–Menten equation using SigmaPlot 10.0.

PTP Activity Assays in the Presence of FlAsH. To measure the effect of FlAsH on TCPTP activity, solutions of TCPTP (2.5 μM) in 1×PTP buffer were incubated in the absence (DMSO vehicle only) or presence of FlAsH (10 μM). After 2.5 h at room temperature, the enzymatic activity in the presence and absence of FlAsH, using either *p*NPP or DADEpYLIPQQG, was assayed as described above.

Dose Dependence of FlAsH Inhibition. FlAsH-concentration-dependence assays were carried out at 22 °C in a total reaction volume of 200 μL containing *p*NPP (concentration equal to K_M for the TCPTP variant being assayed), 50 nM of TCPTP enzyme, and varying concentrations of FlAsH (DMSO only, 25, 50, 100, 150, 200, 300, 400, and 500 nM) in 1×PTP buffer. After a 2.5 h incubation of enzyme and FlAsH (or vehicle), *p*NPP was added. The reactions were quenched at appropriate time points (8–40 min) with 40 μL of 5 M NaOH, and the absorbances at 405 nm were measured. The percentage activity was obtained by dividing the absorbance in the presence of FlAsH by the absorbance of the no-FlAsH control. Apparent inhibition constants were estimated from these data using nonlinear regression analysis (see Supporting Information for details).

Time Course of FlAsH-Induced Inhibition. Inhibition time-course experiments were carried out at 22 °C in a total reaction volume of 200 μL containing *p*NPP (concentration equal to K_M for the TCPTP variant being assayed), 250 nM TCPTP enzyme, and 1 μM of FlAsH (or vehicle) in 1×PTP buffer. Enzyme and FlAsH were mixed and incubated for 1, 3, 5, 10, 15, 20, and 30 min. At the noted time points, *p*NPP was added to initiate PTP activity. The PTP reactions were quenched after 30–60 s, and the absorbances at 405 nm were recorded. The percentage activity at each time point was obtained by dividing the absorbance in the presence of FlAsH by that of a no-FlAsH control.

Fluorescence. TCPTP and FlAsH were diluted (final concentrations: 250 nM and 1 μM, respectively) in 1×PTP buffer to a total volume of 100 μL. With the excitation wavelength at 510 nm, fluorescence spectra from 520 to 560 nm at a scanning speed of 120 nm/min were recorded starting at 0.5 min after the addition of FlAsH. Fluorescence spectra were recorded once every minute for 150 min. The excitation and emission slits used were 4.5 and 10.0 nm, respectively.



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	180	183	184	185	186	187	188	189	190	191
WT	**WPD**	**F**	**G**	**V**	**P**	**E**	**S**	**P**	**A**	**S**
2C−1	WPD	F	G	V	P	**CC**E	S	P	A	S
3C−1	WPD	F	**C**	V	P	**CC**E	S	P	A	S
3C−2	W**C**D	F	G	V	P	**CC**E	S	P	A	S
4C−1	WPD	F	**C**	**C**	P	**CC**E	S	P	A	S
4C−2	WPD	**C**	**C**	V	P	**CC**E	S	P	A	S
4C−3	WPD	F	G	V	P	**CC**E	S**CC**P		A	S
P186C/E187C	WPD	F	G	V	**C**	**C**	S	P	A	S
3C−3	W**C**D	F	G	V	**C**	**C**	S	P	A	S
3C−4	WPD	F	G	V	**C**	**C**	S	P	**C**	S
4C−4	WPD	F	G	V	**C**	**C**	S	P**CC**A		S
2C−2	WPD	F	G	V	**C**	**C**E	S	P	A	S
2C−3	WPD	F	G	V	P	**CC**	S	P	A	S
3C−5	W**C**D	F	G	V	P	**CC**	S	P	A	S
3C−6	WPD	F	**C**	V	P	**CC**	S	P	A	S
E187C/S188C	WPD	F	G	V	P	**C**	**C**	P	A	S
P181C/P186C	W**C**D	F	G	V	**C**	E	S	P	A	S
P181C/E187C	W**C**D	F	G	V	P	**C**	S	P	A	S

FIGURE 2: Design of TCPTP WPD-loop mutants. (A) Three-dimensional structure of TCPTP (PDB code: 1L8K) (*37*). The WPD loop is shown in blue. For perspective, the active-site catalytic cysteine (Cys216) of TCPTP is shown in yellow. (B) Close-up view of the mutagenesis and insertion sites in the TCPTP WPD loop. Side-chain atoms are colored as follows: gray for carbon, red for oxygen, blue for nitrogen. (C) Primary-sequence alignment of the human TCPTP WPD loop with the WPD-loop mutants described in this study.

RESULTS

Design Strategy. To be potentially useful in cellular studies, a ligand-sensitivity engineering strategy must meet two important criteria: a mutation must uniquely confer ligand sensitivity upon the protein of interest, and the sensitizing mutation must not substantially compromise the inherent activity of the target protein (*11, 17*). Our previous work showed that insertion of CCPGCC adjacent to the C-terminal side of the TCPTP's canonical WPD loop (between P186 and E187, see Figure 2) was sufficient for conferring FlAsH sensitivity, suggesting that FlAsH binding may inhibit the proper movement of the WPD loop (*14*). We hypothesized that FlAsH's inhibitory effects may be even greater when a FlAsH-binding peptide is embedded directly into the WPD loop of TCPTP. In preliminary experiments, we found that insertion of cysteine residues within the strongly conserved portion of the WPD loop (between W180 and P186, see Figure 2) severely disables the resulting PTP (data not shown). These findings led us to investigate the

Table 1: Kinetic Constants of Wild-Type and Mutant TCPTPs Assayed with *p*NPP

enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})
wild-type	5.1 ± 0.19	2.5 ± 0.16	2.1 ± 0.060
P181C	9.8 ± 0.47	0.54 ± 0.057	18 ± 1.1
F183C	0.83 ± 0.024	3.1 ± 0.083	0.26 ± 0.0035
G184C	4.4 ± 0.083	1.3 ± 0.094	3.4 ± 0.19
V185C	3.9 ± 0.11	2.7 ± 0.092	1.5 ± 0.020
P186C	3.7 ± 0.17	2.5 ± 0.26	1.5 ± 0.080
E187C	3.5 ± 0.17	2.7 ± 0.20	1.3 ± 0.036
2C-1	9.5 ± 0.24	1.4 ± 0.057	6.7 ± 0.11
2C-2	0.30 ± 0.020	3.7 ± 0.33	0.082 ± 0.0030
2C-3	12 ± 0.71	1.2 ± 0.087	10 ± 0.14
P186C/E187C	1.7 ± 0.038	3.1 ± 0.042	0.53 ± 0.0053
E187C/S188C	5.4 ± 0.22	2.3 ± 0.11	2.4 ± 0.032
P181C/P186C	3.2 ± 0.10	1.9 ± 0.13	1.7 ± 0.058
P181C/E187C	6.4 ± 0.18	0.85 ± 0.059	7.6 ± 0.38
3C-1	3.3 ± 0.066	2.1 ± 0.084	1.6 ± 0.037
3C-2	2.6 ± 0.029	1.4 ± 0.018	1.8 ± 0.020
3C-3	0.13 ± 0.0072	2.7 ± 0.20	0.047 ± 0.0013
3C-4	0.72 ± 0.023	3.5 ± 0.20	0.21 ± 0.0053
3C-5	1.6 ± 0.054	1.4 ± 0.021	1.2 ± 0.032
3C-6	2.4 ± 0.029	1.1 ± 0.030	2.2 ± 0.043
4C-1	1.0 ± 0.021	2.3 ± 0.16	0.44 ± 0.023
4C-2	0.89 ± 0.015	1.7 ± 0.045	0.51 ± 0.0049
4C-3	8.7 ± 0.13	1.6 ± 0.036	5.4 ± 0.063
4C-4	0.079 ± 0.0031	3.8 ± 0.23	0.021 ± 0.00051

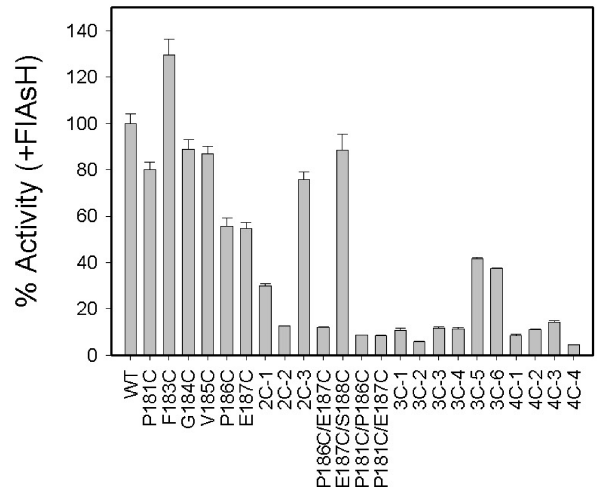

FIGURE 3: FlAsH-dependent inhibition of TCPTP WPD-loop mutants. The indicated enzymes (2.5 μM) were incubated in the absence or presence of FlAsH (10 μM), diluted, and assayed for activity with the artificial PTP substrate *p*-nitrophenyl phosphate at pH 7.0. "% Activity" represents the PTP catalytic efficiency (k_{cat}/K_M) in the presence of FlAsH divided by the control (no-FlAsH) catalytic efficiency of the same TCPTP enzyme.

tolerance of the WPD loop to site-directed mutagenesis, which could be used to embed cysteine-rich units directly into TCPTP's WPD loop while avoiding the insertion of "extra" amino acids in the loop. It has recently been shown that a linear tetra-cysteine sequence is not required for efficient FlAsH/peptide binding; in fact, FlAsH can recognize two independent CC units that are simply brought close together in space, even if they are brought together through intermolecular interactions (*22*). Thus, we devised an incremental mutagenesis approach, designed to independently test the two halves of a $C_1C_2X_nC_3C_4$ motif (i.e., C_1C_2 and C_3C_4). In principle, C_1C_2 motifs that do not disrupt PTP activity could then be combined with nondeleterious C_3C_4 motifs to yield high-activity PTP mutants that contain an intact tetra-cysteine motif. Alternatively, we wished to test if FlAsH sensitivity could be conferred through less radical mutagenic strategies, such as the introduction of tri-cysteine (CX_nCC) or even di-cysteine motifs (CX_nC).

Cysteine Mutations in TCPTP's WPD Loop. To identify cysteine-tolerant positions in TCPTP's WPD loop, we subjected it to cysteine-scanning mutagenesis. Previous alanine-scanning mutagenesis on another classical PTP (SHP1) has shown that, despite its conserved role in PTP mechanisms, the WPD loop displays a high level tolerance to alanine mutations (*23*). Our results with TCPTP, viewed broadly, are consistent with these observations and demonstrate that PTP WPD loops are remarkably tolerant of cysteine mutations, as well. We made six single-cysteine point mutants (P181C, F183C, G184C, V185C, P186C, and E187C TCPTP; see Figure 2), and found that, with the exception of F183C TCPTP (whose activity was reduced substantially compared to wild-type TCPTP, consistent with literature precedent (*24*)) these mutants had catalytic activities very close to that of wild-type TCPTP (turnover numbers within a factor of 2, see Table 1). (It has been shown previously that W180 and D182 are critical for proper PTP activity; thus, these positions were not mutated in our analysis (*20, 25*).) These single-cysteine mutant data validated a potential embedding approach, in which point mutations, or a combination of point mutations and insertions (after P186), could be used to engineer FlAsH-responsive enzymes. We had no expectation that single-cysteine mutants would display significant FlAsH-inhibition. Nevertheless, we found that two of the mutants, P186C and E187C TCPTP, did, indeed, demonstrate modest, yet significant, drops in catalytic efficiency (k_{cat}/K_M) when preincubated with FlAsH (Figure 3), suggesting that combining tolerated cysteine mutations might lead to new modes of engineered FlAsH sensitivity.

Double-Cysteine Mutants. In previous work, we found that insertion of CCPGCC between P186 and E187 was sufficient for conferring strong FlAsH-sensitivity upon TCPTP, albeit with a concomitant loss in inherent PTP activity (*14*). We reasoned that less radical insertions at this same position could potentially yield highly active and FlAsH-sensitive mutants. To test this idea, we inserted two cysteine residues between P186 and E187 to make the insertion mutant designated 2C-1 TCPTP (Figure 2C). We also inserted one cysteine at position 187, combined with point mutation of either P186 or E187, to produce 2C-2 and 2C-3 TCPTP, respectively. Finally, we avoided insertion altogether and introduced two cysteine residues in this region via point mutations: P186C/E187C and E187C/S188C TCPTP.

Kinetic characterization of this initial panel of double-cysteine mutants revealed a wide variety of effects. (For kinetic constants and FlAsH sensitivities of all TCPTP mutants, see Table 1 and Figure 3, respectively.) E187C/S188C and 2C-3 TCPTP possessed wild-type-like activity in the absence of FlAsH, but showed little to no FlAsH sensitivity. 2C-2 TCPTP demonstrated impressive FlAsH sensitivity, but its inherent catalytic efficiency in the absence of FlAsH was severely compromised with respect to wild-type TCPTP. 2C-1 and P186C/E187C TCPTP proved to be more promising: these two enzymes have turnover numbers that are only moderately higher (2C-1) or lower (P186C/E187C) than wild-type TCPTP, and they both demonstrate clear FlAsH sensitivity. 2C-1 TCPTP responds to FlAsH with a 3-fold drop in catalytic efficiency, and P186C/E187C

TCPTP's activity dropped 8-fold in the presence of FlAsH. These results were gratifying validations of the two design principles behind our "minimalist" approach: short insertions (in the case of 2C-1) can be used to replace the six amino acid insertion CCPGCC, and site-directed mutagenesis (in the case of P186C/E187C) can be used in lieu of insertion to yield wild-type-like PTPs that demonstrate significant FlAsH sensitivity.

With the intriguing precedent that strong FlAsH sensitivity could be achieved with no insertional mutagenesis, we generated other double point mutants, with an interest in identifying possible di-cysteine motifs (CX_nC) that are sufficient for conferring FlAsH sensitivity. Since the P186C and E187C single mutants each demonstrated measurable FlAsH sensitivity (see above), we combined them with P181C to make two double mutants, P181C/P186C and P181C/E187C TCPTP. Consistent with the single point-mutation results, we found that P181C/P186C and P181C/E187C TCPTP had catalytic activities that were comparable to that of wild-type in the absence of FlAsH (k_{cat} values of 3.2 s^{-1} and 6.4 s^{-1}, respectively, compared with the wild-type value of 5.1 s^{-1}, see Table 1). Strikingly, P181C/P186C and P181C/E187C also demonstrated strong inhibition upon incubation with FlAsH (Figure 3). The approximately 12-fold drop in catalytic efficiency that these mutants demonstrate upon FlAsH incubation rivals the level of FlAsH-mediated inhibition observed with a previously described PTP construct that contains a six amino acid insertion (*14*). P181C/P186C and P181C/E187C TCPTP demonstrate that strong FlAsH sensitivity can be introduced into enzymes with relatively conservative protein modifications: these two TCPTP variants each possess two cysteine point mutations and no amino acid insertions.

Tri-Cysteine and Tetra-Cysteine Mutants. We next asked whether even more dramatic FlAsH-inhibition effects could be observed when the various sensitizing mutations and insertions were combined. To generate a panel of tri- and tetra-cysteine mutants, we elaborated on three of the CC-containing templates that had demonstrated high activity in the absence of FlAsH: 2C-1 (a CC insertion at position 187), P186C/E187C, and 2C-3 (a single-cysteine insertion, coupled with the E187C mutation). Modification of the 2C-1 template proved to be a robust strategy for generating highly active and FlAsH-sensitive TCPTP mutants. Combining 2C-1 with the G184C, P181C, G184C/V185C, and F183C/G184C mutations, as well as a two-cysteine insertion between S188 and P189, produced five mutants, designated 3C-1, 3C-2, 4C-1, 4C-2, and 4C-3 TCPTP, respectively (Figure 2C). All five mutants were active: 3C-1 and 3C-2 TCPTP have catalytic efficiencies very close to that of wild-type TCPTP; the corresponding values for 4C-1 and 4C-2 were reduced approximately 5-fold with respect to wild-type; and 4C-3 TCPTP demonstrated a 2.6-fold higher efficiency than wild-type (Table 1). Moreover, 3C-1, 3C-2, 4C-1, 4C-2, and 4C-3 TCPTP all demonstrated strong FlAsH-inhibition effects; their catalytic efficiencies were reduced 9-, 17-, 12-, 9-, and 7-fold in the presence of FlAsH, respectively (Figure 2).

Modification of the P186C/E187C and 2C-3 templates, however, did not yield TCPTP mutants that demonstrated both high activity and strong FlAsH sensitivity. Introduction of cysteine residues on the P186C/E187C template proved deleterious, and the three resulting mutants (3C-3, 3C-4, and 4C-4 TCPTP) possessed compromised TCPTP activity (Table 1). By contrast, mutants that derived from elaboration on the 2C-3 template had wild-type-like TCPTP activity, but demonstrated only moderate FlAsH sensitivity (3C-5 and 3C-6 TCPTP, see Figure 3).

Table 2: Kinetic Constants of Wild-Type and FlAsH-Sensitive TCPTPs in the Absence (−) and Presence (+) of FlAsH when Assayed with *p*NPP

enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (mM^{-1} s^{-1})
wild-type (−)	4.4 ± 0.30	2.3 ± 0.24	1.9 ± 0.078
wild-type (+)	4.1 ± 0.066	2.3 ± 0.09	1.8 ± 0.074
P181C/P186C (−)	2.8 ± 0.057	2.0 ± 0.037	1.4 ± 0.020
P181C/P186C (+)	0.31 ± 0.0030	2.6 ± 0.084	0.12 ± 0.0029
P181C/E187C (−)	5.7 ± 0.14	0.82 ± 0.032	7.0 ± 0.10
P181C/E187C (+)	0.72 ± 0.011	1.2 ± 0.032	0.59 ± 0.0065
3C-1 (−)	2.9 ± 0.14	2.1 ± 0.20	1.4 ± 0.068
3C-1 (+)	0.41 ± 0.019	2.8 ± 0.26	0.15 ± 0.0071
3C-2 (−)	1.9 ± 0.058	1.4 ± 0.088	1.4 ± 0.046
3C-2 (+)	0.18 ± 0.0050	2.2 ± 0.071	0.081 ± 0.00064
4C-3 (−)	8.9 ± 0.12	1.5 ± 0.041	5.8 ± 0.090
4C-3 (+)	1.3 ± 0.0068	1.6 ± 0.052	0.82 ± 0.029

In summary, by the incremental approach described above, we obtained five TCPTP mutants (P181C/P186C, P181C/E187C, 3C-1, 3C-2, and 4C-3 TCPTP) that meet the criteria of a ligand-sensitive-engineering strategy: wild-type-like activity in the absence of inhibitor, coupled with a strong and specific response to a compound that leaves the wild-type enzyme unaffected. Each of these mutants appears to be inhibited via a similar allosteric mechanism: while small variations in Michaelis constant (K_M) values were sometimes induced by FlAsH, strong inhibition was, without exception, predominantly due to a decrease in k_{cat} (Table 2). These five high-activity, high-FlAsH-sensitivity mutants were selected from our mutant panel for more detailed characterization.

Catalytic Activity of FlAsH-Sensitive TCPTP Mutants with a Phosphopeptide Substrate. Throughout our initial characterization and selection of FlAsH-sensitive PTPs, we used the artificial substrate *p*-nitrophenyl phosphate (*p*NPP) in our activity assays. Although *p*NPP is widely used for *in vitro* characterization of PTPs (*26*), it is certainly possible that a mutant PTP could appear to be "wild-type-like" with *p*NPP yet fail to demonstrate robust kinetics with a more physiologically relevant substrate. To test whether or not the activities and FlAsH sensitivities of our mutant PTPs were *p*NPP-dependent, we characterized their activity levels with a phosphopeptide substrate, DADEpYLIPQQG, the sequence of which corresponds to the autophosphorylation site of the epidermal growth factor receptor ($EGFR_{988–998}$) (*27*).

When assayed with DADEpYLIPQQG in the absence of FlAsH, the catalytic efficiencies of P181C/P186C, P181C/E187C, 3C-1, 3C-2, and 4C-3 TCPTP are all within a factor of 4 of the wild-type value (Table 3). On the whole the DADEpYLIPQQG data closely mirror the *p*NPP data, although the reduction of inherent activity of the 3C-1 and 3C-2 TCPTP mutants is moderately more pronounced with the phosphopeptide substrate. The other three mutants (P181C/P186C, P181C/E187C, and 4C-3 TCPTP) have turnover numbers and catalytic-efficiency values that are within a factor of 2 of the wild-type numbers (Table 3). Taken together, these data show that the WPD-loop mutants are capable of normal catalytic function on a reasonable model of a potential physiological target of TCPTP.

Table 3: Kinetic Constants of Wild-Type and FlAsH-Sensitive TCPTPs in the Absence (−) and Presence (+) of FlAsH when Assayed with DADEpYLIPQQG

enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} s^{-1})
wild-type (−)	26 ± 1.5	9.8 ± 1.1	2.7 ± 0.14
wild-type (+)	23 ± 1.5	8.9 ± 0.88	2.6 ± 0.14
P181C/P186C (−)	14 ± 0.67	8.1 ± 1.0	1.8 ± 0.14
P181C/P186C (+)	1.9 ± 0.21	13 ± 2.7	0.14 ± 0.013
P181C/E187C (−)	15 ± 0.67	10 ± 1.4	1.5 ± 0.19
P181C/E187C (+)	1.8 ± 0.17	17 ± 3.2	0.11 ± 0.011
3C-1 (−)	13 ± 0.42	11 ± 1.2	1.2 ± 0.091
3C-1 (+)	1.2 ± 0.12	25 ± 1.0	0.048 ± 0.0040
3C-2 (−)	6.3 ± 0.11	8.5 ± 0.44	0.75 ± 0.036
3C-2 (+)	0.75 ± 0.045	17 ± 4.1	0.046 ± 0.010
4C-3 (−)	26 ± 1.5	14 ± 2.0	1.9 ± 0.23
4C-3 (+)	2.7 ± 0.16	15 ± 2.3	0.18 ± 0.040

In the presence of FlAsH, the catalytic efficiency of wild-type TCPTP on DADEpYLIPQQG does not change, showing that the "orthogonal" nature of the FlAsH/mutant-TCPTP interaction is not substrate-dependent (Figure 4A). By contrast, the activities of all five mutants dropped dramatically when incubated with FlAsH (Table 3 and Figure 4B, C). As with *p*NPP as substrate, the inhibitory effects of FlAsH were predominantly due to sharp decreases in k_{cat} values (7- to 11-fold for all five mutants). For 3C-1 and 3C-2 TCPTP, the FlAsH-induced decreases in k_{cat} were coupled with K_M increases of approximately 2-fold, leading to remarkable 25- and 17-fold drops in catalytic efficiency, respectively. On all five of the selected FlAsH-sensitive mutants, the inhibitory effects of FlAsH with DADEpYLIPQQG (Table 3) were comparable to those observed with *p*NPP (Table 2), showing that the effects of FlAsH are enzyme-specific and not dependent on the choice of substrate.

Potency of FlAsH-Induced Inhibition. To determine the potency of FlAsH's inhibitory activity on the engineered TCPTP mutants, we assayed their activities after incubation with varying concentrations of FlAsH. Under the conditions of the assay, wild-type TCPTP showed no significant inhibition at any concentration. By contrast, the activities of all five mutants dropped dramatically in a dose-dependent manner when preincubated with FlAsH (Figure 5). From these data, the 50% inhibitory-concentration (IC_{50}) values could be estimated at approximately 50–100 nM for each mutant. However, FlAsH so potently inhibits all five of the responsive mutants that the IC_{50} values approach the concentration of enzyme in the assay (50 nM enzyme, the lowest concentration that can be reasonably used in the *p*NPP-based assay). Under conditions of such potent inhibition, the measured IC_{50} values are dependent on the enzyme concentration used: only approximately one to two *equivalents* of FlAsH were necessary to inhibit half of the enzyme activity. These data also revealed that the maximum inhibition effect was obtained when FlAsH concentration reached 4–6 equivalents of protein concentration (200–300 nM under the assay conditions).

In order to achieve a more meaningful estimate of FlAsH's inhibitory potency, we measured the apparent inhibition constants (K_I^{app}) for FlAsH with the five sensitized mutants (see Materials and Methods and Supporting Information). Analyses for all five mutants confirmed the low-nanomolar potency of FlAsH: K_I^{app} values for P181C/P186C, P181C/E187C, 3C-1, 3C-2, and 4C-3 TCPTP were measured as 24 ± 1.6 nM, 37 ± 7.0 nM, 35 ± 10 nM, 35 ± 6.6 nM, and 31 ± 8.1 nM, respectively. (As a poor-potency control, we also determined the K_I^{app} value of the single-cysteine mutant E187C TCPTP: 340 ± 220 nM.) Interestingly, FlAsH's affinities for the di-cysteine mutants P181C/P186C and P181C/E187C TCPTP were similar to those for the FlAsH-

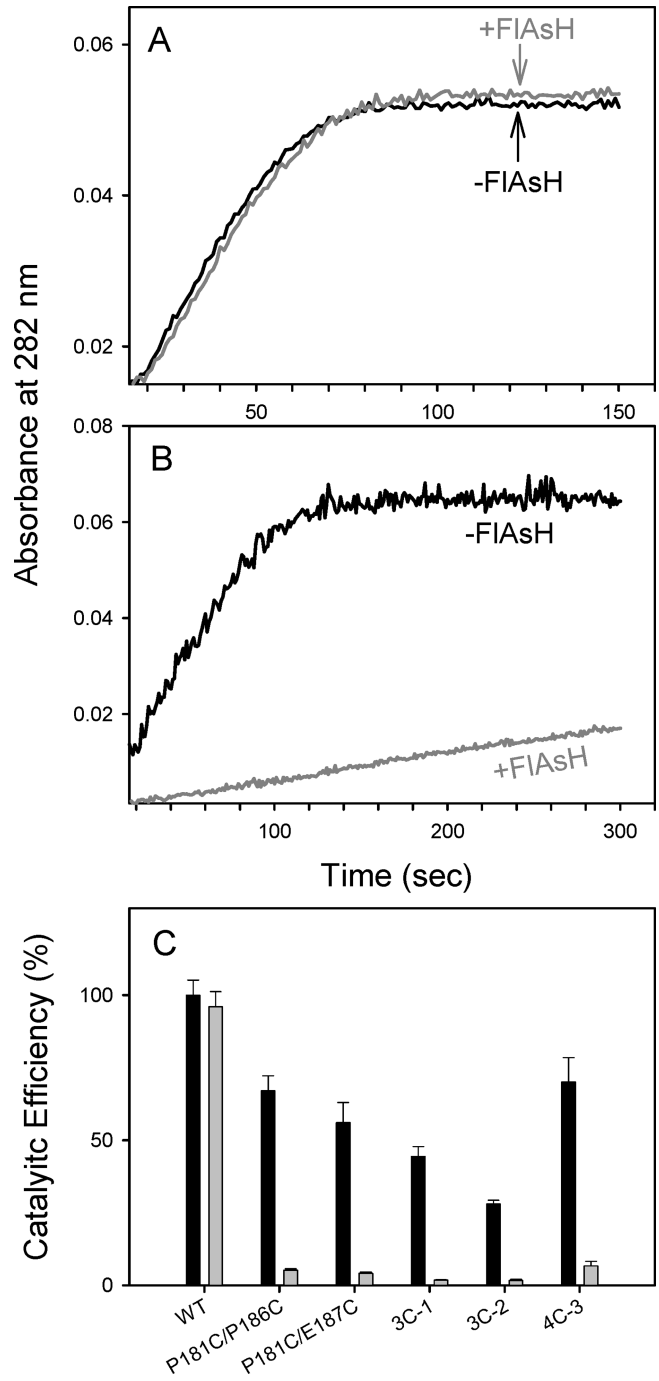

FIGURE 4: FlAsH-dependent inhibition of WPD-loop TCPTP mutants assayed with the phosphopeptide substrate DADEpYLIPQQG. TCPTP enzymes (2.5 μM) were incubated in the absence or presence of FlAsH (10 μM), diluted, and assayed at pH 7.0. (A) Kinetic curves of wild-type TCPTP in the absence (black) and presence (gray) of FlAsH. (B) Kinetic curves of a representative FlAsH-sensitive mutant, P181C/P186C TCPTP, in the absence (black) and presence (gray) of FlAsH. (C) FlAsH-dependent inhibition of P181C/P186C, P181C/E187C, 3C-1, 3C-2, and 4C-3 TCPTP. The black bars represent the catalytic efficiency (k_{cat}/K_M) in the absence of FlAsH, and the gray ones in the presence of FlAsH. All data were normalized relative to the catalytic efficiency of wild-type TCPTP in the absence of FlAsH (100%).

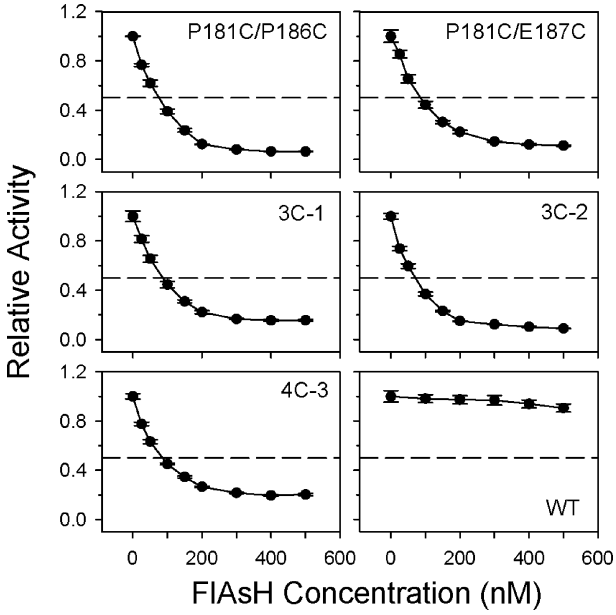

FIGURE 5: Dose-dependent inhibition of the FlAsH-responsive TCPTP mutants. TCPTP activity at the indicated FlAsH concentrations was measured for P181C/P186C, P181C/E187C, 3C-1, 3C-2, 4C-3, and wild-type TCPTP, and normalized to uninhibited (no-FlAsH) controls. Enzyme concentration for all experiments was 50 nM.

sensitive tri-cysteine and tetra-cysteine mutants, showing that no advantage was gained from the addition of more cysteine residues.

Kinetics of FlAsH-Induced Inhibition. FlAsH was originally developed as a protein-visualization tool, and FlAsH's fluorescence increases dramatically upon binding to the sulfhydryl groups of a peptide or protein (*15, 16*). Fluorescence spectroscopy thus affords a straightforward tool for assessing the FlAsH's kinetics of binding to our engineered TCPTP mutants. Figure 6 (left panels) shows the results for these fluorescence experiments. All five FlAsH-sensitive mutants reveal similar kinetic properties, which consist of two phases: immediately upon FlAsH addition, the fluorescence intensity of the TCPTP solution increases rapidly over 10–15 min; subsequently, fluorescence intensity continues to increase over time, but at a much slower rate. These experiments suggest that the majority of FlAsH-induced TCPTP inhibition may be achievable within minutes of addition of the compound to a cell or cellular lysate.

To confirm that fluorescence increases provide a valid assessment of FlAsH's inhibitory kinetics, we also directly measured FlAsH-induced time-dependent losses in PTP activity (Figure 6, right panels). In these experiments, FlAsH-responsive mutants were assayed after incubation with FlAsH for various amounts of time. In good agreement with the fluorescence-based kinetics experiments, it was found for all five mutants that TCPTP activity dropped rapidly within minutes of FlAsH addition, reaching a platform of near maximal inhibition after 10–15 min.

DISCUSSION

Minimization of FlAsH-Binding Determinants. The primary advantage of ligand-sensitivity engineering strategies for the design of selective enzyme–inhibitors lies in the ability to readily "program" a target enzyme for unique

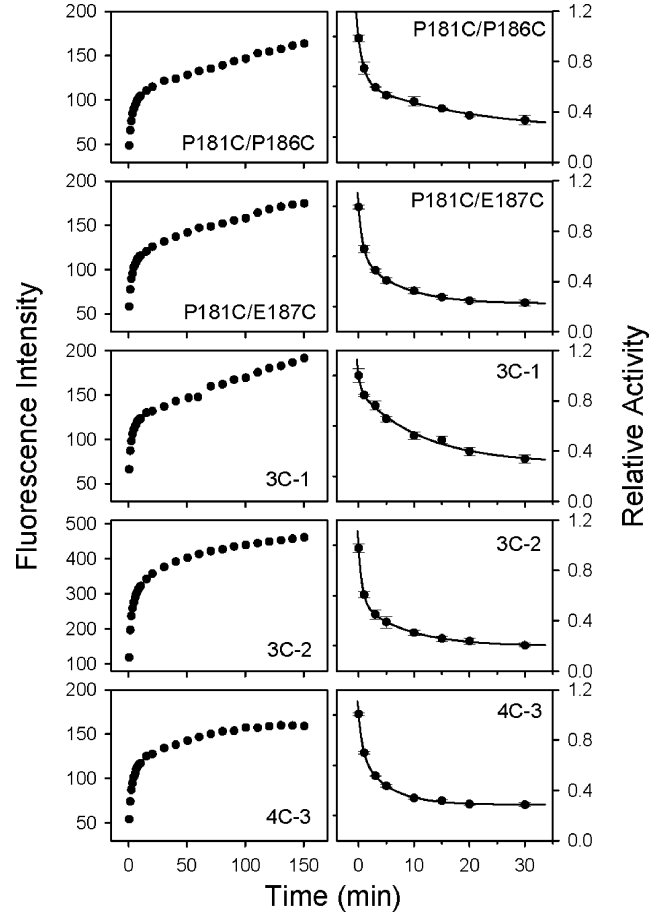

FIGURE 6: Left panels: Time-dependent fluorescence of P181C/P186C, P181C/E187C, 3C-1, 3C-2, and 4C-3 TCPTP. Fluorescence data (excitation and emission wavelengths of 510 and 526 nm, respectively) for solutions of the indicated TCPTP (250 nM) and FlAsH (1 μM) were recorded once every minute starting at 0.5 min after the addition of FlAsH. Right panels: Time-dependent inhibition of P181C/P186C, P181C/E187C, 3C-1, 3C-2, and 4C-3 TCPTP. FlAsH-sensitive TCPTP mutants (250 nM) were incubated with FlAsH (1 μM). At the indicated time points, *p*NPP was added to initiate PTP activity. Percentage activity at each time point was normalized to uninhibited (no-FlAsH) controls. Data were fitted according to two-phase exponential decay using SigmaPlot 10.0.

inhibition by a small molecule. This genetic trick allows for the discovery of selective inhibitors in a much more efficient manner than can be achieved with medicinal chemistry (*6, 7, 28*). In the current work, we show that the WPD loop of a protein tyrosine phosphatase can be readily modified to possess affinity for FlAsH, a small molecule that does not inhibit wild-type phosphatases. We used an incremental approach for the introduction of di-, tri-, and tetra-cysteine motifs into the WPD loop of TCPTP to obtain mutants that inherently possess wild-type-like activity but whose activity is strongly inhibited by FlAsH. In effect, we disassembled the known FlAsH-binding peptide, and recombined promising motifs into minimized "cassettes" for the introduction of FlAsH sensitivity into the target PTP.

In comparison with the domain-wide peptide-insertion strategies that have been used to engineer FlAsH sensitivity in two previous studies (*13, 14*), the approach described here holds a clear advantage. Intuitively, when generating an engineered protein for the purpose of probing its cellular functions, small perturbations are preferable to large ones. We have dramatically scaled down the size of the protein

modification necessary for producing FlAsH-sensitive enzymes: from a six amino acid peptide insertion to as few as two point mutations. Our data show that this "paring down" of a FlAsH-binding motif greatly facilitates the ability to engineer FlAsH-sensitive enzymes that retain their inherent activity. Previously, no FlAsH-responsive enzymes that possess high activity in the absence of the drug were known; in the current study, we have identified and characterized five such variants of an essential tyrosine phosphatase, TCPTP. Among these five TCPTP mutants, only one (4C-3 TCPTP) contained a "complete" (four-cysteine) FlAsH-binding motif. Remarkably, the tetra-cysteine motif conferred no appreciable advantage over the di- (P181C/P186C and P181C/E187C TCPTP) and tri-cysteine mutations (3C-1 and 3C-2 TCPTP). In all measures of the potency and kinetics of FlAsH-induced inhibition (see Figures 5 and 6), the two-cysteine and three-cysteine TCPTP mutants performed equally well as 4C-3 TCPTP. In short, two or three properly situated cysteine residues are sufficient for exerting strong FlAsH-dependent control of protein function.

A primary-sequence comparison of the five FlAsH-responsive mutants yields few general "rules" (Figure 2). Cysteine mutations and insertions in these enzymes were placed over a large range of amino acid positions in the WPD-loop region, and the number of intervening residues between cysteine "patches" varies from two (3C-1, 4C-3) to five (P181C/E187C). What does emerge from a consideration of the entire mutant panel, however, is that P186 and E187 of TCPTP represent a FlAsH-sensitivity hot spot. All five FlAsH-sensitive mutants possess either cysteine mutations at these positions or insertions between them. It has been previously suggested that the residue corresponding to P186 constitutes a "hinge region" for WPD loop movement in another PTP, SHP1 (*23*), and our results hint that this region may play a similarly important role in TCPTP.

Mechanism of FlAsH-Mediated Inhibition. How exactly does TCPTP's WPD loop function in the absence of FlAsH, and how does the binding of FlAsH inhibit its function? We do not currently have satisfactory answers to these questions, but previous studies on the WPD loop of a highly active model PTP (YopH from *Yersinia pestis*) provide some insight. As noted previously, classical PTPs can exist in either the "open" (apo) or "closed" (substrate-bound) forms, which possess dramatically differing WPD-loop conformations. Detailed kinetic and thermodynamic studies suggest that, in the absence of substrate (or other active-site-directed ligand), the open and closed forms of YopH are (roughly speaking) equally stable and rapidly interconverting ($k_{opening} \approx k_{closing} \approx 2.6 \times 10^8\ s^{-1}$) (*29*). After binding of ligand (L), however, the closed form is favored, and the equilibrium population of YopH·L_{closed} is approximately 10-times greater than that of YopH·L_{open} ($k_{opening} \approx 1.1 \times 10^4\ s^{-1}$; $k_{closing} \approx 1.4 \times 10^5\ s^{-1}$ at 30 °C) (*30*). All of these various loop-dynamics processes, however, are much faster than YopH's maximum rate of chemical catalysis with *p*NPP ($k_{cat} \approx 1 \times 10^2\ s^{-1}$ at 30 °C, pH 6.6) (*31*). So, the mechanism by which FlAsH can shut down PTP activity via inhibition of loop dynamics is not readily apparent from the available data. Complicating matters further, YopH shares a rather low degree of sequence identity with mammalian PTPs; and detailed experimental loop-dynamics data are not currently available for TCPTP or its more widely studied homologue PTP1B (*32–35*). At this point, our data cannot speak to the question of whether FlAsH is inhibiting the opening or closing (or both) of the WPD loop, and at what point in the catalytic mechanism FlAsH is exerting its most significant influence. Ultimately, X-ray crystal structures of sensitized PTP mutants in the absence and presence of FlAsH will likely be necessary for generating a more complete picture of FlAsH's mechanism of action.

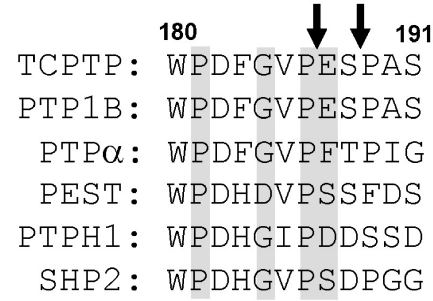

FIGURE 7: WPD-loop primary-sequence alignment for several representative human protein tyrosine phosphatases. Positions of cysteine point mutations in P181C/P186C, P181C/E187C, 3C-1, 3C-2, and/or 4C-3 TCPTP are highlighted. Positions of di-cysteine insertions in 3C-1, 3C-2, and/or 4C-3 TCPTP are marked with arrows. Numbering corresponds to human TCPTP.

Prospects for Application to Other PTPs. A key consideration for the utility of ligand-sensitive engineering approaches is that of within-family generality. Because a significant amount of work goes into the identification of sensitizing mutations and their complementary small-molecule ligands, such approaches are most useful when the sensitizing mutation is applicable across a large family of proteins. For example, the "gold standard" of ligand-sensitive engineering approaches has been described for the protein kinases: targeted mutation of an easily identified "gatekeeper" residue in the kinase active site has been used to sensitize dozens of protein kinases to small-molecule inhibition, greatly facilitating the study of protein-kinase function (*6, 7, 17, 36*). While the work described here has focused on a single phosphatase, TCPTP, the highly conserved nature of the PTP WPD loop augurs well for the prospect of using FlAsH as an inhibitor of many different engineered PTPs. Indeed, the WPD loop is one of the most conserved regions in PTPs, making the identification of analogous sites in other classical PTPs straightforward (*4*). Figure 7 shows a primary-sequence alignment of the WPD-loop region of TCPTP with the corresponding region of several representative PTPs. As can be seen from the alignment, no TCPTP-specific feature was exploited in the design of the FlAsH-sensitive P181C/P186C, P181C/E187C, 3C-1, 3C-2, and 4C-3 TCPTP. The analogous multi-cysteine mutants can be readily designed for any PTP, even ones for which no three-dimensional structure is available (e.g., PEST; see Figure 7).

Prospects for Application to Other Protein Families. Additionally, the general strategy presented here—effecting FlAsH-mediated control of protein function with relatively conservative point mutations—has the potential to be generalized for the introduction of engineered allosteric sites in proteins that are unrelated to PTPs. Our data clearly show that the canonical FlAsH-binding peptide, CCPGCC, is not necessary for the introduction of strong FlAsH sensitivity, and we have no reason to believe that this observation will prove to be PTP-specific. The potential to direct FlAsH to a

given protein target with two point mutations significantly increases the potential applications of the compound as a protein-targeted inhibitor: many families of proteins undergo functionally essential conformational changes; and, in addition to inhibition of enzyme activity, one could imagine specific FlAsH-mediated disruption of other protein functions, such as the formation of protein–protein or protein−nucleic acid interactions. If an artificial FlAsH-binding site can be introduced into a conformationally dynamic and important site, binding of FlAsH may be expected to hinder these conformational changes, inhibiting the protein's function. From a practical viewpoint, point-mutation-based dicysteine motifs (CX_nC) should prove much more broadly useful than CCPGCC insertions, and it is our expectation that mutation-based FlAsH targeting will become a significant addition to the chemical biologist's ever expanding toolbox.

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SUPPORTING INFORMATION AVAILABLE

Sequences of mutagenic primers and a description of the data analysis used for inhibition-constant calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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