Site-Specific Incorporation of Allosteric-Inhibition Sites in a Protein Tyrosine Phosphatase

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Supplementary Information:

Figure S1

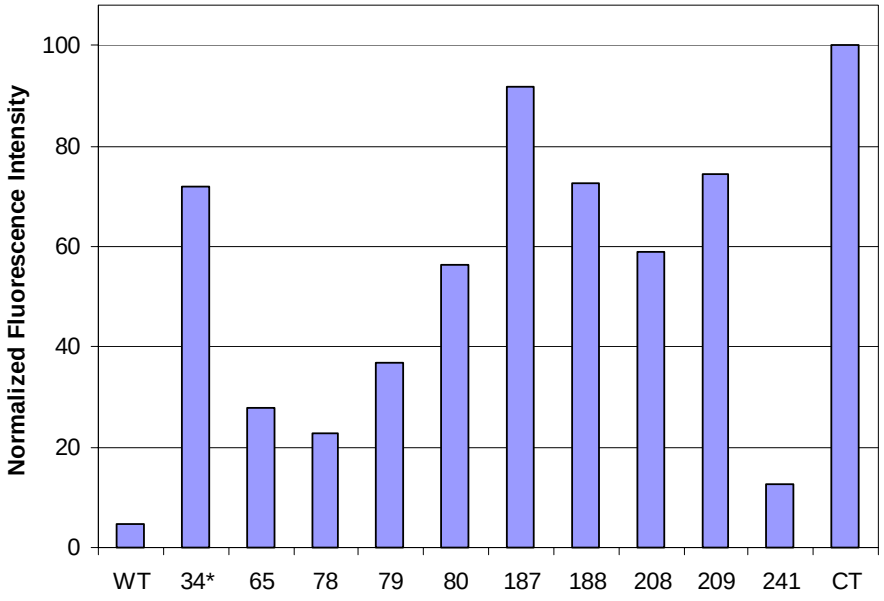

Figure S1. Fluorescence intensity of TCPTP/FlAsH complexes. The indicated TCPTP enzymes (75 nM) were incubated in presence of FlAsH (50 nM) at room temperature for 2.5 hours and assayed for fluorescence intensity (excitation wavelength: 510 nm, emission wavelength: 528 nm). TCPTP-34 is marked with an asterisk because this insertion mutant lacked PTP activity either in the absence or presence of FlAsH; TCPTP-34 thus does not appear in Tables 1 and S1 or Figure 2.

Table S1. Kinetic Constants of TCPTP Insertion Mutants in the Absence and Presence of FlAsH

Protein	no FlAsH			+ FlAsH (10 μM)			% Activity*
	k_{cat} (s^{-1})	K_M for *p*NPP (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_M for *p*NPP (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	
WT TCPTP	3.9±0.20	2.3±0.21	1.7	3.2±0.19	1.8±0.24	1.8	1.1
TCPTP-65	2.8±0.13	2.9±0.16	1.0	2.3±0.11	3.4±0.33	0.68	0.68
TCPTP-78	1.5±0.048	1.8±0.058	0.84	0.87±0.024	1.8±0.082	0.50	0.60
TCPTP-79	5.0±0.043	1.2±0.046	4.1	2.8±0.081	2.1±0.11	1.3	0.32
TCPTP-80	1.1±0.050	2.0±0.24	0.56	0.70±0.039	2.0±0.26	0.36	0.64
TCPTP-187	0.21±0.0084	0.91±0.059	0.23	0.034±0.0031	1.8±0.25	0.019	0.083
TCPTP-188	0.25±0.012	1.1±0.12	0.23	0.15±0.0039	1.1±0.042	0.13	0.57
TCPTP-208	1.3±0.032	3.5±0.14	0.38	0.90±0.10	3.8±0.65	0.24	0.63
TCPTP-209	0.41±0.017	1.8±0.13	0.23	0.34±0.009	2.0±0.057	0.17	0.74
TCPTP-241	2.1±0.15	2.2±0.29	1.0	1.6±0.025	2.8±0.11	0.55	0.55
TCPTP-CT	1.2±0.034	2.2±0.084	0.54	1.3±0.19	3.1±0.79	0.41	0.76

* "% 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.

Material and Methods

FlAsH Synthesis

FlAsH was synthesized essentially as previously described.[1, 2] Briefly, fluorescein mercuric acetate (200 mg, 0.235 mmol) was suspended in dry *N*-methylpyrrolidinone (4 mL). Arsenic trichloride (0.404 mL, 4.7 mmol) was added dropwise, followed by palladium acetate (10 mg) and dry N,N-diisopropylethylamine (0.328 ml, 1.9 mmol). The resulting solution was stirred at room temperature under argon for 3 h. The reaction mixture was then poured into 60 mL of a stirred 9:1 (v/v) mixture of 0.25 M potassium phosphate buffer (pH 7.0)/acetone, to which 1.2 mL of 1,2-ethanedithiol had been freshly added. The resulting solution was extracted three times with $CHCl_3$ (70 mL each); the combined extracts were dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The product was purified by column chromatography over silica gel with a solvent system of 1:9 ethyl acetate-toluene, and subsequently re-purified with a

2:100 ethyl acetate-toluene solvent system. Fractions showing a single spot with an analytical-TLC R_f of 0.51 (stationary phase: silica gel; mobile phase: 1:9 ethyl acetate-toluene) were collected. Solvent was removed and the resulting solid was dissolved in DMSO to prepare a 10 mM stock solution. All spectral characteristics were consistent with previously reported values.[1]

Mutagenesis

A plasmid encoding plasmid TCPTP-His_6, pHEH042[3] (~50 ng), 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 temperature cycler. The reaction mixtures were subjected to 18 cycles of 95 ºC for 30 seconds, 55 ºC for 1 minute, and 68 ºC for 16 minutes. (A 45 ºC annealing temperature was used for the 65, 187, 241, and C-terminal mutants.) Sequences of insertion primers are as follows, 34: CATGACTATCCTTGCTGCCCGGGCTGCTGCCATAGAGTGGCC and GGCCACTCTATGGCAGCAGCCCGGGCAGCAAGGATAGTCATG; 65: CTGCAAAATGCTTGCTGCCCGGGCTGCTGCGAGAATGATTATATTAAT and ATTAATATAATCATTCTCGCAGCAGCCCGGGCAGCAAGCATTTTGCAG; 78: TTAGTTGACATAGAATGCTGCCCGGGCTGCTGCGAGGCACAAAGGAGT and ACTCCTTTGTGCCTCGCAGCAGCCCGGGCAGCATTCTATGTCAACTAA; 79: GTTGACATAGAAGAGTGCTGCCCGGGCTGCTGCGCACAAAGGAGTTAC and GTAACTCCTTTGTGCGCAGCAGCCCGGGCAGCACTCTTCTATGTCAAC; 80: GACATAGAAGAGGCATGCTGCCCGGGCTGCTGCCAAAGGAGTTAC and

GTAACTCCTTTGGCAGCAGCCCGGGCAGCATGCCTCTTCTATGTC; 132: GGCCAACAGATGACTGCTGCCCGGGCTGCTGCCAAGAGATGCTGTTTAAA and TTTAAACAGCATCTCTTGGCAGCAGCCCGGGCAGCAGTCATCTGTTGGCC; 167: TTAGAAAATATCAATAGTTGCTGCCCGGGCTGCTGCGGTGAAACCAGAAC and GTTCTGGTTTCACCGCAGCAGCCCGGGCAGCAACTATTGATATTTTCTAA; 187: GATTTTGGAGTCCCTTGCTGCCCGGGCTGCTGCGAATCACCAGCTTCA and TGAAGCTGGTGATTCGCAGCAGCCCGGGCAGCAAGGGACTCCAAAATC; 188: GGAGTCCCTGAATGCTGCCCGGGCTGCTGCTCACCAGCTTCA and TGAAGCTGGTGAGCAGCAGCCCGGGCAGCATTCAGGGACTCC; 208: TCCTTGAACCCTTGCTGCCCGGGCTGCTGCGACCATGGGCCT and AGGCCCATGGTCGCAGCAGCCCGGGCAGCAAGGGTTCAAGGA; 209: CCTTGAACCCTGACTGCTGCCCGGGCTGCTGCCATGGGCCTGCGG and CCGCAGGCCCATGGCAGCAGCCCGGGCAGCAGTCAGGGTTCAAGG; 241: GGAAAAAGGAGATTGCTGCCCGGGCTGCTGCGATATTAACATAAAACAAG and CTTGTTTTATGTTAATATCGCAGCAGCCCGGGCAGCAATCTCCTTTTTCC; C-terminal: GCGGCCGCACTCGAGTGCTGCCCGGGCTGCTGCCACCACCACCACCAC and GTGGTGGTGGTGGTGGCAGCAGCCCGGGCAGCACTCGAGTGCGGCCGC. At the end of temperature cycling, 10 U (1 μL) of *Dpn* I restriction enzyme were added, and the reaction mixture was incubated at 37 ºC for 1 hour. Following *Dpn* I digestion of parental DNA, the mutant DNAs were ethanol-precipitated and re-dissolved in 3 μL of water. The resulting DNA solutions were used to transform DH5α competent cells.

Plasmid preparations from the resulting ampicillin-resistant colonies were digested with *Sma* I restriction enzymes to test for the inserted sequence. *Sma* I-site-containing plasmids were sequenced over the entire coding region of the TCPTP gene (Cornell Biotechnology Resource Center).

TCPTP expression

TCPTP-His_6-encoding plasmids (wild-type and insertion 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 mid-log phase, and induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 6 hours. Cells were pelleted and frozen at -80 °C. Cell lysis was achieved by incubation of the cell pellets with Bacterial Protein Extraction Reagent (Pierce), and purifications of enzymes were carried out using SwellGel Ni-NTA resin (Pierce) according to the manufacturer's instructions. The protein solutions obtained were concentrated with CentriPrep Centrifugal Filter Devices (Ultracel YM-30, 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 mixed with equal volumes of 60% glycerol and stored at -20 ˚C. Protein concentrations were determined by Bradford assay, and enzyme purities were estimated by SDS-PAGE.

TCPTP Activity Assays

The TCPTP kinetic assays with *para*-nitrophenylphosphate (*p*NPP) were carried out at 22 ˚C in a total reaction volume of 200 μL containing *p*NPP (0.5-10 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 absorbance at 405 nm was measured with a Molecular Devices Versamax plate-reader. The kinetic constants for each enzyme were determined by fitting the data to the Michaelis-Menten equation. Values given in Table 1 represent the averages and standard deviations of three independent experiments.

Enzyme Kinetic 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 hours at room temperature, the solutions were diluted with 1×PTP buffer so that the final concentrations of proteins in the reaction mixtures would be appropriate for PTP assays (50-150 nM). The enzyme activity in the presence and absence of FlAsH was assayed as described above.

Fluorescence Experiments

Solutions of TCPTP (75 nM) in 1×PTP buffer were incubated in the absence (DMSO vehicle only) or presence of FlAsH (50 nM) in Eppendorf Protein LoBind tubes. After 2.5 hours at room temperature, the fluorescence intensities of the protein solutions were measured. The controls were scanned once to use as background. Fluorescence spectra were recorded on a Perkin-Elmer Luminescence Spectrometer LS50B. The excitation wavelength was 510 nm, and the emission spectra were recorded from 520 nm to 560 nm

at a scanning speed of 120 nm/min with the excitation slit at 5.0 nm and the emission slit at 15 nm.

References

(1) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269-272.

(2) Adams, S. R.; Campbell, R. E.; Gross, L. A.; Martin, B. R.; Walkup, G. K.; Yao, Y.; Llopis, J.; Tsien, R. Y. *J. Am. Chem. Soc.* **2002**, *124*, 6063-6076.

(3) Hoffman, H. E.; Blair, E. R.; Johndrow, J. E.; Bishop, A. C. *J. Am. Chem. Soc.* **2005,** *127*, 2824-2825.