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^1H , ^{15}N and ^{13}C sequence specific backbone assignment of the vanadate inhibited Hematopoietic Tyrosine Phosphatase

Luciana ESF Machado^{1, #}, Rebecca Page^{2, #}, and Wolfgang Peti^{1, *, #}

¹Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI 02912, USA

²Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02912, USA

Abstract

The sequence-specific backbone assignment of HePTP (hematopoietic protein tyrosine phosphatase; PTPN7) in presence of vanadate have been determined, based on triple-resonance experiments using uniformly [^{13}C , ^{15}N]-labeled protein. These assignments facilitate further studies of HePTP in the presence of inhibitors to target leukemia and provide further insights into the function of protein tyrosine phosphatases.

Keywords

HePTP; protein tyrosine phosphatase; vanadate; inhibition; protein dynamics; leukemia

Biological context

Hematopoietic Protein Tyrosine Phosphatase (HePTP) or PTPN7 is a non-receptor protein tyrosine phosphatase (PTP) expressed exclusively in the white blood cells of tissues such as the thymus and spleen (Zanke et al. 1992). It functions to negatively regulate T cell activation and proliferation through its ability to dephosphorylate and inactivate the MAP kinases p38 and Erk2 (Muñoz et al. 2003; Francis et al. 2011). Its importance in these processes is evident by the fact that HePTP overexpression has been associated with preleukemic disorder myelodysplastic syndrome, as well as in acute myelogenous leukemia (Zanke et al. 1994). As a consequence, multiple efforts to identify potent strategies and specific inhibitors of HePTP are ongoing (Sergienko et al. 2012; Machado et al. 2017). The sequence specific backbone assignment of free HePTP was previously obtained (Jeeves et al. 2008). Here we investigate the specific backbone assignment of HePTP upon vanadate binding. Vanadate is commonly used as a non-specific inhibitor for phosphatases.

*Correspondence to: Wolfgang Peti; wolfgangpeti@email.arizona.edu; phone: (520) 621-3489.

#current address: Department of Chemistry and Biochemistry, University of Arizona, AZ 85721, USA.

Methods and experiments

DNA coding the human HePTP catalytic domain (residues 44–339; 33.5 kDa) was sub-cloned into RP1B (Peti and Page 2007). For protein expression, plasmid DNA was transformed into *E. coli* BL21 (DE3) RIL cells (Agilent). [^2H , ^{15}N]- or [^2H , ^{15}N , ^{13}C]-labeled HePTP was facilitated by growing cells in D_2O based M9 minimal medium containing selective antibiotics and 4 g/l [^{13}C]-D-glucose and/or 1 g/L $^{15}\text{NH}_4\text{Cl}$ (CIL or Isotec) as the sole carbon and nitrogen sources, respectively. Multiple rounds (0, 25%, 50%, 70% and 100%) of D_2O adaptation were necessary for high-yield expression. Expression was induced by the addition of 1 mM isopropylthio- β -D-galactoside (IPTG). Induction proceeded overnight at 18°C prior to harvesting by centrifugation at $7,647 \times g$ (15 minutes, 4°C). Cell pellets were stored at -80°C until purification.

Cell pellets were resuspended in Lysis Buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole, 0.1 % Triton X-100) and lysed using high pressure homogenization (Avestin C3 EmulsiFlex). The lysate was cleared by centrifugation ($40,905 \times g$, 45 minutes, 4°C). The supernatant was filtered and loaded onto a Ni-NTA column equilibrated in Buffer A (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5 mM imidazole). Protein was eluted in Buffer B (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 300 mM imidazole). The pooled eluted protein was incubated with TEV overnight at 4°C in dialysis buffer (10 mM Tris-HCl pH 8.0, 100 mM NaCl). The next day, a ‘subtraction’ his-tag purification was performed to remove TEV and the cleaved His-tag. Final purification was achieved using size exclusion chromatography (SEC; Superdex 75 26/60 [GE Healthcare]) equilibrated in NMR Buffer (10 mM Tris-HCl pH 7.8, 100 mM NaCl, 0.5 mM TCEP).

NMR spectroscopy

All NMR measurements were performed at 298 K on either a Bruker AvanceII 500 MHz or a Bruker AvanceIIIHD 850 MHz NMR spectrometer both equipped with TCI HCN Z-gradient cryoprobe. The NMR spectra were processed using Topspin 3.5 (Bruker, Billerica, MA) or/and NMRPipe (Delaglio et al. 1995), and analyzed using CARA (www.nmr.ch) or CCPN (Vranken et al. 2005). The sequence specific backbone assignment of HePTP (520 μM) was obtained by recording a 2D [^1H , ^{15}N] TROSY, 3D HNCA, 3D HN(CO)CA and 3D HNCACB spectrum as well as input from (Jeeves et al. 2008). The sequence specific backbone assignment for HePTP (520 μM) in presence of 10400 μM vanadate (1:20) were de novo obtained from a 2D [^1H , ^{15}N] TROSY and 3D HNCA, 3D HN(CO)CA experiment as well as the titration experiment with increasing concentrations of vanadate (1:1, 1:2, 1:5, 1:10 and 1:20 molar ratio) and compared with the sequence specific backbone assignment of HePTP.

Assignment and data deposition

We assigned 87% (236 residues) of free HePTP (44–339; 24 prolines) at pH 7.8. At pH 7.3, Jeeves et al was able to assign 91% of all residues (Jeeves et al. 2008). Residues Y137, D138 (α_2), E178, K180 (E-loop), M204 (β_8), E241 (WPD loop), H262 (loop between α_3 and the PTP loop), V268 (PTP loop), L305 (α_5) and E335 were assigned by Jeeves et al. and are

missing in our assignment. However, we were able to assign R276 and T277 that were previously not assigned by Jeeves. Assignments of residues N44, K74 (α_2'), D94, I95, G97, S100 (SBL loop), E165, E166, V167, I170, V171, M172, L176, K182 (β_7), L215, T216, I217, Q218, Y219, Q220, I229 (β_{10} – β_{11}), A243, L246 (α_3), A272, G275 (PTP loop) and S338 are missing in both assignments. The lack of detectable resonances for ~10% of the residues is most likely due to intermediate conformational exchange (most likely for residues in loops) or to slow H/D back exchange (most likely for residues in β -sheets) after protein expression in D₂O-based expression medium, which broadens peaks beyond detection. The sequence specific backbone assignment for HePTP in presence of vanadate (saturating 1:20 ratio) is 66% complete (179 residues). The fully annotated 2D [¹H, ¹⁵N] TROSY spectrum is shown in Fig. 1A–D. Many HePTP residues, especially at the HePTP active site, the PTP-, the WPD-, the E-loop and the loop connecting helix- α_3 and the PTP-loop are not assigned as their respective NH cross peaks broadened beyond detection, most likely due to intermediate conformational exchange upon vanadate binding. Fig. 2 A shows the 2D [¹H, ¹⁵N] TROSY spectrum of free HePTP and with increasing concentrations of vanadate (1:0, 1:1, 1:10, 1:20 molar ratio). Fig. 2B shows the chemical shift perturbation (CSP) of free HePTP vs HePTP:vanadate (1:20 molar ratio). Fig. 2C shows the CSPs mapped onto the HePTP structure (PDBid: 1ZC0); the regions that are expected in PTPs to change upon active site inhibitor binding are also either broadened beyond detectability (as has been observed for inhibitor binding to another protein tyrosine phosphatase, PTP1B (Choy et al. 2017)) or show CSPs in HePTP. All chemical shifts were deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 27121 and 27122.

Protein dynamics experiments

As it was recently reported that the dynamics of PTP1B (Choy et al. 2017), the prototype non-receptor tyrosine phosphatase, differ in the free and inhibitor bound states, we performed auto-correlated fast timescale ¹⁵N relaxation experiments on free and vanadate-bound (1:20 molar ratio) HePTP, specifically ¹⁵N longitudinal (R_1) and transverse (R_2) relaxation rates and ¹⁵N{¹H} NOE (hetNOE) measurements. T_1 and T_2 experiments were acquired with a recycle delay of 3 s between experiments, and the following relaxation delays for T_1 : 100, 600, 1000, 1600, 1800, 2000, 2400 and 3000 ms; and T_2 : 8.26, 16.52, 24.78, 33.04, 41.3, 59.56, 57.82, 66.08, and 82.6 ms; (85 MHz ¹⁵N Larmor frequency). A recycle delay of 2.5 s between experiments, and the following relaxation delays for T_1 : 100, 400, 700, 900, 1100, 1300, 1600 and 1900 ms; and T_2 : 8.48, 16.96, 33.92, 50.88, 67.84, 76.32, 93.28, 110.24 and 127.2 ms was used at 50 MHz ¹⁵N Larmor frequency. The total length of the T_2 relaxation delay is determined by the length of one Carr–Purcell–Meiboom–Gill cycle multiplied by the number of cycles (1–10 and 1–15 cycles at 85 and 50 MHz ¹⁵N Larmor frequency, respectively). Systematic error in both T_1 and T_2 experiments was estimated from variance averaging of repetition experiments, which were acquired with delays of 600/900 and 2000/1600 ms for T_1 and 24.78/33.92 and 57.82/76.32 ms for T_2 (85/50 MHz ¹⁵N Larmor frequency, respectively). The hetNOE measurements were determined from a pair of interleaved spectra acquired with or without presaturation and a recycle delay of 5 s (50 MHz ¹⁵N Larmor frequency). R_1 and R_2 relaxation rates and the hetNOE were analyzed using CCPN (Vranken et al. 2005).

HePTP exhibits highly uniform ^{15}N dynamics throughout its backbone (average R_1 of $0.45 \pm 0.01/0.85 \pm 0.01$ [85 MHz/50 MHz] and $0.44 \pm 0.01 \text{ s}^{-1}$, average R_2 of 31.4 ± 0.3 and $30 \pm 0.3 \text{ s}^{-1}$, average hetNOE of 0.76 ± 0.01 and 0.79 ± 0.01 for free and vanadate bound HePTP, respectively). Increased dynamics are only seen in two loops: the loop connecting strands $\beta 1$ and $\beta 2$ and the loop connecting helix- $\alpha 3$ and the strand $\beta 12$, which immediately precedes the PTP loop (Fig. 2D,E; orange). Strikingly and in contrast with PTP1B, no changes of fast timescale motions are detected for HePTP when bound to vanadate (1:20 ratio) (Fig. 2D,E, blue).

Acknowledgments

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Figure 1.

(A) Annotated 2D ^1H , ^{15}N TROSY spectrum of the HePTP catalytic domain presence of vanadate (1:20 ratio), pH 7.8, recorded at 298 K on a Bruker AvanceIIIHD 850 MHz NMR spectrometer equipped with TCI HCN Z-gradient cryoprobe. Assigned residues are labeled with the residue name (single letter code) and sequence. (B, C, D) zoom from (A).

Figure 2.

(A) 2D ^1H , ^{15}N TROSY spectrum of the catalytic domain of free HePTP (red) and with increasing concentrations of vanadate (1:1 green; 1:10 cyan and 1:20 blue), pH 7.8, recorded at 298 K on a Bruker AvanceIIIHD 850 MHz NMR spectrometer equipped with TCI HCN Z-gradient cryoprobe. (B) CSP plot of HePTP vs. HePTP with vanadate (1:20 ratio). Residues with CSPs upon vanadate binding ($\sigma = 0.08$ ppm) are shown as black and residues that broadened beyond detectability are shown as red bars. (C) Ribbon representation of HePTP (PDBid: 1ZC0): CSPs (blue), residues broadened beyond detectability (coral) and residues with increased dynamics (orange sticks) are highlighted. The active site Cys270 is shown in sticks in magenta). (D–F) ^{15}N dynamics of free HePTP (red) and in presence of vanadate (blue) (1:20 ratio): (D) ^{15}N R_1 and (E) ^{15}N R_2 relaxation rates (^{15}N 85 MHz Larmor frequency) and (F) hetNOE (^{15}N 50 MHz Larmor frequency). Annotated secondary structure is shown. The dynamic loops are highlighted in orange.