Leveraging Reciprocity to Identify and Characterize Unknown Allosteric Sites in Protein Tyrosine Phosphatases

Danica S. Cui[1], Victor Beaumont[1], Patrick S. Ginther[2], James M. Lipchock[2] and J. Patrick Loria[1,3]

*1 - **Department of Chemistry,** Yale University, New Haven, CT 06511, USA*
*2 - **Department of Chemistry,** Washington College, Chestertown, MD 21620, USA*
*3 - **Department of Molecular Biophysics and Biochemistry,** Yale University, New Haven, CT 06520, USA*

***Correspondence to J. Patrick Loria:** Department of Chemistry, Yale University, New Haven, CT 06511, USA.*
patrick.loria@yale.edu
http://dx.doi.org/10.1016/j.jmb.2017.06.009
Edited by Charalampo Kalodimos

Abstract

Drug-like molecules targeting allosteric sites in proteins are of great therapeutic interest; however, identification of potential sites is not trivial. A straightforward approach to identify hidden allosteric sites is demonstrated in protein tyrosine phosphatases (PTP) by creation of single alanine mutations in the catalytic acid loop of PTP1B and VHR. This approach relies on the reciprocal interactions between an allosteric site and its coupled orthosteric site. The resulting NMR chemical shift perturbations (CSPs) of each mutant reveal clusters of distal residues affected by acid loop mutation. In PTP1B and VHR, two new allosteric clusters were identified in each enzyme. Mutations in these allosteric clusters altered phosphatase activity with changes in k_{cat}/K_M ranging from 30% to nearly 100-fold. This work outlines a simple method for identification of new allosteric sites in PTP, and given the basis of this method in thermodynamics, it is expected to be generally useful in other systems.

Introduction

Protein allostery represents a major mechanism for the regulation of metabolic activity *in vivo*, whereby enzymatic activity or ligand binding affinity is finely tuned through structural or dynamical perturbations at locations that are spatially distinct from the site of activity. Allosteric perturbations can occur as a result of small molecule or macromolecule binding or post-translational modification. It is widely recognized that designed allosteric ligands can have great therapeutic benefit, and thus, extensive research has been focused in this area [1–3]. The potential benefits are far-reaching because while many proteins are not naturally regulated *in vivo* by an allosteric mechanism, it has been speculated that all proteins have potential or hidden allosteric sites and that these locations can be exploited for drug design purposes [4]. Experimental and computational methods have been proposed to identify these unknown sites in allosteric and non-allosteric proteins [5–7], and while some approaches have been successful [6,8], the need for additional methods is clear [6].

It has long been known that amino acid mutations alter the NMR chemical shifts of residues both nearby and distant from the site of mutation [9], which often confounds the interpretation of the mutational study. Rather than a nuisance, these distally perturbed chemical shifts can be exploited for the discovery of new allosteric sites in proteins due to the reciprocity that exists in an allosteric system [10,11]. Reciprocity is based on the thermodynamic premise that for two coupled allosteric sites, perturbation at one site should impact the other site. As such, the effects of an amino acid mutation should propagate to distant sites that are allosterically coupled to the site of mutation. Solution NMR spectroscopy is an exquisitely sensitive technique to detect such changes with atomic resolution. Experimental NMR-based methods such CHESCA and RASSMM have relied on this sensitivity to interrogate and elucidate protein networks [12–14]. However, these methods often require prior knowledge

of known conformational states or are limited to the detection of altered motions in the millisecond timescale. Here, we describe an approach to identify allosteric networks simply by the examination of protein-wide $^{1}H,^{15}N$ chemical shift perturbations (CSPs) for a series of active site mutations. Specifically, analysis of CSP for a series of acid loop alanine mutations in the active sites of protein tyrosine phosphatase (PTP) 1B (PTP1B) and *Vaccinia* H1-related phosphatase (VHR) identified clusters of distally located residues that are coupled to the active sites of these enzymes. Structural and biochemical studies further verify these newly discovered allosteric sites, confirming the validity of this method in addition to providing mechanistic detail of allostery in these enzymes.

PTP1B plays a major role in regulating glucose homeostasis and leptin signaling and has been an active target for the rational design of inhibitors for the treatment of type II diabetes and obesity [15,16]. VHR is involved in cell cycle regulation [17] and is an enzyme of interest for the treatment of cervical [18], breast [19], and prostate cancers [20]. Competitive inhibition of these enzymes has proven to be a poor strategy toward achieving these goals. There are 107 human PTPs with highly conserved catalytic sites, which present a major hurdle for ligand selectivity [21]. Moreover, ligand binding to the active site is driven by electrostatic complementarity, and anionic inhibitors that mimic substrate binding generally have reduced membrane permeability [21]. Thus, recent drug design efforts have focused on allosteric inhibition of PTPs, because this mechanism is not limited by the aforementioned constraints, and human PTPs have diverse surface features outside of the active site, despite a similar three-dimensional architecture [22]. While research is ongoing, the most promising allosteric inhibitor for PTP1B is a benzofuran-based [23] small molecule (BB3) that binds near α-helix 7, approximately 20 Å from the active site [24]. Additionally, another small molecule inhibitor MSI-1436 has been demonstrated to bind near α-helix 7, validating this secondary structure as an important allosteric site [25]. Similar efforts have not yet been successful for VHR. Given the limited success to date, it is clear that future drug discovery efforts focused on PTP1B and VHR would benefit from identification of novel allosteric sites and an improved understanding of potential allosteric networks present within these enzymes.

PTP1B and VHR have two conserved catalytic features, the P-loop and acid loop (Fig. 1). The P-loop is critical for binding of the phosphoryl moiety of the polypeptide substrate and contains the cysteine nucleophile (C215 and C124, respectively), which is essential for covalent catalysis [24,26]. The acid loop (also called the WPD loop based on the one-letter amino acid nomenclature for the three N-terminal residues of this loop in PTP1B) contains a conserved aspartic acid (D181 and D92, respectively) that is important for general acid/base catalysis (Fig. 1). Mutation of this aspartic acid in PTP1B and VHR results in a 100,000-fold and 100-fold reduction in catalytic activity, respectively [27,28]. Research has shown that although the P-loop is immobile on the timescale of the chemical reaction, the acid loop in PTP1B moves 9 Å from an open position to a closed conformation to orient D181 for protonation of the tyrosine leaving group [24,29–31]. There is no published evidence for acid loop motion in VHR, but solution NMR data in our lab indicate that this loop also experiences motions on the millisecond timescale, similar to PTP1B (SI Fig. 1). It was recently shown that the timescale of acid loop closure in PTP1B is closely linked to the timescale of phosphoryl cleavage, corresponding to the first step in the mechanism for class I phosphatases (Fig. 1) [32]. These NMR studies also revealed that the acid loop of PTP1B alternates between open and closed conformations in the absence of substrate. This natural motion of the PTP1B acid loop results in alternating interactions with the main body of PTP1B, specifically with residues in the P-loop and α-helix 4 (R221 and T224), the Q-loop and α-helix 6 (T263, D265 and F269), and the E-loop (M109-E115). We reasoned that perturbation of these interactions would be propagated to distal allosteric sites, as any mutation in the acid loop that affects its conformational exchange motion should lead to changes in the ensemble averaged chemical shifts for any distantly coupled amino acid residues. While the importance of conformational motion exchange in the catalytic mechanism of VHR is less established, we believe that mutation of the acid loop in this enzyme would also aid the discovery of unknown allosteric sites in this enzyme based on the millisecond timescale motions we have observed for its acid loop (SI Fig. 1).

Results and Discussion

Acid loop mutations in PTP1B reveal a hidden allosteric network

To map the hidden allosterically coupled network to the active site of PTP1B, we made 13 single-site alanine mutations at each position of the acid loop (176-YTTWPDFGVPESP-188). By investigating the frequency of CSPs, we aimed to identify regions that are distally linked to the active site. Phosphatase kinetics were measured for each mutant using the *p*-nitrophenylphosphate (pNPP) cleavage assay, and the catalytic efficiencies of the mutants were found to range from a 10^{5}-fold reduction to a 2.4-fold increase, in agreement with many prior studies that highlighted the importance of specific acid loop interactions in regulating the catalytic efficiency of PTP1B (SI Table 1) [27,33,34]. Each mutant and the wild-type enzyme were subsequently analyzed by