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Inactivation of protein tyrosine phosphatases by dietary isothiocyanates

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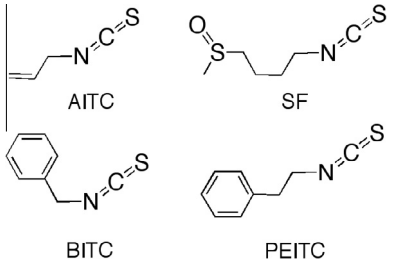
ABSTRACT

Isothiocyanates are bioactive dietary phytochemicals that react readily with protein thiol groups. We find that isothiocyanates are time-dependent inactivators of cysteine-dependent protein tyrosine phosphatases (PTPs). Rate constants for the inactivation of PTP1B and SHP-2 by allyl isothiocyanate and sulforaphane range from 2 to 16 M^{-1} s^{-1}. Results in the context of PTP1B are consistent with a mechanism involving covalent, yet reversible, modification of the enzyme's active site cysteine residue.

Structurally diverse isothiocyanates including allyl isothiocyanate (AITC), phenethylisothiocyanate (PEITC), benzyl isothiocyanate (BITC) and sulforaphane (SF) are released from the corresponding glucosinolate precursors present in plants of the *Brassicales* order, including mustards, wasabi, broccoli, and garden cress.[1] The isothiocyanates display an impressive array of bioactivities including potentiation of insulin signaling,[2] inhibition of cancer cell proliferation,[3] antibacterial properties,[4] TRP channel activation,[5,6] the ability to induce neuritogenesis,[7] and prevention of chemically-induced carcinogenesis.[8,9] In general, isothiocyanates are thiol-reactive chemicals that can modify critical cysteine residues on a variety of proteins.[1,5,6,9–12] In order to gain a better understanding of the molecular mechanisms underlying the myriad bioactivities of the isothiocyanates, it is important to explore potential protein targets of these compounds.



Protein tyrosine phosphatases (PTPs) are a family of cysteine-dependent enzymes that play central roles in mammalian signal transduction.[13] These enzymes possess a highly reactive active site cysteine thiolate residue that serves as a nucleophilic catalyst in the removal of phosphoryl groups from phosphoryltyrosine residues in proteins.[14,15] The results described here provide evidence that isothiocyanates are covalent inactivators of PTPs.

We first examined the ability of various isothiocyanates to inactivate the archetypal member of the PTP enzyme family, PTP1B. This enzyme is involved in a variety of mammalian signaling pathways,[16,17] but is best known for its role as a negative regulator of insulin signaling.[18] Inhibitors of PTP1B potentiate the action of insulin and may be medicinally useful agents for the treatment

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of type 2 diabetes.[18] For these experiments, we employed the catalytic domain (aa 1-322) of recombinant human PTP1B. Incubation of AITC (10–500 μM) with PTP1B (6 nM) in buffers composed of Bis–Tris (50 mM) and Tris (50 mM) or 3,3-dimethylglutarate (50 mM) containing DTPA (diethylenetriaminepentaacetic acid, 1 mM), sodium acetate (100 mM), and Tween 80 (0.05%, v/v) at pH 7.0 and 30 °C caused time- and concentration-dependent loss of enzyme activity (Figs. 1A and S1). Time-dependent inactivation is consistent with a mechanism involving covalent modification of the enzyme.[19] Interestingly, inactivation of PTP1B by concentrations of AITC below ~300 μM clearly did not proceed toward complete inactivation (Fig. 1A). This result signaled that inactivation of PTP1B by AITC may be an equilibrium process involving covalent, yet reversible, enzyme modification (Scheme 1). The chemical reactions of thiols with isothiocyanates can be reversible.[5,6,9,11,12,20,21] We sought evidence that the incomplete inactivation of the enzyme was due to a reversible reaction rather than decomposition of AITC in the reaction mixture. Accordingly, we carried out an inactivation assay in which we added a second aliquot of enzyme after the inactivation process had slowed (Fig. 2). In this experiment, we observed a second inactivation time course that closely mirrored the first, providing evidence that the AITC concentration was not substantially altered at the end of the inactivation process (Fig. 2). To provide further evidence that the inactivation by AITC is reversible, we gel filtered the inactivated enzyme to remove excess AITC and monitored the return of catalytic activity. We found that substantial amounts of activity return over the course of 3 h (Fig. S4).

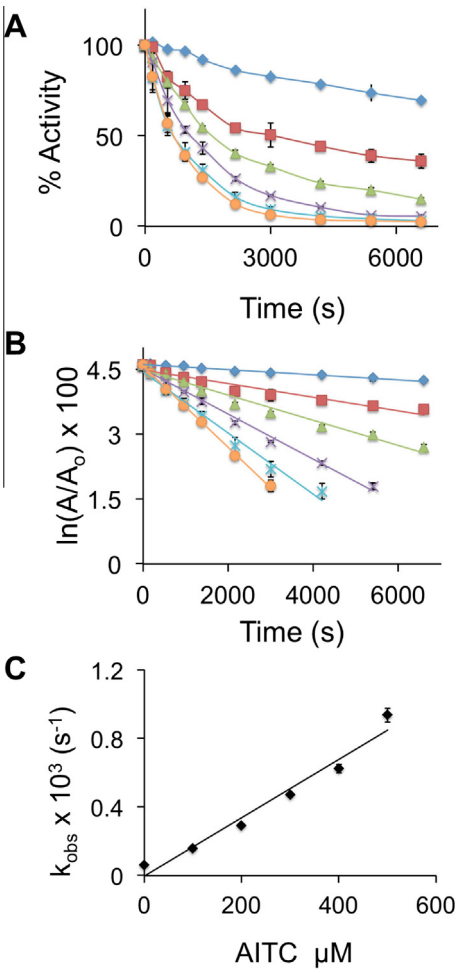


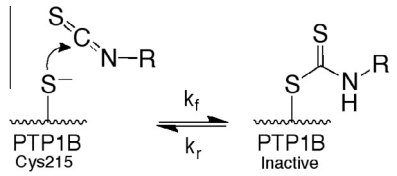
Figure 1. Kinetics of PTP1B inactivation by AITC. Various concentrations of AITC were added to PTP1B (6 nM) in a buffer consisting of 50 mM Bis–Tris (50 mM), Tris (50 mM), DTPA (1 mM), sodium acetate (100 mM), and Tween 80 (0.05%, v/v) at pH 7 and 30 °C. At various time points, aliquots (10 μL) were removed and added to an assay buffer (490 μL) composed of Bis–Tris (50 mM), NaCl (100 mM), DTPA (10 mM), and the substrate *p*-nitrophenyl phosphate (20 mM) at pH 6.0 and 37 °C. After 10 min, the reaction was quenched with NaOH (500 μL of a 2 mM aqueous solution). The amount of *p*-nitrophenol released during the activity assay was then quantitatively determined measuring the absorbance at 405 nm. Panel A. Time courses for inactivation of PTP1B by various concentrations of AITC: no AITC (enzyme alone control), blue diamonds; 100 μM, red squares; 200 μM, green triangles; 300 μM, purple X's; 400 μM, blue asterisk; and 500 μM, orange circles. Panel B. Semi-log replot of the inactivation time courses. The slope of each line corresponds to $-k_{obs}$, the apparent pseudo-first-order rate constant for inactivation of PTP1B by a given concentration of AITC. Panel C. A plot of k_{obs} versus AITC concentration. The linear nature of the plot is consistent with a second-order reaction process and the slope corresponds to the apparent second-order rate constant for inactivation of PTP1B by AITC.

Fitting our inactivation data to the kinetic model for a simple reversible reaction provided values for k_f of 2.3 ± 0.2 M^{-1} s^{-1} and k_r of 2.3 ± 0.9 × 10^{-4} s^{-1} in the Tris buffer system and a k_f of 2.7 ± 0.7 M^{-1} s^{-1} and k_r of 8.3 ± 7.2 × 10^{-5} s^{-1} in the glutarate buffer system. The k_f values for inactivation of PTP1B by AITC are only slightly smaller than the rate constants reported for the inactivation of PTP1B by hydrogen peroxide, a putative endogenous cellular regulator of the enzyme.[22,23] We examined a series of structurally diverse isothiocyanates and found that all were time- and concentration-dependent inactivators of PTP1B (Fig. S1). The widely-studied chemopreventive agent SF[1,8] reversibly inactivates PTP1B with a k_f of 2.4 ± 1.0 M^{-1} s^{-1} and k_r of 1.4 ± 1.2 × 10^{-4} s^{-1} in the glutarate buffer system (Fig. S2).

Inactivation of PTP1B by AITC was slowed by the presence of the reversible, active site-directed inhibitors phosphate (K_i = 17 mM) and arsenate (K_i = 149 μM).[24] Specifically, phosphate (50 mM) and arsenate (50 mM) decreased the apparent second-order rate at which AITC inactivated PTP1B by factors of 5.5 and 8.5, respectively, (Fig. 3A). These results suggested that the inactivation of PTP1B by AITC proceeds via modification of an active site residue.

The active site cysteine thiolate residue of PTP1B is prone to oxidation and the oxidized enzyme is catalytically inactive.[22,23,25–28] In fact, oxidative inactivation of PTP1B and other cysteine-dependent phosphatases arising from the unexpected generation of reactive oxygen species (ROS) via redox cycling or autoxidation of small molecules has been described as an important issue in high throughput screens designed to identify new inhibitors of the enzyme.[29,30] For this reason, we examined whether the inactivation of PTP1B by AITC involved ROS. Toward this end, we examined whether addition of various ROS-degrading enzymes and small molecule ROS scavengers[31] affected the rate of enzyme inactivation by AITC. We found that the presence of the hydrogen peroxide-destroying enzyme, catalase (100 μg/mL), the superoxide-destroying enzyme, superoxide dismutase (10 μg/mL), or the hydroxyl radical scavenger mannitol (500 mM) did not significantly alter the rate at which AITC inactivated PTP1B (Fig. 3B). These results provided evidence that inactivation of PTP1B by AITC does not involve ROS.

We also examined the ability of AITC and SF to inactivate SHP-2, a PTP involved in cytokine and growth factor-stimulated cell proliferation.[32] For these experiments, we employed the catalytic domain (aa 246-527) of the recombinant human enzyme.



Scheme 1.