

Redox-Based Probes for Protein Tyrosine Phosphatases**

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Over the past two decades, it has been established that growth factors, cytokines, and a host of other ligands trigger the production of hydrogen peroxide (H_2O_2) in nonphagocytic cells through their corresponding membrane receptors.^[1] Such H_2O_2 generation has been demonstrated to regulate many basic cellular processes including growth, differentiation, adhesion, migration, senescence, and autophagy.^[2] Once formed, H_2O_2 promotes autophosphorylation of the membrane receptor and induction of the signaling cascade.

Landmark publications from the Finkel and Rhee laboratories were the first to demonstrate an essential role for reactive oxygen species (ROS) growth factor receptor-mediated signal transduction.^[3] As illustrated in Figure 1, ligand stimulation leads to a transient burst of H_2O_2 and a net increase in tyrosine phosphorylation of numerous proteins, including the growth factor receptor itself.^[4] Likewise, application of peroxide scavengers such as *N*-acetyl cysteine or catalase inhibits ligand-induced tyrosine phosphorylation. In large part, these effects are believed to arise from oxidative inhibition of protein tyrosine phosphatases (PTPs), which function as antagonists of protein tyrosine kinases and return membrane receptors to their resting state.^[5]

There are about 80 members of the PTP superfamily, including the tyrosine (Tyr)-specific enzymes and dual-specificity phosphatases (DSPs), which also recognize serine (Ser) and threonine (Thr).^[6] The catalytic activity of PTPs depends upon an invariant active site cysteine (Cys) within the conserved signature motif [His-Cys-(X)₅-Arg-(Ser/Thr)] (His = histidine, Arg = arginine; X = any residue) located at the bottom of the active site pocket.^[7] Owing to the environment of the active site, the catalytic Cys residue exhibits a

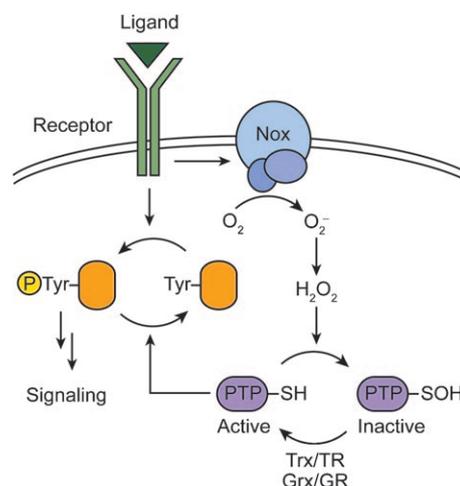


Figure 1. Proposed model for redox-dependent signal transduction. After ligand stimulation the H_2O_2 level increases through cytosolic proteins and subsequent activation of membrane-bound NADPH oxidase (Nox). Increased H_2O_2 production can lead to oxidation of specific reactive Cys residues within proteins, with concomitant modulation of the protein function. In PTPs, oxidation results in inactivation (and unopposed kinase action) until the H_2O_2 level declines and phosphatase activity is restored by reduction of the Cys residue. Trx = thioredoxin, TR = thioredoxin reductase, Grx = glutaredoxin, GR = glutaredoxin reductase.

remarkably low pK_a (4.5 to 5.5) and is present as the thiolate anion at physiological pH. The low pK_a serves to enhance the nucleophilicity of this residue, but also renders it susceptible to oxidation and enzymatic inactivation.^[8] Consequently, oxidative inhibition of PTPs promotes phosphorylation-dependent signaling cascades.

Biochemical evidence indicates that upon exposure to H_2O_2 , the catalytic Cys residue is converted into the sulfenic acid form and results in PTP inactivation (Figure 1).^[9] This oxo form can react with a backbone amide to form a cyclic sulfenyl amide for classical PTPs or an adjacent thiol in DSPs to form an intramolecular disulfide.^[10] The activity of PTPs can be restored through the action of cellular antioxidants, such as the thioredoxin and glutaredoxin reducing systems.^[5,11] Thus, oxidation of the catalytic Cys is reversible and represents a dynamic mechanism of PTP regulation.

Although the model presented in Figure 1 is supported by a number of elegant studies, it is also well-known that the rate of reaction of a PTP with H_2O_2 is about 10^5 times slower than the equivalent reaction with peroxiredoxin, an antioxidant enzyme.^[9,10] This raises the question of whether a nonenzymatic reaction can account for the formation of the sulfenic acid in PTPs.^[12] This apparent discrepancy may reflect the possibility that enzymatic H_2O_2 generation needs to occur in close proximity to PTPs so that the concentration of the

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[**] We acknowledge funding from the Camille and Henry Dreyfus Teacher-Scholar Awards Program (to K.S.C.) and the American Heart Association Scientist Development Award (award number 0835419N to K.S.C.). We thank Prof. M. Saper for providing the YopH expression plasmid and for helpful discussion, Jesse Song for providing technical assistance, and Prof. A. Barrios for helpful discussion.

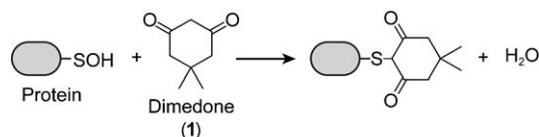


Supporting information for this article (including all experimental procedures) is available on the WWW under <http://dx.doi.org/10.1002/anie.201007871>.

oxidant is high enough to make the rate of the uncatalyzed reaction physiologically relevant, or oxidation of the Cys residue may be catalyzed by another enzyme. Evidence to support or revise these proposals will require a better understanding of the mechanisms that underlie cellular compartmentalization, information about the extent of in situ oxidation of PTPs, and/or the discovery of a specific Cys oxidase.

These outstanding questions, coupled with the physiological importance of PTPs, and their therapeutic relevance to diseases such as cancer and diabetes have motivated the development of methods for monitoring reversible PTP oxidation.^[13] The majority of these approaches rely on the loss of reactivity with thiol-modifying reagents or the restoration of labeling by reducing agents. These techniques require that free thiols are completely blocked by alkylating agents prior to the reduction step and are thus limited to enrichment from protein extracts. Methods to decrease oxidation artifacts after cell lysis have been reported;^[13b] however, they require specialized reagents or equipment, and other issues such as loss of labile modifications and temporal resolution are not addressed.

Direct labeling methods that exploit the selective reaction of sulfenic acid and 5,5-dimethyl-1,3-cyclohexanedione (dimedone, **1**) and related analogues have also been used to monitor PTP oxidation (Scheme 1).^[14] Recently, we have reported the development of azide- and alkyne-based ana-



Scheme 1. Selective reaction between a protein sulfenic acid and dimedone (**1**).

logues of dimedone, such as DAz-1 (**2**, Scheme 1) that enable trapping and tagging of sulfenic acid modified proteins directly in cells.^[14b,15] The azide or alkyne chemical reporter group can be used for bioorthogonal Staudinger and Huisgen [3+2] cycloaddition coupling reactions for downstream analysis of labeled proteins. These reagents have been successfully deployed for global profiling of protein sulfenic acid modifications.^[15,16] Although dimedone-based probes have shown wide utility for investigating Cys oxidation, the modest reaction rate and the low cellular abundance of signaling proteins have hampered the detection of oxidized PTPs.^[16,17] Herein, we present a solution to this issue and report on the design of new redox-based probes (RBPs) for direct detection of PTP oxidation with significantly increased sensitivity and selectivity.

Our overall strategy for targeting reversible PTP oxidation is to create a trifunctional probe that consists of: 1) a “warhead” bearing a cyclic 1,3-diketone group that forms a covalent adduct with the active-site cysteine of PTPs, 2) a module that directs binding to the PTP target, and 3) a reporter tag used for the identification, purification, or direct visualization of the probe-labeled proteins (Figure 2a). This

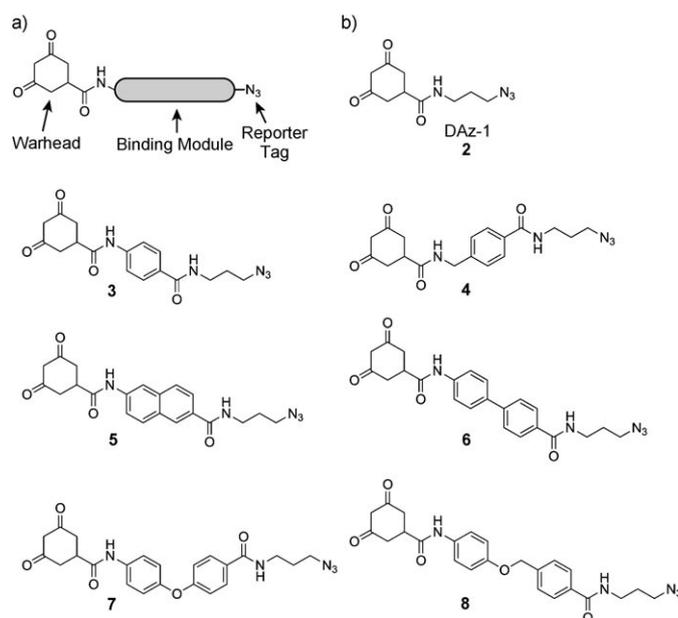


Figure 2. Redox-based probes (RBPs) for detecting reversible PTP oxidation. a) The design strategy for RBP includes the cyclic 1,3-diketone warhead, a synthetic module that directs binding to the PTP active site, and an azide reporter tag for downstream analysis. b) Structures of the parent compound DAz-1 (**2**) and the focused RBP library (**3–8**) synthesized and evaluated in this study.

approach to detect PTP oxidation is inspired by the extensive use of activity-based probes to monitor phosphatase and protease activity.^[18] We anticipated that integrating a binding module into the dimedone scaffold would provide a redox-based probe, termed RBP, designed to react only with the oxidized form of PTPs. Modification of PTP targets by such probes would provide a direct measure of oxidation and enable their purification and identification, thereby aiding in the elucidation of the biological roles of PTP oxidation in physiological and pathophysiological redox-mediated signal transduction pathways. Along these lines, we selected a series of synthetic binding modules known to target the active site of a broad range of enzymes from the PTP superfamily (Figure 2b). The phenyl (**3**) and benzyl (**4**) derivatives are modeled after the phosphotyrosine ring present in the native substrate. Additional scaffolds that are common to many known PTP inhibitors include naphthalene (in **5**), biphenyl (in **6**), diphenyl ether (in **7**), and benzy phenyl ether (in **8**).^[19] Accordingly, we prepared compounds **3–8** (see the Supporting Information) and evaluated their target-binding affinity and ability to monitor PTP oxidation.

To test our compounds of interest, we used recombinant *Yersinia* phosphatase (YopH). The enzyme is a representative member of the classical PTP superfamily and has been extensively characterized in vitro.^[20] Like other PTPs, YopH harbors a catalytic Cys residue in its active site, Cys403 that is characterized by a low pK_a (ca. 5). Since sulfenic acid modification of YopH had not been previously documented, initial experiments were directed toward determining whether Cys403 was susceptible to oxidation by H₂O₂. Analogous to other redox-sensitive PTPs, we expected that

oxidation of Cys403 would manifest itself as a loss of catalytic activity. We evaluated this proposal in steady-state assays using the fluorogenic substrate, 4-methylumbelliferyl phosphate (4-MUP). As shown in Figure 3a, the YopH activity was detected as fluorescence signal owing to dephosphoryla-

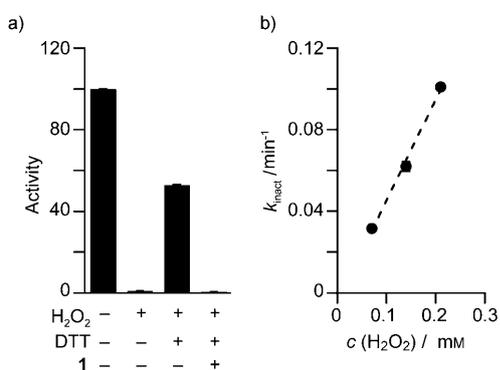


Figure 3. Analysis of YopH PTP oxidation by H₂O₂. a) YopH was inactivated by the addition of H₂O₂ in the absence of a reducing agent. H₂O₂-inactivated YopH could be reactivated through DTT treatment. Dimedone (**1**) forms a covalent adduct with the sulfenic acid form of YopH, which prevents reactivation of YopH by DTT. b) The observed rate of YopH inactivation plotted as a function of the H₂O₂ concentration. The second-order rate constant for oxidation of Cys403 by H₂O₂ was about 10 M⁻¹ s⁻¹.

tion of 4-MUP. Exposure to 100 equivalents of H₂O₂ for 1 h abolished the phosphatase activity and subsequent addition of dithiothreitol (DTT) resulted in substantial restoration of the PTP function. These results are indicative of reversible modification of Cys403 with sulfenic acid. To verify the identity of the modification at the active-site Cys, oxidized YopH was incubated with dimedone, which reacts with sulfenic acids to form a covalent adduct that is nonreducible by thiols. As expected, dimedone treatment inhibited the PTP activity, which could not be restored by incubation with DTT. Mass spectrometry analysis also confirmed formation of the adduct in a stoichiometry of 1:1 (see Figure S1 in the Supporting Information). Having established that YopH is susceptible to oxidation, we determined the second-order rate constant for the reaction of Cys403 with H₂O₂ to be about 10 M⁻¹ s⁻¹, consistent with values obtained for other PTPs (Figure 3b).^[9]

Next, we examined the ability of compounds **3–8**, and the parent compound DAz-1, to reversibly inhibit activity of YopH. To this end, the dissociation constants (K_i) for inhibition were determined by conducting the assays under reducing conditions, in the absence of H₂O₂. The resulting data fit well to a simple model of competitive inhibition^[21] and the K_i values are summarized in Table 1. No significant inhibition by DAz-1 was observed at concentrations up to 12 mM,^[22] consistent with the absence of an active-site-targeted binding module. The phenyl compound **3** was moderately active, with a 130-fold increase in the binding affinity relative to DAz-1. By contrast, the benzyl analogue **4** exhibited weak binding. The naphthalene analogue **5** showed a 255-fold increase in the affinity relative to DAz-1. The

Table 1: Inhibition constants for DAz-1 **2** and compounds **3–8** with YopH.^[a]

Entry	K_i [μM]
DAz-1 2	> 12000
3	162.1
4	2710
5	47.1
6	2.9
7	21.4 ^[b]
8	113.3 ^[b]

[a] K_i values represent the average of at least three independent experiments and the standard deviation was $\leq 25\%$. [b] Compounds exhibited aggregation-based inhibition.

biphenyl derivative **6** conferred a 4135-fold increase in the binding affinity and was the most potent inhibitor of the series ($K_i = 2.9 \mu\text{M}$). Finally, the diphenyl ether in **7** and the benzyl phenyl ether in **8** showed K_i values in the mid-micromolar range.

In many cases, protein aggregation is a common mechanism of promiscuous chemical inhibitors.^[23] The kinetic assays were therefore repeated with 0.02% Triton-X 100 detergent. Inhibition by **2–6** was found to be independent of the enzyme and detergent concentration. By contrast, compounds **7** and **8** both demonstrated a >15-fold decrease in potency when detergent was present in the assay buffer. These data are consistent with aggregation-based inhibition of YopH by **7** and **8**. Promiscuous inhibition has been observed previously in scaffolds that contain the diphenyl ether or benzyl phenyl ether functional groups.^[23,24]

Compounds **3–8** were then tested for detecting sulfenic acid modification of YopH, and compared against DAz-1. For these experiments, the PTP was oxidized and then incubated for 15 min with each probe. The bioorthogonally labeled protein was detected through reaction of the azido group with phosphine-biotin (p-biotin) through the Staudinger ligation.^[25] The products of these reactions were resolved by SDS-PAGE and analyzed by streptavidin-HRP western blot. Figure 4a shows that the majority of compounds exhibited an increase in labeling relative to DAz-1.^[26] The most significant increase in YopH sulfenic acid detection was observed for **3**, **5**, and **6**. Notably, probe **6** exhibited the most potent K_i value and also showed the most robust detection of oxidized YopH. Naphthalene derivative **5**, which displayed the second highest binding activity, also demonstrated enhanced sulfenic acid detection. By contrast, compounds **4**, **7**, and **8** showed moderate to no reaction with the oxidized protein. The apparent lack of reactivity for compounds **7** and **8** is most likely due to the formation of promiscuous aggregates. Additional control experiments also verified that chemical reduction of YopH or pretreatment of the oxidized protein with dimedone inhibited incorporation of the azido probe, as expected (see Figure S4 in the Supporting Information).

Our next goal was to assess the selectivity of probes **5** and **6**, relative to DAz-1, for detecting sulfenic acid modifications in non-PTP proteins. For this purpose, we used the metabolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This protein forms a sulfenic acid modification

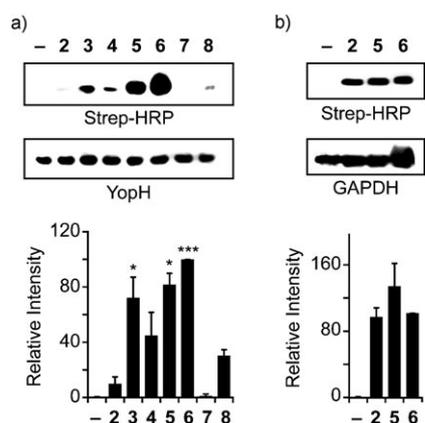


Figure 4. Analysis of RBP selectivity for oxidized YopH. a) RBPs detect sulfenic acid in oxidized YopH with increased sensitivity over the parent compound DAz-1 **2**. YopH was oxidized with H₂O₂ and incubated with compounds **2–8** or DMSO alone (–). Top: Following p-biotin conjugation, reactions were analyzed by streptavidin-HRP western blot. Bottom: Equal loading was verified by Ponceau S staining. b) RBPs do not increase the general sulfenic acid reactivity. Top: GAPDH was incubated with **2**, **5**, **6**, or DMSO alone (–) followed by conjugation to p-biotin and analyzed by streptavidin-HRP western blot. Bottom: Equal loading was verified by reprobing the blot with an antibody against GAPDH. The bar graphs summarize the relative signal intensities from replicate experiments quantified by densitometry, normalized to **6**, and analyzed by Student's *t*-test. Error bars represent standard errors of the mean: *** indicates that $P < 0.001$ and *) indicates that $P < 0.05$ when compared against detection with the parent compound, DAz-1 **2**.

at Cys149, which has been detected in previous studies by dimedone and related azido analogues.^[16,27] Oxidized GAPDH was incubated with DAz-1, **5**, or **6** followed by p-biotin conjugation and western blot analysis. Figure 4b shows that sulfenic acid modification of GAPDH is detected with equal efficiency by the azido probe. Similar results were obtained with the thiol peroxidase, Gpx3 (data not shown). Finally, we examined the ability of compounds **5** and **6** to inhibit and detect reversible oxidation of PTP1B, another redox-sensitive tyrosine phosphatase.^[8] Compounds **5** and **6** inhibited PTP1B with K_i values of $(0.67 \pm 0.19) \mu\text{M}$ and $(61.5 \pm 7.0) \mu\text{M}$, respectively. By contrast, PTP1B activity was not affected by DAz-1. Importantly, **5** and **6** showed enhanced detection of oxidized PTP1B, relative to DAz-1 (see Figure S5 in the Supporting Information). Taken together, these data establish that **5** and **6** are RBPs against YopH and PTP1B, thereby validating our general approach.

To gain molecular insight into interactions between YopH and RBP **6**, we performed docking simulations using AutoDock.^[28] As shown in Figure 5, the two oxygen atoms of the cyclic diketone are predicted to form hydrogen bonds with several active-site residues. In addition, the docking analysis suggests that aromatic stacking interactions can occur between the phenyl rings of RBP **6** and the side chain of Phe229. The remaining portion of the compound (i.e., the chemical reporter group) appears largely exposed to the solvent. Future structural studies of YopH in complex with RBP **6** should provide further insights into interactions that can facilitate active-site targeting. Interestingly, the biphenyl

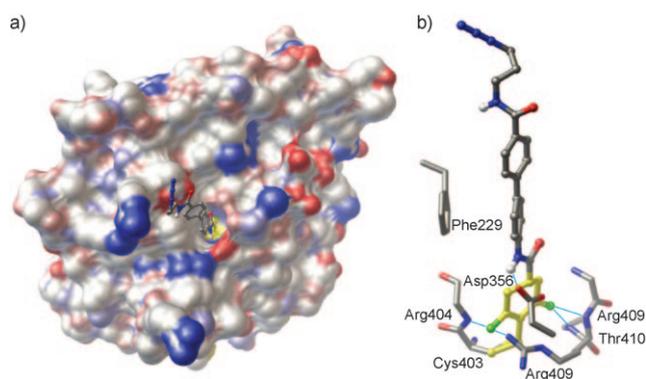


Figure 5. The binding conformation of RBP **6** with YopH predicted by AutoDock. a) Molecular surface representation of YopH (PDB code: 3BLT) colored by the polarity of the atoms. RBP **6** is given in a ball-and-stick representation. b) Detailed view of hypothetical interactions between YopH and RBP **6**. The cyclic diketone is shown with carbon in yellow and oxygen in green. Hydrogen bonds are shown as thin blue lines. Images were created with the Python Molecular Viewer.^[30]

group present in RBP **6** has been identified as a core structure in many bioactive compounds and is considered a privileged scaffold.^[29] Along these lines, an opportunity for future design will be to evaluate the potency of substituted biphenyls and create molecules that interact more closely with the PTP surface.

In summary, we have developed a suite of redox-based probes that target the active site of YopH and PTP1B, which are both members of the classical PTP superfamily. Although indirect and direct methods to detect protein sulfenic acids have previously been reported,^[15,27a,31] our study provides the first example of a cyclic 1,3-diketone, dimedone-like warhead to develop probes with enhanced targeting specificity. In terms of reversible inhibition, the potency of RBPs **5** and **6** are comparable or exceed that of other known YopH and PTP1B inhibitors.^[19] Future studies will focus on the application of RBPs to investigate PTP regulation and redox signaling in living systems. Finally, we note that the strategy employed to identify RBPs in this study may also represent an attractive starting point into the inhibitor- or tool-development cycle for other classes of proteins with redox-sensitive Cys residues.

Received: December 14, 2010

Published online: April 18, 2011

Keywords: chemoselectivity · cysteine oxidation · protein modifications · redox chemistry · tyrosine phosphatase

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