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Redox Regulation of Protein Tyrosine Phosphatase 1B (PTP1B) by Peroxymonophosphate ($=O_3POOH$)

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Abstract

Reversible phosphorylation of tyrosine residues serves as a biochemical “switch” that alters the functional properties of many proteins involved in cellular signal transduction processes. Protein tyrosine phosphatases (PTPs) catalyze the removal of phosphoryl groups from tyrosine residues in target proteins thereby playing a central role in the regulation of diverse cellular processes including glucose metabolism, cell cycle control, and immune responses. Accordingly, small molecules capable of inactivating PTPs may find use as therapeutic agents and tools for the study of diverse signal transduction pathways. In the work reported here, peroxymonophosphate ($^2-O_3POOH$) was reported to be an exceptional inactivator of PTP1B, an archtypical member of the PTP enzyme family ($K_I = 6.6 \times 10^{-7}$ M and $k_{inact} = 0.043$ s⁻¹). In this regard, peroxymonophosphate is over 7,000 times more potent than hydrogen peroxide, the endogenous regulator of PTP1B. Inactivation of PTP1B by peroxymonophosphate is active site directed and, like that by hydrogen peroxide, is readily reversed by treatment with dithiothreitol (5 mM). Together the findings suggest that peroxymonophosphate oxidizes the active site cysteine residue of PTP1B to the sulfenic acid oxidation state. Importantly, peroxymonophosphate (100 nM) yields substantial inactivation of PTP1B even in the presence of physiologically-relevant concentrations of the biological thiol glutathione (1 mM). Collectively, these properties may make peroxymonophosphate a useful tool for probing the role of cysteine-dependent PTPs in various signal transduction pathways. Finally, it is interesting to note that peroxymonophosphate may be biosynthetically accessible via the reaction of endogenous hydrogen peroxide with phosphoryl donors. Peroxymonophosphate possesses key properties expected for the endogenous signaling molecule involved in the redox regulation of PTP activity.

Reversible phosphorylation of tyrosine residues serves as a biochemical “switch” that alters the functional properties of many proteins involved in cellular signal transduction processes.^{1,2} The phosphorylation status of tyrosine residues in target proteins is controlled by the opposing actions of protein tyrosine kinases that catalyze the addition of phosphoryl groups and protein tyrosine phosphatases (PTPs) that catalyze their removal.² Thus, PTPs play a central role in the regulation of diverse cellular processes including glucose metabolism, cell cycle control, and immune responses.³

The catalytic activities of many tyrosine kinases and phosphatases are strictly regulated to control the intensity and duration of cellular responses to various stimuli.^{2,4} For example,

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Supporting Information Available: Experimental protocols for all experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

exposure of cells to insulin, growth factors, or cytokines activates kinases that add phosphoryl groups to tyrosine residues on target proteins.⁴ In some cases, this kinase action is potentiated by a rapid (2–5 min onset), transient inactivation of the tyrosine phosphatases that are responsible for removal of these phosphoryl groups.^{5,6} This involves downstream activation of NADPH oxidases that produce an intracellular burst of H₂O₂.⁷ The H₂O₂, in turn, leads to inactivation of select PTPs via oxidation of their catalytic cysteine thiol residues to the sulfenic acid oxidation state (Scheme 1).^{5,7} Oxidative inactivation of PTPs inside cells is transient because thiol-mediated reduction of the oxidized cysteine residue slowly regenerates the active form of the enzyme.^{5–7}

Interestingly, despite clear evidence for its involvement in the intracellular regulation of PTPs, *in vitro* experiments reveal that H₂O₂, at physiological concentrations, is a rather sluggish PTP inactivator.⁵ Specifically, based upon the reported⁵ rate constants for *in vitro* inactivation of PTPs by H₂O₂ (e.g. $k = 9 \text{ M}^{-1} \text{ s}^{-1}$ for PTP1B), one can calculate that the half-life for inactivation of these enzymes by a steady-state concentration of 1 μM H₂O₂ will be approximately 20 h.^{8,9}

For some applications, it may be desirable to identify small molecules that mimic the ability of hydrogen peroxide to effect transient, thiol-reversible, oxidative inactivation of PTPs. In general, PTP inactivators have potential both as therapeutic agents and tools for the study of signal transduction pathways.¹⁰ Here, we set out to develop a redox regulator of PTP activity that is more potent than hydrogen peroxide. Toward this end, we envisioned that peroxyphosphonate (**1**; Scheme 1) might be an exceptional PTP inactivator, in which noncovalent association of the phosphoryl group with the highly conserved phosphate binding pocket found at the active site of all PTPs³ would serve to guide the peroxy moiety into position for efficient reaction with the catalytic cysteine residue (inset, Scheme 1).

In the studies described here, we employed the catalytic subunit of human PTP1B (a.a. 1–322) as an archetypal member of the PTP family of enzymes.³ Peroxyphosphonate (**1**) was prepared via electrolysis of potassium phosphate to generate peroxydiphosphate, followed by acid hydrolysis.¹¹ The resulting peroxyphosphonate was characterized by ³¹P-NMR and mass spectrometry. We find that peroxyphosphonate is, indeed, a superior inactivator of PTP1B with a K_I of $6.6 \pm 1.5 \times 10^{-7} \text{ M}$ and a k_{inact} of $0.043 \pm 0.008 \text{ s}^{-1}$ (Figure 1A). Thus, peroxyphosphonate ($k_{\text{inact}}/K_I = 65,553 \text{ M}^{-1} \text{ s}^{-1}$) is over 7,000 times more potent than H₂O₂ ($9 \text{ M}^{-1} \text{ s}^{-1}$) as a PTP1B inactivator. The saturation kinetics observed for the inactivation of PTP1B by peroxyphosphonate offers evidence that the phosphoryl group of peroxyphosphonate does, indeed, provide noncovalent binding affinity for the enzyme active site. In contrast, H₂O₂ does not possess noncovalent affinity for PTP1B and inactivates the enzyme via a simple second-order reaction process.⁵

Consistent with a process involving covalent modification of the enzyme, inactivation of PTP1B by peroxyphosphonate is time-dependent and is not reversed by gel filtration of the inactivated enzyme. The presence of the competitive PTP1B inhibitor phosphate¹³ markedly slows inactivation by peroxyphosphonate, indicating that the process is active-site directed. Significantly, inactivation of PTP1B (1 μM , 1 min) by peroxyphosphonate is readily reversed (88% recovery of activity) by treatment of the inactivated enzyme with dithiothreitol (Figure 1B). Together, the results suggest that peroxyphosphonate inactivates PTP1B via conversion of the active site cysteine residue to the sulfenic acid oxidation state as shown Scheme 1.¹⁴

In principle, PTP1B could catalyze the hydrolysis of peroxyphosphonate to H₂O₂ and inorganic phosphate. However, two lines of evidence suggest that such an enzyme-catalyzed decomposition of peroxyphosphonate does not occur. First, addition of the H₂O₂-destroying

enzyme catalase has no effect on the inactivation of PTP1B by peroxyphosphate. Second, titration of the enzyme with peroxyphosphate reveals a turnover number¹⁵ of approximately one (Figure 2), indicating that a single equivalent of peroxyphosphate is sufficient to inactivate PTP1B.

Mammalian cells contain millimolar concentrations of thiols that have the potential to decompose peroxides.^{16,17} Therefore, we investigated whether peroxyphosphate retains the ability to inactivate PTP1B in the presence of the biological thiol glutathione. We find that peroxyphosphate (100 nM) causes substantial inactivation of PTP1B (21 ± 3% activity remaining) within 5 min even in the presence of physiologically-relevant concentrations of glutathione (1 mM). This result shows that peroxyphosphate reacts selectively with the active site cysteine thiolate of PTP1B over solution thiols.

In conclusion, we find that peroxyphosphate is a potent oxidative inactivator of the protein tyrosine phosphatase PTP1B. In this regard, peroxyphosphate is far more potent than H₂O₂. Inactivation of PTP1B by peroxyphosphate, like that by H₂O₂, is readily reversed by treatment of the inactivated enzyme with thiol. A few other oxidative PTP inactivators are known¹⁸ but none (other than the endogenous signaling agents, H₂O₂ and nitric oxide) have been shown to yield thiol-reversible inactivation.^{5,19} Importantly, the inactivation of PTP1B by nanomolar concentrations of peroxyphosphate proceeds effectively in the presence of physiologically-relevant concentrations of the biological thiol glutathione. Collectively, these properties may make peroxyphosphate a useful tool for probing the role of cysteine-dependent PTPs in various signal transduction pathways.

Finally, an additional interesting facet of peroxyphosphate is that this molecule could be biologically accessible via phosphorylation of H₂O₂. In addition to its aforementioned role as an intracellular signaling agent, H₂O₂ is produced inside cells as a byproduct of aerobic metabolism and as a result of various disease states.^{7,20} Phosphoryl transfer to oxygen nucleophiles is a ubiquitous reaction in biology²¹ and H₂O₂, though present at relatively low concentrations, is a substantially better nucleophile than water.²² It remains to be seen whether peroxyphosphate can be generated through spontaneous or enzyme-catalyzed reactions of H₂O₂ with phosphoryl donors under physiological conditions; however, the results reported here suggest that nanomolar concentrations of peroxyphosphate could effect reversible down-regulation of cellular PTP activity within minutes. In this regard, peroxyphosphate possesses key properties expected for an endogenous signaling molecule involved in the redox regulation of PTP activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Panel A. Inactivation progress curves showing time-dependent inactivation of PTP1B by peroxymonophosphate (**1**). PTP1B (25 pmol) was incubated with various concentrations of **1** (250–1500 nM) at 25 °C in aqueous buffer (50 mM Tris, 50 mM Bis-Tris, 100 mM NaOAc, pH 7) containing the substrate *p*-nitrophenylphosphate (*p*-NPP, 10 mM) and enzyme-catalyzed release of *p*-nitrophenolate ion from the substrate was monitored at 410 nm. The inactivation constants k_{inact} and K_I were calculated from the data as described by Voet and coworkers¹² (see Supporting Information. **Panel B.** Reaction progress curves showing inactivation of PTP1B by **1** and reactivation of the inactivated enzyme by treatment with thiol. The enzyme PTP1B (100 pmol) was inactivated by **1** (2 μ M) in aqueous buffer (50 mM Tris, 50 mM Bis-Tris, 100 mM NaOAc, pH 7) at 25 °C containing the substrate *p*-NPP (10 mM). When the enzyme inactivation was complete, dithiothreitol (DTT) was added to a final concentration of 5 mM. Control experiments show that DTT does not react with *p*-NPP.

Figure 2.

Turnover number for the inactivation of PTP1B by peroxyphosphate (**1**). PTP1B (35 pmol) was incubated with various concentrations of **1** for 10 min at 25 °C in 50 mM Tris, 50 mM Bis-Tris, 100 mM NaOAc, pH 7. The amount of remaining enzyme activity (relative to a control sample containing no inactivator) was then observed following addition of *p*-NPP (10 mM final). The turnover number is determined from the x-intercept of the plot (0.86 ± 0.2).

Scheme 1.