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Redox Regulation of Protein Tyrosine Phosphatase 1B by Peroxymonophosphate ($^{=}O_3POOH$)

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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]

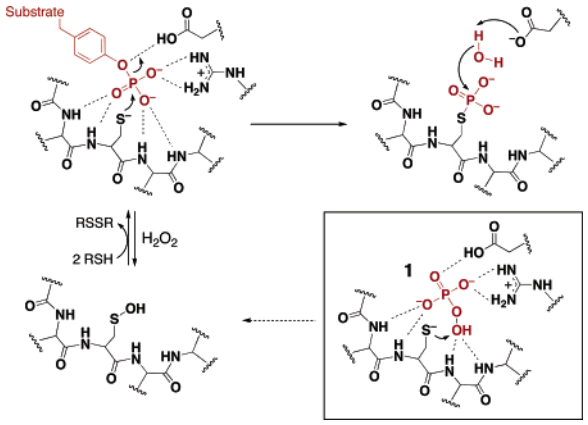
The catalytic activities of many tyrosine kinases and phosphatases are strictly regulated to control the intensity and duration of cellular responses to various stimuli.[2,4] For example, exposure of cells to insulin, growth factors, or cytokines activates kinases that add phosphoryl groups to tyrosine 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 H_2O_2.[7] The H_2O_2, 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 H_2O_2, at physiological concentrations, is a rather sluggish PTP inactivator.[5] Specifically, based upon the reported[5] rate constants for in vitro inactivation of PTPs by H_2O_2 (e.g., $k = 9\ M^{-1}\ s^{-1}$ for PTP1B), one can calculate that the half-life for inactivation of these enzymes by a steady-state concentration of 1 μM H_2O_2 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 as 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 peroxymonophosphate (**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 PTPs[3] would serve to guide the peroxyl 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 (aa 1−322) as an archetypal member of the PTP family of enzymes.[3] Peroxymonophosphate (**1**) was prepared via electrolysis of potassium phosphate to generate peroxydiphosphate, followed by acid hydrolysis.[11] The resulting peroxymonophosphate was characterized by ^{31}P NMR and mass spectrometry. We find that peroxymonophosphate is, indeed, a superior inactivator of PTP1B with a K_I of 6.6 ± 1.5 × 10^{-7} M and a k_{inact} of 0.043 ± 0.008 s^{-1} (Figure 1A). Thus, peroxymonophosphate (k_{inact}/K_I = 65 553 $M^{-1}\ s^{-1}$) is over 7000 times more potent than H_2O_2 (9 $M^{-1}\ s^{-1}$) as a PTP1B inactivator. The saturation kinetics observed for the inactivation of PTP1B by peroxymonophosphate offers evidence that the phosphoryl group of peroxymonophosphate does, indeed, provide noncovalent binding affinity for the enzyme active site. In contrast, H_2O_2 does not possess noncovalent affinity for PTP1B and inactivates the enzyme via a simple second-order reaction process.[5]

Scheme 1



Consistent with a process involving covalent modification of the enzyme, inactivation of PTP1B by peroxymonophosphate is time-dependent and is not reversed by gel filtration of the inactivated enzyme. The presence of the competitive PTP1B inhibitor phosphate[13] markedly slows inactivation by peroxymonophosphate, indicating that the process is active site directed. Significantly, inactivation of PTP1B (1 μM, 1 min) by peroxyphosphate is readily reversed (88% recovery of activity) by treatment of the inactivated enzyme with dithiothreitol (Figure 1B). Together, the results suggest that peroxymonophosphate 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 peroxymonophosphate to H_2O_2 and inorganic phosphate. However, two lines of evidence suggest that such an enzyme-catalyzed decomposition of peroxymonophosphate does not occur. First, addition of the H_2O_2-destroying enzyme catalase has no effect on the inactivation of PTP1B by peroxymonophosphate. Second, titration of the enzyme with peroxymonophosphate reveals a turnover number[15] of approximately one (Figure 2), indicating that a single

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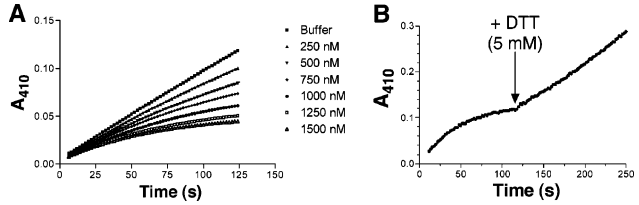

Figure 1. (A) Inactivation progress curves showing time-dependent inactivation of PTP1B by peroxymonophosphate (**1**). PTP1B (25 pmol) was incubated with various concentrations of **1** (250−1500 nM) at 25 °C in aqueous buffer (50 mM Tris, 50 mM bis-Tris, 100 mM NaOAc, pH 7) containing the substrate *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 co-workers[12] (see Supporting Information. (B) Reaction progress curves showing inactivation of PTP1B by **1** and reactivation of the inactive enzyme by treatment with thiol. The enzyme PTP1B (100 pmol) was inactivated by **1** (2 μM) 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.

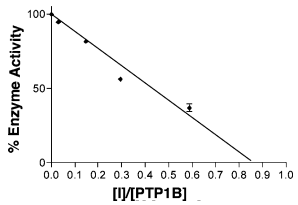

Figure 2. Turnover number for the inactivation of PTP1B by peroxymonophosphate (**1**). PTP1B (35 pmol) was incubated with various concentrations of **1** for 10 min at 25 °C in 50 mM Tris, 50 mM bis-Tris, 100 mM NaOAc, pH 7. The amount of remaining enzyme activity (relative to a control sample containing 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 ± 0.03).

equivalent of peroxymonophosphate 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 peroxymonophosphate retains the ability to inactivate PTP1B in the presence of the biological thiol glutathione. We find that peroxymonophosphate (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 peroxymonophosphate reacts selectively with the active site cysteine thiolate of PTP1B over solution thiols.

In conclusion, we find that peroxymonophosphate is a potent oxidative inactivator of the protein tyrosine phosphatase PTP1B. In this regard, peroxymonophosphate is far more potent than H_2O_2. Inactivation of PTP1B by peroxymonophosphate, like that by H_2O_2, is readily reversed by treatment of the inactivated enzyme with thiol. A few other oxidative PTP inactivators are known,[18] but none (other than the endogenous signaling agents, H_2O_2 and nitric oxide) have been shown to yield thiol-reversible inactivation.[5,19] Importantly, the inactivation of PTP1B by nanomolar concentrations of peroxymonophosphate proceeds effectively in the presence of physiologically relevant concentrations of the biological thiol glutathione. Collectively, these properties may make peroxymonophosphate a useful tool for probing the role of cysteine-dependent PTPs in various signal transduction pathways.

Finally, an additional interesting facet of peroxymonophosphate is that this molecule could be biologically accessible via phosphorylation of H_2O_2. In addition to its aforementioned role as an intracellular signaling agent, H_2O_2 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 biology[21] and H_2O_2, though present at relatively low concentrations, is a substantially better nucleophile than water.[22] It remains to be seen whether peroxymonophosphate can be generated through spontaneous or enzyme-catalyzed reactions of H_2O_2 with phosphoryl donors under physiological conditions; however, the results reported here suggest that nanomolar concentrations of peroxymonophosphate could effect reversible down-regulation of cellular PTP activity within minutes. In this regard, peroxymonophosphate possesses key properties expected for an endogenous signaling molecule involved in the redox regulation of PTP activity.

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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.

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