Biologically relevant chemical properties of peroxymonophosphate ($=O_3POOH$)

Jason N. LaButti, Kent S. Gates *

Departments of Chemistry and Biochemistry, University of Missouri, 125 Chemistry Building, Columbia, MO 65211, USA

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ABSTRACT

It has been suggested that peroxymonophosphate could serve as an endogenous hydrogen peroxide-derived regulator of cellular protein tyrosine phosphatase activity under physiological or pathophysiological conditions. To facilitate further consideration of the potential role of peroxymonophosphate in biological systems we present studies related to the preparation, characterization, stability, and fluorometric detection of this agent.

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Hydrogen peroxide has emerged as a signaling agent that regulates cellular responses to a variety of extracellular stimuli including insulin, epidermal growth factor, platelet-derived growth factor, endothelin-1, and B-cell receptor stimulation.[1] H_2O_2 is generated by NADPH oxidase enzymes (Nox), which are activated by the binding of these growth factors and cytokines to cell surface receptors.[2] Protein tyrosine phosphatases (PTPs) are important cellular targets of hydrogen peroxide.[1,3–6] PTPs work in tandem with protein tyrosine kinases to regulate the phosphorylation status of proteins involved in critical signal transduction pathways.[7–9] Accordingly, peroxide-mediated inactivation of target PTPs, involving oxidation of the catalytic cysteine residue in these enzymes,[3,4,10,11] has the potential to profoundly influence the duration and intensity of cellular responses to various stimuli.

Cellular responses to hydrogen peroxide are generally rapid, occurring within 5–10 min.[12,13] Interestingly, kinetic measurements on isolated PTPs suggest that, at the low concentrations generated during cell signaling processes, H_2O_2 is a rather slow PTP inactivator.[14] For example, the inactivation of the archtypal member of the PTP class of enzymes, PTP1B,[9] by a steady state concentration of 1 µM H_2O_2 is predicted to occur with a half-life of about 20 h. It is possible that co-localization of Nox and PTP enzymes inside cells produces a high local concentration of H_2O_2 near the phosphatase target, thus leading to rapid enzyme inactivation.[15,16] Alternatively, we have considered that the kinetic discrepancy might be explained by a scenario in which intracellular hydrogen peroxide is converted either spontaneously or enzymatically into inorganic peroxides such as peroxymonophosphate, peroxymonosulfate, or peroxymonocarbonate that are more potent oxidizing agents than the parent peroxide.[17] In the case of peroxymonophos-phate, this would involve the reaction of hydrogen peroxide with one of the many phosphoryl donors, such as adenosine triphosphate, that are present inside cells (Scheme 1). Phosphoryl transfer to oxygen nucleophiles is a common reaction in biological systems.[18] Phosphoryl transfer to H_2O_2 may be possible even in the presence of vast excesses of water due to the exceptional nucleophilicity of peroxide.[19] The hydrogen peroxide anion, HOO^-, is much more nucleophilic than HO^- ($k_{HOO^-}/k_{HO^-} \sim 100$).[19] Reactions with peroxide are further facilitated by the fact that a greater fraction exists in the reactive, anionic form at physiological pH (H_2O_2, $pK_a = 11.6$; H_2O, $pK_a = 15.7$).[19]

Importantly, we recently showed that peroxymonophosphate inactivates PTP1B about 10,000 times faster than H_2O_2.[17] Accordingly, nanomolar concentrations of peroxymonophosphate (20–40 nM) are capable of inactivating PTPs within the biologically relevant time frame of 5–10 min. To facilitate further consideration of the potential role of peroxymonophosphate in biological systems we present studies related to the preparation, characterization, stability, and detection of this agent.

Peroxymonophosphate was prepared by a modification of the methods described by Griffith and Battaglia.[20,21] Briefly, an aqueous solution of potassium phosphate (4.12 M), potassium hydroxide (6.6 mM), and potassium fluoride (3.9 M) was electrolyzed at 10 ℃, 10 V, 400 mA, for 6 h on three consecutive days to generate crude potassium peroxydiphosphate as a precipitate.[21] Lithium peroxydiphosphate was then prepared by a metathesis reaction
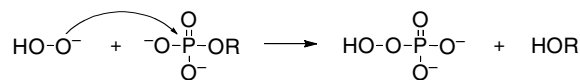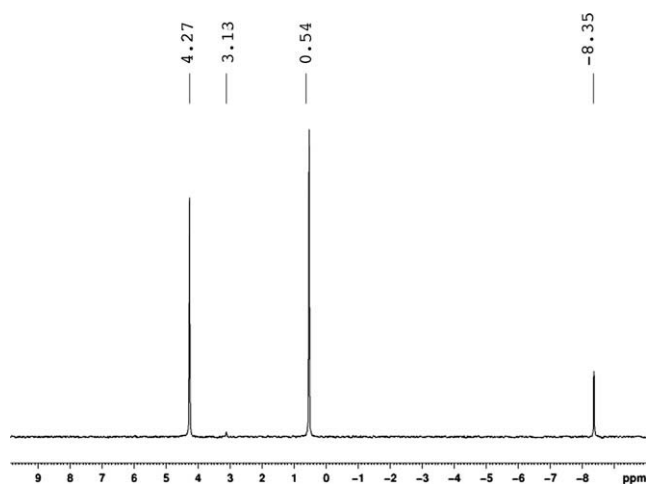
Scheme 1. Phosphoryl transfer to hydrogen peroxide has the potential to produce peroxymonophosphate in cells.

* Corresponding author. Tel.: +1 573 882 6763; fax: +1 573 882 2754.
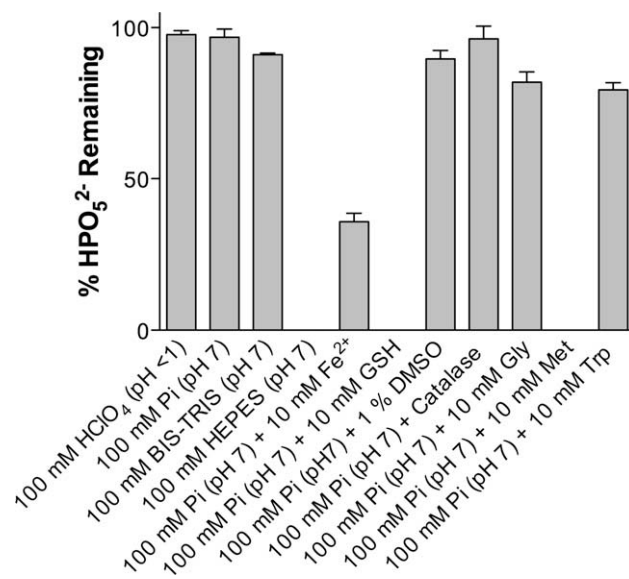E-mail address: GatesK@missouri.edu (K.S. Gates).

and recrystallized from methanol–water at 45 °C to give pure material.[21] Incubation of a solution of lithium peroxydiphosphate (200 mM) in perchloric acid (1 M) at 50 °C for 1 h yields a mixture of lithium phosphate and lithium peroxymonophosphate.[20] It is worth noting that we found commercially available peroxydiphosphate to be unsuitable in this preparation due to the very impure nature of the material.

The product was characterized by ^{31}P NMR and mass spectrometry. The ^{31}P NMR shift of the product is pH-dependent giving a resonance at 4.27 ppm in 0.5 M $HClO_4$ versus 85% phosphoric acid as an external standard (Fig. 1). With careful selection of instrument settings,[22] it is possible to use ^{31}P NMR to quantitatively measure the concentration of peroxymonophosphate in solutions by comparison to an internal diphenyl phosphate standard of known concentration. Alternatively, iodometric titration can be used to measure the concentration of peroxymonophosphate in solutions.[20] Using electrospray mass spectrometry operating in the positive ion mode, the monoisotopic molecular ion was detected at 114.7 $(M+H)^+$ (calculated 115.0). MS/MS analysis of the ion at 114.68 reveals consecutive neutral losses of 17 mass units, as expected for a phosphate derivative. On a practical note, we prefer the preparation of peroxymonophosphate described above over a more recent method involving reaction of 70% H_2O_2 with phosphorus pentoxide.[23] We found that the reaction using 70% H_2O_2 was difficult to control, sometimes leading to vigorous exotherms as noted by the original authors.[23] More importantly, ^{31}P NMR analysis revealed that the 70% H_2O_2 process, in our hands, produced mixtures that may include peroxydiphosphate and diperoxyphosphate derivatives alongside the desired peroxymonophosphate.

We examined the stability of peroxymonophosphate in the presence of several commonly used buffers and biologically relevant substrates. We find that peroxymonophosphate is quite stable in $HClO_4$ (100 mM) over the course of 1 h at 24 °C (Fig. 2). Similarly, peroxymonophosphate is stable in sodium phosphate (100 mM, pH 7) and bis–tris buffer (100 mM, pH 7) under these conditions. In contrast, peroxymonophosphate is completely destroyed upon incubation with Hepes buffer (100 mM, pH 7) for 1 h at 24 °C. Similarly, addition of the biological thiol, glutathione (10 mM), to a sodium phosphate buffered solution leads to complete decomposition of the peroxymonophosphate. The sulfide-containing amino acid methionine also destroys peroxymonophosphate. Tryptophan and glycine lead to only small amounts of peroxymonophosphate decomposition. Addition of $FeSO_4$ (10 mM) results in a 60%

Figure 2. The stability of peroxymonophosphate (HPO_5^{2-}) in the presence of biologically relevant additives. A solution of HPO_5^{2-} (10 mM final concentration) was combined with additive and the pH was adjusted with concentrated NaOH. After standing for 1 h at room temperature, the % remaining HPO_5^{2-} was determined by quantitative ^{31}P NMR (peak area) using a freshly made solution of HPO_5^{2-} in 100 mM $HClO_4$ as a control. Values shown are the averages of three independent experiments and the error bars depict the standard error in the measurements.

decrease in the concentration of peroxymonophosphate. Neither 1% dimethyl sulfoxide (140 mM) nor the hydrogen peroxide-destroying enzyme catalase have significant effects on the stability of peroxymonophosphate under these conditions.

Finally, we examined the potential of 3-oxo-3H-phenoxazin-7-yl pinacolatoboron (PC-1, **1**) to serve as a fluorescent sensor of peroxymonophosphate in biochemical and biological systems. Compound **1** was designed by Chang and coworkers as an intracellular sensor for H_2O_2.[24] Their clever design capitalizes on the fact that reaction of peroxide with the boronate ester group in **1** leads to release of the highly fluorescent resorufin dye (Scheme 2). Peroxymonophosphate is a more reactive oxidizing agent than hydrogen peroxide thus, we anticipated that peroxymonophosphate might convert **1** to its fluorescent form. Indeed, we find that peroxymonophosphate rapidly "lights up" solutions of **1** in bis–tris buffer (50 mM, pH 7) at 22 °C. The rate constant for the reaction of peroxymonophosphate with **1** is $1447 \pm 52 \, M^{-1} \, s^{-1}$ (Fig. 3). For comparison, we determined that H_2O_2 converts **1** to the fluorescent product with a rate constant of $1.21 \pm 0.17 \, M^{-1} \, s^{-1}$.

In summary, we have conducted the first survey of the reactivity of peroxymonophosphate under biologically relevant conditions. Peroxymonophosphate is substantially more reactive than hydrogen peroxide as an oxidant.[17,25] Nonetheless, we find that the selectivity of peroxymonophosphate towards reaction with various biochemicals, in many regards, mirrors that of H_2O_2. Peroxymonophosphate is stable in perchloric acid, sodium phosphate buffer, and bis–tris buffer. These results are consistent with those of Battaglia and Edwards who reported the half-life of peroxymonophosphate to be 12.5 h in 4 M $HClO_4$ (rate of decomposition, $k = 1.54 \times 10^{-5} \, s^{-1}$).[20]

Peroxymonophosphate is unstable in the presence of Fe(II). Presumably this breakdown involves a Fenton-type reaction analogous to the well known metal-mediated decomposition of H_2O_2.[26] In addition, we showed that Hepes buffer, the biological thiol glutathione, and the sulfide-containing amino acid methio-

Figure 1. ^{31}P NMR of peroxymonophosphate in $HClO_4$ (0.5 M). The NMR was calibrated against an external standard of 85% phosphoric acid. Peroxymonophosphate appears at 4.27 ppm, phosphate at 0.59 ppm, and diphenyl phosphate added as an internal standard at −8.35 ppm. The small peak at 3.13 ppm is assigned to a trace of peroxydiphosphate. This impurity disappears as the sample ages.
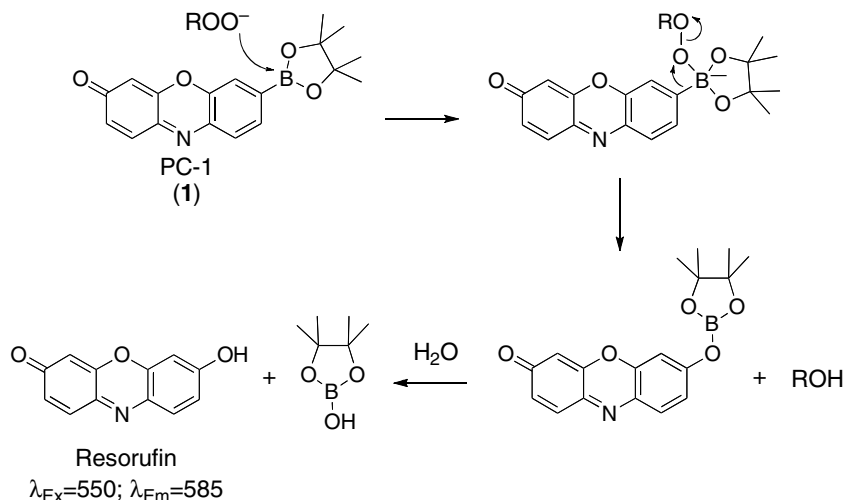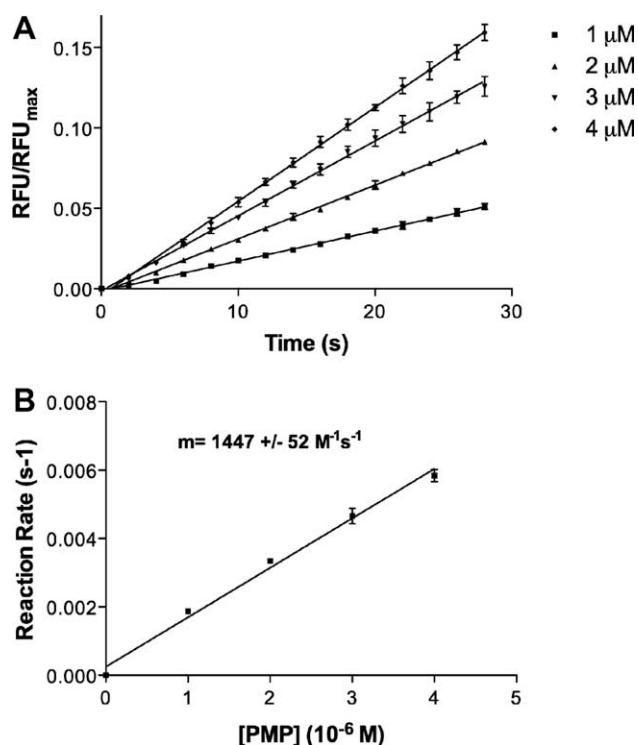
Scheme 2. Oxidative deboronation of PC-1 (**1**).

Figure 3. Kinetics of the reaction of peroxymonophosphate with PC-1 (**1**). Various amounts of peroxymonophosphate were added to a 100 nM solution of **1** in 50 mM bis–tris, pH 7.0 at 22 °C. (A) The resulting pseudo-first-order rate of fluorescence increase was immediately measured using an SLM-AMINCO model 8100 spectrofluorimeter (Ex: 550 nm, Em: 585 nm, 4 nm slit widths). (B) Replotting the pseudo-first-order rates derived from this data gives the apparent second order rate of reaction. Values shown in both graphs are averages of three independent experiments in which the error bars depict the standard error in the measurements.

nine completely decompose peroxymonophosphate. Again this is analogous to the reactivity of H_2O_2 with Hepes,[27] glutathione,[28] and methionine.[29] Our result with methionine in pH 7 buffer is consistent with previous work showing a facile reaction of peroxymonophosphate with aryl sulfides in acetonitrile–water mixtures.[30] We observe that, DMSO glycine, and the indole-containing amino acid tryptophan do not cause substantial decomposition of peroxymonophosphate in neutral aqueous buffer. Others

have reported reactions of peroxymonophosphate with sulfoxides and indoles;[31,32] however, these earlier studies were conducted under conditions where the protonated species H_3PO_5 was predominant. In contrast, with pK_a values of 1.0, 5.5, and 12.8, peroxymonophosphate exists primarily as the dianion under the conditions of our experiments (pH 7).[20,32] A number of studies show that the reactivity of peroxymonophosphate is pH-dependent, increasing at lower pH values as the oxygens become increasingly protonated.[25,30,31] A most striking difference between H_2O_2 and peroxymonophosphate, is revealed by our observation that the H_2O_2-destroying enzyme catalase does not decompose peroxymonophosphate. The inability of catalase to decompose peroxymonophosphate is in alignment with the observation that another bulky hydroperoxide, t-butyl hydroperoxide, is a poor substrate for the enzyme.[33,34]

Peroxymonophosphate readily "lights up" the fluorescent peroxide sensor, PC-1. The reaction of PC-1 with peroxymonophosphate is approximately 1200 times faster than that with H_2O_2. This finding highlights the possibility that boronate ester probes might preferentially detect a secondary, H_2O_2-derived oxidant such as peroxymonophosphate, if this species were generated inside cells. Taken together, elements of the work described here provide a foundation for the development of assays designed to detect spontaneous or enzyme-catalyzed conversion of H_2O_2 to peroxymonophosphate in biochemical or biological systems. After incubation of H_2O_2 with a phosphoryl donor substrate (perhaps in the presence of a putative enzymatic catalyst for the reaction), catalase can be used to destroy excess H_2O_2. Catalase treatment leaves peroxymonophosphate intact (see data in Fig. 2) and addition of the peroxide sensor PC-1 provides a means for highly sensitive detection of peroxymonophosphate produced under a given set of reaction conditions. This general approach might be amenable to either a high-throughput microplate reader or in-gel assays that search for peroxymonophosphate-producing enzymes in a proteome. Such tools will help explore the possibility that peroxymonophosphate participates in the regulation or dysregulation of cell signaling processes under physiological or pathophysiological conditions.

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