Biologically relevant chemical properties of peroxymonophosphate (=O₃POOH)

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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, endothelial growth factor, platelet-derived growth factor, endothelin-1, and B-cell receptor stimulation. H₂O₂ is generated by NADPH oxidase enzymes (Nox), which are activated by the binding of these growth factors and cytokines to cell surface receptors. Protein tyrosine phosphatases (PTPs) are important cellular targets of hydrogen peroxide. PTPs work in tandem with protein tyrosine kinases to regulate the phosphorylation status of proteins involved in critical signal transduction pathways. Accordingly, peroxide-mediated inactivation of target PTPs, involving oxidation of the catalytic cysteine residue in these enzymes, 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. Interestingly, kinetic measurements on isolated PTPs suggest that, at the low concentrations generated during cell signaling processes, H₂O₂ is a rather slow PTP inactivator. For example, the inactivation of the archtypal member of the PTP class of enzymes, PTP1B, by a steady state concentration of 1 μM H₂O₂ 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 H₂O₂ near the phosphatase target, thus leading to rapid enzyme inactivation. 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. In the case of peroxymonophosphate, 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. Phosphoryl transfer to H₂O₂ may be possible even in the presence of vast excesses of water due to the exceptional nucleophilicity of peroxide. The hydrogen peroxide anion, HOO⁻, is much more nucleophilic than HO⁻ (k_{HOO⁻}/k_{HO⁻} ~ 100). Reactions with peroxide are further facilitated by the fact that a greater fraction exists in the reactive, anionic form at physiological pH (H₂O₂, pKₐ = 11.6; H₂O, pKₐ = 15.7).

Importantly, we recently showed that peroxymonophosphate inactivates PTP1B about 10,000 times faster than H₂O₂. 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. 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 °C, 10 V, 400 mA, for 6 h on three consecutive days to generate crude potassium peroxymonophosphate as a precipitate. Lithium peroxydiphosphate was then prepared by a metathesis reaction
and recrystallized from methanol–water at 45 C to give pure material. Incubation of a solution of lithium peroxypentaphosphate (200 mM) in perchloric acid (1 M) at 50 C for 1 h yields a mixture of lithium phosphate and lithium peroxypentaphosphate. It is worth noting that we found commercially available peroxypentaphosphate to be unsuitable in this preparation due to the very impure nature of the material.

The product was characterized by 31P NMR and mass spectrometry. The 31P NMR shift of the product is pH-dependent giving a resonance at 4.27 ppm in 0.5 M HClO4 versus 85% phosphoric acid. On a practical note, we prefer the preparation of peroxymono-phosphate described above over a more recent method involving an internal standard at 0.59 ppm. However, we find that peroxymono-phosphate 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 peroxypentaphosphate. This impurity disappears as the sample ages.

31P NMR analysis revealed that the 70% H2O2 process, similarly, 31P NMR analysis revealed that the 70% H2O2 process, in our hands, produced mixtures that may include peroxypentaphosphate and diperoxypentaphosphate derivatives alongside the desired peroxypentaphosphate.

We examined the stability of peroxypentaphosphate in the presence of several commonly used buffers and biologically relevant substrates. We find that peroxypentaphosphate is quite stable in HClO4 (100 mM) over the course of 1 h at 24 C (Fig. 2). Similarly, peroxypentaphosphate is stable in sodium phosphate (100 mM, pH 7) and bis–tris buffer (100 mM, pH 7) under these conditions. In contrast, peroxypentaphosphate 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 peroxypentaphosphate. The sulfide-containing amino acid methionine also destroys peroxypentaphosphate. Tryptophan and glycine lead to only small amounts of peroxypentaphosphate decomposition. Addition of FeSO4 (10 mM) results in a 60% decrease in the concentration of peroxypentaphosphate. Neither 1% dimethyl sulfoxide (140 mM) nor the hydrogen peroxide-deestroying enzyme catalase have significant effects on the stability of peroxypentaphosphate under these conditions.

Finally, we examined the potential of 3-oxo-3H-phenoxazin-7-yl pinacolatoboron (PC-1, I) to serve as a fluorescent sensor of peroxypentaphosphate in biochemical and biological systems. Compound 1 was designed by Chang and coworkers as an intracellular sensor for H2O2. 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). Peroxypentaphosphate is a more reactive oxidizing agent than hydrogen peroxide thus, we anticipated that peroxypentaphosphate might convert 1 to its fluorescent form. Indeed, we find that peroxypentaphosphate rapidly “lights up” solutions of 1 in bis–tris buffer (50 mM, pH 7) at 22 C. The rate constant for the reaction of peroxypentaphosphate with 1 is 1447 ± 52 M⁻¹ s⁻¹ (Fig. 3). For comparison, we determined that H2O2 converts 1 to the fluorescent product with a rate constant of 1.21 ± 0.17 M⁻¹ s⁻¹.

In summary, we have conducted the first survey of the reactivity of peroxypentaphosphate under biologically relevant conditions. Peroxypentaphosphate is substantially more reactive than hydrogen peroxide as an oxidant. Nonetheless, we find that the selectivity of peroxypentaphosphate towards reaction with various biochemicals is in many regards, mirrors that of H2O2. Peroxypentaphosphate 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 peroxypentaphosphate to be 12.5 h in 4 M HClO4 (rate of decomposition, k = 1.54 × 10⁻⁵ s⁻¹).

Peroxypentaphosphate 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 H2O2. In addition, we showed that Hepes buffer, the biological thiol glutathione, and the sulfide-containing amino acid methio-
nine completely decompose peroxymonophosphate. Again this is analogous to the reactivity of H$_2$O$_2$ 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$_3$PO$_5$ 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$_2$O$_2$ and peroxymonophosphate, is revealed by our observation that the H$_2$O$_2$-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$_2$O$_2$. This finding highlights the possibility that boronate ester probes might preferentially detect a secondary, H$_2$O$_2$-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$_2$O$_2$ to peroxymonophosphate in biochemical or biological systems. After incubation of H$_2$O$_2$ 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$_2$O$_2$. 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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References and notes

14. Quantitative 31P NMR spectra were collected with a Bruker ARX-250 equipped with a 5 mm QMP probe utilizing the following parameters: Inverse gated acquisition-30°C pulse, 12.5-s relaxation delay (D1), 1.47-s acquisition, 16 scan average. For a similar example of quantitative analysis using 31P NMR, see: Lerman, C. L.; Cohn, M. J. Biol. Chem. 1980, 255, 8756.
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