

The Biological Buffer Bicarbonate/CO₂ Potentiates H₂O₂-Mediated Inactivation of Protein Tyrosine Phosphatases

Haiying Zhou,[§] Harkewal Singh,[§] Zachary D. Parsons,[§] Sarah M. Lewis,[§] Sanjib Bhattacharya,[§] Derrick R. Seiner,[§] Jason N. LaButti,[§] Thomas J. Reilly,[†] John J. Tanner,^{*,§,‡} and Kent S. Gates^{*,§,‡}

[§]Department of Chemistry, [†]Department of Biochemistry, and [‡]Department of Veterinary Pathobiology and Veterinary Medical Diagnostic Laboratory, University of Missouri, Columbia, Missouri 65211, United States

S Supporting Information

ABSTRACT: Hydrogen peroxide is a cell signaling agent that inactivates protein tyrosine phosphatases (PTPs) via oxidation of their catalytic cysteine residue. PTPs are inactivated rapidly during H₂O₂-mediated cellular signal transduction processes, but, paradoxically, hydrogen peroxide is a rather sluggish PTP inactivator *in vitro*. Here we present evidence that the biological buffer bicarbonate/CO₂ potentiates the ability of H₂O₂ to inactivate PTPs. The results of biochemical experiments and high-resolution crystallographic analysis are consistent with a mechanism involving oxidation of the catalytic cysteine residue by peroxydicarbonate generated via the reaction of H₂O₂ with HCO₃⁻/CO₂.

Hydrogen peroxide is a signaling agent that mediates cellular responses to growth factors, hormones, and cytokines such as platelet-derived growth factor, epidermal growth factor, vascular endothelial growth factor, insulin, tumor necrosis factor- α , and interleukin-1 β .^{1,2} Protein tyrosine phosphatases (PTPs) are important targets of H₂O₂ produced during signal transduction processes.^{2,3} PTPs help regulate a variety of critical mammalian signaling pathways by catalyzing the removal of phosphoryl groups from phosphotyrosine residues on target proteins. H₂O₂ inactivates PTPs via oxidation of the catalytic cysteine residue in these enzymes, leading to elevated levels of tyrosine phosphorylation on key signaling proteins.^{3–7} Reactions with cellular thiols ultimately return oxidized PTPs to the catalytically active forms.^{3–6,8}

Peroxide-mediated inactivation of intracellular PTPs during signaling events typically occurs rapidly (5–15 min).^{5,9,10} Paradoxically, the rate constants measured for *in vitro* inactivation of purified PTPs by H₂O₂ (e.g., 10–40 M⁻¹ s⁻¹ for PTP1B)⁴ suggest that the loss of enzyme activity should be rather sluggish ($t_{1/2}$ = 5–200 h) at the low cellular concentrations of H₂O₂ thought to exist during signaling events (0.1–1 μ M).^{11,12} This kinetic discrepancy led us and others to consider the possibility that H₂O₂ may undergo spontaneous or enzymatic conversion to more reactive oxidizing agents that effect rapid intracellular inactivation of PTPs.^{11–14}

Along these lines, we set out to explore a potential role for the biological bicarbonate/CO₂ buffer system in H₂O₂-mediated signal transduction. H₂O₂ reacts with bicarbonate/CO₂ to generate the highly reactive oxidant, peroxydicarbonate (Figure 1a).^{15–17}

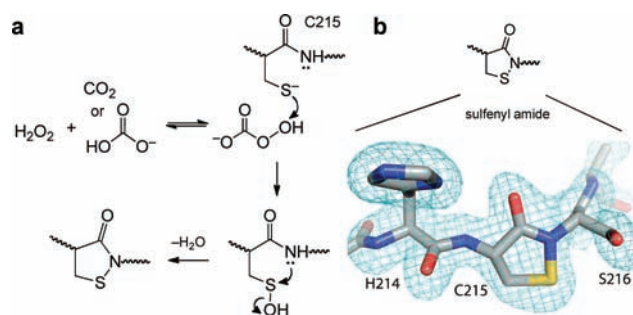


Figure 1. (a) Possible mechanism for the inactivation of PTP1B by H₂O₂–HCO₃⁻. (b) Treatment of PTP1B with H₂O₂–HCO₃⁻ yields the oxidized, sulfenyl amide form of the enzyme. The structure of the oxidized enzyme was solved at 1.7 Å resolution (pdb code 3SME). The image shows a simulated annealing σ_A -weighted $F_o - F_c$ omit map contoured at 3.0 σ , covering Cys215 and flanking residues.

This process may be catalyzed by thiols and sulfides.^{15–17} Peroxydicarbonate is an acyl peroxide,¹⁸ and on the basis of our recent studies of organic acyl peroxides,¹³ we anticipated that this species might be a potent PTP inactivator.

To address this question, we employed the catalytic domain (aa 1–322) of recombinant human PTP1B, as an archetypal member of the PTP family. First, we confirmed that H₂O₂ alone causes time-dependent inactivation of PTP1B with an apparent second-order rate constant of 24 \pm 3 M⁻¹ s⁻¹ at 25 °C, pH 7.⁴ The extracellular and intracellular concentrations of bicarbonate are 25 and 14.4 mM, respectively,¹⁶ and we examined the effects of bicarbonate within this general concentration range. We found that the presence of potassium bicarbonate markedly increased the rate of time-dependent PTP1B inactivation by H₂O₂. For example, potassium bicarbonate increased the apparent second-order rate constants for inactivation of PTP1B by H₂O₂ to 202 \pm 4 and 330 \pm 11 M⁻¹ s⁻¹ at concentrations of 25 and 50 mM, respectively (25 °C, pH 7, Figure 2). At physiological temperature (37 °C), the rate of inactivation by the H₂O₂–KHCO₃ system increases further to 396 \pm 10 M⁻¹ s⁻¹ (KHCO₃, 25 mM, pH 7, Figure S9). Other bicarbonate salts (NaHCO₃ and NH₄HCO₃) produced similar effects (Figure S21). Preincubation of H₂O₂ and KHCO₃ (up to 2 h) prior to addition of the enzyme did not significantly alter the observed rate of inactivation (Figure S10). Control experiments showed that KCl (25 mM), NaCl (25 mM),

Received: August 15, 2011

Published: September 13, 2011

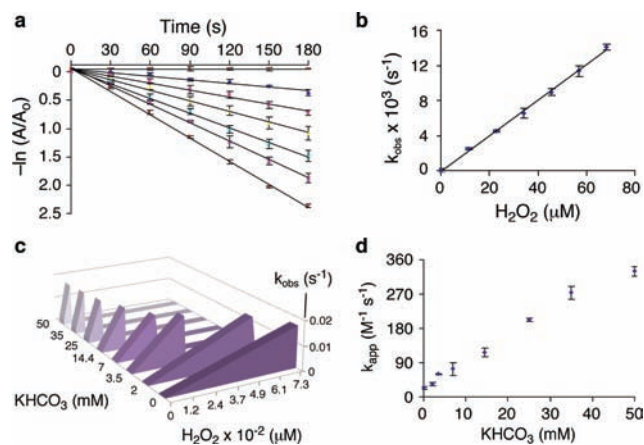


Figure 2. Kinetics of PTP1B inactivation by H_2O_2 – KHCO_3 . (a) A semi-log plot showing time-dependent loss of PTP1B activity in the presence of various concentrations of H_2O_2 (the lines correspond to 0, 12, 23, 33, 45, 57, and $68 \mu\text{M}$ H_2O_2 from top to bottom) at a single concentration of KHCO_3 (25 mM). Inactivation assays were carried out as described in the Supporting Information. The pseudo-first-order rate constant for inactivation at each concentration of H_2O_2 (k_{obs}) was calculated from the slope of the line. (b) The apparent second-order rate constant for inactivation by the H_2O_2 – KHCO_3 (25 mM) system was obtained from the slope of the replot of k_{obs} values obtained from the data shown in panel a versus H_2O_2 concentration. (c) Plots of k_{obs} versus H_2O_2 concentration in the presence of various concentrations of KHCO_3 (see also Figures S1–S8). This plot provides a graphical depiction of how, as KHCO_3 concentration increases, markedly lower concentrations of H_2O_2 are required to achieve a given rate of PTP1B inactivation. (d) Plot of the apparent second-order rate constants for the inactivation of PTP1B by H_2O_2 in the presence of various concentrations of KHCO_3 .

or MgCl_2 (2 mM) did not significantly alter the rate at which H_2O_2 inactivated PTP1B (Figures S11–S13). This indicates that the effect of bicarbonate on the peroxide-mediated inactivation of PTP1B was not merely an ionic strength effect. It is important to emphasize that KHCO_3 alone did not cause time-dependent inactivation of PTP1B at 24 °C (Figure 2a). The time-dependent nature of the inactivation observed here is consistent with a process involving covalent chemical modification of the enzyme.

The cellular milieu contains millimolar concentrations of thiols such as glutathione, which can decompose peroxides.¹² Therefore, we examined the effects of glutathione on the inactivation of PTP1B by H_2O_2 – KHCO_3 . We find that the H_2O_2 – KHCO_3 system causes rapid and complete loss of enzyme activity in the presence of glutathione (1 mM), with only an approximately 2-fold decrease in the observed rate of inactivation (Figure 3a).

Time-dependent inactivation of PTP1B by the H_2O_2 – KHCO_3 system was slowed by competitive inhibitors (Figures S14 and S15). For example, phosphate (50 mM) slowed inactivation by a factor of 1.7 ± 0.1 . Activity did not return to the inactivated enzyme following gel filtration or dialysis to remove H_2O_2 and KHCO_3 (Figures S22 and S23). These results suggest that inactivation of PTP1B by H_2O_2 – KHCO_3 involves covalent modification of an active-site residue. Catalytic activity was recovered upon treatment of the inactivated enzyme with thiols such as dithiothreitol (DTT, Figures 3b and S24). For example, when the enzyme was inactivated by treatment with H_2O_2 – KHCO_3 (34 μM and 25 mM,

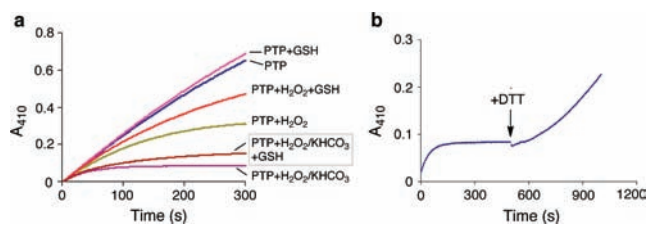


Figure 3. Effect of added thiol on the inactivation of PTP1B by H_2O_2 – KHCO_3 and reactivation of inactivated enzyme by dithiothreitol. (a) The inactivation of PTP1B by H_2O_2 – KHCO_3 proceeds in the presence of glutathione. Thiol-free PTP1B (16.7 nM) was inactivated by treatment with H_2O_2 (100 μM) and KHCO_3 (25 mM) in buffer containing sodium acetate (100 mM), bis-Tris (50 mM, pH 7.0), Tris (50 mM), DTPA (50 μM), Tween-80 (0.0005%), and *p*-NPP (10 mM) in a quartz cuvette. Enzyme activity in the sample was continuously monitored by following the release of *p*-nitrophenolate at 410 nm. Reactions contained the indicated combinations of H_2O_2 (150 μM), glutathione (GSH, 1 mM), and KHCO_3 (25 mM). (b) Catalytic activity can be recovered by treatment of inactivated enzyme with dithiothreitol (DTT). Enzyme activity was continuously monitored as described above. Thiol-free PTP1B (16.7 nM) was inactivated by treatment with H_2O_2 (100 μM) and KHCO_3 (25 mM) as described above. When enzyme inactivation was complete, DTT (5 μL of a 1 M solution in H_2O) was added directly to the cuvette to yield a final concentration of 5 mM DTT, and the recovery of enzyme activity was measured by observing the turnover of substrate at 410 nm (control reactions showed that DTT alone does not yield significant release of *p*-nitrophenolate).

respectively, for 3 min to yield 80% inactivation), almost all (98%) of the initial activity was recovered by treatment with DTT (50 mM, 30 min, 25 °C). The thiol-reversible nature of the inactivation reaction is consistent with a mechanism involving oxidation of the enzyme's catalytic cysteine residue.^{3,6,8} Indeed, crystallographic analysis of PTP1B treated with H_2O_2 – KHCO_3 produced a 1.7 Å resolution structure showing that the catalytic cysteine residue was oxidized to the cyclic sulfenyl amide residue observed previously for this enzyme (Figure 1b and Supporting Information).^{3,6–8} There was no evidence in the electron density map for oxidation at any other residue in the enzyme. Peroxycarbonate can decompose to yield highly reactive oxygen radicals in the presence of transition metals.^{16,19} However, addition of radical scavengers or chelators of adventitious trace metals had no effect on the inactivation of PTP1B by the H_2O_2 – KHCO_3 system (Figures S16–S18), suggesting that the enzyme inactivation process described here proceeds via a two-electron oxidation mechanism as shown in Figure 1a.

We also examined the ability of bicarbonate to potentiate H_2O_2 -mediated inactivation of SHP-2, a different member of the PTP enzyme family. The intracellular activity of SHP-2 is thought to be redox regulated in response to platelet-derived growth factor, endothelin-1, and T-cell receptor stimulation.¹ Our experiments employed the catalytic domain (aa 246–527) of the recombinant human enzyme. We found that the inactivation of SHP-2 by H_2O_2 occurs with an apparent second-order rate constant of $15 \pm 2 \text{ M}^{-1} \text{ s}^{-1}$ (25 °C, pH 7, Figure S19).²⁰ The presence of potassium bicarbonate (25 mM) increased the apparent second-order rate constant for inactivation of SHP-2 to $167 \pm 12 \text{ M}^{-1} \text{ s}^{-1}$, an 11-fold rate increase (Figure S20). Similar to PTP1B, the inactivation process was slowed by the competitive inhibitor sodium phosphate, and enzyme activity was recovered by treatment of the inactivated enzyme with DTT (Figures S25 and S26).

Finally, for the purposes of comparison, we measured the ability of H₂O₂ alone, or the H₂O₂–KHCO₃ system, to inactivate a different type of cysteine-dependent enzyme, papain. We found that H₂O₂ alone inactivates this cysteine protease with an observed second-order rate constant of $43 \pm 7 \text{ M}^{-1} \text{ s}^{-1}$, whereas inactivation of papain by the H₂O₂–KHCO₃ (25 mM) system occurs with an observed rate constant of $83 \pm 9 \text{ M}^{-1} \text{ s}^{-1}$ (Figures S27 and S28). The ~2-fold enhancement in the rate of papain inactivation engendered by bicarbonate is modest compared to its effect on the oxidation of PTPs and is similar to the 2-fold enhancement exerted by bicarbonate (25 mM) on the observed rate of peroxide-mediated oxidation of low-molecular-weight thiols.²¹ The larger effect of bicarbonate on the H₂O₂-mediated inactivation of PTPs suggests that this may be a mechanism-based inactivation process, in which the anion-binding pocket and general acid–base residues at the active site of the enzyme²² catalyze oxidation of the active-site cysteine by H₂O₂–KHCO₃.

In summary, we report that the biological buffer system bicarbonate/CO₂ selectively potentiates the ability of H₂O₂ to inactivate PTPs. Bicarbonate/CO₂ enables steady-state concentrations of H₂O₂ in the low micromolar range to inactivate PTPs within the biologically relevant time frame of 10–15 min. Still, the rate constants reported here do not rise to the levels reported for other cellular H₂O₂ sensors such as the peroxiredoxins.^{3,12,23,24} Therefore, spontaneous conversion of the second messenger H₂O₂ to peroxymonocarbonate may work in tandem with colocalization,²⁵ compartmentalization, or other means²⁶ of generating localized H₂O₂ concentration gradients to effect rapid, transient down-regulation of selected PTPs during signal transduction processes. The chemistry described here, in some regards, is reminiscent of the abilities of superoxide and CO₂ to modulate the properties of the cell signaling agent nitric oxide.²⁷

■ ASSOCIATED CONTENT

S Supporting Information. Methods and results of inactivation and mechanism experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

gatesk@missouri.edu; tannerjj@missouri.edu

■ ACKNOWLEDGMENT

We thank the Diabetes Action Research and Education Foundation and the NIH for partial support of this work (KSG CA83925 and 119131). H.S. was supported by a predoctoral fellowship from National Institutes of Health grant DK071510 and a Chancellor's Dissertation Completion Fellowship from the University of Missouri–Columbia. We thank Dr. Jay Nix of ALS beamline 4.2.2 for assistance with data collection. Part of this work was performed at the Advanced Light Source. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. This paper is dedicated to Prof. Richard B. Silverman on the occasion of his 65th birthday.

■ REFERENCES

- (1) Tonks, N. K. *Nat. Rev. Cell. Biol.* **2006**, *7*, 833–846.
- (2) Rhee, S. G. *Science* **2006**, *312*, 1882–1883.
- (3) Tanner, J. J.; Parson, Z. D.; Cummings, A. H.; Zhou, H.; Gates, K. S. *Antioxid. Redox Signal.* **2011**, *15*, 77–97.
- (4) (a) Denu, J. M.; Tanner, K. G. *Biochemistry* **1998**, *37*, 5633–5642. (b) Heffetz, D.; Bushkin, I.; Dror, R.; Zick, Y. *J. Biol. Chem.* **1990**, *265*, 2896–2902.
- (5) Lee, S. R.; Kwon, K. S.; Kim, S. R.; Rhee, S. G. *J. Biol. Chem.* **1998**, *273*, 15366–15372.
- (6) Sivaramakrishnan, S.; Keerthi, K.; Gates, K. S. *J. Am. Chem. Soc.* **2005**, *127*, 10830–10831.
- (7) (a) Salmeen, A.; Anderson, J. N.; Myers, M. P.; Meng, T.-C.; Hinks, J. A.; Tonks, N. K.; Barford, D. *Nature* **2003**, *423*, 769–773. (b) van Montfort, R. L. M.; Congreeve, M.; Tisi, D.; Carr, R.; Jhoti, H. *Nature* **2003**, *423*, 773–777.
- (8) Sivaramakrishnan, S.; Cummings, A. H.; Gates, K. S. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 444–447.
- (9) (a) Mahedev, K.; Zilbering, A.; Zhu, L.; Goldstein, B. J. *J. Biol. Chem.* **2001**, *276*, 21938–21942. (b) Kwon, J.; Lee, S.-R.; Yang, K.-S.; Ahn, Y.; Kim, Y. J.; Stadtman, E. R.; Rhee, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 16419–16424.
- (10) Meng, T.-C.; Buckley, D. A.; Galic, S.; Tiganis, T.; Tonks, N. K. *J. Biol. Chem.* **2004**, *279*, 37716–37725.
- (11) Stone, J. R. *Antioxid. Redox Signal.* **2006**, *8*, 243–270.
- (12) Winterbourn, C. C. *Nat. Chem. Biol.* **2008**, *4*, 278–286.
- (13) Bhattacharya, S.; LaButti, J. N.; Seiner, D. R.; Gates, K. S. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5856–5859.
- (14) (a) LaButti, J. N.; Chowdhury, G.; Reilly, T. J.; Gates, K. S. *J. Am. Chem. Soc.* **2007**, *129*, 5320–5321. (b) Seiner, D. R.; LaButti, J. N.; Gates, K. S. *Chem. Res. Toxicol.* **2007**, *20*, 1315–1320.
- (15) (a) Regino, C. A. S.; Richardson, D. E. *Inorg. Chim. Acta* **2007**, *360*, 3971–3977. (b) Bonini, M. G.; Fernandes, D. C.; Augusto, O. *Biochemistry* **2004**, *43*, 344–351.
- (16) Medinas, D. B.; Cerchiaro, G.; Trindade, D. F.; Augusto, O. *IUBMB Life* **2007**, *59*, 255–262.
- (17) Bakhmutova-Albert, E. V.; Yao, H.; Denevan, D. E.; Richardson, D. E. *Inorg. Chem.* **2010**, *49*, 11287–11296.
- (18) Adam, A.; Mehta, M. *Angew. Chem., Int. Ed.* **1998**, *37*, 1387–1388.
- (19) (a) Lane, B. S.; Vogt, M.; DeRose, V. J.; Burgess, K. *J. Am. Chem. Soc.* **2002**, *124*, 11946–11954. (b) Arai, H.; Berlett, B. S.; Chock, P. B.; Stadtman, E. R. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 10472–10477.
- (20) Chen, C.-Y.; Willard, D.; Rudolph, J. *Biochemistry* **2009**, *48*, 1399–1409.
- (21) Trindade, D. F.; Cerchiaro, G.; Augusto, O. *Chem. Res. Toxicol.* **2006**, *19*, 1475–1482.
- (22) Zhang, Z.-Y. *Acc. Chem. Res.* **2003**, *36*, 385–392.
- (23) Reddie, K. G.; Carroll, K. S. *Curr. Opin. Chem. Biol.* **2008**, *12*, 746–754.
- (24) Hall, A.; Parsonage, D.; Poole, L. B.; Karplus, P. A. *J. Mol. Biol.* **2010**, *402*, 194–209.
- (25) Chen, K.; Kirber, M. T.; Yang, Y.; Keaney, J. F. *J. Cell. Biol.* **2008**, *181*, 1129–1139.
- (26) Woo, H. A.; Yim, S. H.; Shin, D. H.; Kang, D.; Yu, D.-Y.; Rhee, S. G. *Cell* **2010**, *140*, 517–528.
- (27) Szabó, C.; Ischiropoulos, H.; Radi, R. *Nat. Rev. Drug Discovery* **2007**, *6*, 662–680.