The decomposition of peroxynitrite does not yield nitroxyl anion and singlet oxygen

Gábor Merényi*†, Johan Lind*, Gidon Czapski‡, and Sara Goldstein†‡

*Department of Chemistry, Nuclear Chemistry, The Royal Institute of Technology, S-10044 Stockholm 70, Sweden; and ‡Department of Physical Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Communicated by Raphael D. Levine, The Hebrew University of Jerusalem, Jerusalem, Israel, May 22, 2000 (received for review April 12, 2000)

In a recent article [Khan, A. U., Kovacic, D., Kolbanovsky, A., Desai, M., Frenkel, K. & Geacintov, N. E. (2000) *Proc. Natl. Acad. Sci. USA* 97, 2984–2989], the authors claimed that $ONOO^-$, after protonation to ONOOH, decomposes into ^1HNO and $^1O_2$ according to a spin-conserved unimolecular mechanism. This claim was based partially on their observation that nitrosylhemoglobin is formed via the reaction of peroxynitrite with methemoglobin at neutral pH. However, thermochemical considerations show that the yields of $^1O_2$ and ^1HNO are about 23 orders of magnitude lower than those of $^{\bullet}NO_2$ and $^{\bullet}OH$, which are formed via the homolysis of ONOOH. We also show that methemoglobin does not form with peroxynitrite any spectrally detectable product, but with contaminations of nitrite and H_2O_2 present in the peroxynitrite sample. Thus, there is no need to modify the present view of the mechanism of ONOOH decomposition, according to which initial homolysis into a radical pair, $[ONO^{\bullet}\ ^{\bullet}OH]_{cage}$, is followed by the diffusion of about 30% of the radicals out of the cage, while the rest recombines to nitric acid in the solvent cage.

Although Mahoney (1) had demonstrated as early as in 1970 that the decomposition of ONOOH yields about 30% $^{\bullet}NO_2$ and $^{\bullet}OH$ free radicals, a great confusion ensued during the next two decades because of misinterpretations of inconclusive experiments, sometimes stimulated by improper thermodynamic estimations (2–7). Fortunately, by the end of the nineties, the radical mechanism was readopted after judicious experiments in several laboratories (8–11). Additional insight gained during these later investigations allowed the pH dependence of the product yield of peroxynitrite decomposition to be explained, i.e., the decomposition of peroxynitrite in acidic solutions yields nitrate as a final product, but as the pH is raised, O_2 and nitrite in a 1:2 ratio are formed at the expense of nitrate, reaching *ca.* 40% O_2 at pH 9 (12, 13). The slowly returning faith in the radical model was disturbed recently by Khan *et al.* (14), who reported that the decomposition of peroxynitrite at neutral pH forms high yields of NO^- and $^1O_2$. In this work, the authors claim that nitrosylhemoglobin (HbNO) is formed via the reaction of peroxynitrite with methemoglobin (MetHb) at neutral pH. They also report that the decomposition of peroxynitrite in the presence of 9,10-diphenylanthracene (DPA) forms substantial amounts of the transannular peroxide of DPA. Because DPA is considered a specific reagent for $^1O_2$, the authors suggested that $ONOO^-$, after protonation to ONOOH, decomposes into $^1O_2$ and ^1HNO according to a spin-conserved unimolecular mechanism. However, in what follows, we shall demonstrate that the claim of the authors contradicts accepted thermodynamical data. Experimentally, we were unable to reproduce, with pure peroxynitrite, those experiments that the authors present as evidence for NO^- formation. We therefore conclude that the apparent formation of NO^- results from impurities in the authors' peroxynitrite sample and have nothing to do with the chemistry of peroxynitrite.

Materials and Methods

Materials. All chemicals were of analytical grade and were used as received. Solutions were prepared with distilled water, which was purified further by using a Milli-Q water purification system from Millipore. Bovine MetHb (Sigma) was purified by passing through a Sephadex G-25 column and using 100 mM phosphate buffer (pH 7) as the eluant. The concentration of MetHb was determined from its absorption at 406 nm by using $\varepsilon = 154\ mM^{-1}\cdot cm^{-1}$ (15). Fresh solutions of peroxynitrite were prepared before use by reacting nitrite with acidified hydrogen peroxide at room temperature in a quenched-flow system. This system was optimized as we described recently (16) to produce high yields of $ONOO^-$ with known contaminations by residual nitrite, nitrate, and H_2O_2. Minimization of residual nitrite and H_2O_2 is crucial for studying the reaction of peroxynitrite with MetHb, because the latter is known to react with these contaminants (see below) (17–19). A syringe pump (WPI Instruments, Waltham, MA; model SP 230IW) was used to inject 0.63 M $NaNO_2$ and 0.60 M H_2O_2 in 0.7 M $HClO_4$ into the first mixing chamber through tangential inlets. The combined solutions were allowed to react in a delay line connected to a second mixing chamber, where 3.6 M NaOH was injected with the same flow rate (45 ml/min) to quench the reaction. The yield of $ONOO^-$ depends on the time of quenching (16) and was determined from its absorption at 302 nm by using $\varepsilon = 1{,}670\ M^{-1}\cdot cm^{-1}$ (20). Under the conditions used, the stock solution of peroxynitrite contained 8% nitrite, 32% nitrate, and practically no residual H_2O_2.

Methods. Spectral properties of MetHb upon the addition of various additives were monitored by recording the UV-visible absorption in the Soret region (350–450 nm) with a 1-mm optical path length cuvette or a 1-cm cuvette in the visible region (450–700 nm) by using a Hewlett—Packard 8453 UV-visible diode array spectrophotometer.

A solution of MetHb in 100 mM phosphate buffer at pH 7 (4.8 ml) was vortexed rapidly with 0.2 ml of 0.1 M NaOH without and with various concentrations of peroxynitrite, $NaNO_2$, H_2O_2, KNO_3, or H_2O_2 and peroxynitrite. The final pH was 7.2. The absorption spectrum was recorded immediately after mixing. A solution of 100 mM phosphate buffer at pH 7 was used as a blank. Efficient mixing of MetHb with these additives is obtained by using a vortex because of the relatively long half-life of peroxynitrite, about 2 s at pH 7.2 (2, 3), and the relatively slow reactions of MetHb with nitrite (17) and H_2O_2 (18) at neutral pH.

Results

The spectra obtained upon the addition of various concentrations of peroxynitrite, nitrite, and H_2O_2 to 36 μM MetHb at pH 7.2 (96 mM phosphate buffer) are given in Figs. 1—3. No effect

Abbreviations: DPA, 9,10-diphenylanthracene; MetHb, methemoglobin; HbNO, nitrosylhemoglobin.

†To whom reprint requests should be addressed. E-mail: gm@nuchem.kth.se or sarag@vms.huji.ac.il.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.150238197. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.150238197

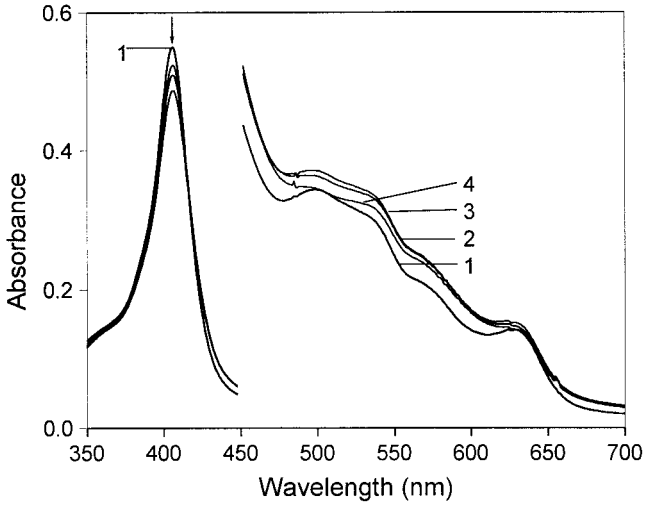

Fig. 1. Absorption spectral changes of 36 μM MetHb in 96 mM phosphate buffer (pH 7.2) in the presence of peroxynitrite. 1, [peroxynitrite] = 0; 2, [peroxynitrite] = 84 μM; 3, [peroxynitrite] = 168 μM; 4, [peroxynitrite] = 336 μM.

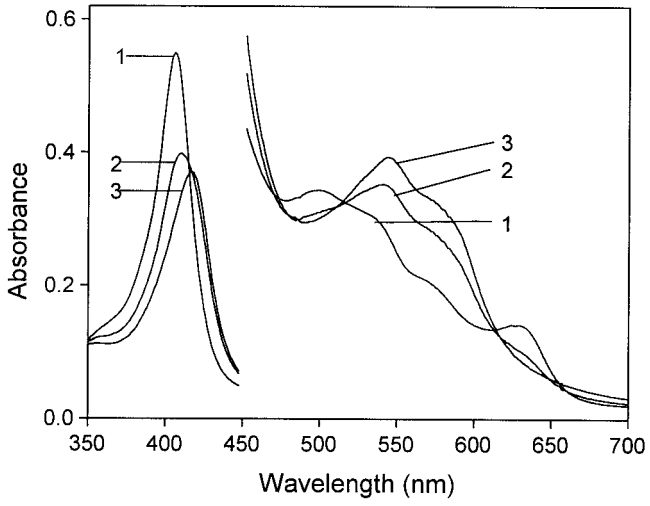

Fig. 3. Absorption spectral changes of 36 μM MetHb in 96 mM phosphate buffer (pH 7.2) in the presence of H_2O_2. 1, $[H_2O_2]$ = 0; 2, $[H_2O_2]$ = 50 μM; 3, $[H_2O_2]$ = 510 μM.

on the spectrum of MetHb was observed upon addition of 1.5 mM nitrate (results not shown). The spectral changes in the visible region upon the addition of up to 9-fold excess of peroxynitrite over MetHb are insignificant (Fig. 1). This result is in agreement with a previous report (21), where minimal spectral changes of human MetHb were observed in the visible region upon the addition of 5-fold excess of peroxynitrite at pH 7.4 and 37°C. Furthermore, only a minor decrease of the Soret band at 406 nm was observed upon increasing [peroxynitrite] (Fig. 1). No new band at 417 nm was observed, as should be the case if HbNO had been formed (22).

When the concentration of NO_2^- exceeded 1 mM, new bands appeared at 411 nm in the Soret region and at 540 and 570 nm in the visible region, whereas the characteristic band of MetHb at 626 nm did not disappear (Fig. 2). Khan *et al.* (14) have shown a negligible effect of 100 mM nitrite on the visible spectrum of MetHb, which contradicts the literature results (17) as well as those presented by us in Fig. 2.

The effect of H_2O_2 on the spectrum of MetHb was observed at relatively low concentrations of H_2O_2 in both spectral regions (Fig. 3). New bands appeared at 417 nm in the Soret region and at 540 and 570 nm in the visible region, and the characteristic band of MetHb at 626 nm disappeared. The reaction of peroxynitrite with H_2O_2 takes place indirectly (10), and, therefore, mixtures of peroxynitrite and H_2O_2 at pH 13 are stable. The effect of the addition of such a mixture on the absorption of MetHb was compared with that of the addition of H_2O_2 alone (Fig. 4). The resulting spectra demonstrate that the characteristic band of MetHb at 626 nm does not disappear when a mixture of H_2O_2 and peroxynitrite is added to MetHb, whereas new bands are formed at 540 and 570 nm.

Discussion

The claim that $^1O_2$ and ^{1}HNO are the major products of the decomposition of ONOOH implies that the accepted Gibbs free energy of formation in water of at least one of the four species, ^{1}HNO, $^1O_2$, $^{\bullet}NO_2$, or $^{\bullet}$OH, is in error by more than 130 kJ/mol.

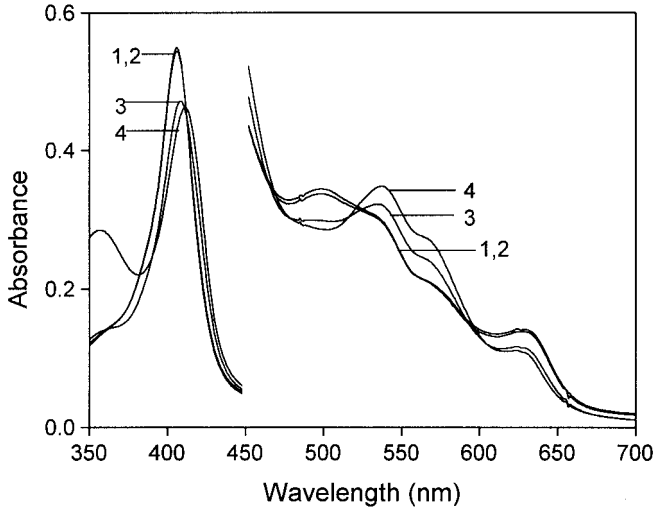

Fig. 2. Absorption spectral changes of 36 μM MetHb in 96 mM phosphate buffer (pH 7.2) in the presence of nitrite. 1, [nitrite] = 0; 2, [nitrite] = 0.26 mM; 3, [nitrite] = 6.4 mM; 4, [nitrite] = 80 mM.

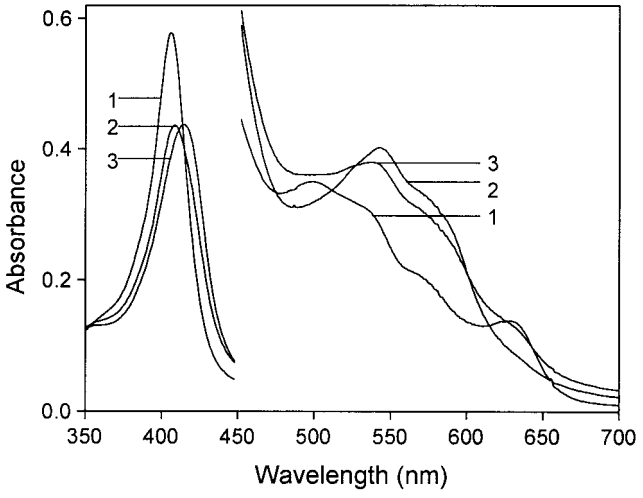

Fig. 4. Absorption spectral changes of 36 μM MetHb in 96 mM phosphate buffer (pH 7.2) in the absence (line 1) and presence (line 2) of 250 μM H_2O_2 or a mixture of 250 μM H_2O_2 and 177 μM peroxynitrite (line 3).

This can be seen as follows. Assuming that ONOOH decomposes via both reactions 1 and 2, one obtains that $RT\ln(K_1/K_2) = \Delta G^{o}_2 - \Delta G^{o}_1$.

$$ONOOH \rightleftharpoons {}^1HNO + {}^1O_2 \qquad K_1 = k_1/k_{-1} \qquad [1]$$

$$ONOOH \rightleftharpoons {}^{\cdot}NO_2 + {}^{\cdot}OH \qquad K_2 = k_2/k_{-2} \qquad [2]$$

Using the literature values of $\Delta_f G^o$ in water for ^1HNO (109 kJ/mol), $^1O_2$ (112 kJ/mol), $^{\cdot}NO_2$ (63 kJ/mol), and $^{\cdot}OH$ (26 kJ/mol) (23), we calculate $K_1/K_2 \approx 10^{-23}$. The rate constant of reaction 2 has been determined to be $k_{-2} = 5 \times 10^9\ M^{-1}\cdot s^{-1}$ by using pulse radiolysis (9, 24). Because this value is close to the expected diffusion-controlled value, the as yet unknown rate constant k_{-1} cannot exceed it. Consequently, k_1/k_2 should be smaller than 10^{-23}. This implies that during the decomposition of ONOOH the yield of $^1O_2$ and ^1HNO must be lower by at least 23 orders of magnitude than the corresponding yield of $^{\cdot}NO_2$ and $^{\cdot}OH$, currently accepted to be about 30% (9–11). For the $^1O_2$ yield to be even 1% of the radical formation during peroxynitrite decomposition, the thermochemistry has to be revised by as much as 120 kJ/mol. Note that the above considerations are completely independent of the thermochemistry of ONOOH/$ONOO^-$ (9). Of the four Gibbs energies, the one for ^1HNO is the least certain, because it is based on the interpretation of the predissociation spectrum of HNO in the gas phase and also on the estimation that the free energy of its hydration is identical to that of HClO (23). These are probably good estimates and hardly can be wrong by more than a few tens of kilojoules per mole. Thus, unless the authors claim a huge revision of the thermochemistry, their interpretation of the unimolecular decomposition of ONOOH to yield $^1O_2$ and ^1HNO would appear absurd.

Khan *et al.* (14) reported that when small aliquots of concentrated alkaline $ONOO^-$ solutions were added to MetHb solution (final pH 7.0–7.2), the NO^- formed was trapped as HbNO. The identification of HbNO was based on the comparison between the spectra obtained in the reaction of MetHb with the same amount of peroxynitrite or trioxidinitrate (Angeli's salt) in neutral pH, where two new bands appeared at 540 and 572 nm but, at 626 nm, did not disappear (14). However, as reported previously for human MetHb (21), and confirmed in this study for bovine MetHb, the reaction of MetHb with an excess of peroxynitrite has only a minor effect on the spectrum of MetHb. Furthermore, we have shown that a visible spectrum similar to that reported by Khan *et al.* (14) is obtained when MetHb reacts with a large excess of nitrite or a mixture of peroxynitrite and H_2O_2 (Figs. 2 and 4). Khan *et al.* (14) did not report on the effect that peroxynitrite had on the Soret band of MetHb. Although not stated explicitly, the peroxynitrite preparation of the authors contains H_2O_2 and NO_2^- well in excess of peroxynitrite, judging by their experimental description (14, 25). Therefore, it is difficult to tell whether the observed spectrum results from contamination of their peroxynitrite sample with nitrite, H_2O_2, or both. A further support for this conclusion is their observation (14) that the resulting spectrum is unaffected by the presence of 25 mM bicarbonate (about 2.3 mM CO_2 at pH 7.2). Under these conditions the reaction of $ONOO^-$ with CO_2 to form $ONOOCO_2^-$ competes efficiently with the self-decomposition of peroxynitrite (26). It has been well established that $ONOOCO_2^-$ decomposes via homolysis into a radical pair, $[ONO^{\cdot}\ {}^{\cdot}CO_3^-]_{cage}$, which is followed by the diffusion of about 30% of the radicals out of the cage, while the rest recombines to nitrate and CO_2 in the solvent cage (10, 27–31). Therefore, NO^- cannot be formed in the presence of bicarbonate, and the reported observation (14) that the spectrum is unaffected by the presence of 25 mM bicarbonate suggests that the latter derives from contamination of the peroxynitrite sample.

It is also worthwhile to emphasize that the suggestion that peroxynitrite decomposes into NO^- and $^1O_2$ contradicts most of the experimental results in the peroxynitrite field. (*i*) The decomposition of peroxynitrite in acidic solutions yields nitrate as a final product, but as the pH is raised, O_2 and nitrite in a 1:2 ratio are formed at the expense of nitrate, reaching *ca.* 40% O_2 at pH 9 (12, 13). (*ii*) The decomposition of peroxynitrite in the presence of bicarbonate yields nitrate as a final product at all pH levels (28). (*iii*) Peroxynitrite oxidizes a large variety of compounds through the intermediates formed during its decomposition (1, 3, 10, 11), and the oxidation yield does not exceed 30%, e.g., with ferrocyanide (11).

We do not know through which reactions or mechanism Khan *et al.* (14) obtained the large amounts of the transannular peroxide of DPA, and we are not even sure that $^1O_2$ is a precursor. One could imagine, for instance, a radical mechanism proceeding via a peroxyl radical intermediate. However, in the present context, this point is not important, because it has nothing to do with the inherent chemistry of peroxynitrite. *A fortiori*, whatever the authors see must be devoid of all biological significance. Clearly, were the authors to unravel, under controlled conditions, the mechanism of $^1O_2$ production, this could have a chemical interest and might reveal hidden aspects of nitrogen–oxygen chemistry.

S.G. and G.C. thank The Israel Science Foundation, and G.M. and J.L. thank The Swedish Natural Science Research Council for their financial support.

1. Mahoney, L. R. (1970) *J. Am. Chem. Soc.* **92,** 4244–4245.
2. Koppenol, W. H., Moreno, J. J., Pryor, W. A., Ischiropoulos, H. & Beckman, J. S. (1992) *Chem. Res. Toxicol.* **5,** 834–842.
3. Pryor, W. A. & Squadrito, G. L. (1995) *Am. J. Physiol.* **288,** L699—L722.
4. Goldstein, S., Squadrito, G. L., Pryor, W. A. & Czapski, G. (1996) *Free Radical Biol. Med.* **21,** 965–974.
5. Merenyi, G. & Lind, J. (1997) *Chem. Res. Toxicol.* **10,** 1216–1220.
6. Koppenol, W. H. & Kissner, R. (1998) *Chem. Res. Toxicol.* **11,** 87–90.
7. Marnett, L. J. (1998) *Chem. Res. Toxicol.* **11,** 709–721.
8. Merenyi, G. & Lind, G. (1998) *Chem. Res. Toxicol.* **11,** 243–246.
9. Merenyi, G., Lind, J., Goldstein, S. & Czapski, G. (1999) *J. Phys. Chem.* **103,** 5685–5691.
10. Hodges, G. R. & Ingold, K. U. (1999) *J. Am. Chem. Soc.* **121,** 10695–10701.
11. Gerasimov, O. V. & Lymar, S. V. (1999) *Inorg. Chem.* **38,** 4317–4321.
12. Pfeiffer, S., Gorren, A. C. F., Schmidt, K., Werner, E. R., Hansert, B., Bohle, D. S. & Mayer, B. (1997) *J. Biol. Chem.* **272,** 3465–3470.
13. Coddington, J. W., Hurst, J. K. & Lymar, S. V. (1999) *J. Am. Chem. Soc.* **121,** 2438–2443.
14. Khan, A. U., Kovacic, D., Kolbanovsky, A., Desai, M., Frenkel, K. & Geacintov, N. E. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2984–2989.
15. Wang, J. H. (1962) in *Oxygenases*, ed. Hayaishi, O. (Academic, New York), p. 472.
16. Saha, A., Goldstein, S., Cabelli, D. & Czapski, G. (1998) *Free Radical Biol. Med.* **24,** 653–659.
17. Antonini, E. & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands* (North—Holland, Amsterdam), p. 280.
18. Kanner, J., Harel, S. & Granit, R. (1991) *Arch. Biochem. Biophys.* **259,** 130–136.
19. Gorbunov, N. V., Osipov, A. N., Day, B. W., Zayas-Rivera, B., Kagan, V. E. & Elsayed, N. M. (1995) *Biochemistry* **34,** 6689–6699.
20. Hughes, M. N. & Nicklin, H. G. (1968) *J. Chem. Soc.* 450–456.
21. Alayash, A. I., Brockner Ryan, B. A. & Cashon, R. E. (1998) *Arch. Biochem. Biophys.* **349,** 65–73.
22. Hoshino, M., Ozawa, K. & Ford, P. C. (1993) *J. Am. Chem. Soc.* **115,** 9568–9575.
23. Stanbury, D. M. (1989) *Adv. Inorg. Chem.* **33,** 69–138.
24. Logager, T. & Sehested, K. (1993) *J. Phys. Chem.* **97,** 6664–6669.
25. Khan, A. U. (1995) *J. Biolumin. Chemilumin.* **10,** 329–333.
26. Lymar, S. V. & Hurst, J. K. (1995) *J. Am. Chem. Soc.* **117,** 8867–8868.
27. Goldstein, S. & Czapski, G. (1997) *Inorg. Chem.* **36,** 5113–5117.
28. Lymar, S. V. & Hurst, J. K. (1998) *Inorg. Chem.* **37,** 294–301.
29. Goldstein, S. & Czapski, G. (1998) *J. Am. Chem. Soc.* **120,** 3458–3463.
30. Boinini, M. G., Radi, R., Ferrer-Sueta, G., Ferreira, A. M. D. C. & Augusto, O. (1999) *J. Biol. Chem.* **274,** 1082–10806.
31. Uppo, R. M. & Pryor, W. A. (1999) *J. Am. Chem. Soc.* **121,** 9738–9739.