Peroxynitrite does not decompose to singlet oxygen ($^1\Delta_g O_2$) and nitroxyl (NO^-)

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According to Khan *et al.* [Khan, A. U., Kovacic, D., Kolbanovskiy, A., Desai, M., Frenkel, K. & Geacintov, N. E. (2000) *Proc. Natl. Acad. Sci. USA* 97, 2984–2989], peroxynitrite (ONOO⁻) decomposes after protonation to singlet oxygen ($^1\Delta_g O_2$) and singlet oxonitrate (nitroxyl, $^1NO^-$) in high yield. They claimed to have observed nitrosyl hemoglobin from the reaction of NO⁻ with methemoglobin; however, contamination with hydrogen peroxide gave rise to ferryl hemoglobin, the spectrum of which was mistakenly assigned to nitrosyl hemoglobin. We have carried out UV–visible and EPR experiments with methemoglobin and hydrogen peroxide-free peroxynitrite and find that no NO⁻ is formed. With this peroxynitrite preparation, no light emission from singlet oxygen at 1270 nm is observed, nor is singlet oxygen chemically trapped; however, singlet oxygen was trapped when hydrogen peroxide was also present, as previously described [Di Mascio, P., Bechara, E. J. H., Medeiros, M. H. G., Briviba, K. & Sies, H. (1994) *FEBS Lett.* 355, 287–289]. Quantum mechanical and thermodynamic calculations show that formation of the postulated intermediate, a cyclic form of peroxynitrous acid (trioxazetidine), and the products $^1NO^-$ and $^1\Delta_g O_2$ requires Gibbs energies of *ca.* +415 kJ·mol⁻¹ and *ca.* +180 kJ·mol⁻¹, respectively. Our results show that the results of Khan *et al.* are best explained by interference from contaminating hydrogen peroxide left from the synthesis of peroxynitrite.

Peroxynitrite [oxoperoxonitrate(1−)] is an inorganic oxidant of biological importance that is produced from superoxide and nitrogen monoxide (1). The mechanisms by which peroxynitrite or peroxynitrous acid [pK$_a$ = 6.7 at 37°C (2)] oxidizes biomolecules are being investigated by various groups. There are two types or reaction: reactions between biomolecules and peroxynitrite (or peroxynitrous acid) that are first-order in peroxynitrite and in the biomolecule, and reactions that are first-order in peroxynitrite and zero-order in the biomolecule. In the latter case, either peroxynitrous acid undergoes homolysis to nitrogen dioxide and the hydroxyl radical (3–7), or it forms an activated intermediate (2, 8). In the absence of oxidizable molecules the radicals recombine to nitrate, or the activated intermediate undergoes an internal isomerization. The rate constant for this process, which is first-order in peroxynitrite, is 3.9 s⁻¹ at 37°C (2). Whether radicals are intermediates is not relevant for the present discussion. All involved agree that at acid pH and at low temperatures peroxynitrous acid isomerizes to nitrate quantitatively, whereas at higher pH nitrite and dioxygen in a 2 to 1 ratio also are found.

Recently, a mechanism was proposed, in which peroxynitrite forms, after protonation, a cyclic intermediate, which dissociates to form oxonitrate(1−)—the commonly used name "nitroxyl" is outdated—and singlet oxygen (9):

$$ONOOH \rightarrow {}^1NO^- + {}^1\Delta_g O_2 + H^+ \qquad [1]$$
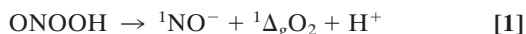
These products have been postulated before by Khan (10). As described in the more recent article (9), Khan *et al.* used methemoglobin (MetHb) to trap oxonitrate(1−), and 9,10-diphenylanthracene and 2,3-dimethyl-2-butene to trap singlet

oxygen. Their mechanism does not explain the quantitative formation of nitrate, and we therefore investigated how these results (9) could have been obtained. We show here that singlet oxygen is not formed when peroxynitrite free of hydrogen peroxide is used, that the evidence for oxonitrate(1−) is based on a misinterpretation of the hemoglobin UV–visible spectra, and that the formation of these products and the postulated cyclic precursor, 4-hydrido-4-azy-1,2,3-trioxy[04]cycle, or trioxazetidine, is thermodynamically unlikely. The experimental evidence presented by Khan *et al.* (9) is best explained by the presence of hydrogen peroxide formed during the synthesis of peroxynitrite.

Materials and Methods

Chemicals. Peroxynitrite was synthesized from sodium nitrite (0.6 M) and hydrogen peroxide (0.65 M) in a quenched-flow reactor (11); excess hydrogen peroxide was used to minimize nitrite contamination (12). To eliminate excess hydrogen peroxide, ONOO⁻ was treated with manganese dioxide. Synthesized ONOO⁻ contained low levels of contaminating hydrogen peroxide (<5 μM) and nitrite (10–30%) that were determined by the titanyl method and by absorbance measurements at 354 nm (ε = 24.6 M⁻¹·cm⁻¹), respectively (13). Peroxynitrite used for the UV–visible experiments was prepared according to ref. 14 and also contained very low levels of the contaminants hydrogen peroxide and nitrite. The concentration of the ONOO⁻ stock solutions was determined spectrophotometrically at 302 nm with an extinction coefficient of 1,670 M⁻¹·cm⁻¹ (15). Sodium trioxodinitrate (Na₂N₂O₃) was purchased from Cayman Chemicals; a stock solution of this salt was prepared in deoxygenated sodium hydroxide (0.01 M) and kept on ice. MetHb was prepared by oxidizing bovine hemoglobin (Sigma) with 20% excess potassium hexacyanoferrate(III) in 0.2 M sodium phosphate buffer, pH 7.4. Hexacyanoferrate(II) and excess hexacyanoferrate(III) were removed by desalting on a G-25 Sephadex column. Heme concentrations were determined spectrophotometrically at 406 nm (ε = 154 mM⁻¹·cm⁻¹) (16). The water-soluble disodium salt of anthracene-9,10-diyldiethyl disulfate (EAS) and the endoperoxide of *N,N'*-di(2,3-dihydroxypropyl)-1,4-naphthalenedipropanamide (DHPNO₂) were synthesized and used according to refs. 17 and 18 and ref. 19, respectively.

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Sodium hypochlorite was analyzed iodometrically. All solutions were prepared with distilled water purified with a Millipore Milli-Q system.

UV–Visible Spectra of MetHb and Peroxynitrite. Absorption spectra were collected with a UVIKON 820 spectrophotometer. An 80 μM MetHb solution in 0.1 M sodium phosphate buffer, pH 7, was placed in a sealable cuvette (path length 1 cm, total volume 3 ml) and deoxygenated by gently bubbling N_2 through it for at least 30 min. Peroxynitrite was added from a deoxygenated stock solution (30 mM in 0.1 M NaOH) by using a gas-tight Hamilton syringe. Hydrogen peroxide was added analogously from a deoxygenated 880 mM stock solution in water.

Electron Paramagnetic Resonance (EPR) Measurements. EPR spectra were recorded with a Bruker EMX instrument at 77 K; this temperature was attained with a fingertip liquid-nitrogen Dewar (20). Peroxynitrite was added to a nitrogen-bubbled solution of MetHb (final volume 0.7 ml), and after 5-min incubation the mixture was transferred into a 1-ml plastic syringe (4 mm inside diameter) and frozen in dry ice/ethanol. Just before EPR analysis, the syringe tip was removed and the hand-warmed sample was pushed to a fingertip Dewar containing liquid nitrogen. The same protocol was used for the reactions with $Na_2N_2O_3$ except that the mixtures were incubated for 20 min at 37°C before freezing. The yield of nitrosyl hemoglobin ($HbFe^{II}NO$) was determined by double integration of the measured EPR signal and subsequent comparison with the doubly integrated signals of samples with known concentrations of $HbFe^{II}NO$ prepared by adding a 5-fold excess of $Na_2N_2O_3$ to MetHb. Under these conditions 99% conversion of MetHb to $HbFe^{II}NO$ was confirmed by the disappearance of the EPR signal of MetHb at $g = 6$.

Monomol Emission of Singlet Oxygen. The infrared photoemission of singlet oxygen at 1,270 nm was measured with a liquid nitrogen-cooled germanium photodetector sensitive in the spectral region from 800 to 1,800 nm with a detector area of 0.25 cm^2 and a sapphire window as described (17). A band-pass filter at 1,270 nm with a 10-nm half-bandwidth was used.

Chemical Trapping of $^1\Delta_gO_2$ and HPLC/Mass Spectrometry Analysis. EAS endoperoxide ($EASO_2$) was separated from EAS by HPLC with a system consisting of LC 10ADVP pumps connected to an automatic SIL-10ADVP injector (Shimadzu, Tokyo). For analytical purposes, the system was equipped with a 150 mm $\times$ 4.6 mm inside diameter (particle size 5 μm) Supelco C_{18} reverse-phase column and a 20 $\times$ 2.1 mm (particle size 5 μm) Supelco C_{18} guard precolumn (Supelco, Bellefonte, PA), connected to a Shimadzu SPD-M10AVVP diode array. The samples were diluted 1:10 and 30 μl was injected. Solvent A was 25 mM ammonium formate, and solvent B was acetonitrile. The compounds were eluted by an increasing linear gradient of B from 15% to 17% during the first 8 min and by keeping it constant thereafter. The flow rate was 1.0 ml/min. Data acquisition was performed by means of Shimadzu CLASS-VP software, version 5.3. Electrospray ionization (ESI) mass spectrometry analyses in the negative mode were performed with a Platform II mass spectrometer (Micromass, Altricham, U.K.). A Shimadzu LC-10AD pump was used to infuse the eluent, a 1:1 mixture of water and acetonitrile, at a final flow rate of 10 μl/min. The samples (20 μl) were injected through a 20-μl Rheodyne loop (Rheodyne; Cotati, CA). The source temperature was maintained at 80°C, and flow rates of the drying and nebulizing gas (nitrogen) were optimized at 300 and 29 liters/h, respectively. The cone voltage was maintained at 10 V, and the capillary and high-voltage electrode potentials were at 3.50 and 0.69 kV, respectively. Full-scan data were acquired over a mass range of
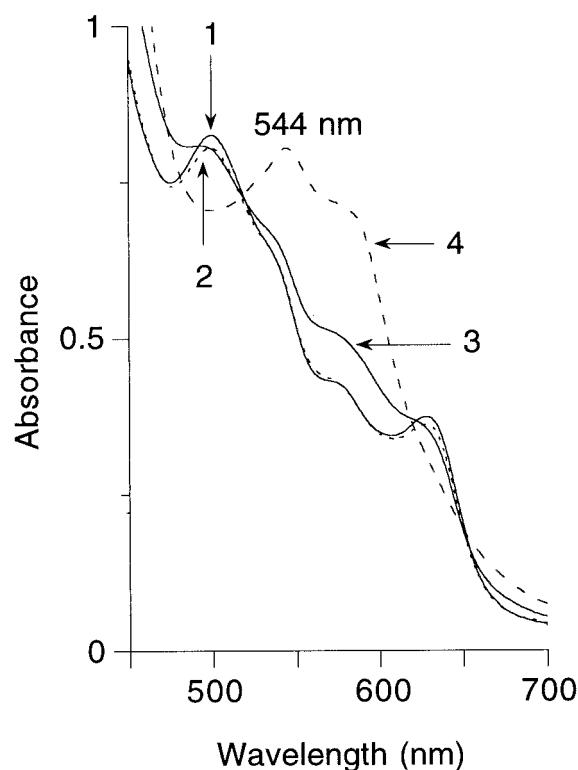
Fig. 1. Absorbance spectra of a solution of 80 μM bovine MetHb in 0.1 M sodium phosphate buffer (pH 7.0) at room temperature under N_2. Trace 1, MetHb solution; trace 2, MetHb + 160 μM $ONOO^-$; trace 3, MetHb + 160 μM $ONOO^- + 400$ μM H_2O_2; trace 4, MetHb + 160 μM $ONOO^- + 960$ μM H_2O_2.

100–400 Da. The data were processed and transformed into values of molecular masses on mass scale by means of the Mass Lynx NT data system 3.20 version (Micromass).

Ab Initio Calculations. Calculations were performed with the GAUSSIAN 94 suite of programs (21) that apply density functional theory methods and a standard 6–311+G(d,p) basis set. We performed gradient-corrected calculations with the functional developed by Becke (22) for the exchange and by Lee *et al.* (23) for the correlation part (BLYP). We also used the hybrid method Becke3-LYP, which combines Becke's three-parameter mixing of exchange terms (24) with the nonlocal part of the LYP correlation functional (B3LYP). To test basis set convergence dependence, additional calculations were performed with the program CPMD (25), which also uses the BLYP functional. Valence orbitals were expanded in a basis of plane waves up to a kinetic energy cutoff of 70 rydberg (150 aJ). Only valence electrons were treated explicitly. To account for the effect of the inner electrons, we applied norm-conserving pseudopotentials of the Martins–Trouiller type (26), with cut-off radii of 0.5 atomic unit (au; 1 au = $0.529 \cdot 10^{-10}$ m) for hydrogen, 1.12 au for nitrogen, and 1.11 au for oxygen.

Results and Discussion

MetHb Experiments. The principal evidence for the formation of oxonitrate(1−) by Khan *et al.* (9) was the putative formation of nitrosyl hemoglobin by the addition of peroxynitrite to MetHb. However, we (27) and others (28, 29) have previously observed that $ONOO^-$ does not react with the iron(III) center of MetHb or metmyoglobin under similar conditions, which we repeated here (Fig. 1, trace 2). Khan *et al.* (9) prepared peroxynitrite by adding solid potassium superoxide to a nitrogen monoxide-

saturated solution. In our experience, such solutions are invariably contaminated with nitrite and with hydrogen peroxide—formed from the spontaneous dismutation of superoxide—at concentrations that exceed the concentration of peroxynitrite. They did not report any efforts to remove hydrogen peroxide, nor did they measure hydrogen peroxide. We found that the simple addition of hydrogen peroxide, which forms ferryl hemoglobin, resulted the spectral features interpreted by Khan *et al.* as resulting from nitrosyl hemoglobin. Indeed, the experiment depicted in figure 1 of the cited paper does not support the generation of NO^-. First, the absorbance spectrum measured after the reaction of MetHb with 2 equivalents of the NO^- source $Na_2N_2O_3$ (spectrum e of figure 1 of ref. 9) does not represent the spectrum of pure $HbFe^{II}NO$, but rather that of a mixture of MetHb and $HbFe^{II}NO$. This conclusion is based on the presence of the characteristic MetHb absorbance band around 500 nm and was not unexpected. As shown by Hollocher and coworkers (30), 5 equivalents of $Na_2N_2O_3$ are needed to quantitatively generate $HbFe^{II}NO$ from MetHb, because of the fast parallel dimerization and dehydration reaction of HNO to yield N_2O. Thus, if this spectrum is considered to be identical to that obtained from the reaction of MetHb with 1 equivalent of peroxynitrite (spectrum d of figure 1 in ref. 9), the yield of NO^- from peroxynitrite *cannot be considered quantitative*. Second, the two spectra mentioned above were obtained by reacting either $Na_2N_2O_3$ or peroxynitrite with MetHb (spectra d and e of figure 1 of ref. 9), are *not* identical: the maximum around 500 nm is present only in the former. The absorbance changes observed by Khan *et al.* (9) upon addition of peroxynitrite to MetHb may derive from the partial formation of oxoiron(IV)Hb ($HbFe^{IV}=O$), generated from the reaction of H_2O_2 with MetHb. As nitrite is known to reduce $HbFe^{IV}=O$ to MetHb (27) and to bind to MetHb, it is not possible to estimate accurately the amounts of nitrite and hydrogen peroxide present in the peroxynitrite solutions used by Khan *et al.*

To further assess whether any trace of nitrosyl hemoglobin could be detected we used low-temperature EPR. No $HbFe^{II}NO$ can be detected by low-temperature EPR of mixtures of 1 mM MetHb and 1 mM $ONOO^-$ under low oxygen tensions (Fig. 2, spectra A and B). As expected, mixtures of 1 mM MetHb and $Na_2N_2O_3$ produced an EPR signal composite of those of hexa- and pentacoordinated $HbFe^{II}NO$ complexes (Fig. 2, spectra C and D). As previously reported, the yields of the detected complexes increased with increasing $HN_2O_3^-$ concentration (30). Under our experimental conditions, addition of either 0.1 or 1 mM $HN_2O_3^-$ to 1 mM MetHb generated 60 and 430 μM $HbFe^{II}NO$, respectively. Consequently, even if only 10% of 1 mM $ONOO^-$ decayed to produce 100 μM NO^-, the EPR signal of the subsequently formed $HbFe^{II}NO$ would be clearly detectable as it was in mixtures of 0.1 mM $HN_2O_3^-$ and 1 mM MetHb (Fig. 2C).

Both UV–visible and EPR experiments demonstrate that virtually no oxonitrate(1−) is produced during peroxynitrite decay.

Singlet Oxygen Detection. We could also find no evidence for the formation of singlet oxygen produced by peroxynitrite decay unless there was hydrogen peroxide present. First, we monitored directly the monomol emission of singlet oxygen at 1,270 nm. Addition of a solution of acetic acid to a 3-ml solution of peroxynitrite (25 mM; pH 14) gave no signal (data not shown), whereas there was a strong signal at 1,270 nm when *tert*-butyl hydroperoxide (25 mM) (see figure 3 of ref. 31) or hydrogen peroxide (32, 33) was also present.

Detection of $^1\Delta_gO_2$ from the decay of high concentrations of peroxynitrite in a two-phase system by Khan *et al.* (9) could be due to the reaction between peroxynitrite and contaminant hydrogen peroxide (33). Indeed, experiments with the water-
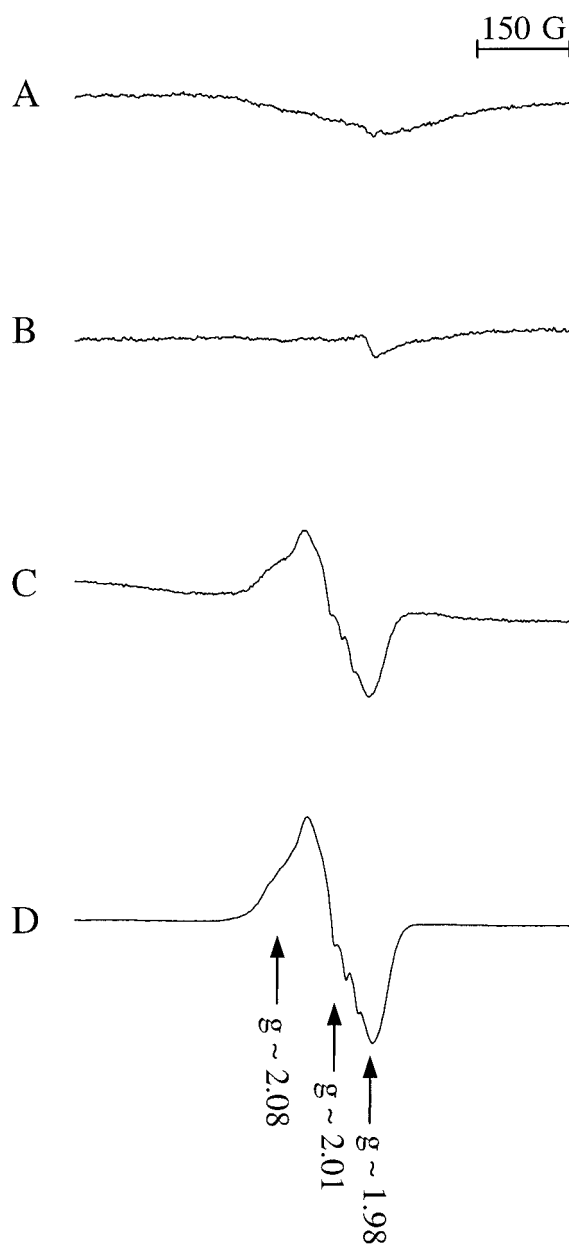
Fig. 2. Representative low-temperature EPR spectra obtained from mixtures of 1 mM MetHb and $ONOO^-$ or $HN_2O_3^-$ under reduced oxygen tensions. A, MetHb; B, MetHb + 1 mM $ONOO^-$; C, MetHb + 0.1 mM $HN_2O_3^-$; and D, MetHb + 1 mM $HN_2O_3^-$. The incubation mixtures were prepared and EPR analyses were performed as described in *Materials and Methods*. Instrumental conditions: power, 20 mW; modulation amplitude, 5 G; scan rate, 7.4 G/s; gain, 1.42×10^4, except for D, where the gain was 2.83×10^3.

soluble $^1\Delta_gO_2$ chemical probe EAS (Reaction **2**) (17, 18) provided no evidence for the formation of singlet oxygen during decay of peroxynitrite when hydrogen peroxide was absent (Fig. 3).
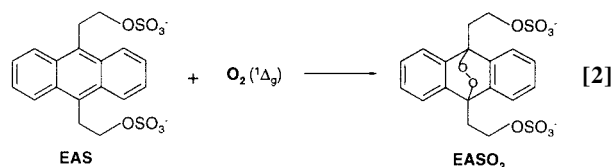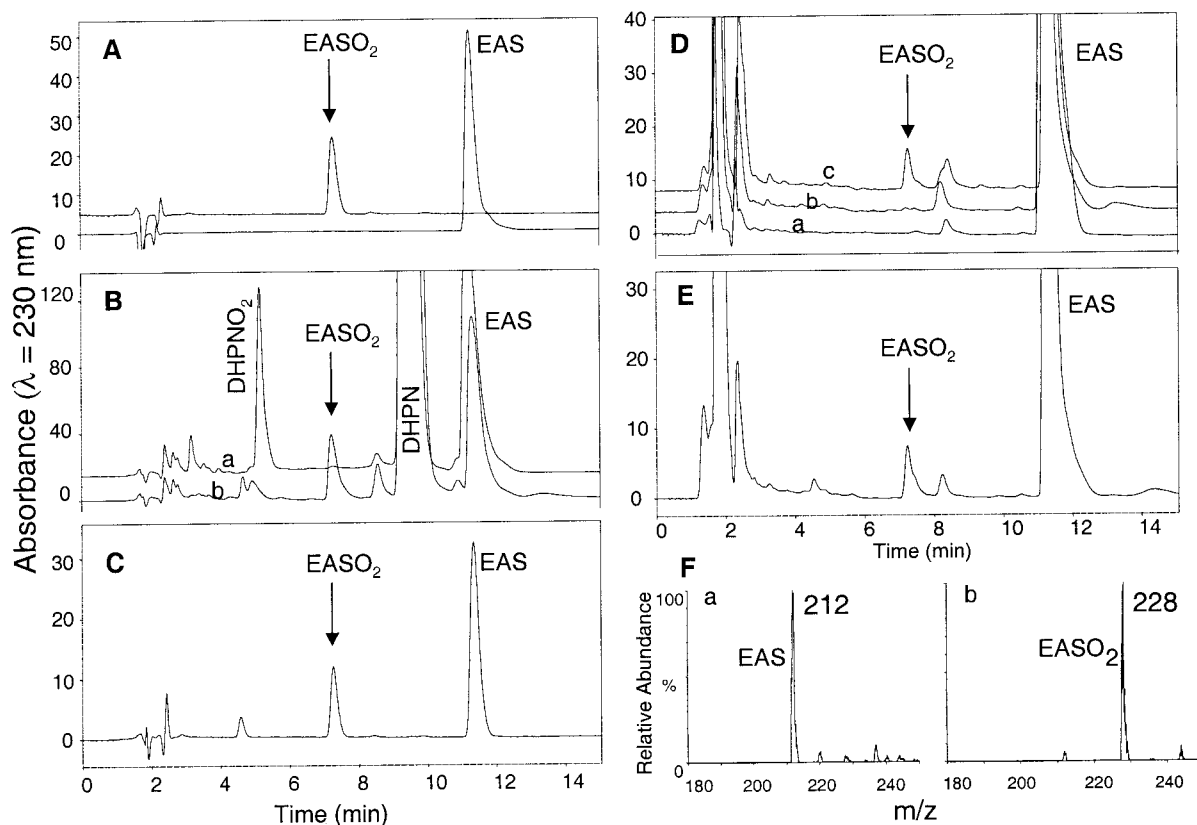
Fig. 3. Chemical detection of $^1\Delta_gO_2$ by formation of EASO$_2$. (*A–E*) Gradient reverse-phase HPLC. (*A*) 0.09 mM EAS and 0.033 mM EASO$_2$. (*B*) Trace a, 10 mM DHPNO$_2$ and 8 mM EASO$_2$; trace b, same as trace a after incubation at 40°C for 2 h in water. (*C*) Six aliquots of 10 μl of both 10 mM OCl$^-$ and H$_2$O$_2$ were added to 8 mM EAS in 150 mM sodium phosphate buffer, pH 7.4, such that the final concentrations of hypochlorite and hydrogen peroxide (if unreacted) were 1 mM each. (*D*) Trace a, 1 mM ONOO$^-$ and 8 mM EAS; trace b, 15 mM ONOO$^-$ and 8 mM EAS; and trace c, 15 mM ONOO$^-$, 8 mM EAS, and 0.012 mM EASO$_2$, all in 150 mM phosphate buffer, pH 7.4. (*E*) 100 mM H$_2$O$_2$, 15 mM ONOO$^-$, and 8 mM EAS. (*F*) Mass spectra of EAS (*a*) and EASO$_2$ (*b*). For the experiments described in *D*, trace b, and *E*, a 138 mM solution of ONOO$^-$ was infused at a rate of 8 μl/min into a mixture containing 8 mM EAS in 150 mM phosphate buffer, pH 7.4, with (*E*) or without (*D*, trace b) 100 mM H$_2$O$_2$. When ONOO$^-$ and EAS were mixed (final concentrations 1 and 8 mM, respectively) in 150 mM sodium acetate buffer, pH 5.5, no EASO$_2$ was detected (data not shown).

EAS and EASO$_2$ were analyzed by gradient reverse-phase HPLC (Fig. 3*A*) and electrospray tandem mass spectrometry (Fig. 3*F*). The mass spectrum of EAS recorded in the negative mode exhibits a major $[M - 2H]^{2-}$ ion at $m/z = 212$ (Fig. 3*Fa*), corresponding to the doubly charged molecule. The spectrum of EASO$_2$ exhibits, as expected, an intense $[M - 2H]^{2-}$ ion at $m/z = 228$ (Fig. 3*Fb*). For comparison, we showed that EASO$_2$ is formed from the reaction of EAS with singlet oxygen generated either by thermal decomposition of the endoperoxide DHPNO$_2$ (10 mM) (19) (Fig. 3*B*) or by 1 mM OCl$^-$/H$_2$O$_2$ system (Fig. 3*C*). In contrast, neither 1 mM nor 15 mM ONOO$^-$ led to the formation of detectable amounts of EASO$_2$ (Fig. 3*D*, trace a, and trace b, respectively) as proved by co-injection of EASO$_2$ in the 15 mM ONOO$^-$ assay (Fig. 3*D* trace c). Peroxynitrite in the presence of high concentrations of H$_2$O$_2$ produced EASO$_2$ (Fig. 3*E*), as already described by Di Mascio *et al.* (32, 33).

Theoretical Considerations. Khan *et al.* (9) propose a strained four-membered structure, 4-hydrido-4-azy-1,2,3-trioxy[04]cycle, or trioxazetidine, for the peroxynitrous acid that then undergoes a simultaneous double homolysis to form ^{1}HNO and ^{1}O$_2$. We estimated the energy of this four-membered structure with three *ab initio* calculations. All identify the—nonplanar—ring structure as a local minimum of the potential energy surface, albeit at a very high energy compared with the ground state of

ONOOH: The G94/BLYP, G94/B3LYP, and CPMD/BLYP differences between the ring structure and *cis*-peroxynitrous acid are +433, +429, and +406 kJ·mol^{-1}, respectively. Although the results apply to the gas phase, these differences should be good approximations for the aqueous phase. These values are nearly 4 times higher than the experimentally measured activation enthalpy of +86.5 kJ·mol^{-1} (34).

A thermodynamic analysis shows that formation of oxonitrate(1−) and singlet oxygen from peroxynitrous acid, Reaction **1**, is most unlikely. The Gibbs energies of formation of ONOO$^-$, ^{1}NO$^-$, and $^1\Delta_gO_2$ have been calculated as +59 to +71 kJ·mol^{-1} (2, 35), +136 kJ·mol^{-1} (36), and +112 kJ·mol^{-1} (37), respectively. The latter value includes a Gibbs solvation energy, which was taken to be the same as that of triplet oxygen. This results in a Gibbs energy of reaction between +176 and +188 kJ·mol^{-1}. These results are shown in Fig. 4.
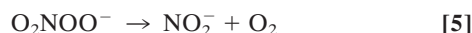
From ^{1}NO$^-$ and ^{1}O$_2$ to NO$_3^-$. The proposed mechanism, which involves the oxidation of oxonitrate(1−) to nitrite, does not explain the quantitative formation of nitrate, observed when peroxynitrous acid isomerizes at low pH and low temperature. As Khan *et al.* (9) themselves point out, oxidizing agents would be required, whereas experimentally there is no need for such agents. Furthermore, oxonitrate(1−) would react with itself to yield dinitrogen monoxide, which has not been observed.

Singlet Dioxygen Formation. The simple explanation for the results of Khan *et al.* (9) is that hydrogen peroxide was present in the
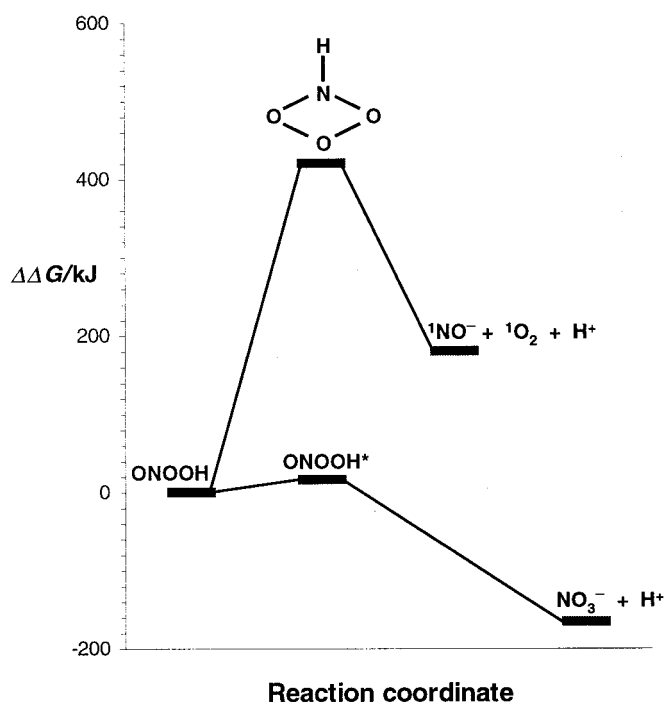
Fig. 4. Gibbs energy differences between the postulated intermediates (9), nitrate and *cis*-peroxynitrous acid. The experimentally observed energetics of the isomerization pathway to nitrate are also indicated (2, 34).

peroxynitrite preparation. The formation of singlet oxygen from peroxynitrite and hydrogen peroxide was described in 1994 (32). Radi and coworkers (38) have found that the reaction of hydrogen peroxide and peroxynitrous acid or peroxynitrite yields dioxygen. To increase the yield of the latter a large excess of hydrogen peroxide had to be used. The mechanism of dioxygen formation (39, 40) involves the one-electron oxidation of hydrogen peroxide by peroxynitrous acid (Reaction **3**), the formation of dioxoperoxonitrate(1−) (O_2NOO^-) from nitrogen dioxide and superoxide (Reaction **4**), followed by heterolysis to produce nitrite and dioxygen (Reaction **5**):

$$ONOOH + HOOH \rightarrow NO_2^{.} + O_2^{.-} + H^+ + H_2O \quad [3]$$

$$NO_2^{.} + O_2^{.-} \rightarrow O_2NOO^- \quad [4]$$

$$O_2NOO^- \rightarrow NO_2^- + O_2 \quad [5]$$

Given the Gibbs energy of formation of dioxoperoxonitrate(1−), +39 kJ·mol^{-1} (41), nitrite, −32.2 kJ·mol^{-1} (42), and of singlet oxygen, +94.6 kJ·mol^{-1} (37), formation of singlet oxygen in Reaction **5** is not favorable, $\Delta_{rxn}G° = +23.0$ kJ·mol^{-1}. However, a direct oxidation of superoxide ($\Delta_fG° =$

+31.8 kJ·mol^{-1}; ref. 37) by nitrogen dioxide ($\Delta_fG° = +63.0$ kJ·mol^{-1}; ref. 36), Reaction 6,

$$NO_2^{.} + O_2^{.-} \rightarrow {}^1\Delta_gO_2 + NO_2^- \quad [6]$$
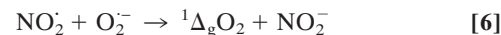
without dioxoperoxonitrate(1−) as an intermediate would allow thermodynamically the formation of singlet oxygen, with a Gibbs energy change of −32.4 kJ·mol^{-1}.

Relevance of Isomerization Intermediates to Biology. The main sinks for peroxynitrite in a biological milieu are the bimolecular reactions with carbon dioxide (43, 44) sulfur- or selenium-containing compounds (45–48), and metalloproteins (27, 49–51). In these reactions peroxynitrite is estimated to disappear at rates in excess of 70–80 s^{-1}, approximately 40 times faster than the isomerization, which involves either an activated intermediate, radicals, or, according to Khan *et al.* (9), $^1NO^-$ and singlet oxygen. Thus, these species do not contribute significantly to the biological reactivity of peroxynitrite, except for environments of low pH, such as those found in the phagosomes of phagocytic cells.

Conclusions

We presented above seven arguments against the conclusion of Khan *et al.* (9) that singlet oxonitrate(1−) and singlet oxygen are intermediates in the isomerization of peroxynitrous acid to nitrate and a proton. Furthermore, the formation of these, or any other intermediates, is of limited relevance to biology.

This publication on the formation of singlet oxygen from peroxynitrite is reminiscent of a previous erroneous claim by Khan and Kasha (52) in 1994 that singlet oxygen is formed from the reaction of superoxide with hydrogen peroxide. This reaction, too, has a positive Gibbs energy, but more importantly, in 1947 George (53), and later several other groups (54–59) showed that, compared with the rate of superoxide dismutation (60), the reaction, even with triplet oxygen as a product, is too slow to be relevant.

We found another criticism (61) of the work of Khan *et al.* (9) while this paper was under review. It mentions two of the seven arguments discussed above. Although these authors, too, come to the conclusion that hydrogen peroxide was present during the experiments of Khan *et al.* (9), they could not account for the formation of singlet oxygen, although its formation from peroxynitrite and hydrogen peroxide has been described in the literature (32, 33) and one of their own works (41) provided a valuable clue for a possible mechanism of this reaction.

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