Formation of Peroxynitrite from Reaction of Nitroxyl Anion with Molecular Oxygen*  

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Peroxynitrite (ONOO\(^{-}\)/ONOO\(^{\cdot}\)) is generally expected to be formed in vivo from the diffusion-controlled reaction between superoxide (O\(_2^\cdot\)) and nitric oxide (NO). In the present paper we show that under aerobic conditions the nitroxyl anion (NO\(^{-}\)), released from Angeli’s salt (disodium diazen-1-ium-1,2,2-triolate, ON=NOO\(^{-}\)), generated peroxynitrite with a yield of about 65%. Simultaneously, hydroxyl radicals are formed from the nitroxyl anion with a yield of about 3% via a minor, peroxynitrite-independent pathway. Further experiments clearly underline that the chemistry of NO\(^{-}\) in the presence of oxygen is mainly characterized by peroxynitrite and not by HO\(^{\cdot}\) radicals. Quantum-chemical calculations predict that peroxynitrite formation should proceed via intermediary formation of NO and O\(_2^\cdot\) probably by an electron-transfer mechanism. This prediction is supported by the fact that H\(_2\)O\(_2\) is formed during the decay of NO\(^{-}\) in the presence of superoxide dismutase (Cu(II),Zn-SOD). Since the nitroxyl anion may be released endogenously by a variety of biomolecules, substantial amounts of peroxynitrite might be formed in vivo via NO\(^{-}\) in addition to the “classical” NO + O\(_2^\cdot\) pathway.

A further source of endogenous peroxynitrite may be the nitroxyl anion (NO\(^{-}\)). This anion has been reported to be generated in vivo from reduction of NO by Cu(I),Zn-SOD (24), hemoglobin (25), and cytochrome c\(^{2+}\) (26), respectively. There are also reports that the NOS-catalyzed oxidation of L-arginine leads to initial formation of NO\(^{-}\) and not NO radical (27), however, this finding is subject to controversial discussion (28). Nitroxyl anion might be further formed from reaction of S-nitrosothiols with thiols (29–31), although this reaction is less well understood. Donald et al. (32) have proven that the photochemical decomposition of Angeli’s salt, a chemical NO\(^{-}\) donor compound (33), in fact yields peroxynitrite under aerobic conditions (Reaction 1). This photochemical process is probably the reason why NO\(^{-}\) has often been referred to as a peroxynitrite-yielding compound, even in textbooks of inorganic chemistry (34).

\[
\text{NO}^{-} + \text{O}_2 \rightarrow \text{ONOO}^{-}  
\]

**REACTION 1**

Only very low yields of nitrated products have been observed from NO\(^{-}\)-induced reactions (35, 36). From these facts it was concluded that during thermal decomposition of Angeli’s salt only a small amount of peroxynitrite is generated (36). Recently, two research groups (37, 38) apparently disproved the capability of NO\(^{-}\) to generate peroxynitrite under more physiological conditions. They reported, for example, that typical peroxynitrite-mediated reactions, e.g. nitrosation reactions, could not be observed (37) and that NADPH could be oxidized by NO\(^{-}\) under hypoxic conditions (38). Unfortunately, these experiments were performed in the presence of organic buffer compounds (Good’s buffer) which are known to effectively react with peroxynitrite (39, 40). Thus, the formation of peroxynitrite by NO\(^{-}\) very easily may have been masked.

In the present study, we demonstrate that in the presence of molecular oxygen NO\(^{-}\) indeed mainly yields peroxynitrite and that NO\(^{-}\) additionally produces HO\(^{\cdot}\) radicals via a minor, peroxynitrite-independent pathway. Furthermore, we present a key experiment which suggests the intermediacy of O\(_2^\cdot\) during the NO\(^{-}\)-mediated formation of peroxynitrite.

**EXPERIMENTAL PROCEDURES**  
**MATERIALS**—Catalase from beef liver (EC 1.11.1.6), copper-zinc superoxide dismutase from bovine erythrocytes (EC 1.15.1.1), and NADH were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Manganese dioxide, benzoic acid, and hydrogen peroxide were from Sigma (Deisenhofen, Germany). Angeli’s salt came from Alexis-Deutschland (Grüenberg, Germany). DHR and DAN were obtained from Molecular Probes (Leiden, The Netherlands). Commercially available mixtures of oxygen 5.0, nitrogen 5.0, carbon dioxide 4.6 (20.5% O\(_2\), 74.5% N\(_2\), 5% CO\(_2\)) were purchased from Messer-Griesheim (Oberhausen, Germany); 5.0 and 4.6 mean purities of 99.999 and 99.996%, respectively. SIN-1 and its decomposition product SIN-1C were generously provided by Drs. K.

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Nitroxyl Anion-derived Radicals

Experimental Conditions—SIN-1 was added to 1 ml of phosphate buffer and incubated in 12-well cell culture plates (volume of each well 7 ml, Falcon, Heidelberg, Germany). Under HCO3-/CO2-free conditions, these plates were placed in an air-tight vessel (10 liters). During the first 15 min of each experiment, these vessels were flushed (5 liters/min with synthetic air in a warming incubator (Heraeus, Hamburg, Germany). The experiments with authentic peroxynitrite (2 μl of 25–125 mM ONOO− in 0.5 mM NaOH was added to 1 ml of reaction solution) and with Angeli’s salt (1 ml of reaction solution) were performed in reaction tubes (20 ml) (Eppendorf, Hamburg, Germany) by using the drop-tube Vortex mixer technique as described (40). Under HCO3-/CO2-free conditions, the experiments with authentic peroxynitrite and Angeli’s salt were performed in a glove-bag (Roth, Karlsruhe, Germany) under synthetic air.

Determination of Peroxynitrite/SIN-1/Angeli’s Salt-driven Hydroxyla-
tion of Benzoic Acid—Peroxynitrite, SIN-1, and Angeli’s salt (each 50–600 μM)-dependent hydroxylation of BA (5 mM) were employed. After vortex-
ing, the samples were kept for 2 min (in the case of peroxynitrite), 4 h (in the case of SIN-1), and 30 min (in the case of Angeli’s salt) at 37 °C, respectively. The product formed was measured by reading its fluorescence with excitation at 290 nm and emission at 410 nm (37).

Determination of Rhodamine 123—Formation of RH was quantified spectrophotometrically at 500 nm (εmax = 78,800 M−1 cm−1) (42).

Determination of Angeli’s Salt-driven Nitrosation Reactions—Ange-
li’s salt (50–600 μM)-dependent nitrosation of DAN (200 mM) was em-
ployed. After vortexing, the samples were incubated for 30 min at 37 °C. NaOH (0.5 mM) was added (5:1, v/v, final pH 11–11.5). The product formed, i.e. NAT, was quantified by reading its fluorescence with excita-
tion at 375 nm and emission at 415 nm (44). Standard calibration curves were prepared from known amounts of NAT.

Determination of NADH—NADH was quantified by reading its fluo-
rescence with excitation at 339 nm and emission at 460 nm (45). Standard calibration curves were prepared from known amounts of NADP/H. Additionally, the oxidation of NADP/H was followed photomet-
rically at 340 nm using Δεmax = 6200 M−1 cm−1 (45). Both methods gave identical results, therefore, only one parameter, the decrease of fluorescence, will be shown here.

Determination of H2O2 and of O2—Hydrogen peroxide was quantified by two techniques. In peroxidase assays, horseradish peroxidase-cata-
lyzed formation of a colored product was measured. 4-Aminonitroprpyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid were used as peroxi-
dase substrates. The quinonemine dye formed from these substrates was measured spectrophotometrically at 546 nm (46) and/or with the PCM (50) procedure incorporated in Gaussian 98W. To verify the accuracy of the force field parameterization, "natural" parameters were used.

Determination of Nitrate—The nitrate yields from decomposition of Angeli’s salt (100 and 200 μM) were quantified by the use of nitrate reductase in conjunction with the Griess assay. The Griess assay was carried out as described elsewhere (46).

Quantum-chemical Calculations—Density functional theory and ab initio calculations were performed with the Gaussian 98W (Revision A.9) suite of programs (47). Geometries were fully optimized to station-
ary points, using the CBS-Q3B methodology in the density functional theory calculations and single-excitation CI calculations and second-
order Moller-Plesset (48) (MP2) calculations with the 6–31+G(d) basis set on the ab initio level. Calculation of UV-VIS absorption spectra was performed by single point energy calculations on the CBS-Q3B-optimized structure using the protocol of the time-dependent density func-
tional theory method (49). Aqueous solvent interactions were evaluated with the PCM (50) procedure incorporated in Gaussian 98W. To verify whether an electron transfer between NO− and O2 would be thermo-
dynamically feasible, geometry optimizations and frequency calcula-
tions were done using the MP2 approximation. Molecular interactions were then evaluated with the IPCM (51) procedure.

RESULTS

Formation of Peroxynitrite from Nitroxyl Anion—To prove whether ONOO− is formed during the decay of NO− in the presence of oxygen, we attempted to identify peroxynitrite by UV-visible spectroscopy. As the half-life of ONOO− is short at physiological pH values and since UV light induces the decom-
position of Angeli’s salt to peroxynitrite (32), 8 samples of 5 mM...
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TABLE I
Quantum chemically calculated UV-visible absorption of ONOO⁻ isomers

Geometries of cis-ONOO⁻, trans-ONOO⁻, and 2,4-cyclo-ONOO⁻ were fully optimized to stationary points using the CBS-QB3 methodology. Calculation of UV-visible absorption spectrum was performed by single point energy calculations on the CBS-QB3 optimized structure using the protocol of the time-dependent TDDFT (49) method. Aqueous solvent interactions were evaluated with the PCM solvation model on the CBS-B3 basis set (TD PCM B3LYP/CBS-B3/CBS-QB3).

| Compound          | Experiment UV-visible absorption | Calculation Absorption coefficient, ε | Oscillator strength, f |
|-------------------|----------------------------------|--------------------------------------|-----------------------|
|                   | UV-visible absorption             |                                      |                       |
|                   | nm                               |                                      |                       |
| cis-ONOO⁻         | 302                              | 303                                  | 1670 ± 50             | 0.0928                |
| trans-ONOO⁻       |                                   | 451                                  | 0.0005                |
|                   |                                   | 374                                  | 0.0005                |
|                   |                                   | 320                                  | 0.0005                |
| 2,4-cyclo-ONOO⁻   | 352                              | 310                                  | 0.0003                |
|                   |                                   | 259                                  | 0.0026                |

*Ref. 54.

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Capabilities of Angeli’s salt to oxidize both NADH and dihydrodedomine 123 and to generate nitrate. Various concentrations of either SIN-1 or Angeli’s salt (each 0–25 μM) in potassium phosphate buffer (50 mM, pH 7.5, HCO₃⁻/CO₂ (25 mM/5%)), 37 °C) were incubated for 1 h to oxidize either DHR or NADH (each 50 μM), respectively. A, oxidation of DHR. B, oxidation of NADH. Residual NADH was quantified by reading its fluorescence with excitation at 339 nm and emission at 460 nm. These values were corrected for autoxidation of NADH. In the absence of SIN-1/Angeli’s salt, about 97% of the initial NADH concentration (50 μM) could still be detected after 1 h of incubation. C, Angeli’s salt (100 μM) was incubated for 1 h in potassium phosphate buffer (50 mM, 37 °C) at various pH values (6–8) in the absence and presence HCO₃⁻/CO₂ (25 mM/5%). Formation of nitrate was quantified by using nitrreductase in conjunction with the Griess assay. Each value represents the mean ± S.D. of three experiments performed in duplicate.

Angeli’s salt from the same stock solution were incubated in parallel runs in the dark at 37 °C at pH 12.25 (Fig. 1A). The decay of Angeli’s salt is slow at these experimental conditions (t₁/₂ = 12 h), thus, reaction times of several hours were necessary to monitor the significant changes of the optical density. The initial absorbance at the beginning of the experiment was 0.35 ± 0.05 at 302 nm. With increasing reaction time, the optical density at 302 nm increased continuously to reach a maximum value of 0.88 ± 0.08 after 6 h, followed by a further decrease of the absorption at longer reaction periods. After 24 h the extinction value had dropped to 0.4 ± 0.05, clearly showing that a relatively long-lived intermediate has been formed during the decay of Angeli’s salt. The absorption at 302 nm had decayed completely when the reaction solution was briefly (20–30 s) bubbled with CO₂ after 5 h of incubation (data not shown). These observations strongly indicated the intermediary formation of ONOO⁻. In fact, when the initial UV spectrum was subtracted from the one observed after 4 h of incubation, the resulting difference spectrum exhibited an absorption spectrum with a maximum at 302 nm (Fig. 1, A and B), similar to what has been reported for peroxynitrite (52). This was verified by comparison with the UV spectrum of authentic ONOO⁻ (Fig. 1C). The scatter of the difference spectrum at shorter wavelengths (λ < 295 nm) derives from the strong absorption of Angeli’s salt in this wavelength region. The absorbance at 302 nm has been attributed to the cis-conformer of peroxynitrite (53, 54). This is excellently supported by time-dependent density functional theory calculations (Table I), which show that the trans-conformer of ONOO⁻ should absorb at longer wavelengths (λₘₚ = 374 nm). Thus, cis-ONOO⁻ is produced from the NO²⁻ donating compound Angeli’s salt during its decay in aerobic solution.

**TABLE II**
Yields of Angeli’s salt-derived O₂ consumption

Angeli’s salt (0–200 μM) was added under normoxic conditions to 50 mM potassium phosphate buffer (37 °C, pH 7.5). O₂ was measured polarographically with a Clark-type electrode. Data are mean ± S.D. of 12 independent experiments.

| Conditions    | O₂ (μM) | ΔO₂ (μM) | Yield (%) |
|---------------|---------|----------|-----------|
| No additives  | 225.0 ± 4 |         |           |
| Angeli’s salt (50 μM) | 190.4 ± 8 | −34.6 | 69.2       |
| Angeli’s salt (100 μM) | 154.8 ± 5  | −70.2  | 70.2       |
| Angeli’s salt (150 μM) | 127.9 ± 8   | −87.1  | 64.7       |
| Angeli’s salt (200 μM) | 96.7 ± 12.5 | −128.3 | 64.2       |

* Ref. 54.

* Yield (%) = ΔO₂ × 100/[Angeli’s salt].

The above observations qualitatively prove the formation of peroxynitrite from NO⁻; the yield of this reaction remained to be established. To this end, the potential of Angeli’s salt to oxidize both DHR and NADH were compared with those of the NO/O₂⁻ releasing compound SIN-1. Increasing concentrations (0–25 μM) of SIN-1, as well as of Angeli’s salt stimulated the oxidation of both DHR and NADH (each 50 μM) in a linear fashion (Fig. 2, A and B). While SIN-1 oxidized DHR and NADH with yields of 80 and 85%, respectively, Angeli’s salt was found to be significantly less effective, oxidizing DHR and NADH with yields of 60 and 65%, respectively. Thus, the production of peroxynitrite from Angeli’s salt is only 65 ± 5% of that from SIN-1. This fact implied that in the absence of suitable targets (i.e. HEPES, DHR, and NADH) the formation of nitrate from Angeli’s salt should be in the same range. In fact, about 65 μM nitrate was formed during the decay of 100 μM Angeli’s salt at pH 7.5 irrespective of the presence of
Interestingly, Angeli’s salt-derived formation of nitrate was maximal at physiological pH values. Control experiments revealed that nitrate was not a contaminant of the applied Angeli’s salt because nitrate could not be detected after decomposition of Angeli’s salt (100 μM/Cu²⁺/H₂O₂ at pH 12.25 in the presence of 20 μM Cu²⁺). Consequently, Angeli’s salt had consumed oxygen with an efficiency of about 65%. To verify this, the oxygen uptake induced by Angeli’s salt (50–200 μM) was determined polarographically (Table II). As expected, the amount of O₂ consumed by NO⁻ was found to be around 65% of the employed amount of Angeli’s salt. Thus, the nitroxyl anion yields peroxynitrite with a yield of about 65%. Our oxygen uptake experiments are in disagreement with data of Miranda et al. (37), who observed a 1:1 stoichiometry between NO⁻ (or rather Angeli’s salt) and O₂. However, it should be remembered that the experiments of Miranda et al. (37) were performed in the presence of HEPES, which is known to effectively react with peroxynitrite, thereby further increasing the uptake of O₂ (40).

### Peroxynitrite-independent Formation of Hydroxyl Radicals from the Nitroxyl Anion

Since only ~65% of the generated NO⁻ was converted into peroxynitrite, the question arose whether other (reactive) intermediates were formed from the remaining 35% of NO⁻. Recently, two research groups (55, 56) reported that NO⁻ released from Angeli’s salt should generate hydroxyl radicals. As peroxynitrite is known to produce HO⁻ radicals (57, 58) with an efficiency of about 28% (9, 59), it is unclear whether the HO⁻ radicals detected by these groups may have derived exclusively from peroxynitrite. To check on this important point, the Angeli’s salt (0–500 μM)-induced hydroxylation of benzoic acid (5 mM) was studied and compared with that of authentic peroxynitrite (0–500 μM) (Fig. 3, A and B). In the absence of CO₂ peroxynitrite-dependent hydroxylation of BA increased in a linear fashion with increasing peroxynitrite concentration (Fig. 3A). However, when CO₂ was added to the reaction mixture, the hydroxylation of BA was inhibited in a concentration-dependent manner (Fig. 3B). These results suggest that Angeli’s salt can generate hydroxyl radicals independently of peroxynitrite formation.
Angeli's salt hydroxylates BA in an almost identical, nonlinear manner with increasing concentrations of peroxynitrite. In the presence of CO2, however, peroxynitrite-mediated hydroxylation of BA was inhibited by about 99%. The effect of CO2 to strongly suppress peroxynitrite-derived formation of hydroxyl radicals is in full agreement with recent reports (57, 60). Moreover, this effect of CO2 offers the possibility to distinguish between HO· released from peroxynitrite and HO· radicals released from other sources. Similar to peroxynitrite, Angeli's salt-mediated hydroxylation of BA increased with increasing concentration of Angeli's salt in the absence of CO2 although not in a strictly linear fashion (Fig. 3B). The efficacy of Angeli's salt to hydroxylate BA decreased with increasing concentration compared with authentic peroxynitrite. This result again is in disagreement with observations by Miranda et al. (37) who found that Angeli's salt was much more effective in hydroxylating BA than authentic peroxynitrite. Again, the usage of HEPES as buffer compound and the fact that no attention was given to exclude traces of CO2 may explain this discrepancy. Most interestingly, however, the presence of CO2 did not completely inhibit the hydroxylation of BA by Angeli's salt. Depending on the initial concentration of Angeli's salt, about 14–29% of the amount of the hydroxylated product that was found in the absence of CO2 was still formed in its presence. In comparison to peroxynitrite generated in situ from SIN-1 (Fig. 3C), Angeli's salt was only slightly more effective in hydroxylating BA. The observation that in the absence of CO2 authentic peroxynitrite hydroxylates BA in a linear manner, whereas peroxynitrite generated in situ from both SIN-1 and

![Graph](image_url)

**Fig. 4. Angeli's salt-mediated nitrosation of 2,3-diaminonaphthalene.** Angeli's salt (500 μM) and DAN (200 μM) were incubated for 30 min in potassium phosphate buffer (50 mM, pH 7.5, HCO3/CO2 (25 mM/5%), 37 °C). A, representative emission spectrum of the product from the reaction between Angeli's salt and DAN. B, emission spectrum of authentic 2,3-naphthotriazole.

**TABLE III**

| Angeli's salt | Absence of CO2 | Presence of CO2 |
|--------------|----------------|-----------------|
| μM | μM | % | μM | μM | % |
| 0 | 3.4 ± 0.3 | 6.8 | 5.0 ± 0.4 | 10.0 |
| 50 | 6.9 ± 0.7 | 6.9 | 10.7 ± 2.0 | 10.7 |
| 100 | 15.1 ± 2.2 | 7.6 | 22.7 ± 1.1 | 11.4 |
| 200 | 29.5 ± 1.6 | 7.4 | 41.3 ± 1.4 | 10.3 |
| 500 | 39.2 ± 0.8 | 6.5 | 58.4 ± 2.2 | 9.7 |

*Yield (%) = NAT × 100/[Angeli's salt].

Angeli's salt hydroxylates BA in an almost identical, nonlinear manner with the intermediary of the same reactive species formed from these peroxynitrite generating systems. For instance, as NO2O3 is known to effectively react with ONOO− (61), one might speculate that such an intermediate decreases the hydroxylation capabilities of in situ generated peroxynitrite. To further verify that in fact HO· radicals were produced independently from peroxynitrite, competition experiments with Angeli's salt (500 μM), BA (5 mM), and the hydroxyl radical scavenger Me6SO (0–10 mM) were performed in the presence of CO2 (Fig. 3D). These experiments demonstrated that low concentrations of Me6SO effectively inhibited in an apparently exponential manner the NO•−-derived hydroxylation of BA. Thus, NO•− very likely generates HO· radicals via a peroxynitrite-independent pathway. However, as the Angeli's salt-induced hydroxylation of BA was only to ~71% inhibited by Me6SO, a hydroxylating species other than the HO· radical may be additionally generated with low yields by the nitroxyl anion. Since the yield of HO· radicals from peroxynitrite is ~28% (9, 59), and because NO•− generates peroxynitrite with a yield of ~65%, and because the hydroxyl radicals from this in situ generated peroxynitrite hydroxylated BA in a range from ~66 to 71%, and because the peroxynitrite-independent hydroxylation of BA is only to 71% induced by HO· radicals, it can be estimated that the NO•−-derived yield of HO· radicals generated via the peroxynitrite-independent pathway is about 3 ± 1.5%. To clarify whether this pathway is additionally oxygen dependent, Angeli's salt was decomposed at various pH values in the absence (hypoxia) or presence of oxygen (normoxia) and CO2 (Fig. 3E). In the absence of O2 the yield of HO· radicals from Angeli's salt increased with increasing H+ concentration, in agreement with findings of Stoyanovsky et al. (55). Furthermore, at atmospheric O2 levels and in the presence of CO2, Angeli's salt was in regard to the O2-free solution only about half as effective to hydroxylate BA. Thus, under conditions where the nitroxyl anion cannot react with oxygen, the NO•−-derived yield of HO· radicals increased to ~8% at physiological pH values.

**Angeli's Salt-derived Nitrosation of 2,3-Diaminonaphthalene**—Since there is a peroxynitrite-independent production of HO· radicals, one may question that in the presence of oxygen the chemistry of NO•− is generally dominated by peroxynitrite. A NO•−-mediated nitration of tyrosine would, for instance, indicate that peroxynitrite is the attacking species, but unfortunately, in situ generated peroxynitrite does not effectively nitrate tyrosine (62, 63). On the other hand, as in situ generated peroxynitrite is able to induce nitrosation reactions (16, 17, 21), the nitroxyl anion released from Angeli's salt is expected to provoke nitrosation reactions when its chemistry is indeed governed by peroxynitrite. To verify this assumption, the reaction between Angeli's salt (500 μM) and DAN (200 μM) was
studied in the presence of CO₂. The product of this reaction was identified as 2,3-naphthotriazole (NAT) because its emission spectrum (Fig. 4A) was found to be identical with that of authentic NAT (Fig. 4B) (44). Thus, peroxynitrite in situ generated from the nitroxyl anion is able to induce nitrosation reactions. To rule out the possibility that only minor amounts of NAT were formed from reaction of Angeli’s salt with DAN, the yield of this reaction was also determined (Table III). Angeli’s salt concentrations in the range from 50 to 600 μM produced NAT with yields of about 7 and 10% in the absence and presence of CO₂, respectively. Since such yields are very typical for peroxynitrite-induced reactions, one must conclude that the chemistry of the nitroxyl anion is mainly characterized by peroxynitrite and with a minor contribution by independently generated HO• radicals.

**Putative Mechanism by Which Nitroxyl Anion Generates Peroxynitrite**—The question arises how the formation of cis-ONOO− from NO− and O₂ may proceed. It has often been suggested that these two molecules, both having a triplet ground state (64), react with each other, thereby directly generating ONOO− (24, 30, 32, 36, 56, 65).

\[ ^3\text{NO}^- + ^3\text{O}_2 \rightarrow \text{cis-ONOO}^- \]

**REACTION 2**

In fact, the reaction between \(^3\text{NO}^-\) and \(^3\text{O}_2\) appears to be thermodynamically feasible, because MP2 ab initio calculations as well as CBS-QB3 calculations in conjunction with the Isodensity Polarized Continuum Model (IPCM) for solvation predicted both an exergonic reaction in the gas phase as well as in aqueous solution (Table IV). These MP2/IPCM calculations also support an outer sphere electron transfer between \(^3\text{NO}^-\) and \(^3\text{O}_2\), i.e. causing solvent-separated \(^3\text{NO}^- + ^3\text{O}_2\) to be a thermodynamically feasible process.

\[ ^3\text{NO}^- + ^3\text{O}_2 \rightarrow ^3\text{NO}^- + ^3\text{O}_2 \]

**REACTION 3**

The subsequent formation of cis-ONOO− is, of course, exothermic (Table IV).

\[ ^3\text{NO}^- + ^3\text{O}_2 \rightarrow \text{cis-ONOO}^- \]

**REACTION 4**

To the best of our knowledge, Reaction 3 was first proposed in 1927 by Andrussov (66). In 1966 Fehsenfeld et al. (67) verified that Reaction 3 indeed proceeds in the gas phase at room temperature. Some years ago, the electron transfer was mentioned with little modifications by the Ignarro group (68), but its significance under physiological conditions has often been questioned by other researchers. Although Reaction 3 appears to be thermodynamically feasible, any experimental indications that it really proceeds in aqueous solution are as yet missing. Provided that in Reaction 3 \(^3\text{NO}^-\) is really released, the addition of superoxide dismutase is expected to strongly increase the yield of \(^3\text{NO}\) during the decay of Angeli’s salt.

\[ 2^3\text{NO}^- + 2^3\text{O}_2^- + \text{Cu(II)}, \text{Zn-SOD} + 2^1\text{H}^+ \rightarrow 2^3\text{NO}^- + 2^1\text{H}_2\text{O}_2 + ^3\text{O}_2^- + \text{Cu(II)}, \text{Zn-SOD} \]

**REACTION 5**

As in case of Reaction 2 no free \(^3\text{NO}\) would be released, the presence or absence of \(^3\text{NO}\) may be taken as a mechanistic probe. In fact, there are several reports in the literature that superoxide dismutase stimulates the formation of \(^3\text{NO}\) from \(^3\text{NO}^-\) (e.g. Refs. 24 and 27). Contrary to our hypothesis, this superoxide dismutase-dependent formation of \(^3\text{NO}\) has been exclusively interpreted in terms of \(^3\text{NO}\) being a substrate for Cu(II),Zn-SOD, i.e. being oxidized by Cu(II),Zn-SOD. However, if superoxide dismutase scavenges \(^3\text{O}_2^-\) rather than \(^3\text{NO}^-\), \(^1\text{H}_2\text{O}_2\) must be formed as a product (see Reaction 5). To clarify this, the superoxide dismutase-dependent formation of \(^1\text{H}_2\text{O}_2\) from Angeli’s salt was studied in the absence and presence of CO₂ (Fig. 5). In the absence of Cu(II),Zn-SOD only about 0.6 μM \(^1\text{H}_2\text{O}_2\) was found regardless of the presence of CO₂. The addition of 100 units/ml superoxide dismutase, however, clearly stimulated the formation of \(^1\text{H}_2\text{O}_2\). In the absence of CO₂, 39.8 ± 2.4 μM \(^1\text{H}_2\text{O}_2\) was found, and in its presence a somewhat lower amount of 30.6 ± 1.5 μM was detected. Increasing the superoxide dismutase activity to 500 units/ml further increased the yields of \(^1\text{H}_2\text{O}_2\) to 46.3 ± 1.8 μM and 36.4 ± 2.3 μM in the

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**TABLE IV**

Quantum chemically calculated reaction energies for reaction of the nitroxyl anion with oxygen

| Reaction | \(\Delta G(H_4)\) | \(\Delta G(G_4)\) | \(\Delta G(\text{aq})^a\) |
|----------|-----------------|-----------------|-----------------|
| \(^3\text{NO}^- + ^3\text{O}_2^-\) | -34.1 | -23.5 | -16.3 |
| \(^3\text{NO}^- + ^3\text{O}_2^-\) | -4.2 | -3.8 | -6.8 |
| \(^3\text{NO}^- + ^3\text{O}_2^-\) | -29.9 | -19.7 | -9.5 |

^a Aqueous solvent correction for the gas phase values.

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2 H.-G. Korth, M. Kirsch, H. de Groot, and R. Sustmann, unpublished results.
absence and presence of CO₂, respectively. The Cu(II),Zn-SOD-induced formation of H₂O₂ during the decay of Angeli’s salt was verified by two independent methods, namely peroxidase assay and catalase assay. Either replacing Cu(II),Zn-SOD by an equivalent amount of albumin (Fig. 5) or treating Cu(II),Zn-SOD with authentic peroxynitrite (500 M, data not shown) strongly decreased H₂O₂ formation to ≤4 M. These results strongly indicate that Reaction 3 is indeed operating.

Effect of Superoxide Dismutase on Nitroxyl Anion-mediated Oxidation Reactions — The above described action of Cu(II),Zn-SOD implied that nitroxyl anion-related formation of peroxynitrite is inhibited by superoxide dismutase. To further support this assumption, the inhibitory effects of Cu(II),Zn-SOD on both SIN-1- and Angeli’s salt-mediated oxidation of both DHR and NADH were studied in the absence and presence of CO₂.

As expected, Cu(II),Zn-SOD inhibited these oxidations with increasing activity of superoxide dismutase (Fig. 6, A–D). Cu(II),Zn-SOD was generally somewhat more effective in inhibiting SIN-1-dependent oxidation reactions than those mediated by Angeli’s salt. This might reflect either the likely differences in the kinetics of O₂⁻ formation or the property of the nitroxyl anion to additionally oxidize these target molecules by releasing HO· radicals via superoxide-independent pathways.

Effect of Nitric Oxide on Nitroxyl Anion-mediated Oxidation Reactions — An anonymous referee mentioned that “NO” and nitric oxide would be present simultaneously” under various conditions, therefore, the nitroxyl anion chemistry presented so far might be of minor importance for biological systems because the following reaction sequence is known to rapidly proceed (69, 70).

\[
\text{NO}^+ + \text{NO} = \text{N}_2\text{O}_3^-
\]

**REACTION 6**

\[
\text{N}_2\text{O}_3^- + \text{NO} \rightarrow \text{N}_2\text{O}_5^-
\]

**REACTION 7**

Provided that such a reaction sequence is indeed effectively operating, nitroxyl anion-derived formation of peroxynitrite, and thus of nitrate (see Fig. 2C), should be effectively inhibited by nitric oxide. To check on this, the inhibitory effects of DEA-NONOate (0–150 M) on Angeli’s salt (200 M)-mediated formation of nitrate were studied. Since both compounds have nearly the same half-life at the selected experimental conditions, nitric oxide and nitroxyl anion should be generated with approximately the same rate, providing the optimum conditions for the suggested reaction sequence. In line with the reviewer’s proposal, nitric oxide inhibited in a linear manner nitroxyl anion-derived formation of nitrate (Fig. 7A). Keeping in mind that one molecule of DEA-NONOate releases about two equivalents of NO it can be estimated that the nitroxyl anion-induced formation of nitrate is half-maximally inhibited at a relatively high [NO/\(\text{NO}^-\)] ratio of 3.5. On the other hand, as it is well known that the chemical power of NO/O₂ releasing systems is strongly diminished when the NO flux is twice as much as the O₂⁻ flux (63, 71, 72), one might argue that this should be especially true for NO/NO⁻ releasing systems. This, however, is not the case. As shown in Fig. 7B, low amounts of DEA-NONOate (5–50 M) strongly increase the capabilities of Angeli’s salt (250 M) to hydroxylate benzoic acid (5 mM) in the presence of CO₂. Even at high concentrations of DEA-NONOate, i.e. 250 M, Angeli’s salt-mediated hydroxylation of BA is only partially inhibited. In contrast, the ‘NO/O₂'-generating system SIN-1 did not effectively hydroxylate BA in the presence of CO₂ especially when nitric oxide was additionally released by spermine-NONOate. Similar to the hydroxylation of benzoic acid, Angeli’s salt (75 M) and low amounts of DEA-NONOate (5–50 M) synergistically acted on the oxidation of 150 M NADH (Fig. 7C). Thus, the chemical power of the nitroxyl anion is strongly increased by nitric oxide when the
Nitroxyl Anion-derived Radicals

DISCUSSION

Contrary to the observations presented above, formation of peroxynitrite during decay of Angeli’s salt in the presence of oxygen, i.e. from reaction of NO$^\cdot$ and O$_2$, has not been reported in previous studies (37, 38). In the present study we avoided the use of tertiary amines as both buffer compounds and heavy metal chelators because it has been shown that oxidizing species stimulate the artificial generation of O$_2^\cdot$ in the presence of these amines (8, 40). The so formed superoxide reacts in a diffusion-controlled manner with the NO$_2^\cdot$ radicals produced from peroxynitrite, thus yielding peroxynitrate (O$_2$NOO$^-$) (8, 14, 17). Peroxynitrate is at physiological pH values of low reactivity in terms of nitration/oxygenation of prototypical biomolecules (14, 17, 73). As a consequence, NO$_2^\cdot$-mediated nitration and nitrosation reactions are largely suppressed when O$_2^\cdot$ is simultaneously formed. Presumably because of these chain reactions, the nitroxyl anion-derived formation of peroxynitrite was not observed in the above mentioned studies, where 10 mM HEPES or 500 mM triethanolamine (37, 38), respectively, were employed.

Due to spin conservation, the nitroxyl anion released from Angeli’s salt is initially produced in the singlet state (32). Reaction of $^1$NO$^\cdot$ with $^3$O$_2$ is a spin-forbidden process, thus, $^1$NO$^\cdot$ should not react fast with $^3$O$_2$. Furthermore, the reaction of $^1$NO$^\cdot$ with $^3$O$_2$ would lead to triplet peroxynitrite, $^3$ONO$^-$, which, according to ab initio calculations is not a stable molecule (energy minimum).$^3$ On the other hand, $^1$NO$^\cdot$ is isoelectronic to singlet oxygen. As singlet oxygen decays within a few ms to $^3$O$_2$ (74), a similar behavior can be expected for $^1$NO$^\cdot$ (Fig. 8). We favor an electron transfer from $^3$NO$^-$ to $^3$O$_2$ with peroxynitrite being formed from the subsequent reaction of NO with $^3$O$_2$. The present data do not allow one to say whether it takes place via an outer sphere or an inner sphere electron transfer mechanism. One may doubt that superoxide dismutase may effectively scavenge the NO$^\cdot$-derived O$_2^\cdot$ radicals, because Litechev and Fridovich (65) estimated the rate constant for the oxidation of $^3$NO$^-$ by Cu(II),Zn-SOD to $^4$NOO$^-$ at $4 \times 10^9$ M$^{-1}$ s$^{-1}$, i.e. this rate constant is about 100-fold higher than the rate constant of the reaction between NO$^\cdot$ and $^3$O$_2$ (75). On the other hand, Murphy and Sies (24) demonstrated that Cu(I),Zn-SOD can re-reduce NO to NO$^\cdot$, and therefore, the reaction between NO$^\cdot$ and $^3$O$_2$ may even proceed in the presence of Cu(II),Zn-SOD. The work of Murphy and Sies (24) is often referred to for demonstrating the direct reaction between Cu(II),Zn-SOD and NO$^\cdot$. These authors observed that under aerobic conditions Cu(II),Zn-SOD stimulates the formation of NO from NO$^\cdot$ and hypothesized “that SOD accepts an electron from NO$^\cdot$, converting it to NO,” although a reaction between Cu(II),Zn-SOD and NO$^\cdot$ seems to be chemically feasible, there is yet no convincing proof that such a reaction really takes place. According to our data, the superoxide dismutase-dependent release of NO from NO$^\cdot$ can satisfactorily be explained by the scavenging of O$_2^\cdot$ by Cu(II),Zn-SOD. Schmidt et al. (27) reported that the Cu(II),Zn-SOD to NO$^\cdot$ ratio must be about 50 under aerobic conditions for achieving a quantitative conversion of NO$^\cdot$ to NO. However, such a high ratio is in fact required when superoxide dismutase would scavenge all O$_2^\cdot$ formed to prevent the diffusion-controlled reaction between NO$^\cdot$ and O$_2^\cdot$.

Our data support the conclusion of Stoyanovsky et al. (55), that NO$^\cdot$ forms hydroxyl radicals via a pathway independent of peroxynitrite. The mechanism offered by these authors appears plausible (Fig. 8). A recent theoretical study indicates that the $pK_a$ value of HNO is at about 7 (64) rather than at 4.7, as generally assumed (76). Provided that the theoretical $pK_a$
value can be verified by experiment, the dimerization of two HNO molecules and the subsequent generation of HO· radicals should even occur at physiological pH values. We estimated that NO· yields HO· radicals under normoxic with an efficiency of only ~3% via the peroxynitrite-independent pathway. Given the fact that the reaction of 3NO· with O2 strictly depends on the availability of O2, and as the O2 concentration in various tissues is significantly lower than under our experimental conditions, the NO·-mediated production of hydroxyl radicals might be expected to be increased under hypoxic conditions (up to 8% of the NO· yield at physiological oxygen concentrations).

Although there can be no doubt that peroxynitrite and radicals derived from it effectively damage biomolecules and a variety of cell types, there is still some uncertainty about the cytotoxic significance of peroxynitrite in vivo, especially when NO and O2· are generated from independent sources. There is evidence that the chemical power of in situ generated peroxynitrite is maximal, when the ratio ([NO]/[O2]) is about one (63, 71, 72). An increase of either the NO flux or of the O2· flux sharply limits the capability of peroxynitrite to attack biomolecules. Therefore, it is most likely that the ([NO]/[O2]) ratio also rules the cytotoxic potential of in situ generated peroxynitrite. Since it is highly unrealistic that the NO fluxes in vivo are always identical to the O2· fluxes, the formation of peroxynitrite from independent sources of NO and O2· cannot fully account for the damaging potential which is so far attributed to peroxynitrite. Otherwise, when both radicals were produced from one source, e.g. SIN-1, the ratio ([NO]/[O2]) is about one and the damaging potential should then rise to a maximum. This is the case when the nitroxyl anion transfers an electron to molecular oxygen (Fig. 8). While increasing NO concentrations continuously decrease the chemical power of a NO/O2· flux (63, 71, 72), a similar behavior cannot simply be expected for NO/NO· releasing systems, because several additional reactions (e.g. Reactions 6–8) may simultaneously operate. For instance, the chemical reactivity of NO· (hydroxylation of BA, oxidation of NADH) was strongly increased at a ratio ([NO]/[NO·]) ≤ 1. This might be related to the reactivity of the protonated form of the intermediate N2O2H· (Reaction 9) or by further release of HO· radicals (Reaction 10). In fact, quantum-chemical calculations at the CBS-QB3 level of theory in conjunction with the IPCM model for solvation (IPCM-B3LYP/CBS-B3/CBS-QB3) predict that the homolysis of N2O2H· is an exergonic reaction (ΔH°(anion) = -28.1 kcal/mol).

\[
\text{N}_2\text{O}_2\text{H} + \text{H}_2\text{O}^+ \rightarrow \text{N}_2\text{O}_2^+ + \text{H}_2\text{O}
\]

**REACTION 9**

\[
\text{N}_2\text{O}_2\text{H} \rightarrow \text{N}_2\text{O} + \text{HO}·
\]

**REACTION 10**

One might then further speculate that at higher [NO] concentrations ([NO]/[NO·] ≥ 1) the intermediate N2O2H· is trapped by a second NO molecule (Reaction 7) so that the chemical power of NO· is then down-regulated by nitric oxide. Due to the above interactions, we postulate that the nitroxyl anion is a major, if not the most important source of peroxynitrite in vivo.

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Formation of Peroxynitrite from Reaction of Nitroxyl Anion with Molecular Oxygen
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