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Generation of peroxynitrite from reaction of *N*-acetyl-*N*-nitrosotryptophan with hydrogen peroxide over a wide range of pH values

Michael Kirsch*[a] and Manfred Lehnig[b]

[a] *Institut für Physiologische Chemie, Universitätsklinikum Essen, Hufelandstrasse 55, D-45122, Essen, Germany. E-mail: michael.kirsch@uni-essen.de; Fax: +49 201 723 5943; Tel: +49 201 723 4108*

[b] *Organische Chemie, Fachbereich Chemie, Universität Dortmund, D-44221, Dortmund, Germany*

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The novel reaction of *N*-acetyl-*N*-nitrosotryptophan (NANT) with hydrogen peroxide to yield peroxynitrite is demonstrated. Quantum chemical calculations performed at CBS-QB3 level of theory predicted that the reaction of *N*-nitrosoindole with both H_2O_2 and its corresponding anion is thermodynamically feasible. At pH 13, the formation of peroxynitrite from the bimolecular reaction of NANT with H_2O_2 is unequivocally demonstrated by ^{15}N NMR spectrometry. In order to prove the intermediacy of peroxynitrite from the NANT–H_2O_2 system at neutral (7.4) and acidic pH (4.5), the characteristic pattern of CIDNP (chemically induced dynamic nuclear polarization) signals were recorded, *i.e.* enhanced absorption in the ^{15}N NMR signal of nitrate and emission in the ^{15}N NMR signal of nitrite. Most interestingly, the NANT–H_2O_2 system nitrated *N*-acetyltyrosine at pH 4 *via* recombination of freely diffusing nitrogen dioxide and tyrosyl radicals, but nitration was negligible at pH 7.4. Since the combination between NANT and H_2O_2 is slow, endogenous *N*-nitrosotryptophan residues cannot act as a “carrier” for peroxynitrite.

Introduction

The transfer of the nitroso-function from (peptide-bound) *S*-nitrosocysteine to protein-bound cysteine is believed to be mainly responsible for the transport and targeting of nitric oxide in signal transduction.[1–3] Such a transnitrosation reaction prevents the undesirable, spontaneous scavenging of nitric oxide by a variety of iron hemoproteins.[4] Recently we[5–7] mentioned that *N*-terminal-blocked *N*-nitrosotryptophan compounds may be additionally involved in the storage, transport and targeting of nitric oxide, although the general chemical capabilities of the *N*-nitrosated indole systems in regard to *S*-nitrosothiols are less well explored.

Peroxynitrite ($ONOO^-/ONOOH$) can be formed at physiological pH from the diffusion-controlled recombination reaction between superoxide ($O_2^{\bullet-}$) and nitric oxide ($^{\bullet}NO$).[8] The decay of peroxynitrite is highly complex, even in purified buffer solutions, but the yields of the products NO_2^-, NO_3^- and O_2 over a broad pH range can be satisfactorily explained with the intermediacy of $^{\bullet}NO_2$ and $HO^{\bullet}$ radicals,[9] which are formed by O–O bond homolysis/cage recombination of peroxynitrous acid to yield about 28% of cage-escaped hydroxyl and nitrogen dioxide radicals.[10,11] The suggested pathological activity of $ONOO^-$ is closely related to its reaction with CO_2,[12] thereby producing the highly reactive radicals $CO_3^{\bullet-}$[13,14] and $^{\bullet}NO_2$[15] with a yield of about 30–35%.[10,16,17] This may be relevant to the fact that nitration of tyrosine is evident in more than 80 diseases.[18]

In 1901, Baeyer and Villiger[19] observed that peroxynitrite is formed from reaction of nitrite with H_2O_2 under acidic conditions. At pH 3–5 H_2O_2 is most likely nitrosated by N_2O_3 formed from nitrous acid. Both the intermediacy of peroxynitrite as well as its nitrating action towards various substrates could be detected by the occurrence of CIDNP effects.[20–22] Noticeably, other nitrosating compounds like *S*-nitrosothiols[23] and *O*-alkylnitrites[24] are only effective in generating peroxynitrite from H_2O_2 at alkaline pH (≥10) values.

In the present study, we demonstrate the *in situ* generation of peroxynitrite from reaction of *N*-acetyl-*N*-nitrosotryptophan (NANT) with H_2O_2 over a wide pH range (4–13). Interestingly, the efficacy of the NANT–H_2O_2 system to nitrate *N*-acetyltyrosine is very low at pH 7.4 but is significantly increased at pH 4.

Results and discussion

The formation of peroxynitrite at alkaline pH

In order to elucidate whether *N*-acetyl-^{15}*N*-nitrosotryptophan nitrosates H_2O_2 to yield peroxynitrite at pH 13, the reaction was analyzed by ^{15}N NMR spectrometry at rt (Figs. 1A and 1B). After about two hours of reaction (Fig. 1A) the characteristic ^{15}N NMR resonance line of peroxynitrite appeared at 174 ppm in the spectrum.[25] The accompanying resonance lines at 164 and 178 ppm, respectively, are due to the *E*- and *Z*-conformation of the starting material *N*-acetyl-^{15}*N*-nitrosotryptophan. Other resonance lines at both 228 ppm and −5 ppm demonstrated the formation of the expected products nitrite and nitrate, respectively. After a reaction period of 22 h (Fig. 1B) the intensities of the resonance lines of *N*-acetyl-^{15}*N*-nitrosotryptophan and peroxynitrite diminished, whereas that of nitrite and nitrate were increased.

Although the foregoing observations unequivocally demonstrate the formation of peroxynitrite from reaction of *N*-acetyl-^{15}*N*-nitrosotryptophan with H_2O_2 at pH ≥ 13, one might ask whether this is also true for more acid pH values, because the *S*-nitrosothiol-mediated nitrosation of H_2O_2 only occurs at alkaline pH values.[23] In order to evaluate whether the reaction between *N*-nitrosotryptophan derivatives and hydrogen peroxide is thermodynamical feasible, quantum chemical calculations were employed at the CBS-QB3 level of theory with *N*-nitrosoindole and *S*-nitrosocysteine as typical “nitroso” compounds (Table 1). These calculations predicted that the transfer of the nitroso function from *N*-nitrosoindole to either hydrogen peroxide or its corresponding anion are exergonic reactions and thus are expected to be thermodynamically feasible. The reliability of these calculations for such a problem is shown by the fact that CBS-QB3 correctly predicted the experimental observation that

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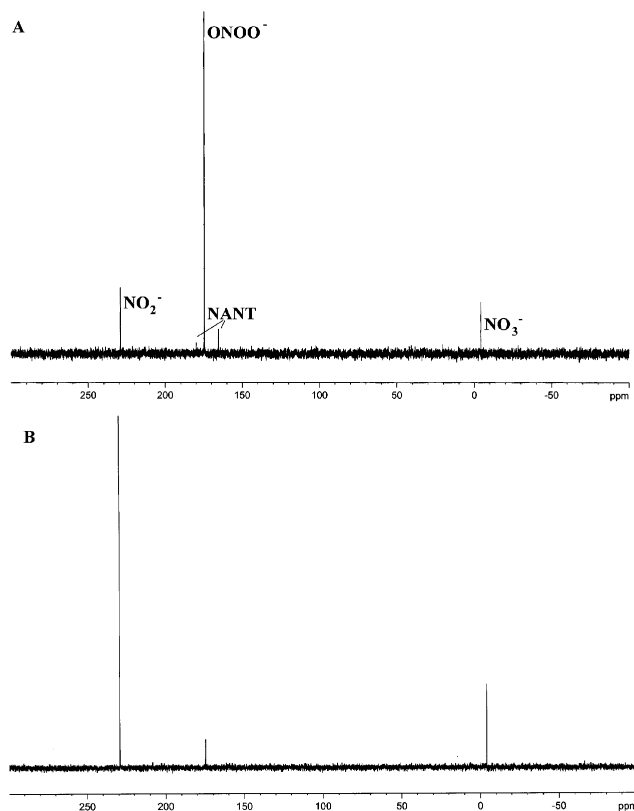
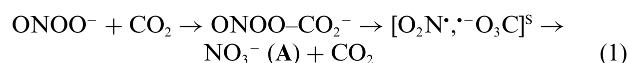


Fig. 1 *N*-Acetyl-^{15}N-nitrosotryptophan (150 mM) was mixed with H_2O_2 (750 mM) at rt in 0.2 M NaOH to reach a final pH of 13. Sample aliquots were supplemented with 10% D_2O and analyzed by ^{15}N NMR spectrometry; (A) ^{15}N NMR spectrum after 2 h of reaction, (B) ^{15}N NMR spectrum after 22 h of reaction. Chemical shifts (δ) are given in ppm relative to neat nitromethane ($\delta = 0$) as an external standard.

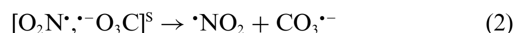
S-nitrosocysteine only reacts with the deprotonated form of hydrogen peroxide to yield peroxynitrite.[23]

The formation of peroxynitrite at neutral and mild acidic pH

Due to the short lifetime of peroxynitrite at pH values <8, its stationary concentrations remain beyond levels which can be detected by conventional ^{15}N NMR spectrometry. Therefore, we used CIDNP as a second analytical tool in order to demonstrated experimentally the intermediacy of peroxynitrite. Recently, Butler *et al.*[25] reported that during the decay of ^{15}N-enriched peroxynitrite at alkaline pH (0.15 M NaOH and pH 7.6) two distinct CIDNP effects in the ^{15}N NMR spectra can be monitored; that is, enhanced absorption in the nitrate signal and emission in the signal of nitrite. It was further deduced that both CIDNP effects are due to the presence of a so-called radical pair formed from a singlet precursor, *i.e.* peroxynitrous acid (ONOOH), although the precise details are not known.[25] Under our experimental conditions the peroxynitrite–CO_2 adduct should act as the singlet precursor for the radicals nitrogen dioxide and trioxocarbonate(1⁻).[15]



$$ONOO^- + CO_2 \rightarrow ONOO\text{–}CO_2^- \rightarrow [O_2N^\bullet,{}^{\bullet -}O_3C]^S \rightarrow NO_3^- \ (\mathbf{A}) + CO_2 \quad (1)$$



$$[O_2N^\bullet,{}^{\bullet -}O_3C]^S \rightarrow {}^\bullet NO_2 + CO_3^{\bullet -} \quad (2)$$

It should be remembered that dimerisation of two nitrogen dioxide radicals and subsequent hydrolysis to nitrate and nitrite cannot lead to CIDNP because two different radicals are needed to build-up the imbalance in the Boltzmann distribution of the nuclear spins.[26] Furthermore, such a reaction would lead to emission in the resonances of nitrate and nitrite because these products would be formed from recombination of freely diffusing nitrogen dioxide radicals. However, provided that peroxynitrite is intermediately formed during reaction of *N*-acetyl-^{15}N-nitrosotryptophan with H_2O_2 at pH 7.4, the enhanced absorption in the signal of nitrate and emission in the signal of nitrite must be recorded. In fact, when the reaction between *N*-acetyl-^{15}N-nitrosotryptophan (Table 2) and H_2O_2 was started at this pH enhanced absorption in the ^{15}N NMR signal of nitrate and emission in the ^{15}N NMR signal of nitrite were observed during the course of the reaction (Fig. 2). As expected, CIDNP effects were not present at the end of the reaction (data not shown). Hence, the intermediacy of peroxynitrite from the *N*-acetyl-^{15}N-nitrosotryptophan–H_2O_2 system is unequivocally demonstrated by this pattern of CIDNP.

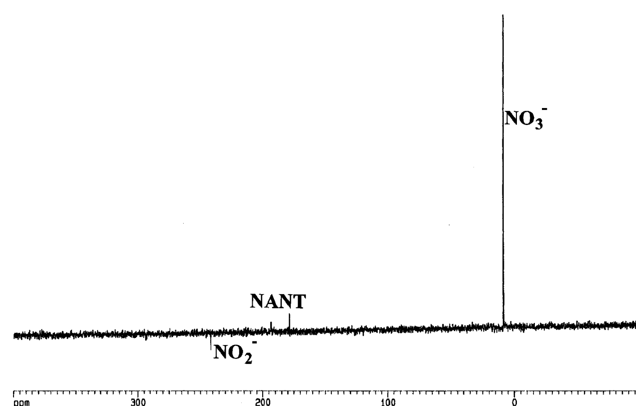


Fig. 2 ^{15}N NMR spectra during reaction of *N*-acetyl-^{15}N-nitroso-tryptophan with H_2O_2 and $NaHCO_3$ in H_2O–D_2O at pH 7.4 taken with single scans (pulse angles 90°) 20 min after adding of H_2O_2 to the solution. Chemical shifts are given in δ values relative to [^{15}N]nitrobenzene dissolved in acetonitrile as an external reference. δ (nitrobenzene) = −9.6 ppm relative to neat nitromethane.

Significantly different spectra were recorded when the reaction was performed at pH 4.5 (Fig 3A–D). Now the emission in the ^{15}N NMR signal in nitrite is no longer observed and the build-up and lifetime of enhanced absorption in the ^{15}N NMR resonance

Table 1 Quantum-chemically calculated Gibbs reaction energies and aqueous solvation energies

Reaction[a]	$\Delta_R G_g{}^b$/kcal mol⁻¹	$\Delta_R G_{solv}{}^c$/kcal mol⁻¹	$\Delta_R G_{aq}{}^d$/kcal mol⁻¹
Z-NOindole + H_2O_2 → indole + ONOOH	−0.8	−0.1	−0.9
E-NOindole + H_2O_2 → indole + ONOOH	−0.8	−0.2	−1.0
Z-NOindole + HO_2^- → indole + $ONOO^-$	−30.9	11.9	−19.0
E-NOindole + HO_2^- → indole + $ONOO^-$	−30.9	11.8	−19.1
Cys_SNO + H_2O_2 → Cys_SH + ONOOH	4.0	3.4	7.4
Cys_SNO + HO_2^- → Cys_SH + $ONOO^-$	−26.1	15.4	−10.7
Cys_SNO + HO_2^- → Cys_S^- + ONOOH	−29.8	17.2	−12.6

[a] Thermodynamic properties were calculated using the Complete Basis Set (CBS-QB3) methodology. [b] Gas phase data. [c] Solvation corrections from (U)HF/6–31(+)G(d)//CBS-QB3 single point calculations, with the CPCM-UAHF solvation model for water.[39] [d] $\Delta_R G_{aq} = \Delta_R G_g + \Delta_R G_{solv}$

Table 2 ^{15}N CIDNP during reactions of *N*-acetyl-*N*-nitrosotryptophan (NANT) with H_2O_2 in phosphate buffer (0.3 M) and $NaHCO_3$ (50 mM)

Entrance	Conditions	^{15}N NMR signals[a] (ppm)	CIDNP[b]	Half-life	Yield[c] (%)
1	NANT (160 mM)	9 ($^{15}NO_3^-$)	A	30 min	70
	T = 22 °C, pH = 7.5	178 (NANT)	N		
	Absence of Tyrac	192 (NANT)	N		
	3 M H_2O_2, Fig. 2	242 ($^{15}NO_2^-$)	E		30
2	NANT (160 mM)	9 ($^{15}NO_3^-$)	A	See text	100
	T = 22 °C, pH = 7.5	178 (NANT)	N		
	Absence of Tyrac, 1 M H_2O_2	192 (NANT)	N		
3	NANT (160 mM)	9 ($^{15}NO_3^-$)	A	30 min	90
	T = 22 °C, pH = 7.5	178 (NANT)	N		
	Tyrac (50 mM),	192 (NANT)	N		
	1 M H_2O_2	242 ($^{15}NO_2^-$)	E		10
		5 (3-NO_2–Tyrac)	N		<0.1
4	NANT (160 mM)	178 (NANT)	N		
	T = 22 °C, pH = 4.5	192 (NANT)	N		
	Tyrac (50 mM), absence of H_2O_2	242 ($^{15}NO_2^-$)	N		
7	NANT (160 mM)	9 ($^{15}NO_3^-$)	A	10 min	99
	T = 22 °C, pH = 4.0	178 (NANT)	N		
	Tyrac (50 mM)	192 (NANT)	N		
	1 M H_2O_2, Fig. 5	242 ($^{15}NO_2^-$)	E		
		5 (3-NO_2–Tyrac)	E		1
		22 (1-NO_2–Tyrac)	E		

[a] δ in ppm against ^{15}N nitrobenzene, positive δ values downfield. [b] E, emission; A, enhanced absorption; N, no CIDNP effect. The CIDNP effects are observed during the reaction. [c] Yields were evaluated from ^{15}N NMR spectra taken after reaction.

line of nitrate correlated with the intermediary formation of nitrite. It should be noted that the missing CIDNP effect in nitrite at this pH has also been observed when peroxynitrite was generated *in situ* by the $^{15}NO_2^-$–H_2O_2 system.[15,20] The increase in the enhanced absorption in the ^{15}N NMR signal of nitrate, which reflects the rate of peroxynitrite release, is easily explained by the occurrence of two independent peroxynitrite-generating reactions. At the beginning of the reaction, *i.e.* when the concentration of *N*-acetyl-^{15}*N*-nitrosotryptophan is high (Fig. 3A), peroxynitrite is solely produced by the reaction of NANT with H_2O_2. As the reaction proceeds some nitrite is formed and peroxynitrite is now additionally generated by the Bayer–Villiger reaction, thereby further increasing the enhanced absorption in the ^{15}N NMR signal of nitrate (Fig. 3B–4D). As long as NANT is available, the stationary concentration of nitrite increased, indicating that the NO_2^-–H_2O_2 reaction is somewhat slower than the NANT–H_2O_2 reaction. When all of the NANT is consumed, peroxynitrite is now exclusively formed from nitrite. The enhanced absorption in the nitrate signal decays when all of the nitrite has been oxidized to nitrate (data not shown). Thus, the rate and the yield of peroxynitrite generated *in situ* from the NANT–H_2O_2 system increased at pH 4.5 compared to pH 7.5.

H_2O_2 dependent and H_2O_2 independent decay of *N*-acetyl-*N*-nitrosotryptophan

In order to find additional evidence for these effects, the H_2O_2 (100 mM)-induced decay of *N*-acetyl-*N*-nitrosotryptophan (100 μM) was spectrophotometrically monitored at the pH values 11, 7.4 and 4.5, respectively (Fig. 4A–4D). At pH 11, *N*-acetyl-*N*-nitrosotryptophan was found to be stable in the absence of H_2O_2 (data not shown). Addition of H_2O_2 led to a moderately fast decay of the absorption of NANT, accompanied by a shift of the absorption maximum from 335 nm to about 320 nm (Fig. 4A). This shift can be explained by the formation of peroxynitrite, which is fairly stable at pH 11 and absorbs at λ_{max} = 302 nm (absorption coefficients: ε_{335} = 6100 M^{-1} cm^{-1} for *N*-acetyl-*N*-nitrosotryptophan and ε_{302} = 1670 M^{-1} cm^{-1} for $ONOO^-$). At pH 7.4, *N*-acetyl-*N*-nitrosotryptophan slowly decays in the absence of H_2O_2 (Fig. 4B), but the decay was accelerated in the presence of hydrogen peroxide (Fig. 4C). Recently, we[5] found that the first-order decay of *N*-acetyl-*N*-nitrosotryptophan at pH 7.4 can be described by the Arrhenius parameters $A = 1.7 \pm 0.3 \times 10^5$ s^{-1} and $E_a = 13.2$ kcal mol^{-1}. In agreement with the short lifetime of peroxynitrite at pH 7.4, the UV-vis maximum of *N*-acetyl-*N*-nitrosotryptophan was not shifted to shorter wavelengths during reaction with H_2O_2 (Figs. 4B and 4C). At pH 4.5, the self-decay of *N*-acetyl-*N*-nitrosotryptophan is further accelerated in the absence of H_2O_2 (Fig. 4D) but only slightly faster in its presence (data not shown). The prominent decay in the absence of H_2O_2 is in agreement with earlier reports,[27–29] which found that the denitrosation of *N*-acetyl-*N*-nitrosotryptophan into *N*-acetyltryptophan and nitrite is an acid-catalyzed process in aqueous solution. Thus, the nitrite donating capabilities of *N*-acetyl-*N*-nitrosotryptophan in acidic solution directs the H_2O_2-dependent formation of peroxynitrite toward the Baeyer–Villiger reaction. Due to this change in mechanism, the rate of peroxynitrite generation is higher at pH 4.5 than at pH 7.4. Because of the described effects observed at pH 11 and pH 4.5, respectively, the Arrhenius parameters of the reaction under pseudo first-order conditions (100 μM NANT, 100 mM H_2O_2) were only determined at pH 7.4 and were corrected for the self-decay. In fact, first-order decay kinetics were observed at five temperatures (15 °C–45 °C) and from the Arrhenius plot of the rate data (data not shown) an activation barrier of $E_a = 19.3 \pm 0.2$ kcal mol^{-1} and an *A*-factor of $1.9 \pm 0.3 \times 10^{10}$ s^{-1} was extracted. Thus, at 37 °C *N*-acetyl-*N*-nitrosotryptophan would decay under such conditions with a half-life of *ca.* 26 min. Provided that *N*-nitrosotryptophan derivatives may be formed *in vivo* they would not react with physiological amounts of hydrogen peroxide (about 10 nM)[30] because the hydrolysis reaction,[5] the *S*-nitrosothiol yielding reaction with thiols[6] and the nitric oxide yielding reaction with ascorbate[5] are then much faster. By these means, *N*-nitrosotryptophan derivatives are expected to act *in vivo* as nitric oxide donating compounds but not as “peroxynitrite carrier”.

The nitration of tyrosine

Equipped with our novel method for *in situ* generation of peroxynitrite, we checked whether the NANT–H_2O_2 system would be able to nitrate *N*-acetyltyrosine. The capability of *in situ* generated peroxynitrite to nitrate tyrosine is the subject of controversy. Mayer and coworkers presented evidence that neither the peroxynitrite generating system SIN-1 nor the xanthine oxidase–Spe-NO couple can effectively nitrate tyrosine.[31–33]

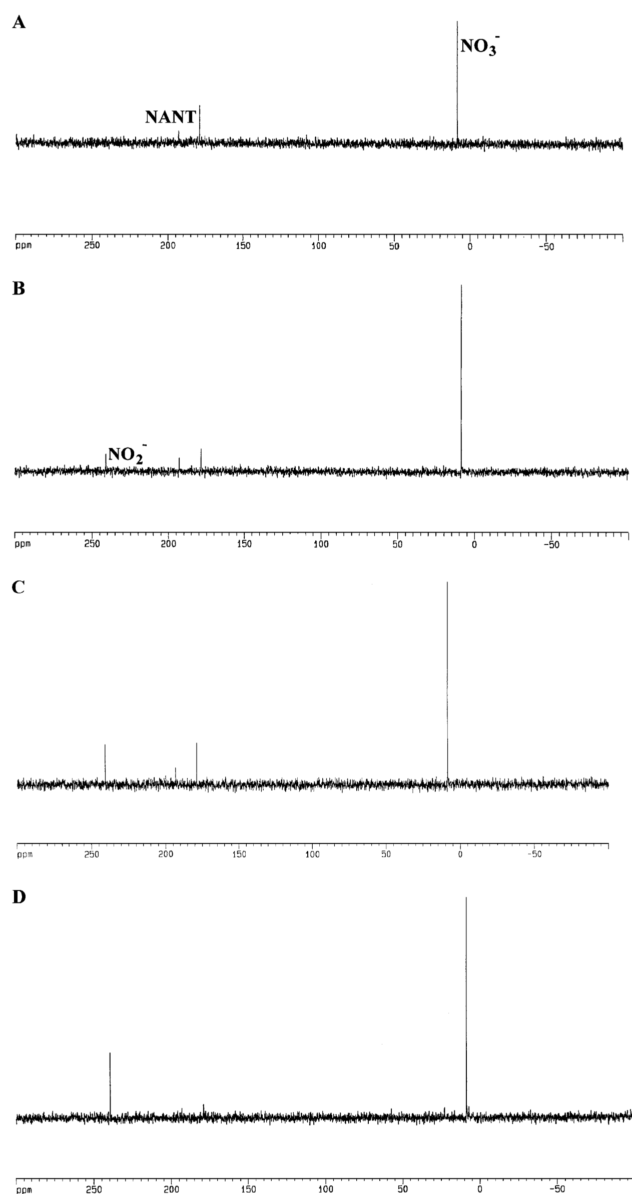
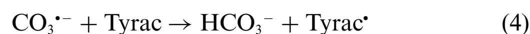


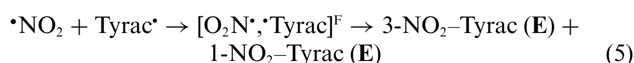
Fig. 3 ^{15}N NMR spectra during reaction of *N*-acetyl-^{15}*N*-nitrosotryptophan with H_2O_2 and $NaHCO_3$ in H_2O–D_2O at pH 4.5 taken with single scans (pulse angles 90°); (A) 2 min, (B) 5 min, (C) 7 min, (D) 16 min after adding of H_2O_2 to the solution. Chemical shifts are given in δ values relative to [^{15}N]nitrobenzene dissolved in acetonitrile as an external reference. δ (nitrobenzene) = −9.6 ppm relative to neat nitromethane.

In addition, the SOTS-1–Spe-NO system also failed to nitrate tyrosine at substantial yields at physiological pH.[34] In line with these findings, CIDNP effects in the ^{15}N NMR resonance lines of *N*-acetyl-^{15}*N*-nitrotyrosine were not observed during reaction of *N*-acetyl-^{15}*N*-nitrosotryptophan–H_2O_2 with *N*-acetyltyrosine at pH 7.4 (Fig. 5A). However, the ^{15}N NMR resonance lines of nitrite and nitrate exhibited now very intense CIDNP effects in line with observations of Butler *et al.*[25] The results from the Mayer group were strongly attacked by others, due to seemingly flawed experimental conditions, namely inactivation of the enzyme xanthine oxidase by peroxynitrite,[35] generation of highly effective scavengers from the xanthine oxidase system[18] and break down of the $O_2^{\bullet-}$ flux in xanthine oxidase–SIN-1 systems due to oxygen depletion.[18] However, as pointed out by Hodges *et al.*,[34] the low yields of nitrotyrosine from *in situ* generated peroxynitrite in regard to preformed peroxynitrite can simply be related to the bimolecular character of the reaction between nitrogen dioxide and tyrosyl radical, *i.e.* at low $^{\bullet}NO_2$ fluxes, virtually all of the $^{\bullet}NO_2$ is trapped by reaction with tyrosine rather than with recombination of tyrosyl radicals. In fact, at pH 4 where the flux of peroxynitrite from the NANT–H_2O_2 system is higher than at pH 7.4 *N*-acetyl-tyrosine was nitrated *via* the usual radical pathway (Fig. 5B). Emission in the ^{15}N NMR resonance lines of both *N*-acetyl-3-^{15}*N*-nitrotyrosine and the *ipso*-intermediate[25] (*i.e.* *N*-acetyl-1-^{15}*N*-nitrotyrosine) demonstrated the intermediacy of a radical pair $[O_2N^{\bullet},{}^{\bullet}Tyrac]^F$ formed by diffusive encounters of nitrogen dioxide with *N*-acetyl-tyrosyl radicals.

$$^{\bullet}NO_2 + Tyrac \rightarrow H^+ + NO_2^- + Tyrac^{\bullet} \quad (3)$$



$$CO_3^{\bullet-} + Tyrac \rightarrow HCO_3^- + Tyrac^{\bullet} \quad (4)$$



$$^{\bullet}NO_2 + Tyrac^{\bullet} \rightarrow [O_2N^{\bullet},{}^{\bullet}Tyrac]^F \rightarrow 3\text{-}NO_2\text{–}Tyrac\ (\mathbf{E}) + 1\text{-}NO_2\text{–}Tyrac\ (\mathbf{E}) \quad (5)$$

At the end of the reaction *N*-acetyl-3-^{15}*N*-nitrotyrosine was identified by ^{15}N NMR spectrometry (Fig. 5C). Since identical experimental conditions were employed at 7.4 and 4.5, respectively, the absence of nitrotyrosine formation at pH 7.4 cannot be related to artificial experimental conditions of our peroxynitrite generating system. Thus, the simple view that *in situ* generated peroxynitrite directly nitrates tyrosine *in vivo* is in question and further investigations are necessary for a satisfactory explanation of endogenous 3-nitrotyrosine formation.

Conclusions

A novel chemical capability of a *N*-nitrosoindole compound (NANT) was presented, *i.e.* the transnitrosation of H_2O_2. The NANT–H_2O_2 system generated peroxynitrite over a wide pH range (4–13) at a low rate. The NANT–H_2O_2-dependent formation of peroxynitrite was demonstrated by ^{15}N NMR spectrometry at pH 13 as well as by the occurrence of CIDNP in the presence of CO_2 at pH 7.4 and pH 4–4.5, respectively. The reaction between NANT and H_2O_2 is so slow that *N*-nitrosotryptophan derivatives cannot act as "peroxynitrite carriers" *in vivo*. In agreement with earlier reports, peroxynitrite *in situ* generated failed to nitrate a tyrosine derivative at physiological pH. Since peroxynitrite *in situ* generated by the NANT–H_2O_2 system nitrates tyrosine at pH 4, the endogenous 3-nitrotyrosine formation remains to be more complicated than believed hitherto.

Experimental

Chemicals and solution

$Na^{15}NO_2$ labeled with 99.3% ^{15}N was purchased from Aldrich/Isotec Inc. (Taufkirchen, Germany). All other chemicals were from Sigma (Taufkirchen, Germany) and were of the highest purity available.

In the preparation of the solutions, care was taken to exclude possible contamination by carbon dioxide and transition metal ions. The following procedures were all performed under nitrogen and at reduced incident light. Doubly distilled water was bubbled (2 L min^{-1}) with nitrogen (5.0) at rt for 20 min and was then treated with the heavy metal ion scavenger resin Chelex 100 (0.5 g in 10 mL) by gently shaking for 18 h in the dark. After separation from the resin by low-speed centrifugation for 5 min and careful decanting, the water was again bubbled with nitrogen for 20 min. This water was used for the preparation of all other solutions. In order to avoid recontamination by traces of heavy metal ions on dissolution of the solid salts, the final solutions were again treated with Chelex 100 (0.5 g in 10 mL) as above.

N-Acetyl-*N*-nitrosotryptophan was prepared according to a procedure originally described by Bonnett and Holleyhead,[36] which was improved by us. In brief, D,L-*N*-acetyltryptophan (526 mg) and sodium nitrite (162 mg $Na^{14}NO_2$ or 165 mg $Na^{15}NO_2$) were stirred in purified water (20 mL) for 2 h in the

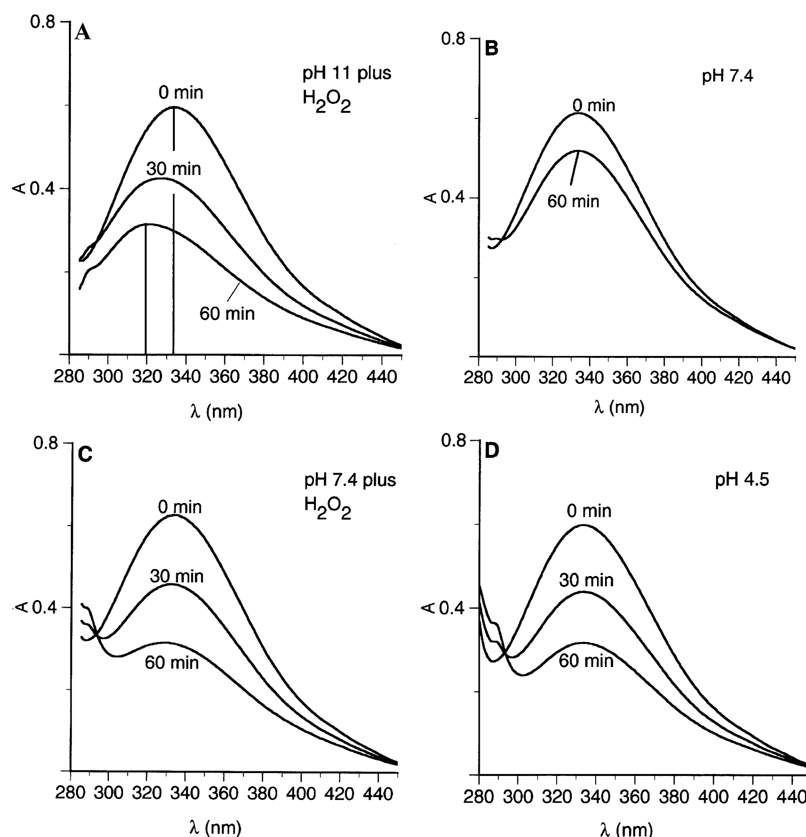


Fig. 4 UV-vis spectra during decay of *N*-acetyl-^{15}*N*-nitrosotryptophan at various pH values in the absence and in the presence of H_2O_2; (A) pH 11 plus H_2O_2, (B) pH 7.4, (C) pH 7.4 plus H_2O_2, (D) pH 4.5.

dark at rt. The yellow reaction mixture was cooled to 1 °C and cold HCl (10 mL, 1 M, 1 °C) was added. A yellow, organic precipitate was formed, which was immediately extracted with ethyl acetate (60 mL, 1 °C). The cold organic layer was separated and taken to evaporation at rt under a reduced pressure (18 Torr) to yield about 500 mg of D,L-*N*-acetyl-*N*-nitrosotryptophan (ε_{335}(ethanol) = 6100 M^{-1} cm^{-1}).[36]

^{15}N NMR identification of peroxynitrite

During reaction of *N*-acetyl-^{15}*N*-nitrosotryptophan (150 mM) with H_2O_2 (750 M) at pH 13, sample aliquots were supplemented with 10% D_2O and analyzed by ^{15}N NMR spectrometry. Peroxynitrite, nitrite, nitrate and *N*-acetyl-^{15}*N*-nitrosotryptophan were identified by 50.67 MHz ^{15}N NMR spectrometry on a Bruker AVANCE DRX 500 instrument. Chemical shifts (δ) are given in ppm relative to neat nitromethane ($\delta = 0$) as an external standard.

Decay kinetic experiments

The decay kinetics of *N*-acetyl-*N*-nitrosotryptophan (100 μM) were determined in air tight quartz cuvettes by rapid-scan monitoring the UV-vis absorption at λ_{max} = 335 nm at 12–30 time steps with an Analytic Jena Specord S100 diode array spectrometer (Jena, Germany). In between the recordings the samples were protected from light. These experiments were performed in the absence and in the presence of H_2O_2 (100 mM) at various pH values (11, 7.4 and 4.5). At pH 7.4 the decay kinetics of *N*-acetyl-*N*-nitrosotryptophan (100 μM) were additionally observed at various temperatures (15–45 °C). Temperature control was maintained at ±0.1 °C.

CIDNP measurements

The ^{15}N CIDNP experiments were performed as reported before.[37] The reaction mixtures were prepared in 10 mm NMR tubes by adding H_2O_2 (1–3 M) to solutions of *N*-acetyl-^{15}*N*-nitrosotryptophan (0.1 M) in H_2O–D_2O (9 : 1) containing phosphate buffer (0.3 M) and $NaHCO_3$ (0.05 M) at various pH values in the absence and in the presence of *N*-acetyl-tyrosine. The pH values given in Fig. 2–5 refer to the final pH at the end of the reaction. The tubes were quickly transferred into the probe head of the ^{15}N NMR spectrometer (Bruker DPX-300) and locked within 1 min after mixing of the reactants (internal lock: D_2O). The ^{15}N NMR spectra were then taken by using single pulses with pulse angles of 90°. Chemical shifts are given in δ values relative to [^{15}N]nitrobenzene dissolved in acetonitrile as an external reference. δ (nitrobenzene) = −9.6 ppm relative to neat nitromethane.

Quantum-chemical calculations

Complete Basis Set (CBS-QB3) computations were carried out with the Gaussian 03 (Revision C.02) suite of programs.[38] Gibbs free energies of solvation for water were estimated for the optimized gas-phase geometries with the CPCM-UAHF procedure incorporated in Gaussian 03. The PCM/(U)HF/6–31 + G(d) and the CBS-QB3 methodology are known to provide thermochemical estimates within "chemical accuracy" (±1 kcal mol^{-1}).[39,40]

Abbreviations

The abbreviations used are: NANT, *N*-acetyl-*N*-nitrosotryptophan; CIDNP, Chemically Induced Dynamic Nuclear Polarization; peroxynitrite, $ONOO^-$/ONOOH; Tyrac, *N*-acetyl-tyrosine; Tyrac$^•$, phenoxyl-type radical of *N*-acetyl-tyrosine; SIN-1, 3-morpholino-sydnonimine; Spe-NO, (*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1,2-diolate.

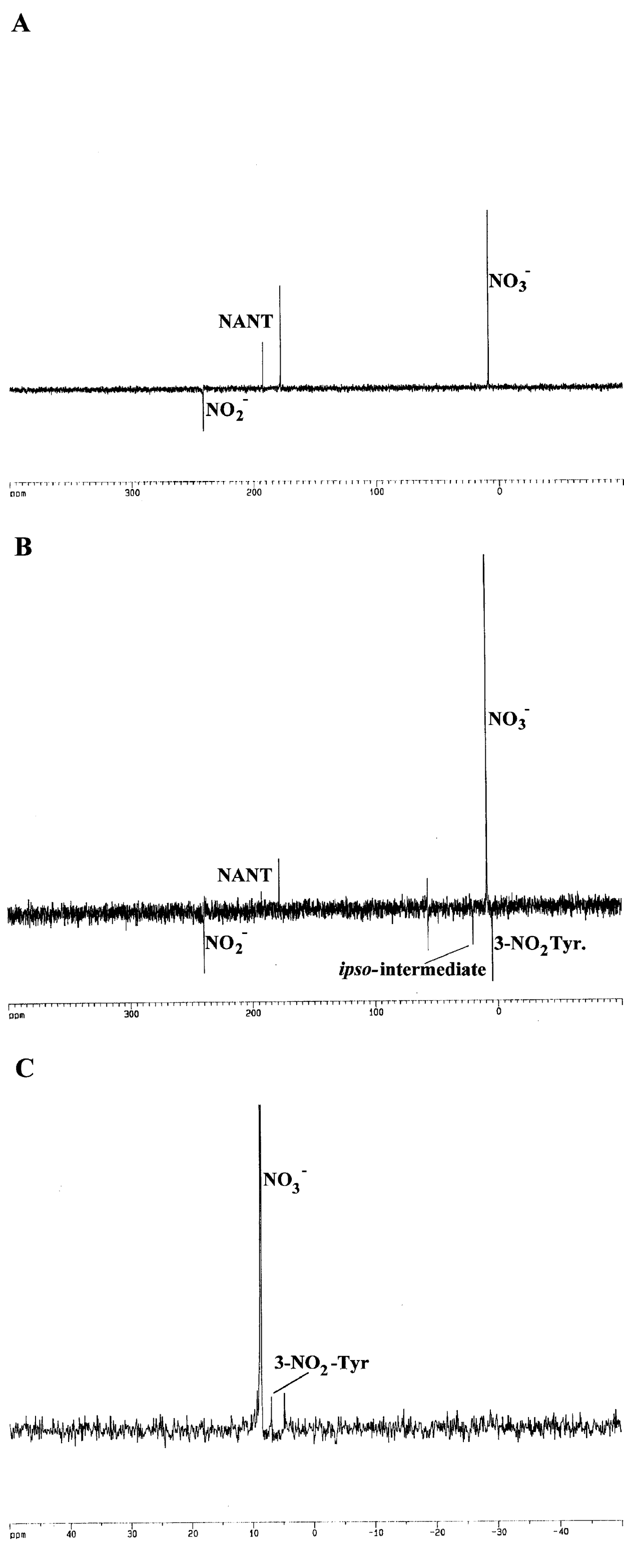


Fig. 5 ^{15}N NMR spectra during reaction of *N*-acetyl-^{15}*N*-nitroso-tryptophan with H_2O_2 and *N*-acetyl-tyrosine–$NaHCO_3$ in H_2O–D_2O at two pH values taken with single scans (pulse angles 90°); (A) 5 min at pH 7.4, (B) 5 min at pH 4, (C) 45 min after adding of H_2O_2 to the solution at pH 4. Chemical shifts are given in δ values relative to [^{15}N]nitrobenzene dissolved in acetonitrile as an external reference. δ (nitrobenzene) = −9.6 ppm relative to neat nitromethane.

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