^{15}N CIDNP during reaction of the oxoperoxonitrate–CO_2 adduct with bovine albumin and L-tyrosine derivatives

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During the reaction of the oxoperoxonitrate–CO_2 adduct with bovine albumin (ALB), the ^{15}N NMR signals of nitrated tyrosyl residues in ALB (3-NO_2-ALB) appear as emission indicating the nitration reaction to be a radical mechanism. The nuclear polarisations are built up by radical pairs $[ALB^{\bullet}\ {}^{\bullet}NO_2]^F$ generated by free radical encounters of nitrogen dioxide $^{\bullet}NO_2$ and phenoxyl type radicals $ALB^{\bullet}$ formed by hydrogen abstraction from the phenolic residues in ALB. Analogous effects are observed during reaction with valyltyrosylvaline (Val-Tyr-Val) and *N*-acetyl-L-tyrosine (Tyrac). During nitration of Val-Tyr-Val and Tyrac with nitric acid, identical ^{15}N CIDNP effects are observed in the nitration products. The reactions are inhibited in the presence of sodium azide or sulfamic acid indicating a nitrous acid catalysed reaction. A comparison of the ^{15}N CIDNP intensities reveals that the radical reaction with the oxoperoxonitrate–CO_2 adduct is the main, if not the only nitration reaction.

Introduction

Oxoperoxonitrate (O=N–OOH/O=N–OO^-, pK = 6.5) is able to nitrate L-tyrosine (Tyr) leading to 3-nitro-L-tyrosine (3-NO_2-Tyr) and tyrosine residues in proteins in the 3-position. It may be formed by recombination of nitric oxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) radicals in biological systems under conditions of oxidative stress [eqns. (1) and (2)].[1] In the presence of CO_2, the

$$NO^{\bullet} + O_2^{\bullet -} \longrightarrow O{=}N{-}OO^- \qquad (1)$$

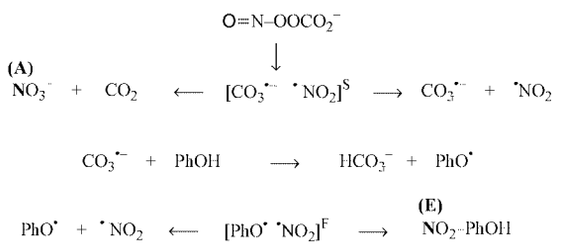
$$O{=}N{-}OOH + Tyr \longrightarrow 3\text{-}NO_2\text{-}Tyr + H_2O \qquad (2)$$

reactivity is improved *via* formation of an oxoperoxonitrate–CO_2 adduct ($O{=}N{-}OOCO_2^-$) as a reactive intermediate [eqns. (3) and (4)].[2] Under physiological conditions ([CO_2] ~ 1.3 mM), nitration mainly occurs *via* eqn. (4).[2,3]

$$O{=}N{-}OO^- + CO_2 \longrightarrow O{=}N{-}OOCO_2^- \qquad (3)$$

$$O{=}N{-}OOCO_2^- + Tyr \longrightarrow 3\text{-}NO_2\text{-}Tyr + CO_2 + OH^- \qquad (4)$$

Tyrosine nitration is a useful marker of oxidation by oxoperoxonitrate in tissues. The oxoperoxonitrate-mediated nitration of tyrosine residues results in changes or even loss of activity in enzymes.[4,5] The mechanism of the nitration reaction *in vivo* is still under discussion.[6] To demonstrate the occurrence of free radicals *in vitro*, EPR and ^{15}N CIDNP (chemically induced dynamic nuclear polarisation) investigations have been performed.[7–9] ^{15}N CIDNP was first applied by Ridd and coworkers for studying nitration reactions of activated arenes.[10] During the reaction of oxoperoxonitrate with L-tyrosine in the probe of a ^{15}N NMR spectrometer, the ^{15}N NMR signal of 3-nitro-L-tyrosine (3-NO_2-Tyr) appears as emission (E).[9] Additionally, the signals due to nitrate (NO_3^-) and nitrite (NO_2^-) show enhanced absorption (A) and emission, respectively.[8,9] In the presence of CO_2, 3-NO_2-Tyr and NO_3^- also show emission and enhanced absorption. NO_2^- does not show CIDNP.[9] The effects observed in the presence of CO_2 are explained according to Scheme 1 (PhOH has to be replaced by Tyr, $NO_2C_6H_4OH$ by 3-NO_2-Tyr and $PhO^{\bullet}$ by tyrosyl radicals Tyr$^{\bullet}$). The oxoperoxonitrate–CO_2 adduct decomposes *via* homolytic O–O bond scission leading to singlet (S) pairs $[CO_3^{\bullet -}\ {}^{\bullet}NO_2]^S$. Free radicals forming the pairs either react to form cage products CO_2 and NO_3^- or escape from the pairs. Freely diffusing radical ions $CO_3^{\bullet -}$ abstract hydrogen from Tyr giving tyrosyl radicals Tyr$^{\bullet}$. Independently generated radicals $CO_3^{\bullet -}$ and $^{\bullet}NO_2$ form free radical (F) pairs $[Tyr^{\bullet}\ {}^{\bullet}NO_2]^F$ by diffusion leading to 3-NO_2-Tyr as a cage product. The ^{15}N CIDNP effect observed for NO_3^- is built up by radical pairs $[CO_3^{\bullet -}\ {}^{\bullet}NO_2]^S$, the polarisation of the ^{15}N nuclei in 3-NO_2-Tyr by radical pairs $[Tyr^{\bullet}\ {}^{\bullet}NO_2]^F$.



Scheme 1

The first purpose of this work is to investigate ^{15}N CIDNP effects during the reaction of the oxoperoxonitrate–CO_2 adduct with a protein in order to show directly the occurrence of radical nitration in a biological system. Bovine albumin (ALB) has been chosen for this purpose, as reactions of albumins with oxoperoxonitrate have been studied thoroughly.[4,7a,11] In addition, EPR investigations during the reaction will be described.

The second purpose is to investigate the importance of the radical mechanism during nitration of tyrosine residues. In general, the observation of CIDNP effects proves the occurrence of free radical reactions, but the possibility that these reactions are only side reactions and that the nitration products are mainly formed *via* a non-radical reaction mechanism cannot be excluded. The magnitude of CIDNP effects is described by enhancement factors E, the ratio between the nuclear polarisation immediately after reaction and in thermal equilibrium with the solvent.[12] The low solubility of Tyr in water did not allow the performance of such experiments with Tyr itself.[9] Valyltyrosylvaline (Val-Tyr-Val) and *N*-acetyl-L-tyrosine

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Table 1 ^{15}N CIDNP during reaction of the oxoperoxonitrate–CO_2 adduct and nitric acid with ALB, Val-Tyr-Val and Tyrac at 300 K

System	^{15}N NMR signals[a]	CIDNP[b]
$Na^{15}NO_2$ (0.25 M), H_2O_2 (1 M), $NaHCO_3$ (0.05 M) with ALB (15%) in H_2O,[c] pH = 4.5, see Fig. 1	349 (NO_4^-)	E
	372 (3-NO_2-ALB)	E
	376 (NO_3^-)	A
	607 (NO_2^-)[d]	N
$Na^{15}NO_2$ (0.25 M), H_2O_2 (1 M), $NaHCO_3$ (0.05 M) with Val-Tyr-Val (0.015 M) in H_2O,[c] pH = 4.5, see Fig. 2	372 (3-NO_2-Val-Tyr-Val)	E
	376 (NO_3^-)	A
	386 (1-NO_2-Val-Tyr-Val)	E
	607 (NO_2^-)[d]	E
$Na^{15}NO_2$ (0.05 M), H_2O_2 (1 M), $NaHCO_3$ (0.05 M) with Tyrac (0.05 M) in H_2O,[c] pH = 5.25	374 (3-NO_2-Tyrac)	E
	378 (NO_3^-)	A
	387 (1-NO_2-Tyrac)	N
	609 (NO_2^-)[d]	E
HNO_3 (0.5 M),[e] H_2SO_4 (2 M) with Val-Tyr-Val (0.015 M) in H_2O	374 (HNO_3)	N
	371 (3-NO_2-Tyrac)	E
	384 (1-NO_2-Tyrac)	E
HNO_3 (0.25 M)[e] with Tyrac (0.05 M) in AcOH, see Fig. 3	354 (HNO_3)	N
	369 (3-NO_2-Tyrac)	E
	384 (1-NO_2-Tyrac)	E

[a] δ in ppm against $^{15}NH_3$, positive δ values downfield, D_2O (10%) as lock. [b] E, emission; A, enhanced absorption; N, no ^{15}N CIDNP effect. [c] Decontaminated potassium phosphate buffer (0.3 M). [d] Not shown in the figures. [e] Nitric acid labelled with ^{15}N (60.3%).

(Tyrac) have been chosen; both can be dissolved in sufficient amounts. Additionally, these compounds are treated as model systems for tyrosyl residues in albumin which itself was not investigated further. A direct comparison of the experimental values with enhancement factors calculated by using different descriptions of the radical pair theory of CIDNP must be treated with caution. The radical pair models contain parameters describing the diffusion behaviour of the radicals within the pair and T_2 values of the radical electrons which are not known very well. In order to avoid this difficulty, E values were determined during nitration with the oxoperoxonitrate–CO_2 adduct and compared with those derived from nitration reactions using nitric acid as the nitrating agent. This well-known reaction is catalysed by nitrous acid giving $^{\bullet}NO_2$ with nitric acid and phenoxyl type radicals with phenolic compounds (see Scheme 2).[13]

$$NO_3^- + H^+ + HNO_2 \rightleftharpoons 2\ {}^{\bullet}NO_2 + H_2O$$

$$2\ HNO_2 \rightleftharpoons NO^{\bullet} + {}^{\bullet}NO_2 + H_2O$$

$$HNO_2 + PhOH \longrightarrow PhO^{\bullet} + NO^{\bullet} + H_2O$$

$$PhO^{\bullet} + {}^{\bullet}NO_2 \longrightarrow [PhO^{\bullet}\ {}^{\bullet}NO_2]^F \xrightarrow{\textbf{(E)}} \mathbf{N}O_2\text{–}PhOH$$

Scheme 2

It has been shown by ^{15}N CIDNP that the nitration products are formed *via* radical pairs $[PhO^{\bullet}\ {}^{\bullet}NO_2]^F$ [10,14] which are the same as those observed during nitration reactions with the oxoperoxonitrate–CO_2 adduct (see Scheme 1). The magnitudes of the ^{15}N CIDNP effects in the nitration products should be the same. A nitrous acid catalysed nitration can be inhibited by application of nitrous acid scavengers such as sodium azide or sulfamic acid.

Results and discussion

^{15}N CIDNP during the reaction of the oxoperoxonitrate–CO_2 adduct with ALB

In Fig. 1a, a ^{15}N NMR spectrum is shown taken after adding H_2O_2 to a solution of ALB (15%), $Na^{15}NO_2$ (0.25 M) and $NaHCO_3$ (0.05 M) in H_2O at pH = 4.5 within 200 s (75 scans). Further details are listed in Table 1. The spectrum shows a strong absorption signal at $\delta = 376$, a broad emission signal at $\delta = 372$ and a small emission signal at $\delta = 349$. The signals at $\delta = 376$ and $\delta = 372$ have also been observed with L-tyrosine.[9] They are assigned to nitrate (NO_3^-) and tyrosine residues of

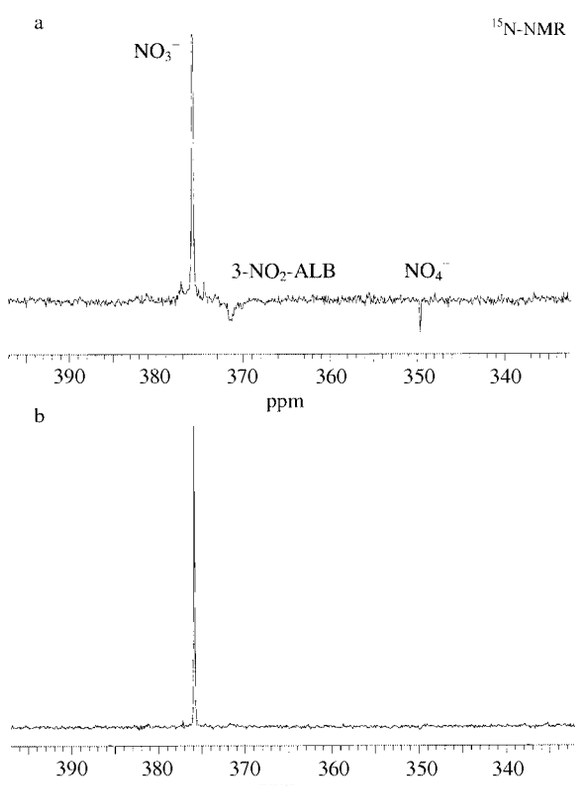

Fig. 1 ^{15}N NMR spectra of bovine albumin with the oxoperoxonitrate–CO_2 adduct in H_2O at pH = 4.5 taken with 90° pulses (a) one minute after mixing the reactants (75 scans, acquisition time 0.7 s, delay time 2 s), (b) ten minutes later (7500 scans). For details, see Table 1.

ALB nitrated in the *o*-position to the hydroxy groups (3-NO_2-ALB). The emission line at $\delta = 349$ is assigned to dioxoperoxonitrate (NO_4^-) by comparison with a reported value of $\delta = 347.7$.[15] The ^{15}N NMR signal of NO_2^- at $\delta = 607$ does not show CIDNP. Similar spectra are observed within 10 min after mixing the reactants. After that, only a weak absorption signal of NO_3^- appears indicating the end of the reaction. A spectrum taken with 7500 scans after the reaction is given in Fig. 1b. It shows signals due to NO_3^- and 3-NO_2-ALB. NO_4^- cannot be detected after the reaction which indicates that NO_4^- is not stable under the reaction conditions. The emission signal of 3-NO_2-ALB is not observed while applying single scans because of the small yield of 3-NO_2-ALB compared with NO_3^- (<1%)

which is a consequence of the small concentration of tyrosyl residues in the reaction mixture. The emission signal does not appear under conditions used previously (pH = 5.25, [Na-$^{15}NO_2$] = 0.05 M).[9] Because of the much larger reaction times (about 1 h) and the lower concentrations of $Na^{15}NO_2$, the reaction rates are smaller by a factor of about 25 under these conditions. The emission signal of NO_4^- is not observed under the former conditions either.

The ^{15}N CIDNP effects in NO_3^- and 3-NO_2-ALB are explained by analogy to those observed during the reaction with Tyr (see Scheme 1).[9] PhOH has to be replaced by tyrosyl residues in ALB, PhO$^\bullet$ by phenoxyl-type radicals attached to the protein (ALB$^\bullet$). The reaction of NO_2^- with H_2O_2 leads to oxoperoxonitrate as an intermediate (eqn. (5));[16] the oxoperoxonitrate–CO_2 adduct is then formed following eqn. (3).

$$HNO_2 + H_2O_2 \longrightarrow O{=}NOO^- + H^+ + H_2O \qquad (5)$$

The enhanced absorption in NO_3^- is built up by radical pairs $[CO_3^{\bullet -}\ {}^\bullet NO_2]^S$ which are formed by O–O bond scission in the oxoperoxonitrate–CO_2 adduct, the emission of 3-NO_2-ALB in radical pairs $[ALB^\bullet\ {}^\bullet NO_2]^F$ is generated by free radical encounters of $^\bullet NO_2$ and phenoxyl type radicals ALB$^\bullet$ attached to the protein. The emission signal of NO_4^- is also observed in the absence of albumin. The polarisation might be built up by F pairs formed by diffusive encounters of $O_2^{\bullet -}$ and $^\bullet NO_2$ [eqn. (6)] leading to emission by the cage product according to Kaptein's first rule as $g(^\bullet NO_2) < g(O_2^{\bullet -})$.[17,18] This explanation is analogous to that of the ^{15}N CIDNP effects observed in nitrated arenes.[10b] However, it is possible that eqn. (6) does not

$$O_2^{\bullet -} + {}^\bullet NO_2 \longrightarrow [O_2^{\bullet -}\ {}^\bullet NO_2]^F \xrightarrow{\textbf{(E)}} NO_4^- \qquad (6)$$

lead to ^{15}N CIDNP because of the linearity of $O_2^{\bullet -}$. As a consequence of this, $O_2^{\bullet -}$ has degenerate orbitals in the ground state leading to a low value of the transverse relaxation time T_2 of the radical electrons. The ^{15}N CIDNP effect in NO_4^- might also be an escape type polarisation built up in geminate radical pairs $[CO_3^{\bullet -}\ {}^\bullet NO_2]^S$ (Scheme 1) and transferred into NO_4^- *via* eqn. (6). $O_2^{\bullet -}$ is formed by reaction of oxoperoxonitrate with H_2O_2 [eqn. (7)][19] or of radical anions $CO_3^{\bullet -}$ with H_2O_2 [eqn. (8)].[20]

$$O{=}N{-}OOH + H_2O_2 \longrightarrow O_2^{\bullet -} + {}^\bullet NO_2 + H_2O + H^+ \qquad (7)$$

$$CO_3^{\bullet -} + H_2O_2 \longrightarrow H_2O + O_2^{\bullet -} + CO_2 \qquad (8)$$

The broad ^{15}N NMR signal of 3-NO_2-ALB might be a superposition of singlets with slightly different chemical shifts as ALB contains 20 different tyrosine residues. It is not possible to distinguish between them at the moment which excludes the possibility of performing more quantitative experiments during the nitration of ALB. The emission signal might be caused not only by nitrated tyrosyl residues. To prove this, L-tryptophan and L-histidine were investigated under the same conditions. Both systems show ^{15}N CIDNP effects, but they are different from those observed with ALB or Tyr. The effects have not yet been analysed in detail. Concerning possible nitration reactions of phenyl residues, it has already been shown that nitration of phenylacetic acid does not occur during reaction with the oxoperoxonitrate–CO_2 adduct.[9]

It has been investigated in further experiments as to whether ^{15}N CIDNP effects are caused by reaction with oxoperoxonitrate without the formation of the oxoperoxonitrate–CO_2 adduct. For this purpose, the reaction was performed in the absence of $NaHCO_3$. During the beginning of the reaction, a weak emission signal of 3-NO_2-ALB is observed, analogous to the emission of 3-NO_2-Tyr observed during reaction of oxoperoxonitrate with Tyr in the absence of CO_2.[9] After a few minutes, the solution becomes highly viscous, probably because of S–S bond formation during the reaction of oxoperoxonitrate with sulfanyl residues [eqns. (9)–(11)].[11a,c]

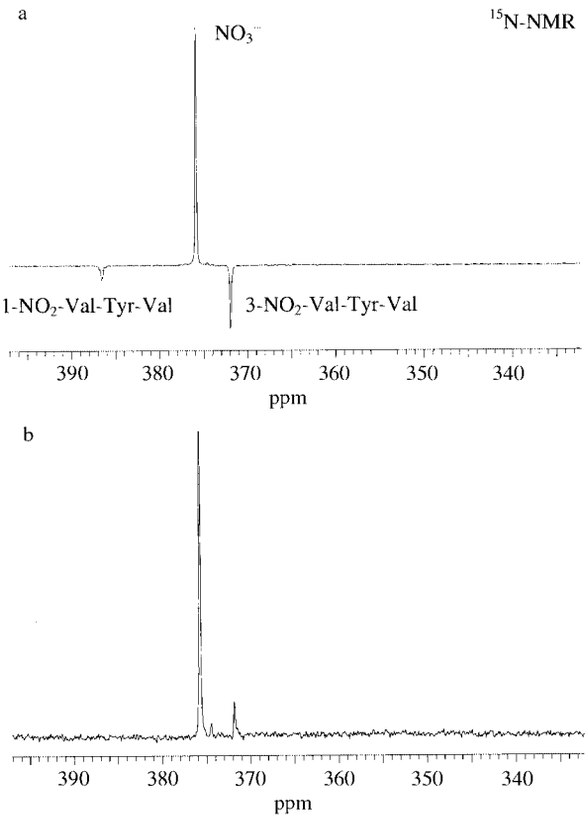

Fig. 2 ^{15}N NMR spectra of valyltyrosylvaline with the oxoperoxonitrate–CO_2 adduct in H_2O at pH = 4.5 taken with 90° pulses (a) 1 min after mixing the reactants (single scan), (b) 300 min later (138 scans, delay time 120 s). For details, see Tables 1 and 2.

$$O{=}NOOH \longrightarrow {}^\bullet NO_2 + {}^\bullet OH \qquad (9)$$

$$^\bullet OH + R{-}SH \longrightarrow H_2O + RS^\bullet \qquad (10)$$

$$2\,RS^\bullet \longrightarrow RS{-}SR \qquad (11)$$

After treatment of human blood plasma or serum albumin with authentic oxoperoxonitrate at pH = 7.1, broad, weak EPR signals were detected and assigned to protein radicals centered on Tyr residues.[7a] Under the experimental conditions used in the present work, EPR signals could not be detected which rules out the formation of persistent radicals in appreciable concentrations.

^{15}N CIDNP during the reaction of the oxoperoxonitrate–CO_2 adduct and nitric acid with Val-Tyr-Val and Tyrac

During the reaction of the oxoperoxonitrate–CO_2 adduct with Val-Tyr-Val, an enhanced absorption signal and two emission signals are observed at δ = 376, 372 and 386. Additionally, the ^{15}N NMR signal of NO_2^- shows emission. Typical spectra during and after reaction are given in Fig. 2a and 2b, further details of the reactions and a description of the ^{15}N CIDNP effects are in Table 1. The signal at δ = 376 is assigned to NO_3^-. The enhanced absorption is built up in radical pairs $[CO_3^{\bullet -}\ {}^\bullet NO_2]^S$ as described before (see Scheme 1). The emission signal at δ = 372 is assigned to the *o*-nitration products of the phenoxyl residues in Val-Tyr-Val (3-NO_2-Val-Tyr-Val) by analogy to the assignment of the emission signal at δ = 372 to 3-NO_2-Tyr observed during the reaction of the oxoperoxonitrate–CO_2 adduct with Tyr.[9]

The ^{15}N CIDNP effects are built up by radical pairs [Val-Tyr-Val$^\bullet$ $^\bullet NO_2]^F$ and are explained following Scheme 1. PhOH has

to be replaced by Val-Tyr-Val, PhO$^{\bullet}$ by the phenoxyl type radical Val-Tyr-Val$^{\bullet}$ formed by hydrogen abstraction from the phenolic residue. The signal at $\delta = 386$ is assigned to a 1-nitrocyclohexa-2,5-dien-4-one residue formed as an unstable intermediate by recombination of $^{\bullet}NO_2$ and the phenoxyl residue in Val-Tyr-Val$^{\bullet}$ in the *p*-position to the hydroxy group. Similar effects have been observed during nitration of various phenolic compounds.[9,14,21] The emission in NO_2^- might be an escape-type polarisation built up in radical pairs $[CO_3^{\bullet -}\ ^{\bullet}NO_2]^S$ and transferred into NO_2^- by reaction of $^{\bullet}NO_2$ with Tyr residues [eqn. (12)].[22] The formation of Tyr$^{\bullet}$ according to eqn. (12) should be less efficient than by reaction with $CO_3^{\bullet -}$ [eqn. (13)],

$$\overset{\textbf{(E)}}{^{\bullet}NO_2} + Tyr \longrightarrow H^+ + \overset{\textbf{(E)}}{NO_2^-} + Tyr^{\bullet} \qquad (12)$$

$$CO_3^{\bullet -} + Tyr \longrightarrow HCO_3^- + Tyr^{\bullet} \qquad (13)$$

but possible ($k_{12} = 10^4$–10^5 M^{-1} s^{-1}, $k_{13} = 4.5 \times 10^7$ M^{-1} s^{-1}).[22,23] The emission line due to NO_4^- which has been observed during the reaction of the oxoperoxonitrate–CO_2 adduct with ALB is not detected. The formation of NO_4^- *via* eqn. (6) might be hindered by Tyr residues in Val-Tyr-Val which scavenge $^{\bullet}NO_2$ according to eqn. (12).

^{15}N CIDNP signals observed during the reaction of Tyrac with the oxoperoxonitrate–CO_2 adduct are listed in Table 1. They are analogous to those observed with Val-Tyr-Val. Emission signals observed at $\delta = 374$ and $\delta = 387$ are assigned to the nitration products of Tyrac (3-NO_2-Tyrac, 1-NO_2-Tyrac), an enhanced absorption signal at $\delta = 378$ to NO_3^- again. NO_2^- also appears as emission. The effects are explained using Scheme 1, with PhOH replaced by Tyrac and PhO$^{\bullet}$ by Tyrac$^{\bullet}$, and eqn. (12).

Table 2 ^{15}N NMR signal intensities I^a of nitration products during the reaction of (A) ValTyrVal (0.015 M) with $Na^{15}NO_2$ (0.25 M), H_2O_2 (1 M) and $NaHCO_3$ (0.05 M) in H_2O at pH = 4.5, (B) Tyrac (0.05 M) with $Na^{15}NO_2$ (0.05 M), H_2O_2 (1 M) and $NaHCO_3$ (0.05 M) in H_2O at pH = 5.25

A		B	
t^b/min	I(3-NO_2-Val-Tyr-Val)	t^b/min	I(3-NO_2-Tyrac)
1	−73	2	−6
2	−64	7	−7
3	−40	12	−15
4	−25	17	−14
5	−18	22	−10
6	−13	27	−9
7	−9	32	−6
8	−6	42	−4
9	−2	52	−2
10	0	62	0
300	0.6[c]	300	0.7[d]

[a] Determined from signal-to-noise ratios using single 90° pulses. [b] Time after mixing the reactants (min). [c] From ^{15}N NMR spectra taken after the reaction (138 scans, delay time 120 s). [d] From ^{15}N NMR spectra taken after the reaction (51 scans, delay time 120 s).

^{15}N CIDNP intensities I recorded during and after reaction are listed in Table 2. The ^{15}N CIDNP effects are much more intense than those observed with ALB and Tyr because of the higher concentrations of Tyr residues in the Val-Tyr-Val and the Tyrac solutions. This allows a determination of enhancement factors. E values determined according to eqn. (14) are listed in Table 3. The values are in the order of −1300 and are comparable to those observed during reaction of the oxoperoxonitrate–CO_2 adduct with phenol.[9]

If nitric acid is used as the nitrating agent, the nitration products 1-NO_2-Val-Tyr-Val, 3-NO_2-Val-Tyr-Val, 1-NO_2-Tyrac and 3-NO_2-Tyrac appear as emission again (see Table 1 and Fig. 3). This indicates the presence of radical pairs [Val-Tyr-Val$^{\bullet}$ $^{\bullet}NO_2]^F$ and [Tyrac$^{\bullet}$ $^{\bullet}NO_2]^F$ during nitration with nitric acid, too. Analogous effects have been found during nitration of phenolic compounds with nitric and nitrous acid and explained *via* nitrous acid catalysis (see Scheme 2). The time dependence of the ^{15}N CIDNP signals of 3-NO_2-Val-Tyr-Val and 3-NO_2-Tyrac is given in Table 4, enhancement factors E in Table 3. The E values are also in the order of −1300 and comparable to those found experimentally in 2-nitrophenol during reaction of phenol with nitric acid in acetic acid.[14] They differ from that observed in 4-nitrophenol which is partially formed by nitro-

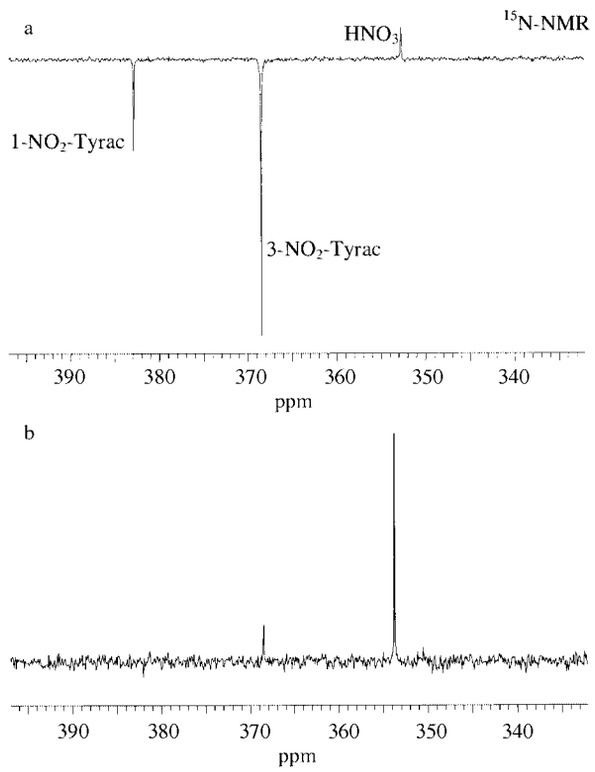

Fig. 3 ^{15}N NMR spectra of *N*-acetyl-L-tyrosine with HNO_3 in AcOH taken with 90° pulses (a) 4 min after mixing the reactants (single scan), (b) 300 min later (52 scans, delay time 120 s). For details, see Tables 1 and 3.

Table 3 Enhancement factors E of ^{15}N CIDNP effects in nitration products of Val-Tyr-Val, Tyrac and phenol

System	Compound	T_1/s	E
Val-Tyr-Val with O=N–$OOCO_2^-$	3-NO_2-Val-Tyr-Val	18	−1390
Tyrac with O=N–$OOCO_2^-$	3-NO_2-Tyrac	24	−1350
Val-Tyr-Val with HNO_3	3-NO_2-Val-Tyr-Val	14	−1290
Tyrac with HNO_3	3-NO_2-Tyrac	16	−1440
Phenol with O=N–$OOCO_2^-$ [a]	2-NO_2-PhOH	90	−1050
	4-NO_2-PhOH	54	−1340
Phenol with HNO_3 [b]	2-NO_2-PhOH	90	−1180
	4-NO_2-PhOH	54	−650

[a] See ref. 9. [b] See ref. 14*c*.

Table 4 ^{15}N NMR signal intensities I^a of nitration products during reaction of (A) Val-Tyr-Val (0.015 M) with HNO_3 (0.5 M, 60.3% ^{15}N) and H_2SO_4 (2 M) in H_2O, (B) Tyrac (0.05 M) with HNO_3 (0.25 M, 60.3% ^{15}N) in AcOH

A		B	
t^b/min	I(3-NO_2-Val-Tyr-Val)	t^b/min	I(3-NO_2-Tyrac)
1	0	2	−3
2	−3	3	−15
3	−10	4	−80
7	−10	5	−250
12	−8	6	−400
18	−6	7	−300
22	−4	8	−80
30	−3	9	−16
40	−2	10	−4
50	0	11	0
300	0.63[c]	300	3.0[d]

[a] Determined from signal-to-noise ratios using single 90° pulses. [b] Time after mixing the reactants (min). [c] From ^{15}N NMR spectra taken after the reaction (360 scans, delay time 120 s). [d] From ^{15}N NMR spectra taken after the reaction (52 scans, delay time 120 s).

sation followed by oxidation, a reaction which does not lead to ^{15}N CIDNP. Nitrosations of phenol in the *p*-position and of 1,2-diphenylenediamine have also been found during the reaction with oxoperoxonitrate.[24] The nitration reaction is suppressed by sodium azide and sulfamic acid added to the reactants in concentrations of 0.1 M. It is concluded that the nitrous acid catalysed nitration is the only reaction leading to nitration products. The similarity of the E values observed during nitration with nitric acid and with the oxoperoxonitrate–CO_2 adduct shows that the radical nitration with the oxoperoxonitrate–CO_2 adduct is the only, or at least the main reaction.

Conclusions

The appearance of ^{15}N CIDNP effects during the reaction of the oxoperoxonitrate–CO_2 adduct with ALB, Val-Tyr-Val and Tyrac indicates a radical mechanism of the nitration reaction. The nitration occurs *via* recombination of $^{\bullet}NO_2$ and phenoxyl-type radicals formed by reaction with $CO_3^{\bullet -}$. Identical ^{15}N CIDNP effects are observed during nitration of Val-Tyr-Val and Tyrac with nitric acid. These reactions are inhibited by addition of nitrous acid scavengers showing that the recombination of $^{\bullet}NO_2$ and phenoxyl-type radicals is the only path leading to nitration products. The comparison of the ^{15}N CIDNP intensities reveals that the radical mechanism is the main, if not the only path leading to nitration products during the reaction of Val-Tyr-Val and Tyrac with the oxoperoxonitrate–CO_2 adduct, too.

It cannot be concluded from the observation of ^{15}N CIDNP effects that nitrations *in vivo* always occur *via* radical decomposition of the oxoperoxonitrate–CO_2 adduct; indeed, enzymatic reactions of oxoperoxonitrate have been proven in certain cases.[6,25] However, selectivity and reactivity of the nitration agent *in vivo* correspond to that of $^{\bullet}NO_2$ and not to that of nitration agents such as NO_2^+, NO^+ or $NO^{\bullet}$ indicating the presence of $^{\bullet}NO_2$ during the nitration reaction.

Experimental

All chemicals were commercially available and of analytical reagent grade and used without further purification. Metal contaminations in water were removed by incubation with Resign Chelex 100 for 15 h.[26] Bovine albumin was 30% in H_2O (Sigma), valyltyrosylvaline was present as 0.5 mol per mol acetate (Sigma), *N*-acetyl-L-tyrosine was 98% min. (Fluka). Nitric acid was 9.4 M in H_2O and labelled with 60.3 atom% ^{15}N (Isotec Inc.), sodium nitrite with 99% min. ^{15}N (Isotec Inc.). Oxoperoxonitrate was generated *in situ* in 10 mm NMR tubes or in a 5 mm flat EPR cell by adding H_2O_2 (1 M) to a solution of $Na^{15}NO_2$ (0.25 M or 0.05 M) in potassium phosphate buffer (0.3 M). The oxoperoxonitrate–CO_2 adduct was generated by adding $NaHCO_3$ (0.05 M) to the solution. The pH value was determined using a pH-Meter CG 825 (Schott GmbH).

The ^{15}N CIDNP experiments were performed as described in the literature.[9,14] After mixing the reactants in the NMR tubes, they were quickly transferred into the probe of the ^{15}N NMR spectrometer (BRUKER DPX-300) and locked within 1 min (internal lock: 10% D_2O). The first ^{15}N NMR spectra were recorded 1 min or later after mixing the reactants by using single pulses with pulse angles of 90°. After that, ^{15}N NMR spectra were recorded until there was no further change in the spectrum. ^{15}N NMR signal intensities I during reaction and I_o after reaction were taken from the spectra by determining the signal-to-noise ratios. This procedure is equivalent to an integrating process if the linewidths of the signals do not change during the reaction which was the case for the nitration products. To improve the signal-to-noise ratios, a large number of scans has been used in several cases. Nuclear relaxation times T_1 were determined after completion of the reactions by applying $\pi - \pi/2$ pulse sequences. Enhancement factors E were determined using eqn. (14), where I_i is the intensity of the *ith*

$$E = \Sigma\, I_i \Delta t(i, i + 1)/I_o T_1 \qquad (14)$$

measurement, $\Delta t(i, i + 1)$ the time interval between the *ith* and the $(i + 1)th$ measurement. T_1 is the longitudinal relaxation time of the ^{15}N nuclei. The summation was carried out as long as the ^{15}N CIDNP effects were observed. Chemical shifts were determined against [^{15}N]nitrobenzene dissolved in acetonitrile used as an external reference and given against $^{15}NH_3$.

EPR experiments were performed in a similar manner. After mixing the reactants in the EPR tubes, the probes were transferred into the cavity, and EPR spectra were run within 10 min. A VARIAN E-109 machine was used.

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