Inhibition of Peroxynitrite-Induced Nitration of Tyrosine by Glutathione in the Presence of Carbon Dioxide through both Radical Repair and Peroxynitrate Formation

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Abstract: Peroxynitrite ($ONOO^-$/ONOOH) is assumed to react preferentially with carbon dioxide in vivo to produce nitrogen dioxide ($NO_2^{\bullet}$) and trioxocarbonate(1−) ($CO_3^{\bullet-}$) radicals. We have studied the mechanism by which glutathione (GSH) inhibits the $NO_2^{\bullet}/CO_3^{\bullet-}$-mediated formation of 3-nitrotyrosine. We found that even low concentrations of GSH strongly inhibit peroxynitrite-dependent tyrosine consumption ($IC_{50} = 660\,\mu M$) as well as 3-nitrotyrosine formation ($IC_{50} = 265\,\mu M$). From the determination of the level of oxygen produced or consumed under various initial conditions, it is inferred that GSH inhibits peroxynitrite-induced tyrosine consumption by re-reducing (repairing) the intermediate tyrosyl radicals. An additional protective pathway is mediated by the glutathiyl radical ($GS^{\bullet}$) through reduction of dioxygen to superoxide ($O_2^{\bullet-}$) and reaction with $NO_2^{\bullet}$ to form peroxynitrate (O_2NOOH/O_2NOO^-), which is largely unreactive towards tyrosine. Thus, GSH is highly effective in protecting tyrosine against an attack by peroxynitrite in the presence of CO_2. Consequently, formation of 3-nitrotyrosine by freely diffusing $NO_2^{\bullet}$ radicals is highly unlikely at physiological levels of GSH.

Keywords: antioxidants · nitrogen oxides · peroxynitrite · radical reactions · tyrosine nitration

Introduction

Oxoperoxonitrate(1−) ($ONOO^-$) can be formed in vivo from the diffusion-controlled reaction ($k = 3.9-19 \times 10^9\,M^{-1}s^{-1}$) of superoxide ($O_2^{\bullet-}$) with nitric oxide (nitrogen monoxide, $^{\bullet}NO$).[1, 2] The pathological activity of this anion and its conjugated acid [hydrogen oxoperoxonitrate(1−), peroxynitrous acid, ONOOH], often collectively referred to as peroxynitrite, is presumably based on its ability to oxidize protein and non-protein sulfhydryls,[3] membrane phospholipids,[4] low-density lipoproteins,[5] and NAD(P)H.[6] Additionally, peroxynitrite and activated $^{\bullet}NO/O_2^{\bullet-}$-releasing alveolar macrophages exhibit nitrating activity.[7] In fact, the formation of NO_2-Tyr[8] is evident in a variety of tissue injuries.[9–12]

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Though exhibiting only a modest reactivity towards peroxynitrite [k(peroxynitrite + glutathione) $= 1350\,M^{-1}s^{-1}$[13]], glutathione (GSH) is considered to be a major scavenger of intracellularly operating peroxynitrite[3] due to its high intracellular concentrations (5–10 mM). However, this conclusion has largely been based on experiments performed in the absence of CO_2. Since the rate of the reaction of CO_2 (relevant concentrations in vivo ≈ 1.3 mM[14]) with $ONOO^-$ clearly exceeds both the rate of reaction of ONOOH with most biological substrates and the rate of release of free $HO^{\bullet}$ and $NO_2^{\bullet}$ radicals[15] through homolysis of ONOOH, the ability of GSH to scavenge ONOOH appears to be irrelevant under typical physiological conditions. Thus, the role of GSH as an antioxidant towards peroxynitrite remains an open question. The reaction of CO_2 with $ONOO^-$ presumably generates an adduct, 1-carboxylato-2-nitrosodioxidane ($ONOOCO_2^-$), which fragments with an efficiency of about 30–35 %[16–18] into free $CO_3^{\bullet-}$[19, 20] and $NO_2^{\bullet}$[21] radicals. Due to the fact that i) $CO_3^{\bullet-}$ reacts several orders of magnitude more rapidly with important biological molecules, such as tyrosine, tryptophan, ascorbate, and NAD(P)H,[1, 6, 22] than it does with GSH;[1] and ii) $NO_2^{\bullet}$ only reacts rapidly with the thiolate form of thiols,[23] GSH ($pK_a = 9.2$), with its limited scavenging ability, should be rather ineffective in protecting molecules against radicals released from the putative

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$ONOO^-/CO_2$ adduct. Indeed, we have recently demonstrated the inefficiency of GSH in preventing NAD(P)H oxidation by peroxynitrite.[6] However, ascorbate was, quite unexpectedly, found to be a more effective antioxidant because it diminished the oxidative power of peroxynitrite-derived radicals by both terminating the attacking entities and by re-reducing the intermediate substrate radical.[24] Additionally, we identified a novel protection pathway, that is, the intermediate formation of peroxynitrate from the diffusion-controlled reaction of $NO_2^\bullet$ with $O_2^{\bullet-}$. In apparent contrast to the inefficiency of GSH in inhibiting peroxynitrite-dependent oxidation reactions, GSH turned out to be highly effective in inhibiting peroxynitrite-mediated nitration reactions in the presence of CO_2. In the present paper, we report on these findings and clarify the underlying mechanism by which GSH exerts its protective, antioxidative function.

Results

Protective effect of GSH on peroxynitrite-induced formation of nitrotyrosine: In the absence of CO_2, about 70 μM NO_2-Tyr was formed from the reaction of peroxynitrite with tyrosine (1 mM each) (Table 1). This result is virtually identical to data from earlier reports.[6, 25] Ascorbate was found to be more potent than GSH in terms of the amount required to inhibit peroxynitrite-derived NO_2-Tyr formation to half of the maximum value. These well-known effects[26] are explained by i) the diffusion-controlled reactions of the $HO^\bullet$ radical with both ascorbate and GSH, ii) the high reactivity of ascorbate towards both $NO_2^\bullet$ and tyrosyl radicals,[1, 27] and iii) the moderate reactivity of GSH towards ONOOH. Surprisingly, the presence of CO_2 did not suppress the ability of GSH to inhibit peroxynitrite-dependent nitration reactions (Table 1). Since a direct reaction of GSH with ONOOH can safely be neglected in the presence of CO_2, we hypothesized that GSH prevents NO_2-Tyr formation by re-reducing the intermediate tyrosyl radical. Similar effects have been observed with *para*-hydroxyphenylacetic acid (*p*-HPA) and tryptophan as substrates.

Table 1. Effect of ascorbate and glutathione on peroxynitrite-induced nitrations.

Product	presence of HCO_3^-/CO_2	yield[a] [μM]	ASC[b] IC_{50} [μM]	GSH
3-nitrotyrosine	no	71.2 ± 2.9	180 ± 10	405 ± 13
	yes	191.6 ± 3.4	325 ± 11	350 ± 15
3-(NO_2)-4-HPA	no	80.2 ± 1.7	155 ± 10	326 ± 12
	yes	191.3 ± 3.5	340 ± 11	415 ± 13
6-nitrotryptophan	no	90.7 ± 3.1	275 ± 11	390 ± 13
	yes	169.5 ± 3.3	370 ± 11	345 ± 13

[a] In the absence of both ASC and GSH. [b] ASC = ascorbate.

Evidence for a glutathione-mediated repair of tyrosyl radicals: The "repair" of tyrosyl radicals by GSH can be rationalized by the following reaction sequence [Eqs. (1)–(3)]:

$$\text{tyrosyl radical} + GS(H) \rightarrow \text{tyrosine} + GS^\bullet \quad (1)$$

$$GS^\bullet + GS^- \rightleftharpoons GSSG^{\bullet-} \quad (2)$$

$$GSSG^{\bullet-} + O_2 \rightarrow GSSG + O_2^{\bullet-} \quad (3)$$

Provided that the prevention of NO_2-Tyr formation by GSH is mediated by this mechanism, peroxynitrite-dependent tyrosine consumption, in other words NO_2-Tyr and dityrosine formation,[28] can be expected to be inhibited by GSH as well. To find out whether this is indeed the case, the experiments with peroxynitrite and tyrosine (1 mM each) in the presence of CO_2 and various GSH concentrations were repeated, and the reaction solutions were analyzed by capillary zone electrophoresis (Figure 1). In the absence of GSH, tyrosine consumption (431.8 ± 37.4 μM) was about 2.4 times higher than NO_2-Tyr formation (180.5 ± 11.3 μM) upon addition of peroxynitrite. The yield of NO_2-Tyr was in good agreement with the data obtained from absorbance measurements (Table 1 and refs. [6, 25]). As expected, GSH was found to effectively inhibit peroxynitrite-mediated consumption of tyrosine as well as the formation of NO_2-Tyr [Eqs. (1)–(3)]. In good accord with the above-mentioned tyrosine/NO_2-Tyr ratio, the IC_{50} value (IC_{50} = inhibitor concentration necessary to inhibit the formation of the product half-maximal) for tyrosine consumption (IC_{50} = 664 ± 12 μM) was found to be about 2.5 times higher than that for NO_2-Tyr formation (IC_{50} = 266 ± 13 μM). In contrast, ascorbate was found to inhibit both the peroxynitrite-induced consumption of tyrosine (IC_{50} = 232 ± 10 μM) and NO_2-Tyr formation (IC_{50} = 237 ± 10 μM) with the same efficiency. Since GSH reacts with the $CO_3^{\bullet-}$ radical much more slowly than tyrosine does (see Discussion), the inhibition of tyrosine consumption by GSH is unlikely to result from effective trapping of $CO_3^{\bullet-}$, but instead can be explained by a GSH-mediated repair function.

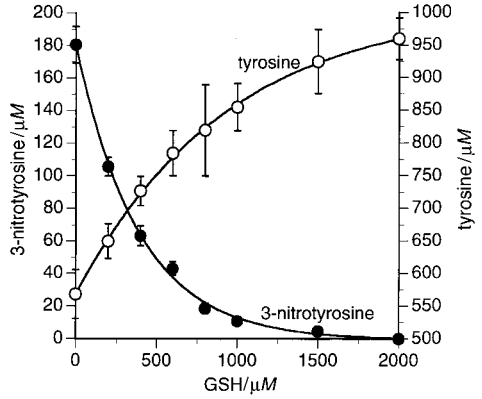

Figure 1. Peroxynitrite (1 mM) was added by vortexing to potassium phosphate buffer (50 mM, pH 7.5, 0.1 mM DTPA, 37 °C, 25 mM/5 % HCO_3^-/CO_2), which contained tyrosine (1 mM) and varying concentrations (0–2 mM) of GSH. The remaining tyrosine and the NO_2-Tyr formed were quantified by capillary zone electrophoresis. Each value represents the mean ± standard deviation of three experiments performed in duplicate.

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Consequently, glutathiyl radicals should have been formed [Eqs. (1)–(3)] under the aforementioned conditions. Accordingly, after mixing of peroxynitrite with GSH at pH 7.5 in the presence of both 50 mM HCO_3^- and 100 mM DMPO, the characteristic ESR spectrum of the DMPO-glutathiyl radical adduct (Figure 2) was observed "instantaneously", that is to say within 40 s. The formation of this DMPO adduct has also been reported after mixing peroxynitrite with GSH in the absence of HCO_3^-/CO_2.[29] The experimental ESR spectrum could be satisfactorily reproduced by simulation with the assumption that two conformers of the DMPO-glutathiyl radical adduct are present. In the presence of additional tyrosine (1 mM), a virtually identical ESR spectrum was observed (not shown). Control experiments revealed that tyrosyl radicals were not trapped by DMPO under these conditions.

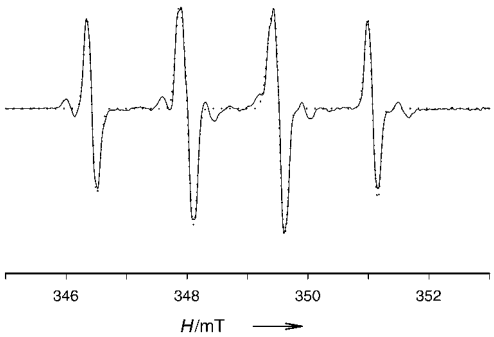

Figure 2. ESR spectra were obtained upon incubating DMPO (100 mM), HCO_3^- (50 mM), GSH (10 mM), and peroxynitrite (1 mM) in potassium phosphate buffer (50 mM, pH 7.5) at room temperature. The dotted lines shows a computer simulation of the spectra. Recording conditions: microwave frequency, 9.8 GHz; modulation, 0.04 mT; signal gain, 5×10^5; sweep range, 20 mT; sweep time, 4 min.

Oxygen can interfere with this system through two pathways,[30] either by reversible addition to the glutathiyl radical and/or, as outlined in Equations (1)–(3), by reaction with the $GSSG^{\bullet -}$ radical. Since only the latter reaction generates $O_2^{\bullet -}$, we attempted to identify this intermediate. After the addition of peroxynitrite (1 mM) to a solution containing both GSH (1 mM) and cytochrome c^{3+} (20 μM) or cytochrome c^{3+} plus SOD (100 units mL^{-1}), 7.9 ± 0.3 and 6.4 ± 0.2 μM $O_2^{\bullet -}$ were found in the absence and in the presence of HCO_3^-/CO_2, respectively (average of three experiments performed in duplicate). Thus, an attack of peroxynitrite, or rather of peroxynitrite-derived radicals on GSH indeed produces $O_2^{\bullet -}$. In the presence of tyrosine, however, the formation of $O_2^{\bullet -}$ could not be verified (≤ 0.2 μM detection limit). A reason for this may be a preferred reaction of $GS^{\bullet}$ with the formed cytochrome c^{2+},[31] because the amount of $GS^{\bullet}$ should be increased in the presence of tyrosine. Thus, our probe is rapidly destroyed by $GS^{\bullet}$.

To unequivocally prove that GSH saves tyrosine by re-reducing the tyrosyl radical, we studied whether oxygen release from 1 mM peroxynitrite was affected by the presence of tyrosine and GSH, respectively. In the absence of HCO_3^-/CO_2, about 172 μM O_2 was released from the peroxynitrite (Table 2), in excellent agreement with previous reports.[32–34] With added tyrosine (1 mM), oxygen production from the same initial amount of peroxynitrite was markedly reduced to ~18 μM O_2. The situation changed again dramatically with GSH as an additive. Irrespective of the presence of tyrosine, O_2 production could not be monitored, but rather most of the atmospheric O_2 level (≈180 μM) was consumed then. In the presence of CO_2, only around 15 μM O_2 was formed from 1 mM peroxynitrite, and this yield proved to be insensitive to the presence of tyrosine (1 mM). However, by replacing tyrosine with GSH (1 mM), an O_2 uptake of about 60 μM was evident. Interestingly, the O_2 uptake further increased to around 105 μM when tyrosine was also present.

Indications for glutathione-derived formation of peroxynitrate: The results reported above leave no doubt that GSH is capable of effectively reconverting the tyrosyl radical into tyrosine. The question remains as to whether this is also the mechanism by which 3-nitrotyrosine formation is inhibited by GSH (remember that the IC_{50} value for 3-NO_2-Tyr is about 2.5 times smaller than that for tyrosine consumption). One explanation for 3-NO_2-Tyr inhibition could be that $NO_2^{\bullet}$ might be capable of directly reacting with GSH. If this was to be the case, then $NO_2^{\bullet}$ would be reduced to nitrite, and, hence, GSH can be expected to initiate an emissive CIDNP effect in the ^{15}N NMR spectrum of $^{15}NO_2^-$ formed from the freely diffusing $^{15}NO_2^{\bullet}$ radicals. This, however, is not observed (spectrum not shown).

Table 2. Effects of glutathione and tyrosine on O_2 formation from peroxynitrite.

Additives (1 mM each)	Absence of HCO_3^-/CO_2		Presence of HCO_3^-/CO_2	
	$[O_2]_{total}$[a] [μM]	$\Delta[O_2]$[b] [μM]	$[O_2]_{total}$ [μM]	$\Delta[O_2]$[b] [μM]
none (atmospheric O_2 saturation level)	225.4 ± 2.9		210.3 ± 2.8	
peroxynitrite	397.2 ± 9.6	171.8	225.9 ± 2.7	15.9
peroxynitrite + tyrosine	243.3 ± 4.8	17.9	225.4 ± 1.0	15.4
peroxynitrite + GSH	43.9 ± 12.8	− 181.5	155.1 ± 5.6	− 60.2
peroxynitrite + GSH + tyrosine	52.1 ± 14.9	− 173.3	105.1 ± 5.7	− 105.2

[a] Each value represents the mean ± standard deviation of six experiments. [b] $[O_2]$(+ additive) − $[O_2]$(no additive).

As we could find no evidence of GSH being able to trap $NO_2^{\bullet}$, quantum chemical DFT (density functional theory) calculations were performed with cysteine as a model for GSH.

These calculations (Table 3) were likewise unsupportive of the view that the $NO_2^{\bullet}$ radical should effectively attack cysteine. However, the calculations predicted that an attack of $NO_2^{\bullet}$ on the thiolate form of cysteine should be energetically favorable. Therefore, one may suggest that the GS^- anion [pK_a(GSH) = 9.2] is the species which actually deactivates the $NO_2^{\bullet}$ radical. To clarify this point, tyrosine was nitrated with

Table 3. Quantum-chemically calculated reaction energies [kcal mol^{-1}].

Reaction[a]	$\Delta_R E_{298}^{\text{gas phase}}$	$\Delta_R E_{298}^{\text{water}}$ PCM	IPCM
Cys–SH + $NO_2^\bullet \rightarrow$ Cys–S$^\bullet$ + HNO_2	10.4	4.2	6.3
Cys–SH + $NO_2^\bullet \rightarrow$ Cys–SH$^{\bullet+}$ + NO_2^-	142.7	25.4	27.9
Cys–S$^-$ + $NO_2^\bullet \rightarrow$ Cys–S$^\bullet$ + NO_2^-	2.2	−5.8	−14.0

[a] Geometries of cysteine (Cys–SH), cysteine thiolate (Cys–S$^-$), $NO_2^\bullet$, and NO_2^- were fully optimized to stationary points using density functional theory (DFT) with nonlocal corrections (B3LYP) on the 6–31 + G(d) basis set. As these calculations refer only to the conditions in the gas phase, single-point calculations were performed at the B3LYP/6–31 + G(d)// B3LYP/6–31 + G(d) level for water with either the PCM[63] or the IPCM[64] solvation model.

peroxynitrite (1 mM each) at various pH values (6.5–8) in the presence of CO_2 (Table 4). The yield of NO_2-Tyr was found to be pH-dependent, with a maximum of 192 μM at pH 7.5. This result is in line with the data obtained by Lemercier et al.[35] At pH 8, peroxynitrite-mediated NO_2-Tyr formation was found to be inhibited by about 85% upon addition of 1 mM GSH. The protective effect exerted by GSH decreased with decreasing pH value, but around 55% protection was still observed at pH 6.5. Since the level of protection at pH 6.5 is still about 64% of that at pH 8 and because the GS$^-$ concentration at pH 6.5 is only 3% of that at pH 8, the GSH-mediated inhibition of peroxynitrite-derived NO_2-Tyr formation cannot be exclusively attributed to the trapping of $NO_2^\bullet$ radicals by GS$^-$.

In view of the fact that $O_2^{\bullet-}$ is produced in an environment in which $NO_2^\bullet$ is also an important intermediate, the formation of peroxynitrate (O_2NOO^-) must be taken into account, because these radicals react with each other at a diffusion-controlled rate.[1] Peroxynitrate is then deactivated by decomposition into NO_2^- and O_2 ($k_{dec} = 0.7\ s^{-1}$).[36] Since

Table 4. Effects of glutathione and pH on peroxynitrite-induced nitration of tyrosine in the presence of HCO_3^-/CO_2.

Peroxynitrite (1 mM) + additive	[NO_2-Tyr][a] [μM]	Protection[b] [%]	[GS$^-$][c] [%]
pH 6.5			0.20
none	104.1 ± 7		
GSH(0.5 mM)	66.5 ± 5	36.1	
GSH(1 mM)	47.3 ± 2	54.6	
pH 7.0			0.63
none	127.7 ± 11		
GSH(0.5 mM)	74.3 ± 7	41.8	
GSH(1 mM)	50.2 ± 2	60.7	
pH 7.5			1.96
none	192.1 ± 6		
GSH(0.5 mM)	77.1 ± 6	59.9	
GSH(1 mM)	39.6 ± 3	79.4	
pH 8.0			5.94
none	168.8 ± 7		
GSH(0.5 mM)	55.9 ± 5	66.9	
GSH(1 mM)	25.2 ± 2	85.1	

[a] Each value represents the mean ± standard deviation of three experiments performed in duplicate. [b] Percentage of reduction of NO_2-Tyr yield. [c] Amount of free GS$^-$ at the particular pH value, calculated from pK_a(GSH) = 9.2.

no specific probe for peroxynitrate is presently available, we checked for the possible intermediacy of this compound by performing a kinetic simulation (Table 5) with the kinetic scheme given in Table 6 (see Experimental Section). The

Table 5. The kinetic analysis of the experimental values, which was performed with 5×10^7 reacting molecules, a fixed pH value of 7.5, and with the following initial concentrations: $[ONOO^-]_0 = 1$ mM, $[GSH]_0 = 1$ mM, $[GS^-]_0 = 19.5$ μM, $[CO_2]_0 = 1.3$ mM, and $[O_2]_0 = 225$ μM.

Reaction	Rate constant [$M^{-1}s^{-1}$]	Estimated contribution of $NO_2^\bullet$ radical decay[a] [%]
$NO_2^\bullet$ + $GS^- \rightarrow NO_2^-$ + GS$^\bullet$	2.4×10^8	28.5
$NO_2^\bullet$ + $O_2^{\bullet-} \rightarrow O_2NOO^-$	4.5×10^9	40.9
$NO_2^\bullet$ + GSO$^\bullet \rightarrow$ GS(O)ONO	4.5×10^9	5.9
$2NO_2^\bullet$ (+ H_2O)[b] $\rightarrow NO_2^-$ + NO_3^-	1.0×10^3	0.6
$NO_2^\bullet$ + $CO_3^{\bullet-} \rightarrow NO_3^-$ + CO_2	4.6×10^8	3.4
$NO_2^\bullet$ + GS$^\bullet \rightarrow$ $GSNO_2$	3.0×10^9	20.6
$NO_2^\bullet$ + HO$^\bullet \rightarrow$ ONOOH	4.5×10^9	0.1
$NO_2^\bullet$ + HO$^\bullet \rightarrow NO_3^-$ + H^+	4.5×10^9	0.1

[a] Since various reactions yield NO_3^-, the contribution of a particular pathway of $NO_2^\bullet$ decomposition cannot necessarily be estimated from the product yields. To overcome this disadvantage, each reaction of interest was assigned as additionally generating one specific generic product, namely NO_2 decay I, NO_2 decay II, …NO_2 decay IX. In the case of N_2O_4, it was assumed that $2NO_2$ decay V were generated. The sum of NO_2 decay I to NO_2 decay IX was found to be 329 μM, that is, the yield of $NO_2^\bullet$ from reaction of 1 mM peroxynitrite with carbon dioxide (experimental yield 33%). The contribution of the nine $NO_2^\bullet$ decomposition pathways could then be calculated. For example, the contribution of the GS$^-$-derived pathway, that is, NO_2 decay I, of $NO_2^\bullet$ deactivation is calculated by: % contribution (GS$^-$) = 100 × NO_2 decay I/329 μM. [b] This reaction is believed to involve N_2O_4.

kinetic analysis predicted that under the applied conditions the $NO_2^\bullet$ radical mainly decays (≈41%) through formation of peroxynitrate, but that other pathways also make significant contributions to this process (Table 5).

Peroxynitrate has been reported to be in partial equilibrium with $NO_2^\bullet$ and $O_2^{\bullet-}$,[37] thus, one might even assume that O_2NOO^- promotes the action of $NO_2^\bullet$. However, the equilibrium constant ($K = 2.3 \times 10^{-10}\ M^{-1}$)[37] is too low to generate a significant equilibrium concentration of $NO_2^\bullet$. To further substantiate whether O_2NOO^- can play a protective role, the effect of peroxynitrate on both tyrosine consumption and NO_2-Tyr formation was analyzed by capillary zone electrophoresis. After mixing peroxynitrate (1.57 mM) with tyrosine (1 mM), NO_2-Tyr was not formed above our detection limit of 8 μM, and the recovery of tyrosine was found to be 96 ± 1% (average of three experiments performed in duplicate), irrespective of the presence of CO_2. Thus, the $NO_2^\bullet$/$O_2^{\bullet-}$-mediated formation of O_2NOO^- indeed represents an additional pathway for the inactivation of $NO_2^\bullet$ radicals towards attack on tyrosine.

Discussion

It has been unequivocally proven by ^{15}N CIDNP experiments that the peroxynitrite-induced nitration of tyrosine in the presence of CO_2 proceeds via freely diffusing $CO_3^{\bullet-}$ and $NO_2^\bullet$

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Table 6. Kinetic scheme used in the simulation to model the pathways of $NO_2^{\bullet}$ radical decay in the reaction of glutathione with peroxynitrite in the presence of CO_2 at pH 7.5.

Entry	Reaction	Rate constants [$M^{-1}s^{-1}$] or [s^{-1}]	Ref.
1	$ONOO^- + CO_2 \rightarrow ONOOCO_2^-$	5.8×10^4	[15]
2	$ONOOCO_2^- \rightarrow NO_3^- + CO_2$	6.7×10^5	[a]
3	$ONOOCO_2^- \rightarrow NO_2^{\bullet} + CO_3^{\bullet-}$	3.3×10^5	[a]
4	$CO_3^{\bullet-} + GSH \rightarrow GS^{\bullet} + HCO_3^-$	5.3×10^6	[1]
5	$GSH\ (+\ H_2O) \rightarrow GS^- + H_3O^+$	63.1	[b]
6	$GS^- + H_3O^+ \rightarrow GSH + H_2O$	1.0×10^{11}	[b]
7	$GS^- + NO_2^{\bullet} \rightarrow GS^{\bullet} + NO_2^-$	2.4×10^8	[1]
8	$GS^- + CO_3^{\bullet-} \rightarrow GS^{\bullet} + CO_3^{2-}$	7.1×10^8	[1]
9	$2\ GS^{\bullet} \rightarrow GSSG$	5.0×10^9	[30]
10	$GS^{\bullet} + GS^- \rightarrow GSSG^{\bullet-}$	6.0×10^8	[30]
11	$GSSG^{\bullet-} \rightarrow GS^{\bullet} + GS^-$	1.6×10^5	[15]
12	$GSSG^{\bullet-} + O_2 \rightarrow GSSG + O_2^{\bullet-}$	5.0×10^9	[30]
13	$O_2^{\bullet-} + NO_2^{\bullet} \rightarrow O_2NOO^-$	4.5×10^9	[1] (8)
14	$O_2NOO^- \rightarrow O_2 + NO_2^-$	0.7	[36]
15	$GSH + O_2^{\bullet-} \rightarrow GSO^{\bullet} + HO^-$	1.0×10^3	[65]
16	$GS^{\bullet} + O_2 \rightarrow GSOO^{\bullet}$	2.0×10^9	[30]
17	$GSOO^{\bullet} \rightarrow GS^{\bullet} + O_2$	6.0×10^5	[30]
18	$GSOO^{\bullet} + GSH \rightarrow GSO^{\bullet} + GSOH$	2.0×10^6	[c]
19	$2\,GSO^{\bullet} \rightarrow$ non-radical products	6.0×10^7	[c]
20	$GSO^{\bullet} + NO_2^{\bullet} \rightarrow GS(O)ONO$	4.5×10^9	[d]
21	$GS(O)ONO + (H_2O) \rightarrow$ non-radical products	7.0×10^2	[e]
22	$HO^{\bullet} + GSH \rightarrow GS^{\bullet} + H_2O$	1.3×10^{10}	[1]
23	$HO^{\bullet} + O_2^{\bullet-} \rightarrow HO^- + O_2$	1.1×10^{10}	[1]
24	$HO^{\bullet} + NO_2^- \rightarrow NO_2^{\bullet} + HO^-$	6.0×10^9	[1]
25	$HO^{\bullet} + GSSG \rightarrow GSSG^{\bullet+} + HO^-$	7.1×10^9	[1]
26	$HO^{\bullet} + NO_2^{\bullet} \rightarrow H^+ + NO_3^-$	4.5×10^9	[f]
27	$HO^{\bullet} + NO_2^{\bullet} \rightarrow ONOOH$	4.5×10^9	[f]
28	$2\,NO_2^{\bullet} \rightarrow N_2O_4$	4.5×10^8	[66]
29	$N_2O_4 \rightarrow 2\,NO_2^{\bullet}$	6.9×10^3	[66]
30	$N_2O_4\ (+\ H_2O) \rightarrow NO_2^- + NO_3^- + 2\,H^+$	1.0×10^3	[1]
31	$NO_2^{\bullet} + GS^{\bullet} \rightarrow GSNO_2$	3.0×10^9	[g] (12)
32	$NO_2^{\bullet} + CO_3^{\bullet-} \rightarrow NO_3^- + CO_2$	4.6×10^8	[67]
33	$CO_3^{\bullet-} + H_2O_2 \rightarrow CO_3^{2-} + O_2^{\bullet-} + 2\,H^+$	9.8×10^5	[68]
34	$CO_3^{\bullet-} + O_2^{\bullet-} \rightarrow CO_3^{2-} + O_2$	6.5×10^8	[1]
35	$2\,O_2^{\bullet-}\ (+\ 2\,H^+) \rightarrow H_2O_2 + O_2$	2.3×10^5	[1]
36	$O_2^{\bullet-} + Cu^{2+}\text{-SOD} \rightarrow O_2 + Cu^{1+}\text{-SOD}$	1.9×10^9	[1]
37	$O_2^{\bullet-} + Cu^{1+}\text{-SOD}\ (+2\,H^+) \rightarrow H_2O_2 + Cu^{2+}\text{-SOD}$	1.9×10^9	[h]
38	$ONOO^- + H_3O^+ \rightarrow ONOOH + H_2O$	1.0×10^{11}	[b]
39	$ONOOH + H_2O \rightarrow ONOO^- + H_3O^+$	15849	[b]
40	$ONOOH \rightarrow NO_3^- + H^+$	0.7	[i]
41	$ONOOH \rightarrow NO_2^{\bullet} + HO^{\bullet}$	0.3	[i]
42	$ONOOH + GSH \rightarrow H_2O + GSNO_2$	1350	[13, 69]

[a] These rate constants correspond to the known $NO_2^{\bullet}/CO_3^{\bullet-}$ yields of about 33% and an estimated upper limit of the lifetime of $ONOOCO_2$ of 1 μs.[67] [b] The rate constants for deprotonation of GSH and ONOOH were calculated from the pK_a values of 9.2 and 6.8, respectively, and from the reasonable assumption that a typical rate constant for protonation lies in the range of $1-10 \times 10^{10}\,M^{-1}s^{-1}$.[70] [c] The unknown rate constants for the reactions of $GSOO^{\bullet}$ with GSH and for the self-reaction of the $GSO^{\bullet}$ radicals were assumed to be similar to the rate constants for the reaction of 2-mercaptoethanol thiylperoxide with 2-mercaptoethanol[30] and of the dimerization of *tert*-butyl sulfinyl radicals,[71] respectively. [d] This reaction and the corresponding rate constant was suggested by an anonymous referee. We calculated the thermochemistry of this reaction by using density functional theory at the B3LYP/6–31 + G(d) level of theory as: $\Delta_R E_{298} = -14.3$ kcal mol^{-1}, $\Delta_R H_{298} = -14.8$ kcal mol^{-1}, $\Delta_R G_{298} = -1.9$ kcal mol^{-1}. [e] The unknown rate constant for hydrolysis of GS(O)ONO was estimated to be similar to the rate constants for simple alkyl nitrites.[72] [f] From $k(26+27) = 1 \times 10^{10}\,M^{-1}s^{-1}$[66] (26 and 27 refer to the entry number) and the assumption $k(26)/k(27) \approx 1$. [g] The rate constant for the recombination of the $NO_2^{\bullet}$ radical with the glutathiyl radical was estimated to be similar to that for the recombination of $NO_2^{\bullet}$ with the tyrosyl radical, that is, $k(NO_2^{\bullet} + \text{tyrosyl radical}) = 3 \times 10^9\,M^{-1}s^{-1}$.[1] [h] Only the rate-limiting step of the catalysis is known. For the sake of simplicity, the second step was assumed to proceed at the same rate. [i] These rate constants correspond to the known yield $NO_2^{\bullet}/HO^{\bullet}$ of $28 \pm 2\%$[18, 73] obtained from peroxynitrous acid homolysis and the apparent rate constant for the decay of peroxynitrite at 298 K ($k_{exp} = 1.2\ s^{-1}$).[2]

radicals,[21, 38] whereby tyrosine should be preferably attacked by $CO_3^{\bullet-}$ with subsequent recombination of the tyrosyl radical with $NO_2^{\bullet}$ [Eqs. (4)–(8)]:

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \quad (4)$$

$$ONOOCO_2^- \rightarrow CO_3^{\bullet-} + NO_2^{\bullet} \quad (5)$$

$$CO_3^{\bullet-} + \text{tyrosine} \rightarrow HCO_3^- + \text{tyrosyl radical} \quad (6)$$

$$NO_2^{\bullet} + \text{tyrosine} \rightarrow NO_2^- + \text{tyrosyl radical} \quad (7)$$

$$NO_2^{\bullet} + \text{tyrosyl radical} \rightarrow NO_2\text{-Tyr} \quad (8)$$

Moreover, $NO_2^{\bullet}$ itself should also be able to oxidize tyrosine, albeit with a somewhat lower rate [k(tyrosine + $NO_2^{\bullet}) = 3.2 \times 10^5\,M^{-1}s^{-1}$] than oxidation by $CO_3^{\bullet-}$.[39] In fact, $NO_2^{\bullet}$ attacks a variety of phenols by such a radical mechanism.[40] Thus, an effective antioxidant should provide two means of preventing peroxynitrite-mediated nitration of tyrosine, firstly by scavenging both the $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ radicals, and, secondly, by regenerating tyrosine through the re-reduction of tyrosyl radicals. According to the known reactivity of $CO_3^{\bullet-}$ towards tyrosine ($k = 4.7 \times 10^7\,M^{-1}s^{-1}$)[41] and GSH ($k = 5.3 \times 10^6\,M^{-1}s^{-1}$),[41] effective prevention of a $CO_3^{\bullet-}$ attack on tyrosine would only be expected to occur at a [GSH]/[tyrosine] concentration ratio > 10. In marked contrast to this expectation, however, the GSH concentration found to be necessary to inhibit tyrosine consumption to half of its maximum level was only 66% of that of the concentration of tyrosine. This fact, in combination with the observation that the GSH-mediated inhibition of tyrosine consumption (Figure 1) is accompanied by an enhanced oxygen uptake (Table 2), is strongly suggestive of a GSH-derived repair function. In line with this conclusion, Sturgeon et al. also reported on the ability of GSH to repair tyrosyl radicals generated by the action of horseradish peroxidase.[42] However, as GSH was found to inhibit the formation of NO_2-Tyr more effectively than the consumption of tyrosine, the repair function of GSH cannot be the only protective pathway. Since ESR trapping experiments showed that $GS^{\bullet}$ radicals are formed from the reaction of peroxynitrite with GSH, they are likely to be involved in the deactivation process for $NO_2^{\bullet}$. We believe that the $GS^{\bullet}$ radical-derived pathway for deactivation of $NO_2^{\bullet}$ involves the formation of peroxynitrate, since we found that peroxynitrate does not attack tyrosine. From our recent observations that $O_2^{\bullet-}$ traps $NO_2^{\bullet}$ in a peroxynitrite-containing environment[38] and that peroxynitrate oxidizes NADH with only low efficiency,[24] it appears very likely that the intermediate formation of O_2NOO^-/O_2NOOH is a general and effective deactivation mechanism for $NO_2^{\bullet}$. However, the situation in vivo is different from that in vitro in that: i) GSH is rapidly reconverted to the reduced state, ii) the oxygen concentration is maintained at significantly lower levels (≈ 40 μM), and iii) high concentrations of Cu,Zn-superoxide dismutase are present in the cytosol. Considering these facts and assuming a reasonable flux of peroxynitrite of 10 μM s^{-1} at pH 7.4,[7] we again applied the kinetic model to mimic the in vivo situation.[43] Interestingly, under such conditions, the kinetic model predicts that the $NO_2^{\bullet}$ radicals

should be almost exclusively deactivated (≈98%) by reaction with the thiolate form of glutathione (GS^-). The situation may be somewhat different in mitochondria, because it is known that recombinant human Mn-SOD is readily inactivated by peroxynitrite.[44] For such a situation, in which SOD is not available, the kinetic simulation predicts that the $NO_2^\bullet$ radicals will be deactivated by reaction with GS^- as well as by formation of peroxynitrate with nearly the same efficiency (53 and 47%, respectively).

In man, intracellular GSH is present at concentrations of 1–10 mM (see, for example, ref. [45]), whereas the intracellular concentration of ascorbate lies in the range 0.5–2 mM.[46–48] Thus, the GSH concentration can be tenfold higher than that of ascorbate. At pH 7, the $CO_3^{\bullet-}$ radical reacts about 260 times more rapidly with ascorbate than with GSH. Thus, at a [GSH]/[ascorbate] ratio of 10:1, ascorbate should preferably (>96%) terminate the $CO_3^{\bullet-}$ radical. On the other hand, there is evidence that ascorbate may repair certain substrate radicals faster than GSH.[49] Two research groups concluded that ascorbate completely outcompetes GSH in repairing phenoxyl radicals, because both oxygen and GSH consumption were found to be suppressed in the presence of ascorbate.[42, 49] However, both groups overlooked the fact that ascorbate rapidly repairs (reduces) glutathiyl radicals [$k(\text{ascorbate} + GS^\bullet) = 6 \times 10^8\,M^{-1}s^{-1}$],[30] and by this means inhibits both oxygen and GSH consumption. Our data suggest that GSH is only three times less effective than ascorbate in inhibiting $CO_3^{\bullet-}$ and $NO_2^\bullet$ radical-driven tyrosine consumption. Thus, at an intracellular [GSH]/[ascorbate] ratio of 10:1, GSH should make a significant contribution to the repair of tyrosyl radicals.

In the present study, compared with a (patho)physiological situation, we employed relatively high peroxynitrite concentrations in order to increase the reliability of the quantitative analysis of tyrosine consumption and NO_2-Tyr formation. As the yield of NO_2-Tyr is predominantly determined by the [peroxynitrite]/[tyrosine] ratio,[28, 50] low steady-state concentrations of peroxynitrite may lead to a sizable consumption of tyrosine, but should not be effective in producing NO_2-Tyr. Because physiological GSH concentrations completely inhibit both tyrosine consumption as well as NO_2-Tyr formation even at physiologically unrealistic high levels of peroxynitrite, we conclude that any non-enzymatic pathway for tyrosine consumption in which tyrosyl radicals are involved is highly unlikely in the presence of such GSH concentrations. It has been suggested that catalase,[51] Cu,Zn-superoxide dismutase,[52] heme-thiolate,[53] and manganese/iron porphyrins[54] catalyze peroxynitrite-mediated nitration reactions. However, to the best of our knowledge, there has as yet been no conclusive proof that these catalysts induce nitration reactions in the presence of both CO_2 and physiological concentrations of GSH. Thus, NO_2-Tyr formation by the action of freely diffusing $CO_3^{\bullet-}$ and $NO_2^\bullet$ radicals is only possible when GSH is either not available (e.g. in membranes or in plasma) or if its concentration is strongly decreased in pathological situations.

Experimental Section

Materials: Catalase from beef liver (EC 1.11.1.6) and copper-zinc superoxide dismutase from bovine erythrocytes (EC 1.15.1.1) were obtained from Roche (Mannheim, Germany). Manganese dioxide, hydrogen peroxide, DTPA, GSH, cysteine, and H_2O_2 were purchased from Sigma (Deisenhofen, Germany). Ascorbic acid, H_3PO_4 (supra pure), and $HClO_4$ (supra pure) were obtained from Merck (Darmstadt, Germany). Mixtures of oxygen 5.0 and nitrogen 5.0 (20.5% O_2/79.5% N_2, "synthetic air"), oxygen 5.0, nitrogen 5.0, and carbon dioxide 4.6 (20.5% O_2/74.5% N_2/5% CO_2) were purchased from Messer-Griessheim (Oberhausen, Germany; "5.0" and "4.6" mean purities of 99.999% and 99.996%, respectively). Peroxynitric acid (O_2NOOH) solutions (1.57±0.02 M) were freshly prepared on a daily basis as recently described elsewhere.[24, 55] Oxoperoxonitrate(1–) (0.73 M) was prepared by the reaction of isoamyl nitrite with hydrogen peroxide [0.12 mol isoamyl nitrite, 100 mL H_2O_2 (1 M) plus DTPA (2 mM)] and purified (sixfold extraction with *n*-hexane, solvent extraction, removal of excess H_2O_2 by passage over MnO_2, N_2-purging) as described by Uppu and Pryor.[56] The peroxide thus obtained was divided into 200 μL aliquots and stored in vials (Eppendorf, Hamburg, Germany) at −79 °C. All other chemicals were of the highest purity commercially available. The program Chemical Kinetics Simulator 1.01 (CKS) was kindly donated by International Business Machines Corporation (http://www.almaden.ibm.com/st/msim/).

Solutions: Care was taken to exclude possible contamination by bicarbonate/carbon dioxide and transition metals. So-called "synthetic air" was bubbled (2 L min^{-1}) through doubly-distilled water at room temperature for 20 min. This water was used in the synthesis of oxoperoxonitrate(1–) and for all other solutions. Traces of transition metal ions were removed from the final phosphate buffer solutions (10 mL, 50 mM) by gently shaking with the heavy metal scavenger resin Chelex 100 (0.4 g) for 18 h in the dark. After low-speed centrifugation for 5 min, the solutions were carefully decanted from the resin. In the course of the resin treatment, the pH increased by about 0.25 units. Thereafter, the various additives (DTPA, tyrosine, GSH) were added. The pH was readjusted to 7.5 by the addition of phosphoric acid (50 mM) at 37 °C and synthetic air or the CO_2 mixture was again bubbled (2 L min^{-1}) through the solutions for 20 min. In the case of bubbling with the CO_2 mixture, the pH had to be readjusted to 7.5 once more.

Experimental conditions: The experiments with peroxynitrite (2 μL of 0.5 M $ONOO^-$ in 0.5 M NaOH was added to 1 mL of buffer solution) and with peroxynitric acid (1 μL of 1.57 M O_2NOOH was added to 1 mL of buffer solution) were performed in reaction tubes (1.4 mL, Eppendorf, Hamburg, Germany) by using the drop-tube vortex mixer technique as described previously.[32] Under HCO_3^-/CO_2-free conditions, these experiments were performed in a glove-bag (Roth, Karlsruhe, Germany) under synthetic air.

Determination of peroxynitric acid: The concentration of the peroxynitric acid stock solution (0.2 μL) was quantified by the amount of O_2 released in 100 mM potassium phosphate buffer (1 mL) at pH 11. O_2 was determined polarographically with a Clark-type oxygen electrode (Saur, Reutlingen, Germany).

Determination of $O_2^{\bullet-}$: Superoxide radicals were determined by the modified ferricytochrome *c* assay of McCord and Fridovich.[57] Peroxynitrite (1 mM) was vortexed into the reaction solution in the presence of both GSH (1 mM) and cytochrome c^{3+} (20 μM) and in the absence and presence of SOD (625 nM = 100 units mL^{-1}). GSH was added in excess to prevent reaction of (residual) peroxynitrite with SOD and the cytochrome c^{2+} formed. The resulting mixture was kept at 37 °C for 2 min. Cytochrome c^{2+} formation was quantified by measuring its absorbance at 550 nm ($\Delta\varepsilon_{550} = 21\,000\,M^{-1}cm^{-1}$).[58] The difference in cytochrome *c* reduction in the presence and absence of SOD was used to calculate the amount of trapped $O_2^{\bullet-}$.

Determination of peroxynitrite-driven nitration reactions: Peroxynitrite (1 mM) was vortexed into potassium phosphate buffer solutions (50 mM, 37 °C, pH 7.5) that contained the substrates, namely *para*-hydroxyphenylacetic acid (*p*-HPA), tyrosine, or tryptophan (each 1 mM), and varying concentrations of either ascorbate or glutathione (0–1000 μM) in the absence and in the presence of HCO_3^-/CO_2 (25 mM/5%). After vortexing, the samples were allowed to stand for 2 min. In the case of tryptophan, the absorbance of 6-nitrotryptophan[59] was detected photometrically at 400 nm ($\varepsilon_M = 5200\,M^{-1}cm^{-1}$). With *p*-HPA and tyrosine as substrates, 0.5–1 M NaOH was added (4:1 *v/v*, final pH 11–11.5). The final products, that is, 3-nitro-4-hydroxyphenylacetic acid and 3-nitrotyrosine, respectively, were detected by UV/Vis spectroscopy at 430 nm ($\varepsilon_M = 4400\,M^{-1}cm^{-1}$)[56] and at

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428 nm ($\varepsilon_M = 4200\,M^{-1}cm^{-1}$),[60] respectively. Each value represents the mean ± standard deviation of four experiments performed in duplicate.

Capillary zone electrophoresis measurements: Tyrosine and 3-nitrotyrosine were quantified on a Beckman P/ACE 5000 apparatus. Separation conditions for tyrosine and NO_2-Tyr were as follows: fused silica capillary (50 cm effective length, 75 μm internal diameter), hydrodynamic injection for 5 s, temperature 30 °C, voltage 18 kV, normal polarity, UV detection at 214 nm. A mixture of 50 mM sodium phosphate, 25 mM sodium borate, and 50 mM sodium dodecyl sulfate (pH 9.0) was used as the electrolyte system. To each sample, 0.2 mM of *p*-hydroxybenzoic acid was added as an internal standard.

ESR measurements: ESR spectra were recorded at ambient temperature on a Bruker ESP-300E X-band spectrometer (Bruker, Rheinstetten, Germany) equipped with a TM_{110} wide-bore cavity. Solutions were prepared from 1 mL of buffer solution (pH 7.5) containing HCO_3^- (50 M) and GSH (10 mM). Peroxynitrite (1 mM) was added to each solution by vortexing as described above. The reaction solutions were quickly transferred to an aqueous solution quartz cell (Willmad, Buena, N.J., USA). The first spectra were recorded as soon as possible, that is, within 1 min, and thereafter at 5 min intervals. Recording conditions: microwave frequency, 9.8 GHz; modulation, 0.04 mT; signal gain, 5×10^5; sweep range, 20 mT; sweep time, 4 min. Simulated spectra were generated using the WinSim program.[61]

CIDNP measurements: For these experiments, peroxynitrite was generated in 10 mm NMR tubes by adding H_2O_2 (1M) to a solution of $Na^{15}NO_2$ (50 mM) in potassium phosphate buffer (300 mM, pH 4.5) in the absence and in the presence of various additives (50 mM HCO_3^-; 10 or 200 mM GSH). After mixing, the NMR tubes were quickly transferred to the probe head of an NMR spectrometer (BRUKER DPX-300) and locked within 1 min (internal lock: 10 % D_2O). The first ^{15}N NMR spectrum was recorded 1 min or later after mixing the reactants by using single pulses with pulse angles of 90°. Subsequent ^{15}N NMR spectra were recorded at intervals of 3–12 min until no further spectral changes were observed.

Quantum chemical calculations: Density functional theory (DFT) calculations were carried out with the Gaussian 98W (Revision A.9) suite of programs.[62] Geometries were fully optimized to stationary points using the B3LYP method on the 6–31 + G(d) basis set. Molecular interactions were evaluated on the optimized gas-phase geometries with both the PCM[63] and the IPCM[64] procedures incorporated in Gaussian 98W.

Kinetic analysis: By far the most common computational methods of numerical simulations of chemical reactions use a deterministic approach, in other words one in which the time dependencies of the species concentrations is written as a set of coupled differential equations that are then numerically integrated in an iterative process. The stochastic simulation method, which is used in the software package "Chemical Kinetics Simulator 1.01" applied here, simulates a reaction scheme by using probabilities for the reaction of the various species derived from the rate laws of each step in the mechanism. The set of reactions and their corresponding rate constants used in the kinetic simulation are collected in Table 6. In order to obtain reliable product yields, the number of molecules employed must be sufficiently high. In the present kinetic analysis the simulation was performed by assuming $5-10 \times 10^7$ reacting molecules. Increasing the number of reacting molecules by a factor of ten led to identical results.

Final remark: While this paper was in press, Bonini and Augusto reported on the peroxynitrite-dependent formation of $RSSR^{\bullet-}$ and $RSO^{\bullet}$ radicals, respectively.[74]

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