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Electrophilic Sulfhydration of 8-Nitro-cGMP Involves Sulfane Sulfur.

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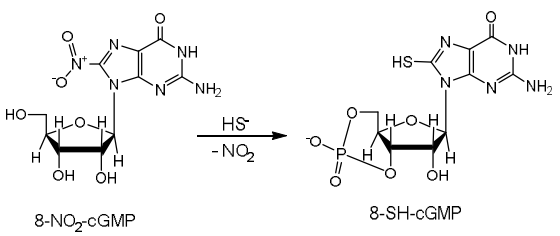
The formation of 8-SH-cGMP from the reaction between hydrogen sulfide and 8-nitro-guanosine-3',5'-cyclic monophosphate in the presence of thiols does not take place by nucleophilic attack of the hydrosulfide anion, as previously proposed, but first involves the formation of reactive species containing sulfane sulfur, like persulfides.

Introduction

Hydrogen sulfide (H_2S)[†] is proposed to be the third gaseous transmitter in mammals, along with nitric oxide (NO) and carbon monoxide (CO).[1-3] It was found to serve as a vasodilator and a novel neuromodulator, to possess pro- and anti-oxidant functions and to be an endogenous regulator of inflammatory response, playing both pro- and anti- inflammatory roles in different systems and situations. However, the molecular basis for these biological activities are still unclear, even if its signaling properties have been ascribed to several reactions. For instance, H_2S signals through "S-sulfhydration"[4-5] of targeted proteins, is involved in a cross talk with NO by reacting with nitrosothiols to produce $H(S)_xNO$ (x=1,2) derivatives[6-7] and H_2S also reacts with electrophiles.[8] 8-nitro-guanosine-3',5'-cyclic monophosphate (8-NO_2-cGMP) is an electrophilic second messenger known to intervene in the post-translational modification of cysteine residues (S-guanylation) of redox-sensor proteins such as Keap1 and H-Ras.[9-10] More recently, H_2S was reported to react with 8-NO_2-cGMP to yield HS-cGMP, through "electrophilic sulfhydration" (Scheme 1),[8] a mechanism proposed to terminate this electrophile-mediated signaling.



Scheme 1. Electrophilic sulfhydration of 8-NO_2-cGMP.[10]

This reaction was shown to be very sluggish, but to proceed properly in the presence of both cysteine (Cys-SH) and transition metals or metalloproteins. Consequently, a mechanism involving the coordination of both HS^- and Cys-S^- to the metal species followed by the nucleophilic displacement of nitrite by the hydrosulfide anion was proposed.[8] However, this pathway is questionable due to the lack of such a precedent (nucleophilic substitution of a nitro group by HS^-) in the literature. Furthermore, nitro groups are known to be reduced to the corresponding amine by hydrogen sulfide (Zinin reaction).[11] This reaction was for instance recently applied to design H_2S-specific fluorescent sensors.[12-13] At last, the transformation of a nitro group into a thiol moiety has been described, but it occurs through a multi-step reactions mechanism.[14-17]

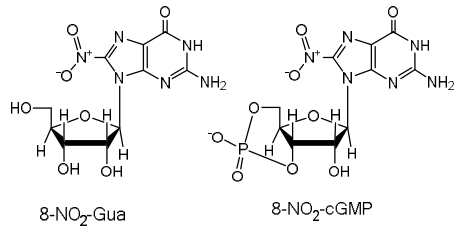
In this work, we show that H_2S reacts with 8-nitro-guanosine (8-NO_2-Gua, used as a model of 8-NO_2-cGMP) or 8-NO_2-cGMP. The reaction is inhibited by dioxygen, but it leads to the reduction of the nitro compounds into their corresponding amino analogs, *i.e.* 8-amino-guanosine (8-NH_2-Gua) and 8-amino-guanosine-3',5'-cyclic monophosphate (8-NH_2-cGMP), under hypoxic conditions. In the presence of additional thiols, these amino derivatives are side-products of the reaction while 8-SH-Gua or 8-SH-cGMP represent the main products along with the S-guanylation derivatives. A mechanism that involves the intermediate formation of reactive species containing sulfane sulfur, is discussed to account for these various reactivities.[††]

Results and Dicussion.

Reaction between 8-NO_2-Gua and H_2S.

Owing to the difficulty to synthesize and purify 8-NO_2-cGMP, we first decided to use the readily avalaible[18] analogous 8-NO_2-Gua (Scheme 2) as a model. Its shows similar reactivity with

Cys-SH than 8-NO_2-cGMP,[9] yielding the guanylated product 8-Cys-Gua along with nitrite release, under the original conditions used to study the S-guanylation of 8-NO_2-cGMP.[9] As shown in Figure 1a, the substitution of the nitro group (λ = 398 nm) by cysteine to form 8-Cys-Gua (λ = 270 nm) can easily be followed by UV-Visible spectroscopy.



Scheme 2. 8-nitro-guanosines derivatives used in this study.

Under similar conditions, the reaction between 8-NO_2-Gua and H_2S leads instead to the formation of a species absorbing at 290 nm (*vs* 270 nm for the S-guanylation product), and exhibits a different kinetic profile with the appearance of a lag phase. Its length is dependent upon the dioxygen content of the solution and can therefore be decreased by bubbling argon into the reaction mixture before the reaction takes place (Figure 1b, recorded under anaerobic conditions). The influence of dioxygen on the reaction is further illustrated in Figure S1a. While the reactivity between H_2S and 8-NO_2-Gua is slow under aerobic conditions, in agreement with the absence of reaction between hydrogen sulfide and 8-NO_2-cGMP without catalysts,[8] it is drastically accelerated under anaerobic conditions. Interestingly, the reaction between Cys-SH and the nitro derivative is only slightly impacted by the presence of dioxygen (Figure S1b), suggesting two different mechanisms for the reaction between 8-NO_2-Gua and H_2S or Cys-SH. This was confirmed by the absence of nitrite release during the reaction with H_2S, whereas almost quantitative yields were observed by the Griess assay in the presence of cysteine.

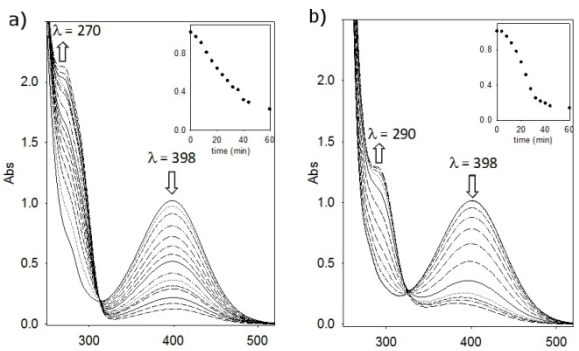


Figure 1. UV-Visible spectra recorded under anaerobic conditions every 4 minutes after the addition of Cys-SH (a) or H_2S (b) (10 mM final concentrations) to a 125 µM solution of 8-NO_2-Gua in phosphate buffer (100 mM, pH = 7.4, 200 µM DTPA). Insert: plot of the absorbance at 398 nm *vs* time.

To further investigate the mechanism involved during the reactivity of H_2S with 8-NO_2-Gua, we measured the oxygen consumption by polarography and observed a fast depletion of oxygen in the reaction mixture (Figure S2). A direct reaction between hydrogen sulfide and dioxygen cannot account for the above observations as H_2S only significantly reacts with O_2 in solution in the presence of trace elements,[19] which are removed in our experiments by using the chelating agent DTPA.[20] 8-NO_2-Gua, like many nitro-containing molecules,[21] mediates the production of superoxide in the presence of reductases, *e.g.* NADPH-dependent reductases.[22] In these systems, the reductases provide one electron to generate the radical-anion [8-NO2-Gua]$^{\bullet-}$ that subsequently reduces dioxygen to form superoxide. Accordingly, a similar pathway could most certainly account for the dioxygen depletion observed in the presence of both 8-NO_2-Gua and H_2S (Figure S2). Indeed, mixing these two reactants resulted in the formation of an EPR-active species (see Figure 2), characterized as the radical anion [8-NO_2-Gua]$^{\bullet-}$. Unfortunately, we were unable to detect the formation of superoxide using spin-trapping techniques,[23] probably because of the faster reaction between superoxide and hydrogen sulfide.[24-25] Under identical conditions, no EPR-active species was detected using cysteine instead of hydrogen sulfide.

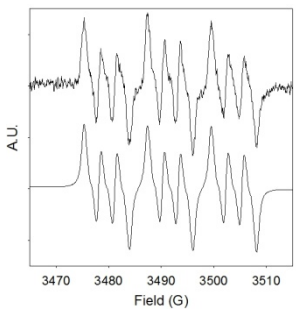


Figure 2. Room temperature EPR spectrum recorded after mixing 8-NO_2-Gua (500 µM) with H_2S (10 mM) in phosphate buffer (100 mM, pH = 7.4) (top), and simulated spectrum (bottom) obtained with the following parameters: $aN_{(NO2)}$ = 12.1 G, $aN_{(N7)}$ = 3.1 G, $aN_{(N9)}$ = 0.8 G, $aH_{(ribose)}$ = 0.4 G.[26]

The sensitivity of the reaction between 8-NO_2-Gua and H_2S to dioxygen is then likely explained by reactions 1 and 2.

$$8\text{-}NO_2\text{-}Gua + HS^- \rightarrow [8\text{-}NO_2\text{-}Gua]^{\bullet-} + H^+ + S^{\bullet-} \quad [1]$$

$$[8\text{-}NO_2\text{-}Gua]^{\bullet-} + O_2 \rightarrow 8\text{-}NO_2\text{-}Gua + O_2^{\bullet-} \quad [2]$$

It has to be noted that the radical anion $S^{\bullet-}$ can itself enter into several kinetically favored reactions, some accelerating the dioxygen depletion.[27] For instance, $S^{\bullet-}$ can directly react with O_2 (reaction 3, $k = 7.5\times10^9$ $M^{-1}s^{-1}$), with HS^- to generate the perthiyl radical anion $HSS^{\bullet2-}$ (reaction 4, $k_{forward} = 5.4\times10^9$ $M^{-1}s^{-1}$, $k_{reverse} = 5.3\times10^5$ s^{-1}) or two radical anions $S^{\bullet-}$ can recombine to produce the disulfane HSSH (reaction 5, $k = 6.5\times10^9$ $M^{-1}s^{-1}$). The radical anion $HSS^{\bullet2}$ can also react with oxygen, producing superoxide and the disulfane anion (reaction 6, $k = 4\times10^8$ $M^{-1}s^{-1}$). Finally, $HSS^{\bullet2-}$ can react with $S^{\bullet-}$ to generate the disulfane HSSH and HS^- (reaction 7, $k = 9\times10^9$ $M^{-1}s^{-1}$).

$S^{\bullet-} + O_2 + H^+ \rightarrow HSOO^{\bullet} \rightarrow H^+ + SO_2^{\bullet-}$ [3]
$S^{\bullet-} + HS^- \leftrightarrow HSS^{\bullet 2-}$ [4]
$S^{\bullet-} + S^{\bullet-} + 2\ H^+ \rightarrow HSSH$ [5]
$HSS^{\bullet 2-} + O_2 \rightarrow HSS^- + O_2^{\bullet-}$ [6]
$HSS^{\bullet 2-} + S^{\bullet-} + 2\ H^+ \rightarrow HSSH + HS^-$ [7]

Thus, dioxygen prevents the conversion of 8-NO_2-Gua by H_2S. However, under anaerobic conditions, this useless redox cycling is prevented and 8-NO_2-Gua is fully converted into a new species unambiguously identified as the reduction product 8-NH_2-Gua by HPLC-HRMS analysis (Figures S3). Although the 6-electrons conversion of a nitro moiety into an amino one is a complex process,[28] especially when the reductant is a sulfide derivative,[29] the following sequence can however be proposed on the basis of the results discussed above: as previously described, the one electron reduction of the nitro group by HS^- produces the nitro radical anion and the radical anion $S^{\bullet-}$. The latter can lead to the formation (reactions 5, 7) of disulfane HSSH (and its deprotonated forms HSS^- and S^{2-}, owing to the lower pKa of HSSH *vs* H_2S), a much better reducing agent that H_2S and its conjugated base HS^-.[30] Likely intermediates in the $NO_2 \rightarrow NH_2$ conversion are the nitro and hydroxylamine derivatives (equations 8-9).

$[8\text{-}NO_2\text{-}Gua]^{\bullet-} + e^- + 2H^+ \rightarrow 8\text{-}NO\text{-}Gua + H_2O$ [8]
$8\text{-}NO\text{-}Gua + 2e^- + 2H^+ \rightarrow 8\text{-}NHOH\text{-}Gua$ [9]

This mechanism is in agreement with the cyclic voltammetry study of 8-NO_2-Gua depicted in Figure S4, which indicates that nitro-guanosine electrochemically behaves like most of the aromatic nitro drugs studied to date.[21] The voltammogram recorded at low sweep rate (0.05 $V.s^{-1}$) in phosphate buffer (pH 7.4) shows a single irreversible cathodic peak, assigned to the 4-electrons steps of reactions 1,8 and 9. At higher sweep rate (5 $V.s^{-1}$), two irreversible peaks are observed (assigned to 1- and 3-electrons processes, corresponding to reactions 1 and 8-9, respectively). The one-electron peak is pH-independent, and becomes quasi-reversible at pH = 10.3, with a E° of *ca.* 0.3V *vs* NHE, which compares well with values reported for the generation of the radical anion derivative of similar compounds.[31]

At last, the hydroxylamine derivative is further reduced to generate the amino derivative (reaction 8).

$8\text{-}NHOH\text{-}Gua + 2e^- + 2H^+ \rightarrow 8\text{-}NH_2\text{-}Gua + H_2O$ [10]

Reaction between 8-NO_2-Gua and H_2S, in the presence of thiols.

In the original report of the formation of 8-SH-cGMP, the thiol used along with the metal catalysts was cysteine. In our study, we used both cysteine and penicillamine (Pen-SH), as we found that Pen-SH is inert towards 8-NO_2-Gua due to steric hindrance in comparison to Cys-SH. The use of Pen-SH thus limits the number of products formed during the reactions and simplifies mechanistic considerations. As observed by HPLC, the addition of thiols into the reaction mixture previously composed of H_2S and 8-NO_2-Gua (typically, 100 µM for 8-NO_2-Gua and 1 mM of thiols and H_2S were used in the following experiments) led to drastic changes in the end-products of the reaction (Figure 3). First, the reactions are less sensitive to the presence of dioxygen as the transformation of 8-NO_2-Gua noticeably proceeds under aerobic conditions, with conversion yields ranging from 60 % (Pen-SH + H_2S) to 75 % (Cys-SH + H_2S). Second, 8-NH_2-Gua is always detected in the reaction mixture but as a "side-product" (7-11 %). Thus, the main products are 8-Cys-S-Gua or 8-SH-Gua. On one hand, 8-SH-Gua is exclusively formed when Pen-SH is used as a thiol (53 and 68 % under aerobic or anaerobic conditions, respectively). On another hand, its formation (28 and 37 % under aerobic or anaerobic conditions, respectively) is accompanied by the generation of the S-guanylated compound 8-Cys-S-Gua (41 and 45 % under aerobic or anaerobic conditions, respectively) when Cys-SH is used as a thiol. These latter observations suggest that a similar reactive species is involved into the formation of 8-SH-Gua, whether Pen-SH or Cys-SH are used as thiols in addition to H_2S. However, in the case of Cys-SH, the reactive species enters in competition with Cys-SH to transform 8-NO_2-Gua.

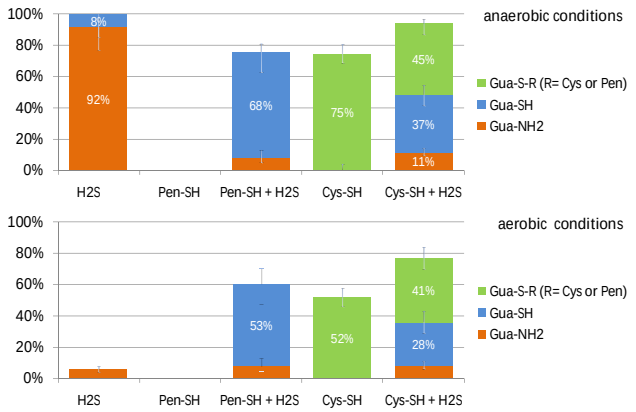


Figure 3. Products yields of the reactions between Gua-NO_2 and mixture of thiols and H_2S ([Gua-NO_2]: 100 µM, [H_2S] : 1 mM [R-SH]: 1 mM, in 20 mM Tris buffer containing 200 µM DTPA at pH 7.1 at 37°C for 3 h)

Interestingly, in the experiments with Pen-SH, we were able to detect the presence of small amounts of the derivative 8-Pen-S-S-Gua by HPLC-HRMS (Figure S5). Additionally, nitrite, di- and polysulfides (essentially as trisulfide) were also detected at the end of the reaction. These results suggest that a persulfide acts as the reactive species to form 8-SH-Gua by nucleophilic displacement of the nitrite. The likely involvement of a persulfide as a reactive species was confirmed by the recovery of more than 90% of the starting 8-NO_2-Gua when the reaction is conducted in the presence of 10 mM of cyanide anion, a classical acceptor of sulfane sulfur.

On the basis of these results, we propose the simplified mechanism described in Figure 4 to rationalize the reactivities of 8-NO_2-Gua. In this mechanism, the one electron reduction of the nitro group by HS^- serves as a starting point. The nitro radical anion will thus either produce superoxide (reaction 2) or

form the amino derivative (reactions 8-10) always observed in our experiments (Figure 4), while the radical anion $S^{\bullet-}$ will participate into the formation of the reactive persulfide species. This latter can be formed by at least two alternative mechanisms: (i) disulfane HSSH can be produced by reactions 4-7 and react with the thiolate RS^- to generate the persulfide anion RSS^- (reaction 11). This very good nucleophile then substitutes the nitro group of 8-NO_2-Gua to form the RSS-Gua derivative and nitrite (reaction 12). RSS-Gua is then easily reduced by either RS^- or RSS^- (reaction 13), the later accounting for trisulfide formation observed in our experiments. (ii) the radical anion $S^{\bullet-}$ can also directly react with the thiolate anion to generate the persulfide radical anion $RSS^{\bullet 2-}$ (reaction 14). This latter could then react with $S^{\bullet-}$ (or another oxidant) to generate the persulfide anion (reaction 15) that will then enter into the reactions 12 and 13 described above.

Thus, the addition of thiol shifts the reactivity from a reduction (likely involving HSSH) to a substitution (involving RSSH).

$$HSSH + RS^- \rightarrow RSS^- + H^+ + HS^- \quad [11]$$
$$RSS^- + 8\text{-}NO_2\text{-}Gua \rightarrow RSS\text{-}Gua + NO_2^- \quad [12]$$
$$RSS\text{-}Gua + R(S)_x^- + H^+ \rightarrow HS\text{-}Gua + RS(S)_xR \ (x=1,2) \quad [13]$$
$$RS^- + S^{\bullet-} \rightarrow RSS^{\bullet 2-} \quad [14]$$
$$RSS^{\bullet 2-} + S^{\bullet-} + H^+ \rightarrow RSS^- + HS^- \quad [15]$$

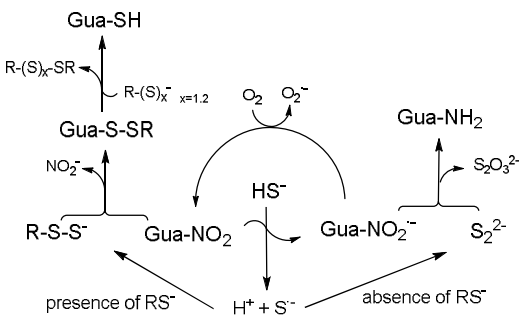


Figure 4. Simplified mechanisms accounting for the various reactivities of 8-NO_2-Gua with H_2S discussed in this work.

It must be noted that, in contrast to the previous study by Nishida *et al.* [8], we did not employ any transition metal salts or metalloproteins to achieve the conversion of 8-NO_2-Gua into 8-SH-Gua. However, as most of the metal catalysts used in the previous study were redox active, they would nicely fit into our mechanisms involving sulfhydryl radical species formation as a starting point.[32]

Extension to 8-NO_2-cGMP.

Finally, similar results were obtained with 8-NO_2-cGMP, confirming that 8-NO_2-Gua was a good mechanistic model. Thus, 8-NH_2-cGMP was the only derivative detected following the incubation of the nitro derivative with H_2S alone. Importantly, this amino derivative has been shown to be an intermediate metabolite in the recycling of 8-NO_2-cGMP into cGMP by a nitric oxide dependent reductive pathway in cells.[33] It is noteworthy that if the mechanistic details for the conversion of 8-NH_2-cGMP to cGMP are well understood, the first step of the recycling, that is the reduction of the nitro derivative into its amino analog, is still not elucidated.

When Pen-SH was added to the reaction mixture, 8-SH-cGMP was recovered as the main product (see Figure S6).

Conclusion

These results highlight the versatile reactivity of the second messenger 8-NO_2-cGMP. They also confirm that various biologically relevant reactions imputed to H_2S are in fact likely to involve derivatives containing « sulfane sulfur(s) » as the true reactive species.[34-37]

Material and Methods

See Supporting Information for full details.

Unless otherwise stated, reaction conditions for the reactivity studies with 8-NO_2-Gua and 8-NO_2-cGMP were the following: in 1.5 mL vials were successively added 1 mL of 20 mM Tris buffer containing 200 µM of DTPA at pH 7.1 at 37°C, 7 µL of a solution of nitro derivative (15 mM stock solution in 50/50 DMSO/water), 10 µL of thiol (100 mM stock solutions) and 10 µL of H_2S (100 mM stock solutions, prepared by dissolving 7 mg of anhydrous sodium hydrosulfide in 1.25 mL of buffer). After incubation for 3 hours at 37°C, the mixtures were analyzed by HPLC-(HR)MS. The terms "Anaerobic conditions" refer to the use of carefully degassed buffer and layering of the solution with argon.

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Notes and references

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† At physiological pH, H_2S dissociates into an equilibrium between H_2S and HS^-, with $pKa(H_2S/HS^-) = 6.97$ (the second $pKa(HS^-/S^{2-})$ is well above 14). In this paper "H_2S" refers to the mixture of the two forms obtained by dissolving the salt NaSH in a buffer solution, their respective proportions being fixed by the pH of the buffer.

†† During the writing of this paper, Akaike and *al.* nicely showed that 8-SH-cGMP (up to 3 µM after 3 hours in Tris buffer pH 7.4, 37°C) is formed when 8-NO_2-cGMP (1 mM) is reacted with a thiol (GSH, 100

μM), H_2S (100 μM) and a NO donor (P-NONOate, 100 μM). They proposed the implication of persulfides/polysulfides in this transformation.[36]

Electronic Supplementary Information (ESI) available: Additional figures discussed in the text. See DOI: 10.1039/b000000x/

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