New insights into the *S*-nitrosothiol–ascorbate reaction. The formation of nitroxyl

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Although the ascorbate-dependent reduction of *S*-nitrosothiol to the corresponding thiol function is frequently used for analyzing *S*-nitrosocysteinyl residues in proteins, it proceeds with low yields. Our re-investigation of the ascorbate–*S*-nitrosothiol reaction demonstrates now the intermediacy of nitroxyl ($HNO/^3NO^-$) that is highly effective in oxidizing thiols. The occurrence of the HNO reporter molecule, *i.e.*, N_2O, during and after reaction was unequivocally demonstrated by ^{15}N NMR. The yield of HNO from the *S*-nitrosoglutathione–ascorbate reaction was determined with the aid of a Mn(III)-complex to 60%, a value that was significantly higher than the one of nitric oxide formation (48%) at physiological pH. The same HNO yield was observed with a *S*-nitrosothiol bound to a protein (*i.e.*, *S*-nitroso-papain). With the known chemistry of nitroxyl, it was possible to optimize the experimental conditions so that the GSNO–ascorbate reaction yielded stoichiometric amounts of glutathione after a reaction period of 1 min.

Introduction

The important physiological mediator nitric oxide ($^{\bullet}NO$)[1,2] is a short-lived intermediate in blood because it can be both oxidized by oxyhemoglobin[3] and rapidly scavenged by the deoxygenated heme of hemoglobin to yield an iron(II)-nitrosyl complex.[4] It has been suggested that this iron(II)-nitrosyl entity transnitrosates β93 cysteine (reaction (1)).[5]

$$NO\text{-}Fe^{3+}\text{-Hem-}\beta93cys\_SH \rightarrow Fe^{2+}\text{-Hem-}\beta93cys\_SNO \quad (1)$$

The term "transnitrosation" means a direct transfer of the NO^+ function to a nucleophile Y^- without the occurrence of a second nitrosating intermediate. Since *S*-nitrosothiols *vice versa* can also act as transnitrosating species[6] (reaction (2))

$$RSNO + Y^- \rightarrow RS^- + YNO \quad (2)$$

it has been frequently suggested that *S*-nitrosothiols located in proteins can be involved in the transport and storage of $^{\bullet}NO$ because they can react with low molecular weight thiols *via* reaction (2) (with $Y^- = R'S(H)$). Although nitrosation of hemoglobin at β93 cysteine is the most intriguing and controversially discussed protein modification, there is general agreement that *S*-nitrosothiols are naturally occurring post-translational modifications on proteins and peptides.

Beside thiols, ascorbate (ASC^-; Scheme 1) can be a target nucleophile for *S*-nitrosothiols[7–9] thereby yielding *O*-nitrosoascorbate ($NOASC^-$, reaction (2a)).[10]

$$RSNO + ASC^- \rightarrow RSH + NOASC^- \quad (2a)$$

Reaction (2a) has some impact for analytical purposes because ascorbate reduces *S*-nitrosothiols to thiols in the absence of copper ions.[9,11] (In its presence ascorbate reduces additionally disulfide bonds to thiols *via* a complex mechanism.[12]) Recently we[10] found that at physiological pH values the decay of *O*-nitrosoascorbate proceeds by various competitive pathways and one of them yields dehydroascorbic acid (DHA) and nitroxyl ($HNO/^3NO^-$) (reaction (2b)).

$$NOASC^- + H^+ \rightarrow DHA + HNO \quad (2b)$$

Thus, the thiol-depleting intermediate nitroxyl[13–16] may be generated during the ascorbate-dependent reduction of *S*-nitrosothiols.

Here we demonstrate that the reaction of *S*-nitrosothiols with ascorbate generates in fact nitroxyl with high yields at physiological pH values. In addition, experiments were performed to determine the experimental conditions where the *S*-nitrosothiol moiety is quantitatively reduced to a thiol moiety by ascorbate (reaction (2a)).

Results

Formation of nitroxyl

Provided that the short-lived intermediate nitroxyl is indeed generated from the *S*-nitrosothiol–ascorbate reaction (see reactions (2a) and (2b)), it can decompose at physiological pH values *via* the following pathways:[15,17]

$$2HNO \rightarrow HON{=}NOH \quad (3a)$$

$$HON{=}NOH \rightarrow N_2O + H_2O \quad (3b)$$

$$HNO + {}^{\bullet}NO \rightarrow H^+ + N_2O_2^{\bullet-} \quad (4a)$$

$$N_2O_2^{\bullet-} + {}^{\bullet}NO \rightarrow N_2O + NO_2^- \quad (4b)$$

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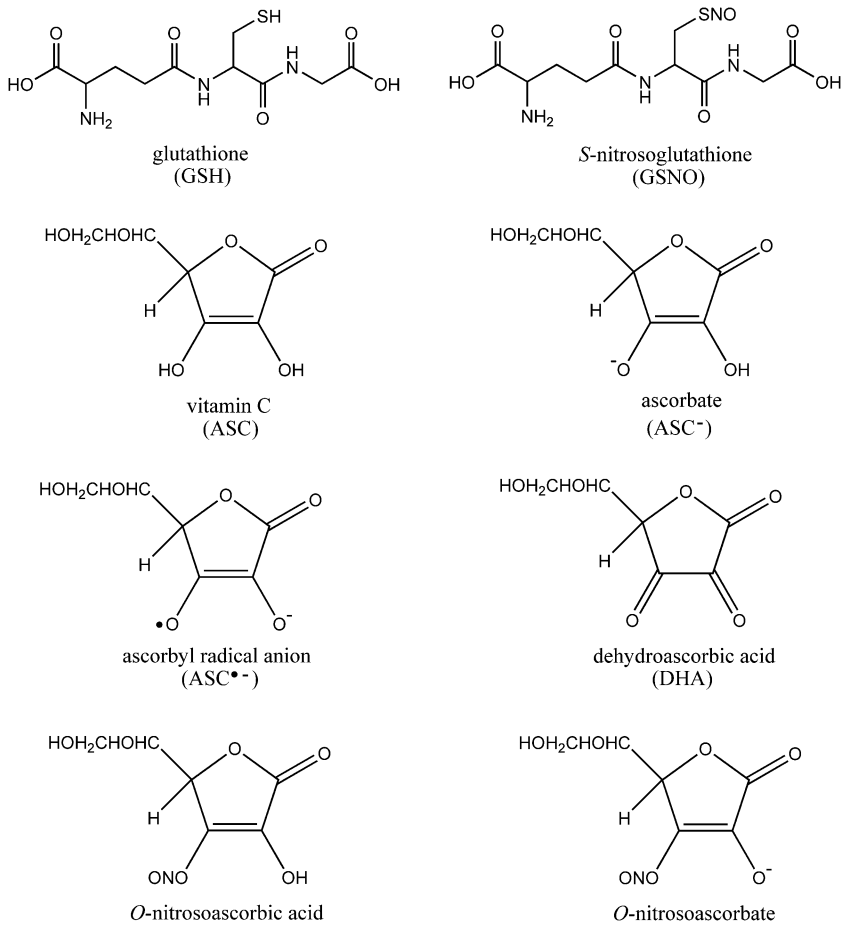


Scheme 1

Since these decomposition pathways yield nitrous oxide as a stable end product, formation of N_2O serves as a reporter molecule for the intermediacy of HNO.[15] In order to demonstrate the intermediary of HNO during the reaction of *S*-nitrosothiol with ascorbate, ^{15}N-enriched *S*-nitrosoglutathione (GS^{15}NO) was mixed with ascorbate and the reaction solution was analyzed by ^{15}N NMR spectrometry (Fig. 1A,B).

During the reaction (Fig. 1A) $^{15}NO_2^-$ ($\delta = 229$ ppm) and $^{15}N_2O$ ($\delta = -147$ ppm and $\delta = -230$ ppm) were detected, *i.e.*, two products typical for the intermediacy of HNO (reactions (3a)–(4b)). After reaction (Fig. 1B) two additional products, namely $^{15}NO_3^-$ ($\delta = -4$ ppm) and $^{15}NH_4^+$ ($\delta = -359$ ppm) were clearly identified. There is evidence that HNO is partially oxidized to nitrate under aerobic conditions[18,19] whereas NH_4^+ may be formed by either GSH-dependent reduction of HNO[20] or reaction of (generated) GSH with (residual) GSNO.[21] In any case, formation of NH_4^+ indicated that GSH is not stoichiometrically formed from the GSNO–ascorbate reaction at a physiological pH value. However, the intermediacy of HNO during that reaction is clearly demonstrated.

Since product yields are generally difficult to be quantified by ^{15}N NMR spectrometry and because "only" the reporter molecule can be analyzed by such a procedure, the foregoing experiments were repeated at much lower concentrations in the presence of the complex Mn^{III}-tetrakis-(1-methyl-4-pyridyl)-porphyrin (Mn^{III}TMPyp, 10 μM). Recently Marti *et al.*[22] introduced Mn^{III}-porphyrin derivatives for the specific detection of nitroxyl and we[23,24] demonstrated that the commercially available Mn^{III}TMPyp works in a similar manner. Because Marti *et al.*[22] mentioned that Mn^{III}-porphyrin derivatives react stoichiometrically with nitroxyl in the absence of molecular oxygen, the following experiments were performed under hypoxic conditions (residual $O_2 < 10$ μM). During reaction of GSNO (5 μM) with ascorbate (2 mM) the HNO-dependent decrease of the Soret band of Mn^{III}TMPyp ($\varepsilon_{463} = 9.2 \times 10^4$ M^{-1} cm^{-1} [25]) occurred instantaneously with concomitant build-up of the absorption of the nitroso complex Mn^{II}TMPyp-NO at $\lambda_{max} = 435$ nm ($\varepsilon_{435} = 1.1 \times 10^5$ M^{-1} cm^{-1} [23]) which specifically indicated the intermediacy of HNO (Fig. 2A). Noticeably, the complex Mn^{II}TMPyp-NO was not formed from a combined action of nitric oxide donor (MAHMA/NO, 10 μM), GSH (10 μM) and ascorbate (2 mM) under such a condition (data not shown).

Since Zhang and Hogg[26] questioned that the *S*-nitrosothiol–ascorbate reaction is effective with *S*-nitrosocysteine residues, the HNO-donating capabilities of GSNO were compared with those of *S*-nitroso-papain (Fig. 2B, 10 μM each). In fact, the ascorbate-mediated release of HNO was slightly faster from GSNO than from *S*-nitroso-papain but the amount of liberated nitroxyl was obviously identical. In order to answer the question about the yield of the formed nitroxyl, the concentration of GSNO was varied (Fig. 2C). The lowest applied GSNO concentration (1.25 μM)

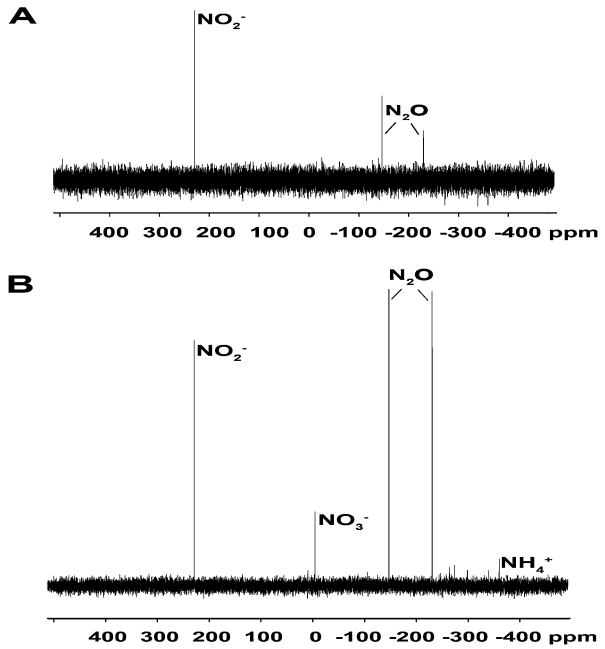


Fig. 1 ^{15}N NMR product analysis of the GS^{15}NO–ascorbate reaction. The analysis was performed in potassium phosphate buffer solution (200 mM, final pH 7.4, 25 °C) with GS^{15}NO and ascorbate (100 mM each). (A) ^{15}N NMR spectrum after a reaction period of 51 min. (B) ^{15}N NMR spectrum after a reaction period of 51 h.

generated about 0.75 μM Mn^{II}TMPyp-NO, *i.e.*, Mn^{II}TMPyp-NO was generated with a yield of about 60%. Increasing the concentration of GSNO up to 7.5 μM increased the concentration of Mn^{II}TMPyp-NO in a strictly linear manner with a slope of 0.6 (again corresponding to a yield of 60%) to reach a value of about 4.5 μM. The Mn^{II}TMPyp-NO concentration leveled off at this value at higher GSNO concentrations. The reasons for this leveling off were not clarified in detail, but it should be noted both that nitroxyl dimerisation (reactions (3)) was the dominant pathway at HNO-donor (Angeli's salt) concentrations >5 μM and that (generated) GSH reacts moderately fast ($k = 2 \times 10^6$ M^{-1} s^{-1}) with HNO.[27]

Formation of nitric oxide

When nitroxyl is formed with a yield of 60% from the GSNO–ascorbate reaction one might argue that generation of nitric oxide should be undetectable because of the •NO-depleting reactions outlined in reactions (4a) and (4b). However, these reactions are in competition with both the self-decay of HNO (reactions (3a) and (3b)) and the •NO-yielding GSNO-nitroxyl reaction (reaction (5)):[20]

$$\text{GSNO} + \text{HNO} \rightarrow \text{GSH} + 2^{\bullet}\text{NO} \quad (5)$$

In order to demonstrate that the intermediary HNO supports partly the *S*-nitrosothiol-dependent release of •NO, the liberation of nitric oxide from the GSNO–ascorbate reaction was monitored in the absence and in the presence of copper, zinc superoxide dismutase (SOD), because SOD is highly effective in oxidizing nitroxyl to nitric oxide under aerobic conditions.[28] Since the experiments had to be carried out in an open experimental setup, only the steady-state equilibrium concentration of •NO with respect to diffusion into the gas phase and autoxidation could be determined. Fig. 3 represented two typical runs.

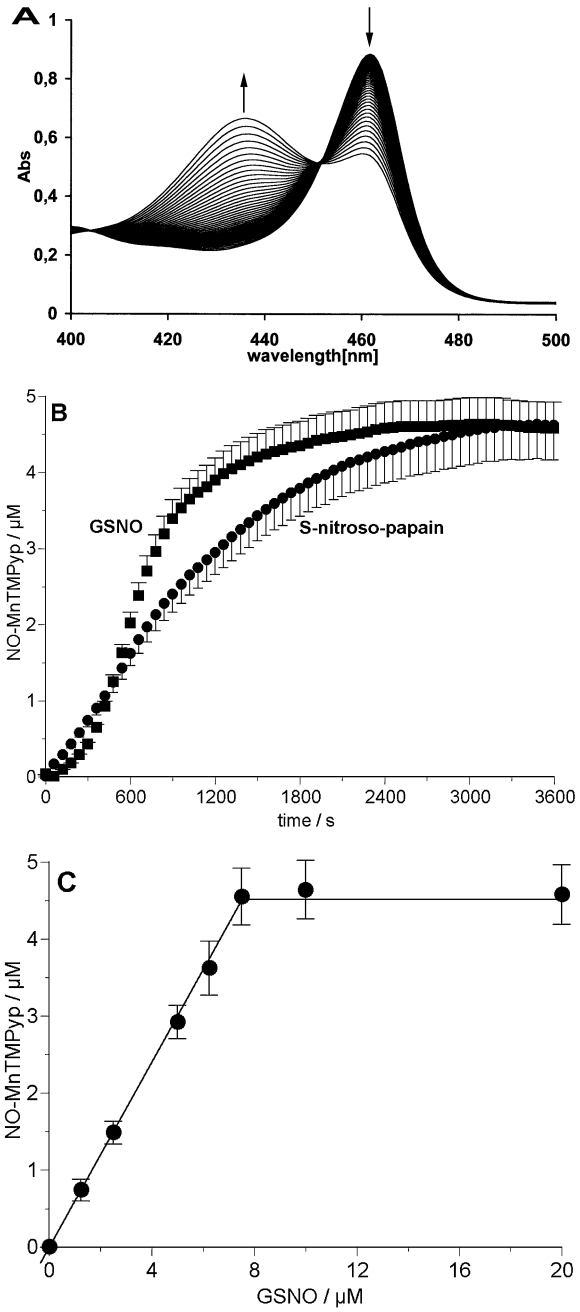


Fig. 2 Quantification of the nitroxyl yield. The reactions were performed in phosphate buffer (50 mM, pH 7.4, 25 °C, 100 μM EDTA, 10 μM Mn^{III}TMPyp, 1 mM ascorbate, hypoxia (O_2 < 10 μM)). Experiments were started by adding ascorbate. (A) Typical kinetics with 10 μM GSNO. The delay between two scans was 60 s. (B) Time-dependent increase of the nitroxyl adduct at $\lambda = 435$ nm from either GSNO or *S*-nitroso-papain (10 μM each). (C) Dependence of the yield of the nitroxyl adduct from the applied GSNO concentration.

In the absence of SOD the steady-state •NO concentration increased in an exponential manner and leveled off to reach a plateau value of 2.8 ± 0.2 μM. In the presence of a high SOD activity (1000 units/ml) a similar increase in the steady-state •NO concentration was evident but the plateau value was with

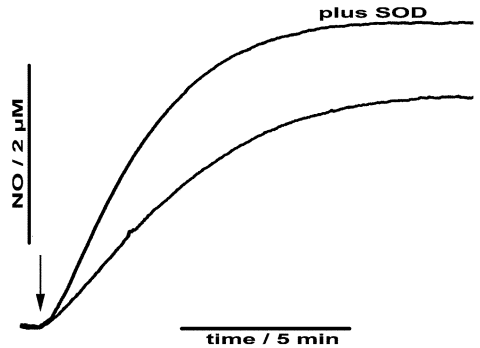


Fig. 3 Nitric oxide generation. Steady-state concentration of nitric oxide generated from reaction of GSNO (200 µM) with ascorbate (2 mM) in phosphate buffer (50 mM, pH 7.4, 25 °C) in the absence and in the presence of SOD (1000 units/ml) were monitored using an •NO-sensitive electrode. The selected data are representative single scans of four independently performed experiments.

3.4 ± 0.3 µM, 21% higher than in the absence of SOD. The same relationship was found for the corresponding final nitrite concentrations with 111 ± 3 µM in the absence of SOD and with 128 ± 3 µM (*i.e.*, an increase of 15%) in the presence of SOD after a reaction period of 2 h. Since HNO is generated with a yield of 60% (see above) and because nitrite is generated with yields of 55.5% (= 111 µM nitrite/200 µM GSNO, absence of SOD) and 64.0% (= 128 µM nitrite/200 µM GSNO, presence of SOD), respectively, only a part of the intermediary nitroxyl can decompose *via* the non-nitrite generating bimolecular self-decay mechanism (reactions (3a) and (3b)). In order to quantify the ascorbate (2 mM)-mediated production of nitric oxide from various concentrations of either GSNO or the nitroxyl donor Angelis's salt (0–7.5 µM) during the entire reaction period, its formation was monitored by means of the •NO scavenger FNOCT-4 which produces the stable fluorescent product FNOCT-4-NOH.[29]

The lowest applied GSNO concentration (0.5 µM) generated FNOCT-4-NOH with a yield of 48% (*i.e.*, about 0.24 µM, Fig. 4). This was obviously the general yield of the GSNO–ascorbate reaction because a stepwise increase of the GSNO concentration to 7.5 µM increased the corresponding FNOCT-4-NOH concentrations in a strictly linear manner with a slope of 0.48. Under such a condition the nitroxyl donor Angelis's salt generated •NO with a yield of only 8.8%, thereby ruling out that HNO was an effective •NO source in the presence of ascorbate. Since the yield of •NO was somewhat lower than the yield of nitrite, a nitrite-yielding pathway other than autoxidation of •NO to N_2O_3 and subsequent hydrolysis of it was additionally operating.

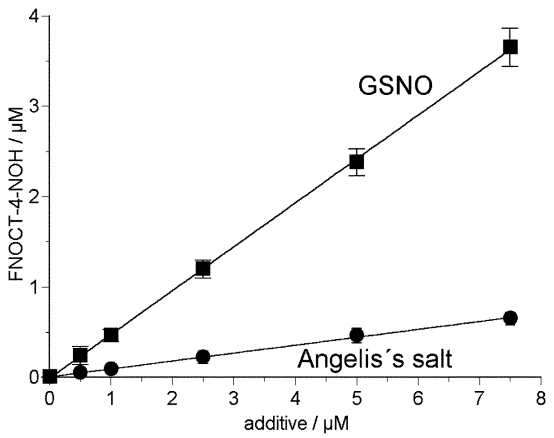


Fig. 4 Quantification of the •NO yield. The reactions were performed in phosphate buffer (50 mM, pH 7.4, 25 °C, 100 µM EDTA, 5 µM FNOCT-4, 1 mM ascorbate, hypoxia (O_2 < 10 µM)) and various concentrations of either GSNO or Angeli's salt. Experiments were started by adding ascorbate.

Formation of thiols

Since most researchers are interested in the yield of thiol from the *S*-nitrosothiol–ascorbate reaction, the pH-dependence of the GSNO–ascorbate (100 µM/2 mM) reaction was analyzed either in the presence of oxygen (Fig. 5A) or under hypoxic conditions (Fig. 5B) with the aid of Ellman's reagent.

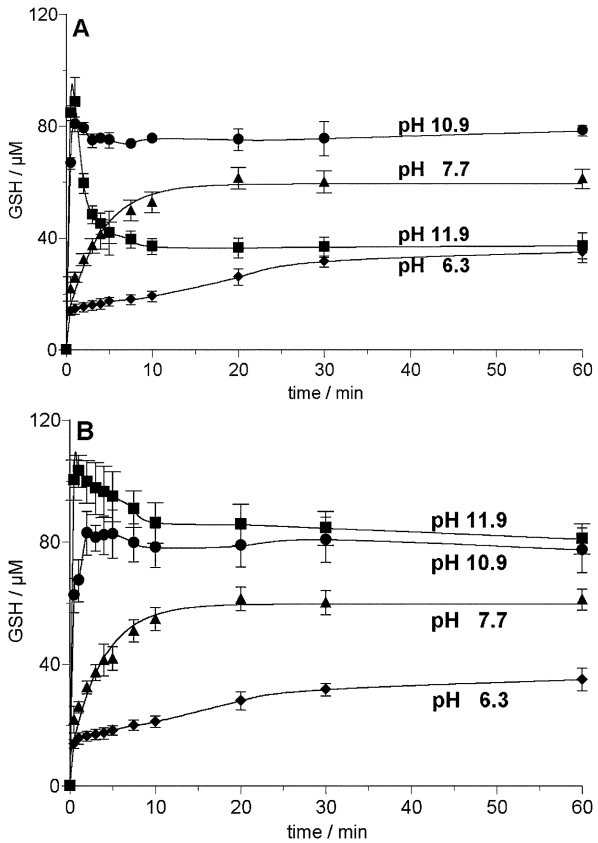


Fig. 5 Quantification of the thiol yield. The reactions were performed in phosphate buffer (50 mM, pH 7.4, 25 °C, 100 µM EDTA, 100 µM GSNO, 1 mM ascorbate) under normoxic and hypoxic (O_2 < 10 µM) conditions, respectively. Experiments were started by adding ascorbate. (A) Normoxia (dissolved O_2 = 225 µM). (B) Hypoxia (dissolved O_2 < 10 µM).

At nearly neutral pH values the yield of GSH increased at prolonged reaction periods and leveled off to 33 ± 1.8 µM (pH 6.3) and 61 ± 2.1 µM (pH 7.7), respectively, after 30 min of reaction regardless of the presence of oxygen. At pH 10.9 the maximal yields of 80 ± 2.7 µM (normoxia) and 82 ± 2.2 µM (hypoxia), respectively, were observed after a reaction period of only 2 min. The GSH yield decreased somewhat at prolonged reaction periods to 75 ± 4.7 µM under normoxia (Fig. 5A) but it was fairly constant under hypoxic conditions (Fig. 5B). The result that alkaline pH values accelerated

the rate of the *S*-nitrosothiol–ascorbate reaction is in agreement with data of Holmes and Williams.[9] The increase in the GSH yield is tentatively explained with a decreased efficacy of the reaction between GSH and HNO. Provided that the glutathione–nitroxyl reaction is indeed inhibited at alkaline pH values, this GSH-depleting reaction should be effectively blocked at pH values above the pK_a value of nitroxyl (pK_a($HNO/^3NO^-$) = 11.4[30]). In fact, at pH 11.9 ascorbate effectively reduced GSNO (90 ± 8.7 μM GSH, Fig. 5A) in the presence of oxygen, and under hypoxic conditions it generated stoichiometrically GSH (100 ± 6.2 μM, Fig. 5B) after a reaction period of only 1 min. Under normoxia (Fig. 5A) the GSH yield dropped down in an exponential manner and leveled off at 36 ± 2.2 μM after a reaction period of 30 min. Control experiments demonstrated that nitroxyl, which yields peroxynitrite at alkaline pH values in the presence of oxygen,[18,30] is most likely responsible for the thiol oxidation because a similar decrease of GSH was observed by mixing GSH (100 μM) with 60 μM of the nitroxyl donor Piloty's acid (that decomposes rapidly at alkaline pH values in contrast to Angelis's salt) at such a condition (data not shown). In line with this conclusion, the decrease of GSH at prolonged reaction times could be largely prevented under hypoxic conditions (Fig. 5B) where only low amounts of peroxynitrite can be formed. It should be noted that the impact of a further increase of the pH value was very limited because of the occurrence of a rapid autoxidation reaction of ascorbate at pH > 12.5. In any case, the highest yields of the GSNO–ascorbate reaction were observed at pH 11.9 after a reaction period of 1 min.

Discussion

S-Nitrosation of protein cysteinyl residues is a reversible post-translational modification involved in physiological regulatory processes. Here we demonstrated that the *S*-nitrosothiol–ascorbate reaction generates mainly the novel product nitroxyl with a yield of approximately 60%. The HNO molecule is highly effective in depleting both thiols[13,31] and $^{\bullet}NO$[32,33] but it is also effective in releasing nitric oxide from *S*-nitrosothiols.[20] Both mechanisms obviously operate under the applied conditions at physiological pH. The moderate SOD-dependent increase of the steady-state $^{\bullet}NO$ equilibrium concentration demonstrates that a part of the intermediary HNO was involved in the decrease of $^{\bullet}NO$ whereas the yields of both $^{\bullet}NO$ (48%) and HNO (60%) indicate that intermediary HNO was partly oxidized to $^{\bullet}NO$. We demonstrated further for the first time that the *S*-nitrosothiol–ascorbate reaction yields stoichiometrically thiols at optimized conditions (pH 11.9, reaction period 1 min, hypoxia).

Chemical mechanism

Two research groups[7,9] claim that the monoanion as well as the dianion of ascorbic acid react in a bimolecular reaction with GSNO to yield GS^-, $^{\bullet}NO$ and ascorbyl radical but they proposed different operating mechanisms. While Smith and Dasgupta[7] suggested an outer sphere electron transfer mechanism between the anions of ascorbate and GSNO (reaction (6a)),

$$GSNO + ASC^- \rightarrow GSH + ASC^{\bullet -} + {}^{\bullet}NO \quad (6a)$$

Holmes and Williams[9] favored the GSNO-dependent transnitrosation of the anions to yield the intermediate *O*-nitrosoascorbate (reactions (6b) and (6c)):

$$GSNO + ASC^- \rightarrow GSH + NOASC^- \quad (6b)$$

$$NOASC^- \rightarrow ASC^{\bullet -} + {}^{\bullet}NO \quad (6c)$$

We recently demonstrated that the ascorbyl radical reacts instantaneously with $^{\bullet}NO$ (reaction (6d)):[10]

$$ASC^{\bullet -} + {}^{\bullet}NO \leftrightarrows NOASC^- \quad (6d)$$

Thus, the occurrence of the key intermediate *O*-nitrosoascorbate ($NOASC^-$) cannot be ruled out with an (hypothetical) operating outer sphere electron transfer mechanism between an *S*-nitrosothiol and ascorbate. The decay of $NOASC^-$ is much more complicated than believed hitherto because it can decay *via* three exergonic, competing pathways, *i.e.*, homolysis of $NOASC^-$ to yield $^{\bullet}NO$ and ascorbyl radical, hydrolysis of $NOASC^-$ to yield ascorbate and nitrite, and after protonation *O*-nitrosoascorbic acid is formed that can fragmentize to yield dehydroascorbate and HNO (Scheme 2, yields are given in parentheses).[10]

Formation of HNO from *O*-nitrosoascorbic acid is predicted by accurately performed quantum chemical calculations to be an exergonic reaction in aqueous solution (–5.3 kcal mol^{-1}).[10] In the present study it is demonstrated that the HNO-yielding pathway is the dominant decomposition channel of *O*-nitrosoascorbate at physiological pH (see Fig. 2C). Nevertheless, as $^{\bullet}NO$ is generated from the homolysis of *O*-nitrosoascorbate as well as from reaction of HNO with GSNO, it is liberated with a yield of about 48%.

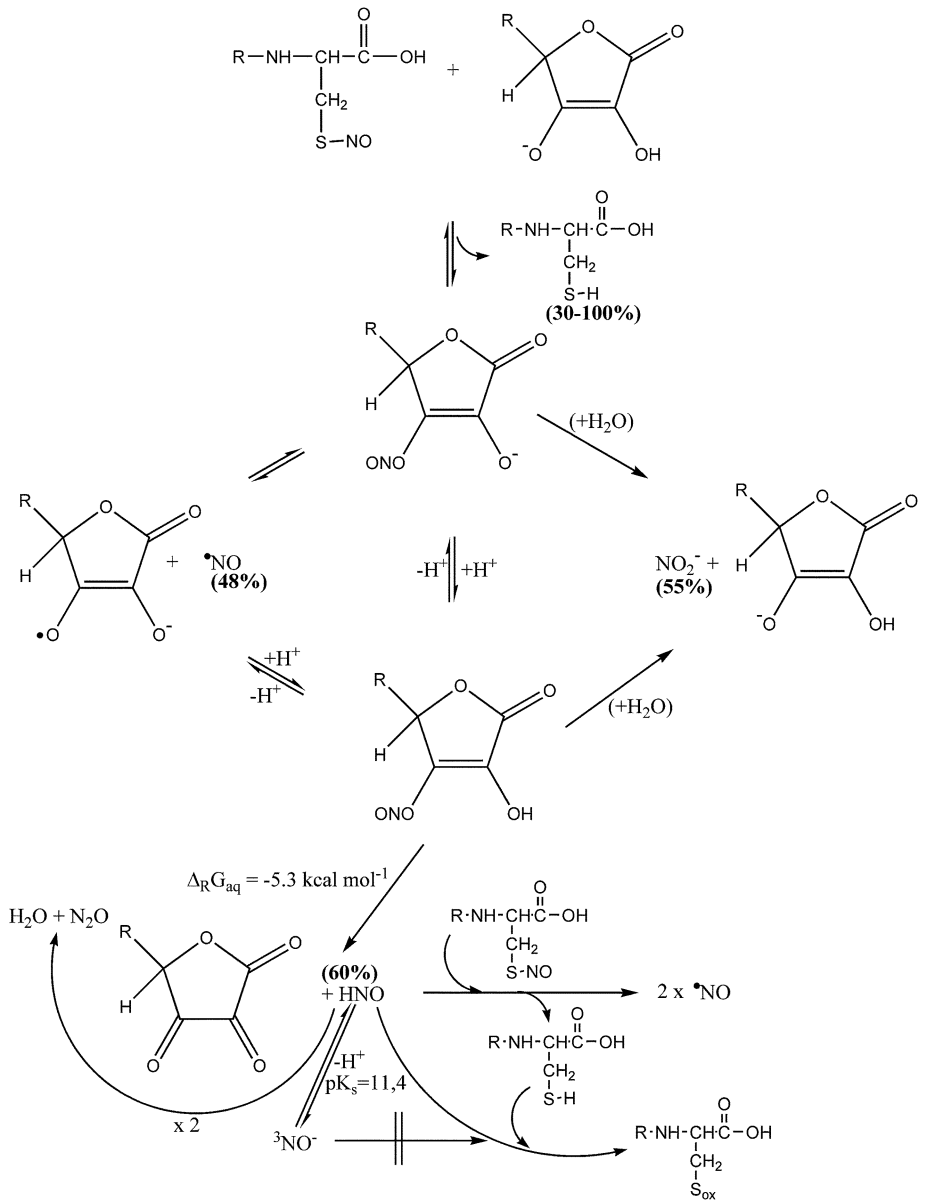
Physiological role of ascorbate

It has been frequently quoted that cuprous ions[34–37] act as catalysts for the liberation of $^{\bullet}NO$ from *S*-nitrosothiols and that ascorbate would maintain the copper ions in the catalytically active redox state by reducing Cu^{2+} to Cu^{1+} (reactions (7a) and (7b)):[36,37]

$$Cu^{2+} + ASC^- \rightarrow Cu^{1+} + ASC^{\bullet -} \quad (7a)$$

$$Cu^{1+} + RSNO \rightarrow Cu^{2+} + RS^- + {}^{\bullet}NO \quad (7b)$$

In fact, $^{\bullet}NO$ release from *S*-nitrosothiols *in vitro* can be conclusively explained with the catalytic action of freely diffusing, redox-active copper ions because such processes are generally abrogated in the presence of the Cu^{1+}-chelator neocuproine.[34,35,37] The question, however, arises whether such a mechanism can operate in blood. Williams demonstrated that the catalytic activity of a physiological copper ion concentration (10 μM) is about 10-fold less effective in the presence of an equimolar concentration of human serum albumin.[35] In line with this observation, the presence of a physiological human serum albumin concentration (700 μM) abolishes the copper ion-catalyzed degradation of both GSNO and *S*-nitroso-*N*-acetylpenicillamine (Kirsch and de Groot, unpublished results). In addition, the *S*-nitroso derivative of human serum albumin was stable in the presence of equimolar copper ion concentrations.[38] As physiological amounts of albumin annul the catalysis of freely diffusing, redox-active copper ions and because a human copper-containing enzyme that needs vitamin C as substrate is yet unknown, ascorbate cannot be involved in



Scheme 2

the endogenous release of •NO from *S*-nitrosothiols in blood *via* maintaining the Cu^{1+} levels.

Since *O*-nitrosoascorbate is in comparison to any *S*-nitrosothiol a short-lived species, it cannot be a transporter for •NO. Furthermore, as the reaction rate constants of *S*-nitrosothiols with thiols[36,39] and thiolate anions[40] are orders of magnitude higher than the one with ascorbate monoanion,[9] *O*-nitrosoascorbate cannot be endogenously generated from reaction of *S*-nitrosothiols with ascorbate. In other words, any endogenous transnitrosation reactions between *S*-nitrosothiols and thiols cannot be hampered by ascorbate. Nevertheless, *O*-nitrosoascorbate may be endogenously formed from reaction of ascorbate with either reactive nitrogen oxygen species[6,41] or a combined reaction of ascorbyl radical generating species (*e.g.*, tocopheroxyl radicals,[42] dehydroascorbate[43]) and •NO so that reaction (6d) can proceed.

Analytical purposes

An actual very popular procedure for the detection of protein-bound *S*-nitrosothiols is the so-called biotin-switch technique.[44] This procedure uses three different steps: *S*-methylthiolation of free thiols with *S*-methyl methanethiosulfonate, reduction of *S*-nitrosothiols to thiols with ascorbate, and *in situ* labeling by *S*-biotinylation of the generated thiols with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide.[11,44] However, as a thiol-depleting species, namely nitroxyl, is intermediary generated with a high yield during the *S*-nitrosothiol–ascorbate reaction, users of the biotin-switch technique should take some action to avoid the nitroxyl-dependent reoxidation of the formed thiol function. In fact, Zhang and Hogg summarized some limitations of the assay and concluded "although the detection methodology is sensitive, it will allow detection of only the tip

of the iceberg".[26] In order to increase the yield of thiols from the *S*-nitrosothiol–ascorbate reaction at about neutral pH values one might suggest to catalyze the decay of nitroxyl with SOD. Such a strategy, however, would ignore the fact that nitroxyl is formed in immediate vicinity to the formed thiol function and that these reactants react moderately fast with each other (k(HNO + thiol) = 3–20 × 10^5 M^{-1} s^{-1}).[15] This and a massive sterical hindrance that is present when the *S*-nitrosothiol is located in a protein would largely suppress the accessibility of SOD on nitroxyl. In addition, as low molecular weight scavengers would be more effective in trapping nitroxyl than SOD, one might improve the scavenging process by applying exceeding $^{\bullet}$NO-levels in order to accelerate the HNO decay *via* Sutton's mechanism (reactions (4a) and (4b)). Unfortunately, such a policy would favor thiol-depleting reactions. At first, the chemical power of HNO is drastically increased by adding stoichiometric amounts of $^{\bullet}$NO.[18] At second, the presence of exceeding $^{\bullet}$NO-levels would suppress the homolytic dissociation of *O*-nitrosoascorbate at the expense of the competitive HNO-yielding pathway. Thus, it might be an improvement that users of the biotin-switch assay reduce *S*-nitrosothiols to thiols with ascorbate under hypoxia at a pH of about 12 because the HNO-dependent degradation of thiols is effectively suppressed at such conditions. HNO is the only known acid that converts from an electronic singlet ground state system to a triplet one during deprotonation[30] so that a reaction between the corresponding anions (GS^- and $^3NO^-$) is a spin-forbidden process. At pH ≈ 12 the $^3NO^-$ anion reacts with molecular oxygen ($^3O_2$) in a diffusion-controlled manner to yield peroxynitrite[30] that can also oxidize thiols *via* various pathways.[45,46] However, formation of peroxynitrite can be effectively limited with the use of hypoxic conditions so that the *S*-nitrosothiol–ascorbate reaction yields stoichiometrically thiol functions. A second major advantage of employing the ascorbate-dependent reduction at alkaline pH values lies in the simple fact that the ascorbate dianion, the dominant ascorbate species at pH 11.9, reacts in regard to ascorbate monoanion 3.5 × 10^5-fold faster with *S*-nitrosothiols.[9] By using the experimental rate constant (k = 220 M^{-1} s^{-1} [9,40]) and an ascorbate concentration of 1 mM at pH 11.9 it can be expected that the *S*-nitrosothiol–ascorbate dianion reaction would be completed within a reaction period of 1 min even at an *S*-nitrosothiol concentration of 5 nm.

Conclusion

In the present investigation, quantification of intermediates and products from the *S*-nitrosothiol–ascorbate reaction in copper ion-depleted solutions were revised. The key observation was the novel release of the intermediate nitroxyl, that is highly effective in decreasing thiol functions, with a high yield. The *S*-nitrosothiol–ascorbate reaction is frequently used for the reduction of *S*-nitrosocysteine residues in proteins to the corresponding cysteine moieties, *i.e.*, the converting reaction of the popular so-called "biotin-switch technique". Since the *S*-nitrosothiol–ascorbate reaction yields both a thiol function and the thiol-depleting intermediate nitroxyl, one might suspect that the converting reaction of the biotin-switch technique needs to be improved. In order to achieve a quantitative yield of thiol functions a change of the processing parameters (pH ≈ 12, hypoxia) might be such an improvement.

Experimental

Chemicals

MAHMA/NO, EDTA, papain, glutathione (GSH), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), naphthylethylendiamine-dichloride, sulfanil-amide and Chelex 100 were purchased from Sigma (Taufkirchen, Germany). $NaNO_2$ was obtained from Merck (Darmstadt, Germany). Angelis's salt, Piloty's acid and Mn(III)-tetrakis(1-methyl-4-pyridyl)porphyrin pentachloride (Mn(III)TMPyp) were from Cayman Chemical (Ann Arbor, Michigan, USA). *S*-Nitrosoglutathione (GSNO) was synthesized as described previously by us.[47] *S*-Nitroso-papain was prepared with a procedure as described by Marley *et al.* for the synthesis of *S*-nitrosoalbumin.[48] FNOCT-4 was a gift from Prof. Dr Dr R. Sustmann (University of Essen, Department of Organic Chemistry, Essen, Germany) that was prepared as described in refs 29 and 49. Stock solutions were freshly prepared on a daily basis and their concentrations were spectrophotometrically determined.

Experimental system

Since transnitrosation reactions are highly sensitive to the presence of metal ions, phosphate buffer solutions (50 mM) were treated with the heavy metal scavenger resin Chelex 100 (0.5 g in 15 ml). The solution was gently shaken, stored overnight, and was then carefully decanted from the resin. Afterwards, the pH of all solutions was readjusted to pH 7.4 ± 0.1 by addition of either 50 mM H_3PO_4 or 50 mM K_3PO_4, respectively. In order to further avoid any interference by traces of transition metals, EDTA (100 μM) was added to all reaction mixtures.

Determination of nitric oxide with an $^{\bullet}$NO-sensitive electrode

Nitric oxide formation was determined using an $^{\bullet}$NO-sensitive electrode (ISO-NO; World precision Instruments, Sarasota, Florida), as described elsewhere.[50] The reaction mixtures were continuously stirred throughout measurements, and the temperature was controlled at 25 ± 1 °C. The electrode was calibrated daily and $^{\bullet}$NO production was quantified according to the manufacturer's instructions employing potassium iodide (100 mM) in H_2SO_4 (0.1 M) as a calibration solution to which various amounts of $NaNO_2$ (0.5 mM) were added. The steady-state concentrations of nitric oxide generated from reaction of GSNO (200 μM) with ascorbate (2 mM) in phosphate buffer (50 mM, pH 7.4, 25 °C) in the absence and in the presence of SOD (1000 units/ml) were monitored.

$^{\bullet}$NO measurement with the fluorescent nitric oxide cheletropic trap (FNOCT-4)

The amount of $^{\bullet}$NO from the GSNO–ascorbate reaction was quantified with FNOCT-4, which directly traps $^{\bullet}$NO to yield the fluorescence dye FNOCT-4-NOH (λ_{em} = 460 ± 5 nm, λ_{ex} = 320 ± 5 nm). A two-point calibration of 5 μM FNOCT-4 was performed by mixing 1 mM ascorbate in the absence and in the presence of 200 μM MAHMA/NO and reading the fluorescence intensity after an incubation period of 30 min at 25 ± 1 °C. The stability of the $^{\bullet}$NO-FNOCT-4 reaction product, FNOCT-4-NOH, was

verified for a period of 4 hours. All experiments with FNOCT-4 were performed under normoxic conditions.

Quantitative detection of nitroxyl

Nitroxyl was determined as originally described by Marti *et al.* for a related Mn^{III}-porphyrin complex.[22] The reactions were performed in phosphate buffer (50 mM, pH 7.4, 25 °C, 100 μM EDTA, 10 μM Mn^{III}TMPyp, 1 mM ascorbate, hypoxia (O_2 < 10 μM)). The experiments were performed with a fixed amount of either GSNO or *S*-nitroso-papain (10 μM each) as well as with various GSNO concentrations (1.25–20 μM). In all cases the reaction was started by adding ascorbate (1 mM). From these probes, spectra were immediately recorded with a gas-tight cuvette by using a SPECTROCORD S 100 spectrometer. On reaction with nitroxyl, the Soret-band of Mn^{III}TMPyp at 463 nm ($\varepsilon_{463} = 9.2 \times 10^4$ M^{-1} cm^{-1} [25]) decreased and that of Mn^{II}TMPyp-NO at 435 nm ($\varepsilon_{435} = 1.1 \times 10^5$ M^{-1} cm^{-1} [23]) increased.

Quantification of thiols

The concentration of thiol functions was quantified as described by Ellmann[51] and improved by us.[24] DTNB (39.6 mg) was dissolved in 50 mM phosphate buffer (10 ml, pH = 7.0). The samples were diluted 10-fold with phosphate buffer (100 mM, pH 7.0, 25 °C, 100 μM EDTA) and to 1 ml of the diluted sample 6.67 μl stock solution of DTNB were added. The optical density of the formed *p*-nitrothiophenol anion ($\varepsilon_{412} = 13\,600$ $M^{-1}cm^{-1}$)[51] was read photometrically after incubation for 20 min at room temperature. The test was calibrated with a solution of pure GSH.

Quantification of nitrite

Nitrite was quantified by using the Griess-reagent (0.1% naphthylethylendiamine-dichloride plus 1% sulfanil-amide in 5% H_3PO_4).[52] At the given time of reaction, the samples were diluted 30-fold with phosphate buffer (50 mM, pH 7.4, 25 °C, 100 μM EDTA), afterwards a 2.5-fold volume of Griess-reagent was added. After 10 min of incubation at room temperature, the optical density was photometrically read at 542 nm. Calibration was carried out with a photometrically controlled solution of pure $NaNO_2$ ($\varepsilon_{354} = 22.9$ $M^{-1}cm^{-1}$).[53]

^{15}N measurements

NMR experiments were performed on a Bruker ADVANCE DRX 500 instrument (Bruker Biospin, Rheinstetten, Germany) at 50.67 MHz. Chemical shifts (δ) are given in ppm relative to neat nitromethane ($\delta = 0$) as external standard.

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