The decomposition of thionitrites

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The mechanism of thionitrite decomposition, both *in vivo* and *in vitro*, remains unclear. Thionitrite stability is highly variable; it is a complex function of thionitrite structure and environmental condition. Several recent advances clarify the role of unimolecular homlytic decomposition, metal-catalyzed reductive decomposition and higher-order enzymatic and non-enzymatic processes to the overall observed stability of thionitrites.

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Introduction

The discovery of the myriad roles of nitric oxide (NO) in biology has led to a remarkable paradigm shift in our understanding of biological systems. Originally considered in the context of vascular tone, NO is now recognized as an important signal in a wide range of complex physiological responses, including immune function, cardiac contractility, intestinal motility and cognition. (The term nitric oxide is used in a generic sense to refer to NO-related molecules that provide biological activity, including redox-related forms and congeners thereof.) Furthermore, deficiency of NO in each of these systems is linked to disease pathogenesis and thus forms a strong rationale for replacement therapy. However, the chemical reactivity of NO poses special challenges for both the regulation of NO bioactivity and the delivery of exogenous NO for therapeutic effect.

NO forms covalent complexes with a wide range of electronegative main-group heteroatoms, including nitrogen (nitrosamines, NONOates), oxygen (nitrites) and sulfur (thionitrites or nitrosothiols). Thionitrites are of clear and well-described importance in biology. In particular, nitrosylated adducts of cysteine, including various nitrosylated cysteine-containing peptides and proteins, are present and active in virtually all species. (We use the term 'nitrosylated' to include all SNO species, regardless of the mechanism of origin. We thus note that certain SNO derivatives may arise from chemistry other than nitrosonium replacement of a proton.) *S*-Nitrosylated compounds have also been proposed as therapeutic agents. From both the perspective of NO biology and the preparation and delivery of NO therapeutics, an understanding of the mechanism of NO release — including the rate of release, the oxidation state of the NO group liberated and the organic by-products of the release — is required. Here, we review the recent literature on the mechanism of thionitrite decomposition. We focus primarily on work reported since 1999, although for both completeness and context some earlier work is included. For a more detailed discussion of earlier literature, readers are directed to several recent reviews [1–3].

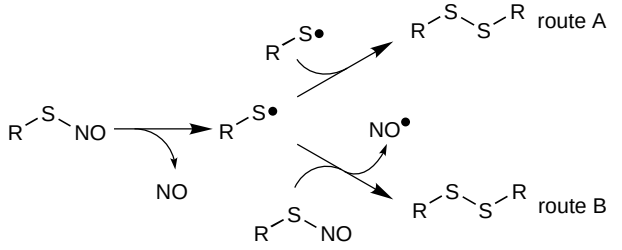
Thionitrite phenomenology

Thionitrites show significant S–N double-bond character, and exist as equilibrating mixtures of *cis*/*trans* isomers [4•]. (It has become common in the thionitrite literature to refer to the geometrical isomers around the S–N bond as *cis* and *trans*, and we retain that convention here. In this nomenclature, the *cis* form of the thionitrite corresponds to the *Z* stereoisomer and the *trans* to the *E* isomer.) Primary and secondary thionitrites selectively populate the electronically favored *cis* form, by a factor of two to five, whereas tertiary thionitrites favor the *trans* isomer by roughly 10:1 [4•,5]. By contrast, *ab initio* studies by Arulsamy and co-workers [6] suggested that the *cis* and *trans* conformers are equienergetic. Confusion exists regarding the 'stability' of thionitrites [7•]. Thermodynamically, primary thionitrites are stabilized relative to tertiary species by roughly 1 kcal mol^{-1}. And yet, tertiary thionitrites are typically much longer-lived than their primary and secondary counterparts. The oft-repeated statement that primary and secondary thionitrites are 'less stable' than tertiary compounds thus refers to a kinetic, rather than thermodynamic, phenomenon. The electronic properties of the two forms are slightly different: primary and secondary thionitrites are thus typically red, whereas tertiary thionitrites are green [4•].

The mechanism of NO-group release from thionitrites is complex. Reported rates of thionitrite decomposition vary widely, and half-lives range from seconds to years, reflecting the profound effect of experimental conditions on reactivity. Although the structure of the thionitrite can be an important component of stability, the relationship between structure and 'stability' is not straightforward. The S–N bond is weak, sterically unencumbered and strongly polarized. Thionitrites are photoreactive, with strong electronic absorbances in the UV range and weaker transitions in the visible spectrum. Given the broad range of prospective reactivities, decompositions can only be compared under equivalent conditions.

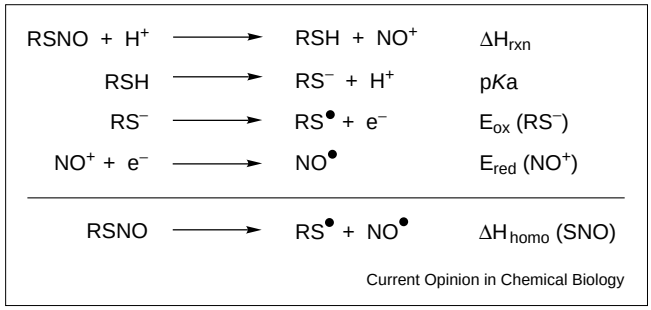
The most general observation is that thionitrites decompose to the corresponding disulfide and NO, although a range of other products — including nitrogen and sulfur in virtually all of their respective oxidation states — have been reported to predominate in biological and complex *in vitro* systems. Our purpose here is to highlight recent mechanistic studies of thionitrite decomposition. We have grouped mechanisms into three classes: unimolecular homolytic scission of the

Figure 1



Homolytic decomposition of thionitrites.

Figure 2



Theremodynamic cycle for determination of SNO bond enthalpies.

S–N bond; metal-catalyzed reductive decomposition; and higher-order decomposition pathways.

Uniomolecular homolytic scission of the S–N bond

Conceptually, the simplest route to thionitrite decomposition involves rupture of the S–N bond. This rupture can occur homolytically or heterolytically; in the latter instance, scission would leave residual electron density on either sulfur or the NO group (nitrosonium or nitroxyl, respectively). The homolytic pathway is by far the route of lowest energy, and unimolecular heterolytic cleavage to yield either nitrosonium or nitroxyl is not significant near physiological temperatures.

The reaction products of unimolecular decomposition are disulfide and NO. Although the reaction of two sulfinyl radicals to yield disulfide has been invoked (Figure 1, route A), bimolecular reaction of two reactive species present at extremely low concentrations is prohibitive. Rather, the nearly barrierless S_H2 reaction of a thiyl radical with concentrated thionitrite is almost certainly responsible for disulfide formation under most *in vitro* conditions (Figure 1, route B).

The relevance of homolytic scission to thionitrite decomposition remains unclear; thionitrites have alternately been described as extremely susceptible [3] and extremely resistant [8] to thermolysis. Estimates place the thionitrite homolytic bond dissociation energy between near 20 and 32 kcal mol^{-1}. This difference is of vital importance to an understanding of thionitrite chemistry and biology; near the low limit of this range, thionitrites are predicted to show half-lives of seconds to minutes near room temperature, whereas at the high limit of the range, stabilities extend to years.

These discrepancies may reflect the activity of low-level contaminants. Indeed, the decomposition of thionitrites is seldom first-order, unless oxygen, metal ions and light are rigorously excluded. Consistent with the notion of a contribution from higher-order decomposition processes, primary thionitrites are less likely to show simple first-order decay kinetics than their more hindered tertiary counterparts.

Wang and co-workers [9•] utilized a thermodynamic cycle to measure homolytic and heterolytic bond dissociation energies for a series of substituted phenylthionitrites, *t*-butylthionitrite and benzyl thionitrite (Figure 2). This methodology requires knowledge of the enthalpy of thiol nitrosylation, thiol pK_a values, the reduction potential of the corresponding thiolate and the reduction potential of nitrosonium. Enthalpies of thiol nitrosylation were evaluated calorimetrically by the addition of nitrosonium perchlorate to the appropriate thiophenol. Bond dissociation energies of near 20 kcal mol^{-1} for aromatic thionitrites, and near 25 kcal mol^{-1} for *t*-butyl and benzyl thionitrite were measured. Density functional theory was also used to examine bond homolysis energies, and yielded values in good agreement with the experimentally derived values.

In contrast, Bartberger *et al.* [10•] deduced bond dissociation energies for methyl, ethyl, isopropyl, *t*-butyl and vinyl thionitrites from direct measurements of first-order decompositions. (It should be noted that decompositions failed to follow first-order kinetics in the absence of added thiol and at temperatures below roughly 65°C.) Evaluation of the temperature dependence of the decomposition yielded enthalpies of activation of 28.1 and 30.9 kcal mol^{-1} for *n*-hexyl and cyclohexylthionitrite, respectively, whereas Arrhenius analysis provided activation energies of 28.9 and 31.6 kcal mol^{-1}. Bond dissociation energies and free energies were calculated using the CBS-QB3 methodology of Petersson; these calculations suggested bond dissociation energies uniformly near 32 kcal mol^{-1} for alkyl thionitrites and 23.3 kcal mol^{-1} for the vinyl analogue.

Most recently, Grossi and Montevecchi [7•] reported significantly lower bond dissociation energies than either previous study, ~22 kcal mol^{-1}. They attributed the higher values to a previously unrecognized reversible out-of-cage recombination of thiyl radical with NO to reform thionitrite. In support of this theory, unimolecular decomposition was inhibited by excess exogenous gaseous NO and both thermal and photochemical decomposition of thionitrites was attenuated in high viscosity polyethylene glycol [11]. But while an in-cage recombination of thiyl radical and NO seems reasonable, out-of-cage recombination is hard to

Table 1

Susceptibility of thionitrites to copper-induced decomposition.

RSNO	$k_2(s^{-1}M^{-1})$
S-nitrosopenicillamine	67 000
S-nitrosysteamine	65 000
S-nitrosocysteine	24 500
SNAP	20
S-nitroso-*N*-acetyl cysteamine	0
S-nitroso-*N*-acetyl cysteine	0
GSNO	0
S-nitrosothioglycolate	300
Methyl *S*-nitrosothioglycolate	0

imagine since it would require reaction of thiyl radical with NO at a rate significantly faster than the S_H2 reaction of thionitrite with thiyl radical. This latter process, however, is barrierless and not substrate-limited.

None of the reports find any effect of thiol structure on the rate of decomposition. In contrast to the postulate of Arulsamy *et al*. [6], there is little reason, *a priori*, to imagine that substitution at the sulfur-bearing carbon (and thus conformation) might affect the C–S homolytic bond strength. The unique steric constraints posed by proteins might provide a special case, however. A recent crystal structure of nitrosylated hemoglobin orients the *S*-nitroso group with a 90° dihedral; this conformation should be destabilized to homolytic scission by at least 10 kcal mol^{-1}, whereas the nitrosylated protein is very stable in this state [12]. It is unclear if this unusual orientation is stabilized by a neighboring tyrosine side chain (e.g. pi-stacking) or if the unusual dihedral is artifactual, arising from fitting to a 180° model.

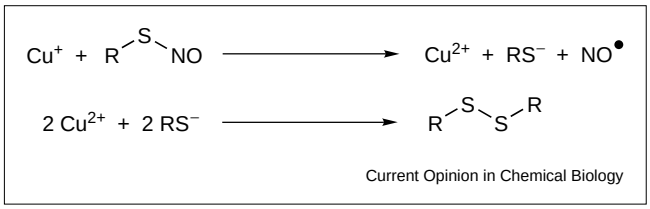
Given the difficulties associated with artificially decreasing the rate of a unimolecular process and the myriad reaction manifolds that might contribute artificially to increase the rate, it is tempting to speculate that the true homolytic bond dissociation energy is near the high end of the proposed range, above roughly 30 kcal mol^{-1}. Still, the issue remains unresolved and thus the importance of homolytic decomposition of thionitrites remains unclear.

Reductive cleavage of the S–N bond: copper-catalyzed thionitrite decomposition

Some thionitrites are decomposed catalytically by copper salts (Table 1). Williams and co-workers have suggested that much of the reported discrepancy on thionitrite decomposition can be rationalized on the basis of adventitious copper contamination present in even deionized and/or distilled water, and propose a mechanism involving reductive cleavage of the S–N bond by Cu(I).

In the absence of light or other species capable of inducing thionitrite decomposition, the observed rate of decomposition is given by the expression:

Figure 3



Catalytic decomposition of thionitrites by copper.

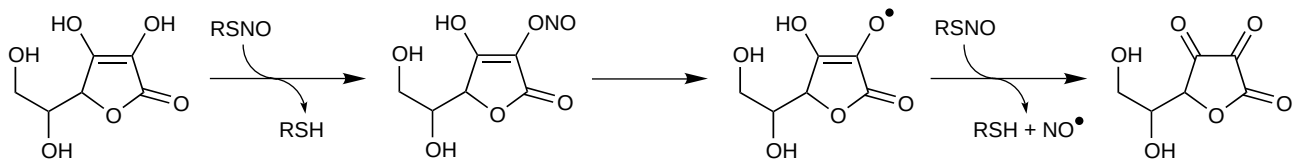
$$\text{Rate} = k_1\,[\text{RSNO}] + k_2\,[\text{RSNO}]\,[\text{Cu(I)}]$$

Where the first term represents unimolecular homolytic decomposition, and is typically considered as negligible (see above discussion). Although the reaction is suppressed by copper chelators, including EDTA and neocuprine, the rate of copper-catalyzed decomposition is strongly dependent on the structure of the thionitrite. No correlation is observed, however, between susceptibility to cuprous decomposition and thionitrite reduction potentials, as would be predicted from a simple outer-sphere reduction mechanism (Figure 3) [9•,13,14]. Additionally, the rate constant for the reaction of small-molecule thionitrites with Cu(I) has recently been reported as essentially diffusion controlled and independent of thionitrite structure. Together, these observations preclude outer-sphere electron transfer as the rate-determining step [15]. Therefore, to account for these data, other rate-determining steps have been postulated, including the regeneration of cuprous ion, the formation of reactive aggregates, and the differential chelating ability of various thionitrites. In this latter motif, reductive cleavage is preceded by metal ion chelation and proceeds through inner-sphere electron transfer, a process not simply proportional to reduction potentials.

More recently, Noble and Williams [16,17] made the unexpected observation that, in some cases, the effect of copper on thionitrite decomposition varied inversely with thionitrite concentration. At millimolar concentrations, both *S*-nitrosoglutathione and *S*-nitrosoglutamylcysteine are unreactive to 10 μM Cu(II), whereas *S*-nitrosocysteine and *S*-nitrosocysteinylglycine decompose rapidly. At micromolar substrate concentrations, all four thionitrites react at approximately the same rate. Additionally, decomposition was halted in the presence of exogenous disulfide. These observations were interpreted in terms of Cu(II) chelation by the product disulfides.

Although it is clear that cuprous ion is an excellent catalyst for small-molecule thionitrite decomposition, its relevance to the *in vivo* decomposition of more complex thionitrites remains unclear. The concentration of free copper is extraordinarily low in most living tissue, estimated at less than one atom per cell. Most protein and peptide thionitrites are not susceptible to limiting copper-catalyzed decomposition.

Figure 4



Ascorbate-induced thionitrite decomposition.

Both silver and mercury ions promote the stoichiometric cleavage of thionitrites, activating the S–N bond to hydrolysis and producing nitrite and the corresponding metal thiolate [18]. Reaction of thionitrites with mercuric ion forms the basis of the well-known Seville assay. Several other metal ions have been examined as possible reagents for thionitrite decomposition, including Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and Cr^{3+}; none show any activity [19]. Some reports suggest weak catalytic decomposition by Fe^{2+}; ferric ion is inactive.

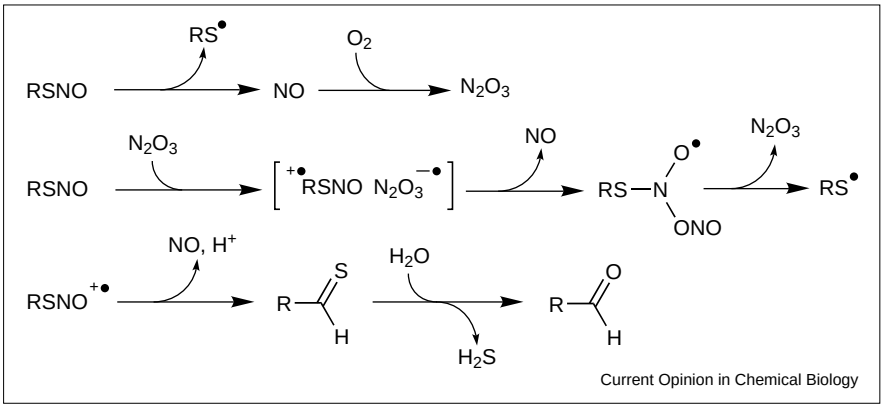
Non-redox bimolecular decomposition pathways

Most thionitrite decompositions are complex both with regard to product distribution and kinetic order. Virtually every conceivable oxidation state of both sulfur and nitrogen have been reported, including thiol, disulfide, sulfinic and sulfonic acids, disulfide sulfoxides and sulfones, dinitrogen, ammonia, hydroxylamine, nitrite and nitrate. In many instances, no single species dominates the product spectrum; frequently, far less than stoichiometric nitrogen is recovered. Powerful and conflicting effects of solvent, temperature, the presence or absence of oxygen and the presence or absence of excess reduced thiol have been reported. Myriad reaction schemes have been proposed; here, we consider a few of the more important, recently described pathways.

Transnitrosation

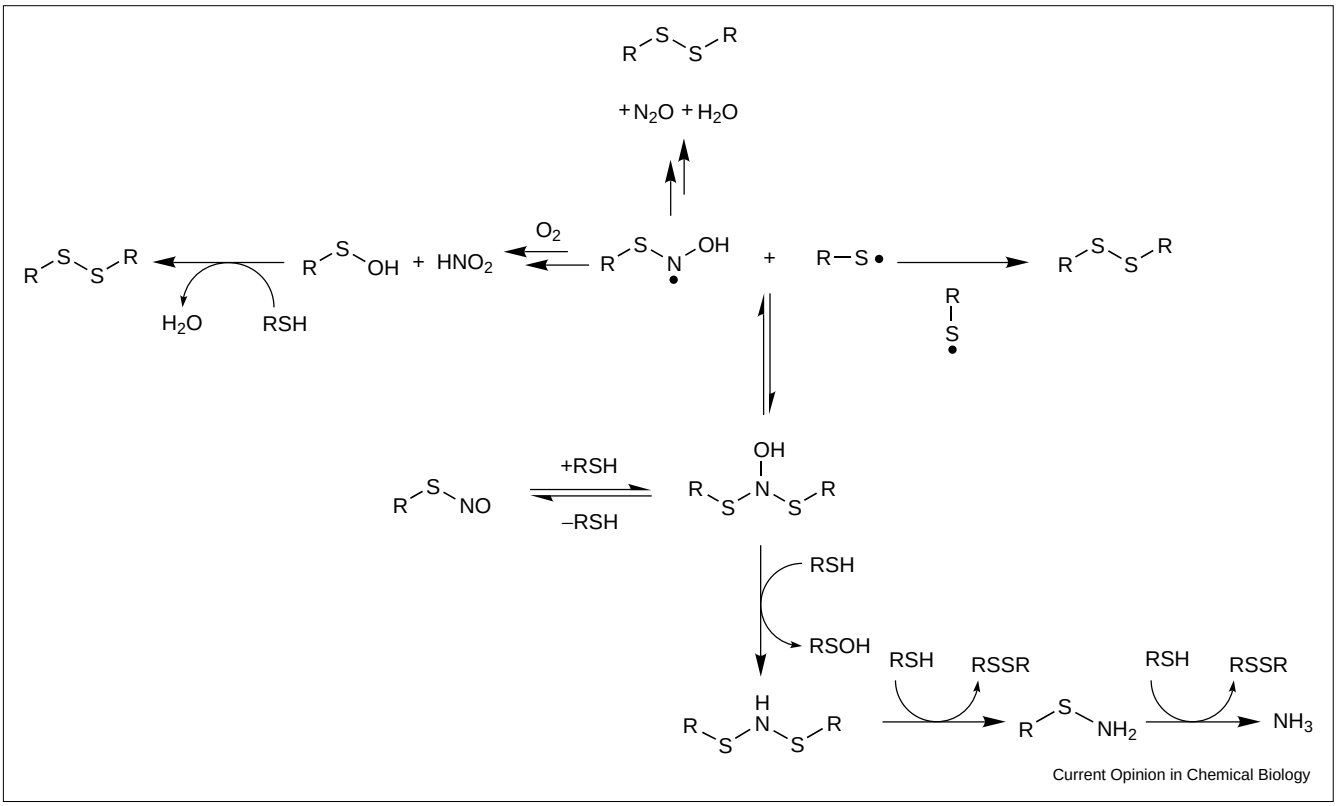
With a strongly polarized S–N bond, an exceptionally good leaving group, and virtually no steric encumbrance, thionitrites are powerful electrophiles. Facile transfer of nitrosonium to almost every reasonable nucleophile, including amines, azide, alcohols, hydroperoxide, other thiols and sulfite, has been reported; decomposition from nitrogenous species other than thionitrite is probably important in many instances [20–23]. Ascorbic acid, which is present in all cells and extracellular fluids, stoichiometrically decomposes thionitrites, although the mechanism of the decomposition remains unclear (Figure 4). Williams and co-workers reported a decomposition dependent on both ascorbate concentration and solution pH. At lower ascorbate concentration (~10^{-4} M^{-1}), decomposition produces disulfide and NO, whereas at higher concentrations and pH values above 7, decomposition yields thiol and NO [24•]. The former reaction is inhibited by EDTA, and is presumably a cuprous-mediated process. Thus, in this concentration domain, the primary role of ascorbate is the formation and/or regeneration of low levels of cuprous ion. The pH dependence of the rate of decomposition at higher ascorbate concentrations is consistent with O-transnitrosation followed by homolytic scission of the O–N bond. Reaction with a second equivalent of thionitrite ultimately yields two equivalents of thiol, two equivalents of NO, and dehydroascorbic acid.

Figure 5



Grossi N_2O_3-mediated chain decomposition.

Figure 6



Tannenbaum decomposition mechanism.

By contrast, Smith and Dasgupta [25] have reported the ascorbate–*S*-nitrosoglutathione system proceeds via outer-sphere electron transfer, rather than by transnitrosation. All three protonation states of ascorbate participate, albeit with markedly different rates.

Oxygen-induced decomposition

The presence of oxygen provides a range of decomposition pathways. Grossi and co-workers have suggested a chain mechanism involving N_2O_3 to rationalize the observation of aldehydic products, the sensitivity of the decomposition to thionitrite steric bulk and the stabilizing effect of *p*-cresol (Figure 5) [7•,14,26]. However, the mechanism invokes several speculative and unprecedented reactions and intermediates, including a one-electron oxidation of thionitrite by N_2O_3 to yield free thionitrite radical cation. Moreover, alternative facile reaction manifolds involving superoxide and/or peroxynitrite are not discounted [27]. It should be emphasized that the reaction of thionitrite and superoxide, which proceeds with a rate constant of 10^5 $M^{-1}s^{-1}$, is unavoidable in most aerobic systems.

The Tannenbaum mechanism, involving decomposition of thionitrite by reduced thiol, is perhaps the most complex suggested to date (Figure 6) [28]. Although it requires several highly speculative steps, including the homolytic scission of the relatively strong *N*-hydroxysulfenamide S–N bond, the mechanism rationalizes the observed product distribution, in particular ammonia, as the predominant nitrogenous product.

The Tannenbaum mechanism also highlights the controversy over the effect of reduced thiol on thionitrite stability, wherein it can participate as a reductant, a nucleophile or a scavenger of free radicals. For example, free reduced thiol induces transnitrosation at rates dependent on both the thiol pK_a and steric bulk. Such transnitrosation can lead to a Curtin–Hammett situation in which decomposition proceeds from the least stable thionitrite. In addition, reports of S-thiylation, with concomitant release of nitroxyl, have appeared [29,30]. These varying results are best rationalized by the following points:

1. Stabilizing effects of thiol in organic solvent or under other conditions where little or no thiolate exists and low level of active oxygen species are thus effectively scavenged.

2. Destabilizing effect of thiolate involving a slow reductive decay to ammonia in aqueous systems, yield notwithstanding.

3. Destabilization under conditions of high thiolate concentration, exemplified in intramolecular thiylation reactions where high effective concentration of thiolate

and thermodynamically advantaged disulfide favor expulsion of nitroxyl.

4. The stabilizing or destabilizing effect of covalent complexes between thiol(ate) and thionitrite (termed nitroxyl disulfide or SNO-II).

Enzymatic decomposition

Liu *et al.* [31•] have shown that glutathione-dependent formaldehyde dehydrogenase (GSNO reductase) reduces *S*-nitrosoglutathione to disulfide and ammonia. The activity is reportedly conserved from bacteria to humans. Sulfenic and sulfinic acids are reported as intermediates [32]. Biochemical studies conducted in animals deficient in the enzyme establish that the activity is the primary *in vivo* mechanism of GSNO decomposition [31•]. In addition, these studies establish the existence of an equilibrium between GSNO and protein SNO, the position of which is dictated by GSNO reductase. A 'lyase' activity that cleaves GSNO and CysNO to NO has also been observed in bacterial extracts and is probably of a similar nature to the copper-independent decomposing activity found in bovine aortic endothelium and neutrophils [33]. Whether the process liberates disulfide or involves a reducing equivalent to generate thiol is unclear. It has been suggested, for example, that copper either bound in a true enzyme pocket or weakly adsorbed at the surface of proteins might be involved in such processes, but this proposition remains speculative [34–36]. In addition, several oxidoreductases, including glutathione peroxidase, xanthine oxidase and thioredoxin, decompose thionitrites *in vitro*, but their physiological relevance is unclear [37–39].

Conclusion

Despite the unambiguous importance of thionitrites as both reservoirs of NO bioactivity *in vivo* and as potential therapeutic products, confusion over the mechanism of thionitrite decomposition persists due, in large measure, to the wide range of experimental conditions employed. The role of unimolecular homolytic scission in biological thionitrite decomposition is in question. Homolytic bond dissociation energies appear too high to explain decomposition on physiological time scales of milliseconds to days. Rather, reductive mechanisms probably dominate the picture. Such pathways include reduction by ascorbate, thiol or superoxide in appropriate physiological context. Some thionitrites are susceptible to cuprous-mediated reductive decomposition, but an example of biological relevance has yet to be identified. Ultimately, *in vivo* reductive decomposition of thionitrite appears to be largely enzymatic in nature.

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