The metabolism of *S*-nitrosothiols in the trypanosomatids: the role of ovothiol A and trypanothione

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It has recently been established that nitrosogluthathione is the preferred substrate of the glutathione-dependent formaldehyde dehydrogenase from divergent organisms. Trypanosomatids produce not only glutathione, but also glutathionylspermidine, trypanothione and ovothiol A. The formaldehyde dehydrogenase activity of *Crithidia fasciculata* was independent of these thiols and extracts possessed very low levels of nitrosothiol reductase activity with glutathione or its spermidine conjugates as the thiol component. Although ovothiol A did not form a stable nitrosothiol, it decomposed the *S*-nitroso groups of nitrosogluthathione (GSNO) and dinitrotrypanothione [T(SNO)₂] with second-order rate constants of 19.12 M⁻¹ · s⁻¹ and 8.67 M⁻¹ · s⁻¹ respectively. The reaction of T(SNO)₂ with ovothiol A, however, accelerated to a rate similar to that seen with GSNO. Ovothiol A can act catalytically to decompose these nitrosothiols, although non-productive mechanisms exist. The catalytic phase of the reaction

was dependent on the production of thiyl radicals, since it was abolished in the presence of 5,5-dimethyl-1-pyrroline-*N*-oxide and the formation of nitric oxide could be detected by means of the conversion of oxyhaemoglobin into methaemoglobin. The rate-limiting step in the catalytic process was the reduction of oxidized ovothiol species and, in this respect, T(SNO)₂ is a more efficient substrate than GSNO. Trypanothione decomposed GSNO with a second-order rate constant of 0.786 M⁻¹ · s⁻¹ and the major nitrogenous end product changed from nitrite to ammonia as the ratio of thiol to nitrosothiol increased. The results indicate that ovothiol A acts in synergy with trypanothione in the decomposition of T(SNO)₂.

Key words: *Crithidia*, dinitrosotrypanothione, nitric oxide, nitrosothiol reductase, *S*-nitrosogluthathione.

INTRODUCTION

Despite recent advances in the development of therapies for diseases caused by trypanosomatids, the therapeutic options are still limited. The available drugs often have undesirable side effects, which limit their utility [1,2]. The search for novel drug targets that can be exploited for the treatment of infectious diseases is, therefore, particularly relevant in the case of the trypanosomatids.

The thiol metabolism of the trypanosomatids [3–6] and of some Gram-positive bacteria, such as the mycobacteria [7–9] and staphylococci [10], deviate significantly from that of their mammalian hosts and has, therefore, attracted attention as potential drug targets. The trypanosomatids are unique in that they convert glutathione, the principal low-molecular-mass thiol of most Gram-negative bacteria and of eukaryotes, into glutathionylspermidine (GSH-SPD) and the bis-GSH-SPD conjugate, trypanothione (T(SH)₂) [3] (Figure 1). Trypanosomatids also produce significant amounts of N¹-methyl-4-mercaptohistidine (ovothiol A; OvSH) [4]. There are pronounced quantitative variations in the levels of glutathione, GSH-SPD, T(SH)₂ and OvSH between different trypanosomatids and also between different life-cycle stages within a single species [6], but this information has not shed any light on the functional benefits that might have led to the development of the unusual thiol metabolism that we see in these organisms.

Following their discovery in the marine invertebrates in 1988 [11], it was proposed that the possession of an abundance of OvSHs in the egg cells and ovaries of marine organisms served to protect their egg cells against reactive oxygen intermediates generated by the respiratory burst that is triggered by fertilization [12]. Recent studies, however, suggested that the possession of OvSH is unlikely to result in enhanced oxidative stress tolerance in the trypanosomatids [6]. At the present time, no enzymes of the OvSH biosynthetic pathway [13] have been purified to homogeneity, there are also no known analogues of the OvSH biosynthetic enzymes in the available databases and, consequently, it has not been possible to investigate the functional relevance of the OvSHs by genetic approaches.

Leishmania and some *Trypanosoma* species are able to proliferate within macrophages by surmounting or evading two crucial components of innate immunity; the phagocyte oxidase (phox) and inducible nitric oxide synthase (NOS2). Early evidence for the importance of nitric oxide in the elimination of intracellular trypanosomatids came from studies with nitric oxide synthase inhibitors [14,15]. Subsequently, experiments with mice deficient in either phox or NOS2 have shown that, while a functional phox contributes to defence of the host against *Leishmania* parasites, the expression of NOS2 is essential for the elimination of these pathogens [16]. It was also shown [17] that down-regulation of T(SH)₂ reductase, the trypanosomal equivalent of glutathione reductase, compromised the survival of

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; Et₃N(MalNPh)CMe, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin; GSH, glutathione; GSH-SPD, glutathionylspermidine; GSNO, nitrosogluthathione; GSSG, glutathione disulphide; GSSOv, glutathione/OvSH mixed disulphide (heterodisulphide); NOS2, inducible nitric oxide synthase; OvSH, ovothiol A; phox, phagocyte oxidase; TFA, trifluoroacetic acid; T(SH)₂, trypanothione; T(SNO)₂, dinitrosotrypanothione.

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Figure 1 Trypanosomatid thiols

Although only the N¹-isomer of GSH-SPD is shown, N⁸-isomers are also present.

Leishmania donovani in interferon- γ -activated macrophages, which implied that the maintenance of an adequate reduced thiol pool is essential for the ability of the parasites to tolerate nitrosative stress.

While the T(SH)₂-based system for the detoxification of reactive oxygen intermediates has been well characterized [18–21], nothing is known at present about the metabolism of reactive nitrogen intermediates in the trypanosomatids. Among these compounds, the most stable naturally occurring forms are the nitrosothiols, which were shown to be parasitocidal when tested against *Leishmania major* [22]. It has been reported that the glutathione-dependent formaldehyde dehydrogenase has potent nitrosothiol reductase activity [23], which is widely distributed in Nature [24]. It was also shown that disruption of the formaldehyde dehydrogenase gene in yeast cells renders them hypersensitive to nitrosative stress [24]. In the present study, we evaluate the capacity of the trypanosomal thiol species to form stable nitrosothiols which can potentially serve as substrates for a putative thiol-dependent formaldehyde dehydrogenase. The presence of an analogous enzyme in the trypanosomatid, *Crithidia fasciculata*, could not be demonstrated. It is, however, well-known that reduced thiols can decompose nitrosothiols and the possibility that the unusual thiols of the trypanosomatids could serve such a function was, therefore, investigated. At the present time, no selective advantage in possessing T(SH)₂ rather

than its precursor, glutathione, as the principal low-molecular-mass thiol in the trypanosomatids has come to light. The results presented here suggest that OvSH and T(SH)₂ act in synergy to provide an efficient non-enzymic route for the detoxification of nitrosothiols to less toxic forms of nitrogen. Such synergistic interactions could provide a rational explanation for the co-existence of OvSH, T(SH)₂ and its biosynthetic precursors as principal low-molecular-mass thiols in the trypanosomes.

EXPERIMENTAL

Materials

Glutathione (GSH), sodium nitrite, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), α -ketoglutarate, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, 4,4'-dithiodipyridine and sulphanilamide were purchased from Sigma Chemical Company, ergothioneine disulphide and GSH-SPD were from Bachem and beef liver glutamate dehydrogenase was from Roche Diagnostics. 7-Diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin [Et₂N(MalNPh)CMe] was synthesized as described in [25].

Cell culture

C. fasciculata was cultured as described in [13].

Preparation of thiols

T(SH)₂ was isolated as described in [26]. Eggs of the South African abalone, *Haliotis midae*, were found to possess significant amounts of OvSH and were used for its isolation. A 20 ml settled bed of abalone eggs was thawed in 10 ml of 0.75 M HClO₄/2 mM EDTA and disrupted by sonication. The sample was centrifuged (48 000 *g* for 20 min at 4 °C using a Beckman JA 30.5 rotor) to remove the insoluble fraction and the supernatant was applied in 5 ml fractions to C18 Sep-Pak cartridges equilibrated with 0.1% trifluoroacetic acid (TFA). The cartridges were rinsed with 5 ml of 0.1% TFA. OvSH was contained in the eluate and was isolated as previously described in [4]. A total of 9.5 μ mol of OvSH was obtained (a yield of 24% in terms of total thiols). Its identity was confirmed by co-migration as the Et₂N(MalNPh)CMe derivatives on reversed-phase HPLC with OvSH in a *C. fasciculata* extract and by spectral analyses of the reduced and oxidized forms.

Reduction of thiols

Thiols were reduced by the addition of one fifth of the volume of 25 mg/ml NaBH₄ for 30 min at 37 °C and the reaction was terminated by the addition of one fifth of the initial volume of 1 M TFA. Thiols were estimated using 4,4'-dithiodipyridine [27].

Preparation of nitrosothiols

Nitrosothiols were prepared by reaction with an equimolar amount of sodium nitrite in 0.1 M HCl for 20 min at room temperature (23 °C) and in the dark. The compounds were quantified by measuring the absorbance at 335 nm (ϵ = 920 M⁻¹·cm⁻¹), which was found to correlate with the initial thiol concentrations. All solutions were freshly prepared for use on the day of the experiment.

Preparation of mononitrosotrypanothione

Reduced T(SH)₂ was reacted with a 50% molar equivalent of sodium nitrite (relative to thiol groups) in 0.1 M HCl for 20 min

at room temperature and in the dark. The sample was applied to a 5 μ m Phenomenex C18 column (250 mm $\times$ 4.6 mm) at a flow rate of 0.8 ml/min and eluted using the following conditions: 5 min at 100 % buffer A, a linear gradient to 70 % buffer A over 45 min, 5 min to 100 % buffer B followed by a return to initial conditions (buffer A: 0.1 % TFA; buffer B: 100 % CH₃CN). The elution of the nitrosylated derivatives was monitored at 335 nm, whereas the elution of T(SH)₂ was monitored by thiol analysis of the eluted fractions. The elution times of T(SH)₂, mononitroso-trypanothione and T(SNO)₂ were 30, 34 and 38 min respectively. The identity of mononitrosotrypanothione was evaluated by calculating the ratio of thiol content to S-nitroso content, measured at 335 nm, and a ratio of 1:1 was obtained.

Enzyme assays

Formaldehyde dehydrogenase

Assays for formaldehyde dehydrogenase were performed essentially as described in [23]. Assay mixtures contained 2 mM formaldehyde, 2.5 mM NAD⁺, 1 mM dithiothreitol and enzyme in 100 mM Tris/HCl, pH 8.35. Thiol-dependent enzyme assays contained, in addition, 500 μ M GSH, 500 μ M GSH-SPD or 250 μ M T(SH)₂. Reactions were followed spectrophotometrically at 30 °C by measuring the increase in absorbance at 340 nm.

Nitrosothiol reductase

Assay mixtures contained 500 μ M nitrosoglutathione (GSNO) or nitrosoglutathionylspermidine or 250 μ M T(SNO)₂, 0.3 mM NADH, 0.5 mM EDTA and enzyme in 100 mM Tris/HCl, pH 7.6. The assay was performed at a temperature of 30 °C and the decrease in absorbance at 340 nm was followed spectrophotometrically. Activities were corrected for NADH oxidase activity. An absorption coefficient of 7.13 mM⁻¹·cm⁻¹, corresponding to the combined spectral changes owing to the utilization of NADH (6.22 mM⁻¹·cm⁻¹) and nitrosothiol (0.91 mM⁻¹·cm⁻¹) was used.

Preparation of Hb

Human Hb was prepared by washing red blood cells in 0.9 % (w/v) NaCl, followed by lysis in deionized water. The lysed cells were centrifuged and Hb was obtained by passing 1 ml aliquots through a 16 mm $\times$ 180 mm Sephadex G-25 column prepared in 25 mM potassium phosphate buffer, pH 7.6. The peak fractions were collected and the oxyHb concentration was determined using the difference absorption coefficients of 8150 M⁻¹·cm⁻¹ (541 nm) or 10487 M⁻¹·cm⁻¹ (576 nm) for the conversion of oxyHb into metHb.

Analytical methods

Thiols were analysed as their Et₃N(MalNPh)CMe derivatives by reversed-phase HPLC on a 5 μ m Luna phenyl-hexyl column (250 mm $\times$ 4.6 mm) essentially as described in [13]. Nitrite was determined by the Griess reaction [28]. Ammonia was estimated spectrophotometrically using an assay based on the reductive amination of α -ketoglutarate by glutamate dehydrogenase. Enzyme assays contained 150 μ M NADH, 0.1 % BSA, 2 mM ADP, 0.5 M α -ketoglutarate, 0.1 mM EDTA and 17.5 units/ μ l glutamate dehydrogenase in 84 mM triethanolamine/HCl, pH 7.5. Reactions were monitored at 37 °C and the decrease in absorbance at 340 nm was found to be dependent on the ammonia concentration in the sample.

Kinetic studies

An Ocean Optics USB2000 diode-array spectrometer fitted with a heating block was used for kinetic experiments. Absorbance changes are shown as (A₀ - A_t), where A₀ is the initial absorbance and A_t the absorbance at time t. All experiments were conducted in 50 mM potassium phosphate buffer, pH 7.4, and 0.5 mM EDTA at 37 °C, unless otherwise stated. The dependence of reaction rates on the pH was determined using glycine/HCl, pH 3.0, sodium succinate, pH 4.0 and 5.0, sodium phosphate buffer, pH 6.0, 7.0 and 8.0, sodium glycinate, pH 9.0 and 10.0, and diethylamine/HCl, pH 11.0 and 12.0, all at a concentration of 50 mM, in the presence of 0.5 mM EDTA. Anaerobic experiments were conducted in cuvettes equipped with rubber septa. Solutions were bubbled extensively with nitrogen and reactions initiated by the addition of GSNO using a gas-tight syringe. Additional information is included in the legends to the Figures.

RESULTS

Absence of thiol-dependent formaldehyde dehydrogenase/nitrosothiol reductase activity in crithidial extracts

GSH, GSH-SPD, T(SH)₂ and OvSH were analysed for their ability to act as cofactors for the formaldehyde dehydrogenase activity present in ammonium sulphate fractions of *C. fasciculata*. Although we observed a thiol-independent formaldehyde dehydrogenase activity in these fractions, no significant improvement in activity was obtained in the presence of the above-mentioned thiols (results not shown). The same fractions were evaluated for their ability to metabolize the nitrosothiols of GSH, GSH-SPD and T(SH)₂, but the activities observed were quite low [0.0027 to 0.0063 unit/mg, where 1 unit is defined as the enzymic capacity to utilize 1 μ mol of NADH/min at 30 °C (results not shown)]. Rat liver GSNO reductase has a K_m of 28 μ M for GSNO and a specific activity of 0.23 unit/mg was obtained for dialysed cytosol at 25 °C [23].

Role of trypanosomatid thiols in the decomposition of nitrosothiols: conversion of the trypanosomatid thiols into nitrosothiols

Owing to their aromatic nature, the mercaptohistidines are chemically distinct from the aliphatic thiols [29]. Whereas the reaction of aliphatic thiols with acidified nitrite is known to result in the formation of relatively stable nitrosothiols, the mercaptohistidines were readily oxidized to the disulphide [30]. The addition of equimolar nitrite to OvSH resulted in a rapid absorbance increase at 246 nm that was complete within the mixing time and could perhaps be indicative of the transient formation of a nitrosothiol, but since no long wavelength absorbance peaks were observed either at 330 nm or in the region of 550 nm (Figure 2), it is possible that this early spectrum already represented that of an ovoidiyl radical, formed by homolytic fission of a nitrosothiol. This was followed by an isosbestic decay to the disulphide, the assignment of which was based on a comparison with the spectrum of an authentic sample of OvSH disulphide. When OvSH was mixed with a 50 % molar equivalent of nitrite, only approx. 50 % of the increase in absorbance at 246 nm was observed, and this was again followed by decay to a spectrum indicative of the disulphide. Even a moderately stable nitrosothiol was, therefore, not produced in the reaction of OvSH with nitrite under acidic conditions. Instead, these results indicate that the reaction of an equimolar amount of OvSH and nitrite resulted in a one-electron reduction of the nitrosonium ion to nitric oxide (reactions 1 and 2 below), in

Figure 2 Reaction of OvSH with acidified nitrite

The reaction was conducted at 25 °C and contained 50 μ M sodium nitrite and 50 μ M OvSH in 0.1 M HCl. The spectra are those of OvSH (·····), immediately after mixing (—), 150 s after mixing (— · — ·) and on completion of the reaction (— — —).

agreement with the results of Amado et al. [30] for a number of heterocyclic thiones, but that OvSH can also mediate a two-electron reduction of the nitrosonium ion to nitroxyl (reaction 3) at nitrite:OvSH ratios of 0.5:1, to again yield the disulphide.

Although long wavelength absorbance changes, characteristic of nitrosothiols, could not be detected, the conversion of OvSH into the disulphide (OvSSOv) by a 50% molar equivalent of nitrite is difficult to rationalize without the intermediate formation of a nitrosothiol, which is converted into the disulphide by nucleophilic attack at its sulphur by the excess thiol, as depicted in reaction 3.

Since $\text{T}(\text{SH})_2$ is a dithiol, it seemed possible that an attempt to convert it into $\text{T}(\text{SNO})_2$ may result, instead, in oxidation to the disulphide. The reaction of $\text{T}(\text{SH})_2$ with acidified nitrite, however, did not seem to differ from that observed with GSH and a stable dinitroso derivative was obtained.

Reaction of OvSH with GSNO

Spectral changes and kinetics of the reaction

The high rate of the OvSH-mediated decomposition of aliphatic nitrosothiols made it possible to study the reactions at concentrations of OvSH where spectral changes in the intense absorbance bands of OvSH in the UV region could be observed. At the present time, this has only been possible in transnitrosation reactions with *S*-nitroso-*N*-acetylpenicillamine as a donor of nitrosonium ions [31].

GSNO and OvSH were both found to be stable for several hours at 37 °C. Complex kinetic behaviour was observed during

Figure 3 Reactions observed during the decomposition of GSNO by OvSH

Reactions were conducted at 37 °C and contained 50 μ M OvSH and 400 μ M GSNO. (A) Difference spectra ($A_0 - A_t$) for the reaction taken at 1, 2.5, 5, 10, 15, 20 and 24.5 min (the arrow indicates the direction of the time-scale). Aliquots of the reaction mixture were removed, at the time points indicated, into an equal volume of 2 mM $\text{Et}_2\text{N}(\text{MalNPh})\text{CMe}$ and analysed by HPLC as described in the Experimental section. (B) The thiols analysed were OvSH (○) and glutathione (●). Inset, reaction kinetics monitored as the decline in absorbance at 280 nm (○) and 360 nm (●), which reflect reactions involving the OvSH and GSNO chromophores respectively.

the reaction of OvSH with GSNO (Figure 3A and Figure 3B inset), which consisted of rapid and slow phases. Spectral changes associated with the rapid phase predominated at 280 nm and mainly reflected changes in the OvSH chromophore whereas the biphasic nature of the reaction was more prominent at 335 nm at the absorbance maximum of GSNO. The absorbance changes at 360 nm primarily reflected changes in the GSNO chromophore and this wavelength was chosen to limit the contribution of the

Figure 4 OxyHb assay of NO[•] release during the OvSH/GSNO reaction

Experiments were performed at 37 °C and were initiated by the addition of OvSH. **(A)** Spectral changes for the conversion of 320 μM oxyHb into metHb during the reaction of 50 μM OvSH with 1 mM GSNO (0.2 cm cuvette). Spectra are shown for $t = 0, 103, 410, 697$ and 1230 s after the addition of OvSH. **(B)** Reactions observed at 280 nm as the absorbance change, $A_0 - A_t$, during the decomposition of 1 mM GSNO by either 50 μM (○) or 100 μM (●) OvSH in the absence of oxyHb (1 cm cuvette). **(C)** Kinetics of NO[•] formation during the reaction of OvSH with 1 mM GSNO in the presence of 320 μM oxyHb. Reactions were initiated at 120 s by the addition of 50 μM (○) or 100 μM (△) OvSH. NO[•] concentrations were based on the difference absorption coefficient of $8150 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 541 nm for the conversion of oxyHb into metHb (0.2 cm cuvette). **(D)** Comparison of the kinetics of the reaction of 100 μM OvSH with 1 mM GSNO in the presence (●) and absence of 320 μM oxyHb (○). GSNO consumption in the absence of oxyHb and NO[•] production in the presence of oxyHb are compared on identical scales of 350 μM.

OvSH chromophore, which is significant in the 335 nm region. Unfortunately, the slow phase of the reaction, observed at 360 nm, did not adhere to an identifiable rate law and, as a result, it was not possible to deconvolve the two phases of the reaction in order to obtain the individual rate constants.

The absorbance changes at 280 nm followed pseudo-first-order kinetics and were linearly dependent on the GSNO concentration. A second-order rate constant of $19.17 \pm 0.28 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($n = 4$) was obtained for the reaction of 46 μM OvSH with variable GSNO. Aliquots of a reaction mixture were removed and immediately reacted with Et₂N(MalNPh)CMe, a maleimide which reacts rapidly with thiols. {Based on previous results, the half-life [26] for the reaction of Et₂N(MalNPh)CMe with OvSH at its initial concentration was estimated to be approx. 0.7 s}. The results of subsequent HPLC analysis (Figure 3B) indicated that thiol equivalents lost as OvSH did not reappear quantitatively in GSH. This implies either that a large proportion of OvSH and GSH is present as the hypothetical transnitrosation intermediate, GS-N(OH)-SOv, shown in reaction 4, or that the

reaction has proceeded further to generate additional reaction intermediates.

Since we were unable to demonstrate the formation of a stable S-nitroso OvSH by the reaction with acidified nitrite, it seemed likely that OvSNO, if transiently formed at neutral pH, would also rapidly decompose, presumably to the ovothiyl radical and nitric oxide. Two lines of evidence supported this possibility.

When oxyHb was included in the reaction mixture, a conversion of Hb into metHb was observed (Figure 4A). The reaction of oxyHb with NO[•] to form metHb and nitrate is considered to be diagnostic for nitric oxide [32,33]. The reaction was markedly biphasic, with the first phase (Figure 4C) roughly corresponding in rate to the rapid phase observed at 280 nm in the traces presented in Figure 4(B), and which can be attributed to changes in the OvSH chromophore. The subsequent slower phase proceeded linearly until a complete conversion of oxyHb into metHb had been achieved (Figure 4C). Even with only

Figure 5 Inhibition of the second catalytic phase of the OvSH/GSNO reaction by DMPO

Reactions were performed at 37 °C and contained 50 μM OvSH, 500 μM GSNO and 0 (●), 0.25 (Δ) or 0.5 M DMPO (○). The absorbance change ($A_0 - A_t$) reflects a decline in absorbance at 360 nm over time.

50 μM OvSH, a complete conversion of 320 μM oxyHb into metHb was observed and the role of OvSH therefore appeared to be catalytic, requiring turnover. The consumption of GSNO in the absence of oxyHb was significantly slower than the rate of NO^* production in its presence, and it is apparent that oxyHb can facilitate the regeneration of OvSH (Figure 4D). By back-extrapolation of the slower, linear phase to the time of addition of OvSH, the amount of NO^* released in the rapid phase was estimated to be roughly stoichiometric with the amount of OvSH added to initiate the reaction. This result also indicated that the decomposition of the intermediate that gave rise to NO^* , presumably OvSNO, was rapid compared with its formation.

In the presence of only OvSH and GSNO, the decomposition of GSNO also exceeded the amount of OvSH, as judged from the absorbance changes at 360 nm ($\epsilon = 679 \text{ M}^{-1} \cdot \text{cm}^{-1}$), thus indicating a recycling of reducing equivalents, which could be more accurately quantified in terms of the recovery of nitrite relative to OvSH added (see below). The second-order rate constant for the GSNO/GSH reaction ($0.008 \text{ M}^{-1} \cdot \text{s}^{-1}$) [34] is at least three orders of magnitude slower than the OvSH/GSNO reaction and it is, therefore, likely that the GSH formed in the transnitrosation reaction contributed only indirectly to the observed decomposition of GSNO, by reducing oxidized OvSH species.

A second line of evidence in support of the formation of NO^* by homolytic fission of OvSNO came from the effect of DMPO on the decomposition of GSNO by OvSH (Figure 5). DMPO is a spin-trapping agent that reacts rapidly with thyl radicals ($k_2 = 10^7\text{--}10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$) [35]. An effect on the reaction rates was only observed at large concentrations of DMPO and, consequently, the reaction could only be monitored at 360 nm, due to the contribution of the DMPO chromophore in the UV region. Concentrations in excess of 0.1 M DMPO were found to result in a progressive inhibition of the second, slower phase of the OvSH/GSNO reaction. This result implied that ovothiyl radicals

Figure 6 Saturation kinetics of OvSH/GSNO reaction in the presence of GSH

Experiments were performed at 37 °C and contained 2 mM GSH, 1 mM GSNO and 0, 25, 50, 75 or 100 μM OvSH (the arrow indicates the direction of increasing OvSH concentration). Inset, plot of GSNO consumed against OvSH concentration.

were generated in the first rapid phase and that further decomposition of GSNO could be aborted by trapping the OvSH as a DMPO adduct.

The ability of organisms to tolerate nitrosative stress differs widely and nitrosothiols or NO^* itself are often only microbicidal in the millimolar range [36]. In an experiment which, therefore, mimics a nitrosative stress situation more closely, the decomposition of 1 mM GSNO in the presence of 2 mM GSH and varying levels of OvSH was monitored at the absorbance maximum of GSNO at 335 nm (Figure 6). Under these conditions, the early stage of the decomposition of GSNO proceeded linearly. It is, however, evident that the dependence of the rate of decomposition on OvSH concentration followed saturation kinetics (Figure 6, inset). This saturation phenomenon was also evident in previous studies [37] of the GSNO/GSH reaction, in which case it could most likely be ascribed to an increasing involvement of the reducing thiol in alternative reaction pathways, to generate an increased proportion of reduced nitrogen species, as the GSH concentration increased. In the OvSH/GSNO reaction, however, a corresponding variation in the products of the reaction was not observed (see below) and the saturation phenomenon is more likely to be the result of the chain-terminating reaction of ovothiyl radicals to form OvSH disulphide.

Nitrogenous products of the OvSH/GSNO reaction

In previous studies, the formation of multiple products has been a prominent feature of the thiol-mediated decomposition of nitrosothiols and complex reaction schemes were proposed to account for this observation. Recent studies of the products formed in the reaction between GSNO and GSH have identified glutathione disulphide (GSSG), glutathione thiosulphinate [GS(O)SG], glutathione sulphinic acid [GS(O)OH] and glutathione sulphinamide [GS(O)NH₂] as the ultimate products de-

Table 1 Formation of nitrite in the presence and absence of oxygen during the reaction of 100 μ M GSNO with different ovothiol A concentrations

Samples were incubated for 20 h at 37 °C in the dark and nitrite was determined as described in the Experimental section. Values are the means of two determinations expressed as a percentage of GSNO provided. n.d., not determined.

Ovothiol A (μ M)	Nitrite (μ M)	
	Aerobic	Anaerobic
0	15	7
10	60	76
25	77	n.d.
50	99	85
100	99	n.d.
200	99	90
500	96	87

rived from the glutathione entity, while the nitroso nitrogen was recovered in glutathione-sulphinamide, hydroxylamine, nitrite, N_2O and ammonia [37,38]. When OvSH was incubated with GSNO, nitrite was recovered as the major product (Table 1). The nitrite yields at low OvSH to GSNO ratios provided further evidence for the recycling of reducing species. Up to 60 μ M nitrite was obtained when 10 μ M OvSH was incubated with 100 μ M GSNO (Table 1). It was suggested that the formation of nitrite from the nitrosothiol/thiol reaction mixtures is brought about by the presence of catalytic amounts of copper [39]. Our experiments were, however, conducted in the presence of 500 μ M EDTA, which practically eliminates the metal-ion-catalysed decomposition of nitrosothiols. The metal-ion-independent reaction of aliphatic thiols with nitrosothiols leads predominantly to the reduction of the nitrosothiol, with the proportion of nitrogen recovered as ammonia increasing as the ratio of thiol to nitrosothiol increases [37,38]. At a ratio of GSH to GSNO of 5:1, approx. 64 % nitrite and 23 % ammonia were recovered under aerobic conditions, in agreement with the results of Singh et al. [37]. In contrast, up to 96 % of the nitrogen in GSNO was recovered as nitrite in the OvSH-catalysed decomposition of GSNO at ratios of OvSH to GSNO of 5:1 (Table 1). The differences in the products obtained can possibly be ascribed to the free energies of homolysis of the S-N bond in an aliphatic thiol, as compared to an aromatic one, such as OvSH, where the thiyl radical is resonance-stabilized. The effect of anaerobiosis was to decrease the nitrite formation at higher OvSH concentrations. Studies on the GSNO/GSH reaction have demonstrated that N_2O is the major nitrogenous product produced at the expense of nitrite under anaerobic conditions [37,38].

Effect of anaerobicity on the OvSH/GSNO reaction

Under anaerobic conditions, the value of the observed rate constant, k_{obs} , obtained for the rapid phase of the reaction seen at 280 nm (0.0178 s^{-1}) was almost 2.5-fold slower than that obtained under aerobic conditions (0.0436 s^{-1}), suggesting that this phase of the reaction consisted of parallel oxygen-dependent and oxygen-independent processes. Surprisingly, however, the rate of the slow phase of the reaction, detected at 360 nm, was unaffected by the presence or absence of oxygen. The overall reaction appeared to be less sensitive to the presence of oxygen than the decomposition of GSNO by ergothioneine, where an 8-fold difference in rates was observed [40], while a decrease of approx. 2-fold in the rate of decomposition of GSNO by GSH was reported [37].

Figure 7 pH-dependence of the reaction of OvSH and GSNO

Reactions were conducted at 37 °C in the presence of 100 μ M OvSH and 1 mM GSNO. (A) Reactions observed at 280 nm for experiments conducted at pH 3 ($\blacktriangle$), 5 ($\square$), 8 ($\blacklozenge$), 9 (∇) and 12 ($\bullet$). (B) Reactions observed at 360 nm for experiments conducted at pH 3 ($\circ$), 7 ($\bullet$), 8 ($\square$), 9 ($\blacksquare$), 10 ($\triangle$) and 12 ($\blacktriangle$).

The influence of pH on the reaction of OvSH with GSNO

Since the reactive species in the transnitrosation reaction is expected to be the thiolate anion [37], the pH dependence of the reaction between OvSH and GSNO was studied and found to be complex. Hence, no attempt was made to obtain rate constants in these experiments (Figure 7). Qualitatively, however, the pH dependence of the OvSH/GSNO reaction provided further

Table 2 Formation of nitrogen products during the reaction of 200 μM GSNO with different T(SH)_2 concentrations

Samples were incubated for 20 h at 37 °C in the dark and ammonia and nitrite were determined as described in the Experimental section. Values are the means of two determinations expressed as a percentage of GSNO provided. n.d., not determined.

T(SH)_2 (μM)	Nitrite (%)	Ammonia (%)	Total nitrogen (%)
0	8	n.d.	n.d.
25	22	n.d.	n.d.
50	38	n.d.	n.d.
100	70	11	81
250	63	40	103
500	36	60	96

evidence of the involvement of several reaction intermediates. Pronounced changes in the kinetics of the reaction and magnitude of spectral changes were observed at 280 nm within pH ranges where neither OvSH nor glutathione in their oxidized or reduced forms are known to have dissociation constants to which these effects could be ascribed. At pH 3, for example, the absorbance of the OvSH chromophore at 280 nm (Figure 7A) decreased rapidly in a process suggestive of zero-order kinetics while obeying first-order kinetics at neutral pH. The spectral changes at 360 nm (Figure 7B), however, did indicate an accelerated decomposition of GSNO with increasing pH. The most significant change in the rate at 360 nm was observed between pH 8.0 to 9.0, a region that corresponds to the pK_a of the glutathione thiol group ($\text{pK}_a = 8.6$). The thiolate anion is the reactive species in thiol/disulphide exchange reactions and the improved ability to reduce OvSH was reflected in an enhanced rate of decomposition of GSNO.

Reaction of T(SH)_2 with GSNO and of OvSH with T(SNO)_2

For the reaction of GSNO with T(SH)_2 , a second-order rate constant of $0.786 \pm 0.048 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($n = 4$) was obtained by measuring the initial rates of absorbance change at 335 nm. GSNO and variable amounts of T(SH)_2 were incubated at 37 °C for 20 h to determine the major nitrogen-containing end products (Table 2). Nitrite was the major end product up to ratios of T(SH)_2 to GSNO of 1:2 [this corresponds to a ratio of 1:1 in terms of the thiol content of T(SH)_2]. At higher ratios of T(SH)_2 to GSNO, ammonia is produced at the expense of nitrite. We observed some 'missing' nitrogen product at ratios of T(SH)_2 to GSNO of 1:2 and it is likely to be present as the *S*-amine or sulphinamide of T(SH)_2 , which are expected to accumulate when the thiol component is not present in excess [37].

The reaction of OvSH with T(SNO)_2 showed more unusual kinetics (Figure 8). These reactions were conducted at a constant initial T(SNO)_2 concentration and variable OvSH concentration. At higher OvSH concentrations, an increasingly pronounced sigmoid reaction profile became apparent at 280 nm (Figure 8A). This reaction profile was less prominent at 360 nm (Figure 8B) and the initial rates were used to estimate the rate constants for the early phase using the rate equation:

$$-d[\text{T(SNO)}_2]/dt = k_2 \cdot [\text{T(SNO)}_2] \cdot [\text{OvSH}]$$

The initial rate constant for the decomposition of *S*-nitroso groups was $8.67 \pm 0.15 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($n = 4$) or somewhat less than half of the rate seen with GSNO as the nitrosonium ion donor, but was linearly dependent on the OvSH concentration. We considered that the acceleration in nitrosothiol decomposition

Figure 8 Kinetics of the reaction of OvSH with T(SNO)_2

Reactions were conducted at 37 °C and contained 250 μM T(SNO)_2 and 50 (○), 100 (●), 150 (△) or 200 μM (▲) OvSH. Reactions are shown as the change in absorbance ($A_0 - A_t$) at 280 nm (A) and 360 nm (B) and initial rates at 360 nm were used to calculate the second-order rate constant.

could be due to the formation of mononitrosotrypanothione in which the thiol group reacts intramolecularly with the nitroso group. An accelerated decomposition would, in that case, only be obtained in a reaction that results in the oxidation of T(SH)_2 to the disulphide and reduction of the nitroso group to nitroxyl. Mononitrosotrypanothione was, therefore, synthesized by the addition of a 50% molar equivalent of nitrite, relative to thiol groups, to T(SH)_2 and isolated by HPLC. Mononitrosotrypanothione was found to decompose with a first-order rate constant of $4.14 \times 10^{-4} \text{ s}^{-1}$, which is too slow to account in itself for the

Figure 9 Comparison of the kinetics of the reaction of OvSH with GSNO and T(SNO)₂

Reactions are shown as the change in absorbance ($A_0 - A_t$) at 360 nm and contained 100 μ M OvSH and 500 μ M GSNO (●) or 250 μ M T(SNO)₂ (○).

Scheme 1 Minimal scheme for the catalytic reaction of OvSH with T(SNO)₂

observed increase in the rate of nitrosothiol decomposition seen in Figure 8(B). Moreover, the sigmoidal traces were also seen at 280 nm, where the spectral changes reflect principally changes in the OvSH chromophore. Perhaps an investigation of the decomposition of a range of nitroso derivatives of dithiols will shed further light on this phenomenon, which is not, at present, understood. When observed at 360 nm, however, the decomposition of T(SNO)₂ proceeded at a much faster rate than that of GSNO (Figure 9) and this is probably due to a more efficient intramolecular reduction of OvSH, presumably present as T(SH)(SSOv) (Scheme 1).

DISCUSSION

S-Nitrosothiols have been extensively studied due to their ability to act as nitric oxide donors and bioregulators. Studies in *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana* and various mammalian sources indicate that the glutathione-dependent formaldehyde dehydrogenases may have an important role in the detoxification of nitrosothiols [23,24,40]. However, only low nitrosothiol reductase activities could be detected in ammonium sulphate fractions of *C. fasciculata*, an organism that is metabolically closely related to the *Leishmania* parasites. The activities observed were more than two orders of magnitude lower than that observed for the mammalian systems at similar stages of purification and are unlikely to have a significant role in the detoxification of nitrosothiols.

A recent study on the decomposition of nitrosothiols by ergothioneine [41] prompted us to investigate the role of the mercaptohistidine, OvSH. The second-order rate constant for the reaction of OvSH with GSNO ($19.2 \text{ M}^{-1} \cdot \text{s}^{-1}$) can be compared with much lower rate constants reported in the literature for ergothioneine ($0.178 \text{ M}^{-1} \cdot \text{s}^{-1}$) [40] and GSH ($0.0083 \text{ M}^{-1} \cdot \text{s}^{-1}$) [34]. It has been proposed that the transnitrosation reaction proceeds via nucleophilic attack of the thiolate anion on the nitrosothiol nitrogen [37] and, consequently, it may be expected that OvSH would be more effective than ergothioneine, which is present predominantly in the thione form.

A scheme to account for the formation of a range of nitrogenous products in various oxidation states, depending on the ratio of thiol to nitrosothiol in nitrosothiol/thiol reactions and the absence or presence of oxygen was earlier proposed by Singh et al. [37]. Scheme 2 is a minimal scheme, adopted from the one proposed by Singh et al. [37], which is consistent with the experimental results obtained in the present case. Two possible routes of decomposition of GSNO by OvSH are proposed. In a pathway which could predominate at low OvSH concentrations, transnitrosation is proposed to result in the formation of the unstable OvSNO which decomposes rapidly to generate the ovothiyl radical and nitric oxide. The ovothiyl radical is proposed to react with a second GSNO to form a second molecule of nitric oxide and the glutathione/OvSH mixed disulphide (heterodisulphide), GSSOv. The observed stoichiometry with which methHb is formed in the initial rapid reaction of Figure 4(C) indicates that this reaction, which involves the formation of a second NO*, must be slow. GSH released in the transnitrosation step regenerates OvSH from OvSSG and produces GSSG. At low concentrations, the role of OvSH in this pathway is catalytic, thus accounting for the production of reaction products substantially in excess of the amount of OvSH, but, at higher concentrations, dimerization of the ovothiyl radical is likely to compete with its reaction with GSNO and this most probably accounts for the observed saturation effect (Figure 6, inset).

It is doubtful that reaction of OvSH at the GSNO sulphur to form a mixed disulphide and nitroxyl has a significant role, since this leads to reactions which do not allow for the observed recycling of OvSH and can, moreover, generate peroxide.

Also shown in Scheme 2 is a pathway of GSNO decomposition that diverges at the level of the transnitrosation intermediate GS-N(OH)-SOv to form GS-N(OH)* and OvS*. The latter was proposed to react with an additional molecule of GSNO to produce the GSSOv heterodisulphide and nitric oxide. The observed dependence on oxygen of the rapid phase of the reaction seen at 280 nm is consistent with the proposed reaction of GSH-N-(OH)* with oxygen to form unstable oxygenated intermediates. These were proposed to react with the thiol, in this

Scheme 2 Reaction mechanisms likely to be involved in the decomposition of GSNO by OvSH

case OvSH, to generate an additional thiyl radical which could decompose an additional GSNO molecule. Although unambiguous evidence for the existence of the intermediates proposed to be formed in the oxygen-dependent decomposition of GSNO is, at the present time, lacking, there is ample experimental evidence for the existence of an oxygen-dependent pathway for the decomposition of GSNO by thiols. In the presence of oxygen and water, nitric oxide reacts quantitatively to produce nitrite [42], the major product of the OvSH/GSNO reaction according to reaction 5:

It is evident that the combination of T(SH)₂ and OvSH in trypanosomatids provides them with a system which is remarkably efficient in the non-enzymic decomposition of nitrosothiols. Moreover, OvSH seemed to be also more efficiently regenerated in the presence of oxyHb than in its absence (Figure 4D) and, in that case, the final nitrogenous product is likely to be nitrate. Further studies are also needed to understand this effect. At the present time, little is known about the possible occurrence of Hb homologues in the trypanosomatids, although such proteins have been found in other protozoa [43]. It is, however, evident that, in trying to understand the reasons for the divergent thiol composition of the trypanosomatids, the answer may reside also in the interaction of the thiol species with each other, an example of which is afforded by the synergistic interaction of OvSH and T(SH)₂ in the decomposition of GSNO.

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