The metabolism of nitrosothiols in the mycobacteria: identification and characterization of S-nitrosomycothiol reductase

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When grown in culture *Mycobacterium smegmatis* metabolized S-nitrosoglutathione to oxidized glutathione and nitrate, which suggested a possible involvement of an S-nitrosothiol reductase and mycobacterial haemoglobin. The mycothiol-dependent formaldehyde dehydrogenase from *M. smegmatis* was purified by a combination of Ni²⁺-IMAC (immobilized metal ion affinity chromatography), hydrophobic interaction, anion-exchange and affinity chromatography. The enzyme had a subunit molecular mass of 38 263 kDa. Steady-state kinetic studies indicated that the enzyme catalyses the NAD⁺-dependent conversion of S-hydroxymethylmycothiol into formic acid and mycothiol by a rapid-equilibrium ordered mechanism. The enzyme also catalysed an NADH-dependent decomposition of S-nitrosomycothiol (MSNO) by a sequential mechanism and with an equimolar stoichiometry of NADH:MSNO, which indicated that the enzyme reduces the nitroso group to the oxidation level of nitroxyl. $V_{\max}$ for the MSNO reductase reaction indicated a turnover per subunit

of approx. 116 700 min⁻¹, which was 76-fold faster than the formaldehyde dehydrogenase activity. A gene, Rv2259, annotated as a class III alcohol dehydrogenase in the *Mycobacterium tuberculosis* genome was cloned and expressed in *M. smegmatis* as the C-terminally His₆-tagged product. The purified recombinant enzyme from *M. tuberculosis* also catalysed both activities. *M. smegmatis* S-nitrosomycothiol reductase converted MSNO into the N-hydroxysulphenamide, which readily rearranged to mycothiolsulphinamide. In the presence of MSNO reductase, *M. tuberculosis* HbN (haemoglobin N) was converted with low efficiency into methHbN [HbN(Fe³⁺)] and this conversion was dependent on turnover of MSNO reductase. These observations suggest a possible route *in vivo* for the dissimilation of S-nitrosoglutathione.

Key words: formaldehyde dehydrogenase, mycobacteria, mycothiol, S-nitrosomycothiol reductase.

INTRODUCTION

Tuberculosis is the leading cause of human death due to a single bacterial infection. It is, nonetheless, thought that a large percentage of mycobacterial infections do not result in active disease, but that the infection persists due to a balance between the antimicrobial mechanisms of the host and the ability of the pathogen to surmount these mechanisms [1,2]. The imposition of oxidative and nitrosative stress by host cells are indispensable components of innate immunity. The proliferation of a pathogen within its host therefore depends on strategies aimed at evading or counteracting the onslaught of ROI (reactive oxygen) and RNI (reactive nitrogen) intermediates. When these processes of detoxification differ from their counterparts in the host, the reactions become of chemotherapeutic interest.

While the detoxification of ROI is brought about by a number of enzymes, which, for the most part, have been well characterized, the production of RNI has only in recent years come to be recognized as indispensable for the defence of the host against a number of pathogens, including *Mycobacterium tuberculosis* [1]. Mechanisms whereby bacterial pathogens protect themselves against nitrosative stress are therefore of interest and such a mechanism was first assigned to the bacterial flavohaemoglobin, which converts nitric oxide into nitrate under aerobic or micro-aerophilic conditions [3–6]. The presence of electron acceptors such as oxygen, however, facilitates the conversion of NO[•] into nitrosating species at the oxidation level of the nitrosonium ion, such as in N₂O₃, and consequently treatment of cells with nitric

oxide or nitric oxide donors results in the accumulation of the more long lived S-nitrosothiols [7,8]. Low-molecular-mass S-nitrosothiols can nitrosate critical thiol groups in proteins by a process of transnitrosation and thus disrupt cellular function. Evidence was presented that GSNO (S-nitrosoglutathione) enters *Mycobacterium smegmatis* through a peptide transporter and is microbicidal [9], while peptide-transporter-deficient mutants of *Mycobacterium bovis* displayed increased virulence in murine macrophages compared with the wild-type [10]. A role in the detoxification of nitrosothiols by bacteria was initially also attributed to the flavohaemoglobin, but it was subsequently found that the glutathione-dependent formaldehyde dehydrogenases from various organisms are considerably more active as GSNO reductases than as S-hydroxymethylglutathione dehydrogenases [11,12]. GSNO reductase activity, therefore, seemed likely to constitute a major route for the detoxification of S-nitrosothiols. Similar enzymes were shown to be present in organisms from widely separated stages of evolutionary development and mutants lacking GSNO reductase were found to be hypersensitive to GSNO [12]. S-nitrosothiol reductase activity thus appeared to be an inherent property of glutathione-dependent formaldehyde dehydrogenases, but mechanistic differences between enzymes from various sources came to light. Such differences were reflected in the stoichiometry with which NADH was utilized relative to GSNO. Thus the enzyme from *Escherichia coli* was proposed to catalyse sequential reductions of the NO group of GSNO to ammonia with NADH and GSH as the electron donors, as shown in Scheme 1(A). The rat liver enzyme, on the other

Abbreviations used: DTT, dithiothreitol; Et₂N(MalNPh)CMe, 7-diethylamino-4-methyl-3-(4'-maleimidyl-phenyl)-coumarin; GSNO, S-nitrosoglutathione; GSSG, glutathionedisulphide; IMAC, immobilized metal ion affinity chromatography; IPTG, isopropyl β-D-thiogalactoside; HbN, haemoglobin N; methHbN, HbN(Fe³⁺); MSH, mycothiol; MSNO, S-nitrosomycothiol; Na-PPI, sodium pyrophosphate; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; TFA, trifluoroacetic acid.

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Scheme 1 Proposed route for the decomposition of GSNO by *E. coli* GSNO reductase (A) and rat liver 'GSNO terminase' (B)

Figure 1 The structure of MSH

hand, was proposed to use equimolar NADH and GSNO for the two-electron reduction of the NO group to the oxidation state of nitroxyl (NO^-), which in the absence of excess GSH rearranged to glutathione sulphinamide as the main product (Scheme 1B). In the presence of high GSH concentrations, however, the main nitrogenous products were hydroxylamine and ammonia and very little nitrite or nitrate could be detected. It is unclear whether the reduction of reaction intermediates by GSH is also enzyme catalysed. Consistent with these properties of the rat liver enzyme, mammalian cells produce variable, but low, amounts of nitrite, but negligible nitrate, when exposed to GSNO [13,14] or compounds which gave rise to GSNO [15]. Paradoxically, glutathione sulphinamide could also be detected in cell-free extracts which had been exposed to GSNO and NADH, but intact cells were found not to accumulate glutathione sulphinamide when exposed to GSNO [11,16]. The products obtained when intact bacteria metabolize GSNO have not been characterized.

From a chemotherapeutic perspective, the discovery of GSNO reductase is of considerable interest, since several pathogenic micro-organisms do not produce glutathione, but alternative low-molecular-mass thiols, as their major redox buffer. In particular, the Actinomycetes, which include the medically important mycobacteria [17,18], produce MSH (mycothiol) (Figure 1), rather than glutathione, and possess an MSH-dependent formaldehyde dehydrogenase [19]. MSH was shown to play a role in the detoxification and export of alkylating agents, such as mercapturic acids [20]. MSH-deficient mutants were found to be hypersensitive to alkylating agents, to a number of antibiotics and to peroxide, but surprisingly, resistant to isoniazid [21,22]. Not all of these effects are presently understood.

In the present study, we report that the metabolism of GSNO by *M. smegmatis* results in the accumulation of stoichiometric amounts of GSSG (glutathionedisulphide) and nitrate in the culture medium. This observation suggested the possible involvement of the mycobacterial equivalent of the ubiquitous

S-nitrosothiol reductase and of HbN (haemoglobin N), a truncated haemoglobin which had been shown to play a role in the metabolism of RNI in the mycobacteria [23,24]. We found that the product of the *adhE2* gene (*Rv2259*) of *M. tuberculosis* is indeed primarily an MSNO (*S*-nitrosomycothiol)-reductase. The enzyme also catalyses the oxidation of formaldehyde, but at a much lower rate. Evidence is presented that the two-electron reduction of MSNO results in the formation of the unstable *N*-hydroxysulphenamide of MSH. HbN is converted into methHbN [HbN (Fe^{3+})] in the presence of MSNO, NADH and MSNO reductase.

EXPERIMENTAL

Materials

MSH was isolated from *M. smegmatis* as described previously [25]. The *E. coli*-mycobacteria shuttle vector pSD26 was a gift from Dr W. R. Jacobs (Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY, U.S.A.). Imidodiacetic acid agarose and Phenyl Sepharose CL-4B were obtained from Sigma Chemical Co., and 5'-AMP-Sepharose was from Pharmacia. The pCRBlunt cloning kit was from Invitrogen. *S*-Nitrosothiols were synthesized by the addition of an equimolar amount of nitrite under acidic conditions (0.1 M HCl) and concentrations were estimated using a molar absorption coefficient of $920 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 335 nm. [^3H]Inositol-labelled MSNO was prepared by labelling *M. smegmatis*. When exponentially growing cells were cultured in the presence of [^3H]inositol (Amersham, 555 GBq/mmol), approx. 65 % of the label was taken up by the cells. Most of the radioactivity was incorporated into material which co-eluted with MSH during HPLC on C_{18} reversed-phase columns. MSH was estimated as described previously [26] and was treated with an equimolar amount of sodium nitrite. The resultant MSNO was freeze-dried and purified by HPLC on a $250 \text{ mm} \times 4.6 \text{ mm}$ Phenomenex Luna C_{18} column. After the sample was injected, the column was eluted at a flow rate of 0.8 ml/min as follows: solvent A for 5 min, followed by a linear gradient of 0–70 % solvent B for 25 min, a linear gradient of 70–100 % solvent B for 5 min, 100 % solvent B for 5 min, then a return to 0 % solvent B [solvent A: 0.1 % TFA (trifluoroacetic acid); solvent B: 1 % TFA in a water:acetonitrile mixture (40:60, by vol.)]. In this system, MSH was eluted at 11.25 min, MSSM at 18.75 min and MSNO at 20.25 min.

Isolation of MSNO reductase/formaldehyde dehydrogenase from *M. smegmatis*

M. smegmatis, either wild-type mc² 155 or constructs containing the *M. tuberculosis adhE2*-encoded formaldehyde dehydrogenase gene inserted into the pSD26 plasmid, was cultured in the medium described by Bornemann et al. [27] and was harvested in the late exponential phase of growth. Frozen cells were stored until used and were then suspended in $3 \times (\text{w/v})$ the amount of 25 mM potassium phosphate buffer, pH 7.5, which also contained 0.5 M NaCl and 2 mM 2-mercaptoethanol (Buffer A). The cells were disrupted by sonication and the lysate was centrifuged at $48000g$ for 60 min at 4°C to obtain a crude extract. Formaldehyde dehydrogenase activity in this extract was MSH-dependent. The crude extract was applied to a $15.5 \text{ cm} \times 1.2 \text{ cm}$ Ni^{2+} -IMAC (immobilized metal ion affinity chromatography) column which had been equilibrated with Buffer A. The column was first eluted with Buffer A, until the absorbance of the effluent at 280 nm had decreased to 0.1, and then a 400-ml linear gradient of 0–200 mM imidazole in Buffer A (pH 7.5) was

Spectra were acquired in the continuum mode and a representative spectrum constructed by addition of the spectra during the elution of the protein, with subtraction of the background. Deconvolution of the multiply charged spectrum was accomplished with the MaxEnt programme.

Analysis of GSNO and the products of its dissimulation

GSNO, GSSG and nitrate were analysed by anion exchange chromatography essentially as described previously [15]. After centrifugation to remove bacteria, samples of the culture medium were applied to a 250 mm × 4.6 mm Partisil 10 SAX column and eluted at a flow rate of 1.5 ml/min as follows: solvent A for 2 min, followed by a linear gradient of 0–50% solvent B for 10 min, a linear gradient of 50–100% solvent B for 3 min, 100% solvent B for 2 min, followed by a return to 0% solvent B (solvent A: 7 mM H₃PO₄; solvent B: 300 mM H₃PO₄ adjusted to pH 2.0 with KOH). Under these conditions, GSNO was eluted at 3.8 min, GSSG at approx. 5.0 min and nitrate at 12.1 min.

RESULTS AND DISCUSSION

Products of the metabolism of GSNO by whole cells of *M. smegmatis*

When GSNO (0.125–2 mM) was added to the culture medium of *M. smegmatis* cells in the mid-exponential phase of growth, a progressive inhibition of growth was observed. Analysis of the culture medium by anion-exchange chromatography indicated that the disappearance of GSNO could be accounted for in the short term by the appearance of equivalent amounts of GSSG and nitrate. More prolonged periods of incubation, however, resulted in a gradual disappearance of the nitrate. Results for the decomposition of 250 µM GSNO are shown in Figure 2. GSNO itself was not significantly degraded when incubated in the growth medium in the absence of the bacteria for the time period which applied to the experiment shown in Figure 2.

The possibility that exposure of *M. smegmatis* to GSNO could result in a partial depletion of MSH, either as a result of the hydrolytic cleavage of MSNO to α-D-Gl [1-D-1-O-(2-amino-2-deoxy-α-D-glucopyranosyl)-myo-inositol] and S-nitrosocysteine by the S-alkylmycothiolamidase, or by conversion into the sulphinamide of MSH by a putative MSNO reductase (see below), was investigated by studying the effects of addition of 1 mM GSNO to the culture medium on the incorporation of [³H]inositol into cellular constituents of *M. smegmatis*. When analysed by HPLC on a C₁₈ reversed-phase column, tritiated MSH represented the major radiolabelled component in perchloric acid-soluble, cell-free extracts of *M. smegmatis*, and no significant decrease in the amount of radiolabel recovered in those fractions which contained MSH, or the appearance of new radiolabelled peaks was observed. In particular, MSH sulphinic acid was not retained on such columns, but no increase in radiolabel eluted in the early fractions was observed.

Purification of the native and recombinant enzymes from *M. smegmatis*

Since it may be possible to reconstitute *in vitro* the conversion of the nitroso group of GSNO into nitrate, which is evidently operative in intact mycobacteria, we decided to try to establish whether the MSH-dependent formaldehyde dehydrogenase of mycobacteria has MSNO reductase activity and whether the production of nitrate could be attributed to a close interaction with HbN, which is known to produce nitrate from NO[•] [31].

Figure 2 Metabolism of GSNO by *M. smegmatis*

M. smegmatis was cultured at 37 °C in Middlebrook 7H9 medium to $A_{600} = 0.55$. At this stage GSNO from a stock solution was added to a final concentration of 250 µM and incubation was continued. Aliquots of the culture were removed at intervals, the cells removed by centrifugation and the supernatant was analysed by HPLC as described in the Experimental section. The symbols indicate GSNO (●), NO₃⁻ (■) and GSSG (▲).

Table 1 Purification table for *M. smegmatis* MSH-dependent formaldehyde dehydrogenase

Crude extract was prepared from 20 g wet weight of cells. 1 unit of activity corresponds to the formation of 1 µmol of NADH/min per mg at 30 °C.

Step	Protein (mg)	Activity (units)	Yield (%)	Specific activity (µmol/min per mg)	Purification
1. Crude extract	1130	47.35	100	0.042	1
2. Ni ²⁺ -IMAC	212	48.79	103	0.230	5
3. Phenyl-Sepharose	30.65	32.77	69	1.069	26
4. Vydac DEAE	6.05	22.62	48	3.739	89
5. Vydac DEAE 2	4.43	19.57	41	4.418	105
6. 5'-AMP-Sepharose	0.852	19.29	41	22.641	540

The MSH-dependent formaldehyde dehydrogenase from wild-type *M. smegmatis* mc² 155 was purified 540-fold in a 41% yield using the six-step procedure summarized in Table 1. The protein was electrophoretically pure as judged by SDS/PAGE (Figure 3). A homologue with 78% predicted protein sequence identity to the Rv2259 gene product is present in the genome of *M. smegmatis* (Tigr *M. smegmatis* contig#3310), but the position of migration of the isolated native MSNO reductase/formaldehyde dehydrogenase corresponded to a molecular mass of 49.4 kDa, as compared with a predicted mass of 38.38 kDa from the gene sequence. The subunit molecular mass of the protein was therefore determined by electrospray MS and a mass of 38 263 Da was obtained. Given that there are still uncertainties in the *M. smegmatis* genome sequence, which has not as yet been completed and annotated, the mass spectral result is in satisfactory agreement with that predicted from the available sequence data.

Following induction of *E. coli* B121(DE3) or B121(DE3)plysS containing the pET23b:Rv2259 plasmid with IPTG, the Rv2259 gene of *M. tuberculosis* was expressed as an intense band which migrated on SDS/PAGE with a molecular mass of approx. 38 kDa when electrophoresed on the Pharmacia PhastSystem. The protein was, however, expressed in an inactive, insoluble form and strategies, such as lowering the temperature during induction of

Figure 3 SDS/PAGE showing purification of native and recombinant MSH-dependent formaldehyde dehydrogenase

The purification of native *M. smegmatis* MSH-dependent formaldehyde dehydrogenase is shown in lanes 2–7 and correspond to the crude extract, Ni^{2+} -IMAC, phenyl-Sepharose, Vydac 1, Vydac 2 and 5'-AMP-Sepharose purification steps. The purification of the recombinant *M. tuberculosis* His₆-tagged enzyme is shown in lanes 8–11. These lanes correspond to the crude soluble extract, membrane fraction, Ni^{2+} -IMAC and 5'-AMP-Sepharose purification steps respectively. Protein bands were visualized by silver staining [51]. Lanes 1 and 12, molecular mass markers.

the gene or decreasing the amount of IPTG used for induction, did not result in recovery of a soluble, active protein.

When expressed in *M. smegmatis* using the shuttle vector pSD26 a variable partitioning of the Rv2259 gene product between the soluble and insoluble fractions was observed. In cells induced with acetamide in the early exponential-phase of growth ($D_{600} < 0.4$) a significant proportion of MSNO reductase/formaldehyde dehydrogenase was expressed as a soluble protein, while induction with acetamide at a later stage of growth resulted in the accumulation of much of the expressed protein in an insoluble, inactive form. MSNO reductase/formaldehyde dehydrogenase present in the soluble fraction after sonication of *M. smegmatis* was eluted in at least four discrete peaks when chromatographed on an Ni^{2+} -IMAC column (Figure 4). Since the *M. smegmatis* and *M. tuberculosis* enzymes exhibit 78% amino acid sequence identity, it is possible that the assembly of the enzyme into its oligomeric form [32] resulted in trimers consisting of mixtures of recombinant His₆-tagged subunits derived from *M. tuberculosis* and wild-type subunits from *M. smegmatis*. The final peak of activity was concentrated and further purified by affinity chromatography on 5'-AMP-Sepharose. The resultant preparation, which presumably consisted of a trimer with three Rv2259 derived subunits, had about 50% of the activity of the wild-type enzyme from *M. smegmatis* in both the formaldehyde dehydrogenase and MSNO reductase assays. During electrophoresis on vertical SDS/PAGE slab gels this protein also migrated as a larger protein than was predicted from the gene sequence (47.2 kDa as opposed to an estimated mass of 38 686 kDa with the His₆-tag) (Figure 3), whereas such an anomaly was not observed on the Pharmacia PhastSystem. The purified enzyme had activity as both a formaldehyde dehydrogenase and MSNO reductase.

High-level expression of HbN from pET17b was obtained in *E. coli* B121(DE3)plysS by induction with IPTG, to the extent that intact cells were visibly reddish in colour. HbN was isolated essentially as described previously [33].

Figure 4 Ni^{2+} -IMAC of recombinant *M. tuberculosis* MSH-dependent formaldehyde dehydrogenase

Activity of the enzyme using the formaldehyde dehydrogenase assay is shown as a bar graph (right-hand side axis). The last 300 ml of the elution pattern consisted of a gradient from 0–200 mM imidazole in 0.5 M NaCl, 25 mM potassium phosphate buffer, pH 7.1, and 2 mM 2-mercaptoethanol.

Activity and stability of the formaldehyde dehydrogenase as a function of pH and storage conditions

The formaldehyde dehydrogenase activity was absolutely dependent on MSH for activity, which was optimal between pH 8.0 and 9.5 in Na-PPi (sodium pyrophosphate) buffer. Buffers containing amine groups gave poor activity, possibly because of Schiff base formation with formaldehyde. The optimum pH for the MSNO reductase was at pH 7.0 in potassium phosphate buffer. The enzyme was reasonably stable at pH values above 7.5, but not below pH 7.0. The recombinant enzyme lost most of its activity when stored in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM DTT, for a period of 4 weeks at 4 °C and also showed poor stability as an $(\text{NH}_4)_2\text{SO}_4$ pellet. Over the period of 4 weeks, about 50% of the activity was retained when the enzyme was stored in 20% (w/v) glycerol or in the presence of 1% serum albumin at –20 °C. In view of these results, we elected to use the more stable MSNO reductase isolated from *M. smegmatis* in further experiments, rather than the heterologously expressed *M. tuberculosis* enzyme.

Initial velocity studies of the MSNO reductase and formaldehyde dehydrogenase activities

The formaldehyde dehydrogenase reaction was saturable by formaldehyde, with half-maximal reaction rate under standard conditions of assay (see the Experimental section) being achieved at 0.83 mM formaldehyde. For the purpose of the present investigation it was assumed that S-hydroxymethylmycothiol, formed non-enzymically, was used as the substrate. Since the chosen level of formaldehyde (6 mM) exceeded the highest level of MSH used in the assays by 30-fold, the actual S-hydroxymethylmycothiol concentrations were in direct proportion to the amount of MSH added. It should be noted that MSH lacks amino groups, which can react with formaldehyde, as was reported for glutathione [34]. When using only 28 μM MSH in the assay, the absorbance was found to increase to a value which exceeded the absorbance change expected for an equimolar utilization of MSH and NAD^+ by a factor of at least 7-fold, at which stage observation was discontinued. The final product of

Figure 5 Double-reciprocal plots for MSH-dependent formaldehyde dehydrogenase

(A) The dependence of the reaction rate in the formaldehyde dehydrogenase assay with MSH as the variable substrate at different fixed concentrations of NAD^+ : 100 μM (●), 125 μM (■), 166 μM (▲), 250 μM (◆) and 500 μM (○) NAD^+ . (B) The dependence of the reaction rate in the formaldehyde dehydrogenase assay with NAD^+ as the variable substrate at different fixed concentrations of MSH: 10 μM (●), 12.5 μM (■), 16.6 μM (▲), 25 μM (◆) and 50 μM (○) MSH. The inset shows the dependence of the slopes on reciprocal of MSH concentration.

the reaction was therefore not *S*-formylmycothiol, since MSH is evidently not consumed, but more probably formate and MSH, the products of its hydrolysis. In view of the known instability of *S*-formyl esters, this result in itself does not preclude the existence in mycobacteria of an *S*-formyl-MSH hydrolase, by analogy to *S*-formylglutathione hydrolase.

Double-reciprocal plots of the dependence of the formaldehyde dehydrogenase reaction on MSH concentration at different fixed concentrations of NAD^+ are shown in Figure 5(A), and with NAD^+ as the variable substrate at different fixed concentrations of MSH in Figure 5(B). As expected, both sets of plots show an intersecting pattern, consistent with a sequential kinetic mechanism. When NAD^+ is the variable substrate, the lines intersect on the y -axis, and a secondary plot of the slopes from Figure 5(B) against the reciprocal of the MSH concentration passes through the origin (Figure 5B, inset). This pattern is indicative of a rapid

Table 2 Kinetic parameters for the formaldehyde dehydrogenase (a) and MSNO (b) reductase reactions

(a) Formaldehyde dehydrogenase

K_{NAD} (μM)	K_{MSH} (μM)	V_{max} ($\mu\text{mol/min} \cdot \text{mg}$)
354 ± 16	17.3 ± 0.8	38.8 ± 0.6

(b) MSNO reductase

K_{NADH} (μM)	K_{NADH} (μM)	MSNO (μM)	V_{max} ($\mu\text{mol/min} \cdot \text{mg}$)
13 ± 6	430 ± 170	1020 ± 390	3050 ± 995

Figure 6 Double-reciprocal plots of the MSNO reductase reaction with NADH as the variable substrate at different fixed levels of MSNO

Assays were conducted as described in the Experimental section and each data point represents the average of duplicate determinations. MSNO concentrations of 60 μM (■), 80 μM (◇), 120 μM (●), 200 μM (▲) and 300 μM (○) were used. The kinetic parameters for the reaction were evaluated by fitting the primary data to the equation for an ordered sequential reaction using the regression wizard in Sigmaplot. The regression lines were plotted using the reciprocals of the predicted rates.

equilibrium ordered reaction [35] for which the initial-velocity equation is:

$$v = V_1 AB / (K_A K_B + K_B A + AB)$$

The kinetic parameters shown in Table 2 were obtained by fitting the primary data to this equation by non-linear regression.

Double-reciprocal plots for the dependence of reaction rate in the MSNO reductase reaction on the substrate concentrations showed intersecting patterns indicative of a sequential mechanism (Figure 6). The kinetic parameters were evaluated by fitting the primary data to the equation for an ordered bireactant system [35]:

$$v = V_1 AB / (K_{ia} K_B + K_B A + K_A B + AB)$$

where K_{ia} is the dissociation constant for binding of substrate A to a bisubstrate enzyme. The high value for the Michaelis constants, given in Table 2, resulted in a considerable error in their estimation, but nonetheless indicated that the reaction velocity of MSNO reductase would saturate only at high concentrations of MSNO, thus allowing responsiveness to a wide range of MSNO concentrations within the cell. From the results presented in Table 1 it can be seen that V_{max} for the *M. smegmatis* enzyme is approx. 76-fold faster as a MSNO reductase than as a formaldehyde dehydrogenase.

Products of the MSNO reductase reaction and attempted simulation of the *in vivo* metabolic route

In view of the fact that intact *M. smegmatis* metabolized GSNO to GSSG and nitrate, it was important to establish (i) whether GSNO is used as a substrate by the MSNO reductase of mycobacteria, (ii) if so, whether transnitrosation of the nitroso group from GSNO to MSH proceeds fast enough not to be rate limiting, (iii) to which redox level MSNO reductase reduces the nitroso group, and (iv) whether this intermediate can be utilized by HbN to produce nitrate.

The stoichiometry with which MSNO reductase utilizes NADH for the reduction of MSNO was determined spectrophotometrically and was found to be equimolar, thus indicating that the nitroso group of MSNO was reduced to the oxidation level of nitroxyl. This interpretation was also subsequently supported by product analysis.

The enzyme was unable to utilize GSNO as a substrate, but upon the addition of MSH, the oxidation of NADH ensued without any evidence that a lag-phase was required for the accumulation of MSNO. Moreover, when MSH was added to an assay mixture containing GSNO and NADH, followed by a short interval of 2 min to allow for the accumulation of MSNO before addition of the enzyme, the reaction progress was no different from that seen when the order of addition of enzyme and MSH was reversed. When using catalytic amounts of enzyme, the transnitrosation reaction between GSNO and MSH was evidently fast enough not to be rate-limiting. In either case, the absorbance change observed at 340 nm corresponded to that expected for an equimolar utilization of NADH and MSH, which is consistent with the formation of the *N*-hydroxysulphenamide of MSH, MS(NHOH), as a primary product of the reduction of MSNO. As a result of the instability of both *N*-hydroxysulphenamides and nitroxyl, the equilibrium constants for the dissociation of *N*-hydroxysulphenamides into nitroxyl and the free thiols [36] are not known, but the failure of MSH to recycle to any significant extent indicated that the equilibrium is far over towards formation of the *N*-hydroxysulphenamide of MSH.

N-Hydroxysulphenamides, however, readily isomerize to the sulphenamides, which may be expected to give rise to ammonia upon hydrolysis, rather than to nitrate, the product of the decomposition of GSNO by intact *M. smegmatis*. As further evidence favouring the proposed reaction course, the MSNO reductase reaction was carried out with [³H]inositol-labelled MSNO as the substrate. The sulphenamide was recovered as the [³H]inositol-labelled acid-hydrolysis product, MSH sulphonic acid, and represented about 88 % of the total radiolabel recovered from an anion-exchange column. It was identified by MS. In the negative electrospray mass spectrum of MSH sulphonic acid (Figure 7) a species at *m/z* 517.02 was assigned to the deprotonated molecular ion. The fragmentation product at *m/z* 453.1 is proposed to have arisen from the loss of SO₂ (*m/z* 64) and the one at 450.99 from the further loss of H₂. The significantly more stable sulphonic acid, an oxidation product of the sulphonic acid, is proposed to be represented by the species observed at *m/z* 533.03. Upon collision-induced dissociation, the latter gave rise to HSO₃⁻ (*m/z* 80.64) as the principal ion, but not to the aforementioned fragmentation products, which seem to have been derived from the sulphonic acid.

We tried to establish whether the *N*-hydroxysulphenamide exists in a reversible equilibrium with free NO⁻ and MSH by studying the spectral changes observed when haemoproteins were included in the reaction mixture. The mycobacterial HbN was of particular interest, since evidence has been presented that it plays a role in the protection of mycobacteria against nitrosative

Figure 7 Negative ion electrospray mass spectrum of MSH sulphonic acid

MSH sulphonic acid was purified from MSNO reductase assay mixtures as described in the Experimental section, freeze-dried and dissolved in 50 % acetonitrile/water. The electrospray mass spectrum was recorded using a cone voltage of 50.

Scheme 2 The conversion of oxygenated HbN into metHbN by nitroxyl

stress [24]. Oxygenated haemoglobins react with nitroxyl to produce nitrate according to the stoichiometry presented in Scheme 2 [37], but these reactions are dramatically quenched by thiols due to an equilibrium which favours formation of the *N*-hydroxysulphenamide. When HbN was included in the MSNO reductase reaction mixture spectral changes in the α - and β -bands of the haem and a new absorbance band at 630 nm, indicative of the formation of metHbN, were observed upon addition of MSNO reductase, and these spectral changes coincided with the oxidation of NADH (Figure 8). The contribution of the oxyHbN-to-metHbN conversion to the absorbance change at 340 nm could be calculated from the spectral change in the β -band of HbN at ~ 545 nm, using the known difference molar absorption coefficients at these wavelengths. This in turn allowed calculation of the contribution of NADH oxidation to the absorbance change at 340 nm and an estimate of MSNO consumed in the reaction (Figure 8B). The amount of NADH or MSNO utilized was found to exceed the oxyHbN-to-metHbN conversion by a factor of 6.4. If we assume a 2:1 stoichiometry in which oxyHbN is utilized for the formation of NO₃⁻, as in Scheme 2, this meant that only 7.76 % of the MSNO reduced could have ended up as NO₃⁻. When 10 μM [³H]inositol-labelled MSNO and an equimolar amount of HbN were used in a similar assay, the amount of MSH recovered, as judged by HPLC, was estimated to correspond to about 6 % of the MSNO whereas the rest of the radiolabel was associated with a more polar compound. When ferri-cytochrome *c*, which is reduced to the ferrous form by nitroxyl [38], was substituted for HbN, no reduction of the haemoprotein, which could be attributed to the MSNO reductase reaction, was observed.

Figure 8 Reaction of the products of MSNO reductase with *M. tuberculosis* truncated haemoglobin (trHb)

Reaction mixtures contained 150 μM NADH and 11 μM HbN in 50 mM NaPP_i (pH 8.7), 500 μM EDTA. (A) This panel illustrates the NADH oxidase activity of HbN (●) and the kinetics of NADH and MSNO (50 μM) utilization by MSNO reductase (0.228 $\mu\text{g}/\text{ml}$) in the presence of trHb (○). (B) Kinetic representation of the efficiency of the oxidation of HbN during the MSNO reductase reaction. The amount of MSNO consumed (●) was calculated using a molar absorption coefficient of 7120 $\text{M}^{-1} \cdot \text{cm}^{-1}$ at 340 nm after subtracting the residual NADH oxidase activity of HbN and the decrease in absorbance at 340 nm due to the oxidation of HbN to the met form ($\epsilon_{340} = 6610 \text{ M}^{-1} \cdot \text{cm}^{-1}$). The extent of HbN oxidation was calculated using a difference molar absorption coefficient of 4750 $\text{M}^{-1} \cdot \text{cm}^{-1}$ at 545 nm (○) and this was used to back-extrapolate the contribution at 340 nm. (C, D) These panels show spectral changes observed in the HbN during the MSNO reductase reaction. The solid line represents the initial spectrum of HbN and the dotted line represents the spectrum observed after 400 s reaction time. Arrows indicate the direction of absorbance change. trHbN, truncated haemoglobin.

Like several other haemoglobins [39] HbN was found to have a low level of NADH oxidase activity associated with it, which at high concentrations of the protein became significant. *S*-Nitrosothiols are decomposed by superoxide, with nitrate as one of the major products [40]. Seen together with the poor efficiency with which metHbN was produced in the MSNO reductase reaction *in vitro*, the requirement of two oxygenated HbN molecules for the production of one NO_3^- and the high background level of NO_3^- production made it impractical to establish the stoichiometry with which nitrate is produced in the NADH-driven MSNO reductase reaction. In the hope that it may be possible to accumulate the *N*-hydroxysulphenamide in the absence of the haemoprotein we attempted an alternative strategy, based on the premise that *N*-hydroxysulphenamides may be relatively stable at pH values in excess of 8.5. The nitrosomycothioli reductase reaction was allowed to proceed in the absence of HbN until a complete conversion of MSNO into products had been achieved, at which point the remaining NADH was oxidized by the addition of pyruvate and lactate dehydrogenase. When HbN was added to this reaction mixture, no alteration in its spectrum could be detected. Evidently the conversion of HbN into metHbN only occurred during turnover of MSNO reductase. Consequently, we concluded that the spectral changes observed when HbN is present in the MSNO reductase assay mixture are due either to the interaction with nitroxyl, derived from the reversible dissociation of the *N*-hydroxysulphenamide, both of which are present as labile, short-lived species, or to

direct transfer of nitroxyl from the reductase to HbN. There is ample precedent for the direct sequestration of toxic metabolites, with examples being afforded in the oxidative demethylation reactions of sarcosine and dimethylglycine dehydrogenases which transfer formaldehyde directly to tetrahydrofolate [41,42], or trimethylamine dehydrogenase, which uses GSH for the same purpose [43]. Under the reaction conditions used *in vitro*, the reaction with HbN as a nitroxyl acceptor competes with the rearrangement of the *N*-hydroxysulphenamide to the more stable, but unreactive sulphinamide of MSH.

Further experiments were undertaken to try to quantify the extent to which the *N*-hydroxysulphenamide dissociates into nitroxyl and MSH. In one such experiment, we endeavored to trap MSH, released in the MSNO reductase reaction, as the highly fluorescent Et₂N(MalNPh)CMe [7-diethylamino-4-methyl-3-(4'-maleimidyl-phenyl)-coumarin] derivative. It was first established that MSNO reductase activity is only inhibited by approx. 20% by 0.5 mM NEM (*N*-ethylamide), and this suggested the feasibility of such an experiment. While the Et₂N(MalNPh)CMe reagent has a solubility limit of only approx. 10 μM in Na-PP_i buffer, pH 8.7, its adduct with MSH is soluble up to at least 100 μM . The MSNO reductase reaction was allowed to proceed in the presence of 200 μM NADH, 100 μM MSNO and a fine suspension of Et₂N(MalNPh)CMe sufficient to derivatize 135 μM MSH. When analysed at different stages during the course of the reaction by direct injection into an HPLC column [17] the amount of MSH recovered as the Et₂N(MalNPh)CMe derivative represented at all times less than 1% of the initial amount of MSNO, not very different from that seen at zero time.

Since the conversion of HbN into metHbN was consistent with either the release of nitroxyl or its direct transfer to these haemoproteins, additional evidence that HbN reacts with nitroxyl in a manner similar to human haemoglobin or myoglobin [37] was sought. Towards this end, the changes in the spectra of HbN and human Hb, resulting from the formation of nitroxyl and cyanide from cyanamide in the presence of catalase and peroxide [44], were compared. Both haemoproteins were converted into the cyanomethemoglobin derivatives under these conditions.

Conclusion

An enzyme which catalysed the reduction of nitrosothiols was first discovered during studies on the metabolism of a xenobiotic. These studies established GSNO as an important intermediate in the catabolism of 1,3-dimethyl-2-cyano-1-nitrosoguanidine [15] and identified GSNO reductase activity as an inherent property of the alcohol dehydrogenase class III isoenzyme [11], which also catalysed a GSH-dependent oxidation of formaldehyde to *S*-formylglutathione. Depending on the presence of GSH, however, GSNO reductase was found to produce different products [11]. The major product produced *in vitro*, glutathione sulphinamide, however, seemed not to be formed in intact cells. Recent studies on the metabolism of GSNO in epithelial cells [14] indicated that the pathway is predominantly reductive in nature. That a disparity exists between the products formed *in vitro* by the *S*-nitrosothiol reductase and those formed *in vivo* when cells are exposed to GSNO, was even more apparent in the case of the mycobacteria. The present data indicated that the product of the *Rv2259* gene of *M. tuberculosis* is an MSNO reductase. A turnover per subunit of 116700 min^{-1} was calculated from the V_{max} given in Table 2, which is considerably higher than that reported for the GSNO reductases from *E. coli* or from rat liver [12]. As in the case of the mammalian enzyme, MSNO reductase produced predominantly MSH-sulphinamide when assayed *in vitro*, but not only could

we find no evidence for the formation of MSH-sulphinamide or its hydrolysis products in intact cells, but the nitroso group was converted into nitrate rather than ammonia.

These results could call into question whether the S-nitrosothiol reductase activities do indeed play a significant role in the metabolism of S-nitrosothiols. The newly discovered S-nitrosothiol reductase activities of the thiol-dependent formaldehyde dehydrogenases, however, are as yet the only enzymic activities which have unambiguously been shown to be involved in the metabolism of S-nitrosothiols. In the case of yeast cells evidence was presented that GSNO reductase plays a crucial role in protection against nitrosative stress [12]. It is, however, of interest that some S-nitrosothiol reductases, including the mycobacterial MSNO reductase, should catalyse a two-electron reduction of the nitroso group of GSNO to the oxidation state of nitroxyl, one of the most toxic of the RNI. It is evident that mycobacteria do not solve this dilemma by converting the N-hydroxysulphenamide into ammonia, but possess an efficient system which produces nitrate as the nitrogenous product when intact cells are exposed to GSNO.

One possible route for the formation of nitrate from GSNO was examined in this study. It is known that oxygenated haemoglobins convert nitric oxide into nitrate in the reaction:

The formation of methaemoglobin, HbFe^{3+} , has therefore been used as a measure of the release of $\text{NO}^{\cdot}$ [45]. Mycobacteria produce two truncated haemoglobins, one of which, HbN, has been shown to play a role in the detoxification of $\text{NO}^{\cdot}$ by mycobacteria [46]. HbN, however, is not an abundant protein and consequently it was proposed that it might have catalytic properties [47]. It should be noted that mycobacteria do not seem to produce the ubiquitous flavohaemoglobin which can indeed perform such an enzymatic function [48]. It, therefore, seemed reasonable to ascertain whether HbN would be able to catalyse the conversion of $\text{NO}^{\cdot}$ into NO_3^- without being oxidized to metHbN, since this process proceeds non-enzymatically via peroxynitrite [49,50]. The present results, however, did not support such an independent enzymic role for HbN. We did, however, observe that HbN was oxidized to metHbN in the presence of NADH, MSNO and MSNO reductase and this occurred only during turnover of the enzyme. The capture of nitroxyl by HbN was, however, quite inefficient, and was estimated to amount to only about 7.8% of the MSNO utilized in the reaction.

While our attempts to reconstitute a system which converts the nitroso group of GSNO into nitrate have therefore met with limited success, this may to a large extent reflect the difficulties inherent in reconstituting in a closed system events which occur in an open system. Thus the relative intracellular concentrations of all the reactants are likely to be very different from those used in our experiments. This applies to the concentration of MSNO as well as that of HbN and MSNO reductase. While we used catalytic amounts of MSNO reductase in our experiments, a study of potential interactions between MSNO reductase and HbN by rapid reaction kinetics, using physiologically relevant levels of the proteins, may be more informative. Additional components may also be required to reconstitute the reaction which occurs *in vivo*. Studies on the effect of disruptions in the *gblN* and *adhE2* genes on the ability of mycobacteria to metabolize GSNO should then be invaluable in establishing that at least these two components are essential for the process.

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