Mycothiol-dependent proteins in actinomycetes

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Abstract

The pseudodisaccharide mycothiol is present in millimolar levels as the dominant thiol in most species of *Actinomycetales*. The primary role of mycothiol is to maintain the intracellular redox homeostasis. As such, it acts as an electron acceptor/donor and serves as a cofactor in detoxification reactions for alkylating agents, free radicals and xenobiotics. In addition, like glutathione, mycothiol may be involved in catabolic processes with an essential role for growth on recalcitrant chemicals such as aromatic compounds. Following a little over a decade of research since the discovery of mycothiol in 1994, we summarize the current knowledge about the role of mycothiol as an enzyme cofactor and consider possible mycothiol-dependent enzymes.

Introduction

Low molecular-weight thiols play a key role in maintaining a reducing environment in the cell, which is necessary for regular metabolic activities to occur. These thiols thus represent a major biological adaptation that is important for the survival of organisms under various endogenous and exogenous stresses. In eukaryotes and gram-negative bacteria, the much-studied tripeptide glutathione (GSH) is the dominant thiol. GSH plays a major role in protecting the cell against oxygen toxicity (Anderson, 1998) by removing reactive oxygen species that may result from atmospheric oxygen and basal metabolic activities in aerobic organisms. In the case of pathogenic microorganisms, toxic oxidants from the host phagocytic response intended to destroy the bacterial invader also result in oxidative stress. In higher organisms and gram-negative bacteria, GSH is involved in the detoxification of exogenous xenobiotic agents. In addition to detoxification, GSH also plays a role in the synthesis of metabolites such as steroids and prostaglandins in higher organisms (Sheehan *et al*., 2001). In gram-negative bacteria, GSH has also been implicated in the growth of these organisms on recalcitrant nutrients by serving as a cofactor for enzymes in various degradation pathways (Vuilleumier & Pagni, 2002).

In eukaryotic parasites, thiols other than GSH, such as trypanothione and ovothiol, are the dominant thiols and thus fulfill the role of GSH in these organisms (Spies & Steenkamp, 1994; Muller *et al*., 2003). These thiols differ in their redox potential, nucleophilicity, and susceptibility to auto-oxidation. In the gram-positive order *Actinomycetales*, which includes the genus *Streptomyces*, predominantly known for its ability to produce large numbers of antibiotics, *Rhodococcus,* recognized for its bioremediation capabilities, and *Mycobacterium,* which counts the pathogens *M. tuberculosis* and *M. leprae* as members, mycothiol (MSH), 1-D-myoinosityl-2-(*n*-acetyl-L-cysteinyl)-amido-2-deoxy-α-D-glucopyranoside (Fig. 1), is the major thiol (Newton *et al*., 1996). While common thiols such as cysteine, Coenzyme A, inorganic sulfide and thiosulfate are also present in actinomycetes, MSH, whose distribution is limited to actinomycetes, serves as the GSH analogue (Newton *et al*., 1996; Fahey, 2001). Another thiol present in actinomycetes, ergothioneine (ESH), is a betaine of 2-thiol-L-histidine. Unlike MSH, ESH has been detected in plants, fungi, animals and bacteria. However, only fungi and actinomycetes are able to synthesize this thiol (Genghof, 1970). The amount of ESH present in actinomycetes is ten-fold lower than that of MSH and its exact function in these bacteria is unknown (Fahey, 2001).

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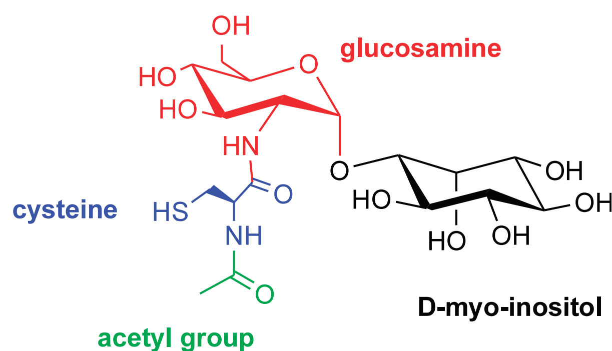

Fig. 1. Structure of mycothiol.

Like GSH, MSH has a functional cysteine moiety but instead of the two amino acids, glycine and glutamic acid, present in GSH, there are two sugar moieties, inositol and *N*-glucosamine (Fig. 1). The $\alpha(1 \rightarrow 1)$ glycosidic link between glucosamine and inositol is comparable in strength to the amide bond connecting the acetyl group to the cysteine and the second amide bond connecting the cysteine to the glucosamine. This stability in the bonds may have played a role in the evolution of MSH as the major intracellular thiol (Newton *et al.*, 1995) in actinomycetes. Moreover, certain actinomycetes such as mycobacteria are known for their complex cell walls consisting of polysaccharides, lipopolysaccharides and complex fatty acids. The preponderance of glucosamine and, in particular, inositol, in the actinomycete cell wall may have favoured the use of a 'sugar' thiol (MSH) for redox control over the 'peptide' (GSH) thiol.

One of the major functions of thiols is to serve as a storage form of cysteine, because cysteine tends to auto-oxidize in a matter of minutes, readily forming toxic peroxy radicals and hydrogen peroxide. MSH undergoes copper-catalyzed auto-oxidation 30-fold more slowly than cysteine and 7-fold more slowly than GSH (Newton *et al.*, 1995). This important difference implies that the ability to cope with oxidative stress is much higher in actinomycetes. In terms of chemical reactivity, MSH is an inherently poor nucleophile compared with ovothiol and GSH, according to Spies & Steenkamp (1994). The physical parameters of mycothiol such as the thiol pKa and the stability of the free radical form have not been elucidated, although the redox potential of mycothiol is expected to be similar to that of glutathione ($E = -0.25$ V).

The biosynthetic pathway of MSH has been elucidated in the last few years (Fig. 2). The first step, the formation of *N*-acetylglucosaminylinositol (Newton *et al.*, 2003, 1999), is catalyzed by the gene product of *mshA*. *N*-acetylglucosaminylinositol is then deacetylated by MSH deacetylase, encoded by *mshB*, to yield glucosaminylinositol (Newton *et al.*, 2000, 2006; Buchmeier *et al.*, 2003; Rawat *et al.*, 2003). The third step, the coupling of cysteine to the 2′ amine of this pseudodisaccharide (Bornemann *et al.*, 1997; Anderberg *et al.*, 1998; Rawat *et al.*, 2002; Sareen *et al.*, 2002, 2003), is catalyzed by an MSH ligase, encoded by *mshC*. The final step is the *N*-acetylation of cysteinylglucosaminylinositol to yield MSH, catalyzed by mycothiol synthase, the product of *mshD* (Koledin *et al.*, 2002; Newton *et al.*, 2005). Although an *M. tuberculosis* mutant disrupted in *mshB* has been generated (Buchmeier *et al.*, 2003), attempts to isolate an *M. tuberculosis mshC* (Sareen *et al.*, 2003) mutant were unsuccessful, suggesting that it is an essential gene in *M. tuberculosis* (Newton *et al.*, 2006). Mutants of the nonpathogenic model organism, *Mycobacterium smegmatis*, that are disrupted in all four steps of the MSH biosynthetic pathway, have been generated and characterized (Newton *et al.*, 2003, 1999; Rawat *et al.*, 2003, 2002). These mutants are sensitive to oxidative stress, antibiotics such as streptomycin and rifampin, and alkylating toxins, indicating that either MSH or MSH-dependent enzymes are involved in protecting the mycobacterial cell against oxidants and toxins [Newton *et al.*, 1999; Rawat *et al.*, 2003, 2002, Rawat & Av-Gay, unpublished observations]. *Streptomyces coelicolor* mutants in the four genes involved in MSH biosynthesis have been isolated (Park *et al.*, 2006), and recently an *mshD* mutant of *Amycolatopsis mediterranei* has been reported (Chen *et al.*, 2005). S1 mapping analysis has demonstrated that three of these genes, *mshA*, *mshC*, *mshD*, are induced under osmotic challenge in *S. coelicolor* (Lee *et al.*, 2005).

In this review, we focus on MSH-dependent enzymes that utilize MSH for their activity either as a cofactor or as a substrate, as illustrated schematically in Fig. 2, which summarizes the current knowledge about MSH metabolism. As most studies on MSH metabolism have been performed on mycobacteria, the majority of the information is drawn from mycobacterial research. We also draw parallels to the extensive field of GSH metabolism whenever possible, because it is likely that MSH and GSH serve similar sets of functions.

Mycothiol disulfide reductase (Mtr)

One of the key enzymes involved in maintaining MSH levels is MSH disulfide reductase, which reduces the oxidized MSH disulfide (Fig. 3). This enzyme is a member of the pyridine nucleotide-disulfide reductase superfamily, to which other disulfide-reducing enzymes such as GSH reductase, trypanothione reductase, and thioredoxin reductase also belong. All of these proteins maintain intracellular redox homeostasis to allow the proper functioning of a variety of biological processes, including enzyme activation, cell-cycle regulation, and DNA synthesis.

The *M. tuberculosis* MSH disulfide reductase (Mtr, encoded by Rv2855) was identified by homology with GSH

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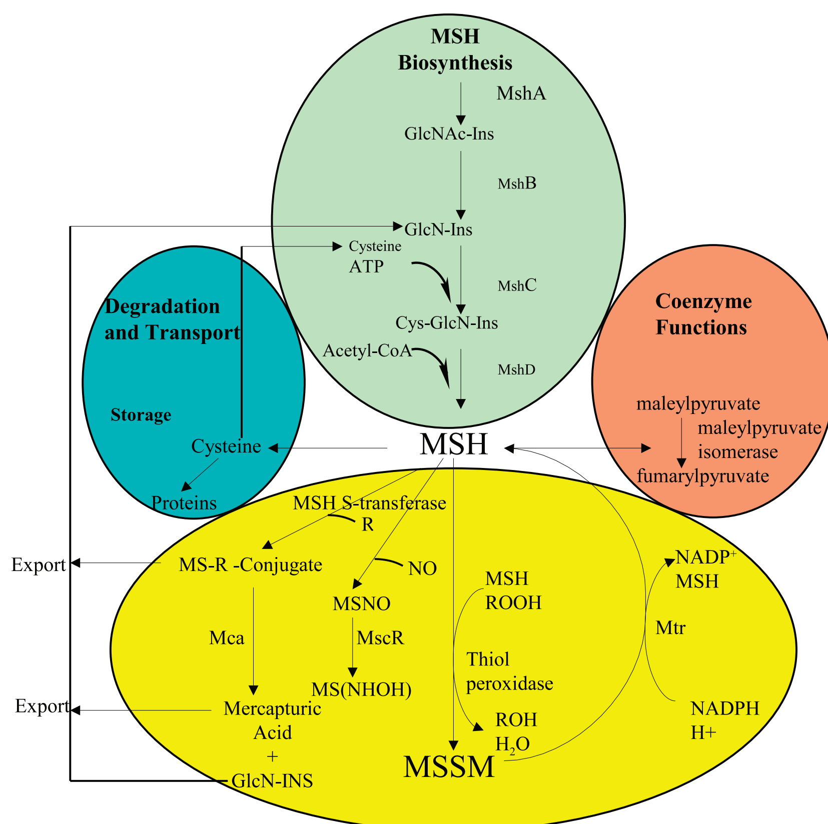

Fig. 2. Overview of mycothiol metabolism. Green, biosynthesis of mycothiol catalyzed by MshA, MshB, MshC, MshD. Blue, degradation reactions to scavenge mycothiol for cysteine in times of nutrient starvation. Yellow, protective reactions catalyzed by: mycothiol amidase (Mca) and putative mycothiol-*S*-tranferases involved in the detoxification of xenobiotic agents; putative thiol transferases, nitrosothiol reductase (MscR) and thiol peroxidases or peroxiredoxins involved in oxidative and nitrosative stress protection and mycothiol reductase (Mtr), which maintains mycothiol in a reduced form within the mycobacterial cell. Red, metabolic reactions catalyzed by enzymes such as maleylpyruvate isomerase requiring mycothiol as a cofactor for growth on diverse carbon sources.

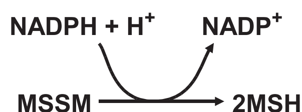

Fig. 3. The reaction catalyzed by mycothiol reductase.

reductase (Patel & Blanchard, 1999). Recombinant Mtr was able to reduce the disulfide of MSH (MSSM) with a concomitant oxidation of NADPH (Patel & Blanchard, 1999) (Fig. 3). Mtr is a homodimeric flavoprotein disulfide isomerase, with monomers of 49.9 kDa, an isoelectric point of 5.43, and requiring FAD as a cofactor. Analysis of the sequence reveals that Mtr contains a pfam00070.11, Pyr_redox pyridine nucleotide-disulphide oxidoreductase domain that is found in both class I and class II oxidoreductases, NADH oxidases and peroxidases, and is actually a small NADH binding domain within a larger FAD binding domain. In addition, the sequence also has a pfam02852.11, Pyr_redox_dim pyridine nucleotide-disulphide oxidoreductase, dimerization domain. Like other such proteins, Mtr contains a redox-active disulfide involved in substrate disulfide reduction and has a bi-bi-ping-pong kinetic mechanism. Blanchard and coworkers (Patel & Blanchard, 1999) reported that the k_{cat} for MSSM is 400 mol per mole of FAD per minute, which is one to two orders slower than other disulfide reductases. Because the auto-oxidation rate of MSH is so much slower than those of cysteine and GSH, it was postulated that this rate may be sufficient to maintain reduced MSH levels in the mycobacterial cell. The Michaelis-Menten constant (*Km*) of this enzyme for MSSM, 73 μM, however, is similar to the *Km* of other reductases for disulfides (Patel & Blanchard, 1999). Blanchard and coworkers further demonstrated that the reductase is active not only against the disulfide of MSH, but also against the disulfide of acetylcysteinylglucosamine (MSH without the inositol moiety). However, the enzyme did not display activity against the disulfide of other thiols such as GSH and trypanathione.

Bioinformatic analysis of *mtr*

The *M. tuberculosis mtr* gene is highly conserved among mycobacteria. For example, the gene product has 71% sequence identity with the *M. smegmatis* orthologue. It is not very highly conserved in other actinomycetes, sequence identities with *M. tuberculosis* Mtr for putative orthologues being 29% for *Nocardia farcinica*, 28% for *Kineococcus radiotolerans*, and 27% for *S. coelicolor* and *Streptomyces avermitilis* (Table 1).

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Table 1. Orthologues of genes coding for mycothiol-dependent proteins identified by BLAST analysis of the *Actinomycetales* genome sequences using *M. tuberculosis* sequences

	Mycothiol reductase (Rv2855) (Mtr)				Nitrosothiol reductase (Rv2259) (MscR)				Mycothiol amidase (Rv1082) (Mca)			
Strains	Accession number	Identity	Positives	Gaps	Accession number	Identity	Positives	Gaps	Accession number	Identity	Positives	Gaps
Mycobacterium bovis ssp. *bovis* AF2122/97	NP_856525	459/459 (100%)	459/459 (100%)	0/459 (0%)	NP_855932	361/361 (100%)	361/361 (100%)	0/361 (0%)	NP_854766	288/288 (100%)	288/288 (100%)	0/288 (0%)
Mycobacterium avium ssp. *paratuberculosis* K10	NP_96187	380/458 (82%)	419/458 (91%)	0/458 (0%)	NP_960942	321/361 (88%)	340/361 (94%)	0/361 (0%)	NP_959963	237/284 (83%)	254/284 (89%)	0/284 (0%)
Mycobacterium smegmatis mc^{2}155	MSMEG2612	329/460 (71%)	382/460 (83%)	1/460 (0%)	MSMEG4346	283/361 (78%)	310/361 (85%)	0/361 (0%)	MSMEG5246	214/288 (74%)	240/288 (83%)	0/288 (0%)
Mycobacterium leprae TN	Pseudogene NP_302544				NP_302213	321/361 (88%)	340/361 (94%)	0/361 (0%)	NP_302547	248/287 (86%)	264/287 (91%)	0/287 (0%)
Rhodococcus RHA1	rha03945	296/462 (64%)	360/462 (77%)	10/462 (2%)	rha04330	281/361 (77%)	317/361 (87%)	0/361 (0%)	rha0877	199/286(69%)	230/286 (80%)	0/286 (0%)
Corynebacterium diphtheriae NCTC 13129	NP_939840	259/461 (56%)	320/461 (69%)	15/461 (3%)	NP_940430	104/345 (30%)	151/345 (43%)	29/345 (8%)	NP_939290	154/286 (53%)	203/286 (70%)	0/286 (0%)
Corynebacterium efficiens YS-314	NP_738506	254/461 (55%)	319/461 (68%)	146/461 (3%)	NP_736940	236/368 (64%)	285/368 (77%)	7/368 (1%)	NP_737662	172/288 (59%)	210/288 (72%)	0/288 (0%)
Corynebacterium glutamicum ATCC 13032	NP_601209	245/461 (53%)	311/461 (68%)	146/461 (3%)	YP_224619	238/366 (65%)	289/366 (78%)	7/366 (1%)	YP_225280	170/288 (59%)	212/288 (73%)	0/288 (0%)
Nocardia farcinica IFM 10152	YP_12148	137/469 (29%)	213/469 (45%)	27/469 (5%)	YP_117432	295/361 (81%)	325/361 (90%)	0/361 (0%)	YP_121040	209/290 (72%)	232/290 (80%)	3/290 (1%)
Kineococcus radiotolerans sp nov.	ZP_00619308	134/474 (28%)	219/474 (46%),	47/474 (9%)	ZP_00618936	269/372 (72%)	300/372 (80%)	11/372 (2%)	ZP_00618112	166/290 (57%)	202/290 (69%)	7/290 (2%)
Streptomyces avermitilis MA-4680	NP_82330	134/484 (27%)	215/484 (44%)	43/484 (8%)	NP_828129	252/357 (70%)	287/357 (80%)	0/357 (0%)	NP_824475 (SAV3299)	149/287 (51%)	194/287 (67%)	4/287 (1%)
Streptomyces coelicolor A3(2)	NP_6276480	129/467 (27%)	195/467 (41%)	24/467 (5%)	NP_625045	269/362 (74%)	308/362 (85%)	1/362 (0%)	NP_629119. (SCO4967)	150/287(52%)	190/287 (66%),	4/287 (1%)

The percentage of identical (Identity) and similar (Positives) amino acids between the *Mycobacterium tuberculosis* protein sequence and the orthologous sequence, and any gaps introduced in the alignment of the sequences are listed.

Mycobacterium tuberculosis Rv sequences were obtained from *http://genolist.pasteur.fr/TubercuList/; Mycobacterium bovis subsp. bovis* AF2122/97, *Mycobacterium avium* subsp. paratuberculosis str. K10, *Mycobacterium smegmatis* mc^{2}155, *Mycobacterium leprae* TN, *Corynebacterium diphtheriae* NCTC 13129, *Corynebacterium efficiens* YS-314, *Corynebacterium glutamicum* ATCC13032 and *Nocardia farcinica* IFM 10152 were obtained from www.tigr.org; *Rhodococcus* RHA1 sequences were obtained from http://www.rhodococcus.ca/; *Kineococcus radiotolerans sp. nov* sequences were obtained from http://genome.ornl.gov/microbial/krad/; *Streptomyces avermitilis* MA-4680 sequences were obtained from http://avermitilis.ls.kitasato-u.ac.jp/; and *Streptomyces coelicolor* A3(2) sequences were obtained from http://streptomyces.org.uk/S.coelicolor/index.html (as of March 26, 2006).

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In *M. tuberculosis*, *mtr* is the second gene in a bicistronic operon (Romero & Karp, 2004); Rv2854, the gene upstream, codes for a 346-amino acid (-aa) protein. Sequence analysis of Rv2854 gene product reveals that it has an alpha/beta hydrolase fold (pfam00561.11), an acyltransferase (COG0596), and a lysophospholipase (COG2267) domain. It is likely that Rv2854 codes for an enzyme such as a lipase or thioesterase that catalyzes the hydrolysis of an ester bond. In GSH-containing organisms, *S*-glutathione thioesterase, *S*-succinylglutathione hydrolase, or *S*-formylglutathione hydrolase are common enzymes; Rv2854 could, thus, be a MSH-based analogue of such enzymes. In fact, an *S*-formylmycothiol hydrolase is expected to be part of a formaldehyde detoxification pathway that is discussed in the next section.

Rv2854 orthologues are present in all actinomycetes and their location next to *mtr* is conserved in all sequenced mycobacteria and corynebacteria with the exception of *M. leprae*, where a possible orthologue is present elsewhere. In *N. farcinica*, *S. coelicolor* and *S. avermitilis*, this gene order is not conserved, and the radiation-resistant actinomycete *K. radiotolerans* does not appear to have an orthologue.

Is mycothiol reductase essential to mycobacteria?

In bacteria such as the gram-negative *Escherichia coli* (Vlamis-Gardikas *et al.*) and the gram-positive *Streptococcus mutans* (Yamamoto *et al.*, 1999), and in the eukaryote *Saccharomyces cerevisiae* (Muller, 1996), mutants disrupted in GSH reductase are viable. They can grow aerobically but show limited or no growth in the presence of diamide, a thiol-specific oxidant (Muller, 1996; Yamamoto *et al.*, 1999; Vlamis-Gardikas *et al.*). Moreover, the *S. cerevisiae* GSH reductase mutant shows increased sensitivity to other oxidants such as hydrogen peroxide and superoxide (Grant *et al.*, 1996; Muller, 1996). Although an *M. tuberculosis* transposon mutant in *mtr* was obtained by McAdam and coworkers (McAdam *et al.*, 2002), indicating that such a mutant is viable, phenotypic characterization of an actinomycete *mtr* mutant has not been yet reported. Application of antisense *M. tuberculosis mtr* oligonucleotides resulted in a 66% reduction in the growth index after 7 days' incubation as compared with the control culture (Hayward *et al.*, 2004). In contrast, high-density Himar-1 transposon mutagenesis (Sassetti *et al.*, 2003) suggested that this gene may be essential in *M. tuberculosis* strain H37Rv. Transcriptional analysis of *Mycobacterium bovis* BCG demonstrated that *mtr* is actively transcribed during growth, but *mtr* mRNA was absent in the stationary phase (Hayward *et al.*, 2004), suggesting that Mtr is required to maintain the redox balance during growth. However, our studies have indicated that MSH levels remain high in stationary phase (Rawat *et al.*, 2003). It is conceivable that another thiol reductase may be able to reduce oxidized MSH, because thioredoxin reductases that are also able to reduce GSH disulfides have been reported (Kanzok *et al.*, 2001; Salinas *et al.*, 2004).

In *M. leprae*, which contains all the components of the MSH biosynthetic pathway, the *mtr* orthologue is a pseudogene, ML1570. Because MSH levels have not been measured in *M. leprae*, it is possible that MSH may not be present in the reduced form. In that case, thioredoxins may compensate for the lack of reduced MSH because these thiols have been shown to reduce dinitrobenzenes and peroxides (Zhang *et al.*, 1999), or the *M. leprae* thioredoxin reductase may serve a dual function as a mycothiol reductase.

NAD/mycothiol-dependent formaldehyde dehydrogenase/nitrosothiol reductase (MscR)

Another enzyme that has a functional analogue in GSH metabolism is NAD/mycothiol-dependent formaldehyde dehydrogenase/nitrosothiol reductase. In normal cells, formaldehyde, which is toxic at low concentrations, is generated as a result of the biological degradation of natural C1 compounds such as methane, methylated amines and sulfur compounds. In GSH-producing organisms, formaldehyde is detoxified by a NAD/GSH-dependent formaldehyde dehydrogenase. GSH reacts spontaneously with formaldehyde to form *S*-hydroxymethyl GSH, a substrate for formaldehyde dehydrogenase, which converts the substrate into a GSH formate ester that is further hydrolyzed into GSH and formate by a thiol esterase. In organisms lacking GSH, another thiol should be able to substitute for GSH. Indeed, the first enzyme that was identified as utilizing MSH as a cofactor was the NAD/MSH-dependent formaldehyde dehydrogenase: Misset-Smits *et al.* (1997) demonstrated that the 'factor' of NAD/factor-dependent formaldehyde dehydrogenase from *Amycolatopsis methanolica* and *Rhodococcus erythropolis* was MSH. An orthologue of the NAD/MSH-dependent formaldehyde dehydrogenase gene (*mscR*, encoded by Rv2259), is present in *M. tuberculosis*. It is assumed that, like GSH, MSH reacts spontaneously to form a *S*-hydroxymethylmycothiol adduct that is converted by the formaldehyde dehydrogenase to a MSH formate ester. Although the thiol esterase motif is present in some *M. tuberculosis* gene products, no thiol esterase activity has been detected, and thus it is likely that an aldehyde dehydogenase converts the MSH formate ester to a carbonate ester, CO_2 and MSH (Fig. 4a). Molecular modelling studies of the GSH- and MSH-dependent formaldehyde dehydrogenases revealed that they are distinct in their catalytic sites (Norin *et al.*, 1997); that is, GSH cannot be substituted for MSH in the reaction catalyzed by MSH-dependent formaldehyde dehydrogenase and vice versa.

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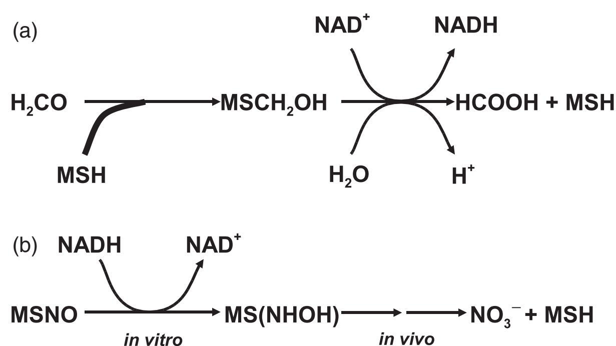

Fig. 4. (a) The formaldehyde dehydrogenase reaction of MscR. (b) The nitrosothiol reductase reaction of MscR, indicating that MSH sulphinamide is formed *in vitro* and that MSNO is eventually converted to nitrate and MSH *in vivo* through unknown reactions.

Through biochemical and genetic studies, it was demonstrated that GSH-dependent formaldehyde dehydrogenases are more active as nitrosoglutathione reductases. The detoxification of nitrosothiols, which damage proteins by nitrosating critical sulfhydryl groups in proteins by a process of transnitrosation, was initially attributed to flavohemoglobins. In 1998, Jensen *et al*. (Jensen *et al*., 1998) demonstrated that the formaldehyde dehydrogenase from rat-liver cytosol also had nitrosothiol reductase activity that reduced nitrosoglutathione (GSNO) to GSH disulfide and ammonia. (Vogt *et al*., 2003) demonstrated that the *M. smegmatis* MscR was also more active as a nitrosothiol reductase than as an MSH-dependent formaldehyde dehydrogenase: the maximum velocity (*Vmax*) for the nitrosomycothiol (MSNO) reductase activity is 76 times faster than the *Vmax* for the formaldehyde dehydrogenase activity, with the reaction velocity of MSNO reductase saturating at a high concentration of MSNO. In fact, the turnover rate per subunit of MSNO reductase was substantially higher than that of GSNO reductases. This paper also reported that the metabolism of MSNO results in the disulfide of MSH and nitrate in *M. smegmatis*, *in vivo*, in contrast to the metabolism of GSNO, which results in the disulfide of GSH and ammonia in *E. coli*. *In vitro*, both nitrosothiol reductases catalyze the formation of either GSH sulphinamide or MSH sulphinamide (Fig. 4b). Hence, although the reactions for the two nitrosothiol reductases are identical, it appears that, *in vivo*, the product of the MSNO reductase, MSH sulphinamide, is processed differently and is converted into nitrate instead of ammonia, indicating a difference in the downstream reactions between MSH-containing actinomycetes and GSH-containing organisms.

Bioinformatic analysis of *mscR*

The *mscR* gene is highly conserved in the *Actinomycetales* (Table 1). *Mycobacterium tuberculosis* MscR shares 78% sequence identity to the *M. smegmatis* MscR, and 70% even with the *S. avermitilis* MscR. Intriguingly, while the *Corynebacterium glutamicum* and *Corynebacterium efficiens* MscR sequences range from 64% to 65% identity to *M. tuberculosis* MscR, *Corynebacterium diphtheriae* MscR has only 30% sequence identity (Table 1). Conceivably, there may be functional differences in the two enzymatic activities of the protein in *C. diphtheriae*.

mscR, the first gene in a bicistronic operon (Patel & Blanchard, 1999), codes for a 361-aa protein of 37.9 kDa with an isoelectric point of 4.5. Rv2260, the gene downstream, codes for a 211-aa protein with sequence identity to other hypothetical proteins in *M. tuberculosis* (gene products of Rv0634c, Rv1637c, Rv3677c, Rv2581c) and to various hydrolases. Himar transposon mutagenesis data suggest that this gene may be essential (Park *et al*., 2006). Rv2260 gene product contains a pfam00753 domain that represents the metallo-beta-lactamase superfamily, which includes zinc-dependent enzymes, such as thioesterases and glyoxalase II proteins, that catalyze the hydrolysis of *S*-D-lactoyl glutathione to form GSH and D-lactic acid. The bicistronic operon arrangement is conserved in mycobacteria with the exception of *M. leprae*, where in the operon the gene next to the *mscR* orthologue, ML1784, is a pseudogene, and the gene product of a more distant gene, ML1391, has the highest sequence identity (31%) to the Rv2260 gene product. Sequenced corynebacteria also maintain this location of Rv2260 next to *mscR*, with the exception of *C. diptheriae*, where the putative *mscR* orthologue is DIP2114 and the putative Rv2260 orthologue, DIP1158, has only 28% sequence identity. Other actinomycetes, such as *Rhodococcus* Rha1, *N. farcinica*, *S. coelicolor* and *S. avermitilis*, maintain the synteny that is present in the *M. tuberculosis mscR* operon. In *K. radiotolerans*, this synteny is not conserved; the putative orthologue of *mscR* (ORF493) is separated from the putative orthologue of Rv2260 (ORF506) by 12 genes. Further studies of the transcriptional regulation of the putative orthologues of *mscR* and Rv2260 in *C. diptheriae* and *K. radiotolerans* will demonstrate whether these genes are coordinately transcribed.

Genetic analysis of *mscR* mutants

Liu *et al*., (2001) generated and characterized nitrosothiol reductase mutants of yeast and mice. They showed that these mutants do not have GSNO-consuming activity and consequently exhibit increases in the cellular quantity of both GSNO and protein *S*-NO. Mutant yeast cells showed increased susceptibility to a nitrosative challenge, while mutant mice lacking GSNO reductase activity exhibited substantial increases in whole-cell *S*-nitrosylation, tissue damage, and mortality following endotoxic or bacterial challenge (Liu *et al*., 2001). In *Cryptococcus neoformans*, an

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intracellular fungal pathogen that replicates in macrophages, both flavohemoglobin denitrosylase and GSNO reductase contribute to *C. neoformans* pathogenesis. Growth of mutants disrupted in flavohemoglobin denitrosylase was inhibited when they were exposed to nitrosative stress and their survival was reduced in activated macrophages, whereas growth of mutants disrupted in nitrosothiol reductase mutants was not affected under the same conditions. Nevertheless, nitrosothiol reductase has a synergistic effect on pathogenicity in this intracellular pathogen, because a double mutant lacking both flavohemoglobin denitrosylase and nitrosothiol reductase activity exhibited increased attenuation in virulence as compared with the mutant disrupted in flavohemoglobin denitrosylase alone (de Jesus-Berrios *et al.*, 2003). We generated an *M. smegmatis* mutant disrupted in formaldehyde dehydrogenase/nitrosothiol reductase and did not observe any difference in the growth rate of this mutant from that of the wild-type strain (data not shown). Inactivating this gene in pathogenic mycobacteria will indicate whether this gene plays a protective role within macrophages.

Mycothiol-*S*-conjugate amidase (Mca), a major detoxification enzyme

Eukaryotes and gram-negative bacteria rely on GSH for protection against electrophilic toxins such as alkylating agents, halogens, and chlorine derivatives. This GSH-dependent detoxification system utilizes glutathione-*S*-transferases (GST) to conjugate the GSH to the toxin through the sulfur group of GSH, forming an *S*-conjugate. In animals, the *S*-conjugate is excreted from the cell by a specific transport system, and transported to other tissues and organs such as the kidney and liver, where the conjugate is hydrolysed, acetylated and excreted as a mercapturic acid derivative. In gram-negative bacteria such as *E. coli*, the *S*-conjugate is excreted into the medium (Kaluzna & Bartoz, 1977).

In mycobacteria, and presumably in other actinomycetes, a single enzymatic activity accomplishes this task of producing a mercapturic acid derivative from the *S*-conjugate. The thiol-specific fluorescent alkylating agent, monobromobimane, is lethal to cultured mammalian cells in the nanomolar range, whereas *M. smegmatis* cells can survive exposure to millimolar levels. Monobromobimane binds to MSH forming a MSH-monobimane adduct that is cleaved to produce glucosaminyl inositol and acetyl cysteinyl bimane, a mercapturic acid, which is rapidly excreted by the cell (Fig. 5) (Newton *et al.*, 2000). In a similar manner, MSH-toxin conjugates are formed with reactive electrophilic substrates. This process may be spontaneous or may be mediated by an enzymatic reaction similar to that of GST. Because MSH-monobimane is not detected in the growth medium, an activity that converts the MSH-toxin conjugate to a mercapturic acid was anticipated. The enzyme responsible for this activity, MSH-S-conjugate amidase (Mca), was purified from *M. smegmatis*. Comparison of the N-terminal sequence indicated that the orthologous gene in the *M. tuberculosis* genome is Rv1082 (Newton *et al.*, 2000). The *M. smegmatis* enzyme had a Km of 95+/−8 μM and k_{cat} of 8 s^{-1} for the MSH-monobimane adduct. Mca is relatively specific for the MSH moiety of the conjugates. If the acetyl or inositol residues are removed, Mca does not have significant activity; however, this enzyme can accommodate rather large groups attached to the sulfur moiety in the MSH-toxin conjugate and is thus flexible for the *S*-conjugate moiety. Purification of *M. smegmatis* amidase on a Sephadex G-100 column demonstrated that Mca is active as a monomer of 36 kDa or perhaps as an aggregate larger than a dimer, because activity was detected in the void volume (Newton *et al.*, 2000). The pH optimum for this enzyme was between 7 and 9, and the activity greatly declined below pH 7.0. Significant inhibition is seen of the amidase activity with cellular levels of MSH. The cloned *M. tuberculosis* amidase has a substrate specificity similar to that of the *M. smegmatis* amidase (Newton *et al.*, 2000; Steffek *et al.*, 2003). The substrates for Mca include the MSH conjugate of cerulenin, an antibiotic that inhibits fatty acid synthetase, and several antibiotic adducts have been identified as Mca substrates. So far, though, the model substrate, monobromobimane, is the best Mca substrate. The search for the natural substrate(s) is still underway.

Bioinformatic analysis of *mca*

The *M. tuberculosis mca* codes for a 32.7-kDa metalloenzyme (Newton *et al.*, 2000; Steffek *et al.*, 2003) with an isoelectric point of 4.9 and a Pfam 02585 domain shared with an uncharacterized lincomycin biosynthesis gene (*lmbE*). BLAST searching indicates that this gene is present in all sequenced mycobacterial species (Table 1). In other actinomycetes, the amino acid identity between Mca orthologues ranges from 51% for *S. avermitilis* to 72% for *N. farcinica*. CLUSTALW alignment revealed at least four major domains that are highly conserved among members of the amidase family. Histidine residues are conserved in three out of the four major domains.

In the *M. tuberculosis* genome, two other paralogues of *mca* exist: Rv0323c and *mshB*. Rv0323c codes for a protein of 24.2 kDa with an isoelectric point of 5.78, is considered nonessential (Sassetti *et al.*, 2003), and interestingly is found only in pathogenic mycobacteria. The second paralogue, *mshB,* codes for a MSH deacetylase that catalyzes the second step of the MSH biosynthesis pathway (Newton *et al.*, 2000, 2006). The amino acid sequences of MshB and Mca are similar (36% identity in 299 amino acids), and MshB has a

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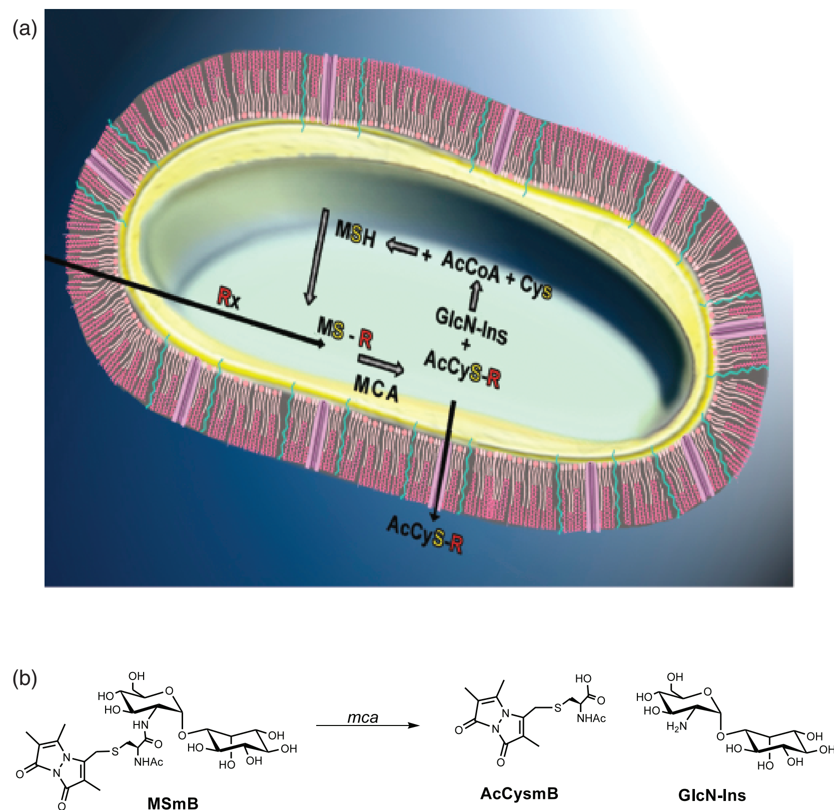

Fig. 5. Model of mycothiol-dependent detoxification. (a) Mycothiol reacts with a substrate R to form a MS-R conjugate. The Mca catalyzes the cleavage of an amide bond to yield a mercapturic acid, AcCys-R, and glucosaminylinositol, GlcN-Ins, which is recycled back to mycothiol. (b) Mca catalyzes the cleavage of the model substrate, mycothiol monobimane, to the mercapturic acid of monobimane and glucosaminylinositol.

Table 2. Orthologues of *mca* in antibiotic biosynthesis operons of actinomycetes

Strain		Accession number	Antiobiotic biosynthesis operon	Identity	Positive	Gaps
Streptomyces atroolivaceus	Ofv (+3)	AF484556.1	Leinamycin	146/282 (51%)	181/282 (64%)	7/282 (2%)
Streptomyces lincolensis 78-11	*lmbE*	CAA55751	Lincomycin	91/278 (32%)	131/278 (47%)	27/278 (9%)
Amycolatopsis mediterranei S699	*rifO*	AAC01723.	Rifamycin	74/246 (30%	117/246 (47%)	28/246 (11%)
Streptomyces rochei	*sttH*	AJ271405.1	Streptothricin	49/163 (30%)	73/163 (44%)	29/163 (17%)
Streptomyces lavendulae	*mitC*	AAD32721	Mitomycin	30/79 (37%)	36/79 (45%)	9/79 (11%)
Micromonospora sp. ML1	*tioW*	CAJ34379	Thiocoraline	52/169 (30%)	75/169 (44%)	21/169 (12%)

The percentage of identical (Identity) and similar (Positive) amino acids between Mca and the corresponding protein encoded by a gene in the antibiotic biosynthesis operon and any gaps added to align the sequences are listed.

weak MSH amidase activity *in vitro* (Newton *et al.*, 2000). Conversely, Mca has also been shown to have deacetylase activity with GlcNAc-Ins, *in vitro* (Newton *et al.*, 2000, 2006), and introduction of the *mca* gene into a *M. smegmatis mshB* mutant partially restored mycothiol levels (Rawat *et al.*, 2003).

The MshB deacetylase crystal structure has been elucidated, and, based on the sequence identity between Mca and MshB, a model has been proposed for the catalytic portion of Mca (Maynes *et al.*, 2003; McCarthy *et al.*, 2004). The pertinent active-site residues, including the metal binding site, the highly conserved His13, His147 and Asp16, align in the two proteins, with the exception of a Ser20 in MshB, which has been replaced with a Lys19. The Ser20 may be important for disaccharide binding in MshB. In Mca, the Lys 19 would impart a positive charge and prevent disaccharide binding; hence, the disaccharide portion of MSH must bind in a different orientation in Mca. The crystal structure of Mca will provide greater insight into the exact binding of MSH conjugates.

Most intriguingly, *mca* orthologues have been found in several antibiotic biosynthesis operons, such as those for avermectin (*Streptomyces avermitilis*), leinamycin (*Streptomyces atroolivaceus*), rifamycin (*Amycolatopsis mediterranei*), lincomycin (*Streptomyces lincolnensis*), erythromycin (*Saccharopolyspora erythraea*), mitomycin (*Streptomyces*

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lavendulae) and thiocoraline, an antitumour agent (*Micromonospora* sp. ML1) (Table 2) (Newton & Fahey, 2002; Lombo *et al.*, 2006). These genes have been annotated as either regulatory proteins, as in the case of *rifO* in the rifamycin biosynthesis cluster (Yu *et al.*, 2001), or proteins of unknown function. In this regard, it should be noted that mercapturic acid derivatives such as seongomycin, a mercapturic acid derivative of kinamycin (Gould *et al.*, 1998), have been found in fermentation broths. These mercapturic acid derivatives are weaker in activity than the parent antibiotic. This indicates that MSH may play a role in the protection of the host cell against either its own antibiotics or antibiotics produced by competitor bacteria (Newton & Fahey, 2002).

Direct experimental proof of the conjugation of an antibiotic to MSH was provided by Steffek *et al.*, (2003), who demonstrated that MSH forms a conjugate with Rifamycin SV that serves as a substrate for *M. tuberculosis* Mca. We extended these studies by showing that *in vivo* treatment of the parent *M. smegmatis* wild-type strain or an *mca* mutant with Rifamycin SV results in a MS-Rifamycin SV adduct, which is then partially converted to mercapturic acid only in the wild-type (Rawat *et al.*, 2004). Yu *et al.* (2001) reported that inactivation of *rifO*, the *M. tuberculosis mca* orthologue in the rifamycin biosynthesis cluster of *A. mediterranei*, does not result in a decrease in rifamycin B formation, but the levels of MS-rifamycin adduct were not measured. *In vivo* studies, similar to the ones performed with *M. smegmatis mca* mutant, that measure the MS-rifamycin adduct and mercapturic acid in the *A. mediterranei rifO* mutant, or amidase activity assays using monobromobimane as a substrate with the purified RifO, would determine if RifO is the functional analogue of the mycobacterial Mca.

mca is the first member of a tricistronic operon (Romero & Karp, 2004), followed by a small gene, Rv1083, whose product is possibly secreted, and Rv1084. The gene product of Rv1084 has a thioredoxin domain as well as a carboxypeptidase domain. The operon arrangement is not fully conserved among actinomycetes. In *M. leprae*, an orthologue of Rv1084 is present downstream but it is a pseudogene. In *M. avium paratuberculosis*, the orthologues of Rv1083 and Rv1084 are located next to each other but not next to the *mca* orthologue, MAP1029. In other actinomycetes, such as *N. farcinica*, the operon has an additional gene, nfa48220, between the orthologues of Rv1083 (nfa48230) and Rv1084 (nfa48210). Similarly, in *S. avermitilis*, SAV3297, coding for a regulatory protein, is between the orthologues of Rv1083 (SAV3298)and Rv1084 (SAV3296), while in *S. coelicolor*, the orthologue of Rv1084, SCO4974 is separated by five genes from the *mca* orthologue (SCO4967) and Rv1083 orthologue (SCO4968). In *K. radiotolerans*, the orthologue of *mca* is ORF1197 and the orthologue of Rv1084 is ORF1199, but there are no orthologues of Rv1083. Remarkably, no orthologues of Rv1083 or of Rv1084 are present in the sequenced corynebacterial genomes.

Mycobacterium smegmatis mca mutant is sensitive to toxins and antibiotics

The previously mentioned *M. smegmatis mca* mutant (Rawat *et al.*, 2004) lacks amidase activity and accumulates MSH adducts of monobromombimane inside the cell, in contrast to the parent strain, which rapidly converts these adducts to mercapturic acids and excretes them into the media. The *mca* mutant is more sensitive to the alkylating agents, iodoacetamide and the GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB), than are mutants lacking MSH. The mutant is also similar in phenotype to mutants lacking MSH in that it shows more sensitivity than the parent strain to several antibiotics, most notably streptomycin. Whether MSH forms adducts with streptomycin and other drugs, as it does with Rifamycin SV, remains to be established.

Inhibitors of Mca as potential drug candidates?

The uniqueness of Mca makes it a promising target for inhibitor development. Bewley and coworkers (Nicholas *et al.*, 2003) have screened 1500 natural product extracts and identified several inhibitors as lead candidates. Two types of bromotyrosine-derived natural products were competitive inhibitors of Mca. Based on the structure of the natural bromotyrosine inhibitor, a family of compounds were synthesized and tested for the ability to act as anti-infective agents against mycobacteria and other gram-positive bacteria (Pick *et al.*, 2006). One lead compound, EXEG1706, has low minimum inhibitory concentrations for methicillin-sensitive and -resistant *Staphylococcus aureus*, vancomycin intermediate *S. aureus*, and the mycobacteria *M. smegmatis* and *M. bovis* BCG, indicating that this class of inhibitors has broad specificity and is not specific for Mca, possibly inhibiting other metalloenzymes present in these bacteria.

Are there MSH transferases (MSTs) in mycobacteria?

GSTs are a group of proteins that are involved in detoxification of endobiotic and xenobiotic compounds by covalently linking GSH to a hydrophobic substrate resulting in more polar GSH conjugates (Sheehan *et al.*, 2001). In general, the enzymes are highly specific for the thiol substrate, GSH, and less specific for the second substrate. GSTs catalyze nucleophilic aromatic substitutions, Michael additions to alpha, beta unsaturated ketones and epoxide ring opening. Natural substrates include 4-hydroxyalkenal, which is formed during lipid peroxidation, steroids, leukotrienes, anthocyanins, and

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organic isothiocyanates in eukaryotes. In prokaryotes, GSTs, such as dichloromethane dehalogenases (Vuilleumier *et al.*, 2001), are associated with catabolic pathways that are involved in the bioremediation of xenobiotics (Vuilleumier, 1997; Vuilleumier & Pagni, 2002).

As a parallel to GSH chemistry, MSH-S-transferases that catalyze the conjugation of MSH to xenobiotics are conceivable. A model substrate such as monobromobimane would not benefit from transferase activity as it readily reacts with MSH. However, more inert electrophiles such as some antibiotics and recalcitrant chemicals, would probably need enzymatic activity to catalyze the conjugation to MSH. Some actinomycetes, including *Rhodococcus* and mycobacteria, are able to grow on toxic compounds such as vinyl chloride, 1,2-dibromoethane, and other haloalkanes. These toxic compounds are first dehalogenated to form epoxides or monohaloaldehydes that are toxic to microorganisms unless they are conjugated to thiols, indicating a requirement for an MSH transferase activity.

A GST has been purified from a *Rhodococcus* strain AD45 that is able to utilize isoprene (van Hylckama Vlieg *et al.*, 1999, 2000) and degrade 1,2-dichloroethylene, toluene, propylene and styrene. Because this GST has a Km for GSH in the millimolar range, we had initially thought that it may indeed be a MSH transferase. Sequencing of the gene cluster involved in isoprene metabolism and the flanking area revealed not only two genes coding for homologues of GSTs but also the presence of genes with sequence similarity to γ-glutamyl cysteine synthetase and a GSH synthetase, enzymes that are needed for GSH biosynthesis. In *Rhodococcus* AD45, γ-glutamyl cysteine synthetase is located upstream and GSH synthetase is located downstream of the gene cluster coding for isopresne metabolism (van Hylckama Vlieg *et al.*, 2000). We have shown that *Rhodococcus* AD45 contains both GSH and MSH, and that the amount of GSH is ten-fold lower than the amount of MSH in rich media such as trypticase soy broth (Rawat *et al.*, manuscript in preparation). It appears that, in *Rhodococcus* AD45, the whole gene cluster of isoprene metabolism, including the genes involved in GSH biosynthesis, has been transferred horizontally (van Hylckama Vlieg *et al.*, 2000).

The *Rhodococcus* AD45 GST genes have, recently, been cloned into a recombinant expression system to engineer a novel pathway for trichloroethylene degradation in *E. coli* (Rui *et al.*, 2004). These GSTs show sequence similarity to other GSTs in the N-terminal portion of the enzyme, which is responsible for binding to GSH. Sequence searches of the available actinomycete genome sequences did not unearth any orthologues, emphasizing the uniqueness of *Rhodococcus* AD45. Similarly, data mining of bacterial sequences using profiles designed to 'fish' for GSTs from completed microbial genomes did not yield any potential candidates in actinomycetes or Archaea (Vuilleumier & Pagni, 2002). This result is not surprising, as the structure of the tripeptide GSH differs considerably from the structure of MSH.

S-conjugate or mercapturic acid transporters

Both the MSH amidase activity, which results in mercapturic acids, and the postulated MSH transferase activity, which would result in MSH-toxin conjugates that may not be converted to mercapturic acids, may require the presence of specific transport systems that are able to remove these compounds from within the cell. In organisms that rely on the GSH-based detoxification system, the GSH conjugates and the mercapturic acids are excreted to avoid long-term damage. In *E. coli*, a conjugate of glutathione and monobromobimane (GS-mB) is excreted by an ATP-binding cassette transporter (Kaluzna & Bartoz, 1977). In humans, multidrug resistance proteins (MRP) and *P*-glycoproteins (PGP) confer resistance to cells to a number of cytostatic drugs by mediating the ATP-dependent transport of glutathione *S*-conjugates (Pulaski *et al.*, 1996). Many isoforms encoded by various genes have been cloned, and orthologues of MRP and PGP have been identified in many species, including yeast, plants, and nematodes. MRP belong to the ABC family of transporters, which have varying degrees of structural and functional relatedness and overlapping substrate specificities for a wide range of compounds including organic anions and weakly cationic hydrophobic compounds besides glutathione *S*-conjugates. van Veen *et al.*, (1998) demonstrated that *lmrA*, a member of the ABC transporter family that mediates antibiotic resistance in *Lactococcus lactis*, conferred multidrug resistance to human lung fibroblast cells, indicating that LmrA is functionally interchangeable with PGP. BLAST analysis using the *L. lactis* LmrA sequence revealed that orthologues of this protein, having a sequence identity of *c.* 30%, are present in actinomycetes. The sequence identity is more pronounced in the C-terminus portion of the protein. In the *M. tuberculosis* genome, the gene products of Rv1272c and Rv1273c have the greatest sequence similarity to LmrA, 31% and 26% respectively, followed by Rv1348 (27%) and Rv1349 (26%), and Rv0194 (24%). All these gene products have the nucleotide binding domains and membrane spanning domains characteristic of ABC transporters (Braibant *et al.*, 2000), of which 26 complete (having one nucleotide binding domain and one membrane spanning domain) and 11 incomplete versions are encoded in the *M. tuberculosis* genome (Braibant *et al.*, 2000). Whether any of these ABC transporters are actually able to transport mercapturic acids or MSH-*S*-conjugates of antibiotics or toxins out of the mycobacterial cell remains to be seen.

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The similarity of the antibiotic resistance phenotype of mutants lacking MSH mutants and *whiB7* mutants is worth noting. Thompson and colleagues (Morris *et al.*, 2005) reported that *whiB7* null mutants of *S. coelicolor* and *M. tuberculosis* are hypersusceptible to antibiotics *in vitro*, and demonstrated that *M. tuberculosis whiB7* is induced by subinhibitory concentrations of antibiotics (erythromycin, tetracycline, and streptomycin). The WhiB7-dependent set of eight transcripts includes tap (Rv1258c), encoding an efflux pump that confers low-level resistance to aminoglycosides and tetracycline, and an unstudied gene encoding a putative macrolide transporter (Rv1473) with an ATP-binding cassette (Morris *et al.*, 2005). The MSH content of the *whiB7* mutant has not been measured, and the authors listed only the top 10 genes that were inducible by antibiotics under WhiB7 control. WhiB7 may work cooperatively with the MSH-dependent Mca detoxification system to confer antibiotic resistance to actinomycetes.

Other mycothiol-dependent proteins

Maleylpyruvate isomerase

Grund *et al.*, (1990) examined the catabolic pathways involved in aromatic compounds such as benzoate, m-hydroxybenzoate, and salicyclate in eight strains of *Streptomyces* and *Amycolatopsis*. In contrast to *S. umbrinus* and *A. mediterranei*, which did not require GSH for the isomerization of maleylpyuvate, *S. ghanaensis* required the addition of GSH to convert maleylpyruvate, the ring fission product of gentisic acid, to fumarylpyruvate in cell extracts that had been depleted of all thiols by the addition of 1 mM *N*-ethylmaleimide. There have been no further reports on this GSH-dependent enzyme from *S. ghanaensis*. As in the case of *Rhodococcus* AD45 GST described in Section 5, it is possible that horizontal transfer of genes coding for the enzymes in the gentisate pathway and glutathione biosynthesis may have occurred from gram-negative bacteria such as *Ralstonia* sp. strain U2 (Zhou *et al.*, 2001) to *S. ghanaensis*.

More recently, Feng *et al.*, (2006) demonstrated that the GSH-independent gentisate pathway in actinomycetes requires MSH as a cofactor for the catalysis of maleylpyruvate to fumarylpyruvate by a maleylpyruvate isomerase, encoded by ncgl2918. This *Corynebacterium glutamicum* enzyme is functionally homologous to GSH-dependent maleylpyruvate isomerase enzymes from *Klebsiella pneumoniae* and *Ralstonia* strains (Zhou *et al.*, 2001), but the sequence similarity between the *C. glutamicum* protein and the other malelylpyruvate isomerase proteins is low. We were unable to identify orthologues of maleylpyruvate isomerase in the mycobacterial strains sequenced thus far, although orthologues were present in the *S. coelicolor* and *S. avermitilis* genomes.

Rv0274

A transposon mutant disrupted in the promoter region of an *M. smegmatis* orthologue of *M. tuberculosis* Rv0274 was discovered in a screen for mutants sensitive to diamide, a thiol-oxidizing agent (Rawat *et al.*, 2002). BLAST analysis indicates that the gene product of Rv0274 is similar to lactoylglutathione lyase/glyoxylases, which catalyze the detoxification of methylglyoxal by conjugation of GSH to methylglyoxal resulting in (R)-*S*-lactoylglutathione. More interestingly, this gene also has 29.1% identity in 151-aa overlap with the gene products of fosfomycin resistance genes that serve as GSTs in the conjugation of GSH to a carbon on the epoxide ring of the fosfomycin. Besides diamide, this mutant is sensitive to CDNB, the model GST substrate, and other alkylating agents, but it is not sensitive to either fosfomycin or methyl glyxoal (van Hylckama Vlieg *et al.*, 1999). MSH transferase assays with the recombinant gene product of Rv0274 using CDNB, fosfomycin, and monochlorobimane, a less reactive bimane than monobromobimane, as substrates were unsuccessful (M. Rawat and G. Newton, unpublished results), contradicting the assumption that it is a MSH transferase. However, as glutathione transferase can conjugate a variety of substrates, it is likely that the correct substrate has yet to be tested. In a microarray analysis of the starvation response of *M. tuberculosis*, Betts *et al.*, (2002) reported that the Rv0274 mRNA was upregulated after 4, 24 and 96 h of starvation, which suggests that Rv0274 may act as a glyoxylase to detoxify the higher concentrations of methylglyoxal that result from an imbalance in the production and metabolism of glycolytic triose phosphates upon transfer to nutrient-poor media. Further studies of Rv0274 are warranted to establish the function of this intriguing gene.

Mycothiol-dependent peroxidases and thiol transferases

We have previously shown that *M. smegmatis* mutants lacking MSH are sensitive to oxidants such as peroxides and redox cycling agents [Newton *et al.*, 1999; Buchmeier *et al.*, 2003; Rawat *et al.*, 2003, 2002, Rawat & Av-Gay, unpublished observations], which cause chemical alterations in proteins, lipids, carbohydrates and nucleic acids in the mycobacterial cell. Like GSH (Chang *et al.*, 1992), MSH appears to react with the napthoquinone redox cycling agent menadione to form a conjugate (M. Rawat, unpublished observations), which may be less toxic to the cell because the menadione MSH conjugate would be expected to redox-cycle at a slower rate. Treatment with another napthoquinone redox-cycling agent, plumbagin, results in a stochiometric decrease in reduced GSH (Inbaraj & Chignell, 2004), and this is also likely to be the case with MSH. It is less clear how MSH protects against organic peroxides, although

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treatment of cells with cumene hydroperoxide results in increased levels of oxidized GSH (Poot *et al*., 1987). Hydrogen peroxide treatment of *M. bovis* BCG that has been transferred to saline solution results in an increase in oxidized MSH, while a similar treatment in normal growth medium does not result in changed MSH levels (Ung & Av-Gay, 2006). In *M. smegmatis*, increasing the concentration of hydrogen peroxide used to treat cells in normal growth medium results in an increase in cell death before a decrease in MSH levels, indicating that MSH is not directly reacting with hydrogen peroxide to protect the cells but that instead an indirect mechanism, probably involving peroxiredoxins or peroxidases, is involved (M. Rawat, unpublished observations).

In *M. tuberculosis*, five genes, *tpx*, *ahpC*, *ahpD*, *ahpE*, *bcpA*, *and bcpB*, have been annotated as encoding peroxiredoxins. There is experimental evidence that AhpC and Tpx both require thioredoxin (Jaeger *et al*., 2003), but that MSH is able to act as an electron donor to these peroxiredoxins has not been conclusively ruled out. X-ray crystallography of AhpC (Guimaraes *et al*., 2005), AhpD (Nunn *et al*., 2002) and AhpE (Li *et al*., 2005) also suggests that thioredoxin is the likely electron donor for these peroxiredoxins. Thus, genes coding for MSH-dependent peroxidases remain to be discovered.

In addition to thiol peroxidases, thiol transferases (or glutaredoxins) play an important role in many organisms in protection against oxidative stress response by catalyzing the reduction of disulfides in proteins, and these enzymes, in turn, are reduced by GSH. In the *M. tuberculosis* genome, three genes have been annotated as glutaredoxins, namely Rv2466c, Rv3198A, and *nrdH*. Furthermore, an arsenate reductase, which appears to require GSH and glutaredoxin for activity, has been characterized from the actinomycete *C. glutamicum* (Ordonez *et al*., 2005). As there is generally no GSH present in actinomycetes, these genes have been annotated as glutaredoxins on the basis of glutaredoxin domains. The mycobacterial 'glutaredoxin', Rv2466c, which has a pfam01323.11 *dsbA*-like thioredoxin domain, is 207 amino acids longer than a typical glutaredoxin or thioredoxin. It has been shown to be regulated by SigH (Raman *et al*., 2001; Kaushal *et al*., 2002; Manganelli *et al*., 2002), the alternative sigma factor that is involved in the oxidative and heat stress response of mycobacteria. Thioredoxin (*trxC*) and thioredoxin reductase (*trxB2*) are also among the 39 genes under the control of this sigma factor (Manganelli *et al*., 2002), but genes involved in MSH biosynthesis and metabolism were not identified among the 39. Similarly, an orthologue of Rv3198A, containing the pfam00462 glutaredoxin domain, has been shown to be under the control of the SigH equivalent in *S. coelicolor*, SigR (Paget *et al*., 2001). Mycothiol levels in the *sigR* mutant were found to be fourfold lower as compared with wild-type *S. coelicolor*, although none of the genes involved in mycothiol biosynthesis is in the SigR regulon (Paget *et al*., 2001). The third 'glutaredoxin', NrdH, is involved in electron transfer to ribonuculeotide reductase, which catalyzes the formation of deoxyribonucleotides for DNA synthesis. Like Rv3198A, the *M. tuberculosis* NrdH has the pfam00462 domain and also 72% sequence identity to NrdH from *Corynebacterium amminiogenesis*, for which a crystal structure is available. In this *C. amminiogenesis* structure there is a domain-swapped thioredoxin-binding pocket (Stehr & Lindqvist, 2004), suggesting that thioredoxin may serve as the electron donor to the oxidized NrdH in actinomycetes instead of MSH. The structure of MSH differs substantially from that of thioredoxin, and thus it is unlikely that MSH would be able to fit in the thioredoxin-binding pocket. That thioredoxin or perhaps thioredoxin reductase may serve to maintain the actinomycete 'glutaredoxins' in a reduced form is an intriguing possibility.

Concluding remarks

In this review, we have discussed genes whose gene products have been identified conclusively to require MSH for activity. These enzymes play important roles in the actinomycetes, as they maintain the redox balance within the cell and protect the cell against nitrosative stress and toxins. In addition, the role of MSH in growth-supporting biodegradative metabolism is beginning to be elucidated with the identification of MSH-dependent enzymes such as maleylpyruvate isomerase. By analogy to GSH-dependent reactions, many more MSH-dependent enzymes such as peroxidases, glyoxylases, and transferases are expected to be discovered. Moreover, GSH not only participates in a wide variety of metabolic processes in the cell, but also has been implicated in redox regulation of a number of processes, including cell signalling, in eukaryotes (Dickinson & Forman, 2002; Masip *et al*., 2006). Whether MSH participates in a similar manner in actinomycetes is an interesting area for further exploration. Perhaps the most exciting expectation is that novel enzymatic activities, such as the MSH-dependent amidase, that require MSH as a cofactor but do not have a counterpart in GSH chemistry will be unearthed in the future. Because MSH or enzymes involved in MSH biosynthesis and metabolism are not present in mammals, and given the emerging importance of MSH in mycobacteria, MSH and reactions involving MSH are potential targets for drugs directed against mycobacteria.

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