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# Accepted Article

**Title:** Thiol redox and pKa properties of mycothiol, the predomiant low molecular weight thiol cofactor in the Actinomycetes

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# Thiol redox and pKa properties of mycothiol, the predomiant low molecular weight thiol cofactor in the Actinomycetes

Sunil V. Sharma,[a] Koen Van Laer,[b][c][d][e] Joris Messens,[b][c][d] Chris J. Hamilton*[a]

Herein, the thiol p$K_a$ and standard redox potential of mycothiol, the major low molecular weight thiol cofactor in the actinomycetes, are reported. The measured standard redox potential reveals substantial discrepancies in one or more of the other previously measured intracellular parameters that are relevant to mycothiol redox biochemistry.

In eukaryotes and most Gram-negative bacteria, glutathione (GSH) is the major low molecular weight (LMW) thiol cofactor (Figure 1), which serves a number of important metabolic functions.[1] Instead of GSH, most Gram-positive bacteria utilise alternative, structurally distinct, LMW thiols[2] [3] [4] [5] (Figure 1). Mycothiol[2] (MSH) is the predominant LMW thiol in the Actinomycetes, which includes several bacteria of medical (*e.g*. *Mycobacterium tuberculosis*) as well as commercial significance in terms of antibiotic production (*e.g*. Streptomyces) and bioremediation (*e.g*. Corynebacteria). Analogous to GSH, MSH plays a central role in oxidative stress management by maintaining an intracellular reducing environment as well as through detoxifying electrophilic xenobiotics. Protein mycothiolation (reversible formation of MS-S-protein disulfides) is also emerging as an important post-translational modification for regulating protein function and protecting exposed cysteine (Cys) residues from irreversible damage during oxidative stress.[6] Moreover, MSH has recently been identified as the sulfur donor in the biosynthesis of the antibiotic lincomycin A.[7] Since first being discovered more than 20 years ago, despite the wealth of knowledge that has so far been unravelled regarding MSH metabolism, its fundamental biophysical properties (*i.e*. thiol p$K_a$ and standard thiol/disulfide redox potential) have never been measured. Herein, these have now been determined.

The thiol p$K_a$ for MSH (8.76) (Table 1) was determined by measuring the pH-dependent changes in absorbance at 232 nm for the thiolate anion (Figure 2A).[8] The MSH thiol is only ~0.17 p*K*a units more acidic than that of GSH (Table 1). Compared to the first microscopic thiol p$K_a$ value of cysteine (p$K_S$), MSH is ~0.4 p$K_a$ units less acidic. This can be explained by the absence of a protonated amino group in MSH, which helps in stabilising the thiolate anion in Cys. The same reasoning can be used to explain the p$K_a$ difference between MSH and the structurally related bacillithiol (BSH). Removal of the inositol aglycone (dMSH) makes the thiol marginally less acidic by ~0.15 p$K_a$ units. This could be due to loss of stabilising effects of intramolecular hydrogen bonding between the inositol hydroxyls and the thiolate anion. This relatively small influence of the aglycone on the thiol p$K_a$ is comparable to that previously observed when the malic acid of BSH was removed[9] (*cf*. BSH and MeO-GlcN-Cys, Table 1).

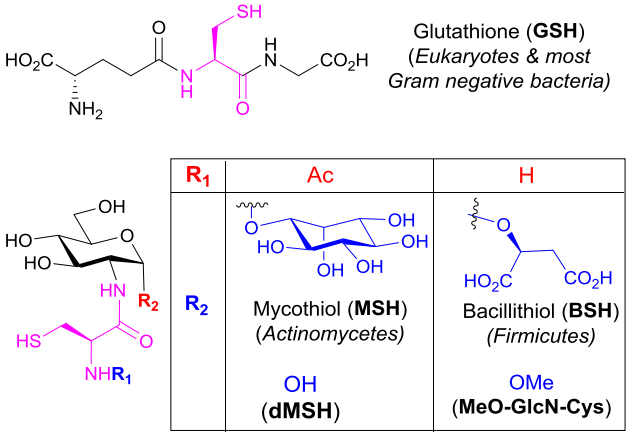


**Figure 1.** Structures of the LMW thiols relevant to this study (distribution of the naturally occurring LMW thiol cofactors (MSH, BSH, GSH) are given in italics).

This thiol p$K_a$ value can now be used to compare the intracellular abundance of MSH, Cys and CoA thiolates under physiological conditions. Across the broad intracellular pH ranges that different actinomycetes have been shown to experience and/or tolerate (~pH 6.1-8.3)[10],[11], the proportion of Cys in its thiolate form is up to two-fold greater than that of MSH (Figure 2B). However, intracellular MSH levels are significantly more abundant than those of Cys (ranging from ~6-fold in *S. coelicolor* to >600-fold in *M. tuberculosis*).[12] Hence, despite the greater thiol acidity of cysteine, MSH is generally present as the most substantially abundant LMW thiolate.

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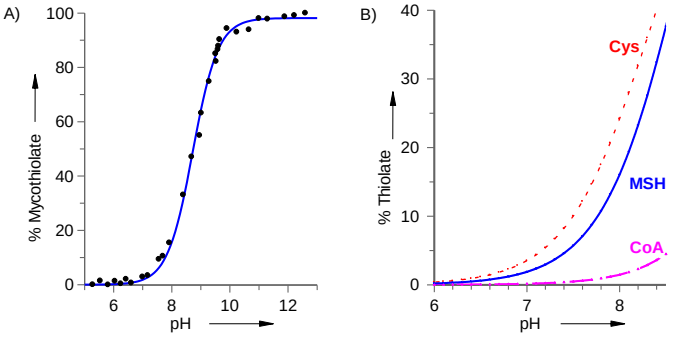


**Figure 2.** A) pH-dependent thiolate titration curve for MSH; (B) Calculated pH-dependent proportions of thiolate forms of MSH, Cys and CoA (based on thiol p$K_a$ values in Table 1).

Whilst many of the metabolic reactions of MSH are facilitated by enzymes (*e.g*. mycoredoxins, mycothiol-*S*-transferases) some biologically relevant carbonyl electrophiles, such as methylglyoxal[13] and formaldehyde, are sufficiently reactive to chemically react with LMW thiols to form hemi-thioacetals, which are then metabolised by glyoxalase[13] and formaldehyde dehydrogenase enzymes,[14] respectively.† Likewise, the initial stages of nitric oxide (NO) detoxification involve chemical reactions with LMW thiols to form *S*-nitroso-thiols, which then serve as substrates for *S*-nitroso-reductases.[14] These non-enzymatic reactions are driven by the more nucleophilic thiolate form of the LMW thiol. The greater intracellular abundance of mycothiolate suggest that it, would be the preferential reactant with such electrophiles *in vivo*.

LMW thiols can protect protein thiols from oxidative damage by the formation of mixed disulfides *via* chemical reaction with protein sulfenic acids and sulfenyl chlorides (*e.g*. produced under peroxide and hypochlorite stress).[15] Such processes protect protein thiols from being further, and irreversibly, oxidised to their sulfinic and sulfonic acid derivatives. To date, a redox proteomics study of *Corynebacterium glutamicum*, has identified 25 different proteins that are exclusively *S*-mycothiolated under hypochlorite (NaOCl) stress while less abundant *S*-cysteinylation only observed in a MSH knockout mutant.[6] The presence of MSH as the most abundant intracellular LMW thiolate anion can account for the exclusive *S*-mycothiolation, rather than *S*-cysteinylation of proteins that is observed during NaOCl stress.[6]

The standard redox potential of MSH ($E^{o'}_{MSSM/MSH}$) was determined by measuring the thiol/disulfide equilibrium constants between MSH and BSH in both the forward (MSH + BSSB) and reverse (MSSM + BSH) reaction. BSH was chosen as the reference thiol in these equilibration experiments because other LMW thiols (GSH, Cys, CoA, penicillamine), failed to give adequate separation of NMR signals that could be used to quantify each of the individual thiol and disulfide components in the equilibrium mixtures. High field (800 MHz) proton NMR provided sufficient resolution of the resonances associated with the cysteinyl alpha protons for BSH, BSSM, MSH and MSSM for their equilibrium ratios to be quantified (Figure 3). The BSH cysteinyl alpha protons in BSSB and BSSM (4.21-4.26 ppm) presented a set of overlapping multiplets, so BSSB was quantified indirectly by subtracting the BSSM integral value (4.78 ppm). These were then used to calculate $E^{o'}_{MSSM/MSH}$ relative to the BSH standard redox potential ($E^{o'}_{BSSB/BSH}$ = -221 mV)[9] using the Nernst equation. The measured standard thiol redox potential of MSH (-230 mV) is only 10 mV less negative (*i.e*. less reducing) than that of GSH (Table 1). Redox potentials are a thermodynamic property based on thiol-disulfide exchange equilibria, but are never at equilibrium in living cells.[16] The actual redox buffering properties of MSH are driven by other factors such as its intracellular abundance, and catalytic efficiencies of MSH specific redox enzymes such as mycoredoxins[17] and mycothiol disulfide reductase,[18] which help maintain high MSH/MSSM ratios and an intracellular reducing environment.[2b] [19]

An intracellular MSH redox potential ($E'_{MSH}$) of -300 mV has recently been measured in the exponential growth phase of a *Mycobacterium smegmatis* strain engineered to express a redox sensitive green fluorescent protein fused to an MSH-specific mycoredoxin (Mrx1-roGFP2).[20] Previously, the intracellular pH (~7.0)[21], MSH levels (~4 mM)††[22] and MSH/MSSM ratios (500:1)[22] have been independently reported. If all five of these values were correct then it should be possible to calculate a comparable value for any one of these reported parameters by substituting the other four into the Nernst equation. The results of these calculations (in bold-type, Table 2) indicate that there should be substantial discrepancies in one or more of the values that have been experimentally determined.

**Table 1.** Thiol p$K_a$ and standard redox potentials for different LMW thiols and their analogues.

| Thiol | p$K_a$ | Ref | $E^{o'}$ (mV) | Ref |
|---|---|---|---|---|
| MSH | 8.76 ± 0.02 | This work | -230 ± 3 | This work |
| dMSH | 8.91 ± 0.02 | This work | | |
| BSH | 7.97[a] | [9] | -221 | [9] |
| | 9.55[b] | | | |
| MeO-GlcN- | 7.79[a] | [9] | | |
| Cys | 9.31[b] | | | |
| GSH | 8.93 | [23] | -240 | [24] |
| Cys | 8.38[a] | [9] | -223 | [25] |
| | 9.94[b] | | | |
| CoA | 9.83 | [26] | -234 | [25] |

[a] The first microscopic thiol dissociation constant (p$K_s$) when the cysteinyl amine is still protonated. [b] The second microscopic thiol dissociation constant (p$K_{ns}$) when the cysteinyl amine is not protonated

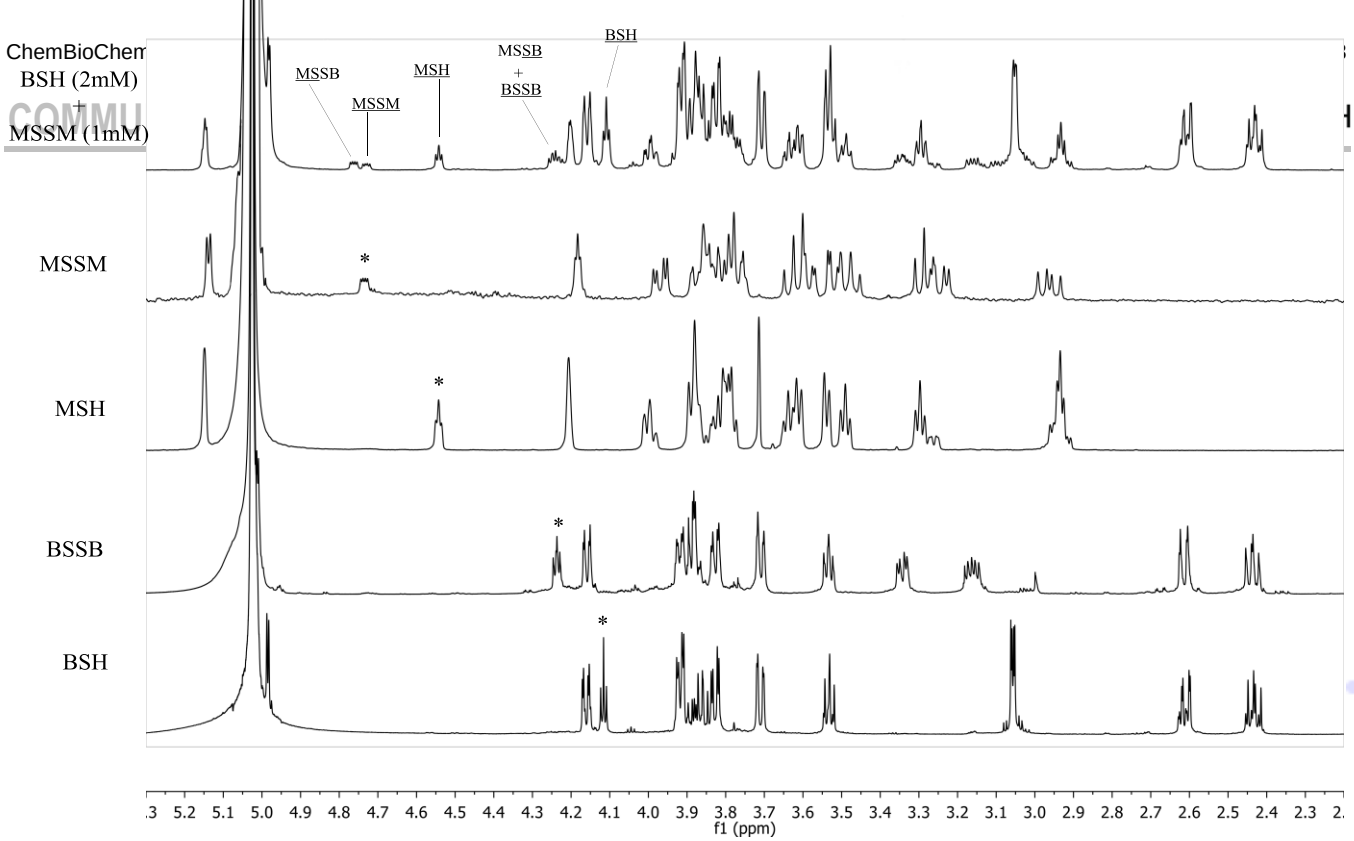


**Figure 3.** Proton NMR spectra of an equilibrated thiol/disulfide mixture of BSH and MSSM. The asterisks in the NMR spectra of the pure thiols and disulfides indicate the cysteinyl CH proton signals that are used to quantify each of these components in the equilibrated mixtures.

A calculated $E'_{MSH}$ value of -300 mV would be possible if $E^{o'}_{MSSM/MSH}$ was -291 mV (*i.e*. 60 mV lower than that which has been measured) (Table 2, entry (i)). (Table S1, supporting information). An $E^{o'}_{MSSM/MSH}$ value of -291 mV would require an equilibrium constant between MSH and BSSB of 0.1, which is 20-fold lower than what is measured in several equilibration studies starting from different MSH and BSSB, or MSSM and BSH ratios, (Table S1, supporting information).

Redox potentials are also pH-dependent[27] and a calculated intracellular pH of 7.9 (Table 2, entry (ii)) is far removed from the experimentally calibrated range that *M. smegmatis* maintains (pH 7.0 ±0.3)[21] when grown in Middlebrook media (at pH 6.9). The calculated MSH concentration of 379 mM (Table 2, entry (iii)) is also not credible as MSH displays feedback inhibition of the *M. smegmatis* glycosyltransferase (MshA), which catalyses the first obligate step of MSH biosynthesis, with an $IC_{50}$ of 3.6 mM.[28]

An $E'_{MSH}$ value of -240 mV would be expected if all of the other independently measured experimental parameters were correct (Table 2, entry (v)), which is 60 mV less reducing than the $E'_{MSH}$ determined by the Mrx-roGFP method.[20] The authors of this study demonstrated that the sensor is responsive to MSH/MSSM *in vitro* and does not equilibrate with other LMW thiols (*i.e*. cystine, GSSG, ergothioneine and 2-hydroxyethyl disulfide) or the cellular thioredoxin pathway.

If all of the other independently measured experimental parameters were correct a much higher MSH:MSSM ratio (~47300:1) would be expected. This is almost two orders of magnitude greater than those previously quantified from cell extracts (Table 2, entry (iv)). It is plausible that experimental artefacts resulting from low levels of MSH oxidation or incomplete N-ethylmaleimide (NEM) derivatisation could lead to substantial underestimation of the true MSH/MSSM ratio. Disulfide levels are quantified by first treating cells with NEM to alkylate all the free MSH. Dithiothreithol (DTT) is then added to reduce the MSSM prior to labelling with the thiol specific fluorophore monobromobimane (mBBr) before quantification by HPLC.[29] During the thiol-capping step with NEM it would only require 0.4% of the free MSH to remain unreacted (or 0.8% to be oxidised to MSSM) for an initial MSH/MSSM ratio of 44000 to give a value of 500. Intracellular GSH/GSSG values of a similar magnitude (~50000:1) have been measured using a glutaredoxin-coupled roGFP system in glutathione-utilising organisms; ratios that far exceed the ~500:1 ratios typically determined by cell-disruptive enzymatic titration or chemical derivatisation methods. [30] It is worth noting that the reported intracellular $E'_{MSH}$ measurements of -300 mV using the Mrx1-roGFP2 method[20] may represent a conservative estimate as previous redox titrations of Mrx1-roGFP2 show to it be almost completely reduced and operating at its limit of detection at -300 mV.[31] This presents the possibility that even more negative intracellular $E'_{MSH}$ values (and plausibly even larger MSH/MSSM ratios) may actually be present.

**Table 2.** Differences between the experimentally measured and calculated values of parameters that influence $E_{MSH}$ in *M. smegmatis*.

| Entry | $E^{o'}_{MSSM/MSH}$ (mV) | pH | MSH (mM) | MSH/MSSM[b] | $E'_{MSH}$ (mV) |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| (i) | **-291**[a] | 7.0[21] | 4[22] | 500[22] | -300[20] |
| (ii) | -230 | **7.9**[a] | 4[22] | 500[22] | -300[20] |
| (iii) | -230 | 7.0[21] | **379**[a] | 500[22] | -300[20] |
| (iv) | -230 | 7.0[21] | 4[22] | **47300**[a] | -300[20] |
| (v) | -230 | 7.0[21] | 4[22] | 500[22] | **-239**[a] |

[a] Values in bold type are calculated values required, alongside the other reported parameters in that row, to satisfy the Nernst equation for the $E_{MSH}$ in *M. smegmatis*:-

Where R = the gas constant (8.314 J $K^{-1}$ $mol^{-1}$); F = the Faraday constant (9.65 x $10^4$ coulombs $mol^{-1}$); n = number of electrons transferred (2) and T = the absolute temperature (310K) at which *M. smegmatis* was grown for the original $E'_{MSH}$ measurements.[20] The change in $E'_{MSH}$ is pH dependent: if the pH is increased by 1 unit at 37 $^{o}$C, this equates to -65.1 mV for a 2 electron, 2 proton thiol-disulfide redox process. [27]

$$E'_{MSH} = E^{o'}_{MSSM/MSH} - \frac{RT}{nF} . ln \frac{[MSH]^2}{[MSSM]} - (pH - 7.0). 65.1\ mV$$

[b] Values reported to two significant figures.

In summary, we report the fundamental biophysical properties (i.e. thiol p$K_a$ and standard redox potential) of MSH. Determination of the standard redox potential of MSH highlights discrepancies amongst the metabolite and biophysical measurements that are of relevance to MSH redox biochemistry. Amongst these, the technical challenges associated with the accurate determination of cellular MSH/MSSM ratios are likely to be the most significant. The significance of, and ways to further minimise artefacts in the methods used to measure parameters such as intracellular MSH/MSSM ratios clearly warrants further investigation.

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## Footnotes

† Although no MSH-dependent glyoxalases have yet been characterised, MSH null mutants display increased sensitivity to methylglyoxal[32] whilst strains of *C. glutamicum* engineered to produce 2-fold greater quantities of MSH also exhibit a 40% increase in tolerance of methylgloxal.[33]

†† This 4 mM MSH concentration is calculated from a measured MSH content of ~15 μmol/g of residual cell weight and a cellular water content of for *M. smegmatis* of 4 μL per mg of residual dry cell weight that are reported in this paper.[22]

**Keywords:** Mycothiol • redox potential • thiol p*K*a • LMW thiols

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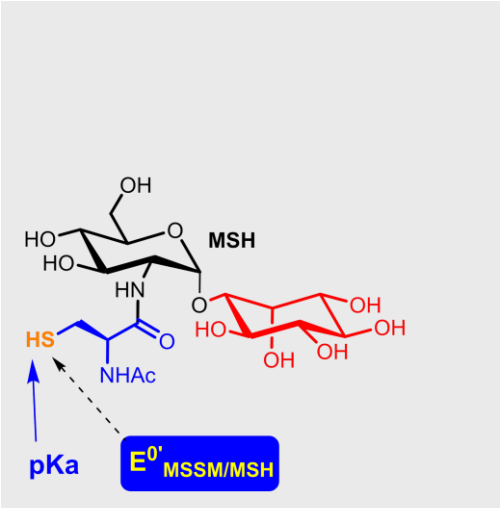
Layout 1:

## COMMUNICATION

**The Bare Essentials.** The thiol pKa and standard thiol/disulfide redox potential for mycothiol are measured and discussed

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