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Ab initio studies of the properties of intracellular thiols ergothioneine and ovothiol

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Abstract—Intracellular naturally occurring aromatic thiols such as ergothioneine and the ovothiols have been shown to play a variety of roles in cellular function. A detailed ab initio electronic structure analysis of these thiols is reported evaluating the thermodynamics of the reactions of these intracellular thiols with alkyl thiols, $HO^{\cdot}$, H_2O_2, ascorbate and their disulfides.

Intracellular thiols are essential for living systems: they are responsible for the maintenance of cellular redox homeostasis and can defend the cell from reactive oxygen and nitrogen species. The best characterized of these thiols is glutathione (γ-Glu-Cys-Gly, GSH), which is the major thiol in eukaryotes, as well as certain bacteria including *Escherichia coli*. GSH belongs to a class of cysteine-based thiols, which includes trypanothione (TSH),[1] found in *Trypanosoma* and *Leishmania*, and mycothiol,[2] found in the *Actinomycetales* bacteria. A second class of intracellular thiols exists, based on histidine, which consists of ergothioneine (ESH, Fig. 1 (**1**)) and the ovothiols ($OSH_{A–C}$, Fig. 2 (**3A**–**C**)).

ESH has been detected in plants, animals, fungi and certain bacteria at millimolar concentrations.[3,4] The biosynthesis of ESH has only been reported in certain fungi[5–8] and the *Actinomycetales* bacteria.[9,10] In animals, including humans, ESH has been found in tissues subject to oxidative stress including the liver, kidneys, red blood cells, seminal fluid and the ocular lens at millimolar concentrations[3,4] and in the brain at micromolar amounts.[11] Unlike cysteine-based thiols, ESH can exist in several tautomeric forms with the thione form (**1A**) predominating at physiological pH. ESH may be important in protection against reactive oxygen species. ESH scavenges the hydroxide radical ($HO^{\cdot}$) in a biologically relevant manner,[12] protects against protein inactivation by HOCl[12] and has been shown to prevent the formation of $^1O_2$ by photosensitizers.[13,14] ESH induces *S*-nitrosoglutathione decomposition faster than GSH and may be responsible for the high rate of *S*-nitrosoglutathione decomposition in whole blood, the liver and kidneys.[15] ESH may assist in the treatment of chronic inflammatory lung diseases such as asthma[16] and the administration of ESH can protect against peroxidation of the kidney and liver.[17] ESH decreases the rate of embryo malformations in diabetic pregnant rats[18] and protects against retinal neuron cell loss caused by over stimulation of the *N*-methyl-D-aspartate subtype of glutamate receptors.[19]

Unlike ESH, the OSHs have a sulfur atom at C^4 of the imidazole ring. The OSH thiols, OSH_A, OSH_B and OSH_C, differ in methylation at the α-amino group (**3A**–**C**).[20] OSHs have been identified in a number of echinoderms and marine invertebrates.[20–24] In sea urchins it is believed that OSH_C is responsible for protection against the oxidative burst caused by fertilization: OSH_C replaces GSH peroxidase as a scavenger of H_2O_2.[25,26] As oxygen centered radicals have been implicated in a number of disease processes,[27–29] there is intense interest in the development of mimetics of GSH peroxidase[30–33] and the development of OSH derivatives to act as medicinally useful antioxidants has been reported.[34,35]

OSH_A has also been detected in the medically relevant genera *Leishmania*[36,37] and *Trypanosoma*,[37] which include the parasites responsible for Chagas disease,[38] African sleeping sickness[39] and leishmaniasis.[40] OSH_A

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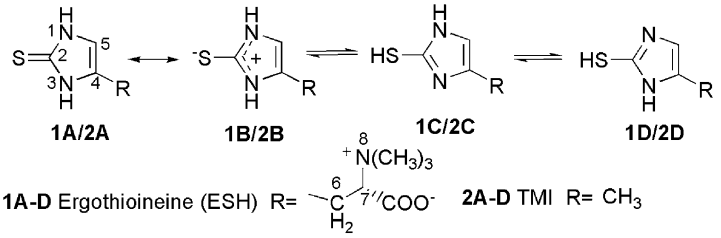


Figure 1. Tautomeric structures of ergothioneine and 2-thiol-4-methyl-imidazole.

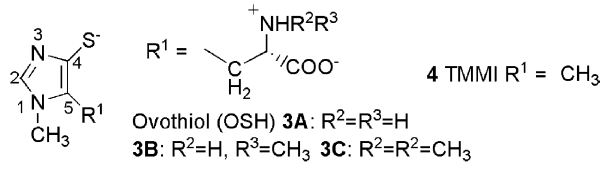


Figure 2. Ovothiols A–C and 4-thio-N^1-methyl-5-methylimidazole.

may be essential for the detoxification of the reactive nitrogen species used by the host in defense against parasitic infection.[41] Detoxification appears to be the major role of these histidine-based thiols, making them biologi cally very important. An ab initio study was undertaken to provide additional insight into their critical biological properties, an aspect not previously explored in detail.

A survey of the importance of basis set, as well as electron correlation in correct modelling of ESH was undertaken. A preliminary scan of geometry optimizations of ESH utilizing RHF, B3LYP and B3PW91 at the 3-21G, 6-31G, 6-311G, cc-pVDZ and cc-pVTZ(-f) levels with the absence/presence of additional diffuse and polarization functions was undertaken in both vacuo and water.[42] As a benchmark for these calculations, we utilized the X-ray structure of ESH obtained from crystals grown from water/ethanol.[42,43] These crystals belong to the $P2_12_12_1$ space group. The structure revealed C^5–C^4–C^6–C^7 and C^4–C^6–C^7–N^8 dihedral angles of −98.7° and 179.9°, respectively. The N^1–C^2–N^3 bond angle was found to be 105.5°. The N^1–C^2 and C^2–N^3 bond lengths were determined to be 1.35 Å. A C^2–S bond length of 1.69 Å was determined, which is intermediate to the C–S single and C–S double bond lengths of 1.56 and 1.82 Å, respectively, indicating the presence of a thione moiety (**1A**). This is in keeping with Raman and ^{13}C spectral data,[44] which indicates that ESH exists predominately in the thione form under physiological conditions, as well as with the crystal structure previously obtained with ESH crystals grown from only water (C^2–S = 1.69 Å).[45]

Based on the values of the C^2–S bond distances and the two dihedral angles mentioned above, increasing basis set size was important in matching the experimental structure data. It was observed that the B3LYP/6-311++G(d,p) basis set successfully predicted the structural characteristics of the crystallized ESH and hence was used as the computational method for this study and all subsequent calculations discussed herein are performed at this level.

Detailed electronic structure calculations of the various tautomers of 2-thiol-4-methylimidazole (TMI, (**2A**–**D**)), an ESH model compound, its disulfide (TMI-S)$_2$, OSH_A, OSH model compound 4-thiol-N^1-methyl-5-methylimidazole (TMMI (**4**)) and its disulfide (TMMI-S)$_2$ were performed in vacuo and in water using the IEFPCM solvation model.[42,46] TMI, TMMI and their respective disulfides were used to simplify the calculations by reducing the conformational variability of the α-amino acid portion of ESH and the OSHs. It has been shown that the amino acid portion of ESH does not affect its chemical reactivity.[47]

Calculations on TMI are in agreement with the ESH crystal structure yielding C^2–S bond lengths of 1.67 Å (gas) and 1.71 Å (water). In both vacuo and water, the thione form **2A** is more stable than structures **2C** and **D** by a minimum of −7.71 kcal/mol at the B3LYP/6-311++G(d,p) level. Since the pKa of ESH had been determined to be 11.5[44] the percentage of thiolate present would be expected to be low (0.003%) at pH 7. Nevertheless, evaluation of the C^2–S bond distances of the thiolate forms of **2C** and **D** in gas/water give 1.73 Å/1.75 Å and N^1–C^2–N^3 bond angles of 108.4°/109.5° (**2C**) and 108.5/109.4° (**2D**), indicating little contribution of a C=S to the structure of deprotonated **2C** and **2B**.

TMMI has two protonatable atoms: N^3 and S, with pKas of 10.3 and 2.6, respectively.[48] At pH 7 the thiolate zwitterionic form would predominate (thiol form contributing 0.002%); however, it is believed that the thiolate anion is the primary reducing agent rather than the zwitterion.[48] Calculations on the anion (gas/water) predict a C^4–S bond distance of 1.75 Å/1.77 Å and the N^1–C^2–N^3 angle (112.6°/111.9°). This indicates the existence of substantial thiolate forms both in vacuo and in water. There is no thiocarbonyl equivalent that is relevant to the chemistry of the OSHs. It is likely that the formation of a disulfide would alter the pKa of N^3, decreasing it close to that of imidazole, mirroring that seen with *S*-methyl-TMMI,[48] therefore the OSH disulfide (OSSO) was modelled as a neutral molecule (TMMI-S)$_2$.

During their biological function these intracellular thiols will be oxidized to their corresponding disulfide forms,[15,26,47,49] yet there has been no evidence for the existence of enzymes catalyzing their reduction back to their free thiol forms. It has been suggested that GSH and TSH may serve as the reductants for these reactions.[25,37,50] The disulfide of ESH (ESSE) has been

found to be unstable under physiological conditions[50] although it has been detected as a transient species.[47] To obtain an estimate of the free energy of the oxidation of OSH and ESH, calculations on the isodesmic reactions using CH_3SH as a model for GSH were undertaken.

$$RSSR + 2\,CH_3SH \rightarrow 2\,RSH + H_3CSSCH_3 \quad (1)$$

The net free energy of the reduction of ESSE via Eq. 1 where RSH is TMI_{thione} was determined to be between −13.12 and −16.43 kcal/mol in the gas phase and −30.97 kcal/mol in water.[42] This indicates that ESSE reduction is most likely a thermodynamically favourable reaction in the presence of millimolar concentrations of GSH and ESH in the cell. An interesting aspect to this calculation is how this compares with other aromatic thiols. The overall free energy change of Eq. 1 with diphenyldisulfide producing thiophenol (gas) was calculated to be −1.80 kcal/mol, a thermodynamically less favourable reaction. To determine what additional feature in the structure of ESH produces such a substantially different overall thermodynamic reaction, Eq. 1 was broken down into its component steps (Eqs. 2 and 3).

$$(TMI\text{-}S)_2 + 2\,CH_3SH \rightarrow 2\,TMI_{thiol} + H_3CSSCH_3 \quad (2)$$

$$2\,TMI_{thiol} \rightarrow 2\,TMI_{thione} \quad (3)$$

The overall free energies for Eq. 2 were determined to be between −1 and 3.15 kcal/mol (gas) and −3.7 and 3.47 kcal/mol (aqueous) depending on the protonation states of $(TMI\text{-}S)_2$ and TMI_{thiol}.[42] Interestingly, the overall free energy for Eq. 3 was determined to be between −15.43 and −17.11 kcal/mol in the gas phase and between −26.53 and −27.06 kcal/mol in aqueous phase.[42] As shown, the first step of the reaction is comparable in magnitude to that for other aromatic disulfides that are reduced by alkyl thiols. However, the major contribution to the free energy change in Eq. 1 appears to be due to the tautomerization step of the thiol form of ESH to its thione form. This is reminiscent of the thermodynamics of the hydrolysis of phosphoenolpyruvate to pyruvate and phosphate wherein the free energy associated with tautomerization of enolpyruvate to pyruvate (−6.9 kcal/mol[51]) is a major contributor to the overall free energy of that reaction.

The overall reaction of OSSO with GSH yields OSH and GSSG (modelled by TMMI and CH_3SH (Eq. 1)) and is favourable for the formation of the zwitterion of TMMI (−6.40 kcal/mol, aqueous).[42] The increased stability of OSSO, predicted by calculation, is consistent with the identification of its disulfide in vivo. It is clear that under identical conditions ESSE would be less thermodynamically stable than OSSO.

OSH is believed to be oxidized to OSSO during the detoxification of H_2O_2 formed during sea urchin egg fertilization, where substantial amounts of H_2O_2 are used for cell wall polymerization.[25] In trypanosomatids OSH and TSH have been shown to contribute to the reduction of H_2O_2.[37] Comparison calculations of the reactions of CH_3SH, TMI and TMMI with H_2O_2 (Eq. 4) indicate that the reaction of ESH with H_2O_2 will likely be less thermodynamically favourable. In agreement with OSH experimental data, it was found that the thiolate of TMMI had the most favourable reaction with H_2O_2 (−431.69 kcal/mol, gas), which was 5–9-fold greater than reactions with TMI and CH_3SH. All three thiols studied show favourable free energies of reaction with H_2O_2 and have been shown experimentally to react with H_2O_2.[50]

$$2\,RSH + H_2O_2 \rightarrow RSSR + 2\,H_2O \quad (4)$$

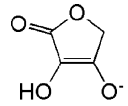
$$RSH + HO^{\cdot} \rightarrow RS^{\cdot} + H_2O \quad (5)$$

$$RS^{-} + HO^{\cdot} + H_3O^{+} \rightarrow RS^{\cdot} + 2\,H_2O \quad (6)$$

$$2\,RSH + 2\,HO^{\cdot} \rightarrow RSSR + 2\,H_2O \quad (7)$$

$$RS^{\cdot} + \mathbf{5} \rightarrow RS^{-} + \mathbf{6} \quad (8)$$

The $HO^{\cdot}$ radical has been shown to be extremely reactive to cellular components.[29] The overall reactions for CH_3SH, TMI and TMMI with $HO^{\cdot}$ have been calculated and the results indicate that all three intracellular thiols are likely to be thermodynamically capable of decomposition of the $HO^{\cdot}$ and could contribute to detoxification of $HO^{\cdot}$ (Eqs. 5–7).[42] Analysis of the radical TMI produced by the above reactions indicate that in vacuo spin density is localized over the S, C^5 and N^3 atoms in the radical of **2C** and over the S, C^4 and N^1 atoms in the radical of **2D**, while in water the spin density on the nitrogen atoms decreases, leaving most of the spin density on C^5 and S in the radical of **2C**, and C^4 and S in the radical of **2D**.[42] In the case of TMMI, in both the in vacuo and water phases, most of the spin density is found on C^5 and S. This data is in qualitative agreement with previous AMI calculations for OSH derivatives.[35]



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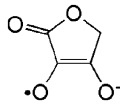

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Ascorbic acid has been shown to be an important protecting agent to reactive oxygen species.[29] Ascorbate and the ascorbate radical were modelled using **5** and **6**. The reduction of the thiyl radicals of TMI, TMMI and CH_3SH with the formation of the ascorbate radical was thermodynamically favourable, indicating that ascorbate may play a role in returning ESH, OSH and GSH to their reduced, biologically active form.[42]

In conclusion, a computational approach has explored the thermodynamics of several of the important reactions that ESH and OSH may undergo in the cell. A key aspect of this work is the indication that the thermo-

dynamics of ESH disulfide reduction is affected in a major fashion by the conversion of the thiol to the lower energy thione serving to drive the overall thermodynamics of the reaction. The theoretical calculations presented in the present work should contribute to our further understanding of the fundamental chemistry and biochemistry of these important intracellular thiols found in biological systems.

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Supplementary data

Additional information including tables and spin density figures can be found in the online version. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.01.014.

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