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# Expanding the paradigm of thiol redox in the thermophilic root of life

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ABSTRACT

*Background:* The current paradigm of intracellular redox chemistry maintains that cells establish a reducing environment maintained by a pool of small molecule and protein thiol to protect against oxidative damage. This strategy is conserved in mesophilic organisms from all domains of life, but has been confounded in thermophilic organisms where evidence suggests that intracellular proteins have abundant disulfides.
*Methods:* Chemical labeling and 2-dimensional gel electrophoresis were used to capture disulfide bonding in the proteome of the model thermophile *Sulfolobus solfataricus*. The redox poise of the metabolome was characterized using both chemical labeling and untargeted liquid chromatography mass spectrometry. Gene annotation was undertaken using support vector machine based pattern recognition.
*Results:* Proteomic analysis indicated the intracellular protein thiol of *S. solfataricus* was primarily in the disulfide form. Metabolic characterization revealed a lack of reduced small molecule thiol. Glutathione was found primarily in the oxidized state (GSSG), at relatively low concentration. Combined with genetic analysis, this evidence shows that pathways for synthesis of glutathione do exist in the archaeal domain.
*Conclusions:* In observed thermophilic organisms, thiol abundance and redox poise suggest that this system is not directly utilized for protection against oxidative damage. Instead, a more oxidized intracellular environment promotes disulfide bonding, a critical adaptation for protein thermostability.
*General significance:* Based on the placement of thermophilic archaea close to the last universal common ancestor in rRNA phylogenies, we hypothesize that thiol-based redox systems are derived from metabolic pathways originally tasked with promoting protein stability.



## 1. Introduction

Intracellular oxidation–reduction potential must be carefully balanced to support oxidative metabolic processes, while protecting from detrimental effects of free radical damage. It is generally accepted that the cytoplasm is kept in a reducing (electron rich) state, with small variations between different cell types [1]. The cytoplasmic redox potential is typically quantified by measuring relative amounts of protein disulfide (PSSP) to thiol (PSH) in equilibrium with compounds of known redox potential, such as oxidized (GSSG) and reduced glutathione (GSH) [2–5]. In the cytoplasm of eukaryotic and most gram negative prokaryotes, GSH and PSH are the predominant forms and exist at significant concentrations [5–7]. Whereas, GSSG and PSSP are primarily present in more oxidizing environments, such as extracellular space, mitochondria [4,8–10] or as catalytic intermediates.

In contrast, structural and genomic evidence suggests thermophiles and their viruses favor higher ratios of PSSP to PSH. The abundance of PSSP is proposed to be an adaptation that increases protein stability at elevated temperatures [11,12]. The genomic analyses show that this adaptation is exclusive to thermophilic organisms, with the highest proposed PSSP/PSH ratios observed in the hyperthermophilic Crenarchaea [12–17]. *Sulfolobus solfataricus* is a hyperthermophilic Crenarchaeon living optimally at 80 °C and a pH of 3, and has been adopted as a model for study of high temperature adaptation [18–24]. Previous studies of *S. solfataricus* and phylogenetically related thermophiles failed to find glutathione [25,26], raising the question, how do these organisms regulate intracellular redox and protein disulfide formation? Organisms without glutathione typically have an alternative small molecule thiol fulfilling this role [6,25–27]; however both proteomic and genomic evidence have failed to provide evidence of common thiol-biosynthesis pathways in *S. solfataricus* and its thermophilic relatives [14,25].

The maintenance of thiol redox homeostasis is fundamentally important to an organism's ability to protect against oxidative damage. PSSP/PSH and GSSG/GSH ratios are often used as a proxy for estimating intracellular redox state and tracking changes [2–5]. *S. Solfataricus*

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utilizes a novel enzyme system for regulating oxidative stress [22,28], but no evidence supports the role of intracellular thiol in this process. Herein we provide evidence that both metabolites and proteins of *S. solfataricus* are tuned to operate in a noncanonical redox environment. We find that *S. solfataricus* does utilize glutathione, although primarily in the oxidized form. Because the balance of oxidized to reduced protein and small molecule thiol is tilted toward oxidized PSSP and GSSG, this strongly suggests a more oxidized intracellular environment. Together these results characterize a phenotype which lies outside the traditional paradigm, and provides an expanded model of redox homeostasis in thermophilic organisms.

## 2. Materials and methods

### 2.1. Growth of microorganisms

*S. solfataricus P2* (ATCC) was grown aerobically in liquid DSMZ media 182 (22.78 mM $KH_2PO_4$, 18.90 mM $(NH_4)_2SO_4$, 0.81 mM $MgSO_4$, 1.7 mM $CaCl_2$, 0.2% Yeast Extract) (Media 1), or with the addition of 0.1% glucose as carbon source (Media 2), pH adjusted to 2.8 with 6 M $H_2SO_4$. Media was analyzed to ensure no glutathione or small molecule contaminant affected results. Batch cultures of *S. solfataricus* were grown with media in long neck Erlenmeyer flasks at 80 °C. One liter of media was inoculated with 10 ml of log phase ($OD_{650}$ 0.33) culture and divided evenly between ten, 1-liter long neck flasks. At ($OD_{650}$, 0.35), 50 ml of each 0.25 liter culture was removed and placed in a 50 ml falcon tube as growth controls. Cells were collected at exponential, late exponential and stationary growth phases for metabolite extraction. Liquid cultures of recombinant *Escherichia coli* (Strain: BL21-D3) were grown in Luria-Bertani media (1% Peptone, 0.5% Yeast Extract and 1% NaCl, pH = 7) in 1 l shaker flasks at 37 °C. For oxidative stress experiment, $H_2O_2$ was administered to a final concentration of 30 μM (n = 10). One liter of media was inoculated with 10 ml of log phase ($OD_{650}$ 0.33) *S. solfataricus* culture and divided evenly between four, 1-liter long neck flasks. At ($OD_{650}$, 0.35), 50 ml of each 0.25 liter culture was removed and placed in a 50 ml falcon tube as stressed growth controls. An additional 50 ml aliquot was collected from each culture at 30 min post $H_2O_2$ inoculation and used for metabolite extraction.

### 2.2. Zdye maleimide probe for thiol labeling

Cells were lysed with combination of freeze/thaw cycles. Protein sample extracts were prepared from cell suspensions in ice cold, phosphate buffer solution (pH = 6.5, 1 mM EDTA). All buffers were degassed and kept on ice during extraction to help ensure lack of thiol/disulfide exchange. Proteins were purified and concentrated with 5-fold volume of cold acetone (−80 °C). Protein concentration was measured with RC/DC Protein Assay Kit (Bio-Rad). For each experiment, 50 μg of protein was used. Three replicates were reduced using 20 mM tributylphosphine (Sigma) for 20 min at room temperature, alongside three unreduced controls. Dye labeling was performed using a fluorescent Zdye (ZB-M LC-01-56, and BDR-III-172) coupled to a maleimide, in a method modified from [23]. Dye was added to a final concentration of 5 μM in PBS, pH 6.5, for 20 min at room temperature. Reactions were quenched with 5-fold excess acetone (−80 °C). Samples were centrifuged at 15,000 ×g and the protein pellet was resuspended in 2D gel-loading buffer containing 40 mM DTT. Total protein internal standards were labeled with CyDyes using minimal labeling methods according to the manufacturer's protocol (GE Healthcare). 50 μg of protein extract was labeled separately at 0 °C in the dark for 30 min with 400 pmoles of N-hydroxysuccinimide esters of cyanine dyes (Cy5) dissolved in 99.8% DMF (Sigma). Labeling reactions were quenched by the addition of 1 μl of a 10 mM L-lysine solution (Sigma) and left on ice for 10 min. Cy5, reduced and unreduced Zdye samples (ZB-M LC-01-56) were combined appropriately and mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS). 2-DE was performed as described elsewhere [23] using precast IPG strips (pH 3–11 NL, 24 cm length; GE Healthcare) in the first dimension (IEF). Labeled samples were combined with a maximum of 450 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer pH = 3–11 NL, 40 mM DTT, and a trace of bromophenol blue) and loaded onto IPG strips. 150 μg of protein was loaded onto each strip and IEF was carried out with the IPGPhor II (GE Healthcare). Focusing was carried out at 20 °C with a maximum of 50 μA/strip. Active rehydration was achieved by applying 50 V for 12 h. This was followed by a stepwise progression of 500 V for 500 Vh, gradient ramp from 500 to 1000 V for 1 h, gradient ramp from 3000 to 5000 V for 1 h, gradient ramp from 5000 to 8000 V for 1 h, the 8000 V constant for 44,000 Vh. After IEF separation, the strips were equilibrated twice for 15 min with 50 mM Tris–HCl, 6 M Urea, 30% glycerol, 2% SDS and a trace of bromophenol blue, pH 8.8). The first equilibration solution contained 65 mM DTT, and the second 150 mM iodoacetamide. The strips were sealed on the top of the gels using a sealing solution (0.75% agarose in SDS-Tris–HCl buffer). The second-dimension SDS-PAGE was performed in a Dalt II (GE Healthcare), using 1 mm thick, 24-cm, 13% polyacrylamide gels, and electrophoresis were carried out at a constant current (45 min at 2 W/gel, then at 1 W/gel for ~16 h at 25 °C). 2D gels were scanned on a Typhoon Trio Imager according to manufacturer's protocol (GE Healthcare) at 100 μm resolution with excitation wavelength of 532 nm with a long pass emission wavelength of 570 nm and 488 nm with 520 nm emission for Z-Dye, and 633 nm with long pass emission of 670 nm for Cy5. The number of visible protein spots was counted for direct comparison; fluorescent measurements and image processing was done using ImageJ [29].

### 2.3. Metabolite extraction

A survey of the *S. solfataricus* metabolome was done by extracting metabolites using 60% aqueous (v/v) EtOH, 50% aqueous (v/v) MeOH or MeOH/Chloroform (1 mM EDTA) solvents in an effort to ensure global coverage. Chloroform methanol extraction was a modification from [30]. Briefly, cell pellets were resuspended in 1.5 ml of cold MeOH and transferred to glass tubes, after which 1.5 ml of cold chloroform was added. All samples were then shaken at 0 °C for 2 h using a custom made orbital shaker. The samples were then centrifuged (2000 ×g, 15 min, −9 °C). Upper MeOH/chloroform layer was removed and 50% aqueous MeOH (v/v) was added to cellular debris and vortexed for 30 s. After centrifugation the upper phase was pooled with the first extracts. Proteins were precipitated using 5:1 dilution with −80 °C acetone and centrifuged. Upper phase was collected and subsequently dried and resuspended in 50% MeOH. MeOH/$H_2O$ and EtOH/$H_2O$ extraction was a modification from [31]. Briefly each sample was resuspended in 1.5 ml 60% aqueous EtOH or 50% MeOH (v/v) respectively, 1 mM EDTA, vortexed for 30s, and sample was frozen in liquid nitrogen and thawed to room temperature three times. Samples were subsequently sonicated 60% duty cycle maximum power level for 5 min on ice. The sample was incubated 1 h at −20 °C followed by a 15 min centrifugation at 13,000 ×g and supernatant was pooled. Cell debris was subsequently washed with 1 ml extraction solvent, vortexed for 30 s and centrifuged. Proteins were precipitated using 5:1 dilution with −80C acetone and centrifuged. Upper phase was collected and subsequently dried and resuspended in 50% MeOH. *Ignicoccus hospitalis* and *E. coli* metabolites were extracted using identical 50% MeOH method.

### 2.4. LCMS based metabolome analysis

Analyses were performed using a 1290 UPLC coupled to a 6538 UHD Accurate-Mass Q-TOF (Agilent Technologies). The system was operated in positive and negative electrospray ionization modes. Vials containing extracted metabolites and standard mixtures were kept at −80 °C prior to LCMS analysis. Metabolites were separated using a reverse-phase Kinetix 1.7 μm C18, 100A, 150 mm × 2.1 mm (Reverse Phase), or a Cogent Diamond Hydride, 150 mm (HILIC). In positive ionization mode, A = 0.1% formic acid in water B = 0.1% formic acid in acetonitrile. For



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