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Sulfur-dependent microbial lifestyles: deceptively flexible roles for biochemically versatile enzymes

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Abstract

A wide group of microbes are able to "make a living" on Earth by basing their energetic metabolism on inorganic sulfur compounds. Because of their range of stable redox states, sulfur and inorganic sulfur compounds can be utilized as either oxidants or reductants in a diverse array of energy-conserving reactions. In this review the major enzymes and basic chemistry of sulfur-based respiration and chemolithotrophy are outlined. The reversibility and versatility of these enzymes, however, means that they can often be used in multiple ways, and several cases are discussed in which enzymes which are considered to be hallmarks of a particular respiratory or lithotrophic process have been found to be used in other, often opposing, metabolic processes. These results emphasize the importance of taking into account the geochemistry, biochemistry and microbiology of an organism and/or environment when trying to interpret the function of a particular sulfur-dependent redox enzyme.

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> " . . . the sulfur in these organisms is the sole respiratory source, and in that sense plays the same role as that of carbohydrate in other organisms . . . [1] . . . A complete synthesis of organic material by the action of living organisms has been accomplished on our planet independent of solar energy [2]."
>
> Sergei Winogradsky

Introduction

In 1887, the incomparable Winogradsky noted that cells of the (relatively) large bacterium *Beggiatoa* utilized granules of sulfur in a manner similar to the utilization of starch granules by yeast [1]. In a series of elegant experiments originally designed to confirm the idea that individual species of bacteria existed that exhibited defined characteristics (known as *monomorphism*) Winogradsky not only provided support that the microbial community was made up of a diverse array of defined species, he also demonstrated the first known case of chemolithotrophy, at the same time establishing the fields of geomicrobiology and microbial ecology [3]. The importance of the microbes and enzymes capable of sulfur-based chemolithoautotrophy and photoautotrophy (using sulfur compounds as energy and/or electron sources, respectively, in the oxidative direction) and sulfur-based respiration (in the reductive direction) on the formation and maintenance of the biosphere and geosphere is only beginning to be understood [4]. The biochemical foundations for these reactions are reasonably well understood, and monoculture and community culture techniques have been used to characterize microbes responsible for the reactions.

Sulfur-dependent microbial lifestyle

With the exponential development of molecular techniques and cataloging of enzymes and pathways (in databases such as KEGG [5], etc.), it has become practically possible to detect many or most of the genes, and, therefore, potential enzymes and pathways/microbial functions, present in an environmental sample (e.g. Ref. [6]). Through further analysis, it is possible to assign those genes to organisms, and even to assemble genomes of organisms present (e.g. Refs. [7•,8,9•,10]). The result of this is that it now appears technically possible to determine the biochemical pathways present in an environmental sample, and, therefore, what types of sulfur chemistry will be performed by individual microbes or communities of organisms. Many caveats should be considered when trying to infer which functional pathways and metabolisms are present in an organism or environment through these types of analyses. One of the most significant considerations is that many of the enzymes that are considered to be hallmarks of certain pathways are often functional in either the reverse direction or in other pathways. As an example, the *dsrAB* genes, which are usually considered to be the hallmark of sulfate-respiring microbes, can be used in the reverse direction, and are often found in non-sulfate-respiring organisms, as discussed below. This means that the presence of genes in a genome or metagenome is not a guarantee that a certain microbial metabolism predominates, or is even

significantly present, in a given sample. The observation also emphasizes that the finding of genes, transcripts or proteins in a sample should always be considered in the context of the other metabolites and substrates, proteins, genes and microbes present in a sample, as well the geo or biochemical context of the sample. While the range of '-omic' techniques and systems biology are complementing our chemical understanding of sulfur-based chemolithoautotrophy and respiration, careful enzymology, geochemistry and microbiology are still necessary to provide proof of the systems in operation in microbes and the environment (e.g. see Ref. [11]).

This review will focus on sulfur cycling in the environment via dissimilatory processes and chemolithoautotrophy, emphasizing cases in which enzymes normally considered to be 'hallmarks' for a given inorganic sulfur transformation that have been observed to catalyze other processes. While the focus on this review is somewhat 'gene-heavy,' the reason for the difficulty in assigning function discussed here is a very chemical one: the reversibility of many of the redox reactions involved, as well as the intersections of the chemistry of dissimilatory (i.e. energy conserving), assimilatory (for cell building), detoxification and the somewhat nebulous redox balancing reactions in the cell means that the same reaction can serve many different functions. When combined with the modular nature of the catalysts that nature has provided [12], the biochemistry becomes quite difficult to disentangle.

Sulfur-based chemolithoautotrophy

Biochemical pathways for sulfur-dependent chemolithoautotrophy (essentially, 'eating' sulfide or sulfur) are highly diverse, in terms of phylogeny, subcellular location of enzymes and intermediates, and the intermediates involved, making a brief review of the subject difficult [13]. As a generalization, the process involves the oxidation of sulfide or elemental sulfur to sulfate (S^{2-} or $S^0 \rightarrow {SO_4}^{2-}$), an exergonic process that allows for carbon fixation under the right conditions [14,15]. The predominant genes that allow for autotrophic growth on sulfide are the genes of the *sox* clusters; however, in keeping with the theme of this article, the presence of *sox* genes is no guarantee an organism is capable of sulfur/-ide dependent chemolithoautotrophic metabolism, and sox genes are often used in the respiratory (reductive) direction [16].

The initial oxidation of S^{2-} to S^0 occurs via quinones and is catalyzed by the sulfide:quinone oxidoreductase (SQR). In contrast to the other enzymes discussed here, which are usually confined to the prokaryotes, and often to quite distinct lineages as well, SQR is nearly ubiquitous (the exception being plants). While the basic structure and chemical mechanisms for the SQRs, which catalyze the general reaction $S^{2-} + 2e^- \rightarrow S^0$, have been worked out [17,18], there is a diversity in SQR structure and reactivity, and on the basis of these differences, six different groups of the enzyme have been proposed [19,20]. In humans (and eukaryotes in general), it has been proposed to be involved in sulfide signaling processes and maintaining redox balance in the mitochondria, and has been proposed to play a similar role in some bacteria [21,22]. SQR is in the same large family as the Nsr/CoADR/Npsrs discussed below, and has an active site that utilizes both an FAD and a redox-active cysteine pair. The SQRs differ from other members of this family; however, in that they are periplasm-facing peripheral or integral membrane proteins, and they do not utilize NAD(P)H, and, depending on the reaction direction, use either a range of electron donors such as cytochrome c to transfer electrons to their bound quinone, or sulfide. From a geochemical perspective, SQR is considered to function *in vivo* in sulfide oxidation; however, there is transcriptomic evidence that it participates in the reductive direction as well [23••].

Sulfur-based respiration — sulfate reduction

In terms of the respiratory or reductive pathways of sulfur compounds, especially in terms of the magnitude of effect on the geosphere, the dissimilatory sulfate reduction pathway predominates (${SO_4}^{2-} + 8e^- \rightarrow S^{2-}$, via adenosine-5′-phosphosulfate (APS) and sulfite intermediates) [24]. As an example, it is responsible for half of the mineralization (i.e. conversion to inorganic carbon) that occurs at the sea floor [25]. The hallmark for sulfate reduction is generally considered to be the dsrAB sulfite reductase, although the transfer of a sulfane sulfur by DsrC is likely to be the rate-limiting step for the reaction in the cell [26], with a protein trisulfide linking DsrAB to DsrC [27,28]. The *dsrAB* genes code a tetrasiroheme-containing heterotetrameric ($\alpha_2\beta_2$) sulfite dehydrogenase (${SO_3}^{2-} + 6e^- \leftrightarrow H_2S + 3H_2O$), and while it is clear that dsrAB genes that code for the reductive or oxidative reactions are usually phylogenetically distinct [29], it was recently found that genes predicted to code for reductive DsrABs were implicated in sulfide oxidation [30••]. Other enzymes distinct from the DsrABs have also been found to catalyze the dissimilatory reduction of sulfite, such as the octaheme SirA from the sulfite (but not sulfate) reducing *Shewanella oneidensis* MR-1 [31].

As an example of the level of complexity at which this type of interchangability can occur, the organization of the genes for the Sat protein (which catalyzes the formation of APS from ${SO_4}^{2-}$ and ATP), the iron–sulfur cluster containing aprABs (which reduce APS to ${SO_3}^{2-}$) and the Fe–S, heme and FAD-containing membrane protein qmoABC (which transfers e^- from the aprAB to a quinol acceptor) are arranged nearly identically in the genomes of the sulfate-reducer *Desulfotomaculm reducens* and the sulfur oxidizers *Thiobacillus denitrificans* and *Chlorobium tepidum* [12,32,33]. Determination of S isotope fractionation during growth of two sulfate-reducing *Desulfovibrio*

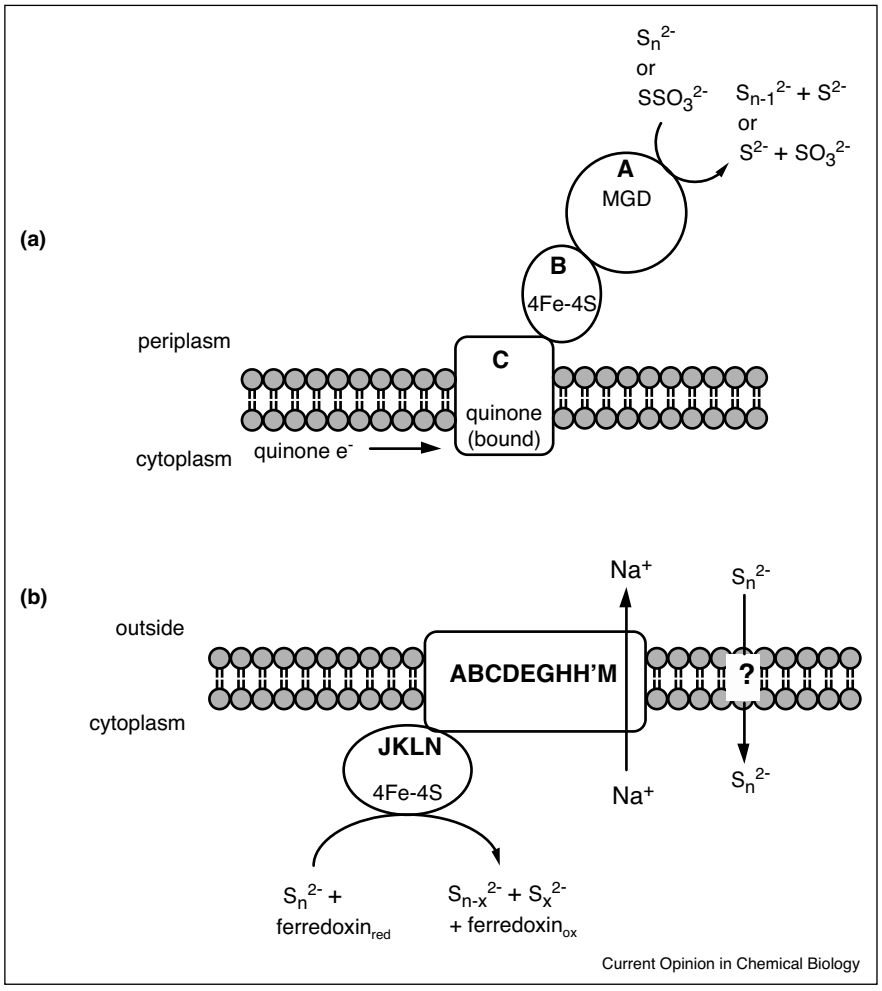
species showed widely different relationships between fractionation and sulfate concentration, a result which suggests that even closely related species may differ significantly in their sulfate transporting and reduction parameters [34••]. Observations such as this point to the difficulty in determining function from genomic and metagenomic data.

Sulfur-based respiration — S^0 reduction

While SO_4^{2-} reduction is the predominant mode of dissimilatory reduction of sulfur compounds, reduction of intermediates such as elemental sulfur, polysulfide and thiosulfate are also important and necessary for functioning of the sulfur cycle. Although controversial, substantial evidence exists that sulfur reduction was the earliest (or one of the earliest) forms of respiration [12,35], and the range of environments, in which sulfur reduction is important is constantly expanding [36]. Four main enzyme types are likely to be important for the apparently simple reduction of $S^0 \rightarrow S^{2-}$: 1) the molybdopterin-dependent trimeric integral membrane complexes, Phs or PsrABC [37,38], 2) The Coenzyme A, NAD(P)H, and FAD-dependent single-cysteine active site members of the larger glutathione reductase family [39], and 3) the FAD and Fe/S-containing heterodimeric sulfide dehydrogenase SudAB [39–41] (not discussed here), 4) and the very recently characterized membrane complex MBX of the *Thermococcales* [42••].

The first of these, the heterotrimeric membrane-bound molybdopterin-dependent Psr or PhsABC, is a member of a larger family of molybdopterin-containing

Figure 1



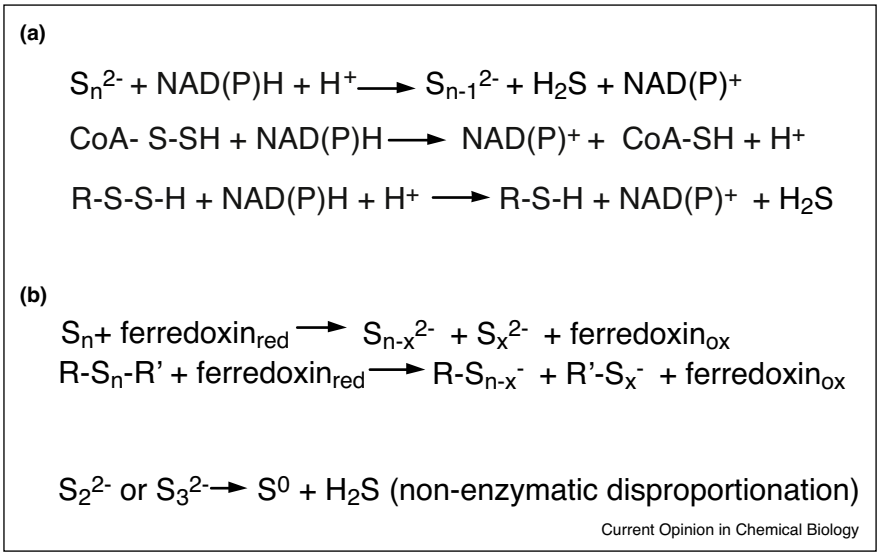
(a) Model for the PsrABC polysulfide reductase ($S_n^{2-} + 2e^- \rightarrow S_{n-1}^{2-} + S^{2-}$, where $n \geq 3$) which has also been shown to act as a thiosulfate reductase ($S\text{-}SO_3^- + 2e^- \rightarrow S^{2-} + SO_3^{2-}$) in some organisms. PsrABC is found throughout the bacterial and archaeal kingdoms. The active site cofactor on PsrA is molybdopterin guanine dinucleotide (MGD). **(b)** Proposed model for the membrane-bound sulfane reductase (MBS) of the *Thermococcales*. Wu *et al.* were able to purify the 13 subunits of holoenzyme complex as well as an active 4 subunits of a soluble cytoplasmic complex (JKLN) which contained 4Fe–4S clusters and the S-reducing active site. The mode of polysulfide transport is not known. Model adapted from Ref. [42••]. This enzyme appears to be limited to the order *Thermococcalles*, which includes *Thermococcus* and *Pyrococcus*.

oxidoreductases in the DMSO reductase family [38,43], and catalyzes the reduction of S^0 to S^{2-}, with the S^0 substrate most likely in the form of polysulfide (S_n^{2-}) (Figure 1a). While it is clear in the case of *Shewanella* that the PsrABC protein is responsible for both sulfur/polysulfide and thiosulfate reduction [44,45], it is less clear whether PsrABC enzymes are consistently multifunctional for thiosulfate and polysulfide [46]. There are also multiple examples of organisms containing PsrABC homologues that are not able to reduce elemental sulfur — *Archaeoglobus fulgidus*, for example, contains a PsrABC homolog [47], and while it is able to efficiently reduce thiosulfate (and sulfate), it is not able to use elemental sulfur as an electron acceptor [48], and its growth is actually inhibited by sulfur. *A. fulgidus* is probably the most extreme example of the difficulty of predicting sulfur-reducing ability — despite having homologs of the PsrABC, CoADR/Nsr and Npsr/Nsr-rhod (discussed below), it is unable to perform dissimilatory sulfur reduction.

The second family of enzymes is the single-cysteine containing flavoproteins in the PNDOR family, which differ from their relatives in the glutathione-reductase class of enzymes in that their active site contains only one cysteine, with a tightly bound Coenzyme A usually taking up the role of the other active site thiol. The Nsr [39] (also known as CoADR [49]) and the Npsr [50,51] (also known as CoADR-rhod [52]) both catalyze the reduction of polysulfide or poly or persulfide derivatives of thiols such as coenzyme A to S^{2-}, using NAD(P)H as an electron source (Figure 2a). The mechanisms of the two enzymes are very similar, except that in the case of the Npsr an additional rhodanase sulfur transfer domain is present, and the cysteine residue on that domain becomes essential for sulfur transfer [51]. While these enzymes are clearly capable of sulfur reduction, their presence in a genome is no guarantee of the ability to respire sulfur, as seen in the case of the lyme-disease causing *Borrelia burgdorferi*, which contains a CoADR important for growth and anti-oxidant functions that does not function in S^0 reduction [53,54], in *Enterococcus faecalis* [55], and as seen in the case of *A. fulgidus* above. In addition, knockout of the NSR from *Pyrococcus* does not appear to affect growth on S^0 [39]. Recent evidence suggests that it may be possible, in the case of the CoADRs, to make functional conclusions based on structural predictions [56].

The chemistry of sulfur reduction in the model hyperthermophile *Pyrococcus* has remained enigmatic, although a very recent result appears to have answered the question of which of the pyrococcal enzymes able to reduce S compounds *in vitro* is actually responsible for most of the *in vivo* activity. Previously, a range of enzymes, including the NSR, had been implicated as the enzyme responsible for S^0 reduction; however, while the enzyme is certainly capable of that chemistry, removal of that gene or those of other candidates (such as NfnI and II, previously thought to be sulfide dehydrogenases but now shown to be important in redox balancing [57]) did not slow growth on S^0, while an apparent membrane-bound oxidoreductase (MBS) was shown to be essential for S^0-enhanced growth [39]. Using very careful and elegant protein biochemistry, Wu *et al.* were able to show that this oxidoreductase, a

Figure 2



(a) Reactions catalyzed by the enzymes in the single-cysteine containing flavoprotein family (PNDORs), including NADH-dependent polysulfide reductase (Npsr) [51] and the NADH dependent sulfur reductase (NSR) [39], also referred to as CoADR [49]. **(b)** Proposed reactions catalyzed by the newly discovered ferredoxin-dependent sulfane reductase [42••], to date only found in the *Thermococcalles*.

13 subunit membrane protein (Figure 1b), which is homologous to (and likely arose from an operon duplication of) the MBH hydrogenase in these organisms, is able to reduce the sulfane sulfur of dimethyl trisulfide using reduced methyl viologen or ferredoxin as the reductant ($CH_3–S–S–S–CH_3 + MV_{red} \rightarrow MV_{ox} + CH_3–S–SH + CH_3–SH$) [42••]. Sulfide is not a product of the *in vitro* assay, instead, it is proposed that the enzyme reduces anionic or organo-polysulfides to smaller polysulfides, with disulfide and trisulfide nonenzymatically reacting to from S^0 and H_2S [42••] (Figure 2b). While the homologous MBH produces a proton gradient during H_2 formation [58•], the MBS is proposed to generate a Na^+ gradient during sulfur reduction (as shown in Figure 1b), and with stable, active MBS it appears quite likely that determination of the mechanism of ion gradient generation by MBS is within reach [42••].

Conclusions

While many or most of the enzymes important in the transformation of inorganic sulfur compounds have been identified, the reversibility and multiple roles of these enzymes in the reduction or oxidation of sulfur compounds can make it difficult to interpret their *in vivo* functions, even in cases where their *in vitro* biochemistry has been well characterized. This means that care should be taken when correlating the presence of these enzymes, or their activities, in genomes or environmental samples, and that confirmation via a combination of biochemical and microbiological techniques are necessary.

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While this structure is not of the membrane-bound sulfane reductase described in Ref. [42••], its homology to that enzyme means that it is able to provide insights into the mechanism of the sulfur-reducing enzyme.