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MINI-REVIEW

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# Thermodynamic aspects of energy conservation by chemolithotrophic sulfur bacteria in relation to the sulfur oxidation pathways

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**Abstract** The free-energy data on which assessments of the autotrophic growth efficiencies of chemolithotrophic bacteria are commonly based have been reevaluated and new values have been calculated. It has been concluded that many earlier calculations are in error and that many values previously reported in the literature are overestimates of efficiency. A problem posed by the chemolithotrophic sulfur-oxidizing bacteria is the elucidation of the mechanism by which elemental sulfur and the sulfane-sulfur (-S-) of the thionic acids are converted to sulfite. Even after decades of studies on sulfur oxidation by these bacteria, this problem has not been fully resolved although it is widely thought that conversion of sulfur to sulfite is brought about by an oxygenase. The biochemically feasible mechanisms by which sulfur and "sulfane" oxidation to sulfite might occur are reviewed. The possible insight afforded by chemical thermodynamics into the most likely mechanisms for oxidation to sulfate in relation to the efficiency of energy conservation is discussed. Energetic calculations and growth yield data indicate that the energy-yielding oxidation of sulfur and "sulfane" to sulfite, either coupled to energy-conserving electron transport or catalyzed by an oxygenase, could explain divergent growth yields among different sulfur-chemolithotrophs.

**Key words** Chemolithotrophic sulfur oxidation · Sulfur oxygenase · Energy conservation · Thermodynamics · Growth yields · *Thiobacillus* · Archaea

## Introduction

Three distinct but interrelated problems are discussed in this review. The first is a consideration of the energy requirement, in free-energy terms, for the reductive assimilation of carbon dioxide into cellular material; the second is a survey of the mechanisms of oxidation of sulfur and sulfane-sulfur to sulfate by chemolithotrophic bacteria; the third is the question of the efficiency with which energy from inorganic sulfur oxidations is linked to carbon dioxide reduction and growth. An aim of these considerations is to assess how the interrelation of these three topics provides insights into likely mechanisms of sulfur oxidation in different bacteria.

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## Assessing the free-energy requirement for carbon dioxide fixation

Virtually all estimates of growth efficiencies for chemolithotrophs in terms of the coupling of the free-energy available from substrate oxidation to the free-energy requirement for carbon dioxide fixation have used the $\Delta G^{\circ}$ for carbon dioxide fixation calculated by Baas Becking and Parks (1927); see also Kelly (1978, 1990). This was derived from the following equation, which is based on the equation describing fixation by oxygenic phototrophs:

$$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2 \quad (1)$$

The standard Gibbs free-energy requirement for this process (calculated using the $\Delta G_f^{\circ}$ values of Table 1) is +470.6 kJ $(mol\ CO_2)^{-1}$.

The principal process whereby $CO_2$ is fixed (to the level of fructose) in chemolithotrophic sulfur bacteria such as *Thiobacillus* is the Calvin cycle, the overall process of which can be summarized as follows:

$$6\ CO_2 + 18\ ATP + 12\ NADH + 12\ H^+ + 12\ H_2O \rightarrow C_6H_{12}O_6 + 18\ ADP + 12\ NAD^+ + 18P_i \quad (2)$$

where $P_i$ represents $H_3PO_4$.

The $\Delta G^{\circ\prime}$ value for $CO_2$ reduction to the level of hexose by this equation is given by Stryer (1988) as +114 kJ $(mol\ CO_2)^{-1}$ . This value is only one quarter of that for Eq. 1, because it describes the energetics of the actual process of $CO_2$ fixation rather than the overall process of $CO_2$ fix-

**Table 1** Values for Gibbs free energies of formation from the elements for the compounds for which free-energy changes in their reactions are described in Tables 4–6 or in the text [derived from or recalculated from Latimer (1952), Brasted (1961), Thauer et al. (1977), and Bard et al. (1985)]. Values in paranthesis were calculated by the group contribution method of Mavrovouniotis (1990, 1991) or calculated from the data of Mavrovouniotis (1990) (*ND* not determined)

| Compound | Gibbs free energy of formation ($\Delta G_f^\circ$ as kJ mol$^{-1}$) | |
|---|---|---|
| $S_2O_3^{2-}$ | –513.4 | (–501.7) |
| $S_3O_6^{2-}$ | –958.1 | (–944.3) |
| $S_4O_6^{2-}$ | –1,022.2 | (–959.8) |
| $H_2S_4O_6$ | –991.6 | (–1,039.1) |
| $S_5O_6^{2-}$ | –956.0 | (–920.1) |
| $S_6O_6^{2-}$ | ND[a] | (–935.5) |
| $S_7O_6^{2-}$ | ND[a] | (–895.8) |
| $S^{2-}$ | –85.8 | |
| $HS^-$ | –12.1 | |
| $H_2S$ | –27.6 | |
| $SO_3^{2-}$ | –486.6 | (–495.4) |
| $HSO_3^-$ | –527.8 | |
| $H_2SO_3$ | –537.9 | |
| $SO_4^{2-}$ | –744.6 | |
| $HSO_4^-$ | –756.0 | |
| $H_2SO_4$ | –742.0 | |
| COS | –169.2 | |
| $SCN^-$ | +88.7 | |
| HSCN | +92.7 | |
| $OCN^-$ | –98.7 | |
| HOCN | –120.9 | |
| $H_2O$ | –237.2 | (–236.8) |
| $OH^-$ | –157.3 | (–197.1) |
| $CO_2$ | –386.0 | |
| $NO_2^-$ | –37.2 | (–29.3) |
| $NO_3^-$ | –111.0 | (–115.1) |
| $NH_3$ | –26.6 | |
| $NH_4^+$ | –79.5 | (–75.7) |
| $NH_2OH$ | –23.4 | |
| $CN^-$ | +165.7 | |
| $C_6H_{12}O_6$ (fructose) | –915.4 | |

[a] Published data for polythionates with six or more S-atoms have not been found in the literature, so the $\Delta G_f^\circ$ values given above for hexa- and heptathionate ions are those calculated by the group contribution method of Mavrovouniotis (1990, 1991)

ation coupled to the photosynthetic photolysis of water. The higher value (Eq. 1) has, however, been very widely applied to non-photosynthetic autotrophs (Baas Becking and Parks 1927; Kelly 1990) but has recently been questioned as a valid approach to comparing growth efficiencies on substrates such as sulfide or thiosulfate. Nelson and Hagen (1995) have argued that Eq. 1 and the derived $\Delta G^\circ$ for $CO_2$ fixation is invalid, since it is "appropriate only for oxygenic photosynthesis", and that the calculation should regard sulfur compounds rather than water as the source of electrons for $CO_2$ reduction. Their argument for a lower $\Delta G^\circ$ for $CO_2$ fixation is derived from their equation (Nelson and Hagen 1995):

$$6\ CO_2 + 3\ S_2O_3^{2-} + 9\ H_2O \rightarrow C_6H_{12}O_6 + 6\ SO_4^{2-} + 6\ H^+ \quad (3)$$

From this equation, a $\Delta G^\circ$ for $CO_2$ fixation of +101.3 kJ (mol $CO_2$ fixed)$^{-1}$ can be calculated [using the data of Table 1, including the standard $\Delta G_f^\circ$ value for $H^+$ of zero, rather than the pH 7.0 value of –39.87 kJ mol$^{-1}$ (Thauer et al. 1977) used in Nelson and Hagen's (1995) calculation]. This equation is, of course, similar to those originally derived by Van Niel (1931) in his seminal paper on the photosynthesis of green and purple sulfur bacteria in which the formal equivalence of water and an alternative hydrogen donor (such as hydrogen sulfide) was considered:

$$CO_2 + 2\ H_2A \rightarrow (CH_2O) + H_2O + 2\ A \quad (4)$$

$$CO_2 + 2\ H_2O \rightarrow (CH_2O) + H_2O + O_2 \quad (5)$$

$$CO_2 + 2\ H_2S \rightarrow (CH_2O) + H_2O + 2\ S \quad (6)$$

The energetic implications of the possibility that the "coupling" of carbon dioxide fixation to sulfide oxidation (in chemolithotrophs) involves provision of reductant only from the oxidation of sulfide to sulfur (Eq. 6) is considered later.

Van Niel (1931) also considered the case (in purple sulfur bacterial photosynthesis) in which carbon dioxide fixation was coupled to the oxidation of sulfide to sulfate:

$$2\ CO_2 + H_2S + 2\ H_2O \rightarrow 2\ (CH_2O) + H_2SO_4 \quad (7)$$

While Eqs 1 and 3–7 adequately describe the overall stoichiometries of $CO_2$ fixation coupled to photolysis of water or photosynthetic or chemolithotrophic sulfur compound oxidation, they do not provide information about the nature of the biochemical link between the two processes (i.e. ATP and NADH, as in Eq. 2). It has also long been realized that the theoretical thermodynamic energy requirement shows an eightfold difference for $CO_2$ fixation by photosynthesis with water as the electron donor (Eq. 5) as compared to sulfide oxidation to sulfur as the electron donor (Eq. 6). Larsen (1954) has reported the free-energy values for the two processes as +115 kcal (mol $CO_2$)$^{-1}$ for Eq. 5 and +15 kcal (mol $CO_2$)$^{-1}$ for Eq. 6 [i.e. +481 kJ (mol $CO_2$)$^{-1}$ and +63 kJ (mol $CO_2$)$^{-1}$, respectively]. Larsen (1953) has also calculated the $\Delta G_{298}$ for Eq. 3 above as being +29.15 kcal (mol $CO_2$)$^{-1}$ [i.e. +122 kJ (mol $CO_2$)$^{-1}$], which is close to the currently accepted value.

It must be remembered, however, that there is no direct chemical coupling between sulfur compound oxidation and the process of $CO_2$ reduction: the former serves only to generate the NAD(P)H and ATP required for the carboxylation of ribulose bisphosphate and reduction to hexose. $CO_2$ reduction by the Calvin cycle (carbon oxidation state being taken from +4 to 0) in sulfur chemolithotrophs depends on electrons donated by sulfur oxidation (e.g. sulfide-sulfur being taken from a –2 to a +6 oxidation state). The sulfur oxidation process involves the introduction of oxygen from water into combination with the sulfur atoms (with the obvious exception of any direct sulfur oxygenation by molecular oxygen), and $H^+$ and electrons from the overall sulfur oxidation process are used to generate NAD(P)H for the reductive part of the Calvin cycle. Oxygen from water is thereby incorporated into the sulfate produced from substrate oxidation. Some caution is needed in

making deductions from overall free-energy changes for equations such as Eq. 7 since generalized equations for $CO_2$ fixation provide only a guide to the overall process and its energetics (Larsen 1954; Nelson and Hagen 1995), and using $\Delta G^\circ$ values (for both sulfur compound oxidation and $CO_2$ fixation) can only be an approximation of the free-energy values in the biological systems at circumneutral pH and reactant concentrations differing from physicochemical standard conditions. If one makes model calculations for other lithotrophic electron-donating substrates, using for example sulfide, ammonia, hydroxylamine or hydrogen oxidation as reductants, the apparent $\Delta G^\circ$ values for $CO_2$ fixation vary as a consequence of the differing redox potentials of the electron-donating couples (which may be multi-component for sulfur compound and ammomia oxidations; e.g. $HS^-/S^0$, $E_0' = -270$ mV; $S^0/HSO_3^-$, +50 mV; $HS^-/HSO_3^-$, –116 mV; $HSO_3^-/SO_4^{2-}$, –516 mV; $SO_3^{2-}/SO_4^{2-}$, –522 mV; $NH_4^+/NO_2^-$, +344 mV; $NH_4OH/NH_2OH$, +562 mV; $NH_2OH/NO_2^-$, +66 mV; $H_2/H^+$, –414 mV (Thauer et al. 1977; Kelly 1978, 1982; R. K. Thauer, Universität Marburg, Germany, personal communication):

$$6\ CO_2 + 3\ H_2S + 6\ H_2O \rightarrow C_6H_{12}O_6 + 3\ H_2SO_4 \quad [\Delta G^\circ = +113.4\ \text{kJ (mol } CO_2)^{-1}] \tag{8}$$

$$6\ CO_2 + 12\ H_2S \rightarrow C_6H_{12}O_6 + 6\ H_2O + 12S \quad [\Delta G^\circ = +51.4\ \text{kJ (mol } CO_2)^{-1}] \tag{9}$$

$$6\ CO_2 + 4\ NH_3 + 2\ H_2O \rightarrow C_6H_{12}O_6 + 4\ NO_2^- + 4\ H^+ \quad [\Delta G^\circ = +305.4\ \text{kJ (mol } CO_2)^{-1}] \tag{10}$$

$$6\ CO_2 + 6\ NH_2OH \rightarrow C_6H_{12}O_6 + 6\ NO_2^- + 6\ H^+ \quad [\Delta G^\circ = +219.6\ \text{kJ (mol } CO_2)^{-1}] \tag{11}$$

$$6\ CO_2 + 12\ H_2 \rightarrow C_6H_{12}O_6 + 6\ H_2O \quad [\Delta G^\circ = -3.8\ \text{kJ (mol } CO_2)^{-1}] \tag{12}$$

The variation between these values shows that this mode of calculation provides a value for the entire coupled process of substrate oxidation and $CO_2$ fixation, while the absolute $\Delta G^\circ$ for $CO_2$ fixation by the Calvin cycle itself will remain constant regardless of the respiratory substrate used as the source of energy and reductant (i.e. Eq. 2).

One further approach is to deduce the energy cost of the main energy-requiring steps of $CO_2$ fixation for the actual conversion catalyzed by the Calvin cycle:

$$CO_2 + \text{RuBP} + 2\ \text{NAD(P)H} + 2\ H^+ + 3\ \text{ATP} \rightarrow 2\ \text{PGA} + 2\ \text{NAD(P)}^+ + 3\ \text{ADP} + 3\ P_i \tag{13}$$

The energy requirements are indicated by the reductive conversion of carbon dioxide and ribulose bisphosphate (RuBP) to phosphoglycerate (PGA), the free-energy change for ATP hydrolysis, and the NADH/$NAD^+$ couple. Using the calculation methods of Mavrovouniotis (1990, 1991), the reaction of Eq. 13 was estimated to have an approximate $\Delta G^\circ$ value of +109 kJ $mol^{-1}$, which is essentially the same as the literature values presented above, including those for Eqs. 2, 3 and 8 for the overall processes of substrate oxidation and $CO_2$ fixation.

For the purposes of the comparative thermodynamic calculations presented in this review I have chosen to use the mean of the four $\Delta G^\circ$ values presented above for Eqs. 2, 3 and 8, namely +113 kJ $(\text{mol } CO_2)^{-1}$, which is only one quarter of the Eq. 1 value widely used in the literature in estimates of chemolithotrophic growth efficiencies. It is suggested that this value be used as a best approximation in calculations of efficiency of carbon dioxide fixation by chemolithotrophic bacteria growing autotrophically by means of the Calvin cycle. It must be recognized that comparing $\Delta G^\circ$ values for sulfur compound oxidation and $CO_2$ fixation, rather than values for actual intracellular pH and substrate and intermediate concentrations, can only be a guideline to efficiencies of energy conservation. Given the variation and uncertainties of actual intracellular (or periplasmic) conditions, it is, however, the device that has been used in most assessments of chemosynthetic efficiency, and it provides at least a broad indication of the relative magnitude of efficiency.

## Sulfur oxidation pathways in sulfur-chemolithotrophs

Chemolithotrophic sulfur bacteria obtain energy to support autotrophic or mixotrophic growth from the oxidation of elemental sulfur and the reduced inorganic and organic compounds of sulfur, including sulfide, thiosulfate, the polythionic acids, thiocyanate, carbon disulfide, carbonyl sulfide and methylated sulfur compounds (Kelly 1982, 1988). Much work has been done in attempts to elucidate the pathways of oxidation to sulfate and to establish the mechanisms and efficiency of the coupling of the energy released to the growth of the bacteria (Kelly 1990). A factor that impeded progress in solving these problems was the finding that, contrary to early expectations, two or more oxidation pathways exist, with the organisms exploiting these sources of energy being distributed through diverse genera of the proteobacteria and the extremely thermoacidophilic archaea. The best-known examples of sulfur chemolithotrophs are the thiobacilli, some *Paracoccus* and *Xanthobacter* species, *Sulfolobus*, *Acidianus* and *Desulfurolobus*. It is clear that the oxidation pathway and energy conservation mechanisms differ among these organisms. For example, differences exist between *Thiobacillus neapolitanus*, *Thiobacillus denitrificans*, and *Thiobacillus tepidarius* (which may have similar oxidation mechanisms, but differ in energy conservation) and *Paracoccus versutus* (with a different oxidation system), and *Acidianus* or *Desulfurolobus*. The archaeal examples may achieve the same oxidative outcomes as the thiobacilli, but by using possibly unrelated enzyme systems (Kelly 1982, 1988; Kelly et al. 1997).

Production of free elemental sulfur by thiobacilli is a well-known phenomenon recognized in the earliest studies of these organisms [see Kelly (1982) and Kelly et al. (1997) for reviews] and for which both nonbiological and enzyme-catalyzed origins have been proposed (Vishniac and Santer 1957; Trudinger 1964; Kelly 1982; Kelly et al. 1997; Visser et al. 1997). Some early observations suggested that a sulfur-producing metabolic process resulted in the production of equivalent amounts of sulfur and sulfate from thiosulfate [e.g. Nathansohn (1902); see Kelly

(1982)], a view that received support from Trudinger's (1964) demonstration of a possible four-sulfur intermediate of thiosulfate oxidation that gave rise to sulfur on disproportionation. More recently, a flavocytochrome-*c*-linked sulfide dehydrogenase has been shown in an organism similar to *T. neapolitanus* (Visser et al. 1997), confirming that sulfur production could be due to a specific enzyme.

The mechanism for the oxidation of elemental sulfur and thiosulfate commonly presented in reviews implicates a sulfur oxygenase (or "sulfur-oxidizing enzyme") that is postulated to be the key enzyme in the conversion of sulfur or the sulfane-sulfur (outer, -S-) atom of thiosulfate to sulfite in bacteria such as the *Thiobacillus* species, which use these materials as energy substrates for growth. With thiosulfate as substrate, some kind of cleavage is envisaged to produce sulfite and an intermediate formally equivalent to elemental sulfur, which is subsequently oxygenated (as for sulfur as a substrate) to sulfite:

$$[S - SO_3]^{2-} \rightarrow [S] + SO_3^{2-} \quad (14)$$

$$[S] + O_2 + H_2O \rightarrow H_2SO_3 \quad (15)$$

A problem with the assumption that an oxygenase-catalyzed reaction is the exclusive means of sulfite production is that there would seem to be no potential for conservation of metabolically useful energy from the conversion of $S_8$ to sulfite, in which the oxidation state of sulfur is shifted from 0 to +4, or from the production of sulfite from the sulfane-sulfur of thiosulfate, in which the oxidation state shifts from –1 to +4 (Vairavamurthy et al. 1993). As yet, no attempt appears to have been made to determine the contribution in vivo of a sulfur oxygenase by providing bacteria with $^{18}O$-labeled oxygen or water to determine the relative contributions of each to the oxygen recovered in the sulfate produced by oxidation of sulfur (and thiosulfate). It is possible that exchange reactions might obscure the involvement of any direct oxygenation.

## Sulfur oxygenase in *Thiobacillus* species

A specific sulfur-oxidizing enzyme was first demonstrated in several thiobacilli by Isamu Suzuki's group (Suzuki 1965, 1994; Charles and Suzuki 1966; Suzuki and Silver 1966). This enzyme activity was absolutely dependent on the presence of reduced glutathione, and catalyzed this reaction:

$$S_8 + 8\ O_2 + 8\ H_2O \rightarrow 8\ H_2SO_3 \quad (16)$$

Experimentally, the product observed was thiosulfate because of the chemical reaction of the sulfite product with the sulfur substrate:

$$8\ SO_3^{2-} + S_8 \rightarrow 8\ S_2O_3^{2-} \quad (17)$$

The essentiality of reduced glutathione ($GS^-$) in the oxidation of sulfur and the sulfane-sulfur of thiosulfate was attributed to the intermediary formation of glutathione polysulfide ($GSS_n^-$; Suzuki 1965; Suzuki and Silver 1966; Taylor 1968), which acts as the substrate for the oxygenase:

$$S_8 + GS^- \rightarrow GSS_8^- \quad (18)$$

$$GSS_8^- + O_2 + H_2O \rightarrow GSS_7^- + SO_3^{2-} + 2\ H^+ \quad (19)$$

$$[SSO_3]^{2-} + GS^- \rightarrow GSS^- + SO_3^{2-} \quad (20)$$

$$GSS^- + O_2 + H_2O \rightarrow GS^- + SO_3^{2-} + 2\ H^+ \quad (21)$$

It is interesting that reduced glutathione and glutathione persulfides ($GSS_n^-$) are implicated in this oxygenase process since sulfanes (such as $GSS^-$, glutathione trisulfide, thiosulfate and tetrathionate) are also established as remarkably strong activators of cytochrome *c* reduction by reduced glutathione (Prütz 1993), in which sulfane regeneration of the kind envisaged in the above equations also takes place. Rhodanese (thiosulfate sulfur-transferase), which occurs in the sulfur chemolithotrophs, also acts as a cytochrome *c* reductase in the presence of thiosulfate and glutathione, a process that probably also involves the formation of $GSS^-$ (Prütz 1993). Interpretation of glutathione-stimulated reactions in extracts of these organisms may thus require caution in interpretation until the in vivo processes resulting in cytochrome reduction are better understood.

Activity of this sulfur-oxidizing enzyme was relatively low in crude cell-free extracts, ranging from approximately 2 nmol thiosulfate formed $min^{-1}$ (mg protein)$^{-1}$ in *T. thioparus* (Suzuki and Silver 1966) to 11 nmol thiosulfate formed $min^{-1}$ (mg protein)$^{-1}$ in *T. thiooxidans* (Suzuki 1965). These low activities were, however, attributable to attenuation of enzyme function by unidentified inhibitory materials in the crude extracts (Suzuki 1994). The total enzyme activity recovered from *T. thioparus* increased as the crude enzyme was fractionated: thus, initially the crude extract sample could catalyze the conversion of 5.43 μmol sulfur converted to thiosulfate $min^{-1}$, but the pH 5.0 supernatant increased this to 14.86 μmol $min^{-1}$, and the ethanol precipitate from that fraction had further increased the total activity recovered to 24.43 μmol $min^{-1}$. Thus, the potential specific activity of the crude extract was indicated to be almost fivefold the one actually observed (Suzuki and Silver 1966), a phenomenon attributed to unknown inhibitors in the crude extract (Suzuki 1994). In that the three-step purification resulted in an increase in enzyme specific activity from 2 to 228 nmol sulfur converted to thiosulfate $min^{-1}$ (mg protein)$^{-1}$, with the elimination of 94% of the crude extract protein (Suzuki and Silver 1966), some loss of the enzyme protein probably also occurred, meaning that the actual potential activity in the cell could have been more than fivefold the one observed in the crude extract.

## The case of *Thiobacillus denitrificans*

It has been argued (Kelly 1988) that there can be no role for a sulfur oxygenase in anaerobically cultured *T. denitrificans* (which is facultatively denitrifying), and hence the

conversion of sulfur or sulfane cannot involve molecular oxygen. It is probable (but not proven) that this organism uses the same mechanism for sulfur/sulfane oxidation when growing with oxygen as its terminal respiratory oxidant. A siroheme sulfite reductase has been shown in this organism, the in vivo function of which in *T. denitrificans* and possibly in other chemolithotrophs is likely to be the oxidation of sulfur to sulfite (Kelly 1988; Trüper 1994; Hipp et al. 1997). The much greater molar growth yield of *T. denitrificans* as compared to that of the strict aerobes *T. neapolitanus* or *T. thiooxidans* when each is grown autotrophically on thiosulfate could be because of the presence of a non-energy-yielding sulfur oxygenase in the latter, while sulfur oxidation to sulfite in *T. denitrificans* is energy-conserving by electron transport phosphorylation. Thus, in the strict aerobes, the production of sulfite (Eq. 16) would not be coupled to the respiratory chain; but in *T. denitrificans*, electron flow from sulfane-sulfur would be used to reduce respiratory cytochromes:

$$[S - SO_3]^{2-} \rightarrow SO_3^{2-} + [S] \quad (22)$$

$$[S] + 3\ H_2O \rightarrow SO_3^{2-} + 6H^+ + 6\ e^- \quad (23)$$

$$e^- + \text{Ferricytochrome} \rightarrow \text{Ferrocytochrome} \quad (24)$$

Interestingly, we have demonstrated that *T. denitrificans* does in fact contain glutathione-dependent sulfur-oxidizing enzyme activity (Justin and Kelly 1978) and that crude cell-free extracts contain activities comparable to those in *T. thioparus*. Extracts assayed by Warburg manometry and thiosulfate estimation (Suzuki and Silver 1966; Justin and Kelly 1978; Suzuki 1994) consume oxygen and produce thiosulfate in equivalent amounts, as predicted from earlier work (Suzuki 1965, 1994): using extracts prepared from bacteria grown aerobically on thiosulfate in chemostat culture (with dissolved oxygen concentrations poised at ten different values between 14 and 211 μM dissolved $O_2$), the ratio of oxygen consumed/thiosulfate formed was 0.995. Freshly prepared extracts from freshly harvested aerobic chemostat culture (196 μM dissolved $O_2$) gave an activity of 5.1 nmol thiosulfate formed $min^{-1}$ (mg protein)$^{-1}$, and the mean value for six aerobic cultures (127–211 μM dissolved $O_2$) was 3.5 ± 1.5 nmol thiosulfate formed $min^{-1}$ (mg protein)$^{-1}$. The activity in crude extracts was lower in chemostat cultures grown at lower oxygen concentrations: the mean activity for five steady states at 14–86 μM dissolved $O_2$ was 1.2 ± 0.5 nmol thiosulfate formed $min^{-1}$ (mg protein)$^{-1}$, while that for four anaerobic cultures (with nitrite or nitrate as the sole terminal electron acceptor) was 0.96 ± 0.18 nmol thiosulfate formed $min^{-1}$ (mg protein)$^{-1}$. It is particularly noteworthy that activity was present even in cultures grown anaerobically, whether with nitrite or nitrate as the respiratory oxidant, and with either thiosulfate or tetrathionate as the energy substrate for growth. The oxygen/thiosulfate ratio in extracts of organisms from a nitrate-limited anaerobic culture was 1.06, consistent with the same activity measured in the aerobic cultures. The presence of the GSH-dependent oxygenase activity even in anaerobic cells could mean that the enzyme responsible has a function other than as a sulfur oxygenase. Its presence in aerobic bacteria also raises the question: "Does the organism use a different sulfane-oxidation mechanism under aerobic conditions as compared to the one used anaerobically?" Given that the free-energy change for thiosulfate oxidation is essentially identical for oxidation with oxygen or with nitrate (see Tables 4, 5), the aerobic growth yield could be *lower* than the anaerobic one if an oxygenase were involved in the former. In fact, the aerobic $Y_{max}$ is approximately 40% higher than that for growth with nitrate (Table 2), which is more indicative of unavailability of metabolic energy from a terminal nitrate reductase as compared to a proton-pumping terminal oxidase for aerobic growth.

The difference in growth yields between *T. denitrificans* and aerobic thiobacilli cannot be attributed simply to the lack of a functioning oxygenase in anaerobic cultures of the former since the variation among yields of chemolithotrophic thiosulfate-users is strikingly species-specific rather than attributable to the ability to grow with denitrification (Table 2). These yield differences are much more likely to result from differences in the point of entry of electrons into the electron transport chain and, consequently, in the number of coupling sites available to drive ATP synthesis and $NAD^+$ reduction involving energy-dependent flow of electrons from cytochromes (Kelly 1982, 1988, 1990). The standard electrode potentials calculated for the $SO_4^{2-}/HSO_3^-$ and $SO_4^{2-}/SO_3^{2-}$ couples are extremely electronegative ($E_0' = -516$ and $-522$ mV; Thauer et al. 1977; R. K. Thauer, Universität Marburg, Germany, personal communication), which is consistent with the likelihood sulfite oxidation being able to couple at a point on the electron transfer chain at least as high as cytochrome *b*. The $S^0/HS^-$ aqueous potential ($E_0' = -270$ mV; Kelly 1982) is also sufficiently electronegative to couple at the cytochrome *b* or *c* levels.

**Table 2** Growth yields of several chemolithotrophic autotrophs on thiosulfate as the sole energy substrate in chemostat culture under optimum conditions with carbon dioxide as the sole source of carbon

| Organism | Yield [g dry wt. (mol thiosulfate used for energy generation)$^{-1}$] | |
|---|---|---|
| | Respiratory oxidant: | |
| | Oxygen | Nitrate |
| Yields at a dilution rate of 0.03 $h^{-1}$ (Timmer-Ten Hoor 1981) | | |
| *Thiobacillus denitrificans* | 18.54 | 11.60 |
| *Thiomicrospira denitrificans* | 9.38 | 5.72 |
| True growth yields ($Y_{max}$; Kelly 1990) | | |
| *Thiobacillus neapolitanus* | 10.8 | – |
| *Thiobacillus tepidarius* | 14.0 | – |
| *Thiobacillus denitrificans* | 20.6 | 14.5 |

**Table 3** Physical size of sulfur oxygenase enzymes (*nd* not determined)

| Source of enzyme | Molecular mass (Da) | | Reference |
|---|---|---|---|
| | Oligomer | Subunit | |
| *Acidianus brierleyi* | 560,000 | 35,000 | Emmel et al. (1986) |
| *Desulfurolobus ambivalens* | 550,000 | 40,000 (35, 317[a]) | Kletzin (1992, 1994) |
| *Thiobacillus thiooxidans* | 46,000 | 23,000 | Suzuki (1994) |
| *Thiobacillus ferrooxidans* | 40,000 | nd | Suzuki (1994) |

[a] Calculated for the 109 amino acids coded by the *sor* gene (Kletzin 1992)

## Sulfur oxygenase in chemolithotrophic sulfur-oxidizing archaea

Sulfur oxygenase/reductase enzymes have been shown in the facultatively anaerobic archaea *Acidianus brierleyi* and *Desulfurolobus ambivalens* (Emmel et al. 1986; Kletzin 1992, 1994). Unlike the enzyme from thiobacilli, the activities of these enzymes did not require reduced glutathione, and activities present in crude extracts of *Desulfurolobus* were 15- to 80-fold greater than in sulfur-grown thiobacilli, although this comparison must take note of the optimum of 85 °C for the assay of the archaeal enzyme (Suzuki 1994). As in the case of the glutathione-independent sulfur oxidation seen with some thiobacilli (Taylor 1968), the archaeal oxidation was inhibited by thiol-binding reagents (N-ethylmaleimide, *para*-chloromercuribenzoate, and iodoacetate), indicating a role for thiols in the mechanism (Suzuki 1994).

Physically, the archaeal enzyme differs in size and structure from those shown in thiobacilli (Table 3). While it might be argued that the enzyme in the two disparate groups originated from a precursor in a common ancestor, with the thiobacilli having lost the massive oligomeric structure seen in the archaea, it is equally likely that these enzymes have separate evolutionary origins. Sequence analysis of the enzyme(s) from the thiobacilli is not yet available in order for any relatedness to be assessed.

## Observations inconsistent with an oxygenase pathway in some *Thiobacillus* species

Apart from the case of *T. denitrificans*, a number of observations suggest that the oxidation of elemental sulfur involves energy-yielding electron transport rather than an oxygenase. Glutathione-independent sulfur oxidation to sulfate has been shown in cell-free preparations of *T. thiooxidans* (membrane-associated) and *T. neapolitanus* (soluble fraction), and reduced glutathione was in fact inhibitory to the activity in *T. neapolitanus* (Taylor 1968). Evidence has been presented for the involvement of thiol-acceptors in the oxidation by *T. neapolitanus* and for the involvement of cytochrome-dependent electron transport to oxygen. Beffa et al. (1991, 1992 a, b, 1993) have provided evidence that elemental sulfur, or a material equivalent to it, is an intermediate in thiosulfate oxidation by a number of sulfur chemolithotrophs including *T. tepidarius*, *T. novellus* and *P. versutus*. The "sulfur-oxidizing activity" observed by Beffa et al. (1991, 1992 a, b, 1993) with intact organisms was inhibited by myxathiazol and HQNO (which did not affect sulfite oxidation), indicating the involvement of cytochromes $bc_1$ in sulfur oxidation. These extensive observations led Beffa et al. (1993) to conclude that sulfur-oxidizing activity "appears not to be an oxygenase". Similarly, electron transport inhibitor studies have indicated that *T. ferrooxidans* uses $bc_1$-electron transport for sulfur oxidation rather than an oxygenase or the Fe(III)-reducing process postulated by other workers (Corbett and Ingledew 1987; Kelly 1988). The proton translocation studies of Lu and Kelly (1988a,b) are also more consistent with the presence of oxidation processes that differ in electron coupling sites in a species-specific manner, rather than with involvement of an oxygenase.

## Chemical thermodynamics of sulfur transformations

The relationship between the free-energy change (energy output) from the oxidation of their substrates by sulfur-chemolithotrophs, and the free-energy requirement (energy input) for carbon dioxide fixation to support their autotrophic growth has long been used to estimate the efficiency of energy conservation from sulfur oxidations (Baas Becking and Parks 1927; Kelly 1990). This approach, coupled with our detailed biochemical knowledge of the process of $CO_2$ fixation and fundamental studies of the ATP requirement for microbial growth, has proved to be useful in assessments of ATP production during substrate oxidation and in estimating $Y_{ATP}$ (the growth yield per mol ATP available from substrate oxidation; Timmer-Ten Hoor 1981; Kelly 1982, 1990).

In order for such calculations to be valid, the thermodynamic data used must obviously be as accurate as possible. The starting point for all calculations is the Gibbs free energy of formation of the substrate and product compounds being studied ($\Delta G_f^\circ$ in kJ mol$^{-1}$). Table 1 provides the best estimates available from the literature for $\Delta G_f^\circ$ values for most of the substrates and products of the sulfur chemolithotrophs. Included in Table 4 are $\Delta G_f^\circ$ values for thiosulfate and polythionates calculated using the group contribution method of Mavrovouniotis (1990, 1991). This method is most applicable to organic biochemicals and is less accurate for very small molecules, including inorganic ions such as $SO_3^{2-}$ (Mavrovouniotis 1990, 1991). The values calculated for the thionic acid ions by the group contribution method typically differ from previously published values by no more than 1–4%

**Table 4** Free energy changes for inorganic sulfur oxidation and thiosulfate cleavage reactions [taken, or calculated, using free energies of formation from Latimer (1952), Thauer et al. (1977), Bard et al. (1985), and Kelly (1990); Table 1)]. For elemental sulfur, the free energy of formation of rhombic sulfur ($\Delta G_f^\circ = 0$) is used (Latimer 1952). The values for $O_2$, $N_2$, and $H^+$ in aqueous solution are also taken as $\Delta G_f^\circ = 0$ (Latimer 1952; Thauer et al. 1977). $S^0$ indicates elemental sulfur as 1/8 of the $S_8$ ring

| Reaction | $\Delta G^\circ$ [kJ (mol S-substrate)$^{-1}$] |
|---|---|
| $S_2O_3^{2-} + 2\ O_2 + H_2O \rightarrow 2\ SO_4^{2-} + 2\ H^+$ | –738.7 |
| $S_2O_3^{2-} + 2\ O_2 + H_2O \rightarrow 2\ HSO_4^-$ | –761.4 |
| $5\ S_2O_3^{2-} + 8\ NO_3^- + H_2O \rightarrow 10\ SO_4^{2-} + 2\ H^+ + 4\ N_2$ | –750.8 |
| $S_3O_6^{2-} + 2\ O_2 + 2\ H_2O \rightarrow 3\ SO_4^{2-} + 4\ H^+$ | –801.3 |
| $S_4O_6^{2-} + 3.5\ O_2 + 3\ H_2O \rightarrow 4\ SO_4^{2-} + 6\ H^+$ | –1,244.6 |
| $S_5O_6^{2-} + 5\ O_2 + 4\ H_2O \rightarrow 5\ SO_4^{2-} + 8\ H^+$ | –1,818.2 |
| $S_6O_6^{2-} + 6.5\ O_2 + 5\ H_2O \rightarrow 6\ SO_4^{2-} + 10\ H^+$ | –2,346.1 |
| $S_7O_6^{2-} + 8\ O_2 + 6\ H_2O \rightarrow 7\ SO_4^{2-} + 12\ H^+$ | –2,803.2 |
| $S_2O_3^{2-} \rightarrow S^0 + SO_3^{2-}$ | +26.8 |
| $S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$ | +50.4 |
| $S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + HS^- + H^+$ | –6.1 |
| $S_3O_6^{2-} + H_2O \rightarrow SO_4^{2-} + S_2O_3^{2-} + 2\ H^+$ | +62.7 |
| $H_2S + 2\ O_2 \rightarrow H_2SO_4$ | –714.1 |
| $S^{2-} + 2\ O_2 \rightarrow SO_4^{2-}$ | –658.8 |
| $HS^- + 2\ O_2 \rightarrow SO_4^{2-} + H^+$ | –732.6 |
| $S_8 + 12\ O_2 + 8\ H_2O \rightarrow 8\ H_2SO_4$ | –504.8 kJ (mol $S^0$)$^{-1}$ |
| $S^0 + 1.5\ O_2 + H_2O \rightarrow SO_4^{2-} + 2\ H^+$ | –507.4 |
| $S^0 + 1.5\ O_2 + OH^- \rightarrow HSO_4^-$ | –598.7 |
| $H_2S + 0.5\ O_2 \rightarrow S^0 + H_2O$ | –209.3 |
| $S^{2-} + 0.5\ O_2 + 2\ H^+ \rightarrow S^0 + H_2O$ | –151.4 |
| $HS^- + 0.5\ O_2 \rightarrow S^0 + OH^-$ | –145.2 |
| $S_8 + 8\ O_2 + 8\ H_2O \rightarrow 8\ H_2SO_3$ | –300.7 kJ (mol $S^0$)$^{-1}$ |
| $S^0 + O_2 + H_2O \rightarrow SO_3^{2-} + 2\ H^+$ | –249.4 |
| $S^0 + O_2 + H_2O \rightarrow HSO_3^- + H^+$ | –290.6 |
| $H_2SO_3 + 0.5\ O_2 \rightarrow H_2SO_4$ | –204.1 |
| $SO_3^{2-} + 0.5\ O_2 \rightarrow SO_4^{2-}$ | –258.0 |
| $HSO_3^- + 0.5\ O_2 \rightarrow HSO_4^-$ | –228.2 |

(Table 1), indicating the validity of these calculations for polythionates.

The standard free-energy changes [$\Delta G^\circ$, kJ (mol sulfur substrate)$^{-1}$] have been newly calculated for the principal reactions involved in sulfur dissimilation (Tables 4–6). These are regarded as definitive values, with the caveat that values in vivo may deviate in detail from these as a consequence of intracellular concentrations of intermediates and cellular pH deviating from those of standard $\Delta G^\circ$ values. These $\Delta G^\circ$ values are the basis of the thermodynamic analyses presented below and are presented as the best estimates available for comparative assessment of relative amounts of energy available from different oxidations and the growth yields of bacteria using those oxidations.

**Table 5** Mean free-energy changes for the partial and complete aerobic oxidation of sulfur and its compounds (calculated from the ranges of values in Table 4)

| Reaction | $\Delta G^\circ$ (mean ± SE) [kJ (mol S-substrate)$^{-1}$] |
|---|---|
| Thiosulfate to sulfate | –750.1 ± 16.1 |
| Sulfide to sulfate | –701.8 ± 31.4 |
| Sulfide to sulfur | –168.6 ± 28.9 |
| Sulfur to sulfate | –537.9 ± 43.7 |
| Sulfur to sulfite | –280.2 ± 22.2 |
| Sulfite to sulfate | –230.1 ± 22.0 |

## The limits of energy conservation from sulfur oxidation reactions

From the free-energy requirement for the $CO_2$ fixation process, an estimate of the maximum possible growth yield can be deduced from the free-energy changes accompanying sulfur oxidations. Thus, thiosulfate oxidation has a $\Delta G^\circ$ of –750.1 kJ (Table 5), suggesting the possibility of a $Y_{max}$ approaching 750.1/113 or 6.62 mol $CO_2$ assimilated (mol thiosulfate oxidized for energy-coupled processes)$^{-1}$. The maximum experimentally determined aerobic $Y_{max}$ for *T. denitrificans* (Kelly 1990) was 0.81 mol $CO_2$ (mol thiosulfate oxidized for energy-coupled processes)$^{-1}$, or an apparent efficiency of 12.3% conservation of the total free energy nominally available. Similar calculations can be performed for other substrates and *Thiobacillus* species, showing that overall maximum efficiencies of energy conversion fall in the range of 5.6–12.2% (see Table 7).

## Potential thermodynamic efficiency of energy coupling between carbon dioxide fixation and component reactions of sulfur oxidation pathways

The efficiencies given in Table 7, derived from $\Delta G^\circ$ values for the complete oxidation of the substrates, are of limited value since they give no indication of which

intermediate steps provide metabolically useful energy and which do not. The oxidation process for the sulfur substrates can be dissected into a number of potentially energy-linked steps. For sulfide, three steps are the minimum:

$$S^{2-} \xrightarrow{(1)} S^0 \xrightarrow{(2)} SO_3^{2-} \xrightarrow{(3)} SO_4^{2-} \quad (25)$$

The average $\Delta G^\circ$ values for the component steps are summarized in Table 5, but if the oxidation of sulfide involved an oxygenase for step 2, then no metabolically available energy would arise from the conversion of sulfur to sulfite (step 3). The energy-conserving steps (1 and 3) would provide (as $\Delta G^\circ$) –398.7 kJ or sufficient to predict a $Y_{max}$ of 3.53 mol $CO_2$ (mol sulfide oxidized for energy)$^{-1}$. The maximum experimentally determined $Y_{max}$ for *T. tepidarius* (see Table 7) was 0.42 mol $CO_2$ (mol sulfide oxidized for energy)$^{-1}$, or an apparent efficiency of 11.9%.

**Table 6** Free-energy changes for some reactions of organosulfur compounds, which are substrates for some *Thiobacillus thioparus* and some *Paracoccus* strains

| Reaction | $\Delta G^\circ$ [kJ (mol S-substrate)$^{-1}$] |
|---|---|
| $SCN^- + 2\ O_2 + 2\ H_2O \rightarrow SO_4^{2-} + CO_2 + NH_4^+$ | –824.5 |
| $SCN^- + 2\ O_2 + 2\ H_2O \rightarrow HSO_4^- + CO_2 + NH_3$ | –783.0 |
| $HSCN + 2\ O_2 + 2\ H_2O \rightarrow H_2SO_4 + CO_2 + NH_3$ | –772.9 |
| Oxidation of thiocyanate to sulfate | –793.5 ± 22.3 |
| $SCN^- + H_2O \rightarrow H_2S + OCN^-$ | +22.1 |
| $SCN^- + OH^- \rightarrow HS^- + OCN^-$ | +42.2 |
| $HSCN + H_2O \rightarrow H_2S + HOCN$ | +4.1 |
| Hydrolysis of thiocyanate to sulfide | +22.8 ± 15.6 |
| $COS + 2\ O_2 + 2\ H_2O \rightarrow H_2SO_4 + CO_2$ | –721.6 |
| $COS + 2\ O_2 + 2\ H_2O \rightarrow SO_4^{2-} + CO_2 + 2\ H^+$ | –724.3 |
| $COS + 2\ O_2 + H_2O \rightarrow HSO_4^- + CO_2 + H^+$ | –735.7 |
| Oxidation of carbonyl sulfide to sulfate | –727.2 ± 6.1 |
| $COS + H_2O \rightarrow CO_2 + H_2S$ | –7.5 |
| $COS + OH^- \rightarrow CO_2 + HS^-$ | –71.6 |
| Hydrolysis of carbonyl sulfide to sulfide | –39.6 ± 32.1 |

For thiosulfate the following sequence is feasible:

$$S_2O_3^{2-} \xrightarrow{(4)} S^0 + SO_3^{2-} \xrightarrow{(5)} 2\ SO_3^{2-} \xrightarrow{(6)} 2\ SO_4^{2-} \quad (26)$$

The $\Delta G^\circ$ value for step 4 at approximately +26.8 kJ is uncertain because of lack of knowledge of how this step is achieved in vivo (Table 4). Again, if sulfur conversion to sulfite (step 5) is by oxygenation, the useful energy ($\Delta G^\circ$) from thiosulfate oxidation would be from the oxidation of the two sulfite ions only (step 6), namely –460.2 kJ. This would indicate a maximum potential $Y_{max}$ of 4.07 mol $CO_2$ (mol thiosulfate oxidized for energy)$^{-1}$. The maximum experimentally determined aerobic $Y_{max}$ for *T. tepidarius* (see Table 7) was 0.55 mol $CO_2$ (mol thiosulfate oxidized for energy)$^{-1}$, or an apparent efficiency of 13.5%. If the oxidation of the sulfur (from sulfide or thiosulfate: steps 2 and 5 of Eqs. 25 and 26) were also energy-coupled, the efficiencies would fall to 7% (sulfide) and 8.4% (thiosulfate). Making the assumption for *T. denitrificans* that only step 6 of Eq. 26 is energy-conserving, then the $Y_{max}$ of 0.81 mol $CO_2$ (mol thiosulfate)$^{-1}$ indicates an apparent efficiency of 19.7%. If both steps 5 and 6 were energy-coupled, the efficiency would be 12.2%. The "one-sulfur" compounds used by some chemolithotrophs must also pass through sulfide as a degradative intermediate (Table 6), so essentially the same calculations apply to them as for sulfide itself. A similar approach can be used for the polythionates.

## Energetic implications of hydrolytic scission initiating thionate metabolism

Oxidation of thiosulfate and polythionates in the chemolithotrophic thiobacilli (such as *T. neapolitanus*, *T. tepidarius*, *T. thiooxidans* and *T. acidophilus*) is still incompletely understood, but some or all of the following component reactions are probably catalyzed in all these species:

$$2\ S_2O_3^{2-} + H_2O + 0.5\ O_2 \rightarrow S_4O_6^{2-} + 2\ OH^- \quad (27)$$

$$S_3O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + 2\ H^+ \quad (28)$$

**Table 7** Comparison of calculated theoretical maximal growth-linked carbon dioxide fixation and actual values for *Thiobacillus* species grown autotrophically under optimal conditions on inorganic sulfur compounds as the sole energy substrate. The theoretical $CO_2$ fixation was calculated as ($\Delta G^\circ$ for oxidation)/($\Delta G^\circ$ for $CO_2$ fixation) = ($\Delta G^\circ$ for oxidation)/113 = mol $CO_2$ fixed (mol substrate oxidized for energy)$^{-1}$. The observed $CO_2$ fixation was calculated from growth yields in the chemostat as mol $CO_2$ fixed (mol substrate oxidized for energy)$^{-1}$ (Kelly 1990)

| Organism and substrate | $\Delta G^\circ$ for complete oxidation (kJ mol$^{-1}$) | Theoretical $CO_2$ fixation | Observed $CO_2$ fixation | Apparent efficiency of energy conservation (%) |
|---|---|---|---|---|
| *Thiobacillus tepidarius* | | | | |
| Sulfide | –701.8 | 6.21 | 0.46 | 7.4 |
| Thiosulfate | –750.1 | 6.64 | 0.55 | 8.3 |
| Trithionate | –801.3 | 7.09 | 0.58 | 8.2 |
| Tetrathionate | –1,244.6 | 11.01 | 1.04 | 9.4 |
| Hexathionate | –2,346.1 | 20.76 | 1.40 | 6.7 |
| Heptathionate | –2,803.2 | 24.81 | 1.79 | 7.2 |
| *Thiobacillus neapolitanus* | | | | |
| Thiosulfate | –750.1 | 6.64 | 0.40 | 6.0 |
| Trithionate | –801.3 | 7.09 | 0.40 | 5.6 |
| *Thiobacillus denitrificans* | | | | |
| Thiosulfate (aerobic) | –750.8 | 6.64 | 0.81 | 12.2 |
| Thiosulfate (denitrifying) | –750.8 | 6.64 | 0.57 | 8.6 |

$$S_4O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + S^0 + 2\ H^+ \quad (29)$$

$$S_nO_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + 2\ H^+ + n - 3\ S^0 \quad (30)$$

Trithionate hydrolase enzymes have been shown in *T. tepidarius* and *T. acidophilus*, and enzymes disproportionating tetrathionate or pentathionate into thiosulfate and sulfate (7:4 ratio) or into thiosulfate, sulfate and sulfur have been described (Sugio et al. 1996; De Jong et al. 1997; Kelly et al. 1997). If such reactions represent the mainstream degradation route for these compounds, the implication is that the first metabolically oxidizable intermediate would be elemental sulfur, whether the starting material were thiosulfate or a polythionate. This would mean that the only two potentially energy-yielding steps would be sulfur → sulfite and sulfite → sulfate. With the first step catalyzed by an oxygenase, only the latter would be energy-yielding (i.e. only the –230.1 kJ per mol of sulfite produced from the substrate would be coupled to growth), as discussed above. An important energetic implication arises from studies on trithionate and polythionate hydrolases in thiobacilli (Eqs. 21–23) and from the belief that tetrathionate is an obligatory intermediate in thiosulfate oxidation by some chemolithotrophs (Eq. 27). This implies that the only energy-yielding step for thiosulfate and trithionate oxidation would be the metabolism of only one sulfur atom per mol (Eqs. 27 or 28 and 29). If energy conservation was only from sulfite oxidation (with sulfur oxidation to sulfite catalyzed by an oxygenase), the theoretical maximum $CO_2$ fixation supported by oxidation of one sulfite ion ($\Delta G^\circ$ = –230.1 kJ) would be 2.04 mol. The fixation (from $Y_{max}$ values; Table 7) of 0.40, 0.55 and 0.81 mol $CO_2$ (mol thiosulfate)$^{-1}$ for *T. neapolitanus*, *T. tepidarius* and *T. denitrificans* would suggest efficiencies of 19.6, 27.0 and 39.7%. If metabolic energy were conserved from the overall conversion of sulfur to sulfate ($\Delta G^\circ$ = –537 kJ), these relative efficiencies would become 8.4, 11.6 and 17%.

Applying Eq. 30 to all the thionates indicates that the number of oxidizable sulfur atoms produced from each substrate would be one each from thiosulfate and trithionate, two from tetrathionate, three from pentathionate, four from hexathionate, and five from heptathionate. This would lead to the prediction (cf. Table 8) that growth yields would be in the ratio 1:1:2:3:4:5, respectively, for thiosulfate and trithionate through to heptathionate. The actual yields for *T. tepidarius* growing at a fixed dilution rate in the chemostat (Wood and Kelly 1986) were: thiosulfate and trithionate, 9.7 ± 1.0 g mol$^{-1}$; tetrathionate, 19.4 g mol$^{-1}$; hexathionate, 38.8 g mol$^{-1}$; and heptathionate, 48.5 g mol$^{-1}$ or 1:1:2:4:5, as predicted.

It might be asked, why not directly assay the efficiencies of growth on sulfite and elemental sulfur? Unfortunately, reliable data on growth yields are not available: sulfite is readily auto-oxidizable and has not been demonstrated to support growth as a sole substrate. Even in chemostats supplied with sulfite solutions maintained under nitrogen, reliable growth yields cannot be obtained: the yield has been estimated as only approximately 0.59 g dry wt. (mol sulfite)$^{-1}$ for *T. tepidarius* growing under dual substrate limitation with thiosulfate and sulfite (Wood and Kelly 1986). In the case of sulfur, reliable growth yield data are not available, and comparative data for carbon dioxide fixation by *T. neapolitanus* supported by the oxidation of sulfur or thiosulfate has indicated the $^{14}CO_2$ fixed per atom of oxygen consumed to be in the ratio of 1.0:2.6 [calculated from Kelly and Syrett (1966)], suggesting sulfur to be an energy-inefficient substrate under the conditions assayed.

## The energetics of sulfite oxidation: more than one option

The minimalist view of thiosulfate oxidation [involving only three steps (Eq. 26), or four if the conversion of the sulfane-sulfur to sulfite passes through sulfide as an intermediate] was put on a good enzymological basis by the seminal work of Peck [1960: see Kelly (1982)]. This established the adenosine phosphosulfate (APS) pathway for the sulfite generated from thiosulfate (Kelly 1982); in this pathway sulfite is oxidatively condensed with AMP by APS reductase to form adenylyl sulfate (releasing two electrons), which by exchanging its sulfate group with orthophosphate produces sulfate and ADP. This route allows not only for the possibility of electron-transport-linked energy conservation during sulfite oxidation, but also for the generation of ATP (from 2 ADP by adenylate kinase) via the APS intermediate. The presence of APS reductase has been established in several obligately chemolithotrophic thiobacilli (Kelly 1982), and this ancestral enzyme is believed to function in sulfur dissimilation in photolithotrophs, in sulfate-reducing bacteria and archaea, and in chemolithotrophic bacteria (Hipp et al. 1997). The presence or absence of the APS pathway in specific chemolithotrophs might thus affect their relative growth efficiency, and when present would support a maximal level of conservation of the free energy available from sulfite conversion to sulfate. It is noteworthy that APS-independent cytochrome-*c*-reducing (or ferricyanide-reducing) sulfite dehydrogenases exist in several thiobacilli and in *P. versutus*, and that APS reductase is not apparently involved in sulfite oxidation in some of these [see Kelly (1982) and Kelly et al. (1997)].

## Correlation of maximum growth yields with requirements for ATP and NAD(P)H by the Calvin cycle

The growth yield value (Table 7) of 0.81 mol $CO_2$ fixed (mol thiosulfate oxidized for energetic purposes)$^{-1}$ indicates a requirement for a minimum of 3 ATP for $CO_2$ reduction, plus a minimum of 1 ATP for each of the 2 NAD(P)H used (Kelly 1990). Thus, the total requirement is for at least 5 ATP to be generated during the oxidation of 1 mol thiosulfate. If this is generated by electron transport phosphorylation with a maximum coupling efficiency of 2 ATP (electron pair transported to oxygen)$^{-1}$,

then up to six of the eight electrons from thiosulfate oxidation would have to support ATP synthesis. This would be inconsistent with Eqs 27 and 29 if an oxygenase catalysed conversion of the one sulfur to sulfite, since only one electron pair would enter the electron transport chain. Even by Eq. 26, sulfur conversion to sulfite would need to be electron-transport-linked unless the conversion of sulfite to sulfate generated ATP by the APS pathway as well as allowed 2 ATP to be synthesized by electron transport phosphorylation per sulfite oxidized.

## Conclusions and wider significance of the data

The theoretical analysis presented of growth energetics in sulfur chemolithotrophs illustrates the uncertainty that still exists about reactions that are regarded as mainstream routes for sulfur oxidation, e.g. (1) the centrality of the role of the conversion of thiosulfate to tetrathionate, (2) the nature of the products of polythionate hydrolases ($S_4$ and higher), and (3) the role, if any, of the sulfur oxygenase. The thermodynamic considerations are generally consistent with the principal energy-conserving step of sulfur compound oxidation being the oxidation of sulfite to sulfate alone, with sulfur oxidation to sulfite by an oxygenase being energetically feasible. In the case of *T. denitrificans*, however, apparent efficiencies of carbon dioxide fixation of nearly 40%, when calculated on this basis, suggest that sulfur oxidation by a nonoxygenative pathway might operate.

Energetic calculations based on the Baas Becking and Parks (1927) free-energy calculation for carbon dioxide fixation [+470.6 kJ (mol $CO_2$)$^{-1}$; Eq. 1] have led in the past to overestimates of the efficiency of coupling of energy from chemolithotrophic oxidations to autotrophic growth. Observed growth yields for some sulfur and iron bacteria have been interpreted as indicating energy efficiencies exceeding 50% (Jones and Kelly 1983; Kelly 1990; Kelly et al. 1987). Such data should be reexamined, since an apparent efficiency of 100% is actually only 24% when calculated using the value of +113 kJ (mol $CO_2$)$^{-1}$ proposed in this review.

Future work must continue to address the question of how sulfur oxidations actually occur in the sulfur chemolithotrophs, with particular reference to the obligate chemolithotrophs such as *T. thioparus*, *T. neapolitanus* and *T. tepidarius*. These probably have common mechanisms for the oxidation of sulfur and the thionates, but are taxonomically heterogeneous, since analysis of the base sequences of their 16S rRNA genes distributes them between the β- and γ-subdivisions of the proteobacteria, with the facultatively autotrophic species (*T. acidophilus* and *T. novellus*) falling into the α-subdivision (McDonald et al. 1997). The obligate species are biochemically distinct from other sulfur chemolithotrophs such as *P. versutus* (α-proteobacteria), in which a sulfur oxygenase is not involved in the operation of the thiosulfate-oxidizing multienzyme system (Kelly et al. 1997). A molecular approach to the structural relationships between sulfur oxidation enzymes across the species, together with attempts to select mutants (e.g. those lacking the sulfur oxygenase gene), may assist in resolving the nature of the oxidation pathways and the energy-coupled reactions. The possibility of the wider occurrence of siroheme sulfite reductase (as in *T. denitrificans* and *Chromatium vinosum*) among the chemolithotrophs needs investigation: in the case of this being an ancestral enzyme function in sulfur dissimilation (Hipp et al. 1997), it may have a key role in sulfur conversion to sulfite in bacteria additional to *T. denitrificans*. Moreover, if it can be established that sulfur oxygenase is not a key enzyme in chemolithotrophic sulfur oxidation (at least in the proteobacteria), then an alternative function for this enzyme will need to be found. It is possible that the sulfur oxygenation activity is a rogue function of an enzyme that has a different metabolic importance, much as the oxygenase activity of ribulose bisphosphate carboxylase is not its raison d'être.

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## References

Baas Becking LGM, Parks GS (1927) Energy relations in the metabolism of autotrophic bacteria. Physiol Rev 7:85–106

Bard AJ, Parsons R, Jordan J (1985) Standard potentials in aqueous solution. Dekker, New York

Beffa T, Berczy M, Aragno M (1991) Chemolithoautotrophic growth on elemental sulfur ($S^0$) and respiratory oxidation of $S^0$ by *Thiobacillus versutus* and another sulfur-oxidizing bacterium. FEMS Microbiol Lett 84:285–290

Beffa T, Berczy M, Aragno M (1992a) Inhibition of respiratory oxidation of elemental sulfur ($S^0$) and thiosulfate in *Thiobacillus versutus* and another sulfur-oxidizing bacterium. FEMS Microbiol Lett 90:121–128

Beffa T, Fisher C, Aragno M (1992b) Respiratory oxidation of reduced sulfur compounds by intact cells of *Thiobacillus tepidarius* (type strain). Arch Microbiol 158:456–458

Beffa T, Fisher C, Aragno M (1993) Growth and respiratory oxidation of reduced sulfur compounds by intact cells of *Thiobacillus novellus* (type strain) grown on thiosulfate. Curr Microbiol 26:323–326

Brasted RC (1961) Comprehensive organic chemistry, vol 8. Van Nostrand, Princeton, NJ

Charles AM, Suzuki I (1966) Mechanism of thiosulfate oxidation by *Thiobacillus novellus*. Biochim Biophys Acta 128:510–521

Corbett CM, Ingledew WJ (1987) Is $Fe^{3+/2+}$ cycling an intermediate in sulphur oxidation by $Fe^{2+}$-grown *Thiobacillus ferrooxidans*. FEMS Microbiol Lett 41:1–6

De Jong GAH, Hazeu W, Bos P, Kuenen JG (1997) Isolation of the tetrathionate hydrolase from *Thiobacillus acidophilus*. Eur J Biochem 243:678–683

Emmel T, Sand W, König WA, Bock E (1986) Evidence for the existence of a sulphur oxygenase in *Sulfolobus brierleyi*. J Gen Microbiol 132:3415–3420

Hipp WM, Pott AS, Thum-Schmitz N, Faath I, Dahl C, Trüper HG (1997) Towards a phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes. Microbiology 143:2891–2902

Jones CA, Kelly DP (1983) Growth of *Thiobacillus ferrooxidans* on ferrous iron in chemostat culture: influence of product and substrate inhibition. J Chem Tech Biotechnol 33B:241–261

Justin P, Kelly DP (1978) Metabolic changes in *Thiobacillus denitrificans* in anaerobic and aerobic chemostat culture. J Gen Microbiol 107:131–137

Kelly DP (1978) Bioenergetics of chemolithotophic bacteria. In: Bull AT, Meadow PM (eds) Companion to microbiology. Longman. London, pp 363–386

Kelly DP (1982) Biochemistry of the chemolithotrophic oxidation of inorganic sulphur. Philos Trans R Soc Lond Biol 298:499–528

Kelly DP (1988) Oxidation of sulphur compounds. Soc Gen Microbiol Symp 42:65–98

Kelly DP (1990) Energetics of chemolithotrophs. In: Krulwich TA (ed) Bacterial energetics. Academic Press, San Diego, pp 479–503

Kelly DP, Syrett PJ (1966) Energy coupling during sulphur compound oxidation by *Thiobacillus* sp. strain C. J Gen Microbiol 43:109–118

Kelly DP, Mason J, Wood AP (1987) Energy metabolism in chemolithotrophs. In: van Verseveld H, Duine J (eds) Microbial growth on $C_1$ compounds. Nijhoff, Dordrecht, pp 186–194

Kelly DP, Shergill JK, Lu W-P, Wood AP (1997) Oxidative metabolism of inorganic sulfur compounds by bacteria. Antonie Van Leeuwenhoek 71:95–107

Kletzin A (1992) Molecular characterization of the *sor* gene, which encodes the sulfur oxygenase/reductase of the thermoacidophilic archaeum *Desulfurolobus ambivalens*. J Bacteriol 174:5854–5859

Kletzin A (1994) Sulfur oxidation and reduction in archaea – sulfur oxygenase/reductase and hydrogenase from the extremely thermophilic and facultatively anaerobic archaeon *Desulfurolobus ambivalens*. Syst Appl Bacteriol 16:534–543

Larsen H (1953) On the microbiology and biochemistry of the photosynthetic green sulfur bacteria. Det Kgl Norske Videnskabers Selskabs Skrifter, vol 1. Bruns Bokhandel, Trondheim, p 129

Larsen H (1954) The photolitho-autotrophic bacteria and their energy relations. In: Fry BA, Peel JL (eds) Autotrophic micro-organisms, 4th symposium. Society for General Microbiology, Cambridge, pp 186–201

Latimer WM (1952) The oxidation states of the elements and their potentials in aqueous solutions. Prentice-Hall, Englewood Cliffs, NJ

Lu W-P, Kelly DP (1988a) Kinetic and energetic aspects of inorganic sulphur compound oxidation by *Thiobacillus tepidarius*. J Gen Microbiol 134:865–876

Lu W-P, Kelly DP (1988b) Respiration-driven proton translocation in *Thiobacillus versutus* and the role of the periplasmic thiosulphate-oxidizing enzyme system. Arch Microbiol 149: 297–302

Mason J, Kelly DP, Wood AP (1987) Chemolithotrophic and autotrophic growth of *Thermothrix thiopara* and some thiobacilli on thiosulphate and polythionates, and a reassessment of the growth yields of *Thx. thiopara* in chemostat culture. J Gen Microbiol 133:1249–1256

Mavrovouniotis ML (1990) Group contributions for estimating standard Gibbs energies formation of biochemical compounds in aqueous solution. Biotechnol Bioeng 36:1070–1082

Mavrovouniotis ML (1991) Estimation of standard Gibbs energy changes of biotransformations. J Biol Chem 266:14440–14445

McDonald IR, Kelly DP, Murrell JC, Wood AP (1997) Taxonomic relationships of *Thiobacillus halophilus*, *T. aquaesulis*, and other species of *Thiobacillus*, as determined using 16S rDNA sequencing. Arch Microbiol 166:394–398

Nathansohn A (1902) Über eine neue Gruppe von Schwefelbacterien und ihren Stoffwechsel. Mitt zool Stat Neapel 15:655–680

Nelson DC, Hagen KD (1995) Physiology and biochemistry of symbiotic and free-living chemoautotrophic sulfur bacteria. Am Zool 35:91–101

Peck HD (1960) Adenosine 5′-phosphosulfate as an intermediate in the oxidation of thiosulfate by *Thiobacillus thioparus*. Proc Nat Acad Sci USA 46:1053–1057

Prütz WA (1993) Sulfane-activated reduction of cytochrome *c* by glutathione. Free Radic Res Commun 18:159–165

Smith NA, Kelly DP (1988) Oxidation of carbon disulphide as the sole source of energy for the autotrophic growth of *Thiobacillus thioparus* strain TK-m. J Gen Microbiol 134:3041–3048

Stryer L (1988) Biochemistry, 3rd edn. Freeman, New York

Sugio T, Kanao T, Furukawa H, Nagasawa T, Blake RC (1996) Isolation and identification of an iron-oxidizing bacterium which can grow on tetrathionate medium and the properties of a tetrathionate-decomposing enzyme isolated from the bacterium. J Ferm Bioeng 82:233–238

Suzuki I (1965) Oxidation of elemental sulfur by an enzyme system from *Thiobacillus thiooxidans*. Biochim Biophys Acta 104:359–371

Suzuki I (1994) Sulfur-oxidizing enzymes. Methods Enzymol 243:455–462

Suzuki I, Silver M (1966) The initial product and properties of the sulfur-oxidizing enzyme of thiobacilli. Biochim Biophys Acta 122:22–33

Taylor BF (1968) Oxidation of elemental sulfur by an enzyme system from *Thiobacillus neapolitanus*. Biochim Biophys Acta 170:112–122

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100–180

Timmer-Ten Hoor A (1981) Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared with *Thiobacillus denitrificans*. Antonie Van Leeuwenhoek 47:231–243

Trudinger PA (1964) Evidence for a four-sulphur intermediate in thiosulphate oxidation by *Thiobacillus* X. Aust J Biol Sci 17: 577–579

Trüper HG (1994) Reverse siroheme sulfite reductase from *Thiobacillus denitrificans*. Methods Enzymol 243:422–426

Vairavamurthy A, Manowitz B, Luther GW, Jeon Y (1993) Oxidation state of sulfur in thiosulfate and implications for anaerobic energy metabolism. Geochim Cosmochim Acta 57:1619–1623

Van Niel CB (1931) On the morphology and physiology of the purple and green sulfur bacteria. Arch Mikrobiol 3:1–112

Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21:195–213

Visser JM, De Jong GAH, Robertson LA, Kuenen JG (1997) A novel membrane-bound flavocytochrome *c* sulfide dehydrogenase from the colourless sulfur bacterium *Thiobacillus* sp. W5. Arch Microbiol 167:295–301

Wood AP, Kelly DP (1986) Chemolithotrophic metabolism of the newly-isolated moderately thermophilic, obligately autotrophic *Thiobacillus tepidarius*. Arch Microbiol 144:71–77