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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

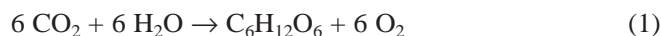
Introduction

Three distinct but interrelated problems are discussed in this review. The first is a consideration of the energy re-

quirement, 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.

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 ΔG° 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:



The standard Gibbs free-energy requirement for this process (calculated using the ΔG_f° values of Table 1) is +470.6 kJ (mol CO₂)⁻¹.

The principal process whereby CO₂ 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:



where P_i represents H₃PO₄.

The $\Delta G^{\circ\prime}$ value for CO₂ reduction to the level of hexose by this equation is given by Stryer (1988) as +114 kJ (mol CO₂)⁻¹. This value is only one quarter of that for Eq. 1, because it describes the energetics of the actual process of CO₂ fixation rather than the overall process of CO₂ fix-

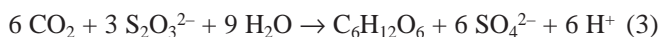
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Table 1 Values for Gibbs free energies of formation from the elements for the compounds for which free-energy changes in their reactions are described in Tables 4–6 or in the text [derived from or recalculated from Latimer (1952), Brasted (1961), Thauer 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)

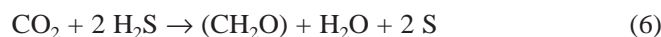
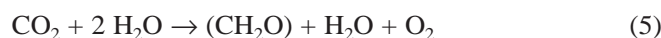
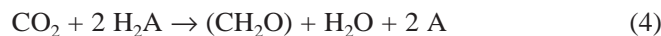
Compound	Gibbs free energy of formation (ΔG_f° as kJ mol ⁻¹)	
S ₂ O ₃ ²⁻	-513.4	(-501.7)
S ₃ O ₆ ²⁻	-958.1	(-944.3)
S ₄ O ₆ ²⁻	-1,022.2	(-959.8)
H ₂ S ₄ O ₆	-991.6	(-1,039.1)
S ₅ O ₆ ²⁻	-956.0	(-920.1)
S ₆ O ₆ ²⁻	ND ^a	(-935.5)
S ₇ O ₆ ²⁻	ND ^a	(-895.8)
S ²⁻	-85.8	
HS ⁻	-12.1	
H ₂ S	-27.6	
SO ₃ ²⁻	-486.6	(-495.4)
HSO ₃ ⁻	-527.8	
H ₂ SO ₃	-537.9	
SO ₄ ²⁻	-744.6	
HSO ₄ ⁻	-756.0	
H ₂ SO ₄	-742.0	
COS	-169.2	
SCN ⁻	+88.7	
HSCN	+92.7	
OCN ⁻	-98.7	
HOCN	-120.9	
H ₂ O	-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 ₂ OH	-23.4	
CN ⁻	+165.7	
C ₆ H ₁₂ O ₆ (fructose)	-915.4	

^aPublished data for polythionates with six or more S-atoms have not been found in the literature, so the ΔG_f° 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 ΔG° for CO₂ 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 CO₂ reduction. Their argument for a lower ΔG° for CO₂ fixation is derived from their equation (Nelson and Hagen 1995):

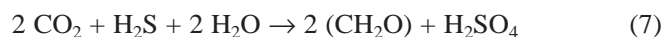


From this equation, a ΔG° for CO₂ fixation of +101.3 kJ (mol CO₂ fixed)⁻¹ can be calculated [using the data of Table 1, including the standard ΔG_f° value for H⁺ of zero, rather than the pH 7.0 value of -39.87 kJ mol⁻¹ (Thauer et al. 1977) used in Nelson and Hagen’s (1995) calculation]. This equation 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:



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.

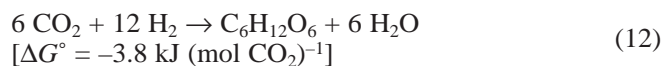
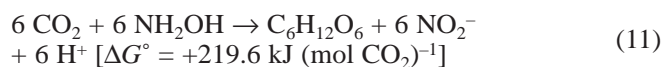
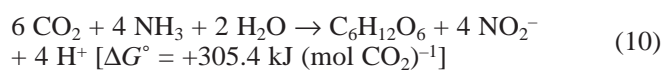
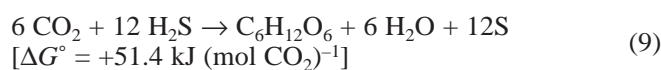
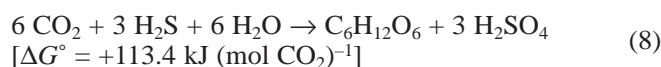
Van Niel (1931) also considered the case (in purple sulfur bacterial photosynthesis) in which carbon dioxide fixation was coupled to the oxidation of sulfide to sulfate:



While Eqs 1 and 3–7 adequately describe the overall stoichiometries of CO₂ 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 CO₂ 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 CO₂)⁻¹ for Eq. 5 and +15 kcal (mol CO₂)⁻¹ for Eq. 6 [i.e. +481 kJ (mol CO₂)⁻¹ and +63 kJ (mol CO₂)⁻¹, respectively]. Larsen (1953) has also calculated the ΔG_{298} for Eq. 3 above as being +29.15 kcal (mol CO₂)⁻¹ [i.e. +122 kJ (mol CO₂)⁻¹], which is close to the currently accepted value.

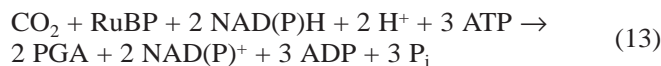
It must be remembered, however, that there is no direct chemical coupling between sulfur compound oxidation and the process of CO₂ reduction: the former serves only to generate the NAD(P)H and ATP required for the carboxylation of ribulose biphosphate and reduction to hexose. CO₂ reduction by the Calvin 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 CO₂ fixation provide only a guide to the overall process and its energetics (Larsen 1954; Nelson and Hagen 1995), and using ΔG° values (for both sulfur compound oxidation and CO₂ 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 ΔG° values for CO₂ fixation vary as a consequence of the differing redox potentials of the electron-donating couples (which may be multi-component for sulfur compound and ammonia oxidations; e.g. HS⁻/S⁰, $E_0' = -270$ mV; S⁰/HSO₃⁻, +50 mV; HS⁻/HSO₃⁻, -116 mV; HSO₃⁻/SO₄²⁻, -516 mV; SO₃²⁻/SO₄²⁻, -522 mV; NH₄⁺/NO₂⁻, +344 mV; NH₄OH/NH₂OH, +562 mV; NH₂OH/NO₂⁻, +66 mV; H₂/H⁺, -414 mV (Thauer et al. 1977; Kelly 1978, 1982; R. K. Thauer, Universität Marburg, Germany, personal communication):



The variation between these values shows that this mode of calculation provides a value for the entire coupled process of substrate oxidation and CO₂ fixation, while the absolute ΔG° for CO₂ 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 CO₂ fixation for the actual conversion catalyzed by the Calvin cycle:



The energy requirements are indicated by the reductive conversion of carbon dioxide and ribulose biphosphate (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 ΔG° 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 CO₂ fixation.

For the purposes of the comparative thermodynamic calculations presented in this review I have chosen to use the mean of the four ΔG° values presented above for Eqs.

2, 3 and 8, namely +113 kJ (mol CO₂)⁻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 ΔG° values for sulfur compound oxidation and CO₂ 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:



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:

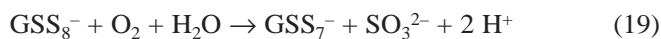


Experimentally, the product observed was thiosulfate because of the chemical reaction of the sulfite product with the sulfur substrate:



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:



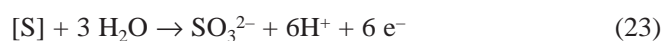
It is interesting that reduced glutathione and glutathione persulfides (GSS_n^-) are implicated in this oxygenase process since sulfanes (such as GSS^- , glutathione trisulfide, thiosulfate and tetrathionate) are also established as remarkably strong activators of cytochrome *c* reduction by reduced 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 $\mu\text{mol min}^{-1}$, and the ethanol precipitate from that fraction had further increased the total activity recovered to 24.43 $\mu\text{mol min}^{-1}$. 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:



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 mea-

sured 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 $\text{SO}_4^{2-}/\text{HSO}_3^-$ and $\text{SO}_4^{2-}/\text{SO}_3^{2-}$ 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)		
<i>Thiobacillus denitrificans</i>	18.54	11.60
<i>Thiomicrospira denitrificans</i>	9.38	5.72
True growth yields (Y_{max} ; Kelly 1990)		
<i>Thiobacillus neapolitanus</i>	10.8	—
<i>Thiobacillus tepidarius</i>	14.0	—
<i>Thiobacillus denitrificans</i>	20.6	14.5

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

Source of enzyme	Molecular mass (Da)		Reference
	Oligomer	Subunit	
<i>Acidianus brierleyi</i>	560,000	35,000	Emmel et al. (1986)
<i>Desulfurolobus ambivalens</i>	550,000	40,000 (35, 317 ^a)	Kletzin (1992, 1994)
<i>Thiobacillus thiooxidans</i>	46,000	23,000	Suzuki (1994)
<i>Thiobacillus ferrooxidans</i>	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 CO₂ 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 (ΔG_f° in kJ mol⁻¹). Table 1 provides the best estimates available from the literature for ΔG_f° values for most of the substrates and products of the sulfur chemolithotrophs. Included in Table 4 are ΔG_f° 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 SO₃²⁻ (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	ΔG° [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 [ΔG° , 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 ΔG° values. These ΔG° 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	ΔG° (mean \pm SE) [kJ (mol S-substrate) ⁻¹]
Thiosulfate to sulfate	-750.1 \pm 16.1
Sulfide to sulfate	-701.8 \pm 31.4
Sulfide to sulfur	-168.6 \pm 28.9
Sulfur to sulfate	-537.9 \pm 43.7
Sulfur to sulfite	-280.2 \pm 22.2
Sulfite to sulfate	-230.1 \pm 22.0

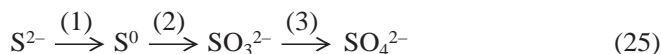
The limits of energy conservation from sulfur oxidation reactions

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 ΔG° 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 ΔG° 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:



The average ΔG° 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 sulfide to sulfite (step 3). The energy-conserving steps (1 and 3) would provide (as ΔG°) -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%.

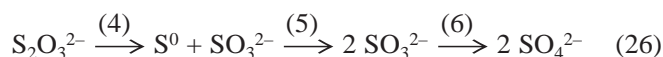
Table 6 Free-energy changes for some reactions of organosulfur compounds, which are substrates for some *Thiobacillus thioparus* and some *Paracoccus* strains

Reaction	ΔG° [kJ (mol S-substrate) $^{-1}$]
$\text{SCN}^- + 2 \text{O}_2 + 2 \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + \text{CO}_2 + \text{NH}_4^+$	-824.5
$\text{SCN}^- + 2 \text{O}_2 + 2 \text{H}_2\text{O} \rightarrow \text{HSO}_4^- + \text{CO}_2 + \text{NH}_3$	-783.0
$\text{HSCN} + 2 \text{O}_2 + 2 \text{H}_2\text{O} \rightarrow \text{H}_2\text{SO}_4 + \text{CO}_2 + \text{NH}_3$	-772.9
Oxidation of thiocyanate to sulfate	-793.5 ± 22.3
$\text{SCN}^- + \text{H}_2\text{O} \rightarrow \text{H}_2\text{S} + \text{OCN}^-$	+22.1
$\text{SCN}^- + \text{OH}^- \rightarrow \text{HS}^- + \text{OCN}^-$	+42.2
$\text{HSCN} + \text{H}_2\text{O} \rightarrow \text{H}_2\text{S} + \text{HOCN}$	+4.1
Hydrolysis of thiocyanate to sulfide	$+22.8 \pm 15.6$
$\text{COS} + 2 \text{O}_2 + 2 \text{H}_2\text{O} \rightarrow \text{H}_2\text{SO}_4 + \text{CO}_2$	-721.6
$\text{COS} + 2 \text{O}_2 + 2 \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + \text{CO}_2 + 2 \text{H}^+$	-724.3
$\text{COS} + 2 \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{HSO}_4^- + \text{CO}_2 + \text{H}^+$	-735.7
Oxidation of carbonyl sulfide to sulfate	-727.2 ± 6.1
$\text{COS} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2\text{S}$	-7.5
$\text{COS} + \text{OH}^- \rightarrow \text{CO}_2 + \text{HS}^-$	-71.6
Hydrolysis of carbonyl sulfide to sulfide	-39.6 ± 32.1

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 $(\Delta G^\circ \text{ for oxidation})/(\Delta G^\circ \text{ for } \text{CO}_2 \text{ fixation}) = (\Delta G^\circ \text{ for oxidation})/113 = \text{mol } \text{CO}_2 \text{ fixed (mol substrate oxidized for energy)}^{-1}$. 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	ΔG° for complete oxidation (kJ mol $^{-1}$)	Theoretical CO_2 fixation	Observed CO_2 fixation	Apparent efficiency of energy conservation (%)
<i>Thiobacillus tepidarius</i>				
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
<i>Thiobacillus neapolitanus</i>				
Thiosulfate	-750.1	6.64	0.40	6.0
Trithionate	-801.3	7.09	0.40	5.6
<i>Thiobacillus denitrificans</i>				
Thiosulfate (aerobic)	-750.8	6.64	0.81	12.2
Thiosulfate (denitrifying)	-750.8	6.64	0.57	8.6

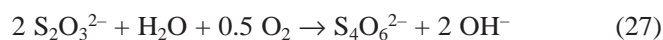
For thiosulfate the following sequence is feasible:



The ΔG° 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 (ΔG°) 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:





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 \rightarrow sulfite and sulfite \rightarrow 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 $^{14}\text{CO}_2$ 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 [$+470.6 \text{ kJ (mol CO}_2\text{)}^{-1}$; 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 $+113 \text{ kJ (mol CO}_2\text{)}^{-1}$ 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 ox-

idation 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 biphosphate carboxylase is not its *raison d'être*.

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