Review Letter

A CHEMIOSMOTIC MODEL FOR SULPHATE RESPIRATION

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1. Introduction

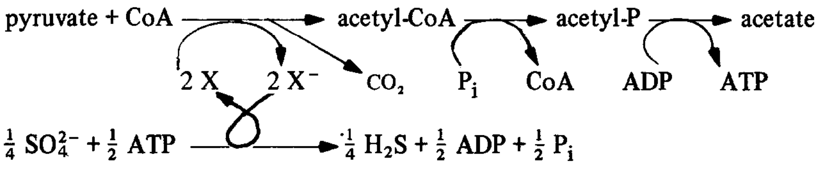
The reduction of sulphate to sulphide is part of normal biosynthesis in all organisms that use sulphate as a source of sulphur. By contrast, the coupling of sulphate reduction to net ATP synthesis (dissimilatory sulphate reduction or sulphate respiration) is confined to about three genera of anaerobic bacteria. The redox proteins and energetics of these bacteria have been the subject of many studies, mostly with species of *Desulfovibrio*, and these have been well summarised by Siegel [1] and Thauer et al. [2]. Most of this work has dealt either with whole cells or with purified proteins, and the middle ground between these levels of investigation has received much less attention. In particular, there has been no concrete proposal for the coupling of electron transport to ATP synthesis. Yet sulphate respirers have membrane-bound redox components which are by no means unusual, including menaquinone, *b*-type cytochromes, and fumarate reductase [2,3]. They also possess an uncoupler-sensitive ATPase [2,4,5]. In other bacteria with these features the chemiosmotic hypothesis has proved very fruitful as a basis for explaining phosphorylation coupled to electron transport [3,6], and this review will attempt to analyse sulphate respiration in the same way.

Sulphate respiration is unusual in that ATP has to be expended to prime the sulphate prior to reduction, thereby raising its extremely low redox potential, $E_{m,7}(SO_4^{2-}/SO_3^{2-}) = -480$ mV [7], and enabling it to act as an oxidant:

$$SO_4^{2-} + ATP^{4-} + H^+ \xrightarrow{\text{ATP-sulphurylase (EC 2.7.7.4)}} APS^{2-} + PP_i^{3-}$$

The pyrophosphate is almost certainly decomposed by pyrophosphatase (EC 3.6.1.1) [2]. It is therefore essential that more than two molecules of ATP can be synthesised by substrate level or electron transport phosphorylation in the course of the 8 e^- reduction of APS to H_2S, and that sulphate respiration is tightly coupled.

The popular modes of growth of these organisms are with lactate or pyruvate as reductant and carbon source, or with hydrogen as reductant and acetate as carbon source [2]. In the latter case, there is no possibility for substrate level phosphorylation. During growth on lactate or pyruvate the only substrate level phosphorylation is that catalysed by acetate kinase (EC 2.7.2.1), since acetate is not catabolised further. Thus with pyruvate as substrate and sulphate reduction simply used as an electron sink, a net synthesis of 0.5 ATP/pyruvate would be possible:



Abbreviation: APS, adenylylsulphate

However, if the additional two electrons from lactate were wasted in the same way, the net ATP yield would be zero.

2. Redox proteins

2.1. *Dehydrogenases*

In *Desulfovibrio*, lactate oxidation is mediated by a membrane-bound dehydrogenase, independent of NAD or NADP [2]. Pyruvate oxidation is brought about by a soluble enzyme, pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) [2]. Hydrogen oxidation is catalysed by hydrogenase. Part of the hydrogenase activity is soluble, and the enzyme has been purified (EC 1.12.2.1), while the rest is membrane-bound [2]. A soluble formate dehydrogenase has also been partly purified [2]. It should be noted that there is not normally a membrane-bound NADH dehydrogenase [2,8], and little is known about the roles of NAD and NADP in catabolism.

2.2. *Reductases*

APS is reduced to sulphite and AMP by APS-reductase (EC 1.8.99.2), a soluble enzyme with bound FAD and non-haem iron [1]. The physiological donor is uncertain, but is not NAD(P)H; reduced methyl viologen is the most popular donor in vitro [1]. Sulphite is reduced by bisulphite reductase (EC 1.8.99.1), so named because its pH optimum is below $pK(SO_3^{2-}/HSO_3^-) = 6.8$ [2]. This enzyme is also soluble; it has sirohaem (an iron-dihydrochlorin) as its prosthetic group [1]. It has been named desulfoviridin in *D. gigas*, desulforubidin in *D. desulfuricans*, and P582 in *Desulfotomaculum*.

Both of these reductases are very similar to their assimilatory counterparts. APS-reductase is widely distributed in photosynthetic organisms, while most non-photosynthetic bacteria have the closely related 5′-phospho-APS-reductase [9,10]. Sulphite reductases with sirohaem are also widespread. In plants 1 e$^-$ donors are effective, as in sulphate respirers, while in most non-photosynthetic organisms the enzyme complex includes a flavoprotein, and NAD(P)H is the donor [9]. It should be emphasised that the assimilatory enzymes have electron donors which rule out energy conservation. The respiratory enzymes have different specifities: this is the key to sulphate respiration.

There has been controversy as to whether bisulphite reductase functions by 6 e$^-$ reduction to H_2S, as for the assimilatory enzyme, the alternative being a 2 e$^-$ reduction to give trithionate ($S_3O_6^{2-}$), reduced further by separate trithionate and thiosulphate reductases. The evidence is thoroughly reviewed by Siegel [1], who concludes that all the bisulphite reductases which have been purified can catalyse the complete 6 e$^-$ reduction, while trithionate and thiosulphate are side-products at high sulphite concentrations from reaction of enzyme-bound intermediates with further sulphite. An indirect argument in favour of trithionate as an obligatory intermediate has been that lactate oxidation with APS or sulphite as electron acceptor has too small a free energy change for coupling to phosphorylation to be possible [1,11]. The redox potentials are: $E_{m,7}$ (pyruvate/lactate) = −190 mV; $E_{m,7}$ (APS/AMP, SO_3^{2-}) = −60 mV; $E_{m,7}(SO_3^{2-}/HS^-) = -116$ mV [1,2]. The line of argument overcomes this problem by postulating that phosphorylation is coupled solely to trithionate reduction, $E_{m,7}(S_3O_6^{2-}/S_2O_3^{2-},SO_3^{2-}) = +230$ mV [2]. However this has not been experimentally demonstrated. In practice, lactate oxidation is followed immediately by pyruvate oxidation, $E_{m,7}$(acetyl-CoA, CO_2/pyruvate, CoA) = −490 mV. If both oxidations were coupled identically to proton pumping and ATP synthesis, the very negative $\Delta G^{0\prime}$ of the second reaction would compensate for the less favourable $\Delta G^{0\prime}$ of the first, making the capacity for ATP synthesis the same as if both donors had $E_{m,7}$(acetyl-CoA, CO_2/lactate, CoA) = −340 mV. With $\Delta E_m \approx$ 225 mV, 1 ATP/2 e$^-$ is just feasible. In the model presented below, only 5/8 (0.625) ATP/2 e$^-$ is predicted for this system. Thus there is no thermodynamic necessity for trithionate. From kinetic considerations the trithionate pathway would be unlikely at low sulphite concentrations since it is effectively termolecular with respect to sulphite: in vitro about 10 mM sulphite and a favourable pH are needed for a good yield [1,2]. The ATP yields given in section 5 show that cells growing on sulphate cannot afford to let sulphite escape unreduced into the medium, and this implies that cytoplasmic sulphite concentrations are likely to be low. In the model presented below, 6 e$^-$ reduction will be assumed to predominate.

Only two other reductases are well characterised. Thiosulphate reductase is a soluble protein [1], while fumarate reductase is membrane-bound; in most media its role is mainly biosynthetic [2].

2.3. *Soluble electron carriers*

There are a profusion of soluble electron carriers in *Desulfovibrio*. Cytochrome c_3 has 4 independently acting haems packed into mol. wt 13 000, all with redox potentials of around –300 mV [12]. Cytochrome *c*-553, 1 haem/molecule, has no known general role [13]. It is partly reduced by ascorbate, suggesting $E_{m,7} \approx +50$ mV, and shows sequence homology with mitochondrial cytochrome *c* [14,15]. Cytochrome cc_3 has been studied less; it has mol. wt ~26 000 [16]. Rubredoxin, $E_{m,7} = -60$ mV, with a 1 Fe active centre, has no clear role [17].

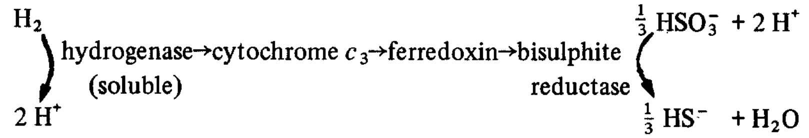
Ferredoxin, with one 4 Fe/4 S centre (as in *Bacillus polymyxa*), and flavodoxin, with FMN, both act as low potential 1 e^- carriers, the latter operating between semiquinone and fully reduced states [17,18]. Their properties seem to be much as in other bacteria that contain both [18,19]. The typical interrelation is that in vitro flavodoxin can substitute for almost all of the reactions of ferredoxin, in some species synthesis of flavodoxin is greatly stimulated by iron deficiency at the expense of ferredoxin (thereby sparing the cells' requirement for iron), while in others this is not so and there may be slight functional differences. *D. gigas* seems to be an example of the first type of behaviour, though only a 5-fold change in ratio has been achieved [20], while in *D. vulgaris* flavodoxin always predominates [21]. In this paper, 'ferredoxin' implies either carrier, in the absence of clear kinetic distinctions.

3. Compartmentation

With so many soluble carriers, it is very important to distinguish those present in the periplasm from those found in the cytoplasm. Work with other bacteria shows that a location in both compartments is unlikely, probably because of the manner of synthesis of proteins which have to be exported to the periplasm [22,23]. The key study is that of Bell et al. [24]. They worked with *D. gigas*, and found that in the course of spheroplast formation the soluble hydrogenase and cytochrome c_3 were released in high yield, but only very small amounts of APS-reductase, bisulphite reductase, ferredoxin, flavodoxin and rubredoxin. These latter proteins were all released when the spheroplasts were broken, indicating a cytoplasmic location. For *D. vulgaris*, similarly, hydrogenase and cytochrome c_3 could be released wtihout bisulphite reductase [25].

These results are not surprising, and are likely to be general, since sulphate reduction is constrained to the cytoplasm by its ATP requirement, while ferredoxin has a central role in bacterial metabolism, including pyruvate:ferredoxin oxidoreductase. A periplasmic *c*-type cytochrome found in anaerobically-grown *Escherichia coli* has a redox potential similar to cytochrome c_3 (–200 mV), and generally similar mediatory properties [2,16,26].

It follows that direct interaction of cytochrome c_3 with ferredoxin, a reaction studied in depth with purified proteins [27], cannot occur under physiological conditions. For the same reason, electron transport chains such as the following [11,28,29] can be ruled out:



With the four purified proteins this works well, but it could not be coupled chemiosmotically to phosphorylation because all the carriers are soluble and thus no proton gradient could be generated.

4. Electron transport chains

4.1. *Hydrogen as reductant*

Hydrogen oxidation is coupled to phosphorylation with fumarate, sulphate (APS) or sulphite as acceptor [2,4,5]. In the case of fumarate reduction, a membrane preparation was active without any soluble proteins [4]. This points to the membrane-bound hydrogenase being active, as in aerobic hydrogen bacteria [30], rather than the periplasmic hydrogenase coupled through cytochrome c_3. All of these hydrogen-driven phosphorylations are uncoupled by catalytic amounts of methyl viologen [4,31]. Methyl viologen is a quaternary ammonium base and its redox potential, $E_m(\text{MeV}^{2+}/\text{MeV}^{+}) = -446$ mV, is independent of pH [32,33]. It could not act as a weak acid type of uncoupler. If H_2 oxidation ($H_2 \rightarrow 2\ H^+ + 2\ e^-$) and substrate reduction ($S + 2\ H^+ + 2\ e^- \rightarrow SH_2$) took place on opposite sides of the membrane, bypassing of membrane-bound electron carriers by methyl viologen would make no difference to proton gradients set up by electron transport. The uncoupling would, however, be readily explained if methyl viologen bypassed H-atom carriers, allowing a membrane-bound hydrogenase sited near the cytoplasmic side of the membrane to function so that protons from H_2 ended up in the cytoplasm, instead of being conveyed to the periplasm by the operation of the membrane-bound chain. Provided a little reduced viologen can be formed externally and then enter, this mechanism is not dependent on the dication being permeant.

4.2. *Cytochrome* c_3

What role does this leave for cytochrome c_3? It is very efficient at scavenging oxygen [13]. It has been shown to equilibrate efficiently with the periplasmic hydrogenase [2,34]. It also accepts electrons from a purified formate dehydrogenase, though it is not known whether this is a periplasmic enzyme [2]. The efficiency of its connection with the main electron transport chain remains to be established. To the author, these properties suggest that cytochrome c_3 can usefully be regarded as a periplasmic analogue of ferredoxin, linking a variety of oxidoreductions which the cell prefers to perform outside the cytoplasm.

In addition, there are other lines of evidence against a central role for cytochrome c_3 in sulphate respiration. Soluble *c*-type cytochromes are absent from the genus *Desulfotomaculum*, in which sulphate respiration is very similar to *Desulfovibrio* [2]. *Desulfuromonas acetoxidans* contains a cytochrome (c_7, *c*-551.5) showing close sequence homology with cytochrome c_3, yet this organism cannot respire sulphate or sulphite, although elemental sulphur can be reduced [35–37]. Similar cytochromes also seem to be present in some photosynthetic bacteria [38].

4.3. *Electron donors for APS and bisulphite reductases*

Lactate oxidation by the membrane-bound lactate dehydrogenase, like hydrogen oxidation, can be coupled to sulphate (APS) and sulphite reduction [1,2]. This leads to the important conclusion that APS-reductase and bisulphite reductase must both be able to accept electrons from the membrane-bound electron transport chain, either directly or through some unidentified intermediate (e.g., rubredoxin).

When pyruvate oxidation is linked to sulphate and sulphite reduction, the initial electron carrier is ferredoxin [2]. This could either react directly with the sulphur reductases, or pass its electron on to the membrane-bound chain. The crucial point is that membrane-bound intermediates could move protons across the membrane in the course of the reaction, whereas direct interaction would rule this out. The direct interaction of ferredoxin with APS-reductase has not been reported, but direct reaction has been demonstrated for bisulphite reductase, as implied in section 3 [39]. Measurement of molar growth yields with pyruvate as substrate has given evidence for electron transport coupled to phosphorylation during growth with sulphate, but not with sulphite [40]. Taken together, these observations point strongly to ferredoxin being able to pass its electron to bisulphite reductase directly, but only to APS-reductase via the membrane bound chain.

There are useful analogies in plant and green bacterial photosynthesis. Ferredoxin is thought to be oxidised by membrane-bound *b*-type cytochromes during cyclic electron flow, while pyridine nucleotides only interact with the electron transport chain through ferredoxin:NADP reductase [19]. Ferredoxin and APS-reductase are present together in *Chlorobium*, but do not interact: when sulphite is electron donor for non-cyclic electron flow to $NADP^+$ its oxidation is accomplished by APS-reductase operating in reverse (with APS as product, converted to ATP and SO_4^{2-} by ADP-sulphurylase (EC 2.7.7.5)), while reduced ferredoxin is an intermediate after the photoreaction [1,19].

5. Stoichiometries for ATP synthesis

The model which has been built up in section 4 has all pairs of oxidants and reductants coupled

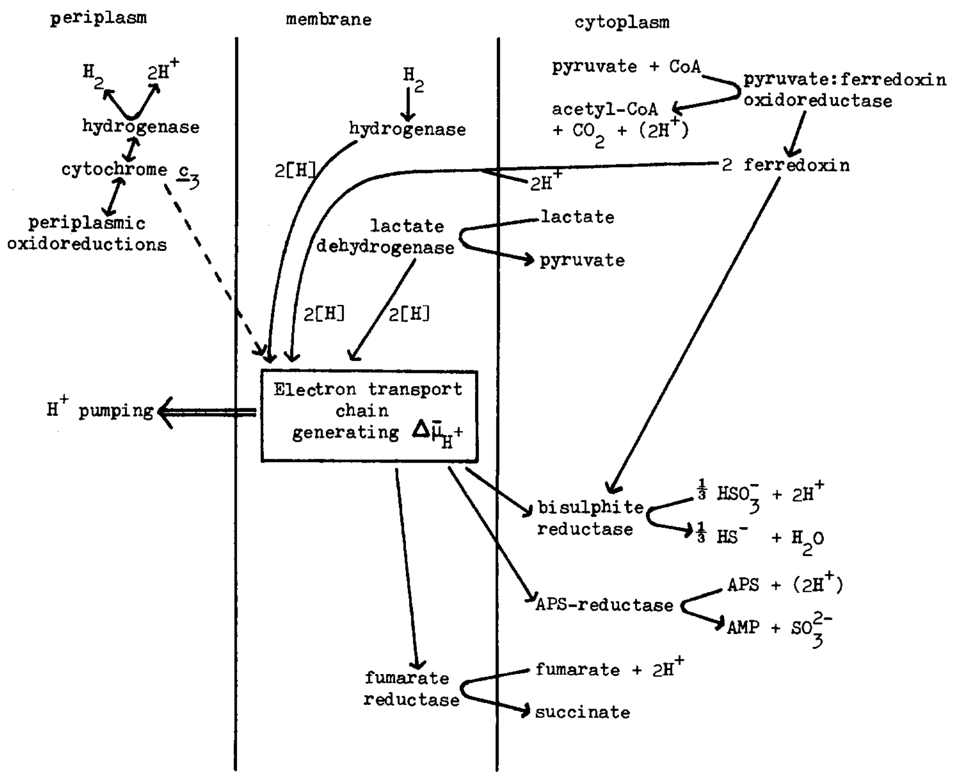


Fig.1. Proposal for electron transport in *Desulfovibrio.* The figure shows a scheme for coupling various 2 equiv. oxidations and reductions to the generation of a proton gradient across the cytoplasmic membrane. The proton numbers in brackets are those for the complete reactions:

$$\text{pyruvate}^- + H_2O \rightarrow \text{acetate}^- + CO_2 + 2\,H^+ + 2\,e^-$$

and

$$SO_4^{2-} + 2\,H^+ + 2\,e^- \rightarrow SO_3^{2-} + H_2O$$

through the membrane-bound chain, except for sulphite reduction with pyruvate as electron donor. The complete scheme is shown in fig.1, which is deliberately vague about the proton pumping mechanism, since all that is known is that the same components are present as in other bacteria (see section 1).

If one assumes that 2 H^+ are lost from the cytoplasm and gained by the periplasm for every 2 e^- passing down the membrane-bound chain, as in fumarate reduction by other bacteria [2], and that 2 H^+ returning through the ATPase lead to synthesis of 1 ATP [6], then provisional estimates can be made for ATP yields from electron transport. The arithmetic is made easier by the fact that all the redox couples balance in terms of protons, when written as in fig.1. Table 1 lists predicted values, in terms of mol ATP/mol reductant, and corrects them for substrate level phosphorylation and sulphate priming (see section 1) to give net ATP yields. These are compared with experimental values. It should be noted that most of the experimental measurements with whole cells have no correction for maintenance energy, while membrane preparations are often poorly coupled. With these factors in mind, agreement is reasonably good, though two experimental values for pyruvate are appreciably higher than the prediction.

Table 1
Experimental and predicted stoichiometries for ATP synthesis

Reductant	Oxidant	ETP predicted from model	SLP minus sulphate priming	Predicted net ATP	Experimental net ATP
		(mol ATP/mol reductant)			
Hydrogen	Sulphate→ sulphide	1.0	−0.5	0.5	$0.24^{c,e}$
Hydrogen	Sulphite→ sulphide	1.0	–	1.0	$0.2^{b,d}$
Hydrogen	Thiosulphate sulphide	0.75^{g}	–	0.75	$0.65^{c,e}$
Hydrogen	Fumarate→ sulphide	1.0	–	1.0	$0.3^{b,d}$
Formate→ CO_2	Sulphate→ sulphide	1.0^{h}	−0.5	0.5	$0.28^{c,f}$
Pyruvate→ acetate	Sulphate→ sulphide	0.25	+0.5	0.75	$0.92^{c,f}$, $0.72^{b,f}$
Lactate→ acetate	Sulphate→ sulphide	1.25	–	1.25	$0.63^{a,f}$, $0.89^{a,f}$ $0.57^{c,f}$, $0.18^{c,f}$ $0.50^{a,f}$, $0.94^{a,f}$

[a] *D. desulfuricans* [41,42]
[b] *D. gigas* [5,8,40]
[c] *D. vulgaris* [40,43]
[d] Membrane preparation [5,8]
[e] Y^{max}_{ATP} taken as 12.8 $g.mol^{-1}$, the mean of 3 values proposed [43]
[f] Y^{max} not computed. Y_{ATP} taken as 10.5 $g.mol^{-1}$, as in [40]
[g] Assuming no protons are pumped in the course of thiosulphate reduction by H_2 ($H_2 + S_2O_3^{2-} \rightarrow SO_3^{2-} + H_2O$), for which $\Delta G^{0\prime}$ is near zero [2]
[h] Assuming that formate oxidation is coupled as for H_2 oxidation

The predicted values for electron transport phosphorylation (ETP) are derived from the scheme of fig.1 as described in the text. They are corrected for substrate level phosphorylation (SLP) and sulphate priming (assuming 2 ATP/SO_4^{2-}), in order to give a net value for ATP synthesis. The experimental values are from growth studies except where stated. The whole cell values are $Y_{reductant}/Y_{ATP}$, or $Y^{max}_{reductant}/Y^{max}_{ATP}$ where available: Y is the molar growth yield in g dry cells/mol reductant or ATP, and Y_{max} is an extrapolation to infinite growth rates in order to eliminate maintenance energy

In making valid predictions there are, however, several further complications which have not been taken into account, and which can only be resolved by further experimentation:

(i) Proton uptake during entry of lactate or pyruvate into the cytoplasm, not compensated during exit of acetate;
(ii) Net proton movements in entry of SO_4^{2-} and exit of HS^- or H_2S ($pK \approx 7$);
(iii) Some measure of direct reaction of ferredoxin with APS-reductase, or with the membrane chain in preference to bisulphite reductase;
(iv) Movement of less than 2 $H^+/2\ e^-$ across the membrane for some or all of the reactions involving membrane-bound components;
(v) Thiosulphate or trithionate formation at high sulphite levels.

At any rate, it is hoped that this analysis will be useful for designing further experiments.

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