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Respiration-driven proton translocation in *Thiobacillus versutus* and the role of the periplasmic thiosulphate-oxidizing enzyme system

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Abstract. The periplasmic location of enzymes A and B of the thiosulphate-oxidizing multienzyme system of *Thiobacillus versutus* has been further confirmed by differential radiolabelling of periplasmic and cytoplasmic proteins. The stoichiometries of respiration-driven proton translocation in *T. versutus* were determined using the oxygen pulse and the initial rate methods. A value for the $\rightarrow H^+/O$ quotient (number of protons translocated per oxygen atom reduced) of about 2.8 was found for the oxidation of thiosulphate, and of about 2.5 for sulphite. The $\rightarrow H^+/O$ quotient for endogenous respiration was about 5.7. The data are shown to be in good agreement with the scheme proposed previously for thiosulphate oxidation by this organism. Proton generation during the oxidation of thiosulphate or sulphite is indicated to occur in the periplasm rather than by pumping across the cytoplasmic membrane. The results also suggest that a $\rightarrow H^+/O$ quotient of six occurs during NADH oxidation (from endogenous metabolism measurements) and that the terminal cytochrome oxidase, aa_3, does not function as a proton pump.

Key word: *Thiobacillus versutus* – Proton translocation – Thiosulphate oxidation – Sulphite oxidation – Periplasmic proteins

Hooper and DiSpirito (1985) have proposed that bacteria which grow on simple reductants, like thiosulphate, generate a proton gradient by oxidizing the substrate extracytoplasmically. Thus protons would be expected to be produced on the periplasmic side of the cytoplasmic membrane in organisms such as thiobacilli, whereas proton and oxygen consumption by terminal oxidases would occur on the cytoplasmic side of the membrane. To date there has been little firm evidence for this hypothesis in the thiobacilli (Hooper and DiSpirito 1985).

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Abbreviations: DCCD, dicyclohexyl carbodiimide; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; HQNO, 2-n-heptyl-4-hydroxyquinoline N-oxide; TMPD, N,N,N′,N′-tetramethyl-p-phenylenediamine; IEF, isoelectric focusing; HIC, hydrophobic interaction chromatography; EAI, ethyl acetimidate hydrochloride; IAI, isethionyl acetimidate

We have shown (Lu and Kelly 1984a) that the chemolithotrophic oxidation of thiosulphate by *Thiobacillus versutus* can be dissected in two component processes, as described by reactions (1) and (2):

$$S_2O_3^{2-} + 5H_2O = 2SO_4^{2-} + 10H^+ + 8e^- \quad (1)$$

$$8H^+ + 8e^- + 2O_2 = 4H_2O \quad (2)$$

Reaction (1) was catalysed by a multienzyme system consisting of four major components (Lu and Kelly 1983b, 1984b), which was located on the periplasmic side of the membrane (Lu 1986). The electrons released from the oxidation reactions coupled with the electron transport chain at the level of cytochrome *c* (Lu and Kelly 1983a) and finally reduced oxygen through the cytochrome oxidase aa_3 (reaction 2) on the cytoplasmic side of the membrane (Lu 1986). Consequently ten protons would be released into the periplasmic space by the operation of these reactions, thus establishing an electrochemical proton gradient by a mechanism entailing an electron motive redox arm without actual proton movement across the membrane (Lu 1986). It has also been shown (Lu and Kelly 1983a) that crude extracts of *T. versutus* catalysed thiosulphate-oxidation-dependent oxidative phosphorylation with a P/O ratio of about one.

The experiments described in the present paper were designed to measure proton translocation in intact *T. versutus*, measured by oxygen pulse and initial rate methods, using thiosulphate, sulphite and endogenous oxidation substrates. The results are analysed for their consistency with the scheme proposed earlier for thiosulphate oxidation (Lu 1986) and for consistency of the thiosulphate-oxidation-linked $\rightarrow H^+/O$ quotient with the equation of reaction (1).

Materials and methods

Growth and harvesting of Thiobacillus versutus

The organism was grown in batch or chemostat culture at 30° C in a medium with thiosulphate as energy source as described previously (Lu 1986; Wood and Kelly 1977). Culture pH was maintained at about 7.5 by manual addition of 2 M Na_2CO_3 to batch cultures, using phenol red as the indicator, or by automatic titration of continuous cultures. The chemostat culture (working volume, 1.5 l) was growth-limited by 30 mM thiosulphate and was maintained at a dilution rate of 0.08 h^{-1}. Organisms collected from batch cultures (about 24 h after inoculation) or directly removed from the chemostat, were washed three times in 1.5 mM glycylglycine buffer, pH 7, containing 150 mM KCl, at 15° C and were used within 3 h.

Differential labelling of cytoplasmic and periplasmic proteins with ^{14}C-imidoesters

These methods were based on Kasprzak and Steenkamp (1984) and Whiteley and Berg (1974). *Thiobacillus versutus* grown in batch culture for 30 h on thiosulphate was harvested and the cells washed with 0.05 M Tris/0.2 M KCl, pH 7.2 and then with 0.1 M phosphate, pH 8.0, containing 0.1 M KCl, Washed cells were suspended in the latter buffer to give 50 mg dry wt/ml, and 0.1 ml of suspension incubated at 20° C for 30 min with either (a) 2.9 mM ^{14}C-ethyl acetimidate hydrochloride (EAI; 57 mCi/mmol) or (b) 4 mM ^{14}C-isethionyl acetimidate (IAI; 28 mCi/mmol). In some experiments, cells were pretreated with unlabelled 9 mM IAI at 20° C for 30 min, centrifuged and washed once with 0.1 M phosphate/0.1 M KCl, pH 8.0, then resuspended in 0.1 ml buffer and treated with labelled IAI or EAI. After labelling, the cells were washed with 0.1 M phosphate/0.2 M KCl, pH 8.0, and twice with 0.1 M Tris/0.01 M EDTA, pH 7.8 and finally suspended in 0.15 ml 20 mM phosphate, pH 7.0. Samples were prepared for SDS-PAGE by adding an equal volume of SDS-buffer (60 mM Tris-HCl, pH 6.8, containing (per l) 30 g SDS, 50 ml 2-mercaptoethanol, 200 ml glycerol, 20 mg bromophenol blue) and boiling for 2 – 5 min. As markers for SDS-PAGE, a molecular weight range mixture of proteins (8 mg/ml) was labelled with ^{14}C-EAI for 30 min and treated with SDS-buffer. Specific markers, labelled in the same way, were enzyme A (IEF fraction; 6 mg/ml) and enzyme B (HIC fraction; 6 mg/ml) purified as described previously (Lu and Kelly 1984a). SDS-treated samples (20 µl for cell suspensions, 4 µl for enzyme A and 2 µl each for enzyme B and the marker protein mixture) were loaded on to 12% polycarylamide SDS gel and run at a constant 40 mA current for 4 h at 4° C. Gels were stained for 2 h in 0.4% (w/v) Coomassie Brilliant Blue G in 30% (v/v) methanol containing 5% (v/v) acetic acid, and destained overnight in the methanol-acetic acid mixture. Labelled protein bands were detected essentially by the scintillation autography method of Laskey (1980). Gels were then soaked in two successive 600 ml lots of DMSO for 30 min each time, then in 20% (w/v) PPO in DMSO (150 ml) for 3 h, followed by 600 ml water for 1 h. Gels were then dried in a Bio-Rad dryer for 1 h at 80° C and exposed for several weeks at −70° C to Kodak Royal X-Omat film in a cassette. Labelled bands and known protein markers were identified by comparison of films and stained gels.

Measurement of respiration-linked proton translocation by the oxygen pulse method

The technique used was essentially that of Scholes and Mitchell (1970). An oxygen electrode vessel (Rank Bros, Bottisham, Cambridge, UK) was used as the reaction chamber, which contained in a 3.5 ml volume incubated at 25° C, a reaction mixture of: 150 mM KCl, 1.5 mM glycylglycine; 20 µg valinomycin (or 60 mM KSCN); 35 µg carbonic anhydrase; 8 – 10 mg dry wt of washed cells; substrate as required: 57 µM thiosulphate, 14 µM sulphite, 125 µM ascorbate with 29 µM TMPD, or 125 µM ferrocyanide; and respiratory inhibitors when used. Suspensions were preincubated for 1 h when valinomycin was present and for 15 min with KSCN. Anaerobiosis was established and maintained by passage of a stream of oxygen-free nitrogen over the surface and by the bacterial endogenous respiration. Although the $\rightarrow H^+/O$ quotients were constant over the pH range 6 – 7.2, all the experiments described in the text were initiated at pH 6.4 – 6.6, exact starting pH being adjusted by the addition of small quantities of oxygen-free solutions of KOH or HCl. Proton translocation was induced by the injection of 10 – 25 µl of air-saturated 150 mM KCl at 25° C. This was a standard solution known to contain 4.8 ng-atoms of oxygen in 10 µl. The resulting acidification of the medium was calibrated by additions of 1 – 5 µl of oxygen-free 10 mM HCl. Acidification following addition of oxygen or HCl was complete within 3 – 5 s and the maximum concentration of H^+ produced by an oxygen pulse was estimated from a semilogarithmic plot against time of the decay of the ΔpH (as shown in Fig. 2D). Routinely extrapolation back to the time taken for half the added oxygen to have been reduced gives the peak of H^+ concentration, and the $\rightarrow H^+/O$ quotient was calculated as maximum H^+ generated divided by the ng-atoms O added (Mitchell and Moyle 1965; Scholes and Mitchell 1970). The change in pH (H^+ translocation) was measured with a CMAWL pH probe (Russell pH Ltd, Fife, UK) on a Pye Unicam PW 9409 pH meter (Philips) and recorded on a JJCR100 potentiometric recorder (Lloyd Instruments Ltd, UK), with full-scale deflection corresponding to a ΔpH of 0.12.

Measurement of respiration driven proton translocation by the initial rate method

This was done essentially as described by Dawson and Jones (1981). Bacterial suspensions were incubated in the same mixture as for the oxygen pulse experiments, but aerated with efficient stirring. Substrate was added immediately after introducing nitrogen gas to the surface of the suspension and the initial rates of acidification and oxygen uptake were recorded simultaneously. The oxygen uptake was recorded with a Euroscribe chart recorder (Gallenkamp, UK) and corrected for the anaerobiosis caused by the nitrogen gassing procedure in the absence of substrate.

Chemicals

Valinomycin, HQNO, antimycin A, rotenone, EAI, IAI and carbonic anhydrase were obtained from Sigma (London) Ltd. Amytal, DCCD and FCCP were from Aldrich (UK). Reagents were obtained from Fisons Ltd or British Drug Houses. ^{14}C-labelled EAI and IAI were from Amersham International PLC.

Results

Confirmation of the periplasmic location of the enzymes A and B of the thiosulphate-oxidizing complex of Thiobacillus versutus

Labelling intact cells with ^{14}C-ethyl acetimidate hydrochloride (EAI), which penetrates the cytoplasmic membrane, resulted in labelling of many cell proteins (Fig. 1, column 3), including enzymes A and B. Pretreatment of the cells with unlabelled isethionyl acetimidate (IAI), which does not penetrate the membrane and binds only to periplasmic and outer membrane proteins, prevented subsequent labelling of enzyme A and B by ^{14}C-EAI (Fig. 1, column 4). This indicated that the enzymes were entirely located on the

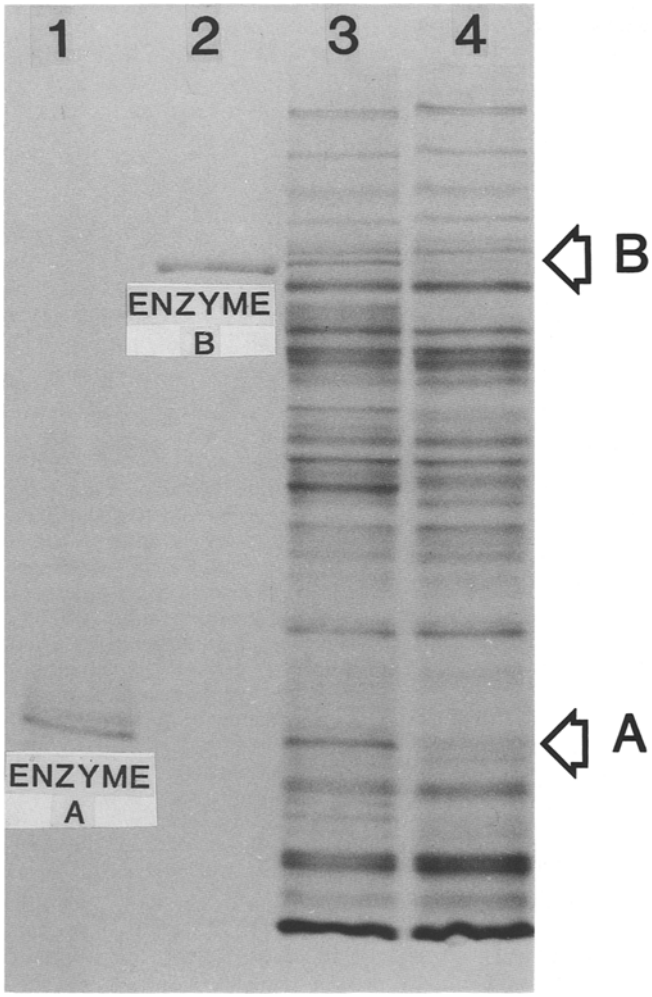

Fig. 1. Differential labelling of periplasmic and cytoplasmic proteins of *Thiobacillus versutus*, using ^{14}C-labelled amidoesters. Experiments were conducted as described in Materials and methods. *1*, marker sample of purified enzyme A, labelled with ^{14}C-EAI; *2*, marker sample of pure enzyme B, labelled with ^{14}C-EAI; *3*, SDS-extract of *T. versutus* following labelling with ^{14}C-EAI; *4*, SDS-extract of *T. versutus* pretreated with unlabelled IAI than labelled with ^{14}C-EAI. '*A*' and '*B*' mark the positions of enzymes *A* and *B* respectively on the gels

periplasmic side of the cell membrane. Comparing the labelling of cells by ^{14}C-EAI and ^{14}C-IAI showed that enzymes A and B were very heavily labelled by IAI, compared to other cell proteins (gels not shown), confirming their periplasmic location.

Respiration-driven proton translocation in batch grown Thiobacilus versutus

Addition of oxygen to anaerobic suspensions of organisms in the absence of exogenous substrate resulted in rapid acidification of the medium (Fig. 2A). The amplitude of the ΔpH was maximal after prior incubation with valinomycin for 45 min, whereas KSCN (up to 60 mM) was a poor membrane potential ($\Delta\Psi$) – collapsing reagent for these cells. The decay of the ΔpH showed first order kinetics with a half-life ($t_{1/2}$) of about 45 s (Fig. 2D). The time taken for 50% reduction of the pulse of 4.8 ng-atoms added was estimated as 0.9 s. Extrapolation of the ΔpH decay plot to zero time (Table 1), 1 s or 2 s gave $\rightarrow H^+/O$ values of 5.68 ± 0.62, 5.60 ± 0.60 and 5.53 ± 0.54 respectively. The uncoupling agent FCCP (1.1 μM) abolished the response of the organisms to subsequent addition of oxygen.

The same preparation showed a $\rightarrow H^+/O$ quotient of 4.3 ± 0.2 (8 determinations) for thiosulphate oxidation in the presence of HQNO (34 μM). Although the thiosulphate-linked respiration rate was about 6–8 times higher than the endogenous rate (Table 1), this high $\rightarrow H^+/O$ ratio for thiosulphate oxidation was likely to be due due in part to a contribution from endogenous substrate oxidation (cf. Scholes and Mitchell 1970), as the endogenous respiration was found extremely difficult to abolish by the addition of respiratory inhibitors, even at high concentrations. For example, addition of HQNO (up to 114 μM), antimycin A (up to 43 μg/ml) or amytal (up to 2 mM) only inhibited about 50% of the endogenous respiration, while at the same time causing physiological side effects that resulted in very low $\rightarrow H^+/O$ ratios (less than one) for thiosulphate oxidation.

Since the pH of stirred aerobic cell suspensions was rather stable, the initial rate of proton translocation could be measured on addition of thiosulphate. By measuring the rate of O_2 uptake and H^+ ejection 4 s after the addition of thiosulphate enabled calculation on the $\rightarrow H^+/O$ quotient as 1.83 ± 0.4 (3 determinations). Since the rate of H^+ ejection was observed to be maximal immediately following thiosulphate addition, and began to decline after 5 s while the rate of O_2 uptake did not reach its maximum rate until 5–6 s after adding thiosulphate, the $\rightarrow H^+/O$ quotient so obtained was rather imprecise. It does, however, suggest the true value to be nearer to 2 than 4.

Proton translocation measured with Thiobacillus versutus taken from thiosulphate-limited chemostat culture (D 0.08 h^{-1})

Organisms harvested from chemostat culture showed a lower endogenous respiration rate (4 nmol O_2 min^{-1} (mg dry wt)$^{-1}$) than those from batch culture (Table 1). The respiration was linked to proton translocation with a $\rightarrow H^+/O$ value of 5.4 ± 0.37 (4 determinations) when measured by the oxygen pulse method. This endogenous respiration could be severely inhibited by the addition of respiratory inhibitors at relatively low concentrations, without significantly affecting the oxidation of thiosulphate or sulphite. HQNO (51 μM), rotenone (86 μM) or amytal (500 μM) were all effective. Amytal was found to have the least side effect and was routinely employed in determination of the thiosulphate- and sulphite-dependent $\rightarrow H^+/O$ quotients. The endogenous metabolism of the cell suspensions incubated either aerobically or anaerobically produced an alkalization of the medium that could be abolished by the addition of respiratory inhibitors. In the presence of inhibitor, the cell suspensions gave virtually no response to an oxygen pulse in the absence of exogenous substrate. In contrast to the batch-grown organisms, valinomycin (up to 8.6 μg/ml) was much less effective than KSCN (60 mM) in collapsing the $\Delta\Psi$, so KSCN was employed in all experiments using chemostat-grown organisms.

Using an anaerobic cell suspension in which endogenous respiration had been inhibited by amytal, a rapid acidification of the medium was produced when a pulse of oxygen

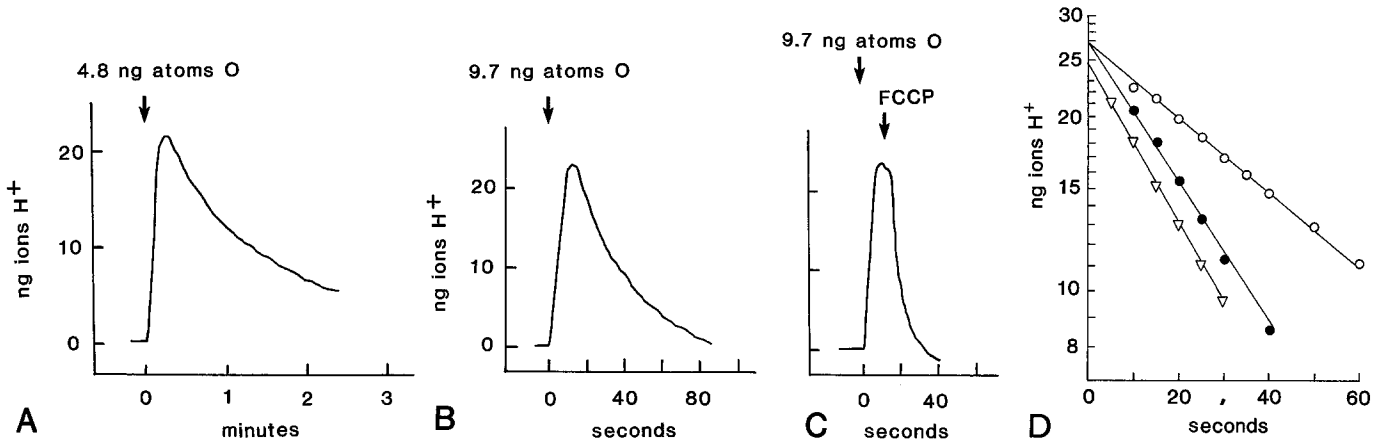

Fig. 2A–D. Proton translocation resulting from the addition of oxygen to anaerobic suspensions of *Thiobacillus versutus*. A detailed description of the procedures is given in the Materials and methods. **(A)** Endogenous respiration-driven proton translocation. Organisms from batch culture on thiosulphate (14 mg dry wt) were incubated at 25°C in 3.5 ml reaction mixture containing 150 mM KCl and 20 ng valinomycin at pH 6.5–6.6; **(B)** Thiosulphate oxidation-driven proton translocation. Organisms from chemostat culture on thiosulphate (8 mg dry wt) were incubated at 25°C in 3.5 ml reaction mixture containing 150 mM KCl, 1.5 mM glycylglycine, 66 mM KSCN and 0.5 mM amytal, at pH 6.4–6.5; **(C)** Dissipation of H^+ by the addition of FCCP (1.1 μM; *arrow*) to a suspension prepared as in **(B)**. The amount of oxygen (ng atoms) added as pulses of air-saturated 150 mM KCl is indicated on the traces in **A–C**. **D** Determination of $\rightarrow H^+/O$ quotients from extrapolation to zero time of the logarithmic decay of the proton gradient generated by the oxygen pulse. Mean values from several experiments for the translocated H^+ concentration 5–60 s following the oxygen pulse are shown for the endogenous respiration- (○), thiosulphate oxidation- (●) and sulphite oxidation (▽)- dependent proton translocation. Extrapolation to zero time indicates the maximum number of H^+ that could have been produced following the addition of 4.84 ng atoms O (endogenous) or 9.68 ng atoms O (thiosulphate and sulphite)

Table 1. Determination of $\rightarrow H^+/O$ quotients and respiration rates for whole cells of *Thiobacillus versutus*. The $\rightarrow H^+/O$ quotients were measured by the oxygen pulse method as described in the text and the legend to Fig. 2. Endogenous values were for organisms from batch cultures grown on thiosulphate. All other values were for organisms from chemostat culture on thiosulphate, and were obtained in the presence of 0.5 mM amytal

Substrate	$\rightarrow H^+/O$ quotient[a]	Respiration rate (nmol O_2 min^{-1} (mg dry wt)$^{-1}$)
Endogenous	5.68±0.62 (9)	15
Thiosulphate	2.85±0.20 (5)	120
Sulphite	2.57±0.22 (5)	12
Thiosulphate (−valinomycin −thiocyanate)[b]	0.65±0.12 (5)	120
Ascorbate/TMPD	0.80±0.08 (4)	130
Ferrocyanide	0	5

[a] Mean values and standard deviation, with the numbers of experiments shown in parenthesis, calculated by extrapolation of decay of ΔpH to zero time, following addition of the oxygen pulse

[b] Measured in the absence of valinomycin or thiocyanate

was added in the presence of thiosulphate (Fig. 2B). This ΔpH was rapidly dissipated when 1 μM FCCP was added (Fig. 2C). The decay of the ΔpH in the absence of FCCP showed first order kinetics with a $t_{1/2}$ of about 25 s (Fig. 2B, D). The time estimated to be required for 50% reduction of the added pulse of 9.7 ng-atoms O was 0.22 s, meaning that extrapolation of the ΔpH decay to zero time gave the best estimate for the $\rightarrow H^+/O$ (Table 1). Comparison of $\rightarrow H^+/O$ calculated at 2 s gave a value of 2.68±0.17 (5 determinations). Using sulphite as the substrate, slightly lower $\rightarrow H^+/O$ values were seen (Table 1). The time for 50% reduction of the 9.7 ng-atom pulse of oxygen was about 1.5 s, and the $\rightarrow H^+/O$ calculated from ΔpH decay at 1.5 s was 2.41±0.20 (5). Neither thiosulphate nor sulphite-dependent proton translocation was significantly affected by DCCD (40 μM).

Proton translocation associated with the oxidation of the nonphysiological substrates, ascorbate/TMPD or ferrocyanide was also measured. The $\rightarrow H^+/O$ quotient for the former was 0.8 by the oxygen pulse method (Table 1) and 0.95±0.05 (2 determinations) using the initial rate method. Ferrocyanide oxidation did not generate a ΔpH (Table 1). Ascorbate/TMPD is known to release one H^+ per two electrons donated to the respiratory chain at the level of cytochrome *c* under physiological conditions, so these values imply that a net proton translocation across the membrane does not accompany the electron transfer from cytochrome *c* to oxygen catalysed by cytochrome oxidase in *T. versutus*.

The low $\rightarrow H^+/O$ quotient for thiosulphate-dependent proton translocation in the absence of $\Delta\Psi$-collapsing reagents (valinomycin or KSCN; Table 1) suggests that delocalized proton translocation accounts for only about a quarter of the total proton release.

Discussion

Results from the oxygen pulse measurements for *Thiobacillus versutus* from continuous culture and the initial rate measurement for organisms from batch culture, indicate that the oxidation of thiosulphate is associated with a $\rightarrow H^+/O$ ratio of around 2.5, as predicted by Eq. (1). The small excess of protons translocated in the oxygen pulse experiments can

probably be attributed to the residual endogenous respiration with a $\rightarrow H^+/O$ of 5–6. The observed $\rightarrow H^+/O$ about 2.5 is in good agreement with the proposal that ten protons should be released on the periplasmic side of the membrane for each mol of thiosulphate oxidized and each two mol of oxygen reduced [Eq. (1 and 2)]. The periplasmic location of the thiosulphate-oxidizing enzyme complex (Lu 1986), which has now been independently confirmed by the protein imidoester-labelling technique, thus allows the generation of 10 $H^+/S_2O_3^{2-}$ in the periplasm, using water as the source of protons [Eq. (1)] (Hooper and DiSpirito 1985; Lu 1986; Lu and Kelly 1984a). Since the cytochrome oxidase-catalysed consumption of oxygen is believed to occur on the cytoplasmic side of the membrane, and must occur after release of protons and electrons by the periplasmic enzyme system, there is both temporal and spatial separation of the processes of H^+ production and O_2 reduction. The observation on proton translocation using ascorbate/TMPD or ferrocyanide as oxidation substrates, and the lack of effect of DCCD (which inhibits both ATPase activity and proton pumping by cytochrome oxidase aa_3; Harold 1986; Casey et al. 1980) indicate that the *T. versutus* cytochrome oxidase does not act as a proton pump during thiosulphate or sulphite oxidation. There would seem to be no possibility of proton extrusion due to quinone or cytochrome *b* as the oxidation of thiosulphate in *T. versutus* is coupled to the electron transport chain at cytochrome *c* (Lu and Kelly 1983a, 1984b; Kula et al. 1982). These observations make it likely that a $\rightarrow H^+/O$ quotient greater than 2.5 could not be produced by the oxidation of thiosulphate alone.

The $\rightarrow H^+/O$ data also support the mechanism of energy conservation involving only one phosphorylation site previously proposed for thiosulphate oxidation by *T. versutus* (Kelly 1982; Lu 1986; Lu and Kelly 1983a), since the formation of four ATP per thiosulphate oxidized (and two O_2 consumed) would indicate that the ATP synthase reaction consumed 2.5 H^+/ATP formed. Values of 2, 3 or 4 H^+ are proposed in the literature with 3 H^+/ATP being most favoured (Harold 1986; Kashket 1982), indicating reasonable consistency between the observed thiosulphate-dependent $\rightarrow H^+/O$ value of 2.5 and ATP/O of 0.71–1.11 (Kelly 1982; Lu and Kelly 1983a).

The $\rightarrow H^+/O$ quotient of about 2.4 for the oxidation of sulphite (by organisms showing low residual endogenous respiration) is reasonably consistent with the equation for the activity of sulphite dehydrogenase (Lu and Kelly 1984c):

$$Na_2SO_3 + H_2O = Na_2SO_4 + 2H^+ + 2e^- \quad (3)$$

This stoichiometry is supported by the demonstration that sulphite oxidation couples to the electron transport chain at the level of *c*-type cytochrome (Lu and Kelly 1983a, 1984c) and occurs on the periplasmic side of the membrane (Lu 1986).

These observations may be compared with $\rightarrow H^+/O$ values of about 2 for thiosulphate, sulphide and sulphite oxidation by *T. neapolitanus* (Drozd 1974, 1976) which exhibits a similar growth yield on thiosulphate to *T. versutus*, as do *T. novellus*, *T. thiooxidans* and *T. ferrooxidans* (Kelly 1982; Leefelt and Matin 1980; Mason et al. 1987). Such comparisons indicate that the efficiency of energy conservation is similar in these bacteria, and could indicate that each couples thiosulphate oxidation to reduction of *c*-type cytochromes and can gain ATP by a phosphorylation system with a P/O ratio of about one. Whether the same energy-conserving mechanism as described here for *T. versutus* applies also to *T. neapolitanus* and other species cannot at present be decided, since the cellular location of their thiosulphate-oxidizing enzyme systems is not known with certainty. If, as is possible in *T. neapolitanus*, thiosulphate oxidation occurs in the cytoplasm rather than periplasmically, there could be the necessity for the cytochrome oxidase to function as a proton pump in order to generate an electrochemical proton gradient. The presence of a proton-translocating cytochrome oxidase in *T. neapolitanus* was implied by the finding of a $\rightarrow H^+/O$ ratio of 1.5 for the oxidation of ascorbate-TMPD (Drozd 1974). Certainly in *T. tepidarius* (Wood and Kelly 1986), evidence is emerging to show that the major enzyme system for the complete oxidation of thiosulpate and tetrathionate to sulphate could be located on the cytoplasmic side of the membrane (Lu and Kelly, in preparation).

Autotrophically-grown *T. versutus* contains NADH dehydrogenase and succinate dehydrogenase (Lu 1986; Kula et al. 1982), ubiquinone (Katayama-Fujimura et al. 1982), two *b*-type cytochromes (Kula et al. 1982), at least four *c*-type cytochromes and aa_3-type cytochrome oxidase (Lu et al. 1984). The present demonstration of a $\rightarrow H^+/O$ quotient approaching six for endogenous respiration is thus consistent with the operation of a proton translocation system for NADH oxidation similar to that reported for *Micrococcus lysodeikticus* (Jones et al. 1975). Conceivably, two of the six protons translocated per electron pair during NADH oxidation with oxygen are expelled at the level of NADH dehydrogenase, and the other four by a protonmotive quinone cycle (Harold 1986). None of those possible proton translocation processes is involved in energy conservation during thiosulphate oxidation for which the more direct and simple device of the periplasmic location of the thiosulphate-oxidizing multienzyme system has been evolved to generate the electrochemical proton gradient.

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