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ATP generation during reduced inorganic sulfur compound oxidation by *Acidithiobacillus caldus* is exclusively due to electron transport phosphorylation

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Abstract The synthesis of adenosine 5-triphosphate (ATP) (increase in phosphorylation potential) during the oxidation of reduced inorganic sulfur compounds was studied in the moderately thermophilic acidophile *Acidithiobacillus caldus* (strain KU) (formerly *Thiobacillus caldus*). The phosphorylation potential increased during the oxidation of all reduced inorganic sulfur compounds tested compared with resting cells. The generation of ATP in whole cells was inhibited by the F_0F_1 ATPase inhibitor oligomycin, electron transport chain inhibitors, valinomycin and potassium ions. There was no increase in the phosphorylation potential, nor synthesis of ATP, in the absence of electron transport. An apparent lack of substrate-level phosphorylation was indicated by the lack of adenosine 5-phosphosulfate reductase in tetrathionate-grown *At. caldus*. Studies were also performed on the synthesis of ATP by membrane vesicles of *At. caldus* when presented with an artificial proton gradient. Complete inhibition of ATP synthesis in these vesicles occurred when they were loaded with N,N-dicyclohexylcarbodiimide (DCCD), but not when they were loaded with oligomycin, vanadate or electron transport chain inhibitors. The data presented here suggest that during the oxidation of reduced inorganic sulfur compounds by *At. caldus*, all ATP is synthesized by oxidative phosphorylation via a membrane-bound F_0F_1 ATPase driven by a proton gradient.

Key words *Acidithiobacillus caldus* · Acidophile · ATP synthesis · Energetics · Sulfur metabolism

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Introduction

The moderately thermophilic acidophile *Acidithiobacillus caldus* (strain KU) is a chemolithoautotroph that obtains energy for growth from reduced inorganic sulfur compounds but not metal sulfides (Hallberg and Lindström 1994). Despite the inability to oxidize metal sulfides, *At. caldus* can grow by the oxidation of sulfur while attached to the surface of pyrite (Edwards et al. 2000). *At. caldus* has been found in the planktonic cell population of two pilot-scale leaching bioreactors created to study the oxidation of arsenical pyrite (Amaro et al. 1994) and chalcopyrite (Goebel and Stackebrandt 1994). Recently, it has been found that *At. caldus* is also present in a full-scale commercial arsenopyrite bioleaching plant used for the recovery of gold (Rawlings 1998).

During the chemical oxidation of metal sulfides by ferric iron, reduced inorganic sulfur compounds are formed (Goldhaber 1983; Schippers and Sand 1999) including thiosulfate, which is rapidly converted to tetrathionate under the acidic conditions in which bioleaching occurs. Tetrathionate and elemental sulfur have been detected in the culture medium during the oxidation of pyrite by a non-sulfur-oxidizing *Leptospirillum* isolate and by the sulfur-oxidizing bacterium *Acidithiobacillus ferrooxidans* (Schippers et al. 1996). The generation of these reduced inorganic compounds during the leaching of metal sulfides could explain the presence of the relatively rapidly growing *At. caldus* in bioleaching systems. It has been previously shown that the presence of *At. caldus* strain BC13 in co-culture with a *L. ferrooxidans*-like strain caused an increased bioleaching rate compared with the *L. ferrooxidans*-like strain alone (Norris 1990). We have also shown that the oxidation of arsenopyrite by the moderately thermophilic *Sulfobacillus thermosulfidooxidans* is enhanced by the presence of *At. caldus* (Dopson and Lindström 1999). These studies indicate that *At. caldus* plays a beneficial role in the bioleaching of metal sulfides rather than merely being present.

The suggestion that *At. caldus* is beneficial to bioleaching systems has led us to investigate the metabolism of this aci-

dophile. We have previously proposed a model showing how *At. caldus* oxidizes the reduced inorganic sulfur compounds (Hallberg et al. 1996). The acidic environment in which acidophiles such as *At. caldus* grow presents a challenge to the bacterial energetics in the form of an extremely large pH gradient that exists over the cytoplasmic membrane (up to 5 pH units). Studies have been carried out to investigate the energetics of acidophiles in whole cells (Cobley and Cox 1983; Matin 1990). However, only one biochemical study has been carried out on the synthesis of adenosine 5-triphosphate (ATP) in an acidophilic organism (Apel et al. 1980). In the present investigation, we show how the moderately thermophilic acidophile *At. caldus* derives energy during reduced inorganic sulfur compound oxidation.

Materials and methods

Chemical reagents

All stock solutions were prepared from commercially available chemicals. The sulfite solution was prepared in 50 mM ethylenediaminetetraacetic acid (EDTA) to prevent autooxidation. The stock solutions of other reduced inorganic sulfur compounds were dissolved in distilled water, except for elemental sulfur, which was dissolved in acetone. The inhibitors and uncouplers utilized were dissolved in ethanol (95% v/v), except for potassium cyanide, which was dissolved in distilled water. Control experiments showed that neither the ethanol nor acetone used to dissolve the various reagents adversely affected *At. caldus* metabolism. All solutions for measurement of the phosphorylation potential (ΔGp) were made with MilliQ Ultra Pure water (Millipore, Bedford, MA, USA).

Adenosine 5-diphosphate (ADP) was purchased from Sigma (St Louis, MO, USA) at a purity of greater than 99%, but it was found that the level of contaminating ATP was still too high to obtain accurate measurements of the ATP produced in vesicles. Therefore the ADP solution was purified using ion exchange chromatography (Cohn and Carter 1950) with the modification that the ADP was eluted with 200 ml of 0.01 M HCl + 0.1 M NaCl. ADP concentration was calculated using the molar extinction co-efficient of 15,400 M^{-1} cm^{-1} at 259 nm. This procedure removed more than 99% of the contaminating ATP.

Bacteria, growth conditions, and preparation of resting cells

At. caldus KU (DSM 8584, ATCC 51756) was grown at 45°C in batch cultures with 5 mM tetrathionate as energy source (Hallberg and Lindström 1994). During the late exponential growth phase (optical density at 440 nm = 0.260 – 0.270 measured on a Hitachi 150–20 spectrophotometer (Tokyo, Japan) in a 1 cm cuvette) bacteria were harvested by centrifugation (10,000 *g* for 10 min), washed and resuspended in β-alanine buffer (50 mM β-alanine adjusted to pH 3 with H_2SO_4). The resulting resting cells were kept on ice and used within 2 h. All the experiments were carried out in triplicate and averages and standard deviations presented, except where stated.

Assay of substrate-dependent oxygen consumption

The oxidation of reduced inorganic sulfur compounds by resting cells of *At. caldus* in the presence and absence of uncouplers and inhibitors was assayed in β-alanine buffer (pH 3) with a Hansatech (King's Lynn, UK) oxygen electrode at 45°C (Hallberg et al. 1996).

Measurement of adenosine 5-phosphosulfate reductase

At. caldus was grown and harvested as stated above and then washed in 50 mM Tris-HCl pH 7.5 containing 50 mM $MgSO_4$. Resting cells were incubated in the presence of lysozyme (100 μg ml^{-1}) for 10 min on ice, followed by sonication (8 × 30 s with 30 s pause). The suspension was then centrifuged for 20 min at 10,000 *g*. Sulfite oxidation was assayed according to the method of Lu and Kelly (1988) except that the reactions were carried out at 45°C and pH 7.5. Adenosine 5-phosphosulfate (APS) reductase activity was assayed by the addition of adenosine 5-monophosphate (AMP) to the sulfite oxidation reaction mixture (Lu and Kelly 1988). Control treatments were carried out in the presence and absence of AMP and these rates were subtracted from those in the presence of crude extract.

Determination of phosphorylation potential of *At. caldus* resting cells

Resting cells of *At. caldus* were resuspended to 10 mg dry wt cells ml^{-1} in a total volume of 1 ml β-alanine buffer (pH 3). The cells were incubated at 45°C for 5 min before addition of the substrate to start the reaction. For the analysis of adenine nucleotides in the presence of inhibitors and uncouplers, they were added to resting cells prior to the 5 min pre-incubation period. Samples of the cell suspensions (150 μl) were removed at the indicated intervals and placed into 150 μl cold 1.2 M $HClO_4$ and the nucleotides measured as described below.

Preparation of membrane vesicles

Vesicle preparation was based on the method previously described for *At. ferrooxidans* (formerly *Thiobacillus ferrooxidans*) (Apel et al. 1980). Spheroplasts were resuspended in 2 ml of 50 mM phosphate buffer (pH 7.5) containing 1 mM purified ADP. EDTA (pH 8) was added to a final concentration of 10 mM and the spheroplasts were broken by sonication (30 s pulse with 30 s pause over 5 min) and then incubated at 37°C with vigorous shaking for 20 min. $MgSO_4$ was added to a final concentration of 15 mM and this mixture was incubated at 37°C for 30 min. DNAase

(300 µg) was added, followed by a further 30 min incubation. This preparation was centrifuged at 3,000 *g* for 15 min to remove whole cells and large debris, and then the supernatant was centrifuged at 40,000 *g* for 30 min. The pellet, containing the vesicles, was washed once with 15 ml cold 10 mM phosphate + 10% (w/v) sucrose + 5 mM $MgCl_2$ buffer at pH 7.5 (PSM buffer) and the vesicles resuspended in 300 µl PSM buffer. The vesicles were stored on ice for no longer than 2 h prior to use. When buffers of varying pH were to be loaded inside the vesicles, the desired buffer + 1 mM ADP was substituted for 50 mM K_2HPO_4 at each of the above vesicle preparation steps. In some cases, ADP or $MgCl_2$ were omitted from the vesicles. The protein concentration of the vesicle preparations was assayed using a Pierce Protein Assay kit (Rockford, Ill.) with bovine serum albumin as standard.

Generation of ATP in membrane vesicles

ATP production in the vesicles was carried out with 1 mg vesicle protein ml^{-1} in stirred PSM buffer in a temperature-controlled chamber at 45°C, unless otherwise stated. Following an equilibration period of 3 min, H_2SO_4 was added to obtain the desired external pH, and 100 µl samples were removed into 100 µl 1.2 M $HClO_4$ at the indicated time intervals following the adjustment of the external pH. For measurement of ATP production in the presence of inhibitors and uncouplers, the chemicals were added prior to the 3 min equilibration period. The pH was adjusted with H_2SO_4 to 2.2 ± 0.1 (the external pH at which the highest concentration of ATP was produced in the absence of poisons) and the ATP concentration determined as described below.

Measurement of adenosine nucleotides and inorganic phosphate

Nucleotides were extracted from whole cells and vesicles with 1.2 M $HClO_4$, and then cell debris was cleared by centrifugation in a bench centrifuge (10,000 *g* for 10 min). The pH of the acid extract was adjusted by dilution in potassium phosphate buffer pH 7.8 (Cox and Henick-Kling 1989). In whole cells, ATP and ADP concentrations were assayed using the BioThema ATP kit SL 144–041 (Dalarö, Sweden) according to the manufacturer's instructions. Inorganic phosphate (P_i) was measured by mixing with ascorbic acid and ammonium molybdate according to the method of Ames (1966). ΔGp was calculated according to the equation $\Delta Gp = [ATP]/[ADP][P_i]$ (M^{-1}) and the standard deviation as the square root of the sum of the squares of the standard deviations of the ATP, ADP and P_i concentrations, each calculated in triplicate. ATP generation in vesicles was measured using a commercially available luciferin–luciferase kit (Sigma) on a Wallac 1409 Liquid Scintillation Counter (Turku, Finland) set to measure ^{14}C. The range of ATP detection was linear from 10^{-13} to 10^{-8} mol.

Results

Effect of cellular poisons on the oxidation of reduced inorganic sulfur compounds by *At. caldus* resting cells

The inhibitors and uncouplers chosen for use in this investigation have been reported to have effects on the bioenergetic parameters of bacterial cells. While the effect on the consumption of oxygen by *At. caldus* of some of the inhibitors used here has been reported previously, for example carbonyl-cyanide-m-chlorophenyl-hydrazone (CCCP) and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) (Hallberg et al. 1996), the effect of the others used in this study are given in Table 1. The F_0F_1 inhibitor, oligomycin, had no effect on the rate or total oxygen consumption with any of the reduced inorganic sulfur compounds tested. Titration of the rate of oxygen consumption by tetrathionate with azide (inhibits cytochrome oxidase aa_3 in the millimolar range) showed approximately 90% inhibition at 1 mM compared with the control without azide. Cyanide (inhibits cytochrome oxidase aa_3 in the micromolar range and cytochrome oxidase *o* in the millimolar range) exhibited 50% inhibition of oxygen consumption rate at approximately 10–100 µM (Fig. 1) and 86% inhibition at a concentration of 100 mM. Myxothiazol (cytochrome bc_1 inhibitor) reduced the rate of O_2 consumption for each of the reduced inorganic sulfur compounds, and also lowered the total O_2 consumed except with sulfite, without stopping the oxidation at any specific oxidation state. Valinomycin (K^+ channel-former), K^+ alone, and valinomycin in conjunction with K^+

Table 1. Oxidation of reduced inorganic sulfur compounds by resting cells of *Acidithiobacillus caldus* in the presence and absence of inhibitors

Inhibitor/uncoupler	Total O_2 consumed (nmol)				
	$S_4O_6^{2-}$ 25 nmol	$S_2O_3^{2-}$ 100 nmol	S^0 90 nmol	SO_3^{2-} 50 nmol	S^{2-} 50 nmol
None	77 ± 3	159 ± 7	126 ± 4	19 ± 2	94 ± 5
Oligomycin (10 µg ml^{-1})	88 ± 2	182 ± 3	134 ± 8	25 ± 1	99 ± 4
Myxothiazol (20 µM)	54 ± 3	123 ± 3	69 ± 4	19 ± 4	69 ± 4
Valinomycin (5 µg ml^{-1})	67 ± 1	163 ± 4	119 ± 10	22 ± 1	90 ± 5
K^+ (300 mM)	81 ± 4	162 ± 4	130 ± 10	25 ± 2	98 ± 2
Valinomycin + K^+	85 ± 3	155 ± 7	144 ± 10	23 ± 2	101 ± 2

The data, Total O_2 consumed (nmol), are mean values ± SD of at least two experiments

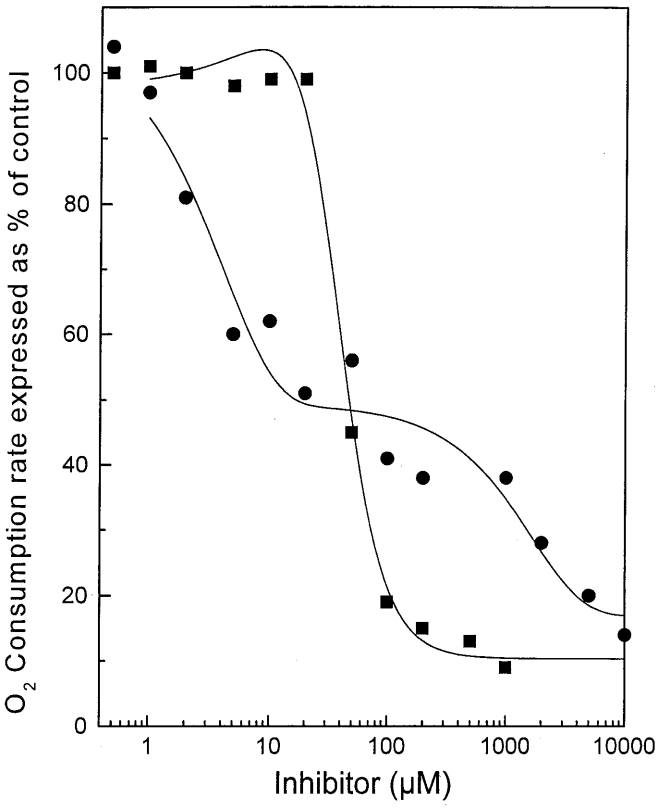

Fig. 1. Inhibition of the rate of oxygen consumption by resting cells of *Acidithiobacillus caldus* in the presence of cyanide (*circles*) and azide (*squares*) with 125 nmol tetrathionate. 100% was defined as the rate of oxygen consumption without the addition of inhibitor. The rate of oxygen consumption in the absence of inhibitor was 393 nmol O_2 min^{-1} per milligram (dry weight) cells

had very little effect on either the rate or total oxygen consumed with any of the reduced inorganic sulfur compounds tested.

Measurement of phosphorylation potential in *At. caldus* resting cells upon addition of reduced inorganic sulfur compounds

In order to measure the amount of ATP produced from the oxidation of some reduced inorganic sulfur compounds, the ΔGp was measured. In the absence of any substrate the ATP, ADP and P_i concentrations yielded a ΔGp of 387 ± 59 M^{-1} at time zero and 468 ± 35 M^{-1} after 2 min (Fig. 2a). In the presence of 125 μM tetrathionate, the ATP concentration doubled while the concentrations of ADP and P_i decreased, resulting in a ΔGp of 7,600 ± 267 M^{-1} after 5 s, decreasing to 802 ± 95 M^{-1} at 2 min (Fig. 2b). Addition of other reduced inorganic sulfur compounds also resulted in an increase in the ΔGp; the highest was for 250 μM thiosulfate (8,075 ± 734 M^{-1}), followed by 125 μM tetrathionate (Fig. 2b), 250 μM sulfide (3,150 ± 170 M^{-1}), 300 μM elemental sulfur (1,277 ± 158 M^{-1}) and 50 μM sulfite (662 ± 40 M^{-1}).

The addition of poisons lowered the ΔGp to varying extents (Table 2), 200 μM CCCP (uncouples respiration from proton movement) not only inhibited any increase in the ΔGp, but also lowered the initial ΔGp to 93 ± 20 M^{-1}. No ATP was produced in the presence of CCCP, as tetrathionate metabolism is completely inhibited by CCCP (Hallberg et al. 1996). Oligomycin not only lowered the rate of increase in ΔGp, but also reduced the peak ATP concentration and ΔGp; this effect was not due to inhibition of tetrathionate metabolism, as oxygen

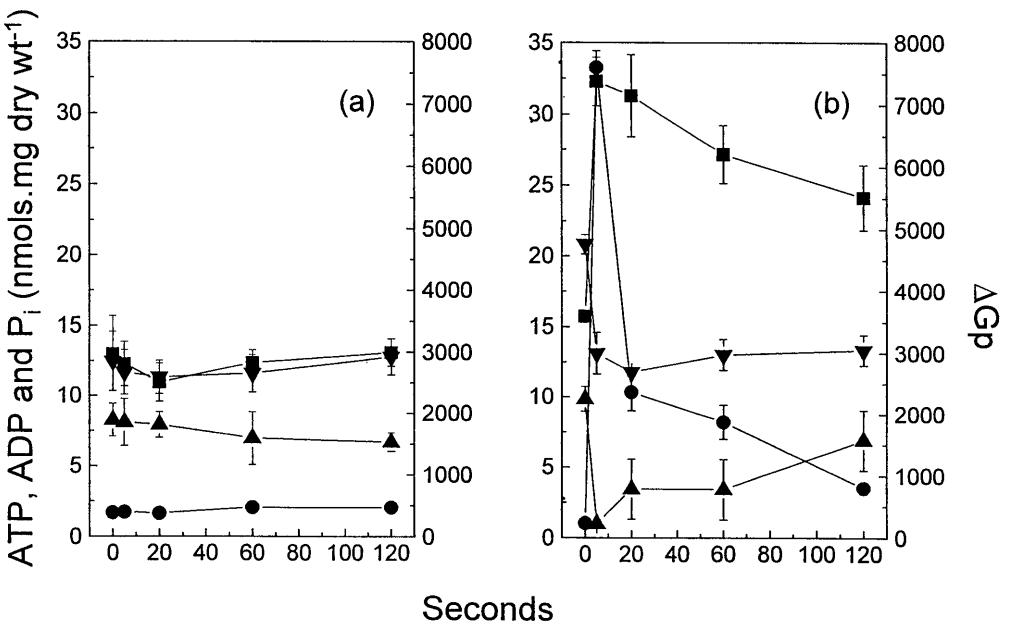

Fig. 2. Changes in adenosine 5-triphosphate (*ATP*; *squares*), adenosine 5-diphosphate (*ADP*; *triangles*) and inorganic phosphate (P_i; *inverted triangles*) (nmol/mg dry weight *At. caldus*) and ΔGp (*circles*) in the absence of substrate (**a**), and in the presence of 125 μM tetrathionate (**b**). *Error bars* indicate the standard deviation ($n = 3$)

Table 2. Mean values of peak phosphorylation potential (ΔGp) in whole cells of *At. caldus* with the addition of 125 μM tetrathionate

Inhibitor	Time to reach peak ΔGp (s)	Peak ΔGp ± SD (M^{-1})
None	5	7,600 ± 267
CCCP[a] (200 μM)	20	100 ± 36
Oligomycin (200 μg ml^{-1})	20	1,223 ± 74
Azide (50 μM)	5	3,505 ± 507
Azide (1 mM)	60	504 ± 114
Cyanide (20 μM)	5	1,002 ± 50
Cyanide (10 mM)	60	474 ± 114
Myxothiazol (20 μM)	5	1,651 ± 71
Valinomycin (25 μg ml^{-1})	5	5,058 ± 163
K^+ (300 mM)	60	153 ± 24
Valinomycin + K^+	60	201 ± 26

Standard deviations were calculated as the square root of the sum of the squares of the adenosine 5-triphosphate (ATP), adenosine 5-diphosphate (ADP), and inorganic phosphate (P_i) concentrations each calculated in triplicate (n = 9)
[a]CCCP, carbonyl-cyanide-m-chlorophenyl-hydrazone

uptake was unaffected. Inhibitors of the electron transport chain also lowered peak ΔGp, and in many cases increased the time it took to reach the peak. This reflects the reduced rate of oxygen consumption by *At. caldus* when incubated in the presence of these inhibitors. Valinomycin (25 μg ml^{-1}) only had a limited affect on the ΔGp, but both 300 mM potassium ions and potassium ions plus valinomycin not only completely inhibited any increase in ΔGp, but also lowered the resting ΔGp.

APS reductase assay

Sulfite-dependent reduction of ferricyanide in the presence and absence of AMP was 28 ± 4 and 30 ± 4 μmol ferricyanide reduced min^{-1} (mg protein)$^{-1}$, respectively. The significance of the variation was analyzed by a Student's *t*-test resulting in a *P* value of 0.34. This indicates that the values were not significantly different, and therefore there was no evidence for sulfite oxidation by an AMP-dependent system.

Synthesis of ATP in membrane vesicles

The results obtained with resting cells of *At. caldus* indicated that ATP is synthesized by an F_0F_1 ATPase during substrate oxidation. We have therefore prepared membrane vesicles of *At. caldus* and investigated the synthesis of ATP by F_0F_1 ATPase in response to artificially created pH gradients. Vesicles prepared with an internal pH of 7.5 were able to synthesize ATP from ADP and PO_4^{3-} when subjected to a pH gradient (Fig. 3a). Most ATP was synthesized in buffer of pH 2.2–2.5, with peak levels being reached after 10 s. No ATP was detected in vesicles prepared without ADP or Mg^{2+}. At an internal pH of 7.5 or 8.0, maximal amounts of ATP were formed when the external pH was adjusted to 2.2.

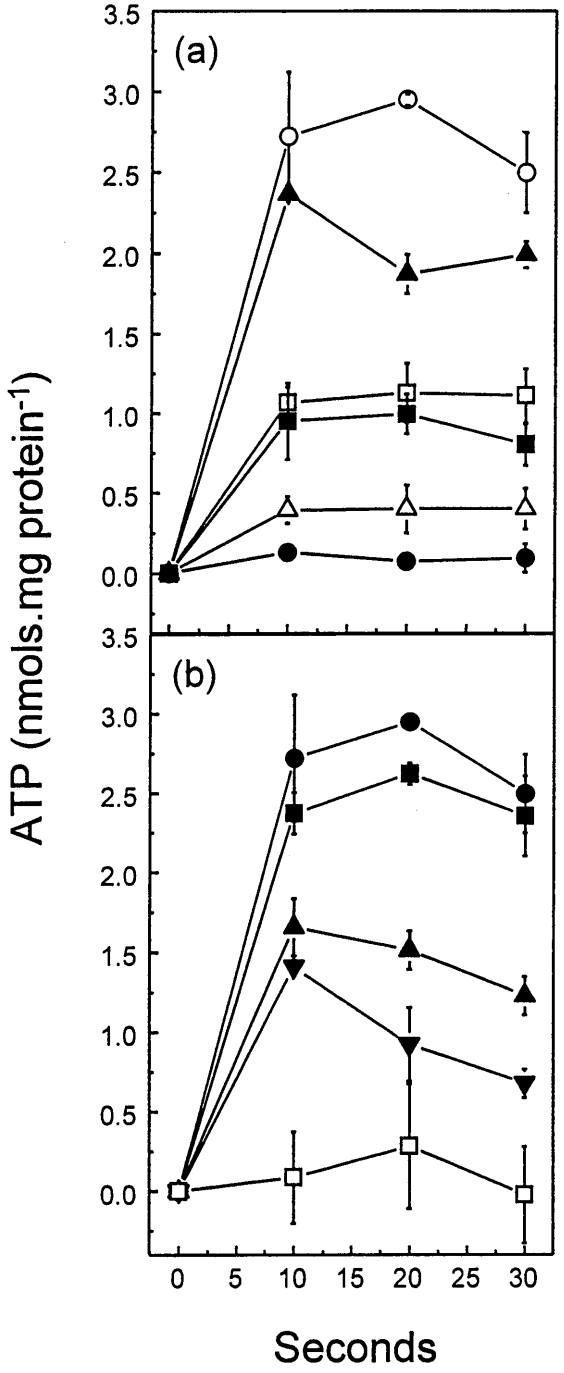

Fig. 3. **a** The influence of external pH on ATP synthesis in *At. caldus* membrane vesicles with an internal pH of 7.5. The external pH was set at 1.0 (*solid circles*), 1.5 (*solid squares*), 2.2 (*open circles*), 2.5 (*solid triangles*), 3.1 (*open squares*), and 5.5 (*open triangles*). **b** The influence of internal pH of *At. caldus* membrane vesicles at an external pH of 2.2. The internal pH in the vesicles was 8.0 (*solid squares*), 7.5 (*solid circles*), 7 (*solid triangles*), 6.5 (*solid inverted triangles*), and 6.0 (*open squares*). The data are mean values obtained from three experiments with the *error bars* showing standard deviations

No ATP synthesis was detected when the internal pH was 6.0 (Fig. 3b).

The effect of temperature on ATP synthesis by the ATPase was also investigated. As would be expected for an

Table 3. Mean values of peak ΔpH-driven ATP production at an external pH of 2.2 ± 0.1 by membrane vesicles loaded with various poisons

Inhibitor	Time to reach peak ATP (s)	Peak ATP ± SD (nmol·mg protein^{-1})
None	20	2.83 ± 0.88
Oligomycin (200 μg ml^{-1})	10	0.58 ± 0.15
DCCD[a] (100 μM)	20	0.09 ± 0.24
Vanadate (1 mM)	5	1.65 ± 0.37
CCCP[b] (20 μM)	5	0.16 ± 0.61
HQNO[c] (10 μM)	10	2.84 ± 0.31
Myxothiazol (20 μM)	5	1.97 ± 0.09
Azide (1 mM)	20	2.07 ± 0.08
Cyanide (10 mM)	5	1.45 ± 0.18
Valinomycin (5 μg ml^{-1})	10	5.47 ± 0.26
K^+ (300 mM)	20	1.64 ± 0.66
Valinomycin + K^+	5	1.43 ± 0.04

ATP values were corrected for the ATP present in the vesicles at the start of the experiment
[a]DCCD, N,N-dicyclohexylcarbodiimide
[b]CCCP, carbonyl-cyanide-m-chlorophenyl-hydrazone
[c]HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide

enzyme from the moderately thermophilic *At. caldus*, the highest ATP synthase activity was found at 45°C (2.95 ± 0.03 nmol ATP·mg protein^{-1}). The enzyme also had high activity at 30°C (2.58 ± 0.18 nmol ATP·mg protein^{-1}), a temperature at which *At. caldus* grows relatively rapidly. The enzyme exhibited a reduced rate of ATP synthesis at 22°C (1.47 ± 0.29 nmol ATP·mg protein^{-1}). At 55°C, very little activity was found (0.60 ± 0.15 nmol ATP·mg protein^{-1}).

Finally, we studied the effect of various inhibitors on the synthesis of ATP by the vesicles. As with the whole cells, ATP synthesis by the membrane vesicles was inhibited by the F_0F_1 ATPase specific inhibitor oligomycin as well as the general ATPase inhibitor N,N′-dicyclohexylcarbodiimide (DCCD). The P-type ATPase inhibitor, vanadate, did not inhibit ATP synthesis by the membrane vesicles (Table 3) to the same extent. Also, when the proton gradient was collapsed by CCCP no ATP was formed. The effect of inhibitors of the electron transport chain was less than that seen in whole cells (Table 3), for example HQNO had no effect on ATP production by membrane vesicles. In contrast to whole cells, some ATP synthesis in vesicles was observed when they were loaded with K^+ and valinomycin plus K^+ (Tables 2 and 3). Valinomycin increased ATP formation compared with the control (Table 3).

Discussion

The bioenergetic parameters that make up the proton motive force have been well studied in acidophiles, owing to the interest generated by the reversed polarity of the membrane potential and the maintenance of a large pH gradient directed into the cytoplasm (Cobley and Cox 1983; Matin 1990). In contrast, little is known about how these bacteria generate ATP. One study has shown that *At. ferrooxidans* generates ATP via an F_0F_1 ATPase (Apel et al. 1980), the genes of which have been cloned and sequenced (Brown and Rawlings 1993). We report here how the moderately thermophilic acidophile *At. caldus* derives energy from the oxidation of reduced inorganic sulfur compounds in the form of ATP.

Resting cells of *At. caldus* were used to investigate the effect of inhibitors and uncouplers on substrate respiration and ATP synthesis. The titration of oxygen consumption rates with cyanide and azide suggested the presence of more than one oxidase, because of the biphasic inhibition by cyanide. The inhibition of the rate of oxygen consumption in the range of 10–100 μM cyanide suggests that *At. caldus* strain KU has a cytochrome *c* oxidase (cytochrome aa_3 or cytochrome cbb_3) and the residual oxygen consumption at >10 mM cyanide suggests the presence of a cyanide-insensitive ubiquinol oxidase.

The oxidation of reduced inorganic sulfur compounds by *At. caldus* resting cells supported ATP synthesis. The very small increase in the ΔGp during sulfite oxidation could have been due to sulfite donating its electrons at the point of cytochrome *c*, and therefore only having a single point of energy conservation (cytochrome oxidase). This has been shown to be the case for other thiobacilli (reviewed in Suzuki 1994). A second possible explanation is that the mean ΔG° [kJ (mol S-substrate)$^{-1}$] for the oxidation of sulfite to sulfate is –230.1 ± 22.0 compared with –750.1 ± 16.1 for thiosulfate (Kelly 1999). The reduction in total O_2 consumed in the presence of myxothiazol for all of the reduced inorganic sulfur compounds except sulfite suggests that the other reduced inorganic sulfur compounds have two sites of energy conservation. They reduce the ubiquinone pool, resulting in energy conservation at the levels of the cytochrome bc_1 complex and the cytochrome oxidase.

ATP production (an increase in ΔGp) in resting cells was inhibited by oligomycin, suggesting that ATP is produced via an F_0F_1 ATPase. The APS reductase experiments were designed to test for the existence of substrate level phosphorylation during sulfite oxidation, as has been proposed for some acidithiobacilli and thiobacilli (Cobley and Cox 1983; Hooper and DiSpirito 1985; Kelly 1999; Kelly et al. 1997). The lack of APS reductase in tetrathionate-grown *At. caldus* KU suggests that all ATP is produced by electron transport phosphorylation.

The uncoupler CCCP not only inhibited an increase in the ΔGp during tetrathionate oxidation, but also lowered the initial ΔGp. This was probably due to the cell expending all of its available energy to maintain pH homeostasis. Addition of electron transport inhibitors also inhibited ATP synthesis during tetrathionate oxidation. Azide and cyanide inhibited an increase in ΔGp, whilst myxothiazol only permitted a small increase. That each of the poisons inhibits electron transport at various points in the electron transport chain indicates that it is a general inhibition of electron transport preventing the synthesis of ATP, and that ATP synthesis does not occur in the absence of electron transport.

In membrane vesicles, ATP could be formed from an artificial pH gradient created across the vesicle membrane. It is probable that the inward flow of protons into the vesicles would have generated a large, inhibitory membrane poten-

tial prior to detectable ATP synthesis. Therefore it is necessary that there was either a counterflow of a cation or a coinflux of an anion for ATP synthesis to have been detected. It is unlikely that, in whole cells, charge balance would be via the inward flow of anions, as their toxicity to acidophiles has been documented (Alexander et al. 1987; Matin 1990), suggesting that charge balance may be achieved via the counterflow of a cation. A cation that could possibly be used for charge balance is K^+, and evidence for this has been suggested for the acidophile *Bacillus coagulans* (Matin 1999). In *At. caldus* membrane vesicles valinomycin caused the outward flow of K^+ (internal K^+ > external K^+) which allowed a greater inward flow of protons through the F_0F_1 ATPase as the H^+/K^+ exchange is electroneutral. This lowered the electrochemical barrier formed by the influx of H^+, suggesting that K^+ may also be used as a counter-ion in *At. caldus*. This hypothesis is also supported by the effects of K^+ and valinomycin + K^+ on whole cells where the resting ΔGp was reduced. A possible cause could be perturbation of the K^+ balance possibly created by an ATP-dependent H^+/K^+ symport that is used to maintain pH homeostasis and an internal positive membrane potential (Matin 1999). Oligomycin, DCCD, and CCCP, but not vanadate, inhibited ATP synthesis in membrane vesicles, supporting the existence of a proton-dependent F_0F_1 ATPase. The electron transport inhibitors that blocked ATP synthesis in whole cells had little effect in membrane vesicles, confirming that their effect on ATP generation during tetrathionate oxidation was due to the absence of electron transport.

The data presented here indicate that all ATP is derived by oxidative phosphorylation in *At. caldus* via an F_0F_1 ATPase. No evidence was found to support substrate-level phosphorylation during sulfite oxidation.

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