

Energy coupling sites in the electron transport chain of *Zymomonas mobilis*

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Received 18 July 1995; revised 25 August 1995; accepted 5 September 1995

Abstract

Energy-coupling sites in the electron transport chain of the obligately fermentative aerotolerant bacterium *Zymomonas mobilis* were examined. The H^+/O stoichiometry of the electron transport chain in intact bacteria oxidizing ethanol was close to 3.3. Cytoplasmic membrane vesicles coupled NADH oxidation to ATP synthesis. With ascorbate/phenazine methosulfate they showed oxygen uptake which was sensitive to antimycin A, but no significant ATP synthesis could be detected. Cells with a defective coupling site I, prepared by cultivation on a sulfate-deficient medium, showed a decreased rotenone sensitivity of respiration, and they lacked almost all the respiration-driven proton translocation and ATP synthesis. We conclude that, despite the reported composition of the electron transport chain, only energy coupling site I was functional in *Z. mobilis*.

Keywords: *Zymomonas mobilis*; Oxidative phosphorylation; Transmembrane pH gradient; Energy-coupling site I; H^+/O stoichiometry

1. Introduction

The obligately fermentative aerotolerant bacterium *Zymomonas mobilis* has an electron transport chain (ETC) consisting of NAD(P)H dehydrogenase, a membrane-bound glucose dehydrogenase, coenzyme Q_{10} , and *b*, *c*, and *d* type cytochromes [1–3]. So far, the energy-generating function of ETC in this bacterium has been a matter of discussion. Oxidative phosphorylation apparently does not operate in growing cultures of *Z. mobilis* as judged from the low aerobic growth yields on glucose (below 10 g dry weight mol^{-1}) [1,4,5]. Recently, however, oxidative phosphorylation activity was demonstrated in starved

cells and cytoplasmic membrane vesicles of *Z. mobilis* [6]. Ethanol and acetaldehyde served as non-fermentable substrates for ATP synthesis in whole cell experiments, while NADH oxidation was shown to drive ADP phosphorylation in cytoplasmic membrane preparations.

The aim of the present paper was to examine the energy-coupling sites of *Z. mobilis* ETC. For this, (i) the proton-translocating stoichiometry of the respiratory chain (H^+/O) was determined, (ii) cytoplasmic membrane vesicles were tested for oxidative phosphorylation with ascorbate/phenazine methosulfate (PMS), (iii) bacteria were cultivated under sulfate deficiency to eliminate coupling site I, and aerobic energy generation in such cells was examined. The results indicate that only energy-coupling site I is operative in *Z. mobilis*.

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2. Materials and methods

2.1. Strain and culture conditions

Zymomonas mobilis ATCC 29191 was maintained and cultivated as described previously [6], with some modifications. The preculture for sulfate-deficient cells was grown on the standard medium with 5% glucose in which magnesium and ammonium sulfates were substituted by the respective chlorides, and only 2.5 g l⁻¹ (instead of 5 g l⁻¹) of Difco yeast extract was added. The sulfate-deficient culture was inoculated with 5% of the preculture and was grown on the same medium. The control cells were cultivated on the standard medium with sulfates and 2.5 g l⁻¹ Difco yeast extract. Cells were harvested in late exponential phase (16–17 h after inoculation) by centrifugation, washed [6], concentrated up to 20 mg dry weight ml⁻¹, and starved on a shaker at 150 rpm for 3–3.5 h. Potassium phosphate buffer, 50 mM (pH 6.5) with 5 mM MgCl₂ · 6H₂O (for sulfate-deficient cells) or with 5 mM MgSO₄ (for control cells) was used both for washing and for starvation. Cultivation and starvation of the cells and all experiments were done at 30°C.

2.2. Preparation of cell-free extracts and cytoplasmic membranes

Cell-free extracts (protein concentration 1.5–2.0 mg ml⁻¹) were prepared by sonication [6]. Cytoplasmic membrane vesicles were obtained from sphaeroplasts prepared by batch cultivation in the presence of glycine and sorbitol [7], harvested by centrifugation at 3000 × g for 15 min, resuspended in a 8–10 times smaller volume of the phosphate buffer with MgSO₄ (see above; buffer was supplemented with 15% sorbitol, 0.1 mg ml⁻¹ dithiothreitol, and 0.1 mg ml⁻¹ DNase), and disrupted by sonication [6]. After removal of unbroken sphaeroplasts by centrifugation at 3000 × g for 15 min, the supernatant was filtered through a Synpor membrane filter (0.22 μm pore diameter Chemapol, Prague) in a 200-ml Amicon filtration unit under 1 atm pressure at 0°C. The membrane vesicles sedimented on the filter were washed with a small volume of the same buffer and,

by using a brush, carefully resuspended to a final protein concentration of 0.2–0.5 mg ml⁻¹. The membrane preparations thus obtained were stored at –20°C for several days without any loss of activity.

2.3. Measurement of respiratory-driven proton translocation

The assay for H⁺/O was essentially as described for *Gluconobacter oxydans* [8]. *Z. mobilis* suspension in 150 mM KCl (6 g dry weight l⁻¹) was equilibrated with 100 mM KSCN at pH 6.5 in a MBR minibioreactor, model MCS-11, working volume 1.5 l. The medium pO₂ was brought to zero by gassing the suspension with N₂. Deoxygenated ethanol was added at a final concentration of 0.5%. Proton expulsion was induced by the addition of 20 ml of air-equilibrated 150 mM KCl solution. pH was monitored with an Ingold pH electrode, model 465, and calibrated by addition of 1 ml deoxygenated 0.1 N HCl.

2.4. Assay of oxidative phosphorylation

Oxidative phosphorylation was measured by ATP synthesis from ADP and inorganic phosphate, and by oxygen utilization in the following reaction mixture which was a modification of that described by Hempfling and Hertzberg [9]: 400 μl phosphate buffer with 15% sorbitol, 20 μl ADP (34 mg ml⁻¹), 5 μl methanol, 100 μl membrane vesicle preparation, and either 20 μl of NADH (0.5 mM final concentration), or 20 μl of ascorbate (20 mM final concentration) + 20 μl of PMS (5 μM final concentration). Oxygen consumption was monitored by a Radiometer Clark type oxygen electrode. Oxygen consumption rates with PMS/ascorbate were corrected for the ETC-independent PMS autoxidation. Samples for ATP determination were fixed in parallel with the oxygen consumption measurements by a transfer of aliquots of the reaction mixture into five times smaller volumes of ice-cold 60% perchloric acid. A correction for the traces of adenylate kinase activity present in the membrane preparations was made by sampling for ATP a reaction mixture without added electron donors. In the whole-cell experi-

ments the samples for ATP determination were fixed in an equal volume of ice-cold 10% trichloroacetic acid.

2.5. Measurement of 9-aminoacridine fluorescence (9-AA)

The fluorescence of 9-AA was measured with excitation at 382 nm and emission at 454 nm [10] using a home-built fluorimeter. A 200- μ l aliquot of the cell suspension starved at pH 6.5 was transferred into the fluorimeter cuvette containing 2.3 ml of phosphate buffer with 30 μ M 9-AA at pH 7.0, thus inducing an inverted transmembrane pH gradient. The biomass concentration in the fluorimeter cuvette was 1.5–1.6 mg dry weight ml⁻¹. A reversible quenching of 9-AA fluorescence was observed after *Z. mobilis* cell addition. The degree of quenching could be varied by changing the magnitude of the induced pH gradient, as demonstrated for *Escherichia coli* [11].

2.6. Analytical assays

Samples for ATP determination fixed in the membrane vesicle experiments were neutralized, centrifuged, and assayed by the hexokinase/glucose-6-phosphate dehydrogenase method [12]. The assay was carried out under nitrogen to avoid the PMS-mediated oxidation of NADPH. For whole cell experiments the ATP samples were assayed by the luciferin–luciferase method [13] using a LKB Wallac model 1251 luminometer. Protein concentration was determined according to Bradford [14]. Cell concentration was determined as optical density at 550 nm and the dry cell mass was calculated using a calibration curve. Standard errors of the mean are given for the experimental data with number of assays in parentheses.

2.7. Chemicals

Firefly lantern extracts, NADP and 9-AA were purchased from Sigma, Deisenhofen, Germany. Hexokinase/glucose-6-phosphate dehydrogenase, NADH and ADP were from Boehringer Mannheim GmbH, Germany. All the other chemicals were of analytical grade from commercial sources.

3. Results

3.1. The stoichiometry of proton translocation

The H⁺/O stoichiometry of the electron transport chain in anaerobically grown, washed *Z. mobilis* cells in the presence of 0.5% ethanol was 3.35 \pm 0.48 (4 repeats).

3.2. Oxidative phosphorylation in membrane vesicles with NADH and ascorbate / PMS

Oxygen consumption in the cytoplasmic membrane vesicles of *Z. mobilis* with ascorbate/PMS as electron donor was sensitive to antimycin A; oxygen consumption was inhibited by more than 80% by 100 nmol (mg protein)⁻¹, implying that cytochrome *b* (and not cytochrome *c*, as in mitochondria [15]) is the main electron acceptor for ascorbate in the ETC of *Z. mobilis*. Therefore, we used ascorbate/PMS to examine the ATP synthesis downstream the cytochrome *b*, in the putative energy-coupling sites II and III.

Respiration rate and ATP synthesis were determined in membrane vesicles prepared from *Z. mobilis* grown anaerobically on the complete medium both with NADH and with ascorbate/PMS as the electron donors (Table 1). In the presence of 5 μ M PMS and 20 mM ascorbate, the oxygen consumption rate, corrected for the ETC-independent PMS autoxidation, was about 2.5 times lower than with 0.5 mM NADH. A similar proportion between NADH oxidase and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine/ascorbate oxidase activities in *Z. mobilis* was observed previously [3]. The rate of ATP synthesis and, hence, the calculated P/O ratios varied for different membrane vesicle preparations. The P/O ratio with NADH for several membrane preparations

Table 1
Oxygen consumption rate and P/O ratio in *Z. mobilis* cytoplasmic membrane vesicles with NADH and ascorbate/PMS

Electron donor	Oxygen consumption (nmol min ⁻¹ (mg protein) ⁻¹)	P/O
NADH	201.5 \pm 25.1 (6)	0.130 \pm 0.056 (3)
Ascorbate/PMS	88.0 \pm 19.7 (5)	0.012 \pm 0.024 (3)

ranged between 0.1 and 0.2, close to the values reported earlier [6]. The P/O ratio obtained with ascorbate/PMS was close to zero (Table 1).

3.3. Growth and respiration of sulfate-deficient *Z. mobilis*

Z. mobilis cells grown on a medium where yeast extract (2.5 g l^{-1}) was the sole source of sulfur differed markedly from the control grown on complete medium. The sulfate-deficient cells showed a higher respiration rate with ethanol (Table 2), while their rotenone sensitivity was lower than that of control cells. At the same time, the sensitivity to antimycin A was not affected. No significant differences in the growth yield or growth rate between the control and sulfate-deficient cells were noticed. These observations are in accordance with earlier reports [16] that sulfate limitation in bacteria causes a selective loss of rotenone sensitivity. This, together with a disappearance of electron paramagnetic resonance signals from the NADH-dehydrogenase-linked FeS cluster N2 [16–18], is indicative for impairment of the energy-coupling site I.

3.4. Transmembrane proton gradient in normal and sulfate-deficient cells

The generation of the transmembrane proton gradient after ethanol addition was monitored in normal and sulfate-deficient cells by 9-AA fluorescence (Fig. 1). Addition of $200 \mu\text{l}$ of the cell suspension, starved at pH 6.5, into a 2.3-ml volume of phosphate buffer with 9-AA at pH 7.0 led to a rapid quenching of fluorescence due to accumulation of 9-AA inside the more acid intracellular space. Subsequent addition of

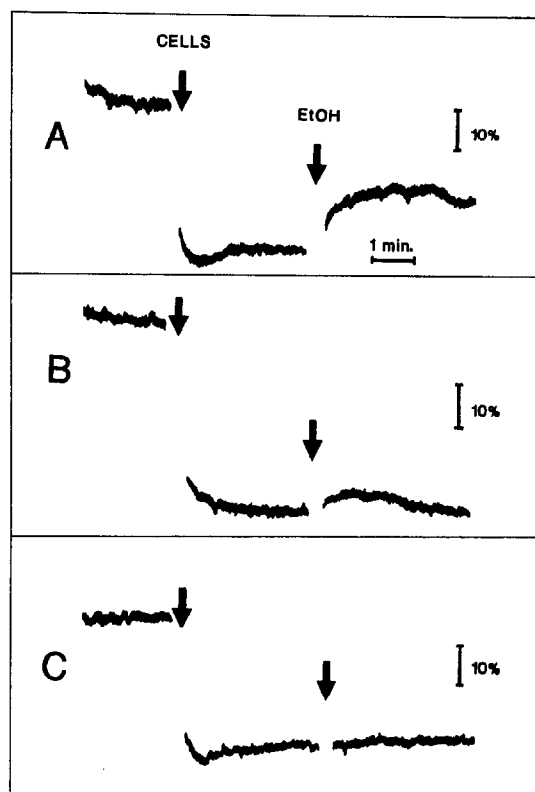


Fig. 1. 9-AA fluorescence in response to ethanol addition to washed and starved *Z. mobilis* cells. (A) control; (B) sulfate-deficient cells; (C) control cells in the presence of 1 mM KCN.

ethanol to a final concentration of 1% caused a partial restoration of the fluorescence intensity in the control cell suspension (Fig. 1A), pointing to an alkalization of the intracellular medium as a result of respiration-driven proton translocation. In the presence of 1 mM KCN, when the respiration was totally inhibited (not shown), the increase of the

Table 2

Respiration rate, growth yield, and inhibitor sensitivity of NADH oxidase in cell-free extracts of *Z. mobilis* cultures grown on complete and sulfate-deficient media

Growth medium	$Y_{x/s}$ (g dry weight mol^{-1})	Respiration with 1% EtOH (nmol min^{-1} ($\text{mg dry weight})^{-1}$)	NADH oxidase activity with inhibitors (% of control)	
			Rotenone ^a	Antimycin A ^a
Sulfate-deficient	3.98	72.9 ± 3.7 (3)	95.2 ± 5.0 (10)	48.4 ± 4.8 (4)
Normal	4.18	63.9 ± 1.1 (3)	81.2 ± 3.4 (10)	46.0 ± 7.0 (5)

^a Inhibitors were added at a concentration of $100 \text{ nmol (mg protein)}^{-1}$.

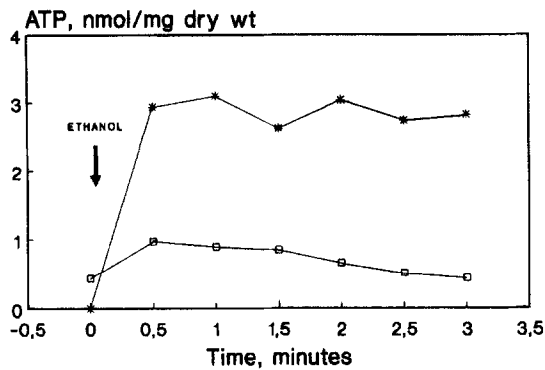


Fig. 2. Time-course of intracellular ATP concentration after ethanol addition to washed and starved *Z. mobilis* cells. (*) control; (□) sulfate-deficient cells.

fluorescence in the control cell suspension was abolished (Fig. 1C). The ethanol-dependent fluorescence increase was strongly reduced in the sulfate-deficient cells (Fig. 1B), although their respiration rate with ethanol was higher than that of the control cells (Table 2).

3.5. ATP synthesis with ethanol in normal and sulfate-deficient cells

Samples for luminometric ATP determination were taken immediately before and at 0.5-min intervals after ethanol was added to an aerated suspension of washed and starved *Z. mobilis* (Fig. 2). An increase of the intracellular ATP concentration up to 3 nmol (mg dry weight)⁻¹ was observed in the control cell suspension. However, in the sulfate-deficient cells, the ATP level rose to only about 0.5 nmol (mg dry weight)⁻¹, and after 2.5–3 min dropped down to the initial level.

4. Discussion

A selective impairment of the energy-coupling site I by sulfate deficiency in the growth medium abolished the respiration-dependent energy generation in *Z. mobilis* cells. Also, when electrons were fed into the ETC downstream of coupling site I using ascorbate/PMS as a substrate in membrane preparations, no significant ATP synthesis was observed. A small, residual ethanol-dependent proton gradient and

some ATP synthesis could still be seen in the sulfate-deficient cells (Fig. 1B; Fig. 2). However, these effects were too small to account for one more fully operative energy-coupling site. The H⁺/O stoichiometry of intact *Z. mobilis* cells oxidizing ethanol did not exceed the values reported for the bacterial energy-coupling site I [18]. Therefore, we concluded that under our experimental conditions the energy-coupling site I was the only functional energy-coupling site in *Z. mobilis*.

The low proton-motive stoichiometry is apparently not consistent with the composition of *Z. mobilis* ETC, which is reported to contain cytochromes *b*, *c*, and *d* [1,2]. However, the existing data on *Z. mobilis* cytochromes and the other ETC components are still incomplete and can be regarded as preliminary. More information about the routes and possible bypasses of electrons in the ETC of *Z. mobilis* would be necessary for understanding why the energy-coupling sites usually associated with cytochromes [19] do not function in this bacterium.

Acknowledgements

The authors wish to thank Prof. Dr. H. Sahn, Prof. Dr. R. Krämer, and Dr. S. Bringer-Meyer for critical reading of the manuscript and for valuable suggestions. This work was supported by a grant from Forschungszentrum Jülich GmbH.

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