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Hypothesis

Ethanol cycle in an ethanologenic bacterium

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Abstract A novel redox cycle is suggested, performing interconversion between acetaldehyde and ethanol in aerobically growing ethanologenic bacterium *Zymomonas mobilis*. It is formed by the two alcohol dehydrogenase (ADH) isoenzymes simultaneously catalyzing opposite reactions. ADH I is catalyzing acetaldehyde reduction. The local reactant ratio at its active site probably is shifted towards ethanol synthesis due to direct channeling of NADH from glycolysis. ADH II is oxidizing ethanol. The net result of the cycle operation is NADH shuttling from glycolysis to the membrane respiratory chain, and ensuring flexible distribution of reducing equivalents between the ADH reaction and respiration.

Key words: Alcohol dehydrogenase; Futile cycle; Respiration; Chemostat; NADH channeling; *Zymomonas mobilis*

1. Introduction

Futile cycles, performing cyclic transformations of metabolites with net energy dissipation, are found both in prokaryotes and eukaryotes. The known futile cycles are driven by ATP or proton motive force and are responsible for uncoupled growth in bacteria, as well as for heat generation in some animal species [1,2]. Here we propose a novel type of an apparently energy non-dissipating redox futile cycle in the respiring ethanologenic bacterium *Zymomonas mobilis*, performing simultaneous acetaldehyde reduction and ethanol oxidation.

In various ethanol producers the last reaction of the fermentative pathway to ethanol is a reversible reduction of acetaldehyde: acetaldehyde+NADH+H^+ ⇄ ethanol+NAD^+. The value of K_{eq} for ethanol oxidation is as low as 6.92×10^{-12} M, hence, the equilibrium is shifted far towards ethanol synthesis [3]. In *Z. mobilis* the reaction is catalyzed by the alcohol dehydrogenase (ADH) isoenzymes ADH I and ADH II [4]. The ADH isoenzymes of *Z. mobilis* are unrelated to each other: ADH I is a member of group I ADHs and contains zinc in its active site, while ADH II belongs to group III ADHs and contains iron [5]. For ADH I, the maximum rate of acetaldehyde reduction exceeds that of ethanol oxidation, reaching its highest value at pH 6.5, which is close to the reported intracellular pH for *Z. mobilis* [6,7]. ADH II oxidizes ethanol faster than it reduces acetaldehyde, and its pH optimum lies in the alkaline region.

Under aerobic conditions a part of NADH, generated in the Entner–Doudoroff glycolytic pathway, is oxidized by respiration. The respiratory chain competes for NADH with the ADH reaction. Relative to anaerobic conditions, this leads to a decrease in the ethanol yield and to acetaldehyde accumulation [8]. Anaerobic *Z. mobilis* cultures produce ethanol from glucose with a high yield, close to the theoretical maximum value of 0.51 g of ethanol produced per g of glucose consumed [9]. However, much lower yields, like 0.17 g g^{-1} [8], or 0.13 g g^{-1} [10], have been reported for aerobic cultures. The low ethanol yield, as well as accumulation of byproducts, more oxidized than ethanol (acetaldehyde, acetone, acetate), indicates that in aerated cultures a substantial or even the major part of NADH is being oxidized in the respiratory chain.

2. How can respiration withdraw a substantial part of NADH?

This is an intriguing question about the aerobic metabolism of *Z. mobilis*, which so far has not been addressed. If both ADH isoenzymes were catalyzing the ethanol synthesis, the activity of the respiratory chain would be too weak to compete with the ADH reaction. Both ADH isoenzymes together represent up to 5% of the soluble cell protein [4,11], ensuring the high specific rate of ethanologenesis in this bacterium. From the data by Neale et al. [4] one can estimate the total activity of both isoenzymes in *Z. mobilis* cell extracts in the direction of acetaldehyde reduction at pH 6.5. It is close to 2.1 U (mg dry weight)$^{-1}$ (or, roughly, around 4 U (mg total protein)$^{-1}$), with approximately equal contributions from each isoenzyme. At the same time, the activity of the NADH oxidase is much lower. For cell-free extracts the reported values are in the range between 0.05 and 0.2 U (mg protein)$^{-1}$ [12–14].

Furthermore, ADH isoenzymes have higher affinities for NADH. In aerobically growing cells the prevailing respiratory NADH dehydrogenase isoenzyme (probably, a homolog of *Escherichia coli* ndh) has its K_m for NADH close to 50–60 μM [15,16], but for ADH I and ADH II the corresponding values (at saturating acetaldehyde concentration) are 27 μM and 12 μM, respectively [11]. Depending on aeration intensity, acetaldehyde in aerobic cultures may reach concentrations from several millimolar to several tens of millimolar (approximately from 0.1 to 2.0 g l^{-1}) [8,10], while the K_m for acetaldehyde is just 86 μM for ADH I and 1.3 mM for ADH II [11].

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Hence, in aerated cells both isoenzymes are operating at near-saturating acetaldehyde concentrations, and the apparent K_m values for NADH in vivo might be fairly close to the reported in vitro data. Since in respect of NADH oxidation both ADH and the respiratory NADH dehydrogenase conform to the Michaelis–Menten kinetics [4,11,16], the respiratory chain can not be expected to outcompete ADH at any intracellular NADH concentration. In order to understand the paradoxic interplay between the ADH reaction and respiration in *Z. mobilis*, a study of the ADH kinetics in vivo under aerobic steady-state conditions was undertaken.

3. ADH reaction in an aerobic chemostat

We investigated the response of the ADH reaction to the addition of a small amount of ethanol in a vigorously aerated chemostat culture at 50% pO_2. Bacteria *Z. mobilis* ATCC 29191 were grown on a medium containing 20 g l^{-1} glucose, 5 g l^{-1} yeast extract and mineral salts [7]. Substrate, product and biomass concentrations were measured, as previously described [10], and the intracellular NAD(P)H concentration was monitored by bioluminescence assay [17] in KOH extracts of cells.

At a flow rate of 0.2 h^{-1}, stationary ethanol and acetaldehyde concentrations were 1.7 g l^{-1} and 0.5 g l^{-1}, respectively. The specific rate of glucose consumption was 31 mmol (g dry weight)$^{-1}$ h^{-1}, and the specific rate of the ethanol production was 7.5 mmol (g dry weight)$^{-1}$ h^{-1}. Although the intracellular NADH level (Fig. 1) was 0.03 nmol (g dry weight)$^{-1}$, which is several times lower than that in anaerobically cultivated *Z. mobilis* [18], the steady-state reactant ratio ([NADH][acetaldehyde][H^+])/([ethanol][NAD^+]) was still above K_{eq}, in full accordance with the observed net ethanol synthesis. For the lowest estimate of the reactant ratio, we assumed that NAD^+ made up most of the 5 mM intracellular NAD(P)H pool, detected in *Z. mobilis* by NMR [19], and that the intracellular pH was 7.0. Taking 3.3 μl (mg dry weight)$^{-1}$ for the intracellular volume [20], we found that the reactant ratio was at least 5.9×10^{-11} M.

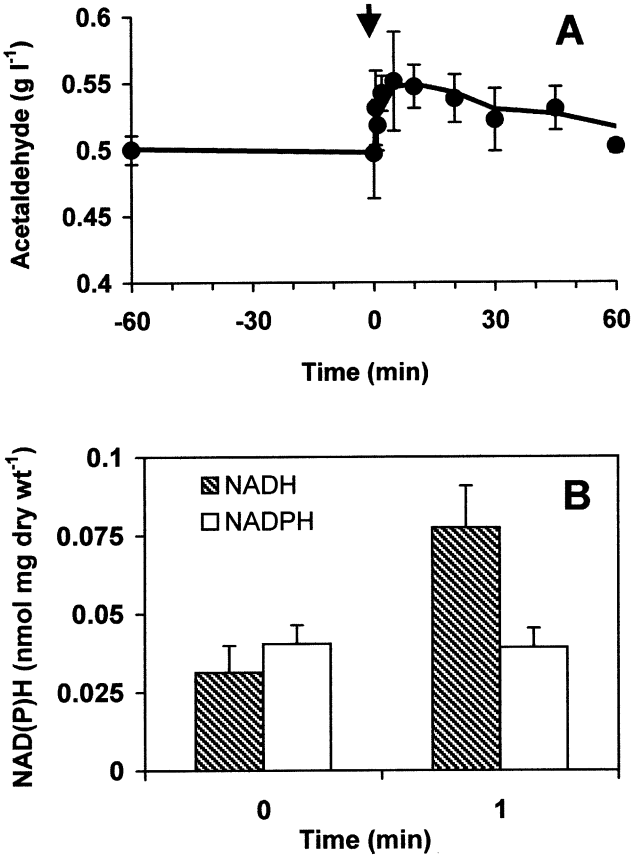

Fig. 1. Response of an aerobic chemostat culture to ethanol addition. Bacteria were cultivated at pH 6, in 1 l working volume at 400 rpm stirring rate and 2 l min^{-1} air flow, ensuring 50% saturation of culture with oxygen. Data points represent means of three perturbations of the steady-state. A: Increase of acetaldehyde concentration after addition of 1 g of ethanol (indicated by an arrow). B: Effect of ethanol addition on the intracellular NADH and NADPH levels.

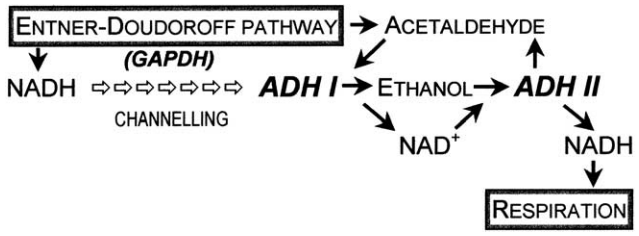

Fig. 2. Proposed scheme of ethanol cycle, explaining the role of ADH II in respiration. NAD(P)H, generated by glucose-6-phosphate dehydrogenase reaction and, presumably, contributing to the bulk intracellular pool, is not shown.

However, the addition of 1 g l^{-1} of ethanol to the culture caused a burst of ethanol oxidation, manifested by a rapid acetaldehyde synthesis and rise of the intracellular NADH concentration (Fig. 1). During the first 2 min the specific rate of acetaldehyde generation increased by more than 30 mmol (g dry weight)$^{-1}$ h^{-1}, which exceeded the stationary rate of the ethanol production four times. Meanwhile, the reactant ratio after the ethanol addition had decreased just 1.6 times, and remained well above K_{eq}. This apparent discrepancy necessarily implies that one of the ADH isoenzymes in *Z. mobilis* is catalyzing ethanol oxidation. To put it another way, the rapid, transient ethanol oxidation reveals a perturbation of a cycle, consisting of an ethanol-synthesizing and ethanol-oxidizing reaction, both being several times faster than the observed net rate of ethanol synthesis.

4. Is ADH II the putative ethanol-oxidizing isoenzyme?

Allyl alcohol-resistant mutants of *Z. mobilis* and yeast are known to be deficient in ADH II [18,21,22]. In our laboratory a mutant strain was obtained by UV mutagenesis and subsequent selection for allyl alcohol resistance, following the procedure of O'Mullan et al. [22]. Respiration of the mutant strain was compared with that of the parent strain, by measuring respiration rates of stationary phase cell suspensions in a potassium phosphate buffer (pH 6.9) supplemented with 4 g l^{-1} glucose, using Clark oxygen electrode [10]. The parent strain cells respired at the specific rate 0.138 (±0.033) U (mg dry weight)$^{-1}$, while the mutant strain showed a much lower respiration rate of just 0.028 (±0.003) U (mg dry weight)$^{-1}$. At the same time, the membrane NADH and NADPH oxidase activities did not differ between both strains (not shown).

If ADH II under aerobic conditions was operating in the

direction of ethanol synthesis (and thus competing with the respiratory chain for NADH), then an elevated, rather than reduced, respiration rate should be expected in an ADH II-deficient mutant. Our finding that the respiration of the mutant was in fact strongly decreased, could be best explained within the framework of the postulated ethanol cycle, with ADH II as the ethanol-oxidizing isoenzyme.

5. Importance of NADH channeling

A simultaneous catalysis of the same reaction in both directions is thermodynamically impossible, unless the isoenzymes are exposed to different reactant concentrations. There is a good reason to think that the ADH isoenzymes in *Z. mobilis* cells are differently supplied with NADH. Direct NADH channeling from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to alcohol dehydrogenase has been the subject of a long-lasting debate in biochemistry [23–25]. The discussions so far have centered upon the results of kinetic experiments with purified, concentrated enzyme solutions, with poor reference to the real intracellular conditions. Notably, in *Z. mobilis*, electron microscopy with gold-labeled antibodies has indicated that ADH I (but not ADH II) forms a supramolecular complex with GAPDH [26], providing an in situ evidence in favor of NADH channeling.

As a result of channeling, the local NADH concentration at the ADH I active site would be kept higher than in the cytosol, promoting acetaldehyde reduction. On the other hand, due to the respiratory chain activity and NADH channeling to ADH I, the local NADH concentration at the ADH II active site might be low enough to enable ethanol oxidation.

6. Conclusions

The proposed cycle is shown in Fig. 2. According to the present hypothesis, ADH II supplies the respiratory chain with NADH, oxidizing part of ethanol made by ADH I, and thus enabling respiration to compete with the ethanol-synthesizing reaction. In contrast to 'classical' futile cycles, the ethanol cycle in *Z. mobilis* is driven by channeling and oxidation of redox equivalents, but not by ATP hydrolysis. First synthesizing an ethanol molecule and then reoxidizing it could make sense for a flexible regulation of respiration: the ADH II-catalyzed reaction might represent an important and so far overlooked checkpoint in the way of NADH flux from the Entner–Doudoroff glycolytic pathway to the respiratory chain.

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