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Intracellular redox state regulates the resistance of *Zymomonas mobilis* alcohol dehydrogenase II to cyanide and oxygen

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Abstract

The variable sensitivity of *Zymomonas mobilis* iron-containing alcohol dehydrogenase isoenzyme (ADH II) to cyanide and oxygen was studied. The cyanide-sensitivity of ADH II was highest in cells grown under conditions of vigorous aeration, in which intracellular NADH concentration was low. Anaerobically grown bacteria, as well as those cultivated aerobically in the presence of cyanide, maintained a higher intracellular NADH pool along with a more cyanide-resistant ADH II. In aerobically grown permeabilized cells, cyanide caused gradual inhibition of ADH II, which was largely prevented by externally added NADH, and, to lesser extent, by NAD. It was demonstrated that cyanide acted as a competitive inhibitor of ADH II, competing with nicotinamide nucleotides. NADH increased both the cyanide resistance and oxygen resistance of ADH II.

Key words: alcohol dehydrogenase, cyanide resistance, oxygen resistance, *Zymomonas mobilis*.

Introduction

There are two cytoplasmic alcohol dehydrogenase (ADH) isoenzymes (EC 1.1.1.1) in the Gram-negative, aerotolerant, ethanol-producing bacterium *Zymomonas mobilis*: a zinc-containing ADH I and an iron-containing ADH II (Kinoshita et al. 1985; Neale et al. 1986; Conway et al. 1987). Both of them are NAD^+-dependent, and together they carry out rapid and efficient synthesis of ethanol, which has attracted the attention of researchers for decades (Rogers et al. 1982; Sprenger 1996; Dien et al. 2003). ADH I is essential in the early stages of culture growth, while ADH II plays a key role later in the fermentation at high ethanol concentrations (O'Mullan et al. 1995). No ADH I-negative mutants have been reported so far, yet ADH II-negative mutants have been obtained and characterized by several authors (Wills et al. 1981; O'Mullan et al. 1995; Delgado et al. 2002). The absence of ADH II results in a prolonged generation time of the mutant strain, as well as impaired growth and ethanol synthesis during the late exponential and early stationary phases of culture (O'Mullan et al. 1995).

However, ethanol synthesis might not be the sole function of ADH II. It seems likely that iron-containing bacterial alcohol dehydrogenases are involved also in respiratory metabolism and oxidative stress response. There is strong evidence from aerobic chemostat experiments (Kalnenieks et al. 2002) that ADH II may function as a component of the

respiratory pathway of *Z. mobilis*. Under vigorous aeration it oxidizes ethanol and supplies the respiratory chain with NADH, while ADH I catalyses ethanol synthesis, thus forming an "ethanol cycle". Notably, iron-containing alcohol dehydrogenases themselves are sensitive to oxygen. Under oxic conditions, ADH II looses much of its activity, apparently due to free radical reactions of oxygen at the active site iron (Tamarit et al. 1997). The same is true for *E. coli* iron-containing isoenzyme ADH-E, homologous to *Z. mobilis* ADH II, which also is highly sensitive to metal-catalyzed oxidation (Membrillo-Hernández et al. 2000). It has been demonstrated that ADH-E needs to be protected against oxidative damage by the chaperone DnaK during aerobic growth (Echave et al. 2002). Besides, ADH-E also acts as a H_2O_2 scavenger and, becoming partially inactivated, protects *E. coli* cells against hydrogen peroxide stress (Echave et al. 2003).

Recently we demonstrated another property of ADH II, traditionally associated with respiratory metabolism – its sensitivity to inhibition by cyanide (Kalnenieks et al. 2003). Inhibition of ADH II at submillimolar cyanide concentrations proceeds gradually, and presumably, reflects slow binding of cyanide to the active site iron. Moreover, the cyanide-sensitivity of ADH II can change. When cells are grown aerobically in the presence of submillimolar cyanide concentrations, ADH II largely looses its sensitivity to cyanide (Kalnenieks et al. 2003). The sensitivity change takes several hours, and the peak of ADH II cyanide resistance coincides with the paradoxical stimulation of aerobic growth in the presence of cyanide (Kalnenieks et al. 2000; 2003). The nature of the variable ADH II cyanide resistance remains an open question. In the present work our aim was to study the mechanism of this phenomenon.

Knowing that cyanide rapidly inhibits the respiratory chain of *Z. mobilis* (Kalnenieks et al. 2000; 2003), a cyanide-dependent shift of intracellular redox cofactor balance could be anticipated as another major effect of this inhibitor. We put forward a working hypothesis postulating that the cyanide-dependent variation of ADH II cyanide-sensitivity was related to the change of the intracellular NADH/NAD^+ ratio. Therefore, in the present work we studied the effects of NADH and NAD^+ on the kinetics of ADH II inhibition by cyanide.

Materials and methods

Bacterial strain and cultivation

Z. mobilis ATCC 29191 was maintained and cultivated at 30 °C in a growth medium containing glucose (50 g l^{-1}), yeast extract ('Difco'; 5 g l^{-1}) and mineral salts, as described previously (Kalnenieks et al. 1993). Aerobic chemostat cultivation was carried out in a 'Labfors' fermenter ('Infors') with 1.5 l culture volume, air flow at 3 l min^{-1} and stirring speed of 400 rpm, pH 6.0 with or without constant cyanide feed (Kalnenieks et al. 2000). Under these conditions a steady oxygen concentration, typically in the range between 40 and 50 % of saturation, was established. Vigorously aerated batch cultivations were carried out on a shaker at 160 rpm in 0.75 l unbaffled flasks containing 50 ml culture. Oxygen supply in these cultures was comparable to that in the aerobic chemostat, as judged from the specific rate of acetaldehyde production, which was similar in both cases (not shown). For cultivations or incubations under moderate aeration, shaking (with smaller amplitude) at 100 to 125 rpm in a water bath was applied to 100 ml unbaffled flasks containing 30 to 50 ml culture. Batch cultures referred to as 'anaerobic' were grown under oxygen-limited conditions in 0.5 l flasks containing 0.4 to 0.5 l culture incubated without shaking.

Permeabilization of cells

Cells were permeabilized following a slightly modified procedure of Osman et al. (1987). Bacteria were pelleted by centrifugation and resuspended at 7 g dry mass ml^{-1} in 30 mM potassium phosphate buffer, pH 6.5, containing 2 mM $MgCl_2$. One milliliter of the obtained suspension was centrifuged, and the pellet was resuspended in 0.2 ml of the same buffer, containing 0.2 mg lysozyme. After that, 15 ml of chloroform was added, the sample was vortexed for 45 s, and placed on ice for 10 min. Then, 0.8 ml of ice-cold buffer was added, and the suspension was used for incubations and measurement of ADH activities, methanol solution of chloramphenicol (10 mg ml^{-1}) was added to prevent *de novo* protein synthesis. As shown previously (Kalnenieks et al. 2003), an advantage of this permeabilization procedure (in contrast to ultrasonic breakage of cells) was that it made ADH accessible to external NADH, while totally inactivating NADH oxidase.

Alcohol dehydrogenase assays

Alcohol dehydrogenase activity was estimated in the direction of ethanol oxidation, as described by Neale et al. (1986). The total ADH activity and the activity of ADH I were measured spectrophotometrically at 340 nm after transfer of an aliquot (10 to 20 ml) of the cell suspension into a cuvette with 1.5 ml of 30 mM Tris-HCl buffer, pH 8.5, containing 1 mM NAD^+. The rate of NADH generation was monitored at room temperature. For measurement of the total activity of both isoenzymes, 1 M ethanol was added to the buffer. Discrimination between the two isoenzyme activities was based on the fact that only ADH I, but not ADH II, could oxidize butanol. For measurement of the ADH I butanol-oxidizing activity, which was taken to be half of the ADH I ethanol-oxidising activity (Kinoshita et al. 1985), 200 mM butanol was added in place of ethanol. ADH II activity was found by subtraction of the estimated ADH I ethanol-oxidizing activity from the total ethanol-oxidizing activity. Control assays showed that without ethanol or butanol in the reaction buffer NADH generation did not take place. Furthermore, permeabilised cells did not oxidise externally added NADH.

Analytical methods

Intracellular NAD(P)H concentrations were determined luminometrically with a LKB 'Wallac 1251' luminometer, using the 'Roche' bacterial luciferase assay, as described previously (Karp et al. 1983; Kalnenieks et al. 2002). Calculation of the intracellular NAD(P)H concentration was based on the published value (3.3 ml mg^{-1} dry mass) of *Z. mobilis* intracellular volume (DiMarco, Romano 1985). The time-course of the intracellular NAD(P)H concentration was monitored fluorimetrically (Chance et al. 1979), using an excitation filter with the spectral maximum around 366 nm, and an emission filter with the maximum around 515 nm. Cell concentration was determined as optical density at 550 nm, and dry cell mass of the suspensions was calculated by reference to a calibration curve. Microsoft Excel software was used for calculations, statistical treatment and plotting of data. If not stated otherwise, all chemicals were purchased from 'Sigma'.

Results

Competitive binding of cyanide at the active site of ADH II

Permeabilized cell suspension, obtained from an aerobically cultivated overnight batch

culture, was incubated at 0 °C for 1 h with added NAD^+ (final concentration 100 or 500 mM) and various potassium cyanide concentrations (0 to 500 mM), and both ADH activities were then measured. The dependence of the calculated initial rates of ADH II-catalyzed ethanol oxidation on the inhibitor concentration at both NAD^+ concentrations was plotted on Dixon coordinates (Fig. 1). According to this plot, cyanide acted as a competitive inhibitor of ethanol oxidation, competing with the oxidized cofactor. The apparent inhibition constant K_i was close to 200 mM.

It was not possible to apply the same straightforward approach to the opposite reaction (acetaldehyde reduction), because under the given assay conditions acetaldehyde rapidly reacted with cyanide (unpublished observation). Therefore, to estimate the competition betwen NADH and cyanide, we used an indirect assay. The permeabilized cell suspension was incubated at 0 °C with various NADH or NAD^+ concentrations (0, 20, 200 and 2000 mM) for 20 minutes. Then cyanide was added to a final concentration of 200 mM, and incubation was continued for an additional 45 min. Control samples were incubated under similar conditions without cyanide addition, and their activity remaining after the incubation period was taken as the reference (100 %) for the data shown in the Fig. 2A. After incubation, ADH activity was monitored exactly as in the previous experiment, but using assay buffer without cyanide, which contained standard amounts of NAD^+ and ethanol (or butanol). It was assumed that the initial rate of ADH II reaction in each sample would depend on cyanide previously bound to the enzyme, and, hence, on the competition between cyanide and the cofactors during the incubation phase.

The results showed that the percentage of ADH II activity that remained after a 45-min incubation of permeabilized cells with 200 mM cyanide, indeed strongly depended on the cofactor concentration during the incubation phase (Fig. 2A). NAD^+ caused a small but significant increase of cyanide resistance of ADH II at 200 and 2000 mM concentrations. NADH had a much more pronounced effect, at higher concentrations turning ADH

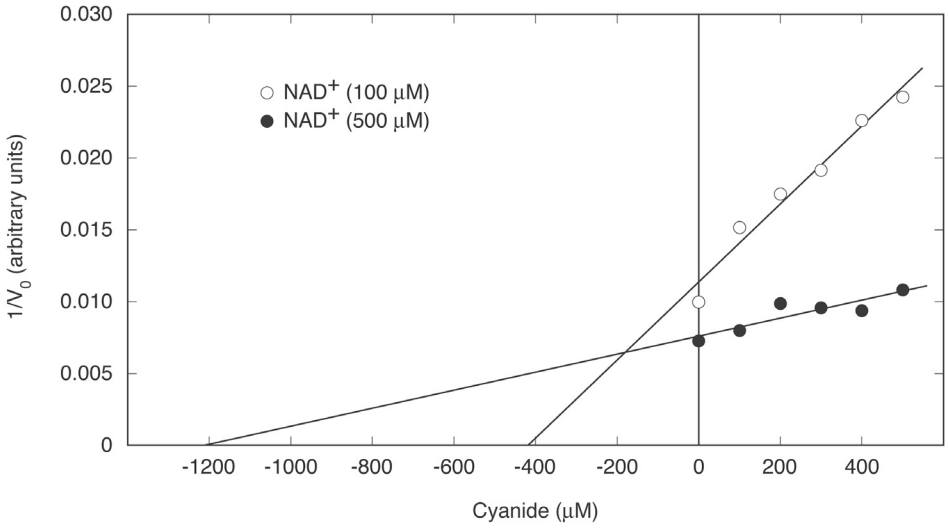

Fig. 1. Dixon plot of ADH II inhibition by cyanide at different NAD^+ concentrations. The plotted values are means of three independent experiments (see comments in the text).

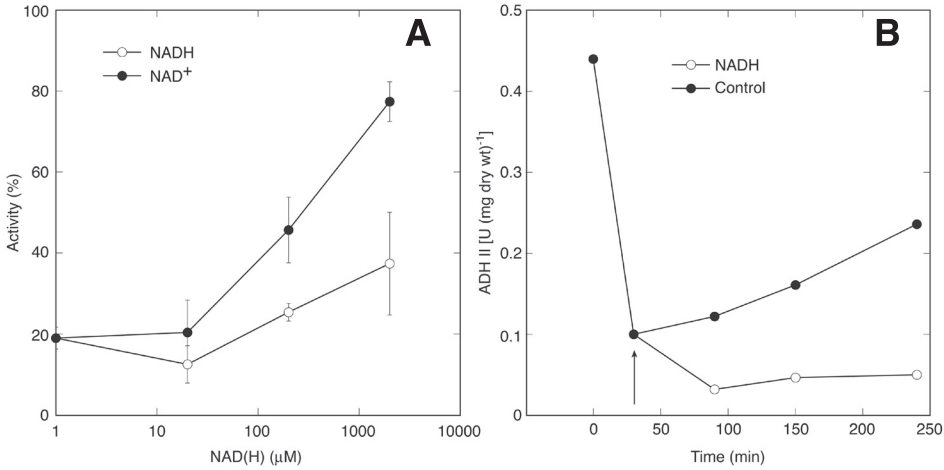

Fig. 2. Elevation of cyanide-resistance of ADH II in the presence of NADH and NAD^+. A, ethanol-oxidising activity of ADH II remaining after a 45-min incubation of aerobically grown permeabilized cell suspension at 0 °C with 200 mM cyanide in the presence of various NADH (open symbols) or NAD^+ (closed symbols) concentrations. In the control samples, incubated without cyanide, the activity of ADH (taken as 100 %) was 0.22 (± 0.06) U mg^{-1} dry mass for ADH I and 0.36 (± 0.20) U mg^{-1} dry mass for ADH II. Mean values of three to five (± SEM) experiments are presented. B, time course of ADH II ethanol-oxidising activity in a permeabilised cell suspension, incubated on a shaker at 15 °C with 200 mM cyanide; 2000 mM NADH added (open symbols), where indicated by an arrow; control (closed symbols) incubated without NADH addition.

II almost insensitive to cyanide. Notably, addition of NADH partially restored ADH II activity. Fig. 2B shows the time-course of gradual restoration of the ADH II activity after NADH addition to permeabilized cells previously treated with cyanide. We speculate that the elevation of cyanide resistance and restoration of ADH II activity can be explained by competition between NADH and cyanide for binding at the active centre of ADH II. The fact that NADH more efficiently competed with cyanide than did NAD^+ was in good agreement with the reported K_M values for both ADH II cofactors (Kinoshita et al. 1985): 12 mM for NADH and 110 mM for NAD^+. In general, this finding means that elevated intracellular NADH concentrations (increased NADH/NAD^+ ratio) can, in principle, increase the apparent cyanide-resistance of ADH II.

Effect of the redox state of culture on the cyanide-sensitivity of ADH II

In order to verify the putative importance of intracellular NADH concentration for ADH II cyanide-resistance in growing cells, bacteria were cultivated under different conditions of aeration and cyanide, and then: (i) cyanide sensitivity of both alcohol dehydrogenases was assayed in permeabilized cell suspensions, and (ii) intracellular NAD(P)H concentrations were determined. The percentage of enzymatic activity remaining after 45 min of incubation of the permeabilized cells with 200 mM cyanide at 0 °C (relative to that of the control sample, incubated for the same period of time without cyanide addition), is presented in Fig. 3A. In agreement with our previous observations (Kalnenieks et al. 2003), ADH I was almost insensitive to cyanide under all experimental conditions. However,

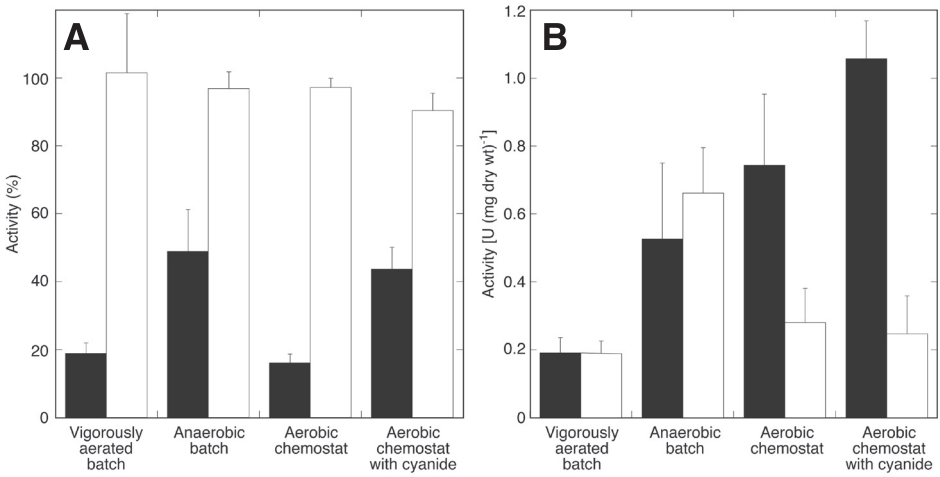

Fig. 3. Dependence of ADH cyanide-sensitivity on culture conditions. Samples for cell permeabilisation were taken from batch cultures in their early stationary phase (after 18 to 20 h of growth), or from steady-state chemostat cultures. Permeabilised cell suspensions were incubated with 200 mM cyanide for 45 min at 0 °C, and the remaining activities (relative to the respective controls without cyanide) of ADH I (empty bars) and ADH II (filled bars) were determined. A, ADH relative activities remaining after incubation with cyanide; B, ADH activities in control samples.

culture aeration strongly affected the cyanide sensitivity of ADH II. It was particularly sensitive to cyanide in aerobically cultivated samples. In permeabilized cells taken from a vigorously aerated chemostat or batch culture, the activity of ADH II after 45 min had decreased to below 20 % of the corresponding control value. At the same time, ADH II in the cells from an anaerobic batch, or from an aerobic cyanide-fed chemostat, retained 40 to 45 % of its activity. Notably, the cyanide resistance of ADH II was not correlated with its absolute activity (Fig. 3B). Elevation of ADH II cyanide resistance in cells grown aerobically with cyanide was demonstrated in our previous paper (Kalnenieks et al. 2003). The present results show that cyanide *per se* is not essential for the increase of ADH II cyanide-resistance. Anaerobic growth conditions led to an equally cyanide-resistant ADH II (Fig. 3), for which the presence of cyanide in the culture medium had no extra effect (not shown).

Also, the intracellular concentrations of reduced nicotinamide nucleotides in *Z. mobilis* depended very much on the culture redox conditions. In Fig. 4, intracellular NAD(P)H concentrations under three different modes of cultivation are presented. The intracellular NADH concentration varied over a range of almost two orders of magnitude, while the NADPH concentration varied to a somewhat lesser extent. A large difference in NADH concentrations (more than an order of magnitude) was seen between a vigorously aerated culture (in a fermenter with 1 l working volume, 3 l min^{-1} air flow, stirring at 400 rpm) and a moderately aerated culture (on a water bath shaker). Doubling of the intracellular NADH concentration (from approx. 300 to 600 mM) took place in 1 h after cyanide addition at a 200-μM final concentration to the moderately aerated batch culture. In good accordance with our data, an intracellular NADH concentration close to 400 mM was reported previously for an anaerobic batch culture during exponential and early stationary

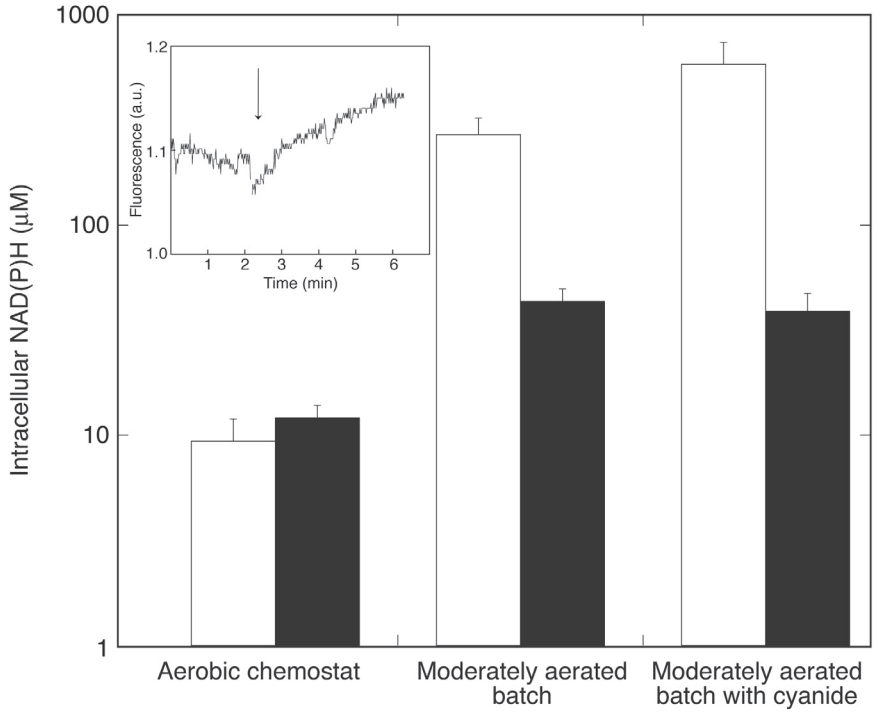

Fig. 4. Intracellular NADH (empty bars) and NADPH (filled bars) concentrations at various cultivation conditions. Mean results of three experiments are presented (± SEM). Inset: the time-course of intracellular NAD(P)H concentration in a suspension of aerobically grown cells after addition of glucose (indicated by the arrow, 1 % final concentration), as monitored fluorimetrically (arbitrary units given).

growth phase (Osman et al. 1987). The characteristic transition times between different NAD(P)H concentration steady-states were in the range of several minutes, as illustrated by the fluorescence data (Fig. 4, inset).

We noted that the range of variation of the ADH II cyanide resistance (Fig. 3) and the corresponding variation of intracellular NADH levels (Fig. 4), fitted reasonably well into the relationship obtained for permeabilized cells with external NADH (Fig. 2A). This finding points to the quantitative relevance of intracellular NADH variation for explaining the observed changes of ADH II cyanide resistance under different growth conditions.

Given that the oxidative damage of ADH II is due to exposure of the active site iron to oxygen (Tamarit et al. 1997), one might speculate that binding of NADH protects the enzyme also against oxidative damage under aerobic conditions. Indeed, data presented in Fig. 5 show a dramatic increase of ADH II resistance to oxygen in permeabilized cells with added external NADH. A suspension of permeabilized cells was incubated at 125 rpm in a water bath shaker at 15 °C. Without addition of external NADH, the activity of ADH II after 4 h of incubation had decreased more than five times, in good agreement with the previously reported results (Tamarit et al. 1997). Yet, even at the lowest concentration of externally added NADH (20 mM), the inactivation of ADH II proceeded much slower:

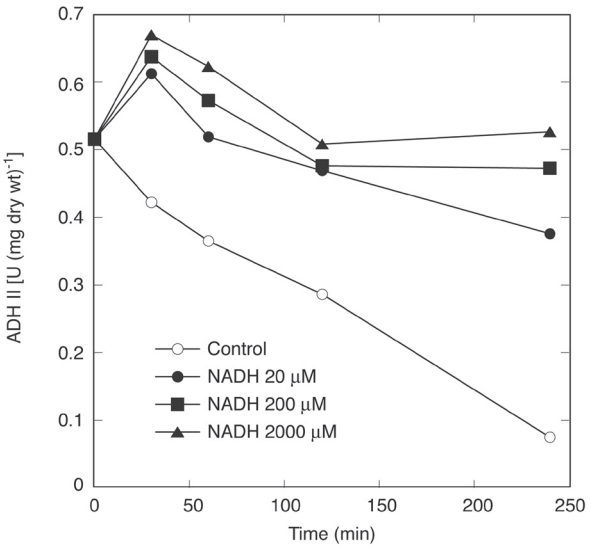

Fig. 5. Time-course of the ethanol-oxidising activity of ADH II in permeabilized cells, incubated on a shaker at 15 °C in the presence of various NADH concentrations. .

only about 20 % of activity was lost after 4 h of incubation. At higher NADH concentrations the activity remained fairly stable.

Discussion

Cyanide is an important ecological factor for microorganisms. It appears in industrial wastewaters, from which various cyanide-degrading species have been isolated (Dubey, Holmes 1995). Many plant species are cyanogenic, particularly in equatorial areas (Francisco, Pinotti 2000): they produce cyanogenic glycosides, which serve to protect the plant against herbivores. Cyanide can be expected to influence also the cyanogenic plant interactions with microorganisms. *Zymomonas mobilis* was first isolated in Mexico from fermenting *Agave americana* juice (Swings, deLey 1977). Although *A. americana* does not belong to the group of cyanogenic plants (Francisco, Pinotti 2000), *Z. mobilis* might well encounter other species in its natural habitat, which are cyanogenic. Therefore, the unusual physiological response of *Z. mobilis* to cyanide might have implications for its natural life cycle, which is poorly investigated.

Z. mobilis responds to cyanide addition to the growth medium in a specific way. Growth in the presence of cyanide does not induce any spectroscopically detectable changes in the *Z. mobilis* respiratory chain (Kalnenieks et al. 2000), nor does it alter the cyanide sensitivity of membrane respiration (Kalnenieks et al. 2003). Instead of expression of a cyanide resistant respiratory pathway, typical for bacteria (Ashcroft, Haddock 1975; Knowles 1976; Kita et al. 1984), *Z. mobilis* elevates the cyanide-resistance of its alcohol dehydrogenase isoenzyme, the iron-dependent cyanide-sensitive ADH II (Kalnenieks et al. 2003). Here we show that the cyanide-sensitivity of ADH II depends on the intracellular redox state, namely, on the concentration of NADH. We hypothesize a simple mechanism

for the variable cyanide-sensitivity of ADH II: the cyanide-resistant (and oxygen-resistant) form of this enzyme is the one with NADH bound to the active site, while the cyanide-sensitive form is the one without a cofactor or, most probably, with a bound NAD^+ (as NAD^+ is more easily replaced by cyanide than is NADH). Under vigorous aeration, ADH II apparently operates in the direction of ethanol oxidation (Kalnenieks et al. 2002) in a microenvironment of high NAD^+ and low NADH concentrations; hence its elevated sensitivity to cyanide in aerobically growing culture is easily explained.

We can summarize the sequence of events after cyanide addition to an aerobic culture and put forward a working hypothesis, explaining the gradual emergence of the cyanide-resistant form of ADH II: (i) inhibition of respiration is the immediate effect of cyanide, (ii) which causes a rise of intracellular NADH level, (iii) the excess NADH occupies the active sites of ADH II, outcompeting cyanide, as well as protecting the enzyme molecule against oxidative damage. The result of these events is a gradual emergence of the "cyanide-resistant" (NADH-bound) form of ADH II, which replaces the inhibited (cyanide-bound) form of ADH II, and, at the same time, is protected against oxygen. Thus, the aerobic culture with cyanide grows in a largely anaerobic manner: it respires slower (Kalnenieks et al. 2000; 2003), yet maintains a larger fraction of active ADH II molecules with bound NADH, obviously participating in ethanol synthesis under oxic conditions.

As far as we know, ADH II is a unique example of an enzyme with an active center, in which nicotinamide cofactors compete for binding with cyanide. Such a dependence of ADH II ligand-binding properties on the intracellular redox cofactors places it in the position of a putative key regulator of respiratory metabolism and ethanologenesis. Interestingly, the NADH/NAD^+ ratio (Leonardo et al. 1996) and, under certain conditions, possibly, *Fnr* (Membrillo-Hernandez, Lin 1999) were demonstrated to act as transcriptional regulators for another iron-containing ADH, the product of the *adhE* gene in *E. coli*. Taking into account the present results, we may speculate that in general the intracellular redox conditions affect the activity of bacterial iron-containing alcohol dehydrogenases at several regulatory levels. The details of transcriptional regulation of *Z. mobilis adhB*, however, still need to be elucidated. Another intriguing question for further study remains, whether ADH II can bind other ligands of physiological significance (e. g. nitric oxide) in a redox cofactor-dependent manner.

Acknowledgements

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Iekššūnas redoks-statuss regulē *Zymomonas mobilis* alkoholdehidrogenāzes II rezistenci pret cianīdu un skābekli

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Kopsavilkums

Darba mērķis bija noskaidrot mehānismu, kas nosaka baktērijas *Zymomonas mobilis* dzelzi saturošās alkoholdehidrogenēzes izoenzīma (ADH II) mainīgo cianīda jutīgumu un jutību pret skābekli. Visaugstāko ADH II cianīda jutīgumu novēroja šūnās, kuras kultivēja intensīvas aerācijas režīmā, kad iekššūnas NADH koncentrācija ir zema. Anaerobi augušajās baktērijās, kā arī tajās, kuras kultivēja aerobi cianīda klātbūtnē, gan ADH II cianīda izturība, gan arī iekššūnas NADH koncentrācija bija augstāka. Aerobi audzētās permeabilizētās šūnās cianīds izraisīja pakāpenisku ADH II inhibēšanu, kuru mazināja NADH un, mazākā mērā, NAD pievienošana. Parādīts, ka cianīds darbojas kā ADH II konkurentais inhibitors, konkurējot ar nikotīnamīda kofaktoriem. NADH palielina kā ADH II izturību pret cianīdu, tā arī tās noturību pret gaisa skābekļa iedarbību.