An iron-activated alcohol dehydrogenase

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Received 14 April 1983

An alcohol dehydrogenase isolated from *Zymomonas mobilis* was found to be activated by ferrous ions but not by zinc, after inactivation with metal-complexing agents. Cobaltous ions also re-activated to a lesser extent. It is suggested that in this species the alcohol dehydrogenase naturally contains iron. Kinetic studies on the iron-treated enzyme indicate an 'alcohol activation' phenomenon, which may have physiological relevance in overcoming product inhibition during fermentation.

Alcohol dehydrogenase Zymomonas mobilis *Iron* *Ferrous* *Cobalt* *Product activation*

1. INTRODUCTION

Alcohol dehydrogenases (EC 1.1.1.1) have been described from several bacteria [1–3], including *Zymomonas mobilis* [4]; in each case the evidence has indicated at least 1 zinc atom present/subunit, as is the case with most of the more extensively studied eukaryotic alcohol dehydrogenases from yeasts [5] and liver [6].

When attempting to purify the *Z. mobilis* enzyme, we noted considerable variation in the specific activity of crude extracts, from 2–20 units/mg; in [4] an initial activity of 1.7 units/mg was reported. Activity was unstable, and although protection could be afforded using dithiothreitol or mercaptoethanol, this was only temporary. However, high activity extracts were made when purifying another enzyme, 6-phosphogluconate dehydratase using ferrous ions in the extraction buffer; this enzyme is known to require ferrous ions for stability. It was then found that alcohol dehydrogenase could also be stabilised and re-activated using reduced iron.

A simple one-step isolation procedure for *Z. mobilis* alcohol dehydrogenase is outlined here, together with observations that indicate the involvement of ferrous ions in its activity. Also, an unusual kinetic behaviour is noted.

2. MATERIALS AND METHODS

Z. mobilis strains ZM1 (ATCC 10988) and ZM4 (CP4) [7] were provided by P.L. Rogers, University of NSW (Sydney). Earlier experiments used the ZM1 cells, but as ZM4 cells contain somewhat more enzyme, the results presented here are for the latter. Cells were grown in 1% yeast extract plus 12% glucose with nutrient salts at 30°C, and were collected when fermentation ceased. The cells were washed by suspension in water, adjusted to pH 6, re-centrifuged and stored frozen.

Cells were extracted by a modified version of the osmotic-shock/lysozyme treatment in [8]. Glycerol replaced sucrose, and ethyl acetate (final conc. 1%) was included. The extraction buffer (4 vol.) consisted of 20 mM K–Mes, 50 mM NaCl, 2 mM $MgCl_2$, 10 mM sodium ascorbate and 0.5 mM freshly dissolved ammonium ferrous sulphate. The pH was maintained at 6.5 during extraction (40 min at 25°C) before centrifuging off cell debris. About 1000 units of enzyme was extracted/g cells.

Cibacron Blue F3GA–Sepharose CL-4B was prepared by the 72 h ambient temperature procedure outlined in [9]. Columns were run at ambient temperature at 30 cm/h.

Alcohol dehydrogenase activity was measured in

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00145793/83/$3.00

0.1 M Tris–HCl (pH 8.5) containing 1 M ethanol and 1 mM NAD^+. Kinetic studies in the direction of alcohol oxidation were carried out in the same buffer. The buffer used for kinetics in the 'reverse' direction was 30 mM K–P_i (pH 7.0).

3. RESULTS

3.1. *Purification of alcohol dehydrogenase*

The buffer used for column chromatography was as above for the extraction procedure. Cibacron Blue F3GA–Sepharose was equilibrated in this buffer, and a column of 3 cm^3 for every g wet wt of cells was prepared. The extract was run into the column, which was then washed extensively with buffer containing an extra 50 mM NaCl. Alcohol dehydrogenase was eluted by inclusion of 1 mM NAD^+ in this buffer. The resulting enzyme was concentrated by ultrafiltration and stored frozen. Specific activities at this stage have been 300–450 units/mg, with over 50% recovery. The best preparations showed >90% homogeneity on polyacrylamide gels.

3.2. *Metal ion requirements of* Z. mobilis *alcohol dehydrogenase*

Samples of the purified enzyme were treated with EDTA (2 mM) or α,α'-dipyridyl (0.5 mM) at pH 6.5. Activity was followed for 30 min, by which time >90% of the activity had been lost in each case (fig.1). To aliquots of the α,α'-dipyridyl-treated sample, excess divalent metal ions were added (to 2 mM) and the activity of the enzyme followed for a further 120 min. This experiment was repeated with the inclusion of 5 mM dithiothreitol. Magnesium, manganous, calcium, cadmium, cupric, nickelous and zinc ions had no effect in restoring the activity; indeed both cupric and zinc ions abolished the small residual activity within a few minutes. Cobaltous ions slowly restored up to half of the original activity, but ferrous ions were most effective. In the presence of dithiothreitol plus ferrous ions all activity was restored within 10 min (fig.1).

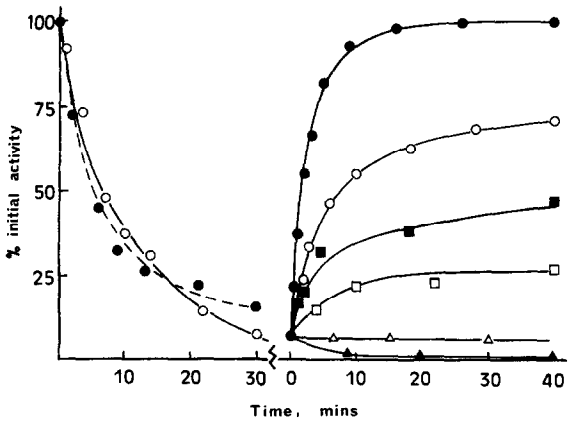

Fig.1. Inactivation and re-activation of *Z. mobilis* alcohol dehydrogenase. Purified enzyme was treated with 0.5 mM α,α'-dipyridyl (○) or with 2 mM EDTA (●) at zero time. After 30 min the α,α'-dipyridyl-treated sample had metal ions added as follows: Fe (○); Fe + 5 mM dithiothreitol (●); Co (□); Co + 5 mM dithiothreitol (■); Mg, Ca, Cd, Ni (△); Zn, Cu (▲).

3.3. *Kinetic studies*

The iron-activated enzyme showed anomalous kinetics with respect to ethanol concentration, with apparent negative co-operativity (fig.2). However, this was not so when varying NAD^+ (K_m = 0.10 mM at 1 M ethanol), acetaldehyde (K_m = 0.3 mM at 0.2 mM NADH), or NADH (K_m ~10 μM at 5 mM acetaldehyde). The maximum activity with acetaldehyde, when multiplied by the total amount of enzyme extracted from the cells, was barely sufficient to account for the rate of ethanol production by the cells. Consequently, the effect of ethanol on the acetaldehyde reduction reaction was tested, and it was found that up to 4-fold activation occurred in the presence of 1 M ethanol, though product inhibition effects undoubtedly caused an underestimation of this factor.

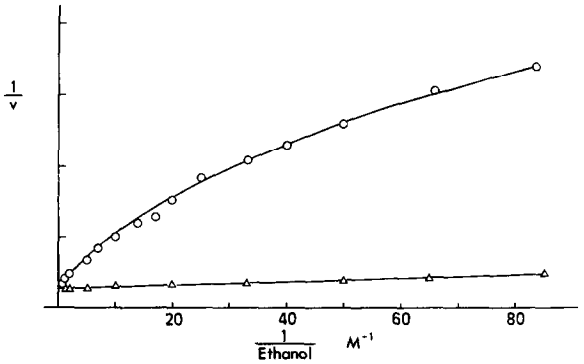

Fig.2. Lineweaver–Burk plot for *Z. mobilis* alcohol dehydrogenase with: varying [ethanol]; pH 8.5; 10 mM NAD^+. The yeast enzyme plot in the same conditions is shown for comparison.

3.4. *Structural studies*

The purified enzyme had an apparent subunit size on SDS–polyacrylamide gel electrophoresis of M_r 38000. This applied to the enzyme from either strain of the bacterium. This value is similar to that reported for the two *Z. mobilis* alcohol dehydrogenases in [4] and slightly smaller than that of the yeast enzyme. In non-dissociating gels our crude extract and purified preparations gave only one band which stained for alcohol dehydrogenase activity. Zinc and iron analyses have shown that iron is certainly present in our preparation after gel filtration, but zinc content was <1 atom/subunit. In view of the necessity of having iron in the buffers during purification, the presence of iron in the final preparation cannot be conclusive of its presence in vivo.

4. DISCUSSION

In [4] two isoenzymes of alcohol dehydrogenase in *Z. mobilis* were reported, one of which was lost when cells were grown in low-sucrose media. The preparation described here was from cells grown on glucose as C-source, and seems to be comparable with their main 'ZADH-I'. Their preparations contained about 1 atom zinc/subunit; however they did not measure iron, nor were they aware of the significance of iron for this enzyme. Yeast alcohol dehydrogenases containing manganese [10], or cobalt [11] have been reported, and these metals, together with cadmium and silver, can be substituted for zinc into the liver enzyme to give active species [12,13]. But there had not been a report of an iron-activated alcohol dehydrogenase, even artificially induced. It is worth noting that the ionic radii of zinc and ferrous are virtually identical. The present results suggest that our *Z. mobilis* enzyme may naturally contain ferrous ions; zinc ions if anything de-activated purified preparations. But further experiments are required to conclusively demonstrate either the presence of iron or the absence of zinc in the natural state.

In [14] it was hypothesised that [Fe(II)] in cells could play a controlling role in metabolism; it remains to be seen whether this could be significant with *Z. mobilis* alcohol dehydrogenase.

The peculiar kinetic characteristics of this enzyme can be attributed to alcohol activation, the simplest interpretation being that alcohol binds to a regulatory site which renders the enzyme several times more active than when the site is not occupied. An alternative explanation would be that this preparation is a mixture of the two isoenzymes in [4], with K_m-values for alcohol of 1.7 and >100 mM. This does not appear to be so, since only one band of activity was found after gel electrophoresis, and there was no comparable 'negative co-operativity' with acetaldehyde as substrate. In view of the extremely low recovery and specific activity of their purified ZADH-II, the kinetic characteristics cannot be directly compared. Indeed, some of the differences between ZADH-I and ZADH-II might be attributable to diferences in iron content. Since the physiological direction of the reaction is the reduction of acetaldehyde, the alcohol effect can be regarded as 'product activation', and may compensate for mass action product inhibition as the level of ethanol builds up during fermentations. The unusual roles of iron and alcohol in the control of this enzyme's activity warrant more extensive investigation.

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