Substrate specificity of nine NAD⁺-dependent alcohol dehydrogenases in *Aspergillus nidulans*

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In *Aspergillus nidulans* three alcohol dehydrogenases (ADHs) have been described. ADHI is induced by ethanol and is the physiological enzyme of ethanol utilization, ADHII has not been attributed a function but is repressed by ethanol. The ALCR regulatory protein acts positively to induce ADHI, and negatively in its control of ADHII. ADHIII is specifically induced by anaerobic stress. We have characterized the substrate specificity of these three enzymes by looking at their staining profile on polyacrylamide gels with a range of alcohols. In addition to these enzymes we have observed six other NAD⁺-dependent ADHs, two of which, propan-2-ol dehydrogenase and pentan-2-ol dehydrogenase, share similar control with ADHII. The inducibility of these enzymes with some alcohols has also been investigated. The profile of ADHs with NADP⁺ as an electron acceptor is also reported.

Keywords: alcohol dehydrogenases, *Aspergillus nidulans*

INTRODUCTION

Aspergillus nidulans is able to grow on ethanol as a carbon source. The ability to utilize ethanol is dependent upon two structural genes, *alcA*, encoding alcohol dehydrogenase I (ADHI) and *aldA*, encoding aldehyde dehydrogenase. *alcA*, *aldA* and *alcR* (the regulatory gene) are under the positive control of ALCR. *alcA* and *alcR* are clustered on linkage group VII, while *aldA* maps on linkage group VIII (Pateman *et al.*, 1983; Sealy-Lewis & Lockington, 1984). Growth of *A. nidulans* on other carbon sources (such as propan-2-ol and butan-2-ol) is poor while many alcohols (such as propan-1-ol) are toxic to the organism. Although many alcohols are poor substrates for the growth of the organism, many of them in addition to ethanol are good inducers of ADHI (Creaser *et al.*, 1985; Felenbok & Sealy-Lewis, 1994).

Two other ADHs have been described in *A. nidulans*. ADHII (structural gene, *alcB*), unlike ADHI, is repressed by ethanol in the growth medium (Sealy-Lewis & Lockington, 1984), whereas ADHIII (structural gene, *alcC*) is produced under conditions of anaerobic stress (Kelly *et al.*, 1990).

In an *alcR125* mutant strain that carries a putative nonsense mutation in *alcR* (Roberts *et al.*, 1979), *alcA* and *aldA* are non-inducible, while ADHII is derepressed in

the presence of ethanol. This led to the proposal that *alcB* is under the negative control of ALCR. The physiological function of ADHII in the cell is not known. *alcA*, *aldA*, *alcR* and *alcB* are subject to carbon catabolite repression (Bailey & Arst, 1975; Pateman *et al.*, 1983; Sealy-Lewis & Lockington, 1984), and in the case of *alcA*, *aldA* and *alcR* this has been shown to be mediated by CREA (Lockington *et al.*, 1987; Felenbok *et al.*, 1989). ADHIII is post-transcriptionally regulated and is thought to be involved in the survival of the organism during periods of waterlogging. Neither ALCR nor CREA are involved in the regulation of ADHIII (structural gene, *alcC*) (Kelly *et al.*, 1990). *alcA*, *aldA*, *alcR* and *alcC* have been cloned and sequenced (Gwynne *et al.*, 1987; Pickett *et al.*, 1987; Felenbok *et al.*, 1988; Kulmburg *et al.*, 1991; McKnight *et al.*, 1985), and more recently *alcB* has been cloned and sequenced (G. Hunter, I. G. Jones & H. M. Sealy-Lewis, unpublished).

One other enzyme has been characterized that is capable of utilizing ethanol as a substrate, namely NADP⁺-dependent glycerol dehydrogenase II (glycerol DHII) which is specifically induced by D-galacturonate (Sealy-Lewis & Fairhurst, 1992).

The three NAD⁺-dependent ADHs of *A. nidulans* and the NADP⁺-dependent glycerol DHII are present under completely different growth conditions in the cell, and moreover they can be visualized as bands of different mobility on native PAGE (Sealy-Lewis & Lockington, 1984; Kelly *et al.*, 1990; Sealy-Lewis & Fairhurst, 1992).

Abbreviations: ADH, alcohol dehydrogenase; glycerol DH, NADP⁺-dependent glycerol dehydrogenase.

We have studied the substrate specificity of these enzymes by comparing their staining with a range of alcohols in polyacrylamide gels. As well as establishing the substrate specificity of the ethanol dehydrogenases mentioned above we were also able to characterize a number of NAD⁺-specific and NADP⁺-specific enzymes that stained with a variety of alcohols. Two enzymes which we have designated propan-2-ol dehydrogenase and pentan-2-ol dehydrogenase are regulated in a similar manner to ADHII, i.e. they are repressed in the presence of ethanol, but are derepressed in the *alcR125* mutant strain.

METHODS

Strains. The markers not specifically detailed below are described in Clutterbuck (1974). The wild-type strain used was *pabaA1* (auxotrophic for *p*-aminobenzoic acid). Strain H444: *biA1*; *alcR125* (auxotrophic for biotin and carrying a nonsense mutation in *alcR*; Roberts *et al.*, 1979). Strain H527: *pabaA1*; *alX4*; *sB43*; *alcA527*. *alcA527* mutants lack any ADHI activity (Sealy-Lewis & Lockington, 1984). Strain H1035: *biA1*; *alcE1*; *alc500*; *alcD1*. The *alcE* and *alcD* mutations confer 100-fold greater ADHII activity than the *alc500*-containing strain (Sealy-Lewis, 1990). *alc500* is a deletion spanning *alcA* and *alcR* (Lockington, 1984). The strain used for looking at ADHIII activity was a multi-copy transformant (4–8 copies) of the *alcC* clone in an *amdS* plasmid pANa3Pv in recipient strain H849 (*pabaA1*; *amdS320* *amdI18*; *alcA527* *amdA7*; Jones & Sealy-Lewis, 1989).

Culture conditions. Media and supplements were as described by Cove (1966). For growth in liquid medium, strains were grown in appropriately supplemented 1% (w/v) glucose medium, 10 mM urea, for 16 h at 37 °C in an orbital shaker (120 r.p.m.). The mycelium was harvested by filtration and transferred to fresh minimal medium containing either glycerol (100 mM), D-galacturonate (50 mM), D-glucose (50 mM), ethanol (100 mM), propan-2-ol (100 mM), pentan-2-ol (50 mM), or 2,3-butanediol (50 mM, Sigma, mixture of DL and meso forms), as well as to medium containing no carbon source. After 6 h incubation the mycelium was harvested by filtration. One of the cultures was transferred to 50 mM glucose for 24 h under anaerobic conditions. Growth conditions for observing ADHII involved transfer to 0.1% fructose for 6 h as described above.

Gel electrophoresis. Gel electrophoresis was performed as described by Sealy-Lewis & Fairhurst (1992). The gel stain was modified from that described for ADH (Sealy-Lewis & Lockington, 1984). Either NAD⁺ or NADP⁺ was added to 0.2 mg ml⁻¹. The following substrates at a final concentration of 100 mM were used: methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, isobutanol, pentan-2-ol, allyl alcohol, glycerol, crotyl alcohol, cinnamyl alcohol, threonine, 2,3-butanediol, 1,2-propanediol, erythritol, and mannitol. Protein concentration was measured according to the microbiuret method (Goa, 1953) and concentrations of protein from different extracts were equalized before loading onto a gel.

RESULTS

Glucose-grown mycelium, from a wild-type strain was transferred to a variety of carbon sources and growth conditions designed to express ADHI (ethanol), ADHII (0.1% fructose), ADHIII (anaerobic conditions), and glycerol DHII (D-galacturonate). Extracts from the mycelium were run on polyacrylamide gels and stained for

Fig. 1. ADH activity of an extract of the wild-type strain on an activity stained gel. Mycelia were grown for 16 h at 37 °C on 1% glucose and transferred to 50 mM 2,3-butanediol for a further 6 h. Each track was loaded with 40 µg protein. The staining solution contained NAD⁺ and a different substrate for each track. Tracks: 1 and 2, isobutanol; 3, butan-1-ol; 4, methanol; 5, crotyl alcohol; 6, cinnamyl alcohol; 7, pentan-2-ol; 8, 2,3-butanediol; 9, no substrate; 10, L-threonine; 11, erythritol. The enzymes are indicated on the left of the gel, and cinnamyl dehydrogenase, propan-2-ol dehydrogenase, and pentan-2-ol dehydrogenase are abbreviated as Cin DH, Prop DH and Pent DH, respectively. The propan-2-ol dehydrogenase was visible on the gel stained with pentan-2-ol, but is very faint on the photograph. Its mobility was between that of cinnamyl dehydrogenase I and II. ADHIII was seen optimally after transfer to anaerobic conditions but was sometimes seen after growth at 37 °C as above because the cultures became anaerobic after prolonged growth (Kelly *et al.*, 1990). This is a representative gel and extracts were grown up more than once and stained with the range of substrates.

activity with the following substrates: methanol, ethanol, propan-1-ol, propan-2-ol, butan-1-ol, butan-2-ol, isobutanol, pentan-2-ol, allyl alcohol, glycerol, crotyl alcohol, cinnamyl alcohol, threonine, 2,3-butanediol, 1,2-propanediol, erythritol and mannitol (see Fig. 1 for a representative gel, and Table 1). Either NAD⁺ or NADP⁺ was used as the electron acceptor. ADHI, ADHII and ADHIII only exhibit activity in the gel stain when NAD⁺ is present, and glycerol DHII only shows activity with NADP⁺. When NADP⁺ was used as the electron acceptor with glycerol as the substrate, an activity band additional to glycerol DHII was seen which corresponded to the previously described glycerol DHI (Hondmann *et al.*, 1991; Sealy-Lewis & Fairhurst, 1992). The latter enzyme is constitutively expressed and was previously shown to have activity with either glycerol or erythritol, but additionally it has some activity with 2,3-butanediol and 1,2-propanediol. Glycerol DHII has a much broader substrate specificity (Sealy-Lewis & Fairhurst, 1992; Table 1). The only other substrate that showed activity in the gel stain when NADP⁺ was used as an electron acceptor was mannitol and there were three bands visible which were constitutively expressed.

The substrate specificity of ADHI, ADHII and ADHIII is shown in Table 1. These ADHs have a broad spectrum of activity. Of the ADHs, ADHI had the most intense staining with a wide range of alcohols. When staining with NAD⁺ as the electron acceptor, and using a range of substrates, there were a number of bands that did not correspond to the three ADHs (ethanol dehydrogenases).

Table 1. Substrate specificity of ADHs in *A. nidulans*

The substrate specificity of the ADHs was determined by comparing the staining in the presence of substrates (all at 100 mM) in extracts from cultures grown under the condition where the ADHs are optimally expressed (see Fig. 1). The staining ability is denoted on a scale + to + + + +. The order of the enzymes in the table follows their increasing mobility on polyacrylamide gels. There are three bands that stained only with NADP⁺ and mannitol that were constitutively expressed but are not shown in the table (see text).

Substrate	NAD ⁺ -dependent alcohol dehydrogenases (in order of mobility on gel)									NADP ⁺ -dependent glycerol dehydrogenases	
	ADHI	ADHIII	Mannitol DH	ADHII	Cinnamyl DHI	Propan-2-ol DH	Cinnamyl DHII	Pentan-2-ol DH	Crotyl DH	Glycerol DHI	Glycerol DHII
Methanol	+	—	—	+	—	—	—	—	—	—	—
Ethanol	++++	±	—	++	—	—	—	—	—	—	++
Propan-1-ol	++++	± or —	—	+++	—	—	—	—	—	—	+++
Propan-2-ol	++++	+	—	±	—	+	—	±	—	—	—
Butan-1-ol	+++	±	—	++	—	—	—	—	—	—	+
Butan-2-ol	+++	+	—	++	—	—	—	—	—	—	+
Iso-butanol	++	+	—	++	—	—	—	—	—	—	+
Pentan-2-ol	++	+	—	+	—	±	—	++	± or —	—	—
Allyl alcohol	++++	+	—	+++	—	—	—	—	—	—	++
Glycerol	±	± or —	—	±	—	—	—	—	—	++++	++
Crotyl alcohol	++++	+	—	+++	+	—	—	—	±	—	+
Cinnamyl alcohol	++	++	—	++	+	—	±	—	—	—	+
Threonine	+	—	—	—	—	—	—	—	—	—	—
2,3-Butanediol	± or —	—	—	±	—	—	—	+	±	+	—
1,2-Propanediol	—	—	—	—	—	—	—	±	—	+	+
Erythritol	± or —	—	—	±	—	—	—	—	—	++	±
Mannitol	—	—	+	—	—	—	—	—	—	—	+

Table 2. Induction/repression of ADHs in *A. nidulans*

The concentration of the inducer/repressor used and the growth conditions for induction are detailed in Methods. The ability of a compound to act as an inducer/repressor was assessed from the staining ability in polyacrylamide gels of an extract made from mycelium grown in the presence of the compound (+ or — indicate the presence/absence of the enzyme under the stated conditions). The NAD⁺-dependent enzymes are listed in increasing order of mobility on the gel. Glycerol DHI has the same mobility as ADHII, and glycerol DHII has approximately the same mobility as cinnamyl DHI.

	Inducer/repressor							
	Ethanol	Fructose	Propan-2-ol	Pentan-2-ol	2,3-Butanediol	Glucose	D-Galacturonate	Anaerobiosis
NAD⁺-dependent enzyme								
ADHI	+	±	+	—	+	—	+	—
ADHIII	—	—	—	—	—	—	—	+
Mannitol DH	+	+	+	+	+	+	+	+
ADHII	—	+	+	+	+	—	±	—
Cinnamyl DHI	+	+	NT	+	+	—	NT	—
Propan-2-ol DH	—	±	—	—	+	—	NT	—
Cinnamyl DHII	—	+	NT	+	+	±	NT	—
Pentan-2-ol DH	±	+	+	+	+	±	NT	±
Crotyl DH	—	+	NT	+	+	—	NT	—
NADP⁺-dependent enzyme								
Glycerol DHI	+	+	+	+	+	+	+	—
Glycerol DHII	—	+	—	—	—	—	+	—

NT, Not tested.

Fig. 2. ADH activity of extracts of the wild-type strain and H444 carrying the *alcR125* mutation on an activity stained gel. The staining solution contained NAD^+ and pentan-2-ol. All mycelia were grown for 16 h at 37 °C on 1% glucose medium transferred for a further 6 h to various media. Tracks: 1–5, H444; 6–10, wild-type. Tracks: 1, 50 mM glucose; 2, 0.1% fructose; 3, 50 mM 2,3-butanediol; 4, 100 mM ethanol; 5, 50 mM pentan-2-ol; 6, 50 mM glucose; 7, 0.1% fructose; 8, 100 mM ethanol; 9, 50 mM 2,3-butanediol; 10, 50 mM pentan-2-ol. Each track contains 30 μg protein. The enzymes are indicated on the left of the gel, and are abbreviated as in the legend to Fig. 1. These are representative gels and mycelia were grown up and run on gels at least twice. ADHIII was generally seen optimally after anaerobic transfer, but it was sometimes seen as above after growth at 37 °C. The propan-2-ol dehydrogenase is only a faint staining band and to see this band more clearly the gel was dried before photography. Under these conditions, above the ADHIII band in H444 additional faint bands can be seen, but normally these were so faint that they were not consistently visible, and they were excluded from the consideration of ADHs.

We have characterized their substrate specificity in the gel stain and given them names on the basis of that substrate which showed the greatest staining activity (Table 1). In all, six new NAD^+ -dependent ADHs were observed: mannitol, propan-2-ol, pentan-2-ol and crotyl dehydrogenase, as well as two cinnamyl dehydrogenases. Of particular interest were propan-2-ol dehydrogenase and pentan-2-ol dehydrogenase; these were present under the same growth conditions as ADHII, i.e. these activities were observed on transfer to 0.1% fructose, and were repressed on transfer to ethanol or glucose. The inducibility of these enzymes on transfer to propan-2-ol, pentan-2-ol and 2,3-butanediol was also investigated since these alcohols were substrates for the enzyme(s) (Table 2).

Propan-2-ol and pentan-2-ol dehydrogenase are under *alcR* control

The wild-type strain was grown in parallel with strains H444, H1035 and H527 (see Methods). These strains were grown on glucose medium and then transferred to media containing 0.1% fructose, ethanol, propan-2-ol, 2,3-butanediol, or glucose and anaerobic conditions. The wild-type strain shows induction of ADHI by ethanol, propan-2-ol and 2,3-butanediol. ADHII, propan-2-ol and 2,3-butanediol dehydrogenases were repressed by ethanol and propan-2-ol but not by 2,3-butanediol (Figs 2 and 3). We have previously reported (Sealy-Lewis & Lockington,

Fig. 3. ADH activity of extracts from the wild-type, H444 and H1035 on polyacrylamide gels. Tracks: 1–3 and 10, wild-type; 4–6, H444; 7–9 H1035. Mycelia were grown on 1% glucose for 16 h at 37 °C and then transferred for a further 6 h to various media. Tracks: 1, 100 mM ethanol; 2, 50 mM 2,3-butanediol; 3, 100 mM propan-2-ol; 4, 100 mM ethanol; 5, 50 mM 2,3-butanediol; 6, 100 mM propan-2-ol; 7, 100 mM ethanol; 8, 50 mM 2,3-butanediol; 9, 100 mM propan-2-ol; 10, wild-type transferred to 50 mM 2,3-butanediol. Tracks 1–9 were stained with NAD^+ and pentan-2-ol. Track 10 was stained with NAD^+ and propan-2-ol. Each track contains 30 μg protein. See legend to Fig. 1 for abbreviations.

1984) that in a strain carrying an *alcA*[−] mutation, such as *alcA527*, the ADHII enzyme is derepressed, and similarly propan-2-ol dehydrogenase and pentan-2-ol dehydrogenase show partial derepression in this mutant on transfer to ethanol medium (data not shown).

In the *alcR125* strain, ADHI is absent because *ALCR* is required for induction of ADHI, but ADHII, propan-2-ol dehydrogenase and 2,3-butanediol dehydrogenase are derepressed on ethanol and propan-2-ol (Fig. 3). In the *alcE1; alc500 alcD1* strain, ADHII and pentan-2-ol dehydrogenase levels were elevated, but the level of propan-2-ol dehydrogenase appeared to be unaffected (Fig. 3). ADHII, propan-2-ol dehydrogenase and pentan-2-ol dehydrogenase are all subject to the same negative control by *ALCR*, and in the case of pentan-2-ol dehydrogenase the *alcD/alcE* mutations which result in elevated levels of ADHII also affect the level of this enzyme. ADHII and propan-2-ol dehydrogenase are completely repressed on transfer to glucose medium, and pentan-2-ol dehydrogenase is present at a low level during growth on glucose medium under both aerobic and, anaerobic conditions (Fig. 2).

DISCUSSION

ADHs have been widely studied in a variety of organisms, and it is common for there to be multiple ADHs within the same organism. There are at least four ADHs in *Saccharomyces cerevisiae* (Ciriacy, 1975; Williamson *et al.*, 1980; Bennetzen & Hall, 1982; Russell & Hall, 1983; Young & Pilgrim, 1985; Williamson & Paquin, 1987). In *A. nidulans*, three NAD^+ -dependent ethanol dehydrogenases have previously been described. ADHI is the physiological enzyme of ethanol utilization, and ADHIII

is produced under conditions of anaerobic stress. The function of ADHII has not been established, but it might be involved in de-toxification of acetaldehyde. In this paper we have demonstrated that in addition to these enzymes there is also a range of ADHs with distinct mobility in polyacrylamide gels, and different substrate specificity from the previously described enzymes. The staining reaction in polyacrylamide gels has allowed detection of very low levels of enzyme activity that would not be detectable in a conventional assay. Their normal role in metabolism is unknown. What is interesting is that at least two of these enzymes are under the same control as ADHII, i.e. they are repressed by ethanol in the wild-type strain, but are derepressed in an *alcR*⁻ mutant strain. Propan-2-ol dehydrogenase, unlike ADHII and pentan-2-ol dehydrogenase, is not induced by pentan-2-ol, and the level of this enzyme is not elevated in the *alcE1*; *alc500* *alcD1* strain (H1035). The pentan-2-ol dehydrogenase, however, has the same profile of inducibility as ADHII and it is also elevated in the *alcE1*; *alc500* *alcD1* strain. The only difference between the two enzymes is that there is low activity of pentan-2-ol dehydrogenase on transfer to glucose or anaerobic glucose medium. It could be argued that this activity is encoded by the *alcB* gene and that it represents a differently processed form of the enzyme or, alternatively, pentan-2-ol dehydrogenase and ADHII represent a monomer and a dimer with different substrate specificity. Whether pentan-2-ol is encoded by a different gene from *alcB* could be resolved if we had a lack of function mutation in *alcB*, but unfortunately, since the function of ADHII is unknown, we have been unable to select one. Now that the *alcB* gene has been cloned, a gene inactivation of *alcB* will answer this question.

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