Purification and preliminary characterization of alcohol dehydrogenase from *Aspergillus nidulans*

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Aspergillus alcohol dehydrogenase is produced in response to growth in the presence of a wide variety of inducers, of which the most effective are short-chain alcohols and ketones, e.g. butan-2-one and propan-2-ol. The enzyme can be readily extracted from fresh or freeze-dried cells and purified to homogeneity on Blue Sepharose in a single step by using specific elution with NAD⁺ and pyrazole. The pure enzyme has M_r 290 000 by electrophoresis or gel filtration; it is a homopolymer with subunit M_r 37 500 by electrophoresis in sodium dodecyl sulphate; its amino acid composition corresponds to M_r 37 900, and the native enzyme contains one zinc atom per subunit. The enzyme is NAD-specific and has a wide substrate activity in the forward and reverse reactions; its activity profile is not identical with those of other alcohol dehydrogenases.

Previous investigations (Pateman *et al.*, 1983) have indicated that the system of genes and enzymes responsible for alcohol metabolism in *Aspergillus nidulans* is of great promise in elucidating metabolic control mechanisms in lower eukaryotes. Two structural genes, coding for alcohol dehydrogenase (ADH) and aldehyde dehydrogenase, were located in the genome, and a regulatory gene, controlling the expression of both structural genes, was found to be adjacent to the structural gene for ADH. Numerous mutants were isolated and characterized. Genomic DNA spanning at least 10 kilobases to either side of known mutations of the ADH structural gene has been cloned, and the region of transcription induced by growth on threonine and repressed by growth on excess glucose defined (C. H. Doy, unpublished work). The present paper describes the purification and some properties of the first enzyme in alcohol metabolism, ADH. A preliminary account of the purification of ADH and aldehyde dehydrogenase from *Aspergillus* has appeared elsewhere (Creaser & Porter, 1982).

ADH enzymes from yeast and mammalian cells have been extensively characterized at the enzymic and structural level (Sund & Theorell, 1963; Brändén *et al.*, 1970) and will provide a solid basis

for comparison of the structure and properties of the *Aspergillus* enzyme.

Experimental

Materials

Bulk chemicals were from Ajax Chemicals (Sydney, N.S.W., Australia); fine chemicals were from Sigma Chemical Co.; electrophoretic and gel-filtration standards, Blue Sepharose and Sephacryl S300 were from Pharmacia; Gradipore gels and electrophoresis equipment were from Gradient Laboratories (Sydney, N.S.W., Australia).

Aspergillus cultures

The strain *Aspergillus nidulans* O51, wild-type with respect to ethanol utilization (Pateman *et al.*, 1983), was used exclusively in the present work. Media and supplements as described by Pontecorvo *et al.* (1953) were basically a salts medium supplemented with urea as a nitrogen source and a low concentration of glucose (4 mM) as carbon and energy source. An inducer for ADH production was also added. It was possible to use ethanol and some other alcohols as combined carbon and energy sources and inducers. Cells were grown for 24 h at 37°C with vigorous aeration, starting from a 10-day-old conidial inoculum. The mycelium was harvested by filtration and washed with distilled water before enzyme extraction.

Abbreviations used: ADH, alcohol dehydrogenase (EC 1.1.1.1); p.i.x.e., proton-induced X-ray emission; SDS, sodium dodecyl sulphate.

Enzyme assay

ADH in solution was assayed by conventional spectrophotometric procedures: the forward reaction by increased absorption at 340 nm and the reverse by diminution. A Pye-Unicam SP.8-100 recording spectrophotometer was used, the reactions being temperature-controlled at 25°C. For alcohol oxidation a 0.5 ml cuvette was used to which was added sequentially 20 μ l of 0.1 M-NAD⁺ solution and 470 μ l of 0.1 M-sodium phosphate buffer, pH 8.3, containing 2 mM-MgCl₂ and 1 mM-dithiothreitol. After addition of 1–10 μ l of enzyme solution the reaction was initiated by rapid addition of 50 μ l of propan-2-ol. Increase in absorption at 340 nm was measured and converted into units (μ mol of NAD⁺ reduced/min). In crude extracts 50 μ l of NAD⁺ solution was added to counteract its possible destruction. For the reverse reaction 2 μ l of NADH (0.1 M) plus 483 μ l of pH 8.3 buffer as above and 10 μ l of enzyme were mixed and the reaction was started by addition of 5 μ l of 1 M-acetaldehyde. Reduction of 340 nm absorption was converted into units as above.

Detection of ADH on polyacrylamide gels was based on the procedure of Fowler *et al.* (1972). After the gels had been run they were immersed in 50 ml of 0.1 M-Tris/HCl buffer, pH 8.1, containing Nitro Blue Tetrazolium (220 mg), phenazine methosulphate (4 mg) and NAD⁺ (100 mg). The reaction was started by addition of 0.5 ml of propan-2-ol.

Protein assay

Protein in solution was measured by the Lowry procedure (Lowry *et al.*, 1951) as modified by Clark & Jacoby (1970), with trichloroacetic acid precipitation to remove sources of error introduced by the routine inclusion of 1 mM-dithiothreitol in enzyme samples. The standard used was bovine serum albumin (fraction V) solution treated in the same way as the unknowns.

Protein bands in polyacrylamide gels were stained with Coomassie Blue/perchlorate (Holbrook & Leaver, 1976) and recorded by using a LKB densitometer.

Amino acid analysis

Protein samples were hydrolysed at 110°C for 24, 48 and 72 h in 6 M-HCl for most amino acids, in 3 M-mercaptoethanesulphonic acid for tryptophan (Penke *et al.*, 1974) and in 6 M-HCl for 24 h after performic oxidation (Hirs, 1967) for cysteine and methionine. Hydrolysates were analysed on a Beckman 119 CL amino acid analyser.

Electrophoresis

Concave-gradient (2.5–27%) gels, 7 cm square, were used in the apparatus supplied by Gradient

Laboratories. These ready to use gels as supplied contained EDTA, and it is essential to remove this as *Aspergillus* ADH is very sensitive to inhibition by EDTA. Accordingly the gels were pre-run for 2 h at 50 V, after which the buffers were discarded and replaced by fresh solutions. The stock gel buffer contains 6 g of Tris and 29 g of glycine per litre adjusted to pH 8.3 with HCl, and is diluted 1:10 to form the running buffer. In addition, the running buffer contains 1 ml of 2 M-MgCl₂ and 100 μ l of 2-mercaptoethanol/l. Samples for electrophoresis were mixed with solid sucrose to increase their density, and Bromothymol Blue was added as a marker. The gels were normally run for 20–24 h in the cold-room at 40 V, then stained for enzyme and protein as above. A mixture of standard proteins was run in flanking lanes to enable the M_r of the ADH to be determined by plots of $\log M_r$ versus mobility. These standards were (M_r values in parentheses) thyroglobulin (669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000) and albumin (67 000).

Electrophoresis of subunits was performed on the same type of gels that had been pre-equilibrated in SDS-containing buffer by running at 70 V for 1 h. The SDS-containing electrophoresis buffer was a solution of 4.84 g of Tris, 1.64 g of sodium acetate and 0.59 g of EDTA per litre adjusted to pH 7.4 with acetic acid, and then 2 g of SDS added per litre. Samples for SDS/polyacrylamide-gel electrophoresis were treated by boiling for 10 min in 100 mM-Tris/HCl buffer, pH 8.1, containing 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 1 mM-EDTA. After cooling, glycerol was added to 50% (v/v) plus 5 μ l of Bromothymol Blue. The gel was normally run at 100 V at room temperature until the blue dye marker was at the edge of the gel. A mixture of standard protein subunits was run in flanking lanes to enable the M_r of the ADH subunit to be determined by plotting $\log M_r$ versus mobility. These were (subunit M_r values in parentheses) phosphorylase *b* (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α -lactalbumin (14 400).

Gel filtration

A column (0.9 cm $\times$ 67 cm) of Sephacryl S300 was used both for analytical determination of M_r and during purification. It was equilibrated with 10 mM-sodium/potassium phosphate buffer, pH 6.5, containing 1 mM-dithiothreitol and 2 mM-MgCl₂. Standards were used to calibrate the column, and they were as follows (M_r values in parentheses): thyroglobulin (669 000), ferritin (440 000), catalase (232 000), lactate dehydrogenase (140 000) and albumin (67 000).

Metal determination

Freeze-dried samples of *Aspergillus* ADH were analysed by p.i.x.e. spectrometry.

Results and discussion

Induction of alcohol dehydrogenase (ADH)

It was previously shown that the formation of *Aspergillus* ADH needed an inducer and was repressed by excess glucose in the medium (Pate-man *et al.*, 1983). Therefore *Aspergillus* was grown under conditions where glucose repression was not acting (4mM), with various potential inducers added at a concentration of 50mM, this being a compromise concentration to enable comparisons to be made. Cells were harvested and crude extracts were assayed for ADH and protein. Results of the various inducer experiments are shown in Table 1. The compound producing the

highest specific activity was butan-2-one (ethyl methyl ketone). Variations from this optimum produced the following results. Increasing the chain length to C₅, pentan-3-one, resulted in a massive loss of inducing ability, but increasing to the C₆ ring ketone, cyclohexanone, gave the same optimal activity as the C₄ butan-2-one. Shortening the carbon skeleton to C₃, acetone, diminished the inducing ability by 22%, and the corresponding secondary alcohols, i.e. propan-2-ol and butan-2-ol, were less effective than their ketones. Normal alcohols such as propan-1-ol and ethanol again were less effective. Increasing the ethanol concentration to 300mM, when it can act as both carbon source and inducer, doubles the inducing ability over that shown in Table 1.

Two further variants of C₄ secondary alcohols are threonine and 3-hydroxybutan-2-one (acetyl-methylcarbinol), which give about 35% of the

Table 1. *Inducers and substrates of Aspergillus ADH*

Inducing ability is shown as relative specific activity (units of enzyme/mg of protein) in crude extracts of cells grown in 4mM-glucose and 50mM-inducer. Activity as substrate is shown as relative rates (%) of reaction of substances under test with pure enzyme compared with propan-2-ol or acetaldehyde. Concentrations were 50mM for oxidation and 5mM for reduction. NAD⁺ concentration was 4mM, that of NADH 0.4mM. Abbreviation: N.T., not tested.

Compound	Inducing ability	Activity as substrate
No inducer	1.7	—
Methanol	0.2	6.3
Ethanol	5.5	95
Propan-1-ol	4.0	100
Propan-2-ol	44.5	100 (406)†
Butan-1-ol	*	131
Butan-2-ol	56	58
2-Methylpropan-2-ol	*	11.3
Cyclohexanol	*	0
Cinnamyl alcohol	*	239
Benzyl alcohol	*	24
Phenethyl alcohol	N.T.	12
But-3-en-2-ol	6.5	80
But-2-en-1-ol	*	80
Serine	5.2	0
Threonine	35	0
3-Hydroxybutan-2-one	34	0 (53)‡
Allyl alcohol	*	98
Lactic acid	2.5	0
3-Hydroxybutyric acid	3.4	0
2-Hydroxybutyric acid	2.6	7.7
Formaldehyde	0.8	23
Acetaldehyde	2.5§	100
Propionaldehyde	*	24
Acetone	78	4.6
Butan-2-one	100	0
Pentan-3-one	8.2	0
Cyclohexanone	98	0
Pyruvic acid	4.1	0
3-Oxobutyric acid	52	0

* Growth inhibition by this inducer.

† Value in parenthesis is rate for saturating propan-2-ol.

‡ Zero value applies to oxidation of alcohol, 53 value applies to reduction of CO group.

§ 2mM Inducer.

inducing activity optimal. However, 2-hydroxybutyrate and 3-hydroxybutyrate are not significant inducers. 3-Oxobutyrate (acetoacetate) is a good inducer, 52% of the optimum. At the maximum concentration that was not growth-inhibitory, 2mM, acetaldehyde would not induce alcohol dehydrogenase. Pateman *et al.* (1983) showed that threonine would induce both ADH and aldehyde dehydrogenase in *Aspergillus*, and it was suggested that its inducing property was due to its conversion into acetaldehyde, which would be the effective inducer, by a proposed threonine aldolase reaction. It would appear unnecessary to propose that threonine induces ADH because it can be converted into acetaldehyde, since it itself acts as a C₄ secondary alcohol.

One can conclude that if ADH formation is controlled by a regulator molecule the effector site of such a molecule would be sterically and chemically adapted for optimal binding of C₄ ketones and close relatives thereof.

Purification of ADH

Two methods of extraction of ADH from *Aspergillus* mycelium were employed, dependent on the amount of material to be processed. For amounts up to about 5g the harvested mycelium was pressed dry between sheets of filter paper, then immersed in liquid N₂ and, when frozen, reduced to a fine powder by mortar and pestle. For larger amounts the pressed mycelial mat was shredded to give flakes and freeze-dried. The completely dry flakes were reduced to a fine powder in a coffee-grinder. On several occasions the two extraction procedures were performed on the same batch of mycelium, and identical yields of enzyme were obtained. With the use of freeze-dried flakes the enzyme content remained constant at -20°C for several months, but the finely powdered mycelium retained its activity for only a few days at -20°C, although it was stable in liquid N₂ for at least a year.

After either procedure the enzyme was extracted at 5°C by dilute buffers before adsorption by Blue Sepharose. Preliminary experiments showed that (1) adsorption of ADH to Blue Sepharose was maximal at pH 6.5, (2) low-ionic-strength buffers were essential, (3) thiol reducing agents were needed for preservation of activity and (4) the ratio of extract to adsorbant was fairly critical. Accordingly, an optimized procedure was adopted as follows. The mycelial powder was extracted in 10mM-sodium/potassium phosphate buffer, pH 6.5, containing 1mM-dithiothreitol and 2mM-MgCl₂ (buffer A) at 5°C in a Waring Blender for 30 min, with 1 min periods of blending every 5 min; 100ml of buffer was used per g of powder. The homogenate was centrifuged at 10000 rev./min

(10240g_{av.}) in a Sorvall RC2B centrifuge at 5°C for 30 min. The supernatant was applied to a column of Blue Sepharose. The Sepharose was normally stored as a moist cake after regeneration and collection on a sintered-glass (porosity 0) filter; 10g of this material was used per g of mycelial powder. The Sepharose was made into any conveniently sized column, consistent with high flow rate, and thoroughly washed with distilled water. It was important to regenerate the Sepharose after use in accordance with the manufacturer's instructions. No conditions were found whereby the Blue Sepharose could be added to the extract and result in any significant absorption of enzyme. The only successful procedure was to run the extract through the Sepharose column, when 80–100% of the ADH in the extract was absorbed. When all the extract had passed through the column, the non-absorbed material was washed off with buffer A until the absorbance at 280 nm of the eluate was the same as the input buffer. At this point the column was eluted with buffer A containing 0.1mM-NAD⁺ and 5mM-pyrazole (cf. Larsson & Mosbach, 1981). A single peak of 280 nm-absorbing material and ADH activity was eluted. This material gave a single coincident band of protein and enzyme on gradient electrophoresis (Fig. 1) and a single protein band after reduction and electrophoresis in SDS/polyacrylamide gels. The yield of enzyme was normally 60–80% of the total adsorbed. The material was normally further processed by concentration and chromatography on Sephacryl S300 with buffer A as eluent, which served to remove NAD⁺ and pyrazole as well as to confirm there was only a

Fig. 1. Gel electrophoresis of a crude extract of *Aspergillus* and of *Aspergillus* ADH in the Blue Sepharose eluate. The tracing shows protein after Coomassie Blue staining. Enzyme activity shown by the arrow in the crude extract is coincident with protein in the purified preparation.

Table 2. *Purification of Aspergillus ADH*

Either 5.3g of dried cells was grown on 4mM-glucose + 50mM-threonine (T) or 4.3g of dried cells was grown on 300mM-ethanol (A). For experimental details of the purification procedure see the text. Values given for enzyme activity after elution from Blue Sepharose are low owing to the presence of pyrazole in the eluting solution.

	Total enzyme (units)		Total protein (mg)		Specific activity (units/mg)		Yield (%)	
	A	T	A	T	A	T	A	T
Cell extract	1984	8118	567	992	3.5	8.2	100	100
Eluate from Blue Sepharose	1350	4730	31	139	43	34	35	41
After chromatography on Sephacryl S300	836	4841	14	85	59	57	47	51

Table 3. *Amino acid composition of Aspergillus ADH*

Duplicate samples of ADH from either ethanol- or threonine-induced cells were hydrolysed as described in the text. Values for most amino acids were averaged; short-analysis-time values were used for serine, threonine and NH_3 , longer hydrolysis times were used for valine and isoleucine. Values found were normalized for glutamic acid = 35 residues and then recorded as integers.

	Composition (nmol of residue/mol)	
	Observed	Integral value
Ala	38.90	39
Arg	12.78	13
Asp	22.60	23
Cys	7.90	8
Glu	35	35
Gly	41.6	42
His	9.37	9
Ile	19.31	19
Leu	27.2	27
Lys	23.3	23
Met	8.95	9
Phe	8.82	9
Pro	21.56	22
Ser	13.06	13
Thr	14.68	15
Trp	2.91	3
Tyr	10.17	10
Val	33.82	34
NH_3	32.2	32
Total amino acids		353

single protein in the preparation. Despite large physical losses in the concentration stage, the overall yield was about 50% or better. Increases in the NAD^+ and pyrazole concentration of 2-fold had no effect on the yield. Increasing the NAD^+ to 5mM or increasing the pH or molarity of the eluting buffer did give marginal improvements in yield, but there were many more proteins eluted than the desired one. A typical balance sheet of ADH purification is presented in Table 2. Threonine-grown cells were routinely used for ADH production, as it was possible to purify aldehyde

dehydrogenase from the same preparation (Creaser & Porter, 1982).

Enzyme properties: physical

A single active species of ADH was found in crude extracts or in pure enzyme preparations by polyacrylamide-gradient-gel electrophoresis. This had M_r 290000. With gel-filtration procedures this M_r was confirmed when 1mM-dithiothreitol or 1.4mM-2-mercaptoethanol was present. Preliminary experiments indicated there is a complex relationship between the quaternary structure of *Aspergillus* ADH and the type and concentration of the thiol reagents present, but it was decided that full investigation lay beyond the scope of this present work.

Only a single subunit of ADH was observed on SDS/polyacrylamide-gel electrophoresis with M_r 37500, indicating that the active enzyme as purified by the above procedure was an octamer.

Amino acid analysis was performed on pure ADH, and the results are given in Table 3. There are no unusual features in its composition, and the data are consistent with a protein of 353 amino acid residues with a unit M_r of 37900, very similar in size to yeast ADH I and ADH II.

Metal analysis of ADH by p.i.x.e. spectrometry gave only zinc as a significant component, and it was present as 1 atom of zinc per 37644 ± 1300 Da of enzyme. It is considered that *Aspergillus* ADH has 1 Zn atom/subunit, as does the yeast enzyme.

In view of the observation that ADH was produced by a variety of possibly unlikely inducers, we considered it desirable to check that it was the same protein. We showed that the ADH from cells grown with either threonine or alcohol as inducer had identical amino acid composition. Furthermore, ADH induced by ethanol, threonine, acetone, serine, propan-1-ol, propan-2-ol or butan-2-one or with no inducer purified in the identical manner had the same single bands in polyacrylamide-gel and SDS/polyacrylamide-gel electrophoresis.

Furthermore, it was found that the same

Table 4. K_m values of *Aspergillus* ADH

K_m values were derived by using standard graphical procedures, with eight concentrations of the varied substrate at 25°C.

Substrate	Concentration range (mM)	Fixed substrate and concentration (mM)		K_m (mM)
Ethanol	0.4–5.0	NAD ⁺	10	0.9
Propan-2-ol	260–2600	NAD ⁺	10	550
Acetaldehyde	0.1–0.625	NADH	0.4	0.35
NAD ⁺	0.2–3.0	Ethanol	50	1.3
	0.2–3.0	Propan-2-ol	1300	1.3
NADH	0.04–0.3	Acetaldehyde	5	0.053

characteristic substrate–activity profile (see below) was shown by ADH induced by acetone, threonine, ethanol or butan-2-one and purified by the same procedure. We conclude only a single species of ADH exists in *Aspergillus*.

Enzyme properties: kinetic

Enzyme activity in the reduction of propan-2-ol or ethanol was tested in a variety of buffers at various pH values. It was found that pH was optimal at 8.2 in phosphate buffer (sodium or potassium) of 0.1M. Glycine/NaOH or pyrophosphate buffers of higher pH were less effective, and Tris/HCl buffer over the pH range 7–9 gave about 70% of the activity as in phosphate buffer of the same pH. The reverse reaction was also assayed in the same 0.1M-phosphate buffer, pH 8.2, that contained 2mM-MgCl₂ and 1mM-dithiothreitol for maximum enzyme activity in both directions.

NAD⁺ was the coenzyme used in the oxidation of ethanol, and NADH in the reduction of acetaldehyde. NADP⁺ and NADPH were inert. A wide variety of substrates were used in both reactions and are shown in Table 1. Again the data are shown for experiments with the same concentration of substrate to facilitate comparisons. The forward reactions of dehydrogenation were carried out at 50mM, and the reverse reactions were normally at 5mM substrate, owing to enzyme inhibition by higher aldehyde concentrations. The most rapidly metabolized substrate in the forward reaction is cinnamyl alcohol, followed by C₃ and C₄ primary or secondary alcohols, and acetaldehyde is the preferred substrate in the reverse reaction. The substrate–activity profile is characteristic of *Aspergillus* ADH; one noteworthy point is the high activity with propan-2-ol, reminiscent of the enzyme from *Euglena gracilis* (Mego & Farb, 1974).

The inducer profile and activity profile are not identical. Several of the better inducers, e.g. butan-2-one, cyclohexanone and threonine, are not metabolized. 3-Hydroxybutan-2-one is a good inducer, and its oxo group is reduced but its alcohol group is not oxidized by the ADH.

One can infer that the active site of *Aspergillus*

ADH is in a similar-sized pocket to the yeast enzyme by reasons of the size of molecule that can be metabolized and hence must enter the pocket. Yeast ADH will not metabolize cyclohexanol, and the *Aspergillus* enzyme will attack neither cyclohexanol or histidinol. The horse enzyme has a bigger pocket and can oxidize cyclohexanol and histidinol.

Several substrates were examined in more detail and their K_m values measured by simple kinetic experiments, and presented in Table 4. Propan-2-ol, which is very rapidly metabolized, has a very high K_m , whereas the K_m for ethanol is low. In the ethanol–acetaldehyde pair the reaction acetaldehyde → ethanol goes more rapidly and the substrates have lower K_m values than in the alcohol → acetaldehyde direction.

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