The Partial Purification and Characterization of Cytosol Alcohol Dehydrogenase from *Astasia*

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1. The cytosol alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) of *Astasia longa* was partially purified and characterized from cells grown in the presence of air+CO₂ (95:5) or of O₂+CO₂ (95:5). 2. Under both these growth conditions, the cells contained a fraction, ADHII, which was characterized by its electrophoretic properties, by a high degree of resistance to heat inactivation, by a sharp pH optimum at 8.2 and by its kinetic properties. The estimated molecular weight of this fraction was approx. 150000, which is similar to that of yeast alcohol dehydrogenase. 3. Cells grown in air+CO₂ (95:5) contain another fraction, ADHI, which can be further separated into two subfractions by polyacrylamide-gel electrophoresis and by DEAE-cellulose chromatography. This was termed fraction 'ADHI-air'. 4. In addition to fraction ADHII, cells grown in the presence of O₂ have a twofold increase in fraction ADHI-air activity as well as two new fractions that could not be demonstrated in air-grown cells. These new fractions which we have called fraction 'ADHI-O₂', account for about 10% of the total activity. 5. The ADHI fractions (air) and (O₂) have similar broad pH-activity curves and similar kinetic properties, both having a lower *K_m* for ethanol and NAD than fraction ADHII. However, they differ from each other with respect to their activity with various substrates. The estimated molecular weight of these two ADHI fractions and their chromatographic behaviour on hydroxyapatite and on DEAE-cellulose also distinguish them.

We became interested in the alcohol dehydrogenases of *Astasia* when we noted that O₂ treatment led to an increase in the alcohol dehydrogenase activity of the cells, although at the same time the ability of the cells to grow and divide was impaired when ethanol was used as the sole carbon source (Bégin-Heick, 1970). We have attempted to determine the nature of the metabolic lesions produced by O₂ (Morosoli & Bégin-Heick, 1973), and we now present results on the partial purification and characterization of the multiple forms of NAD-dependent alcohol dehydrogenase (alcohol-NAD oxidoreductase EC 1.1.1.1) in the cytosol of this organism and the effect of O₂ thereon.

Methods and Results

Reagents

DEAE-cellulose (Whatman DE-52) was obtained from Mandel Scientific Co., Montreal, Que., Canada; Sephadex G-25 was from Pharmacia (Canada), Montreal, Que., Canada; hydroxyapatite, NAD, NADH, cinnamyl alcohol, dithiothreitol, phenazine

methosulphate and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide were from Sigma Chemical Co., St. Louis, Mo., U.S.A.; acrylamide and bisacrylamide were from Eastman Organic Chemical Dept., Rochester, N. Y., U.S.A. The other reagents were of the highest grade available.

Cells

The cells used were *Astasia longa* (Jahn strain) grown axenically on a modified Cramer-Myers medium (Blum & Padilla, 1962) containing 0.05M-ethanol. The cultures were grown in 16-litre incubation vessels (Blum & Padilla, 1962) with either air+CO₂ (95:5) or O₂+CO₂ (95:5) as the gas phase. The air-grown cells were harvested at the end of the exponential growth phase (1.5×10^6 – 2×10^6 cells/ml), and the O₂-grown cells were harvested after 48 h of O₂ treatment under conditions that have been described in detail previously (Morosoli & Bégin-Heick, 1973).

Preparation of cell-free extracts

The cells were harvested by continuous-flow centrifugation in a Sorvall centrifuge at 3000g. They

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were resuspended in a buffered sucrose solution containing 25 mM-Tris (adjusted to pH 7.4 with 0.1 M-HCl), 0.25 M-sucrose and 5 mM-EDTA. The cells were washed once and centrifuged at 3000g for 10 min. They were then resuspended in the same buffer at a concentration of 1 g wet wt. of cells/ml of buffer. The cells were broken for 30 s in a Braun MSK cell homogenizer (Brownwill Scientific Inc., Rochester, N.Y., U.S.A.) with 2 mg of silicone-coated glass beads (0.25–0.3 mm diam., Brownwill Scientific)/g of cells. The cell homogenate was decanted from the glass beads which were then washed with several portions of the buffered sucrose solution. The homogenate and the washings were combined and adjusted to a concentration of 20% (w/v). The total homogenate was centrifuged at 120g for 10 min, then at 3000g for 10 min and finally at 48000g for 20 min. The pellets were discarded and the supernatant from the last centrifugation contained the total alcohol dehydrogenase activity of the cytosol. A typical experiment such as that reported in Table 1 was done with 30 g of cells (wet wt.).

Thermal stability

Crude extracts. The 48000g supernatants obtained from either air-grown or O₂-grown cells were heated for the time specified in a constant-temperature bath at 50°C. They were then cooled for 5 min in ice and centrifuged at 12000g in a refrigerated Sorvall centrifuge. The supernatant fractions were used for electrophoresis and for the spectrophotometric determinations of enzyme activity as described below. In the air-grown cells the uppermost band of activity was denatured very rapidly at 50°C, and after 8 min activity could no longer be detected on the gels. However, in the supernatant obtained from the O₂-treated cells, there was still considerable fraction ADH I activity after 10 min at 50°C (Plate 1). After spectrophotometric assay of the enzyme activity (as described below) it appeared that although the alcohol dehydrogenase activity of the air-grown cells was little affected by heat treatment at 50°C for 2 min, the supernatant from the O₂-grown cells lost one-third of its activity under the same conditions. It also appeared from the electrophoretic behaviour of the crude extract that fraction ADH II was little affected by heat treatment, although it was not possible to evaluate its behaviour by a spectrophotometric assay under these conditions.

Since the above data indicated that the fraction ADH I activity of the O₂-grown cells might contain several subfractions, the time of electrophoresis was doubled to obtain a better resolution of the components; the results of this type of experiment are shown in Fig. 1. The crude supernatant from the air-grown cells (Fig. 1a) separated into three distinct bands, but the supernatant from the O₂-grown cells (Fig. 1b) separated into five distinct bands. Subfrac-

Fig. 1. Electrophoresis on 8% (w/v) polyacrylamide gels of the 48000g supernatant obtained from (a) cells grown in air (b) cells grown in O₂

Electrophoresis was done with a current of 3 mA/gel for 2 h. Each gel received 50 μ l of a dilution of the 48000g supernatant adjusted to a protein concentration of 20 mg/ml. The enzyme activity was determined as described in the text and the coloured bands were read by scanning at 540 nm on a Gilford recording spectrophotometer.

tions b₁ and b₂ were present in both the air- and O₂-grown cells, but subfractions a₁ and a₂ appeared only when the cells were grown in the presence of O₂.

To eliminate the possibility that these various fractions represented only differences in NAD bound, the supernatant fractions were incubated with 1 mM-NAD for 60 min at 25°C to saturate the NAD-binding sites. The supernatants were then electrophoresed. The results obtained were essentially similar to those shown in Plate 1.

EXPLANATION OF PLATE I

Electrophoresis of the supernatant from air-grown cells (a) and from O_2 -grown cells (b) before (i) and after (ii) heat treatment at 50°C

Samples (0.5 ml) containing 20 mg of protein were heated for 8 min. They were cooled in ice for 5 min and then centrifuged at 12000g for 10 min. A portion (50 μ l) of each of the resulting supernatants was electrophoresed on 8% (w/v) polyacrylamide gels. The enzyme activity was determined with cinnamyl alcohol as the substrate. X, alcohol-NADP⁺ oxidoreductase.

Table 1. Fractionation of the alcohol dehydrogenase activity from cells grown in air and in O₂

The various steps are described in detail in the text. One unit of enzyme activity was defined as that amount of enzyme that reduced 1 μ mol of NAD/min at 24°C.

Purification steps	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg of protein)	Total activity (units)
1. Crude supernatant Air	110	57.5	0.04	225
O ₂	110	73.0	0.1	778
2. Supernatant (48000g) Air	73	23.0	0.06	96
O ₂	77	23.5	0.21	370
3. Supernatant (after protamine treatment) Air	68	12.3	0.11	88
O ₂	70	15.5	0.31	336
4. Fraction F55-75% [(NH ₄) ₂ SO ₄ precipitate] Air	2.5	56.0	0.33	46
O ₂	2.5	64.5	1.97	317

Purification of the alcohol dehydrogenases

(a) *Protamine sulphate precipitation.* Protamine sulphate (1mg) was added for each ml of 48000g supernatant fraction. The mixture was stirred for 10min, and then centrifuged at 12000g for 10min. The E_{280}/E_{260} ratio changed from 0.6 to 0.7 in the supernatant, and further addition of protamine sulphate did not cause further precipitation.

(b) *(NH₄)₂SO₄ precipitation.* Solid (NH₄)₂SO₄ was added to the supernatant obtained from the previous step to obtain 55% saturation at 4°C (DiJeso, 1968). The mixture was stirred for 45min at 4°C, left for 15min and then centrifuged at 12000g for 10min. The supernatant fraction resulting from this first precipitation was adjusted to 75% saturation with (NH₄)₂SO₄. It was again stirred for 45min at 5°C, left for 15min and centrifuged at 12000g for 10min. The precipitate from the 55%-75%-satd.-(NH₄)₂SO₄ was resuspended in 2.5ml of 0.1M-sodium phosphate buffer, pH7.6, or 0.1M-Tris-HCl, pH8. The fraction that precipitated between 55%- and 75%-satd. (NH₄)₂SO₄ (fraction F55-75%) contained all the cytosol alcohol dehydrogenases.

The initial purification of the alcohol dehydrogenase activities from air- and O₂-grown cells are described in Table 1. The initial specific activity of the supernatant obtained from O₂-grown cells was about threefold that of the air-grown cells. Both fractions lost more than 50% of their total activity after centrifugation at 48000g owing, at least partially, to the removal of the mitochondria. The total purification obtained by these procedures was tenfold for the air-grown cells and 20-fold for the O₂-grown cells.

Separation of the alcohol dehydrogenase subfractions

(a) *Alcohol dehydrogenase from air-grown cells.* Each portion of fraction F55-75%, containing 30mg of protein, was taken up in 1ml of 0.01M-sodium phosphate buffer, pH7.6. After dialysis against the same buffer for 10h, 0.02ml of a solution of saturated

(NH₄)₂SO₄ at 4°C was added/ml of enzyme solution. This enzyme solution was then mixed with hydroxyapatite (1g/30mg of protein) which had previously been equilibrated with the same buffer (Levin, 1962). The slurry was centrifuged at 120g for 5min. The supernatant contained two-thirds of the total proteins and fraction ADHII. The hydroxyapatite was washed successively with 10ml of 0.01M-sodium phosphate buffer, pH7.6, and twice with 10ml of 0.08M-sodium phosphate buffer, pH7.6. The mixture was centrifuged at 120g for 5min after each addition of buffer. The hydroxyapatite was then stirred for 15min with 0.4M-sodium phosphate buffer, pH7.6, and then centrifuged at 120g for 10min. These steps along with the volume, protein content and total activity of each fraction are summarized in Fig. 2(a). The fraction eluted with 0.4M-sodium phosphate buffer contained fraction ADHI-air. It was precipitated by adding a saturated solution of (NH₄)₂SO₄ at 4°C. The mixture was left for 1h, and then centrifuged at 12000g for 10min. The precipitate was taken up in 1ml of a buffer containing: Tris-HCl, 0.1M, pH8; dithiothreitol, 0.4mM; and ethanol, 1M. The mixture was dialysed for 10h against the same buffer. The solution was then applied to a column (2cm × 60cm) of DEAE-cellulose which had been equilibrated with the same buffer without ethanol. The column was eluted with a linear gradient of 0.1-0.5M-NaCl at pH8. Two fractions were recovered from this last step, one being eluted at 0.175M-NaCl and the second with a peak at 0.188M-NaCl. There was incomplete separation of the fractions and most of the enzyme activity was lost as a result of the DEAE-chromatography step. The methods used for electrophoresis and enzyme activity measurements are described below.

(b) *Alcohol dehydrogenase from O₂-grown cells.* Cells grown in the presence of O₂ for 48h were used for this purification. The cells were treated essentially in the same way as the air-grown cells and the same proportions were observed. After dialysis and addition of (NH₄)₂SO₄ as described above, the 55-75%-satd.-(NH₄)₂SO₄ fraction was applied to a column

Fig. 2. Graphic representation of the purification steps for the alcohol dehydrogenase isolated from (a) air-grown cells and (b) O₂-grown-cells

The details of the procedure are given in the text. The values in parentheses represent the volume (ml), total protein (mg) and total enzyme activity in each fraction (units).

(1cm×2cm diam). of hydroxyapatite. The column was eluted with 20ml of 0.01M-sodium phosphate buffer followed by 20ml of 0.08M-sodium phosphate buffer. These pooled fractions contained fractions ADHII and ADHI-O₂. In contrast, fraction ADHI-air was bound so tightly to the column that it was eluted with 0.4M-sodium phosphate buffer (Fig. 2b). The fraction that contained fractions ADHII and ADHI-O₂ was saturated with (NH₄)₂SO₄ and was centrifuged at 12000g for 10min after being left for 1h. The precipitate was taken up in 2.5ml of 0.01M-sodium phosphate buffer, pH7.6. The sample was then applied to a column (2cm×35cm) of Sephadex G-25 which had been previously equilibrated with 0.01M-sodium phosphate buffer, pH7.6, containing 0.4mM-dithiothreitol. The column was washed with 45ml of buffer and the enzyme fraction was recovered in the next 15ml of buffer. The sample was then applied to a column (1.5cm×10cm) of DEAE-cellulose equilibrated with 0.01M-sodium phosphate buffer, pH7.6, containing 0.1M-dithiothreitol. The column was eluted successively with 30ml portions of NaCl at the following concentrations: 0.03, 0.05, 0.08, 0.12, 0.14 and 0.2M. The only alcohol dehydrogenase activity recovered was eluted between 12 and 24ml of 0.05M-NaCl. This corresponded to fraction ADHI-O₂. The total protein content of the fraction was 18mg and the specific activity was 0.7 unit/mg of protein.

The fractions used for further studies of the physico-chemical properties of the alcohol dehydrogenase were as follows: (a) fraction ADHII: from cells grown in air and eluted from hydroxyapatite at 0.01 M-sodium phosphate; (b) fraction ADHI-air: from cells grown in air and eluted from hydroxyapatite at 0.4M-

sodium phosphate; (c) fraction ADHI-O₂: from cells grown in O₂, eluted from hydroxyapatite at 0.01–0.08M-sodium phosphate and separated from fraction ADHII by chromatography on DEAE-cellulose.

Measurement of enzyme activity

The total incubation volume was 3.0ml and the change in E_{340} at 24°C was measured with a Gilford spectrophotometer (model 2000) fitted with a constant-temperature circulator (Lauda K2/R model, Brinkmann Instruments Inc., Toronto, Canada). Unless otherwise specified in the legends to the Tables and Figures, the alcohol dehydrogenase activity was measured in the direction of ethanol to acetaldehyde as follows: 0.1M-ethanol and 1.2mM-NAD were added to 0.075M-sodium pyrophosphate–0.22M-glycine–0.075M-semicarbazide, pH8.2. One unit of enzyme activity was defined as that amount of enzyme which reduced 1 μ mol of NAD/min. Specific activity was expressed as units/mg of protein.

Dependence of activity on pH

These results are shown in Fig. 3; although fraction ADHII has a sharp pH optimum at pH8.2, the ADHI-air and ADHI-O₂ fractions both show a broad area of activity with a maximum between pH8 and 9.5.

Measurements of the activity with various substrates

The three fractions have different activity profiles for the alcohols that were used as substrates (Table 2). Although all fractions have the highest activity with cinnamyl alcohol, fraction ADHI-air is the most active with this compound. Both fraction ADHI-air and fraction ADHII are more active with allyl alcohol than with ethanol, but fraction ADHI-O₂ is more active with ethanol than with any other alcohol, with the exception of cinnamyl alcohol.

K_m for the substrates

The K_m values of the three fractions for ethanol and NAD, and for acetaldehyde and NADH were measured and the results are shown in Table 3. The results were calculated by the double-reciprocal method of Lineweaver and Burk and by plotting $[S]/v$ against $[S]$ (cf. Dixon & Webb, 1964).

Fraction ADHII has a higher K_m than the other two fractions towards NAD and ethanol, and its K_m for NADH is one-tenth and for acetaldehyde one-hundredth that for NAD and ethanol. Both ADHI fractions have similar K_m values towards both substrate pairs.

Fig. 3. Activity of the alcohol dehydrogenase fractions as a function of pH

The buffers used were 0.1M-Tris-HCl for pH7.0 to 9.0, and 0.1M-glycine-NaOH for pH9.0 to 10.4. All samples contained ethanol (0.1M) and NAD (1.2mM). ○, Fraction ADHI-air; ●, fraction ADHI-O₂; △, fraction ADHII.

Table 2. *Relative activities of the alcohol dehydrogenases of Astasia with various alcohols*

The incubation mixture contained in a volume of 3 ml: sodium pyrophosphate, 0.075M; glycine, 0.022M; semicarbazide, 0.075M, pH 7.2, as well as NAD, 1.2mM and the various alcohols at the concentrations indicated in the Table. Enzyme activity was measured at 25°C. The specific activities of the three fractions for each substrate are reported as the percentage of their activity with ethanol at the same concentration.

Alcohols	Enzyme fraction	...	Specific activity (% of activity with ethanol)		
			Fraction ADHI-air	Fraction ADHI-O ₂	Fraction ADHII
Ethanol (0.1M)			100	100	100
Allyl alcohol (0.1M)			210	80	150
Butan-1-ol (0.1M)			80	40	90
Butan-2-ol (0.1M)			160	40	100
Propan-1-ol (0.1M)			170	60	100
Ethanol (0.5mM)			100	100	100
Cinnamyl alcohol (0.5mM)			410	280	210

Table 3. *K_m and V_{max} values of the alcohol dehydrogenase fractions for their substrates*

The results were analysed by the double-reciprocal method (top value in each case) and by plotting [S]/v against [S] (bottom value in each case).

	NAD		NADH		Ethanol		Acetaldehyde	
	K _m (mM)	V _{max} . (nmol/min)	K _m (mM)	V _{max} . (nmol/min)	K _m (mM)	V _{max} . (nmol/min)	K _m (mM)	V _{max} . (nmol/min)
Fraction ADHII	1.2	53	0.10	76	8.3	13	0.09	27
	1.0	44	0.09	70	11.0	14	0.08	23
Fraction ADHI-air	0.22	32	0.51	140	0.19	30	0.18	27
	0.25	40	0.42	130	0.18	32	0.09	29
Fraction ADHI-O ₂	0.16	20	0.78	200	1.0	23	0.16	19
	0.17	18	0.91	230	0.99	22	0.10	15

Polyacrylamide-gel electrophoresis

Acrylamide and bisacrylamide were purified as described by Loening (1967). All gels contained 5% (w/v) bisacrylamide. The electrophoresis and the development of alcohol dehydrogenase activity on the gels have been described in detail elsewhere (Bégin-Heick & Heick, 1970). To ensure good resolution the gels had to be used within 20min of their preparation. In some cases the gels were scanned after staining, by using the gel-scanning attachment (model 2410) of the Gilford spectrophotometer (model 2000) (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.).

Determination of the molecular weight of the enzyme fractions

This was done by the methods of Hedrick & Smith (1968) and Kingsbury & Masters (1970). The electrophoresis system was that of Davis (1964), and the enzymes were revealed on the gels by the enzymic

method described in detail by Bégin-Heick & Heick (1970). The alcohol dehydrogenase fractions were prepared from cells grown in O₂, through the step of precipitation by 55–75%-satd. (NH₄)₂SO₄. Fraction F55–75% was taken up in 2.5ml of 0.01M-sodium phosphate buffer, pH 7.6, containing 0.1mM-dithiothreitol. The sample was passed through Sephadex G-25 and chromatographed on DEAE-cellulose as described above. The three alcohol dehydrogenase activities were eluted in one fraction by first washing the column with 30ml of the buffer containing 0.03M-NaCl, and then eluting with 30ml of buffer containing 0.16M-NaCl. The fraction thus obtained was diluted with 0.01M-sodium phosphate buffer, pH 7.6, containing 25% (w/v) sucrose to a final concentration of 1mg of protein/ml. Samples (50μl) were added to gels of polyacrylamide concentrations varying between 6 and 9%. The electrophoresis was performed for 45min with a current of 3mA/gel. Standard proteins were electrophoresed under the same conditions (Fig. 4). The standard proteins were revealed on the gels by staining with Amido Schwartz reagent [2g

Fig. 4. (a) Effect of polyacrylamide concentration on the relative mobility of the soluble alcohol dehydrogenases of *Astasia*. (b) Relation between the negative slopes and the molecular weight of the proteins

The cellular extract was purified as described in the Methods and Results section. Samples that were electrophoresed contained 0.05 mg of protein. ○, Fraction ADHII, negative slope 16.8; △, fraction ADHI_{air}, negative slope 17.3; ●, fraction ADHI-O₂, negative slope 31.6. (1) Creatine kinase, mol.wt. 80000, slope = -10.2; (2) albumin (dimeric form) mol.wt. 136000, slope = -15.6; (3) yeast alcohol dehydrogenase, mol.wt. 150000, slope = -17.3; (4) fraction ADHI-O₂, slope = -31.6, mol.wt. approx. 290000; (5) fraction ADHI-air, slope = -17.3, mol. wt. approx. 150000.

of Amido Schwartz/litre of 1M-acetic acid-1M-sodium acetate-1M-glycerol (9:9:2, by vol.]. The gels were stained for 2 min and destained for 24 h in 10% (w/v) acetic acid. When the logarithm of the relative mobility is plotted against the acrylamide concentration the points representing fraction

ADHI-air or fraction ADHII can be joined by parallel lines with slopes of -17.3 and -16.8, respectively. The points representing fraction ADHI-O₂ give a line with slope of -31.6 (Fig. 4a). By comparing these results with those obtained for the standard proteins (Fig. 4b) it can be shown that the molecular weights of fraction ADHII and fraction ADHI-air are similar to that of yeast alcohol dehydrogenase (150000) whereas that of fraction ADHI-O₂ is approximately twice as much (290000).

There can be little doubt that various forms of alcohol dehydrogenase exist in intact *Astasia*. We observed previously with baker's yeast preparations that new electrophoretic bands of alcohol dehydrogenase activity appeared after storing or freeze-drying the extracts (Heick *et al.*, 1969). In contrast the alcohol dehydrogenase of *Astasia* was found to be very labile, the activity being lost after storing or freeze-drying of cell-free extracts so that the various isolation steps had to be performed very quickly and on fresh extracts. Further, the electrophoretic bands of enzymic activity that were recovered after purification were similar to those observed in freshly broken cells.

The purification steps with hydroxyapatite and heat treatment do not lead to a complete separation of the alcohol dehydrogenase activity from other proteins, but these steps do lead to the separation of three forms of alcohol dehydrogenase from each other. Thus by judicious manipulations of the culture conditions and of the carbon source, it might be possible eventually to ascribe a more definite role to the various forms of this enzyme in *Astasia*.

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