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THE SUBCELLULAR LOCALIZATION OF ALCOHOL DEHYDROGENASE ACTIVITIES IN *ASTASIA LONGA*

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SUMMARY

On electrophoresis of whole cell extracts of *Astasia longa* we found two bands of NAD-dependent alcohol dehydrogenase activity (alcohol:NAD oxidoreductase EC 1.1.1.1) and one band of NADP-dependent alcohol dehydrogenase activity (alcohol:NADP oxidoreductase EC 1.1.1.2). These bands were termed alcohol dehydrogenase(NAD) I, alcohol dehydrogenase(NAD) II and alcohol dehydrogenase(NADP), respectively, in the order of their electrophoretic mobilities, alcohol dehydrogenase(NAD) I being the slowest moving component. Upon fractionation of the cells into a particulate and a cytosol fraction, we found that each fraction contained two bands corresponding to alcohol dehydrogenase(NAD) I and alcohol dehydrogenase(NAD) II when the gels were developed with ethanol and NAD as substrates. In the normal cells, alcohol dehydrogenase(NAD) II is predominant in the cytosol and very little alcohol dehydrogenase(NAD) I is seen. In the particulate fraction alcohol dehydrogenase(NAD) I is predominant. O_2 treatment and carbon deprivation increase the activity of alcohol dehydrogenase(NAD) I in the cytosol and this enzyme activity becomes the dominant activity in the cell.

Some properties of these various alcohol dehydrogenase activities have been investigated.

INTRODUCTION

During growth of *Astasia longa* with O_2 as the gas phase, the specific activity of the alcohol dehydrogenase (alcohol:NAD oxidoreductase, EC 1.1.1.1) of cells cultivated with ethanol increases about 10-fold. At the same time, the cells are unable to utilize ethanol for growth and respiration and they behave in many ways like cells which are deprived of a carbon source. Other changes also occur in the cells. The most profound of these changes is the marked inhibition of NADH:cytochrome *c* oxidoreductase activity, which may be the primary event leading to the inhibition of respiration[1]. We have reported also that the alcohol dehydrogenase activity in *Astasia*

Abbreviation: C/E, ratio of the activity with cinnamyl alcohol as the substrate compared to the activity with ethanol as the substrate.

is present in the cytosol as well as the particulate fraction[1]. We propose to report here on the electrophoretic properties and the substrate specificities of alcohol dehydrogenase in the cytosol and the particulate fractions of *A. longa* grown under three different conditions: standard condition with air–CO_2 and ethanol as the substrate; O_2 treatment where the gas phase is O_2–CO_2 (95:5) with ethanol as the substrate; carbon deprivation, where cells which have previously been growing in ethanol are placed in a medium containing no utilizable carbon source.

MATERIALS AND METHODS

Cells

The cells were grown and treated as described previously[1,2].

Preparation of cellular extracts

Cells were harvested in the continuous flow system of the Sorval RC-2B centrifuge and washed once in 0.05 M Tris (pH 7.4) containing 10 mM EDTA. The cell pellets were then suspended in the same buffer (5 ml buffer per ml packed cells) and submitted to sonic oscillations in a Fisher BP2 ultrasonic probe at maximum intensity for 1 min. The resulting homogenate was centrifuged at 100 000 $\times g$ for 30 minutes and the supernatant fraction (whole cell supernatant) was used for enzyme assays and polyacrylamide gel electrophoresis. The particulate fraction was prepared as described previously[1,2]. The final mitochondrial pellet was washed once, suspended in a sufficient volume of Tris buffer at pH 7.9 (Buffer B of DAVIS[3], diluted 1:3) to give a final concentration of 2–3 mg protein per ml and sonicated 30 sec. This sonicate was used for enzyme assays; for gel electrophoresis the supernatant obtained after centrifugation of the sonicate at 100 000 $\times g$ for 30 min was used. Cytosol fraction: The supernatant fraction[1] obtained after the removal of the mitochondria from the whole cell homogenate was centrifuged at 100 000 $\times g$ and the resulting cytosol fraction was used for gel electrophoresis and enzyme assays.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was done using the pH 9.5 system of DAVIS[3] and gel concentrations varying between 7 and 10% (ref. 4). The gels were developed as described previously[5] except that 0.1 M Tris (pH 8.5) was used as the buffer. Except when otherwise specified, cinnamyl alcohol was used as the substrate.

Enzyme assays

NAD-dependent alcohol dehydrogenase was assayed according to the method of BONNICHSEN[6] with the following concentrations of substrates: ethanol, 0.6 M; butanol, 0.35 M; propanol, 0.43 M; allyl alcohol, 0.5 M; amyl alcohol, 0.3 M; decanol, 0.2 M; cinnamyl alcohol, 0.5 mM. NADP-dependent alcohol dehydrogenase was assayed according to the method of BLACK[7] with 0.6 M ethanol or 0.5 mM cinnamyl alcohol.

RESULTS

Fig. 1a shows the growth curve which is characteristic of *Astasia* treated with

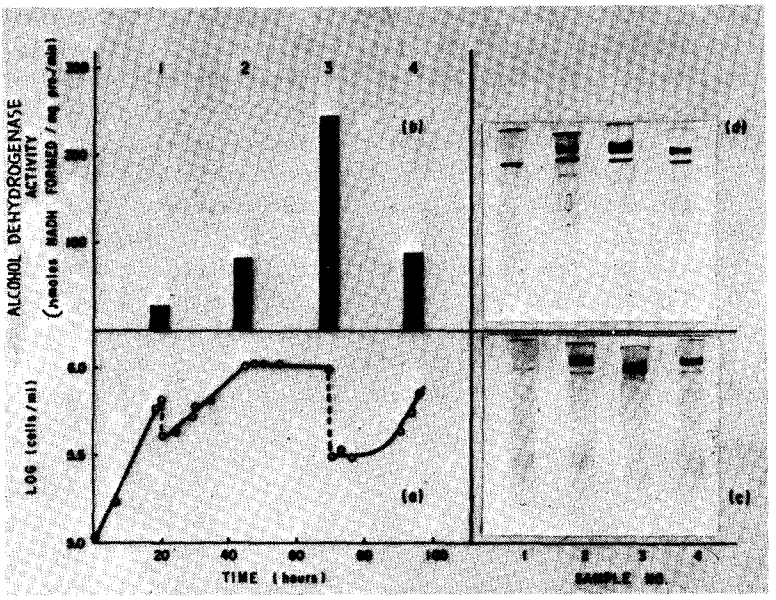

Fig. 1. The growth curve and alcohol dehydrogenase activity of *Astasia* under standard conditions and during O_2 treatment: growth curve (a), specific activity of alcohol dehydrogenase (b), and electrophoretic pattern of alcohol dehydrogenase with ethanol (c) and cinnamyl alcohol (d) as substrate.

O_2 after an initial growth period in air. At the end of the air period (Period I) at 24 and 48 h after onset of O_2 treatment (Period II) and at 24 h after the cells were returned to an air environment (Period III), portions of the culture were harvested. The activities and the electrophoretic properties of the alcohol dehydrogenase of these cells are shown in Figs. 1b–1d. The bands appearing on electrophoresis have been labelled alcohol dehydrogenase(NAD) I and alcohol dehydrogenase(NAD) II in order of their electrophoretic mobility, the slowest band being alcohol dehydrogenase(NAD) I. 48 h after the onset of treatment, the alcohol dehydrogenase(NAD) activity is 10-fold higher than the activity in air cells. On electrophoresis of the air cells only one band of enzymatic activity can be detected which corresponds to alcohol dehydrogenase(NAD) II (Fig. 1c-1). After the gas phase is changed to O_2 a new band with slower electrophoretic mobility (alcohol dehydrogenase I) appears (Fig. 1c-2). The intensity of this alcohol dehydrogenase I increases along with the increase in enzyme activity (Fig. 1c-3) and starts decreasing also with the decrease in total enzyme activity observed when the cells are returned to air again (Fig. 1c-4). Samples 1, 2, 3 and 4 were adjusted to contain the same protein concentration before gel electrophoresis. If the gels are developed with cinnamyl alcohol rather than ethanol, the pattern is similar (Fig. 1d), except for the presence of another faint band of activity, moving faster than the other two. This is due to the alcohol dehydrogenase (NADP) which is slightly reactive in presence of NAD and cinnamyl alcohol.

NAD-dependent alcohol dehydrogenase

Electrophoretic patterns. We have already shown that alcohol dehydrogenase activity was present in the cytosol as well as in the particulate fraction of homogenates

of ethanol-grown *Astasia*. It was therefore interesting to determine whether there were differences in the electrophoretic patterns of the two fractions. Prior to electrophoresis, the fractions to be tested were assayed spectrophotometrically so that essentially the same amount of NAD-dependent alcohol dehydrogenase activity from the cytosol and the particulate fraction was submitted to electrophoresis in all experiments. On 7% gels, only one band of alcohol dehydrogenase activity could be detected in the supernatant fraction from cells grown under standard conditions. This band has the electrophoretic mobility of alcohol dehydrogenase(NAD) II. After O_2 treatment and starvation both alcohol dehydrogenase(NAD) I and alcohol dehydrogenase(NAD) II are detected (Figs. 2a, 2c and 2d). Although activity was detected spectrophotometrically in the particulate fraction, no activity could be detected on 7% gels (Fig. 2f). The addition of 1.4 μM β-mercaptoethanol to the electrophoresis buffer allowed us to reveal activity (Fig. 2g) with the electrophoretic mobility of alcohol

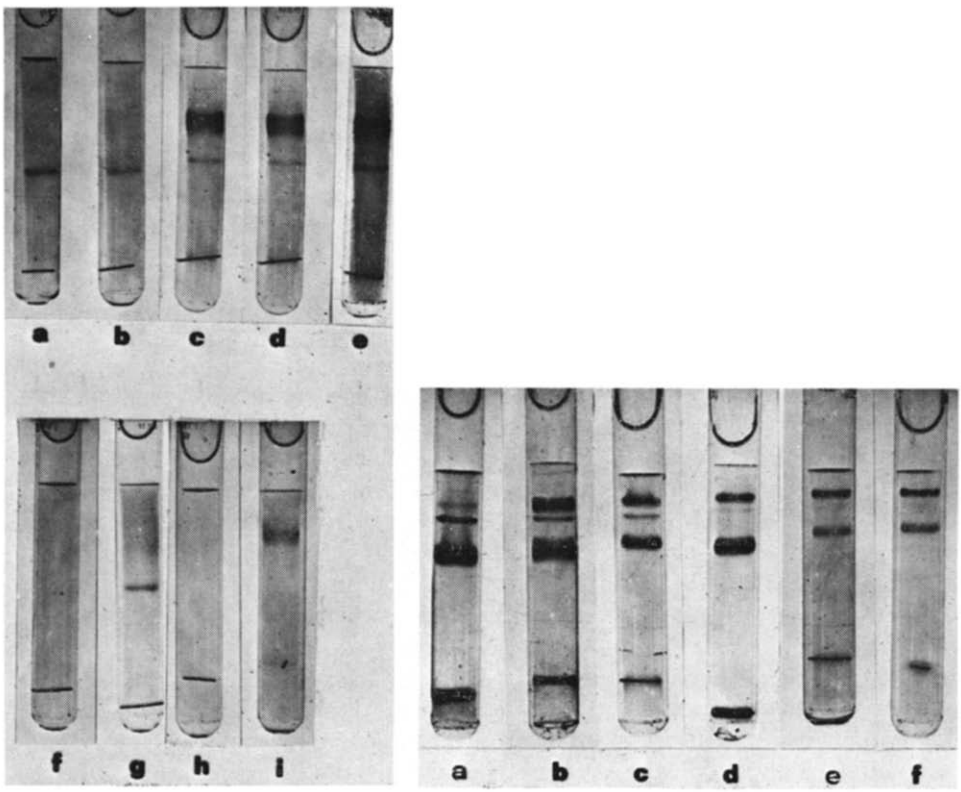

Fig. 2. The electrophoretic pattern of the cytosol and particulate fractions of *Astasia* alcohol dehydrogenase in 7% acrylamide gels. Cinnamyl alcohol was used as the substrate. Cytosol: (a) control, (b) control + mercaptoethanol, (c) O_2-treated, (d) O_2-treated + mercaptoethanol, (e) starved + mercaptoethanol. Particulate fraction: (f) control, (g) control + mercaptoethanol, (h) O_2-treated, (i) O_2-treated + mercaptoethanol.

Fig. 3. The electrophoretic pattern of the supernatant and particulate fractions of *Astasia* alcohol dehydrogenase in 8% acrylamide gels. Cytosol: control (a), O_2-treated (b) and starved (c). Particulate fraction: control (d), O_2-treated (e) and starved (f). Mercaptoethanol was used throughout. The bands are from the top of the gel: alcohol dehydrogenase(NAD) I, alcohol dehydrogenase(NAD) II and alcohol dehydrogenase(NADP).

dehydrogenase(NAD) II in the particulate fraction. After treatment of the cells with O_2, a band with the mobility of alcohol dehydrogenase(NAD) I appears which also requires mercaptoethanol to be active. Mercaptoethanol is not required to demonstrate the alcohol dehydrogenase activities in the cytosol fraction on acrylamide gels (Figs. 2a–2d). Also, no mercaptoethanol is required for the spectrophotometric measurement of the alcohol dehydrogenase activity of either the supernatant or the particulate fractions. The electrophoresis of the supernatant and the particulate fractions on more concentrated gels (8–10%) revealed additional bands of activity and 8% gels were used routinely thereafter. The alcohol dehydrogenase(NAD) activity on polyacrylamide gels corresponds to the two upper bands of each of the gels shown on Fig. 3. It can be seen in Fig. 3a that there is indeed a small amount of alcohol dehydrogenase-(NAD) I in the supernatant fraction of cells grown under standard conditions, but the alcohol dehydrogenase(NAD) II predominates. These gels were developed at the same time with NAD and NADP and the fastest moving band is the NADP-dependent alcohol dehydrogenase. In the particulate fraction of the same cells (Fig. 3d) for the same total activity electrophoresed, the alcohol dehydrogenase(NAD) I band is predominant and there is very little alcohol dehydrogenase(NAD) II activity. Alcohol dehydrogenase(NAD) I is the predominant NAD-dependent alcohol dehydrogenase activity in both the cytosol and particulate fractions of the O_2-treated cells (Figs. 3b and 3e). The electrophoretic patterns of the fractions from starved cells is essentially the same as those from the O_2-treated cells (Figs. 3c and 3f).

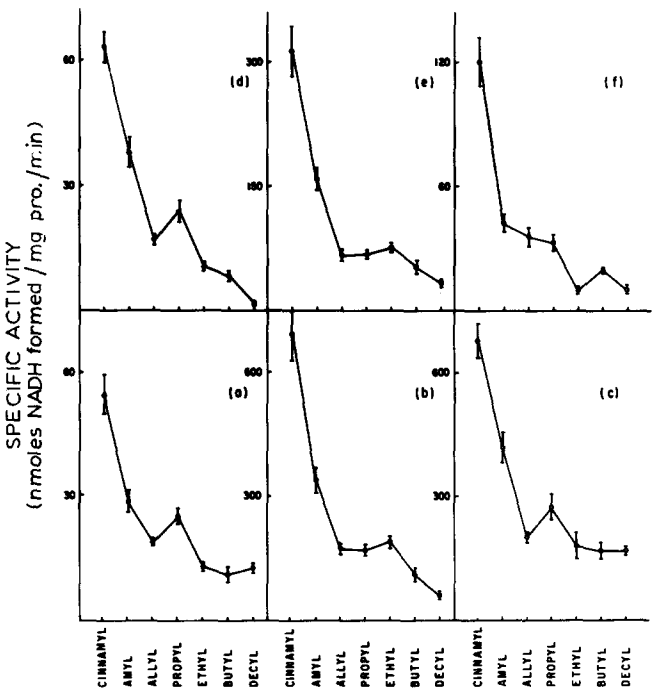

Fig. 4. The specific activity of the NAD-dependent alcohol dehydrogenases of *Astasia* grown under various conditions. Cytosol fraction of control cells (a), O_2-treated cells (b) and starved cells (c). Particulate fraction of control cells (d), O_2-treated cells (e) and starved cells (f).

Relative activities with different substrates. In the cytosol of starved cells, the specific activity of the total NAD-dependent alcohol dehydrogenase activity is approx. 10 times higher than in the cytosol of cells grown under standard conditions. However the relative activities of these two fractions for different alcohols are very similar as shown by the activity profiles (Figs. 4a and 4c). The specific activity of total alcohol dehydrogenase(NAD) activity in the cytosol fraction of O_2-treated cells is about 10-fold higher than in the cytosol of cells grown under standard conditions. The activity profiles of the two samples are also different, due to different relative affinities for propyl, butyl and decyl alcohols (Figs. 4a and 4b). On the other hand, carbon deprivation has little effect on the specific activity of the alcohol dehydrogenase(NAD) of the particulate fraction when ethanol is the substrate; the activity is essentially the same as in the particulate fraction of cells grown under standard conditions. However, the specific activities of the fraction with other alcohols are approximately twice that of the particulate fraction from normal cells. O_2 treatment of the cells leads to a 7-fold increase of the particulate alcohol dehydrogenase activity, and the activity profiles of the particulate fractions obtained from the control and the O_2 cultures are different (Figs. 4d and 4e). It is interesting that in cells grown under standard conditions, the relative affinities of the alcohol dehydrogenase from the cytosol and from the particulate fractions for different alcohols differ with amyl and decyl alcohol (Figs. 4a and 4d). In O_2-treated cells, the profiles are similar (Figs. 4b and 4e), and in starved cells, the profiles are completely different (Figs. 4c and 4f).

NADP-dependent alcohol dehydrogenase

Electrophoretic pattern. The supernatant and the particulate fraction each contain one band of alcohol dehydrogenase(NADP) activity (Figs. 4a and 4d). O_2 treatment and carbon deprivation do not change the relative mobility of the bands.

Specific activity and substrate specificity. In the control cells, there is very little difference between the activities of the supernatant and of the particulate fractions. But while O_2 treatment and carbon deprivation lead to a decrease in the specific activity of the particulate enzyme, these conditions produce an increase in the activity of the supernatant enzyme* (Table I). The ratio of the activities cinnamyl alcohol/

TABLE I

NADP-DEPENDENT ALCOHOL DEHYDROGENASE ACTIVITY OF *A. longa*

The activity is defined as nmoles of NADPH formed per min per mg protein. The standard error of the mean and the number of determinations are given with each value.

Fraction		*Activity*		
		Air	*O_2*	*Starved*
Particulate	Ethanol	1.25 ± 0.15 (17)	0.86 ± 0.02 (6)	0.92 ± 0.05 (10)
	Cinnamyl alcohol	4.45 ± 0.46 (19)	2.08 ± 0.05 (5)	2.10 ± 0.07 (8)
Cytosol	Ethanol	0.91 ± 0.04 (15)	2.45 ± 0.25 (6)	2.92 ± 0.34 (11)
	Cinnamyl alcohol	12.5 ± 1.18 (17)	32.6 ± 1.26 (6)	51.1 ± 4.36 (11)

* The gels shown in Fig. 3 cannot be taken as an illustration of this effect since the amount of enzyme activity submitted to electrophoresis was based only on the alcohol dehydrogenase(NAD) activity.

ethanol (C/E ratio) is between 2 and 3 in the particulate fraction whereas it is between 12 and 15 in the cytosol.

DISCUSSION

The aim of the investigation reported here was to elucidate the effects of O_2 and carbon deprivation on the alcohol dehydrogenase activity of *A. longa* in terms of the presence of different forms of alcohol dehydrogenase and the location of these enzymes within the cell. It is apparent from the results presented above that both NAD- and NADP-dependent alcohol dehydrogenase activities are present in *Astasia* grown on ethanol as the sole carbon source.

Alcohol dehydrogenase(NADP) activity was found in both the cytosol and the particulate fractions. The activity in the two fractions has a similar electrophoretic mobility. However the C/E ratios are distinctly different, respectively 2–3 and 12–15 in the particulate and the cytosol fractions. The C/E ratio has been used often to differentiate between alcohol dehydrogenase isozymes in yeast[5,8,9]. The observation that each compartment possesses an alcohol dehydrogenase(NADP) is further supported by the finding that after O_2 treatment and carbon deprivation the specific activity in the cytosol is increased while that in the particulate fraction is decreased. The C/E ratio characteristic of each fraction was not changed by either treatment.

There are at least two forms of the alcohol dehydrogenase(NAD) in *Astasia*. The electrophoretic patterns of alcohol dehydrogenase activity indicate that in the normal cell, the form which we have termed alcohol dehydrogenase(NAD) I is present mostly in the particulate fraction whereas the form which we called alcohol dehydrogenase(NAD) II is present mainly in the cytosol. However, both forms can be found in each of the two compartments. The role of contamination is difficult to evaluate because the different activities react with the same substrates. Thus the alcohol dehydrogenase(NAD) I activity which is seen on the gels representing the activity of the cytosol fraction of the normal cells may be due to a contamination by the mitochondrial fraction. However, the amount of activity seen on the gels in the alcohol dehydrogenase I region of the cytosol of O_2-treated and starved cells is much too great to be due to contamination. Alcohol dehydrogenase II, the enzyme found in the cytosol of the normal cells, is decreased relative to alcohol dehydrogenase I activity by O_2 and starvation. However, at present there is no evidence that it is directly inhibited by these two treatments.

Thus it seems that normal cells have at least two alcohol dehydrogenase(NAD) activities one which is found associated with the particulate fraction alcohol dehydrogenase(NAD) I and the other which is in the cytosol alcohol dehydrogenase(NAD) II. O_2 treatment and starvation disturb this activity distribution. Cells which are grown in presence of O_2 or which are deprived of ethanol contain an increased amount of alcohol dehydrogenase(NAD) activity. In these cells the electrophoretic patterns show that there is a large increase in activity having the electrophoretic mobility of alcohol dehydrogenase I but located in the cytosol fraction. Thus it appears likely that there is an alcohol dehydrogenase(NAD) in both the cytosol and the particulate fractions having the electrophoretic mobility of alcohol dehydrogenase(NAD) I and that *Astasia* can produce a third NAD-dependent alcohol dehydrogenase under certain conditions. However, as indicated by the activity profiles the effects of O_2

and carbon deprivation are not completely the same. The activity profiles of the cytosol of normal and carbon-deprived cells are similar, but that of the O_2-treated cells differs from both.

Such multiplicity of enzymatic activity is common and isozymes of alcohol dehydrogenase have been described in horse liver[10], Drosophila[11,12] and yeast[5,9,13]. In baker's yeast, there are three different NAD-dependent alcohol dehydrogenase activities, two of these are present in the supernatant and specific metabolic roles have been ascribed to them. The role of the particulate alcohol dehydrogenase of yeast is still obscure[5].

Although both NAD[14]- and NADP[15]-dependent alcohol dehydrogenase activities have been described in *Euglena gracilis*, this is the first time to our knowledge that alcohol dehydrogenase activity is described in *Astasia*. The multiple forms of alcohol dehydrogenase in this organism must play precise roles in the economy of the cells, but the assignment of specific roles for each of the enzymatic activities will have to await the elucidation of the pathways of ethanol metabolism in *Astasia*.

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