Phytochemistry, 1966, Vol. 5, pp. 761 to 765. Pergamon Press Ltd. Printed in England

ALCOHOL DEHYDROGENASE (ALCOHOL:NAD OXIDOREDUCTASE) FROM THE PEA SEEDLING

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Dedicated to Professor HANS BURSTRÖM in honour of his sixtieth birthday

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(*Received* 21 *December* 1965)

Abstract—Alcohol:NAD oxidoreductase (alcohol dehydrogenase, ADH), was partially purified from pea seedlings, and with acetaldehyde gave a Michaelis constant of $4{\cdot}3 \times 10^{-4}$ M. Activity was inhibited by *p*-chloromercuriphenylsulphonic acid, phenylmercuric acetate, *O*-iodosobenzoate, ferron, and other metal-binding agents. Inhibition by thiol reagents was partially prevented by prior incubation with NADH and glutathione. Silver, cadmium and cupric ions are strongly inhibitory; zinc is somewhat inhibitory. Cobaltous, ferrous, and zinc ions counteract the inhibition by *O*-phenanthroline. It is suggested that pea ADH resembles the enzyme from the yeast.

INTRODUCTION

ALCOHOL dehydrogenase (ADH) has been found in various tissues;[1] Stafford and Vennesland obtained a preparation from wheat germ which was inhibited by iodoacetate,[2] as was the preparation from peas.[3] In rice[4] and maize[5] adaptive formation of this enzyme was found. Detailed biochemical studies have been successfully carried out on the alcohol dehydrogenases from yeast and liver,[1] but not with preparations from higher plants. This paper deals with a few properties of ADH from pea seedlings.

RESULTS

General Properties

The activity of the enzyme was found to be proportional to its concentration, and a Lineweaver–Burk plot gave a Michaelis constant (K_m) of $4{\cdot}3 \times 10^{-4}$ M with acetaldehyde as substrate. The optimal range of pH for this enzyme was found to be 7·0 to 7·3, in phosphate buffer.

Inhibition by Thiol Reagents

p-Chloromercuriphenylsulphonic acid (PCMPSA), phenylmercuric acetate (PMA), and *O*-iodosobenzoate all inhibited the enzyme (Table 1). Each was much more inhibitory if preincubated with the enzyme. If on the other hand the enzyme was preincubated with NADH or the substrate before the thiol reagents was added, it was partially protected against inhibition. The enzyme which had been inactivated by preincubation with a thiol reagent

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TABLE 1. PROTECTION BY NADH, ACETALDEHYDE, AND GSH OF THE PEA ADH ACTIVITY AGAINST INHIBITION BY THIOL REAGENTS

Thiol reagent (μM)	Percentage inhibition with following order of addition of reagents:* I,† NADH, A	I, 30 min NADH, A	NADH, 30 min I, A	A, 30 min NADH, I	I, 30 min NADH, A, GSH	I, GSH, 30 min NADH, A
PCMPSA 0·05:	14	66	45	62	60	26
PMA 0·05:	30	66	38	71	57	24
O-iodosobenzoate 500:	20	96	20	71	95	57

Standard assay conditions were used, except of 0·3 ml of NADH solution. Phosphate buffer was used (pH 7·0). Final concentration of GSH was $6·6 \times 10^{-4}$ M. Temp. 25°.

* I, GSH, and A designate inhibitor, reduced glutathione and acetaldehyde respectively. 30 min indicates the time in minutes between addition of reagents.

† Added together in order shown.

could not be reactivated by reduced glutathione (GSH). However, preincubation with GSH afforded some protection against thiol reagents.

Effects of Metal-binding Agents

The strongest inhibitors were 8-hydroxy-7-iodoquinoline-5-sulphonic acid (ferron), potassium thiocyanate, *O*-phenanthroline, sodium azide, sodium sulfide and sodium cyanide (Table 2). This suggests the involvement of a metal component in the pea ADH. Salicylaldoxime, 2,3-dimercaptopropanol (BAL), $\alpha\alpha'$-dipyridyl and sodium diethyldithiocarbamate were less inhibitory, and potassium oxalate and disodium ethylenediamine tetraacetate were

TABLE 2. EFFECT OF METAL-BINDING AGENTS ON THE PEA ADH

Agents	Final concentration (mM)	Inhibition (%) Without preincubation	With preincubation (5 min)
O-Phenanthroline	0·5	53	56
Ferron	0·5	48	84
Thiocyanate	0·5	73	76
2,3-Dimercaptopropanol (BAL)	0·5	31	38
Salicylaldoxime	1·0	44	53
Cyanide	1·0	63	63
Azide	1·0	51	62
Sulfide	1·0	67	69
Ethylenediamine tetraacetate	10·0	0	4
$\alpha\alpha'$-Dipyridyl	1·0	23	29
Oxalate	10·0	0	0
Diethyldithiocarbamate	1·0	8	8

Standard assay conditions were used. Phosphate buffer (pH 7·0). Temp. 23°.

not effective. Preincubation of the enzyme with *O*-phenanthroline, sodium sulfide or potassium thiocyanate resulted in a significant but not very remarkable increase in the inhibitory effect. On the other hand, ferron was much more inhibitory when it was preincubated with the enzyme. The inactivation of pea ADH by ferron can be considerably reduced by preincubation with NADH, but such treatment is less effective with *O*-phenanthroline, BAL, and thiocyanate (Table 3).

TABLE 3. PROTECTION BY NADH OF THE PEA ADH ACTIVITY AGAINST INHIBITION BY FERRON

Agents (mM)	Inhibition (%) I, 30 min NADH, A	Inhibition (%) NADH, 30 min I, A
Ferron 0·5	100	33
O-Phenanthroline 0·5	50	41
BAL 0·5	50	33
Thiocyanate 0·5	73	66

Experimental details were similar to those described for Table 1.

Effects of Inorganic Salts

Table 4 shows that pea ADH is strongly inhibited by silver, cadmium, and cupric ions. It is also sensitive to zinc. Cobaltous, nickel, ferric, magnesium, and calcium ions are far less inhibitory for this enzyme. Disodium molybdate and manganous chloride are slightly stimulatory. Cobaltous, ferrous, or zinc ions more or less reactivate the enzyme which has been inactivated by 10 min of preincubation with *O*-phenanthroline, while cupric, ferric, calcium, manganous, and molybdate ions show no such action (Table 5).

DISCUSSION

The results suggest that pea ADH is probably a metalloprotein. Although the metal may be zinc as is the case with ADH from both yeast and liver because the present enzyme is inhibited by thiocyanate, cyanide, and *O*-phenanthroline,[1, 6] this requires further investigation. The fact that the enzyme has a thiol group has already been demonstrated,[3] and confirmed by the present data (Table 1). The protective action of NADH against either a thiol reagent or ferron may suggest a certain intimate relation of the NADH with the thiol group and/or the metal component in the enzyme.

The alcohol: NADP oxidoreductase from *Leuconostoc mesenteroides*, is very sensitive to inorganic salts[7] and therefore is quite different from yeast ADH. The latter enzyme is more strongly inhibited if preincubated with a certain chelating agent,[6, 8] but the pea enzyme is

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[7] R. D. DEMOSS, *J. Bacteriol.* **68**, 252 (1954).
[8] B. L. VALLEE and F. L. HOCH, *Proc. Natl Acad. Sci.* **41**, 327 (1955).

TABLE 4. EFFECTS OF INORGANIC SALTS

Compounds	Final concentration (mM)	Inhibition (%) 5 min preincubation
$AgNO_3$	0·01	100
$CdSO_4$	0·1	100
$CuCl_2$	0·01	100
$ZnSO_4$	1·0	100
$ZnSO_4$	0·1	90
$ZnSO_4$	0·01	33
$CoCl_2$	1·0	24
$NiCl_2$	1·0	45
$MnCl_2$	1·0	+6*
$FeCl_3$	0·1	23
$MgCl_2$	1·0	0
$CaCl_2$	10·0	10
$CaCl_2$	1·0	0
NaCl	10·0	0
Na_2MoO_4	1·0	+7*
NaF	10·0	0

* Stimulation.

Standard assay conditions were used. Acetate buffer solution (0·2 M, pH 7·2) was used. Temperature was 27°.

TABLE 5. REACTIVATING EFFECT OF METALLIC IONS ON THE *O*-PHENANTHROLINE-INHIBITED PEA ADH

Addition	Decrease of optical density at 340 mμ in 2 min
None	0·084
$FeSO_4$	0·111
$FeCl_3$	0·088
$ZnSO_4$	0·112
$CuCl_2$	0·020
$CaCl_2$	0·078
$MnCl_2$	0·097
$CoCl_2$	0·113
Na_2MoO_4	0·085
No inhibitor and no metal salts (control)	0·147

Standard assay conditions were used, except for the following conditions; enzyme was preincubated (10 min) with *O*-phenanthroline at room temperature before the addition of metallic salts. Final concentration; *O*-phenanthroline was 10^{-3} M, metallic salts were $3{\cdot}3 \times 10^{-4}$ M. Acetate buffer solution (0·2 M, pH 7·2) was used. Temp. 28°.

little effected by preincubation with *O*-phenanthroline, thiocyanate or sulfide; ferron on the other hand exerted a stronger inhibitory effect when preincubated with the enzyme. The dehydrogenases from both yeast and pea seedling are inhibited by silver, cupric, ferric and zinc ions. The enzyme from *L. mesenteroides* is different for it is stimulated by the chlorides

of calcium, magnesium, copper and sodium while the other enzymes are not. It may be concluded, therefore, that the ADH from pea seedlings resembles that from yeast more closely than that from *L. mesenteroides*.

EXPERIMENTAL

Enzyme Preparation

The peas (*Pisum sativum*, Alaska) were placed under running tap water for ~30 hr[9] at 17°. The "seedlings" produced (130 g) were ground for 5 min in a Waring blender with 260 g of ice-cold, 0·1 M Na_2HPO_4. The macerate, squeezed through two layers of cotton cloth, was centrifuged for 30 min at 4500 revs/min and the sediment discarded (Fraction 1, 280 units/mg protein). To each 100 ml of the supernatant was added 2·04 ml of 1 M manganous chloride solution to produce, after standing overnight, a heavy precipitate which was centrifuged off[10] (Fraction 2, 480 units/mg protein). To each 100 ml of the supernatant was added 19·6 g of ammonium sulphate to make it a 0·33% saturated solution. Two to three hours' standing produced a sediment which was discarded (Fraction 3, 440 units/mg protein). The supernatant was treated again with solid ammonium sulphate to make 0·6% saturated solution. After 3 hr the sediment was collected by centrifugation and was dissolved in 30 ml of 0·1 M phosphate buffer solution (pH = 7·0), dialysed twice against each 6 l. of $1·7 \times 10^{-4}$ M phosphate buffer solution of pH, 7·0, and was then free from sulphate and was used in the following experiments (Fraction 4, 0·65 mg protein/ml, 430 units/mg protein). These procedures were followed at temperatures of 0–4°.

Activity

Each reaction mixture contained 2·0 ml of 0·1 M phosphate or acetate buffer solution, 0·2 ml of 0·1% NADH solution, 0·1 ml of enzyme and 0·1 ml of 0·1 M acetaldehyde solution. Either with or without inhibitor the mixture was finally made up to 3·0 ml. A unit activity was defined as that causing 0·01 decrease of absorptivity at 340 mμ in the first minute. A Hitachi EPU type spectrophotometer was used at room temperature.

The enzyme protein was determined by the method of Kalckar.[11]

Chemicals

Disodium salt of NADH (98 ~ 100 per cent in purity), reduced glutathione, and PCMPSA were obtained from the Sigma Chemical Co.; *O*-iodosobenzoate and BAL were from the Nutritional Biochemicals Corps. Acetaldehyde was redistilled before use. This and other chemicals were obtained from commercial sources.

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