CHARACTERIZATION OF ALCOHOL DEHYDROGENASE ISOLATED FROM GERMINATING BEAN (*Vicia faba*) SEEDS

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We have isolated alcohol dehydrogenase (ADH, E.C. 1.1.1.1) from beans germinating 3 days by ammonium sulfate precipitation of the sodium phosphate extract of the homogenate of seeds, followed by chromatography on DEAE-cellulose and gel chromatography on Sephadex G-200 and G-100; both chromatographic operations were repeated twice. The activity of ADH increased 960 times after these procedures. The enzyme whose M_r is 60 000 consists of two identical subunits of $M_r = 30\,000$. Allyl alcohol is oxidized and acetaldehyde reduced at the highest rate of all the alcohols and aldehydes tested. The reaction rate decreases with the increasing length of the carbon chain of the substrate. In contrast, the rate of oxidation of alcohols with a double bond in their molecules increased. The pH-optimum of substrate oxidation (pH 8·5) is different from the pH-optimum of substrate reduction (pH 7·5). From kinetic studies of the effect of pH on V_{max} and K_M the pK-value of amino acids participating on substrate oxidation is 9·2 and 8·5 whereas amino acids with pK 8·3 and 6·8 participate on substrate reduction. We have verified the participation of the SH-groups in experiments with the incativation of ADH by iodoacetate: the inactivation was weaker after the enzyme had been preincubated with NAD or AMP, adenosine or nicotinamide. Likewise pyridoxal phosphate inactivates bean ADH by modifying its ε-amino group of lysine. The degree of inactivation depends also on the pH and the ionic strength of the medium. The protective effect of NAD or its analogs shows that lysine is present in the active center of the enzyme in the coenzyme-binding site.

The germinating seed obtains energy during the first days of germination preceding the rupture of the testa by the degradation of substrates without the participation of oxygen. We have observed that adenosine triphosphate is supplied predominantly as a product of alcoholic fermentation. The terminal step of this degradation of sugars is catalyzed by alcohol dehydrogenase which is present in a series of germinating seeds[1-3]. This study reports on the isolation and characterization of alcohol dehydrogenase from germinating bean (*Vicia faba*) seeds.

EXPERIMENTAL

Isolation of enzyme: ADH was isolated by a procedure involving *a*) homogenization of bean seeds (of commercial Lebanese origin), which had been germinating for three days, with a double volume of 0·2M sodium phosphate buffer at pH 8·5 and containing 10 mM 2-mercaptoethanol,

b) saturation of the supernatant with ammonium sulfate to 35–60%, *c*) chromatography on DEAE-cellulose in 25 mM Tris-acetate buffer at pH 7·0 containing 10 mM 2-mercaptoethanol (elution gradient of 0–3M sodium chloride), *d*) gel filtration over Sephadex G-200, *e*) rechromatography on a column of DEAE-cellulose (using the same buffer and elution gradient as for the first chromatography), *f*) gel filtration over Sephadex G-100, and *g*) rechromatography on DEAE-cellulose (a 1 × 20 cm column instead of the 2·5 × 30 cm column used in the preceding step). All isolation procedures were carried out in a cold room.

Determination of M_r and ADH *subunits*: Gel filtration over a 1·5 × 20 cm column of Sephadex G-100 was used. The elution of 5 mg of sample and of standards (lysozyme, M_r = 17 500, ovalbumin, M_r = 54 000, and serum albumin, M_r = 68 000) was effected by 10 mM Tris-acetate buffer at pH 7·0 containing 10 mM 2-mercaptoethanol.

Preparation of ADH *subunits*: ADH was dissolved in 5 mM Tris-acetate buffer at pH 7·0 containing 20 mM 2-mercaptoethanol and 1% sodium dodecyl sulfate and the solution was heated 3 min at 100°C (ref[4]).

Assay of ADH *activity*: The activity was determined in terms of increase in absorbance at 340 nm in Specord UV-VIS Spectrophotometer (Zeiss, Jena) as described earlier[5].

Polyacrylamide gel electrophoresis was carried out in 7·5% gel according to Sluster[6] yet ommitting the large-pore gel. The proteins were detected by staining with a 1% solution of Amido Black 10B in 7% acetic acid. The gels were destained in 7% acetic acid. ADH activity in the gels was detected by incubation of the gels in a mixture containing 1·5 ml of tetrazolium salt (1 mg/ml), 0·15 ml of phenazine methosulfate (1 mg/ml), 5 mg of NAD added as solid substance, 2 ml of 0·2M glycine–NaOH buffer at pH 9·5, and 0·2 ml of 96% ethanol. The Michaelis constants were determined by the method of Lineweaver and Burke[7].

Modification by pyridoxal-5'-phosphate: The lysine residues of ADH were modified by the procedure of McKinley and Morris[8] using the technique described in our previous paper[5]. The enzyme was mixed with the corresponding volume of the modifying reagent and the mixture was incubated at pH 8·5 in a phosphate buffer at 25°C in a thermostat. Aliquots were withdrawn at intervals and assayed for ADH activity.

Inactivation by iodoacetate: The inactivation mixture contained ADH, phosphate buffer at pH 7·5 and iodoacetate at the corresponding concentration. An aliquot of the mixture was pipetted from 2 ml of the mixture after 20-min incubation at 20°C for assay of ADH activity.

RESULTS

Isolation of Bean ADH *and Structure of Enzyme*

Using the procedure described above involving precipitation of ADH from the phosphate extract of germinating seeds with ammonium sulfate, chromatography and rechromatography on DEAE-cellulose, twice repeated gel filtration over Sephadex, and chromatography on DEAE-cellulose, we obtained an ADH preparation whose activity was increased 960 times (Fig. 1). The electrophoerogram of this preparation showed the presence of one protein band with detectable ADH activity and another weak band located closer to the anode; no ADH activity could be detected in the latter. From the results of the gel filtration experiments the relative

molecular weight of bean ADH is 60 000 ± 5 000. The protein is composed of two identical subunits of M_r = 30 000. The effluent from the Sephadex column contains the dimer in addition to the monomer (Fig. 2); both proteins are lacking alcohol dehydrogenase activity.

Kinetic Studies on Bean ADH

We have examined the substrate specificity of our preparation with 5 alcohols and 4 aldehydes; the decreased solubility of homologs with a longer chain did not permit us to assay ADH activity. The relative rates of oxidation of the alcohols show (Table I) that a double bond in the molecule of the alcohol increases the affinity of the enzyme for such substrates whereas this affinity decreases with the increasing length of the substrate chain. Benzyl alcohol is oxidized by bean ADH. The situation with the aldehydes is different from the alcohols: with the increasing length of the chain the affinity for the enzyme decreases and the same effect has the introduction of a double bond into the molecule of the aldehyde. The best substrate of aldehydes is acetaldehyde, of alcohols it is allyl alcohol.

Effect of pH *on* V_{max} *and* K_M

We have determined the values of the Michaelis constants with 4 alcohols and 3 aldehydes as substrates over the range pH 6·0–10·0. As shown in Table II the pH-value is without effect on the order of the K_M-value (for aldehydes it is 10^{-3} mol l^{-1}, for alcohols 10^{-2} mol l^{-1}). The value of K_M is the lowest at the pH-optimum of the reaction which is 7·5 for the reduction of aldehydes and 8·5 for the oxidation of

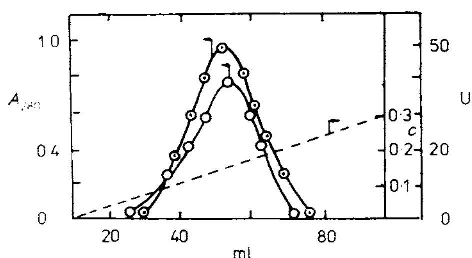

FIG. 1

Rechromatography of bean ADH on column of DEAE-cellulose. x-axis: elution volume in ml; y-axis left: absorbance at 280 nm; y-axis right: units of ADH activity, inset: NaCl concentration in mol l^{-1}

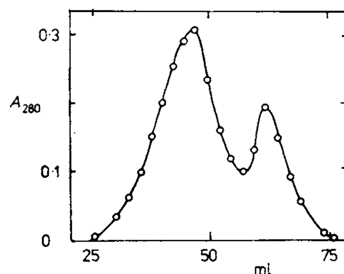

FIG. 2

Gel filtration of bean ADH preincubated with 1% sodium dodecyl sulfate in methanol, 3 min at 100°C. x-axis: elution volume in ml, y-axis: absorbance at 280 nm

alcohols. The K_M values for NAD and NADH are 10^{-4} and 10^{-5} mol l^{-1}, respectively (Table III).

Active Center of Bean ADH

The effect of pH on V_{max} (not shown in the Table) demonstrates that ionisable groups of amino acids participate on the catalysis. For the oxidation of alcohols amino acids with $pK = 8·5$ and $pK = 9·2$ are essential, most likely the SH-group of cysteine and the NH_2-group of lysine or the OH-group of tyrosine, respectively. Amino acids

TABLE I
Relative rate of oxidation of acohols and of reduction of aldehydes by bean ADH

Substrate	Relative conversion rate, %
Ethanol	100
Propanol	68
Isobutanol	40
Allyl alcohol	130
Benzyl alcohol	30
Acetaldehyde	100
Propionaldehyde	46
Butyraldehyde	15
Crotonaldehyde	0

TABLE II
Dependence of K_M-values (in $mmol^{-1}$) for bean ADH on pH

pH	Ethanol	Allyl alcohol	Propyl alcohol	Isobutyl alcohol	Acet-aldehyde	Propion-aldehyde	Butyr-aldehyde
6·0	51	72		—	3·2	6·0	—
6·3	45	65	85	—	2·5	5·0	—
6·5	39	45	78	—	2·0	4·5	0·7
7·0	30	37	65	90	1·8	3·8	0·7
7·5	25	28	50	76	1·5	3·5	0·8
7·9	20	20	45	65	1·8	4·2	0·7
8·5	15	17	32	55	2·4	5·0	0·5
9·5	22	23	45	62	3·8	6·0	—
10·0	33	38	65	80	5·0	—	—

TABLE III

Dependence of K_M (in mmol l^{-1} . 10^{-2}) for bean ADH coenzyme on pH

pH	NAD	NADH
6·0	42	3·7
6·3	36	3·2
6·5	33	2·8
7·0	27	2·2
7·5	23	2·0
7·9	19	2·4
8·5	15	2·8
9·5	25	4·2
10·0	32	5·8

TABLE IV

Inactivation of bean ADH by iodoacetate as function of length of incubation period and concentration of sodium phosphate buffer at pH 7·5. The numbers in the table indicate % of ADH activity

Buffer concentration mol l^{-1}	Length of incubation period, min		
	5	10	20
1	77	70	63
0·1	69	62	56
0·05	49	44	37
0·01	30	30	22

TABLE V

Inactivation of bean ADH by pyridoxal-5-phosphate as function of time and buffer concentration. The numbers in the table indicate % of ADH activity

Concentration of phosphate buffer, mol l^{-1} (pH 8)	Length of incubation period, min		
	5	10	20
1	70	65	60
0·1	60	55	52
0·05	45	35	32
0·01	35	26	20

with pK = 6·8 and pK = 8·3 (Figs 3*a* and 4*a*) participate in the reduction of aldehydes. These are probably the imidazole ring of histidine and again the SH-group of cysteine. In analogy, amino acids with pK = 8·2 and pK = 9·7 play a role in catalysis with the participation of NAD and amino acids with pK = 6·8 and pK = = 8·2 when NADH is involved (Figs 3*b* and 4*b*). Values analogous to those shown in Figs 3 and 4 were obtained when log V_{max} was plotted against pH.

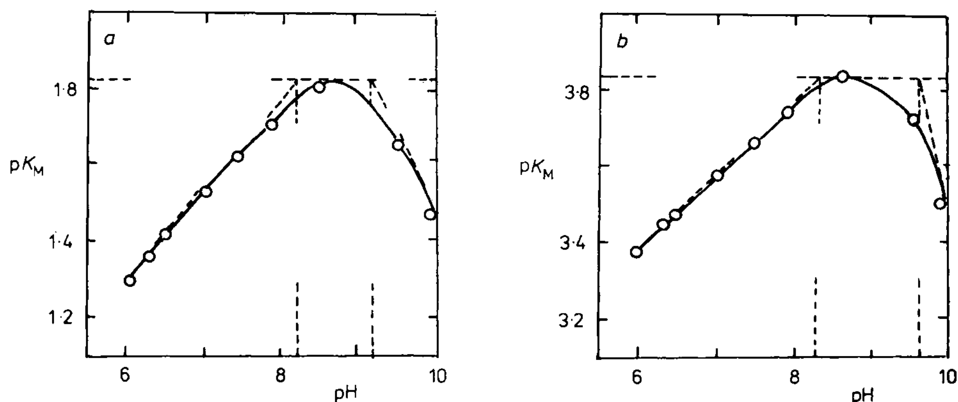

FIG. 3

Effect of pH on reaction catalyzed by bean ADH. Dependence of K_M on pH *a* for ethanol, *b* for NAD. *x*-axis: pH-value, *y*-axis log K_M for ethanol (*a*) and NAD (*b*)

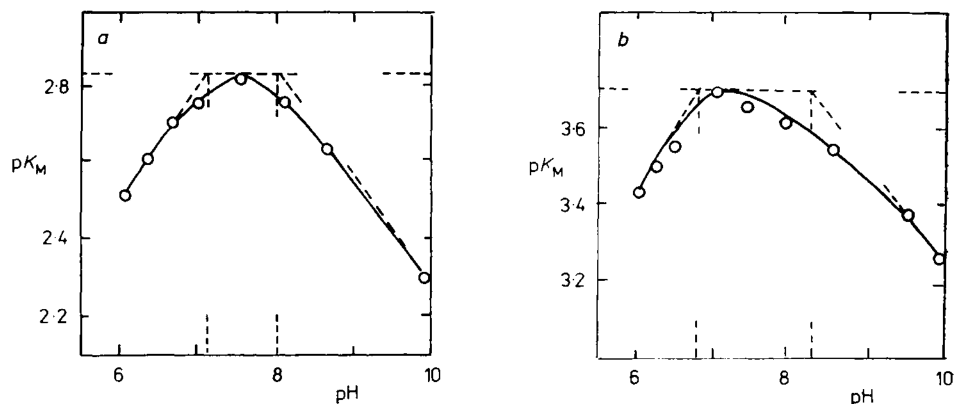

FIG. 4

Effect of pH on reaction catalyzed by bean ADH. Dependence of K_M for acetaldehyde (*a*) and NADH (*b*) on pH. *x*-axis: pH-value, *y*-axis: log K_M for acetaldehyde (*a*) and NADH (*b*)

The alkylation of bean ADH by iodoacetate results in the inactivation of the enzyme, depending on the length of the preincubation period (Table IV). Maximal inactivation is achieved at pH 7·6. NAD protects bean ADH against alkylation by iodoacetate. The protection is competitive with respect to iodoacetate (Fig. 5). ADH is likewise protected against inactivation by AMP and nicotinamide whereas adenosine with respect to NAD, protects bean ADH against inactivation via mixed-type inhibition. Ethanol has no protective effect. Pyridoxal phosphate inhibits bean ADH in a reaction which is maximal after 20 min of action. The inactivation is pH-dependent (maximum at pH 8·5) and is affected by the buffer concentration (Table V). AMP protects strictly competitively ADH against pyridoxal phosphate. Another competitive inhibitor is also NAD (Fig. 6) whereas adenosine inhibits via the mixed type and nicotinamide via the noncompetitive type of inhibition, resp.

DISCUSSION

Bean ADH. which we have been able to purify to homogeneity by gel filtration, has a M_r of 60 000 and consists of two identical subunits of M_r = 30 000 each. It thus resembles a number of other alcohol dehydrogenases from plants[3,9,10] even though there exist ADH's isolated from seeds whose M_r is higher (cf., e.g. ADH from peanut

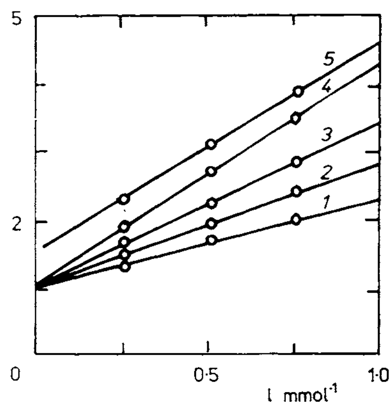

FIG. 5

Double reciprocal plot of dependence of % of bean ADH inactivation on iodoacetate concentration. x-axis: [iodoacetate]$^{-1}$, l mmol^{-1}; y-axis: i(= 100/inactivation in %). *1* In absence of ligand, *2* + 10 mM nicotinamide, *3* + 12 mM NAD, *4* + 10 mM AMP, *5* + 12 mM adenosine

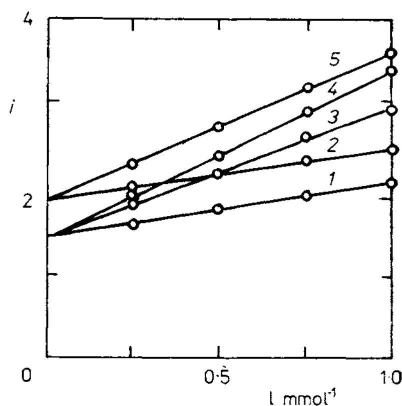

FIG. 6

Double reciprocal plot of dependence of % of bean ADH inactivation on pyridoxal phosphate concentration. x-axis: [pyridoxal phosphate]$^{-1}$, l mmol^{-1}; y-axis: i(= 100/inactivation in %). *1* In absence of ligand, *2* + 10 mM nicotinamide, *3* + 12 mM NAD, *4* + 10 mM AMP, *5* + 12 mM adenosine

seeds with a M_r of 110 000, from tea seeds of M_r = 150 000 (refs[11–13])). Very little is so far known about the subunits of plant ADH's; the only information available is on the enzyme isolated from tea and *Cicer arietinum* which are both dimers composed of identical subunits[14,15]. We observed the presence of both the monomer and of the dimer in the effluent from the Sephadex column. We have not been able so far to determine whether association of subunits took place during the elution or whether the conditions of cleavage were not drastic enough. Bean ADH is more effective in reducing aldehydes than in oxidizing alcohols; the K_M-values for these two substrate types are different. Bean ADH resembles in its specificity other enzymes from plant seeds, the differences being rather quantitative than qualitative[3,10,13]. Likewise the differences in pH-optima for both reduction and oxidation of substrates are frequently observed[3] with plant ADH's. These differences suggest that different amino acid residues are involved in the catalytical process. We observed that the SH-groups of cysteine are important for both reduction and oxidation of substrate and that lysine is essential for its oxidation and histidine for its reduction. From our negative results with N-acetylimidazole we do not consider the participation of tyrosine.

Bean ADH is inhibited by pyridoxal phosphate, the degree of inhibition being the highest after 20 min; longer periods do not lead to a deeper inhibition. The action is reversible: pyridoxal phosphate can be removed by dialysis against phosphate buffer at pH 8·5. The existence of a bond between the 6-amino group of lysine and pyridoxal phosphate was demonstrated by measurement of the absorbance at 326 nm of the ADH-pyridoxal phosphate complex after reduction by sodium borohydride. To examine the role of lysine in the active center of bean ADH the degree of inhibition by pyridoxal phosphate was investigated by preincubation of the enzyme with NAD or its analogs, with adenosine, AMP, and nicotinamide. The preincubation of ADH with AMP, which is competitive with respect to NAD (ref.[16]), prevents the enzyme from inactivation by pyridoxal phosphate; likewise adenosine binds to the ADH protein binding site for NAD yet the protective effect of adenosine against pyridoxal phosphate is of the noncompetitve inhibition type; hence the formation of a ternary ADH–adenosine–pyridoxal phosphate complex may be considered. Since AMP is then more efficient in preventing ADH from inhibition by pyridoxal phosphate than NAD (both inhibitors are competitive) and nicotinamide is a noncompetitive inhibitor, a bond between the adenosinediphosphoribosyl moiety of NAD and the protein has been postulated with the participation of the lysine residue. Cysteine is also present in the active center of bean ADH. The SH-group of cysteine also participates in the binding of NAD to the ADH protein since NAD is a competitive inhibitor with respect to iodoacetate whereas the second substrate of the reaction, ethanol, shows no protective effect against modifiers.

We wish to extent the present scope of our information on the active center of bean ADH to include other data which should enable us not only to obtain charac-

teristics of an important enzyme of higher plants but also to determine whether the bean enzyme resembles other well investigated ADH's, such as horse liver and yeast ADH (refs[17,18]). The basic kinetic data (size of K_M-values, substrate specificity, effect of different inhibitors) have been discussed in our preceding study[3]. Bean ADH resembles from the viewpoint of its function more yeast ADH which, however, is a tetramer of higher M_r than that of the animal dimer enzyme.

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