PEA ALCOHOL DEHYDROGENASE: SUBSTRATE SPECIFICITY AND BINDING OF COENZYME TO ENZYME

Marie Stiborová, Roman Lapka, Noemi Nováková and Sylva Leblová

Department of Biochemistry,
Charles University, 128 40 Prague 2

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Pea alcohol dehydrogenase (EC 1.1.1.1) shows a broad specificity with respect to aldehydes and alcohols. The pH-optimum of substrate oxidation is 8·7 and of substrate reduction 7·0. The enzyme is inhibited by ATP, adenosine, and adenine. The inhibition is competitive with respect to NAD. The inhibition by ATP is pH-dependent. The competitive character of the inhibition by adenine and its derivatives with respect to NAD indicates the importance of the adenine moiety of the coenzyme for its binding to the enzyme. Phenanthroline is a competitive inhibitor with respect to NAD, a mixed inhibitor with respect to ethanol and a noncompetitive inhibitor with respect to acetaldehyde. Experiments carried out simultaneously with ATP and phenanthroline show that the adenine moiety of NAD does not bind *via* the zinc atom to the enzyme protein.

Pea alcohol dehydrogenase is an enzyme catalyzing a redox reaction, namely acetaldehyde reduction to ethanol and *vice versa*. The substrate specificity of the enzyme investigated in terms of determination of relative rates of substrate conversion is relatively broad[1-6]. Pea alcohol dehydrogenase is a NAD-dependent enzyme; the K_m of the reaction with NADP is by two orders higher[6]. The kinetics of the reaction follows the mechanism of Theorell and Chance[7]: the first step is the binding of the coenzyme to the protein, the last step the dissociation of the binary complex and the liberation of the coenzyme[8].

Pyrazole, pyridine, and imidazole[5], berberine and its derivatives, fatty acids and chloride ions[9] are efficient inhibitors of pea ADH. Inhibition studies show the hydrophobicity of the substrate-binding site and indicate that the alcohol- and acetaldehyde--binding sites are not identical[10].

This study extends the present state of our knowledge of pea ADH: the K_m-values of oxidation of alcohols and of reduction of aldehydes have been determined and the mode of binding of the coenzyme to the enzyme elucidated in inhibition studies.

EXPERIMENTAL

The enzyme (ADH) *was isolated* from pea *Pisum arvense* L., cv. Raman-Elita as described in our preceding paper[4]. One activity unit was defined according to Racker[11]. The inhibition constant K_i and the dissociation constant of the enzyme–inhibitor complex were determined by the method of Dixon[12].

If the enzyme (E) *is allowed to react with two inhibitors* (I_1 and I_2), complexes EI_1, EI_2, and eventually EI_1I_2 can be formed. The interaction between the inhibitors in the enzyme–inhibitor complex is reflected by the value of the interaction constant α. This constant equals infinity if I_1 and I_2 react with equal sites of the enzyme. Complex EI_1I_2 is not formed. If inhibitors I_1 and I_2 react with different sites of the enzyme, α is lower than infinity and higher than zero, and complex EI_1I_2 is formed. The inhibitors can in this case react with one another: if α is lower than one, the inhibitors are attracted to one another, if α is higher than one the inhibitors are repulsed, if α equals one the inhibitors do not interact in complex EI_1I_2. The interaction constants α were determined graphically according to Yonetani and Theorell[13].

RESULTS AND DISCUSSION

Substrate Specificity

As shown in Table I, pea ADH oxidizes other alcohols besides ethanol: the K_m-value increases with the increasing chain length but all unsaturated analogs are oxidized faster than saturated ones (with the exception of propargyl alcohol which is not oxidized at all). The best substrate of the pea enzyme is allyl alcohol. Methyl branching strongly increases the K_m-value; because of their poor solubility we could not determine the Michaelis constants with other substrates. Secondary alcohols, diols, alcohols of terpenic, sugar, cyclic, and aromatic character are not substrates for pea ADH.

TABLE I

Substrate Specificity of ADH

Experimental conditions: 0·1 M phosphate buffer, pH 8·7 or 7·0; [NAD, NADH] = 0·5 mM [aldehyde] = 1·6—10 mM; [alcohol] = 50—500 mM; volume 1 ml.

Substrate	K_m, mM			
	pea ADH	liver ADH[a]		yeast ADH[a]
		human	equine	
Ethanol	26	0·40	0·76	17·8
Propanol	49	0·10	0·27	5·7
Butanol	150	0·14	0·25	11·6
Hexanol	600	0·06	0·095	0·38
2-Propen-1-ol	10	0·05	0·12	6·5
Isobutyl alcohol	550	—	—	—
Acetaldehyde	4·0	0·53	0·23	0·32
Propanal	4·7	0·18	0·13	1·7
Butanal	4·8	0·04	0·025	0·7
Isobutanal	14·0	—	—	—

[a] Ref.[22].

As regards similarities in substrate specificity of the two other alcohol dehydrogenases, *i.e.* liver (LADH) or yeast (YADH) alcohol dehydrogenase, the pea enzyme resembles in its K_m-value more YADH than LADH; unlike the pea enzyme the latter also oxidizes alcohols with chains longer than that of ethanol at a higher rate (the length of chain does not play an unambiguous role in oxidations catalyzed by YADH). All three types of enzymes oxidize unsaturated analogs faster than saturated analogs. The measurement of relative rates shows that cyclohexanol is not a substrate of either pea ADH (or LADH) but it is a substrate for YADH. Unlike the pea enzyme LADH oxidizes diols and cyclic and aromatic alcohols[10].

With pea ADH, the reduction of the first three members of aldehydes is characterized by slowly increasing K_m-values. The higher K_m-value obtained with isobutyl aldehyde and the fact that pivalaldehyde is not reduced is in agreement with the data on LADH. The plant enzyme shows an affinity for aldehydes with unbranched chain. Pea ADH resembles in K_m-value YADH rather than LADH. The plant enzyme catalyzes considerably more the reduction of aldehydes than the oxidation of alcohols. The pH-optimum of reduction and oxidation is 7 and 8·7, respectively[14].

Effect of Adenine and Its Derivatives

It has been known that pea ADH is inhibited by ATP; the inhibition is competitive with respect to NAD. The pH-dependence of the inhibition of pea ADH is shown in Table II. Inhibition constants K_i decrease with increasing pH. For the strength of the bond between ATP and the protein moiety of alcohol dehydrogenase are important phosphate groups. The phosphate groups of ATP bearing a negative charge are obviously not in solvate form contrary to NAD which has a positive charge and whose molecule undergoes solvation in alkaline media: this is in accordance with the observed increase of the Michaelis constant for NAD above pH 9 (ref.[8]). The increase of pH causes a decrease of the inhibition constant of the binary complex pea ADH–ATP and by an increase of K_m for NAD. ADP and AMP have a similar effect on rape ADH as ATP; these compounds were therefore not tested with the pea enzyme. The K_i-values for pea and liver ADH are similar; it may be therefore, like in the case of liver ADH, postulated that ATP competes with the binding site of pea ADH for NAD, *i.e.* the site where adenosine diphosphoribose is attached.

The effect of ATP is obviously important also in the regulatory action of the enzyme *in vivo*. A role in this regulation by ATP may also play the pH of the cell which is not constant during anaerobiosis: the first product of the anaerobic mechanism, which appears in plant cell is lactate; this acid makes pH in the cell drop to 6·5. The conversion of pyruvate to lactate is clared at this pH and lactic fermentation is transformed to alcoholic fermentation because the decrease of pH activates pyruvate decarboxylase which catalyzes the conversion of pyruvate into acetaldehyde. This acetaldehyde

acts as an acceptor of the reduction equivalents and thus ethanol is formed: the pH of the plant cell increases to 7 during alcoholic fermentation[15,16].

Whereas the binding of ATP to the enzyme is pH-dependent, adenine and adenosine (Table II) bind independently of the pH of the medium. The competitive character of the inhibition with respect to NAD suggests binding of the adenine moiety of NAD to the protein, obviously to its hydrophobic domain[15,17]. Adenosine is bound a little more firmly than adenine, as follows from the values of the inhibition constants; this finding shows that the binding could be affected by the furanose ring of ribose, as observed with the liver enzyme[16,18].

Inhibition by o-Phenanthroline

Phenanthroline is a known chelating agent. Inhibition studies[4] with pea ADH have shown that the enzyme contains a zinc atom in its molecule[3]. We demonstrated that

TABLE II

Inhibition by Adenine and Its Derivatives

Experimental conditions: 0·1M phosphate buffer, pH 8·5; [NAD] = 0·1—0·78 mM; [ethanol] = = 100 mM; [adenine, adenosine] = 0—4 mM; [ATP] = 0—10 mM; volume of reaction mixture 1 ml.

Inhibitor	K_i, mM		
	pH 7·5	pH 8·5	pH 10·4
Adenine	4·8	5·0	5·0
Adenosine	3·9	4·0	4·0
ATP	12·2	7·5	6·2

TABLE III

Inhibition by *o*-Phenanthroline

Experimental conditions: 0·1M phosphate buffer, pH 7·5 and 8·5; [NAD] = 0·2—0·78 mM; [NADH] = 0·15—0·063 mM; [ethanol] = 20—100 mM; [acetaldehyde] = 1—10 mM; [*o*-phenanthroline] = 1—4 mM; volume of reaction mixture 1 ml.

Substrate	Inhibition type	K_i, mM
Ethanol	mixed	1·35
Acetaldehyde	noncompetitive	1·5
NAD	competitive	0·95
NADH	competitive	1·1

phenanthroline is an inhibitor of pea ADH; the values of the inhibition constants as well as the inhibition type are shown in Table III. Since phenanthroline is a competitive inhibitor with respect to NAD we may postulate that the metal atom participates on the binding of the coenzyme to the enzyme. The mechanism of this binding has not been elucidated even with the widely studied liver enzyme. It has been hypothesized that the binding may involve the adenine moiety or, by contrast, the nicotinamide moiety[19,20] or alternatively phosphate residues. Since this type of inhibition is mixed with respect to ethanol, the binding site for ethanol could be localized either close to the metal binding site or there could be an interaction between the substrate and the zinc atom during the reaction. The inhibition with respect to acetaldehyde is noncompetitive. As stated in our previous study on rape ADH, the behavior of the plant enzyme, as regards the type of inhibition of the coenzyme by phenanthroline, is similar to the behavior of the enzyme from liver and yeast but the type of inhibition with respect to substrates is different[21–24].

Inhibition by Phenanthroline and ATP

We studied the simultaneous effect of ATP and phenanthroline on pea ADH and observed that interaction constant α equals 1·8; hence, the two inhibitors bind to different sites of the enzyme. Inhibition studies carried out with ATP, adenosine, and adenine as competitive inhibitors of NAD show that it is probably the adenine moiety which plays a role in the binding of the coenzyme to the enzyme. The experiments made simultaneously with ATP and phenanthroline permit us to eliminate the possibility that the adenine moiety is bound *via* the metal atom.

Since on the other hand phenanthroline is a competitive inhibitor of NAD we must admit that the coenzyme is also bound *via* the zinc atom. The results presented here permit us to conclude that NAD, the coenzyme of pea ADH, is attached to the protein at two sites: *via* the adenine moiety to the protein without the participation of the zinc atom, and *via* an other part of the molecule of NAD to metal atom present in molecule of pea ADH.

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