EFFECT OF HETEROCYCLIC COMPOUNDS AND 2-MERCAPTOETHANOL ON RAPE ALCOHOL DEHYDROGENASE

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Rape alcohol dehydrogenase (ADH) is inhibited by heterocyclic compounds with a five- to six-membered ring (imidazole, pyrazole, 4-methylpyrazole, 3-methylpyrazole, pyridine, nicotine amide) and by *o*-phenanthroline, a heterocyclic, polycyclic compound. Pyrazole and its derivatives, imidazole and pyridine, are competitive inhibitors with respect to ethanol. Nicotine amide and *o*-phenanthroline behave as mixed inhibitors (competitive — noncompetitive) with respect to the substrate. The addition of Zn^{2+}-ions to the reaction medium interferes with the competition by *o*-phenanthroline. 4-Methylpyrazole and pyrazole are the strongest inhibitors of rape ADH. 2-Mercaptoethanol is an inhibitor of rape ADH which competes with ethanol and the coenzyme. The reversible inhibition by mercaptoethanol changes into the irreversible inactivation of the enzyme. The binding of inhibitors to the zinc atom present in the molecule of rape ADH and the localization of the metal in the enzyme protein have been studied.

Alcohol dehydrogenase (ADH, EC 1.1.1.1) is an enzyme of wide ditribution both in animal and plant tissues. Even though animal ADH is an enzyme whose characteristics are well known[1] far less data are available on ADH's from plants. The role of ADH in germinating seeds of higher plants has been elucidated. The enzyme reoxidizes NADH formed under anaerobic conditions during the early stages of seed germination[2]. The characteristics and the structure of ADH from higher plants have been reported in our previous studies[3-7]. This paper is another contribution to the knowledge of the active center of ADH isolated from rape seeds. Inhibitors binding to the active center of the enzyme have been employed as a tool in these studies.

EXPERIMENTAL

Plant material and chemicals. ADH was isolated from the seeds of rape (*Brassica napus* L., var. Třebíčská). Tris-(hydroxymethyl)-aminomethane and pyrazole were from Merck, AG, Darmstadt, F.R.G.; 2-mercaptoethanol, NAD^+, and nicotine amide from Koch-Light Laboratories Ltd., Colnbrook, England; imidazole, 3-methylpyrazole, and 4-methylpyrazole from Sigma Chemical Corporation, St. Louis, MO, U.S.A. The remaining chemicals were purchased from Lachema, Brno, Czechoslovakia and were of analytical purity grade.

The isolation of rape ADH *and the assay of its enzymatic activity* have been described in our previous paper[7,8]. ADH activity was assayed in a Specord UV-VIS spectrophotometer (Carl Zeiss, Jena, G.D.R.). The specific activity of isolated rape ADH was 4·3 μmol NAD^+ min^{-1} . . mg^{-1}.

Determination of inhibition constants. The inhibition constant is considered the dissociation constant of the enzyme–inhibitor complex. It was determined by the method of Lineweaver and Burk[9] and by the method of Dixon[10].

The interaction constants were assayed graphically according to Yonetani and Theorell[11]. The measurements were made in 2 ml of a reaction medium containing 0·5 mmol l^{-1} NAD^+, 0·1 mol l^{-1} Na-phosphate buffer, pH 8·5, 50 mmol l^{-1} ethanol, 1 μmol l^{-1} ADH, and always two different inhibitors; the concentration of one of the latter was kept constant whereas the concentration of the other one was varied. The interaction constant α provides information on the interaction of the inhibitors with one another in the enzyme–inhibitor complex.

The irreversible inactivation of ADH by 2-mercaptoethanol was effected by dialysis of ADH dissolved in 25 mmol l^{-1} Tris-HCl buffer, pH 7·0 (50 μmol l^{-1}) against the same buffer which moreover contained 1, 10, 100 or 1 000 mmol l^{-1} of 2-mercaptoethanol. At different time intervals (5—86 h) the activity of dialyzed ADH preparations was assayed in 0·01 ml aliquots.

All the measurements were carried out at 20°C.

RESULTS

Effect of Heterocyclic Compounds on Rape ADH

Rape ADH is inhibited by heterocyclic compounds with nitrogen atoms in their molecules. The strongest inhibitor is pyrazole, a five-membered ring heterocyclic compound, and 4-methylpyrazole, its derivative. Pyrazole and its derivatives behave as competitive inhibitors with respect to ethanol and show mixed inhibition of the uncompetitive–noncompetitive type (U–N) with respect to NAD^+, an inhibition which is close to the uncompetitive (U) type. Whereas the inhibition constant with respect to NAD^+ cannot thus be determined, the inhibition constants can be well defined with respect to ethanol (Table I). Pyrazole and its derivatives belong to the strongest inhibitors of rape ADH as regards ethanol oxidation. The inhibitory effect of pyrazole is decreased by the introduction of a methyl group into position 3 of the pyrazole ring and enhanced by its methylation in position 4 (Table I).

The explanation of these inhibition types brought about by pyrazole and its derivatives is the following: The enzyme forms with pyrazole of the various ternary complexes possible probably only the E–NAD^+–pyrazole complex yet not the E–NADH–pyrazole complex.

Heterocyclic compounds with a six-membered ring and a nitrogen atom in their molecules, such as pyridine and nicotine amide, its derivative, are weaker inhibitors of rape ADH. Pyridine acts as a competitive inhibitor both with respect to ethanol and with respect to the coenzyme (Table I). The introduction of an amide group

into position 3 of the pyridine ring leads to a decrease of the inhibition of rape ADH. The values of the inhibition constants for nicotine amide are by one order higher than for pyridine (Table I). Nicotinic acid does not inhibit rape ADH even at a concentration of 0·5 mol l^{-1}.

Imidazole and *o*-phenanthroline of the heterocylic compounds tested also belong to weaker inhibitors. The inhibition constants are given in Table I. Imidazole shows a very interesting inhibition type, both with respect to ethanol and with respect to the coenzyme. As obvious from Fig. 1, it is the CIS type (competitive inhibition and stimulation) reported by Theorell[12]. A characteristic feature of this inhibition type is that at a certain substrate concentration there is no longer inhibition but activation of the reaction. The enzyme forms a binary complex with imidazole and two types of ternary complexes, E–NAD^+–imidazole and also E–NADH–imidazole. The rate of dissociation of the E–NADH–imidazole complex to the enzyme + + NADH + imidazole is higher than the rate of dissociation of the binary complex E–NADH. If this is what really happens then, starting from a certain alcohol concentration, there is no longer inhibition but rather stimulation of ethanol oxidation. This inhibition type is observed with weaker inhibitors only since it requires a strong dissociation of the E–NADH–inhibitor complex. If imidazole formed merely the E–imidazole complex an increase in substrate concentration could cause a suppression of the inhibitor action at the most, not, however, an activation of the reaction.

TABLE I

Inhibition of rape ADH by heterocyclic compounds and 2-mercaptoethanol. Experimental conditions: 0·1 mol l^{-1} Na-phosphate buffer; pH 8·5, 0·1–0·5 mmol l^{-1} NAD^+, 10–100 mmol l^{-1} ethanol, 1 μmol l^{-1} ADH, 0–100 mmol l^{-1} inhibitor

Inhibitor	Type of inhibition with respect to			
	NAD^+	K_i	ethanol	K_i
Imidazole	CIS	2·0	CIS	1·0
Pyrazole	U–N	—	C	0·01
4-Methylpyrazole	U–N	—	C	0·001
3-Methylpyrazole	U–N	—	C	0·14
Pyridine	C	2·0	C	0·9
Nicotine amide	C	32·0	C–N	30·0
Nicotinic acid	does not inhibit			
o-Phenanthroline	C	1·1	C–N	1·2
2-Mercaptoethanol	C	3·3	C	1·1

The inhibition of rape ADH by *o*-phenanthroline is competitive with respect to the coenzyme and mixed (competitive–noncompetitive) with respect to ethanol (Table I). Hence, E–*o*-phenanthroline complexes, in which the inhibitor is bound to the coenzyme binding site, and also the E–NAD^+–*o*-phenanthroline complex are formed. *o*-Phenanthroline also binds in the neighborhood of the ethanol binding site.

Zn^{2+}-Ions restore the rape ADH activity, which had been inhibited by *o*-phenanthroline, as follows: After the addition of a 10 μmol l^{-1} concentration of Zn^{2+}-ions to the enzyme inhibited to 43% by 1 mmol l^{-1} *o*-phenanthroline the enzyme activity increases up to 85% of its original value.

Effect of 2-Mercaptoethanol on Rape ADH

2-Mercaptoethanol is used in the isolation of plant ADH's, as an effective agent protecting the enzyme against oxidation of its essential SH-groups in the active center[2,8,13,14]. We have observed, however, in our preceding study that this compound has also an inhibitory effect on the enzyme. Mercaptoethanol is an inhibitor of rape ADH competitive with respect both to ethanol and to the coenzyme (Table I). Mercaptoethanol is a weaker inhibitor showing inhibition constants of the order of 10^{-3} mol l^{-1} (Table I). The reversible competitive inhibition observed is, however, merely the initial stage of irreversible inhibition of rape ADH. The latter is irreversibly inactivated by mercaptoethanol at concentrations higher than 100 mmol l^{-1}. Total inhibition of rape ADH occurs after 24 h incubation of the enzyme with 1 mol l^{-1} mercaptoethanol (Table II). The enzyme is most stable at mercaptoethanol concentrations of 10 mmol l^{-1}. A decrease of ADH activity is observed at concentrations lower than 10 mmol l^{-1}, probably because of oxidation of the essential SH-group of cysteine in the enzyme molecule.

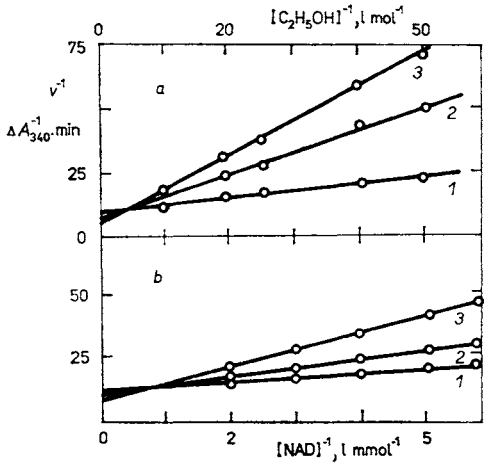

FIG. 1

Plot of reciprocal initial rate, *v*, of reaction catalyzed by rape ADH *versus* *a* reciprocal ethanol concentration and *b* NAD^+ concentration during inhibition by imidazole. Experimental conditions: 0·1 mol l^{-1} phosphate buffer; pH 8·5, 0·01–0·1 mol l^{-1} ethanol, 0·1–0·5 mmol l^{-1} NAD^+, 1 μmol . l^{-1} ADH, *1* in absence of imidazole, *2* in presence of 2 mmol l^{-1} imidazole, *3* in presence of 4 mmol l^{-1} imidazole

Simultaneous Action of Inhibitors Competitive with Ethanol

In an effort to determine where the inhibitors competitive with ethanol are bound *i.e.* with respect to one another and with respect to the substrate binding site, we carried out kinetic measurements always in the presence of two inhibitors in the reaction medium as described under Experimental and the interaction constants were determined.

Of the heterocyclic compounds tested imidazole, *o*-phenanthroline, pyrazole, nicotine amide, and pyridine bind to the same site of the enzyme molecule, as follows from the values of interaction constants which show a value of ∞ for the pairs *o*-phenanthroline–imidazole, *o*-phenanthroline–nicotine amide, *o*-phenanthroline–pyridine and pyrazole–imidazole. Mercaptoethanol binds *via* a reversible bond to the same site; the values of interaction constants for the inhibitor pairs mercaptoethanol–*o*-phenanthroline and mercaptoethanol–pyrazole also equal ∞.

DISCUSSION

Rape ADH is an enzyme containing a metal moiety essential for its activity in the molecule[14]. Like with liver and yeast ADH[1], pea ADH[13] and peanut ADH[15], in rape ADH this metal moiety is probably also zinc[16,17]. This paper describes a study of the effect of heterocyclic compounds containing a nitrogen atom in their molecules and of mercaptoethanol on rape ADH. It is known that some of these compounds

TABLE II

Inactivation of rape ADH by 2-mercaptoethanol. Experimental conditions: ADH (50 μmol l^{-1}) was incubated in 25 mmol l^{-1} Tris-HCl buffer; pH 7·0, containing 0·001—1 mol l^{-1} 2-mercaptoethanol. The ADH activity was assayed after 5—96 h incubation in 0·01 ml aliquots in a medium containing 0·1 mol l^{-1} Na-phosphate buffer, 0·1 mol l^{-1} ethanol, and 5 mmol . l^{-1} NAD^+. The values in the Table indicate ADH activity expressed in % of activity in time 0

Incubation period h	Concentration of 2-mercaptoethanol, mmol l^{-1}			
	1	10	100	1 000
0	100·0	100·0	100·0	100·0
5	100·0	100·0	79·2	28·0
12	100·0	100·0	75·0	8·1
24	100·0	87·5	70·8	0
48	80·0	85·0	70·0	0
72	62·1	84·0	58·0	0
96	50·0	80·0	50·0	0

can form tight complexes with metals and may thus also interact with zinc in the ADH molecule.

Imidazole and pyrazole inhibit rape ADH most likely like liver ADH[18–20], *i.e.* by binding as univalent ligands to Zn^{2+} of the active center. The binding of imidazole and pyrazole to the metal moiety of rape ADH also follows from the measurement of the simultaneous presence of two competitive inhibitors. As the interaction constant shows imidazole and pyrazole bind to the *o*-phenanthroline binding site, which is known as a strong chelating agent forming complexes with metals, mainly with Zn^{2+} (ref.[21]). *o*-Phenanthroline binds to Zn of the active center of ADH as a bivalent ligand[22]. Since these inhibitors are competitive or mixed (C—N) with respect to ethanol or, alternatively, competitive with NAD^+ (Table I) it may be assumed that Zn is localized in the binding site both for NAD^+ and in the substrate-binding site. The NAD^+-binding site, however, is not identical with the substrate binding site[16,17]. The presence of Zn in the coenzyme binding site of rape ADH has been demonstrated in earlier studies[3,16,17]. The interaction of Zn with the carbonyl group of the nicotine amide moiety of the NAD^+ molecule has been hypothesized[4]. This is also evidenced by the observed inhibition by nicotine amide (Table I). The presence of an amide group is obviously necessary for the binding of nicotine amide since nicotinic acid does not inhibit rape ADH. This paper shows moreover that even the substrate interacts while being bound with the Zn atom. The alkyl part of the ethanol molecule is bound to the hydrophobic domain of the enzyme protein[6] and the alkoxide ion most likely interacts with the central Zn atom. An analogy to substrate binding can also be found in the binding of mercaptoethanol which is a typical structural analog of ethanol. The SH-group of mercaptoethanol, however, shows a higher affinity for the central Zn atom than the alkoxide ion. This follows also from the determined value of the inhibition constant for mercaptoethanol (1·1 mmol l^{-1}) which is lower than the K_m-value for ethanol (20 mmol l^{-1}) (ref.[3]). The binding of mercaptoethanol and ethanol to the hydrophobic domain of the enzyme is analogous yet the stronger interaction of the SH-group with Zn strengthens mercaptoethanol binding and hence also the inhibition. The reversible interaction of mercaptoethanol with rape ADH changes into irreversible inactivation of the enzyme is most likely due to demetallization of the enzyme by mercaptoethanol. This fact, however, has to await proof from additional studies.

A comparison of the action of heterocyclic compound with a five-membered and six-membered ring shows that the binding of the former compounds is stronger (lower values of inhibition constants). 4-Methylpyrazole is the strongest inhibitor. The fact that pyrazole is a stronger inhibitor than imidazole can be explained by postulating that the binding of the former is stronger; pyrazole does not react merely with the central Zn atom but also with the nicotine amide moiety of the coenzyme molecule[23].

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