MECHANISM OF ACTION OF HEAVY METALS, S-TRIAZINE HERBICIDES AND NITRATES ON ALCOHOL DEHYDROGENASE FROM RAPE

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Received October 24th, 1984

Heavy metals (Pb^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+}) inhibited alcohol dehydrogenase (ADH) of rape (EC 1.1.1.1.); Pb^{2+} and Cd^{2+} ions affected the imidazole ring of histidine, Cu^{2+} and Zn^{2+} ions interacted with the sulphydryl groups of cysteine in the molecule of the enzyme. The coenzyme protected ADH from inactivation by Pb^{2+} and Cd^{2+}, but did not protect it from Cu^{2+} and Zn^{2+}. Ethanol in a ternary complex ADH–NAD^+–ethanol was a strong protecting agent from Pb^{2+} and Cd^{2+} ions. Nitrates inhibited rape ADH toward any substrate. Sulphates and fluorides had no effect. The S-triazine herbicides studied proved strong inhibitions of rape ADH (their K_i's corresponded to concentrations of the order 10^{-4} mol l^{-1}), occupying the binding site for the coenzyme. The herbicides interacted with the metallic component of the enzyme, to which the nicotinamide part of the coenzyme is bound.

Alcohol dehydrogenase is an enzyme playing an important role in anaerobic degradation of saccharides, present as stock substances in seeds of higher plants. This, the so-called natural anaerobiosis, proceeds at the early stage of germination of seeds of higher plants[1].

The ADH from rape seeds was characterized in our previous papers[2–7]. It is an enzyme covering a wide range of specificity to alcohols and aldehydes[3]. The previous papers deal with the constellation of the active site of the enzyme, and with the nature of binding of the substrates and the coenzyme[3,4,6].

In view of the importance of the enzyme for the germination of seeds, we have investigated how the enzyme is affected by the compounds present in significant concentrations in the environment. These include heavy metals, some herbicides used on a large scale in agriculture (S-triazine herbicides) and some anions, whose concentrations keep increasing. The paper deals with the mechanism of action of these substances and their interaction with the protein part of the enzyme molecule.

EXPERIMENTAL

Plant material and chemicals: seeds of rape *Brassica napus* L. (var. Třebíčská) were used for isolation of the enzyme. All the herbicides were products of the firm Ciba Geigy A. G., Basel, Switzerland; they were: Atrazin (the active component of Zeazin) — 2-chloro-4-ethylamino-6-

-isopropylamino-S-triazine, prometryn — 2-methylthio-4,6-bis(isopropylamino)-S-triazine, terbutylazin — 2-chloro-4-ethylamino-6-tert.butylamino-S-triazine, and simazin (the active compound of Gesatop) — 2-chloro-4,6-bis(ethylamino)-S-triazine. AMP, NAD^+, and 2-mercaptoethanol were from the firm Koch–Light Laboratories Ltd, Colnbrook, England; Sephadex G-25 and G-150 from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE cellulose DE-32 from Whatman–Maidstone, England; NADH and pyrazole from Boehringer Mannheim Gmbh, F.R.G., and the other chemicals (A.R. grade) from Lachema Brno, Czechoslovakia.

Isolation of rape ADH and determination of activity were described previously[7]. The activity was measured employing a spectrophotometer Specord UV-VIS (Carl Zeiss, Jena, G.D.R.).

The inhibition constants, regarded as dissociation constants of enzyme–inhibitor complexes, were determined according to Dixon[8].

The interaction constants were measured as previously described[9] in the reaction medium (volume 2 ml), containing ethanol (100 mmol l^{-1}), NAD (0·5 mmol l^{-1}), Na phosphate buffer pH 8·5 (0·1 mol l^{-1}), atrazin and one competitive inhibitor to NAD (nicotinamide, *o*-phenanthroline, AMP), the concentration of one inhibitor being constant and that of the other varying. The interaction constant α is a parameter indicating how two inhibitors affect each other if both are bound to a molecule of the enzyme.

Inactivation by the heavy metals was measured in 3-ml test tubes, the total volume of the incubation medium being 1 ml. Pipetted into the test tubes were: 0·7 ml of 25 mmol l^{-1} Tris–acetate buffer (pH 6·0—9·0), 0·1 ml of a solution of a metallic ion (suitable conc. in the same buffer), and 0·2 ml of the enzyme in the same buffer (4—5 μmol of the enzyme/l). After a suitable reaction time an aliquot portion (0·1 ml) was pipetted to the reaction medium, containing 0·1 mol l^{-1} Na phosphate buffer pH 8·5, 100 mmol l^{-1} ethanol and 0·5 mmol l^{-1} NAD, and the enzymic activity was immediately measured. The incubation time was 20 min (unless otherwise specified), the temperature was 20°C. The rate constants of the inactivation were calculated from the first-order-kinetics equations. In assessing the effects of NAD, ethanol, pyrazole, and Zn^{2+} on the inactivation rate, a suitable quantity of a given substance was added to the mixture, for the final concentrations to be such as given in the legends for figures and tables.

The compounds used for investigating the inactivation by metallic ions were $ZnSO_4$, 3 $CdSO_4$ . . 8 H_2O, $CuSO_4$. 5 H_2O, and $Pb(NO_3)_2$.

RESULTS

Inactivation by Heavy Metals

ADH of rape has been found to be inhibited by heavy metals (Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}), the reaction being irreversible. The inhibitory action of Pb^{2+}, Cu^{2+}, and Cd^{2+} was stronger than that of Zn^{2+} (Table I).

The inactivation of rape ADH by the heavy metals was dependent on pH (Fig. 1). As can be seen from Fig. 1, the amino acid residues of ADH responsible for the reaction with Cu^{2+} and Zn^{2+} ions have pK values 8·2 and 8·7, respectively. The residue reacting with Cd^{2+} has a pK of 7·3, and the one reacting with Pb^{2+} ions a pK of 7·1. Cu^{2+} and Zn^{2+} ions probably react with the cysteine SH groups of the enzyme, whereas Cd^{2+} and Pb^{2+} with the imidazole ring of histidine.

TABLE I

Rate constants of inactivation of ADH by heavy metals. Experimental conditions: 25 mmol l^{-1} Tris–acetate buffer pH 7·0, 5 μmol l^{-1} ADH; after 20 minutes' incubation an aliquot portion (0·1 ml) was pipetted into the reaction medium, which was 0·1 mol l^{-1} Na phosphate buffer pH 8·5, 0·1 mol l^{-1} ethanol, and 0·5 mmol l^{-1} NAD

Metal	Rate constant of inactivation, s^{-1}
Pb^{2+} (10 μmol l^{-1})	$3·3 . 10^{-4}$
+ 1 mmol l^{-1} NAD	$2·9 . 10^{-4}$
+ 2 mmol l^{-1} NAD	$1·3 . 10^{-4}$
+ 0·1 mol l^{-1} ethanol	$2·5 . 10^{-4}$
+ 1 mmol l^{-1} NAD + 0·1 mol l^{-1} ethanol	$8·1 . 10^{-5}$
+ 1 mmol l^{-1} NAD + 10 μmol l^{-1} pyrazole	$8·1 . 10^{-5}$
Cu^{2+} (10 μmol l^{-1})	$2·9 . 10^{-4}$
+ 2 mmol l^{-1} NAD	$2·9 . 10^{-4}$
+ 2 mmol l^{-1} NAD + 0·1 mol l^{-1} ethanol	$3·1 . 10^{-4}$
+ 10 μmol l^{-1} Zn^{2+}	$3·1 . 10^{-4}$
Cd^{2+} (10 μmol l^{-1})	$1·5 . 10^{-4}$
+ 1 mmol l^{-1} NAD	$8·1 . 10^{-5}$
+ 2 mmol l^{-1} NAD	$1·1 . 10^{-5}$
+ 0·1 mol l^{-1} ethanol	$1·4 . 10^{-4}$
+ 1 mmol l^{-1} NAD + 0·1 mol l^{-1} ethanol	$7·3 . 10^{-6}$
+ 1 mmol l^{-1} NAD + 10 μmol l^{-1} pyrazole	$7·3 . 10^{-6}$
+ 10 μmol l^{-1} Zn^{2+}	$3·4 . 10^{-6}$
Zn^{2+} (100 μmol l^{-1})	$1·7 . 10^{-4}$
+ 2 mmol l^{-1} NAD	$1·7 . 10^{-4}$
+ 2 mmol l^{-1} NAD + 0·1 mol l^{-1} ethanol	$1·5 . 10^{-4}$

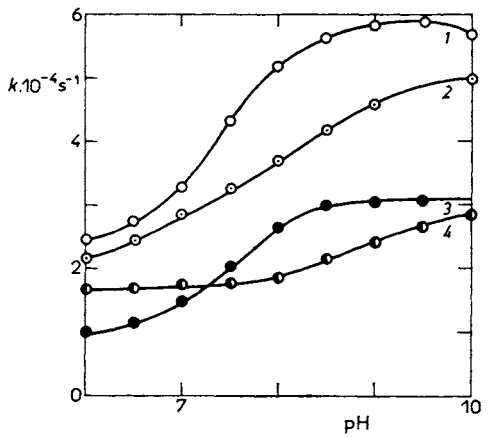

FIG. 1

Rate constant of inactivation of rape ADH by heavy metals in relation to pH. Axis of abscissae: pH; axis of ordinates: rate constant of inactivation (s^{-1}); experimental conditions: 25 mmol l^{-1} Tris–acetate buffer, 10 μmol l^{-1} Pb^{2+}, 10 μmol l^{-1} Cu^{2+}, 10 μmol l^{-1} Cd^{2+}, 100 μmol l^{-1} Zn^{2+}. *1* Pb^{2+}, *2* Cu^{2+}, *3* Cd^{2+}, *4* Zn^{2+}

The inactivation by Cd^{2+} and Pb^{2+} ions appears to be influenced by the coenzyme. NAD protects from inhibition by these metals, in relation to its concentration. If the concentration of NAD was higher than 2 mmol l^{-1}, practically no inactivation by Cd^{2+} was observed, and the inactivation by Pb^{2+} ions was markedly suppressed (Table I). With Cu^{2+} and Zn^{2+} ions, however, the coenzyme exhibited no effect. The inactivation by Cd^{2+} ions was also suppressed by low concentrations of Zn^{2+} ions, but the presence of the latter failed to reduce inhibition by the other ions (Table I). A noteworthy observation was that Zn^{2+} ions added to the enzyme after partial inactivation by Cd^{2+} ions restored its activity to some extent. Consequently, in the presence of Zn^{2+} ions the reaction of ADH with Cd^{2+} was partially reversible (Table II).

A peculiar role in the inactivation due to Cd^{2+} and Pb^{2+} ions was exhibited by ethanol. Although ethanol alone had practically no effect on the inactivation, it suppressed it markedly in the presence of the coenzyme (Table I). Pyrazole, which is an inhibitor of rape ADH and acts competitively with ethanol, behaved likewise (Table I).

Since the studied ions of heavy metals were present in the form of sulphates and a nitrate, we also investigated the effects of these anions on the enzyme. Besides, concentrations of these anions increase in some constituents of the environment (soil, water), so that they can affect the germination of seeds, in which ADH plays an important role.

Effect of Anions

The nitrate anion of $NaNO_3$ inhibits ADH of rape. This inhibition is non-competitive with all substrates (Table III). The values of the inhibition constants for this

TABLE II

Effect of Zn^{2+} ions on rape ADH inactivated by Cd^{2+} ions. Experimental conditions: 25 mmol . l^{-1} Tris–acetate buffer, pH 7·0, 10 μmol l^{-1} Cd^{2+}, 10 μmol l^{-1} Zn^{2+}, 5 μmol l^{-1} ADH. At suitable intervals 0·1-ml portions were pipetted into the reaction medium, which was 0·1 mol . l^{-1} Na phosphate buffer pH 8·5, 0·1 mol l^{-1} ethanol, 0·5 mmol l^{-1} NAD

	Time of inhibition min	Activity %
	0	100·0
+ Zn^{2+}	10	74·1
	15	86·3
	20	92·8
	30	92·8

anion are equal, in the order of magnitude, to the concentration 0·1 mol l^{-1}. Hence it can be concluded that the inactivation of the enzyme produced by $Pb(NO_3)_2$ was due to the cation only.

Sulphates (Na_2SO_4, $(NH_4)_2SO_4$) had no effect on the enzyme even in a concentration of 0·5 mol l^{-1}. Ammonium sulphate is even used for isolation of the enzyme. Consequently, in the inactivation by sulphate of the heavy metals the anions played no part.

Fluoride anions, 0·1 mol l^{-1}, did not affect rape ADH either.

The Action of S-Triazine Herbicides

The triazine herbicides (terbutylazin, simazin, atrazin, prometryn) inhibited rape ADH. The inhibition constants were in all cases roughly equal to a concentration of 0·1 mmol l^{-1} (Table IV). This concentration produced 50% inhibition of the enzyme. The strongest inhibitor was simazin (Table IV).

The inhibition was competitive toward NAD and non-competitive toward ethanol. The competitive character of the inhibition toward the coenzyme of rape ADH is probably due to the similarity of the triazine ring to the nicotinamide moiety of the coenzyme. The latter interacts with the central Zn atom in the active centre of rape ADH[4]. To locate the binding site of triazine herbicides we carried out kinetic measurements with two inhibitors competitive with NAD, and determined the constants of interaction between these inhibitors. The inhibitors used were nicotinamide, *o*-phenanthroline, and AMP along with a representative of the S-triazine herbicides, *viz.* atrazin. The interaction constants are given in Table V. It has been found that atrazin binds to the same site as nicotinamide and *o*-phenanthroline do (the interaction constant equals infinity), and not to the binding site of AMP. This corroborates that triazine herbicides interact with the binding site of the nicotinamide part of the coenzyme, *i.e.* with the central Zn atom, which also binds the inhibitors, *viz.* *o*-phenanthroline and nicotinamide.

TABLE III

Effect of NO_3^- on rape ADH. Experimental conditions: 0·1 mol l^{-1} Na phosphate buffer pH 8·5, 1—100 mmol l^{-1} ethanol, 0·1—1 mmol l^{-1} NAD, 2—200 mmol l^{-1} NO_3^-

Substrate	Type of inhibition	K_i mmol l^{-1}
Ethanol	non-competitive	110
NAD^+	non-competitive	120
Acetaldehyde	non-competitive	140
NADH	non-competitive	120

DISCUSSION

The mechanism of action of toxic substances in the environment has in recent years been the subject of intensive studies. However, there is very little information on the noxious effects of these substances on the metabolism of plants. In view of the important role of ADH in germination of seeds of higher plants we studied the mechanism of action of some toxic compounds on ADH isolated from rape seeds.

Inactivation of rape ADH by heavy metals was observed even at very low concentrations of these metals. A common feature of metallic ions inhibiting this enzyme is their tetrahedral configuration. The heavy metals are known to react specifically with SH groups of enzymes, with the formation of very obscure, undefined complexes. ADH of rape is known to contain cysteine SH groups, which are necessary for its activity[10]. The individual heavy metals differed in their action on ADH. Specific differences were observed between Cd^{2+} and Pb^{2+} on the one hand from

TABLE IV

Action of S-triazine herbicides. Experimental conditions: 0·1 mol l^{-1} Na phosphate buffer pH 8·5, 1—100 mmol l^{-1} ethanol, 0·1—1 mmol l^{-1} NAD, 0—0·2 mmol l^{-1} herbicide

Herbicide	Inhibition toward			
	ethanol		NAD	
	K_i mmol l^{-1}	type of inhibition	K_i mmol l^{-1}	type of inhibition
Atrazin	0·115	non-competitive	0·120	competitive
Prometryn	0·151	non-competitive	0·160	competitive
Terbutylazin	0·195	non-competitive	0·160	competitive
Simazin	0·100	non-competitive	0·085	competitive

TABLE V

Interactive constants. Experimental conditions: 0·1 mol l^{-1} Na phosphate buffer pH 8·5, 0·1 mol l^{-1} ethanol, 0·3 mmol l^{-1} NAD, 0—0·3 mmol l^{-1} atrazin, 0—3 mmol l^{-1} AMP, 0 — 100 mmol l^{-1} nicotinamide, 0—2 mmol l^{-1} *o*-phenanthroline

Pair of inhibitors	Interaction constant
Atrazin–nicotinamide	∞
Atrazin–*o*-phenanthroline	∞
Atrazin–AMP	0·166

Cu^{2+} and Zn^{2+} on the other. The pK values of the groups interacting with Cd^{2+} and Pb^{2+}, being close to 7, suggests that the imidazole ring of histidine is the binding site of these cations. Histidine is known to be present in the active centre of ADH of higher plants, where it probably acts as ligand of the central zinc atom[11] and is a prerequisite for activity of the enzyme. By contrast, the pK values of the groups interacting with Cu^{2+} and Zn^{2+}, close to 8·5, indicate that these metals interact with SH groups of the cysteine residues. Both histidine and cysteine are located in the binding site for the coenzyme, both as ligands of Zn in the active centre of the enzyme[10,11]. Heavy metals probably break this linkage and form chelates of these groups. The binding of the coenzyme probably occurs *via* the imidazole ring of histidine, rather than the SH groups of cysteine, since NAD protects the enzyme from inactivation by Cd^{2+} or Pb^{2+} ions, but does not influence inactivation by Cu^{2+} or Zn^{2+} ions. Histidine is also supposed to play an important role in the formation of the ternary complex enzyme–NAD–ethanol, since the protective action of ethanol against the inactivation is considerable. The weakest inhibitor of the heavy metals tested was Zn^{2+}, which inactivated the enzyme at concentrations an order of magnitude higher than the other metals did. This is probably due to its presence in the active centre, where it works as a catalyst. However, higher concentrations of Zn affect the SH groups, whereby they inactivate the enzyme, perhaps by altering the conformation of its molecule.

The action of nitrates on rape ADH was also inhibiting; a similar observation was described in the case of chloride anions[9]. However, either kind of anion proved inhibitory at concentrations much higher than the heavy metals (the inhibition constants were roughly 0·1 mol l^{-1}). The nitrate anion acted non-competitively toward any substrate, so that they bind to the enzyme elsewhere than in the active site of the enzyme. The binding of NO_3^- alters the structure of the enzyme in such a way as to decrease its activity. By contrast, chloride ions get attached to the binding site for the coenzyme and for ethanol, where they interact with a positively charged residue in an anionic binding site for phosphate of the adenosinediphosphoribosyl part of the coenzyme[9]. Sulphate and fluoride ions do not inhibit the enzyme. The inhibitory action of the triazine herbicides can be ascribed to their competition with the nicotinamide part of the coenzyme. Althouh the inhibition constants are an order of magnitude higher (0·1 mmol l^{-1}) than the concentrations used in the agricultural practice (0·01 mmol l^{-1}), the concentrations of these substances may be higher as they tend to accumulate in the soil and water. Thus the early stage of germination of seeds may be strongly retarded by these herbicides, as a result of reduced activity of ADH, which plays an important part in the germination. This conclusion accords with our previous study[12] on pea ADH which was also inhibited by S-triazine herbicides. However, the inhibition of ADH by triazines appears to be a common phenomenon, since not only ADH from higher plants, but also yeast ADH and especially liver ADH ($K_i \sim 10^{-5}$ mol l^{-1}) are strongly affected by them.

REFERENCES

1. Leblová S., Zimáková I., Barthová J., Ehlichová D.: Biol. Plant. *13*, 33 (1971).
2. Leblová S., Stiborová M.: Physiol. Plant *38*, 76 (1976).
3. Stiborová M., Leblová S.: Biochem. Physiol. Pflanzen *172*, 45 (1978).
4. Leblová S., Stiborová M.: Phytochemistry *17*, 631 (1978).
5. Stiborová M., Leblová S.: Phytochemistry *18*, 23 (1979).
6. Stiborová M., Leblová S.: Biochem. Physiol. Pflanzen *174*, 446 (1979).
7. Stiborová M., Lapka R., Leblová S.: FEBS Lett. *104*, 309 (1979).
8. Dixon M.: Biochem. J. *55*, 170 (1953).
9. Stiborová M., Leblová S.: This Journal *43*, 2217 (1978).
10. Stiborová M., Leblová S.: This Journal *45*, 1601 (1980).
11. Čeřovská N., Barthová J., Leblová S.: This Journal *47*, 1408 (1982).
12. Leblová S., Galociová J., Čeřovská N.: Environmental Res. *30*, 389 (1983).

Translated by J. Salák.