# IODOACETATE INACTIVATION OF RAPE ALCOHOL DEHYDROGENASE

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Iodoacetate inactivates rape alcohol dehydrogenase (ADH, EC 1.1.1.1). The inactivation rate follows the kinetics of the first order, is pH-dependent, and decreases below pH 7·5. Besides irreversible alkylation of the sulfhydryl groups of the enzyme iodoacetate also forms a reversible complex with rape ADH. The coenzyme (NAD) and its analogs (ATP, ADP, AMP) competitively protect the enzyme against alkylation; *o*-phenanthroline also protects the enzyme against alkylation yet noncompetitively with respect to iodoacetate. Imidazole and *o*-phenanthroline compete with one another for binding to the protein molecule of rape ADH. Whereas *o*-phenanthroline decreases the inactivation rate imidazole increases the rate of iodoacetate inactivation.

Iodoacetate is an efficient carboxymethylating agent alkylating the SH-groups of proteins. It alkylates liver ADH (LADH) (ref.[1-3]), yeast ADH (YADH) (ref.[4]), and also pea ADH (ref.[5]). It has not been demonstrated whether or not the alkylation of the sulfhydryl groups of the enzyme protein is also accompanied by reversible binding of the reagent[2].

We studied the effect of iodoacetate on rape ADH which – as follows from its inhibition by sulfhydryl poisons – is an enzyme with SH-groups in its molecule[6].

## EXPERIMENTAL

*Vegetal material and chemicals*: The enzyme was isolated from rape seeds (*Brassica napus* L., var. Třebíčská). ATP, AMP, and NAD were from Koch-Light Lab. Ltd., England, ADP from Calbiochem, San Diego, Cal., U.S.A., and Tris from Merck AG., Darmstadt, GFR. The remaining chemicals (all of analytical purity) were purchased from Lachema, Brno, ČSSR.

*The enzyme* was isolated by the procedure involving extraction with sodium phosphate buffer at pH 8·5, fractionation with ammonium sulfate, chromatography on a column of DEAE-cellulose, and twice repeated gel filtration on Sephadex G-100, as described in detail in paper[7]. The enzyme preparation obtained had a specific activity of 4·258 units/mg protein (μmmol/min . mg).

ADH *activity* was measured in terms of absorbance increase at 366 nm as described in our preceding paper[6]. One activity unit is the quantity of enzyme which converts 1 μmol of NADH in 1 min.

*The protein content* was determined according to Lowry[8] with serum albumin as a standard.

*The kinetic measurements* in the presence of *o*-phenanthroline and imidazole, *i.e.* two inhibitors competitive with respect to the coenzyme, were carried out in 1 ml of a reaction medium containing 100 mM ethanol, 0·5 mM NAD, 0·1M sodium phosphate buffer at pH 8·5, *o*-phenanthroline, and imidazole; the concentration of one inhibitor remained constant and the concentration of the other inhibitor varied. The interaction constant $\alpha$ reflects the interaction of both inhibitors with the enzyme. It was determined by the method described in our preceding paper[9].

*The inactivation* assays were carried out in 3 ml test tubes (total volume 1 ml) at constant pH and ionic strength (0·1M sodium phosphate buffer, pH 7·5). The concentration of ADH was measured kinetically. The initial enzyme concentration varied over the range 5—10 µN. The incubation period was as a rule 20 min unless stated otherwise, the temperature was 20°C. At appropriate times 0·1 ml aliquots of the incubated mixture were pipetted into a reaction medium containing 0·1M sodium phosphate buffer, pH 8·5, 0·6 mM-NAD, and 0·1M ethanol in 1 ml of medium; ADH activity was measured immediately. The inactivation rate constants were calculated from the equations of first order kinetics. When the effect of ATP, ADP and AMP, and of *o*-phenanthroline, NAD and imidazole on the alkylation rate was assayed, an appropriate quantity of the given compound was added to the incubation mixture; the final concentrations are shown in the legends to the corresponding figures.

## RESULTS AND DISCUSSION

### *Inactivation by Iodoacetate*

Iodoacetate efficiently alkylates the SH-groups of rape ADH. The alkylation by iodoacetate is a bimolecular reaction. Since, however, a large excess of the alkylating agent is used, the reaction can be regarded as pseudomonomolecular and can therefore be described by first order reaction equations. This is in accordance with the decrease of rape ADH activity alkylated by iodoacetate: the plot of log % of activity *versus* time is linear (Fig. 1).

TABLE I

Dissociation Constants of Rape ADH Complexes with Various Ligands, Determined in Inactivation Studies with Iodoacetate

Experimental conditions: 0·1M phosphate buffer, pH 7·5; [iodoacetate] = 0-4 mM.

| Ligand | $K_i$, µM |
|---|---|
| ATP | 1 000 |
| ADP | 370 |
| AMP | 72 |
| NAD | 300 |
| *o*-Phenanthroline | 40 |

If the rate constant of inactivation by iodoacetate is plotted *versus* iodoacetate concentration the resulting plot is almost linear (Fig. 2). The line obtained by plotting the reciprocal values, however, does not pass through the origin. This indicates a partial saturation effect by iodoacetate: In the case of rape ADH there is obviously besides alkylation also reversible binding, observed also with liver ADH earlier[2], This phenomenon can be explained best by postulating that iodoacetate alkylation of the SH-groups of the enzyme is paralleled – because of the presence of strongly polarized iodine – by reversible binding similar in character to the binding of chloride ions (negative charge) which form reversible complexes with rape ADH (ref.[9]); the chloride ions react in these complexes with zinc of the active center of the enzyme.

The alkylation caused by iodoacetate depends on the pH of the medium (Fig. 3): the inactivation rate decreases above pH 7·5. We carried out therefore all alkylation studies at constant pH (7·5).

*Effect of* AMP, ADP, *and* ATP *on Inactivation by Iodoacetate*

ATP, ADP, and AMP efficiently prevent rape ADH (similarly to liver ADH, ref.[3]) from iodoacetate inactivation. The protective effect increases in the series ATP-

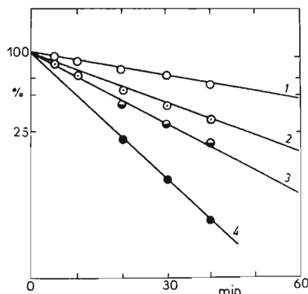


FIG. 1

Plot of Rate of Rape ADH Inactivation by Iodoacetate *versus* Time of ADH Incubation with Iodoacetate

*x* Axis: time of incubation in min, *y* axis: log % of activity. Experimental conditions: 0·1M sodium phosphate buffer, pH 7·5; *1* 1 mM IA, *2* 2 mM IA, *3* 3 mM IA, *4* 4 mM IA.

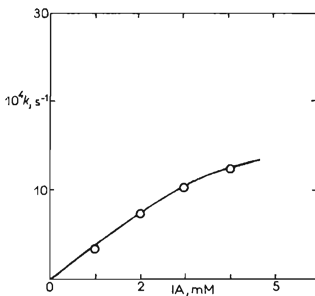


FIG. 2

Plot of Inactivation Rate constant *versus* Iodoacetate Concentration (IA)

*x* Axis: iodoacetate concentration (mM); *y* axis: inactivation rate constant ($s^{-1}$). Experimental conditions: 0·1M sodium phosphate buffer, pH 7·5.

-ADP-AMP as follows from the inhibition constants characterizing the individual nucleotides. The constants are the dissociation constants of the enzyme–nucleotide complex, determined from the protective effect before alkylation by iodoacetate (Table I). All three nucleotides are strictly competitive with respect to iodoacetate (Fig. 4). The observed decrease of the protective effect in the series AMP-ADP-ATP can be ascribed to the hydrophobic character of the phosphate residues.

If we compare the values of inhibition constants obtained in the studies on inactivation by iodoacetate with the values which follow from the kinetic measurements[9] it becomes obvious that they are almost in agreement. The former are a little lower yet the values of inhibition constants obtained in inactivation studies are generally lower, as observed also with LADH (ref.[3]).

*Effect of* NAD *on Inactivation by Iodoacetate*

Besides the adenine nucleotides the coenzyme itself markedly protects the enzyme against inactivation by iodoacetate (Fig. 5). The value of the dissociation constant

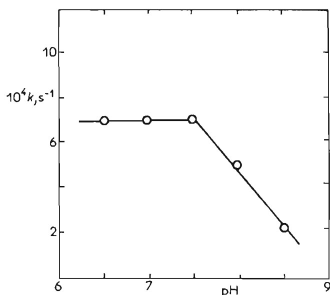


FIG. 3

Plot of Inactivation Rate Constant *versus* pH

*x* Axis: pH; *y* axis: inactivation rate constant ($s^{-1}$). Experimental conditions: 0·1M sodium phosphate buffer; [iodoacetate] = 2 mM.

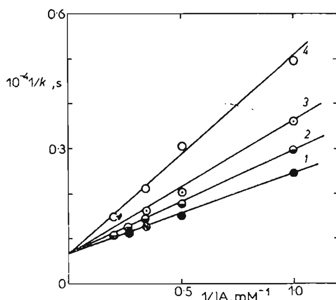


FIG. 4

Plot of Reciprocal Inactivation Rate Constant *versus* Reciprocal Iodoacetate Concentration Characterizing the Protection by Nucleotides

*x* Axis: reciprocal iodoacetate concentration; *y* axis reciprocal inactivation rate constant. Experimental conditions: 0·1M sodium phosphate buffer, pH 7·5; *1* without the protective agent; 2 0·5 mM ATP; *3* 0·3 mM ATP; *4* 0·2 mM AMP.

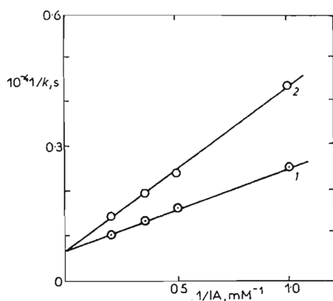


FIG. 5

Plot of Reciprocal Inactivation Rate Constant *versus* Reciprocal Iodoacetate Concentration Characterizing the Protection by NAD

*x* Axis: reciprocal iodoacetate concentration (mM$^{-1}$); *y* axis: reciprocal inactivation rate constant (s). Experimental conditions: 0·1M sodium phosphate buffer, pH 7·5; *1* without NAD; *2* 0·78 mM NAD.

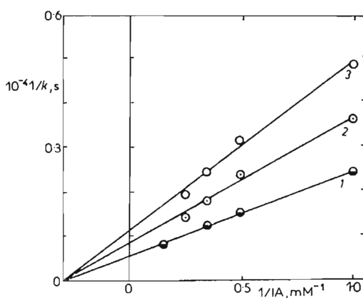


FIG. 6

Plot of Reciprocal Inactivation Rate Constant *versus* Reciprocal Iodoacetate Concentration Characterizing the Protection by *o*--Phenanthroline

*x* Axis: reciprocal iodoacetate concentration (mM$^{-1}$); *y* axis: reciprocal inactivation rate constant (s). Experimental conditions: 0·1M sodium phosphate buffer, pH 7·5; *1* without *o*-phenanthroline; *2* 20 μM *o*-phenanthroline; *3* 40 μM *o*-phenanthroline.

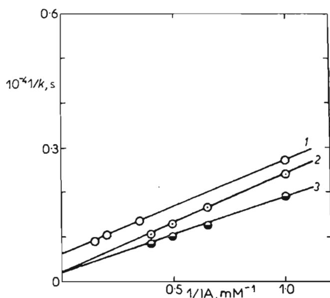


FIG. 7

Plot of Reciprocal Inactivation Rate Constant *versus* Reciprocal Iodoacetate Concentration Characterizing the Action of Imidazole

*x* Axis: reciprocal iodoacetate concentration (mM$^{-1}$); *y* axis: reciprocal inactivation rate constant (s). Experimental conditions: 0·1M sodium phosphate buffer, pH 7·5; *1* without imidazole; *2* 1 mM imidazole; *3* 2 mM imidazole.

of the E–NAD complex determined in inactivation studies (0·3 mM, Table I) is again slightly lower than the value determined kinetically (0·4 mM, ref.[10]). The protection by the coenzyme is, as expected, again competitive with respect to iodoacetate (Fig. 5).

### *Effect of* o-*Phenanthroline and Imidazole on Inactivation by Iodoacetate*

Attention deserves the action of *o*-phenanthroline and imidazole on the inactivation by iodoacetate. Both these compounds are known to bind to the zinc atom of the active center of LADH (ref.[11]), *o*-phenanthroline as a bivalent and imidazole as a univalent ligand. The two compounds inhibit rape ADH and bind to the coenzyme-binding site[6,9]; we found that they compete with one another for the binding site on the enzyme protein, as follows from the kinetic measurements carried out in the presence of *o*-phenanthroline and imidazole, two inhibitors competing with the coenzyme. The interaction constant $\alpha$ which follows from these measurements has an infinite value for this inhibitor pair; *o*-phenanthroline and imidazole therefore bind to the same site of the enzyme protein. They differ, however, in their effect on inactivation by iodoacetate: *o*-phenanthroline decreases the inactivation rate and has therefore a protective effect whereas imidazole increases the inactivation rate (Fig. 6, 7). A 2 mM imidazole concentration increases the inactivation rate almost to the double; *o*-phenanthroline protects the enzyme yet is noncompetitive with respect to iodoacetate (Fig. 6). This can be explained by postulating that *o*-phenanthroline does not prevent iodoacetate from interaction with the enzyme yet somehow protects the essential SH-groups against alkylation, most likely by causing a steric hindrance or perhaps a change in conformation of the enzyme protein; this change may then result in impaired binding of iodoacetate to the essential SH-groups. The stimulation of the inactivation rate caused by imidazole can also be explained by a change in conformation of the enzyme protein which alters the binding conditions. This change, however, is probably of a character completely different from that of the change caused by the binding of *o*-phenanthroline since the change in conformation caused by the binding of imidazole makes the SH-groups of the enzyme more accessible to alkylation by iodoacetate.

The inhibition constant for *o*-phenanthroline determined in experiments with iodoacetate inactivation is shown in Table I. It is again lower, and markedly, than the value established kinetically[9].

The results presented show that the SH-groups of rape ADH which can be alkylated by iodoacetate play a role in the binding of the coenzyme to the enzyme. This is evidenced by the protective effect of the coenzyme and its analogs. The degree of alkylation of SH-groups can also be affected by compounds such as *o*-phenanthroline or imidazole which bind to ADH as zinc ligands at the coenzyme-binding site. This phenomenon indicates the possible presence of SH-groups which can be alkylat-

ed in the neighborhood of the central zinc atom localized in the coenzyme-binding site of rape ADH.

REFERENCES

1. Jörnvall H.: Eur. J. Biochem. *14*, 521 (1970).
2. Reynolds C. H., McKindley-McKee J. S.: Eur. J. Biochem. *10*, 474 (1969).
3. Reynolds C. H., Morris D. L., McKinley-McKee J. S.: Eur. J. Biochem. *14*, 14 (1970).
4. Brändén C.-I., Jörnvall H., Eklund H., Furugren B. in the book: *The Enzymes*, 3rd Ed., Vol. 11, p. 103. Stockholm 1975.
5. Lapka R., Leblová S.: Physiol. Plant. *39*, 86 (1977).
6. Leblová S., Stiborová M.: Physiol. Plant. *38*, 176 (1976).
7. Stiborová M.: *Thesis*. Charles University, Prague 1978.
8. Lowry H. O., Rosebrough N. J., Farr J. L., Randal R. J.: J. Biol. Chem. *193*, 265 (1951).
9. Stiborová M., Leblová S.: This Journal *43*, 2217 (1978).
10. Stiborová M., Leblová S.: Phytochemistry *18*, 23 (1979).
11. Boiwe T., Brändén C.-I.: Eur. J. Biochem. *77*, 173 (1977).

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