CONTRIBUTION TO THE KNOWLEDGE OF THE ROLE OF SH-GROUPS IN THE MOLECULE OF PEA ALCOHOL DEHYDROGENASE

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p-Chloromercuribenzoate irreversibly inactivates alcohol dehydrogenase (ADH) isolated from germinating pea seeds. The reaction follows the first order kinetics. The inactivation of pea ADH is pH-dependent and is maximal at pH 9·0. NAD protects the enzyme from inactivation by *p*-chloromercuribenzoate; the higher the concentration of the coenzyme and the longer the period of incubation of NAD with the enzyme, the lower the degree of inactivation. Ethanol does not prevent the enzyme from inactivation. *o*-Phenanthroline in a concentration of $1 . 10^{-3}$ mol l^{-1} decreases the degree of inactivation of the enzyme by *p*-chloromercuribenzoate by 20%; imidazole is without effect on the reaction. Zn^{2+}-ions in concentration of $1 . 10^{-5}$ mol l^{-1} also partly protect the enzyme from inactivation by *p*-chloromercuribenzoate. The results obtained show that the SH-groups sensitive to labeling with *p*-chloromercuribenzoate are localized in the active center of the enzyme, probably in the coenzyme-binding site. The protective action of Zn^{2+}-ions and of *o*-phenanthroline against this inactivation confirms the assumption that the SH-group acts as a zinc ligand in the active center of the enzyme.

In our earlier kinetic studies we determined the p*K*-values of the groups which play a role in the catalytic function of pea alcohol dehydrogenase[1]. The value of 8·7 belongs most likely to the SH-group of cysteine. The role of SH-groups in animal[2] and microbial[3] alcohol dehydrogenases has been discussed in many papers. The role of these groups in plant alcohol dehydrogenases has not been elucidated completely as yet even though their labeling in the protein by many reagents (iodoacetate, iodoacetamide[4,5], heavy metals[6]) has intensively been investigated in our Laboratory.

This paper continues our earlier measurements. The inactivation of the enzyme by *p*-chloromercuribenzoate provides another piece of evidence of the presence of SH-groups in the molecule of pea ADH. Additional data on the role of SH-groups in the catalytic mechanism of the enzyme are presented.

EXPERIMENTAL

Plant material: The enzyme was isolated from 48-h germinating pea seedlings (*Pisum sativum* L., cv. SALUD).

Chemicals: Tris-(hydroxymethyl)aminomethane, Lachema, Brno; NAD, Koch-Light, Colnbrook, England; *p*-chloromercuribenzoate, Fluka, Buchs, Switzerland; DEAE-cellulose, Whatman, Maidstone, England; Sephadex G-25, G-100, Pharmacia, Uppsala, Sweden; hydroxylapatite, Merck, Darmstadt, FRG; the remaining chemicals were of analytical purity.

Isolation of pea alcohol dehydrogenase (PADH): PADH was isolated by the method described elsewhere[7] from a homogenate of 48-h germinating pea by extraction, salting-out with ammonium sulfate and chromatography of the desalted fraction on DEAE-cellulose, Sephadex G-100, and hydroxylapatite. The enzyme was almost homogeneous on electrophoresis.

Assay of enzyme activity: The enzyme activity was measured as absorbance increase at 340 nm as described elsewhere[8] in a Specord UV-Vis (Carl Zeiss, Jena, GDR) spectrophotometer. Protein concentration was determined by the method of Lowry[9].

Inactivation of enzyme by p-chloromercuribenzoate: The inactivation was carried out in 3 ml incubation tubes. The incubation mixture (total volume 1 ml) contained: 0·1 mol l^{-1} Tris-acetate buffer, pH 6, the enzyme, and different concentrations of the modifying reagent. At time intervals 0·1 ml aliquots of the reaction mixture were withdrawn and transferred to the reaction medium for activity assay. In experiments designed to determine the effect of various ligands on the inactivation of the enzyme by *p*-chloromercuribenzoate the corresponding quantity of the compound examined was added to the reaction mixture, so that its final concentration corresponded to the values given in the figures. The incubation time was 0 to 40 min. The concentration of *p*-chloromercuribenzoate varied over the range $0{\cdot}59-2{\cdot}36 \cdot 10^{-3}$ mol l^{-1}.

RESULTS

Inactivation of PADH *by* p-*Chloromercuribenzoate* (PCMB)

The inactivation of PADH by PCMB is shown in Fig. 1. The reaction is most likely bimolecular; however, as can be seen from the linearity of the semilogarithmic plot of enzyme activity *versus* time, the reaction shows a pseudomonomolecular character. This discrepancy can be accounted for by a considerable excess (approximately 100-fold) of the reagent over the enzyme protein. The reaction can then be described by a first order reaction. Since the straight line of the reciprocal plot passes through the origin, we postulate that the character of inhibition of the enzyme by PCMB is irreversible.

The inactivation of PADH *by* PCMB depends on the pH of the medium (Fig. 2). The pH-value of the group modified is 8·3 thus corresponding to the pK of a sulfhydryl group[10]. The inactivation is maximal at pH 9·0. Fig. 3 shows the time profile of the change of absorbance at 250 nm of the enzyme–PCMB complex. The plot shows that the enzyme is inactivated by one molecule of the modifying reagent.

Modification of PADH *in presence of ligands binding to the active center of the enzyme*: We examined the effect on PADH inactivation by PCMB of some compounds which form complexes with PADH and bind to the neighborhood of the active center of the enzyme molecule.

NAD affects the rate of PADH inactivation by PCMB quite markedly. The effect depends on NAD concentration (Fig. 4) and on the length of the preincubation period (Fig. 5). The longer the preincubation of PADH with NAD the slower the inactivation by PCMB.

Ethanol, unlike NAD, in concentrations higher than K_m ($2 . 10^{-1}$ mol l^{-1}) is practically without any effect on the inactivation of the enzyme by PCMB (Fig. 6).

o-Phenanthroline in a concentration of $1 . 10^{-3}$ mol l^{-1} decreases the degree of inactivation of the enzyme by PCMB by 20% (Fig. 7), whereas imidazole does not affect the reaction.

The protection by Zn^{2+}-ions in a concentration of 10^{-5} mol l^{-1} is shown in Fig. 8. Unlike the protection of the enzyme by NAD, the effect of Zn^{2+}-ions is almost independent of the time of preincubation of the enzyme with this inhibitor.

DISCUSSION

The kinetic measurements of the effect of the pH of the reaction medium on the activity of pea ADH have shown that one of the groups which play a role in the active center of the enzyme during the catalytic process has a p*K*-value of about 8·9 (ref.[1]). This value corresponds to the p*K*-value of the sulfhydryl group of cysteine and indicates the role of these groups in the catalytic process.

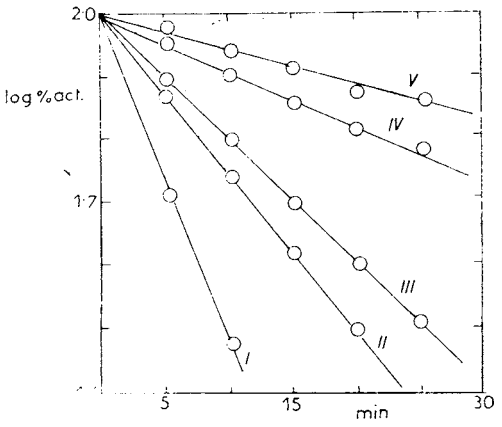

FIG. 1

Dependence of rate of inactivation of pea ADH by *p*-chloromercuribenzoate (PCMB) on time of incubation with PCMB: Tris 0·1 mol l^{-1}, pH 6; *I* $2·36 . 10^{-3}$ mol l^{-1} PCMB; *II* $1·78 . 10^{-3}$ mol l^{-1} PCMB; *III* $1·18 . 10^{-3}$ mol l^{-1} PCMB; *IV* $0·88 . 10^{-3}$ mol l^{-1} PCMB; *V* $0·59 . 10^{-3}$ mol l^{-1} PCMB

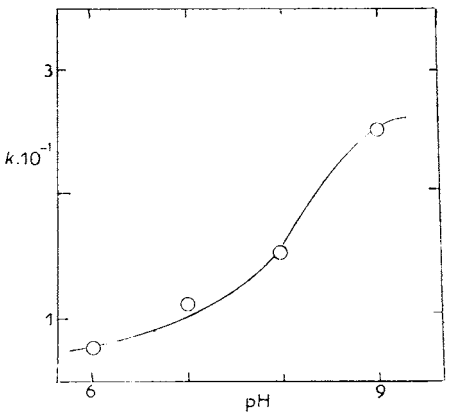

FIG. 2

pH-Profile of inactivation rate constant. Tris 0·1 mol l^{-1}, PCMB $1·78 . 10^{-3}$ mol l^{-1}

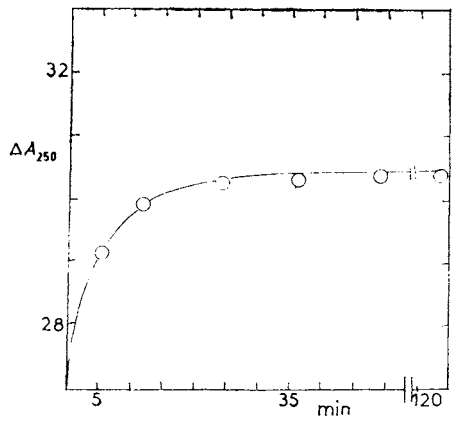

FIG. 3

Time profile of ADH modification by PCMB expressed as change in absorbance at 250 nm. Tris 0·1 mol l^{-1}, pH 6·1; PCMB 0·59 . 10^{-3} mol l^{-1}

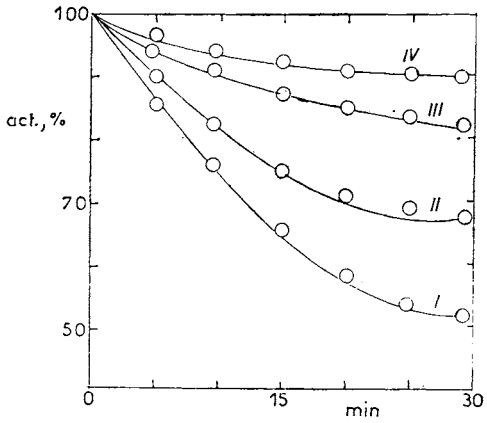

FIG. 4

Effect of NAD on pea ADH inactivation by PCMB. Tris 0·1 mol l^{-1}, pH 6; PCMB 1·78 . 10^{-3} mol l^{-1}. *I* without NAD; *II* 3 . 10^{-5} mol l^{-1} NAD; *III* 5 . 10^{-5} mol l^{-1} NAD; *IV* 1 . 10^{-4} mol l^{-1} NAD; the enzyme was preincubated 20 min with NAD

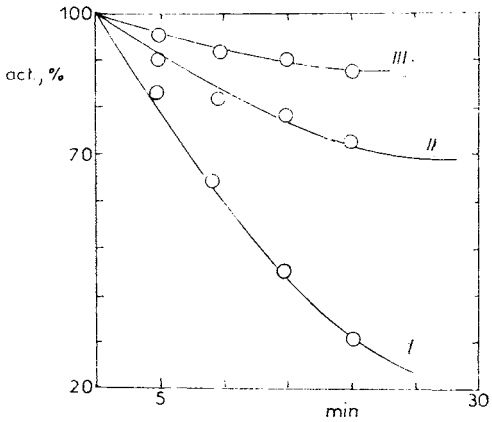

FIG. 5

Dependence of rate of PADH inactivation on time of incubation with PCMB, after preincubation of the enzyme for various periods with 1 . 10^{-4} mol l^{-1} NAD. Tris 0·1 mol l^{-1}, pH 6; *I* without NAD; *II* 5 min preincubation; *III* 20 min preincubation

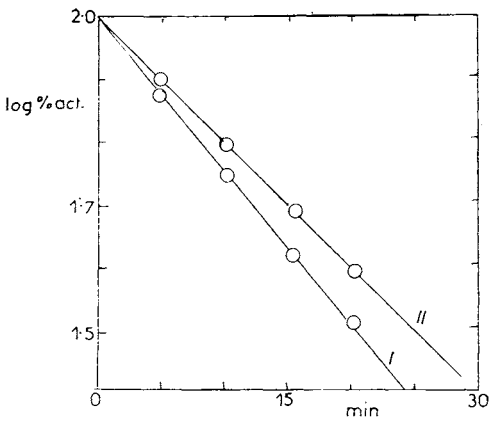

FIG. 6

Protective action of ethanol against pea ADH inactivation by PCMB. Tris 0·1 mol l^{-1}, pH 6·1; *I* without ethanol; *II* 2 . 10^{-1} mol l^{-1} ethanol

Horse liver ADH (LADH) and yeast ADH (YADH) have also been shown[2,3,11] to possess essential SH-groups in their active centers. It has been demonstrated that the essential SH-groups of LADH, whose amino acids of the active center are known, belong to Cys-46 and to Cys-174 which together with His-67 form the ligands of the central zinc atom[12]. Our own measurements of the time profile of changes in A_{250nm}-absorbance of the enzyme during its treatment with PCMB have also demonstrated that pea ADH contains SH-groups. The results of the measurement of the pH-profile of the rate of enzyme inactivation by PCMB have shown that one group only, namely that having a pK of 8·3 is modified. The pK-value of this group indicates that the modification involves one group only, the SH-group of cysteine.

The presence of NAD partly protects the enzyme against inactivation by PCMB even when the NAD concentration is lower (approximately by two thirds) than the K_m-value of the enzyme.

In contrast, ethanol, the second substrate of the reaction, practically does not protect the enzyme against inactivation by PCMB even if the concentration of ethanol is twice the K_m-value of the enzyme. This indicates the localization of the SH-groups in the binding site of the coenzyme.

Interest deserves the action of *o*-phenanthroline and imidazole on the inactivation of pea ADH by PCMB. Both these compounds are well-known chelating agents

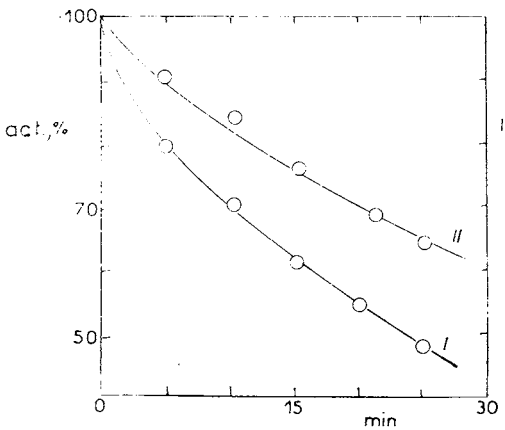

FIG. 7

Effect of *o*-phenanthroline on pea ADH inactivation by PCMB. *I* without phenanthroline; *II* $1 . 10^{-3}$ mol l^{-1} phenanthroline

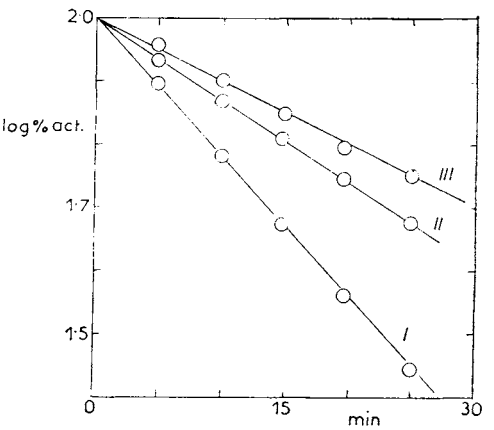

FIG. 8

Effect of Zn^{2+}-ions on inactivation of pea ADH by PCMB. Tris 0·1 mol l^{-1}, pH 6·1; Zn^{2+} $1 . 10^{-5}$ mol l^{-1}. *I* without Zn^{2+}; *II* 5 min preincubation of enzyme with Zn^{2+}; *III* 10 min preincubation

which bind to the zinc atom of LADH (ref.[13]), *o*-phenanthroline as a bivalent ligand and imidazole as a monovalent ligand. Both these compounds inhibit pea ADH and bind to the coenzyme binding site, simultaneously competing for one binding site on the enzyme protein. The effect of these two compounds on the inactivation of pea ADH by PCMB is different: *o*-phenanthroline decreases the inactivation rate thus having a protective effect, whereas imidazole is without effect on this reaction. This can be best explained by assuming that *o*-phenanthroline prevents PCMB from interacting with the enzyme, probably by being either a steric hindrance or by changing the conformation of the enzyme and thus protecting its SH-groups against inactivation. *o*-Phenanthroline, a bivalent ligand, chelates the central zinc atom and also shields the SH-groups of cysteine which probably are ligands of this metal atom. Imidazole, a monovalent zinc ligand, does not sufficiently cover the central metal atom and the SH-groups are still accessible for modification by PCMB.

The inactivation of pea ADH by PCMB shows that the SH-group reacting with this reagent is localized in the active center of the enzyme. The fact that the SH-groups are protected by Zn^{2+}-ions against this inactivation suggests the presence of a modified group of cysteine in the close neighborhood of the zinc atom. The protective action of *o*-phenanthroline against pea ADH inactivation by PCMB confirms the postulate that the SH-group is a ligand of the zinc atom as in the case of LADH (ref.[12]).

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