ROLE OF HISTIDINE IN MOLECULE OF PEA ALCOHOL DEHYDROGENASE

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Alcohol dehydrogenase (E.C.1.1.1.1) from germinating pea seedlings was modified by treatment with diethyl pyrocarbonate. The inactivation rate is proportional to the molar concentration of the modifying agent; the inactivation was almost complete in fifty minutes at a diethyl pyrocarbonate concentration of $5 \cdot 10^{-6}$ mol/l. The histidine content calculated from the absorbance difference at 240 nm was 3·43 residues per molecule of native and 4·75 residues per molecule of demetalized enzyme. A correlation of the absorbance difference at 240 nm with a 100% loss of enzymatic activity shows that 1·22 histidine residue is essential for the activity of alcohol dehydrogenase. The dependence of the inactivation rate constant on the pH of the medium indicates that the treatment of pea alcohol dehydrogenase with diethyl pyrocarbonate results in the modification of one group only with a pK of 7·1, well corresponding to the imidazole group of histidine. The enzyme is partially protected against inactivation by NADH at a concentration close to the Michaelis constant for NADH. The treatment of the ethoxyformylated enzyme with hydroxylamine followed by dialysis restored the activity of pea alcohol dehydrogenase by 88%.

A characteristic feature of germinating seeds is an intensive metabolic activity. Many plants undergo a short anaerobiosis period during germination[1]. An important role in the energetic metabolism of this period plays alcohol dehydrogenase[1] (ADH). Kinetic measurements of the effect of pH and temperature on the activity of pea alcohol dehydrogenase (PADH) showed that the active center of the enzyme involves besides the SH-groups of cysteine also the imidazole groups of histidine[9]. This paper presents the results of kinetic measurements with ADH modified by diethyl pyrocarbonate.

EXPERIMENTAL

Plant material: The enzyme[7] was isolated from pea seedlings (*Pisum arvense* L., *cv. Jupiter*) which had germinated 48 h.

Chemicals: Tris(hydroxymethyl)aminomethane, was from Lachema, Brno, NAD from Koch-Light, England, NADH from Reanal, Hungary, diethyl pyrocarbonate from Fluka, FRG, DEAE-cellulose DE-32 from Whatman, England, Sephadex G-25 and G-200 from Pharmacia Fine Chemicals, Sweden. The remaining chemicals were of analytical purity.

Isolation of pea ADH *and determination of its enzymatic activity*: Pea ADH was isolated by the method described earlier[2]; the plant extract was fractionated by ammonium sulfate and the material desalted on Sephadex G-25 was chromatographed on DEAE-cellulose and Sephadex G-200. The enzyme preparation had an enzymatic activity of 70 000 U/mg. One activity unit was defined as the change in absorbance at 366 nm of 0·01 in two minutes. The protein concentration was determined by the method of Lowry[3].

Modification of pea ADH *by diethyl pyrocarbonate*: The histidine residues were modified by the method of Wallis and Holbrook[4]. ADH was mixed with the corresponding quantity of diethyl pyrocarbonate solution in absolute ethanol and the mixture was thermostated at 25°C. Aliquots of the mixture were removed at intervals. Simultaneously a blank experiment was carried out with a sample which contained ethanol instead of the diethylpyrocarbonate solution. The number of ethoxyformyl groups contained in the protein as ethoxyformylimidazole was determined from the difference in absorbance at 240 nm of the modified and unmodified enzyme. The molar extinction coefficient of ethoxyformylimidazole was 3 500 l mol^{-1} cm^{-1} (ref.[5]). The deacylation of the modified enzyme by hydroxylamine was performed by the method of Choong and coworkers[6].

Dependence of ADH *inactivation on* pH *of reaction medium*: The dissociation constant of the group modified can be calculated from the relation by Ehrlich and Colman[7]

$$k - k_{min} = k_{max} - k_{min}/(1 + [H^+]/K_H) ,$$

where k designates the inactivation rate constant at a H^+-concentration of the medium used for the measurement, k_{max} the rate constant of inactivation of the unprotonated enzyme, k_{min} the inactivation rate constant of the protonated enzyme, and K_H the dissociation constant of the group modified.

Demetalization of ADH: The enzyme preparation was dialyzed 20 h against 0·025 mol/l of Tris acetate buffer at pH 5, containing 0·01 mol/l of mercaptoethanol and 10^{-2} mol/l of EDTA. The latter was removed by 48 h dialysis against 0·1 mol/l of Tris-acetate buffer at pH 6·4 and the enzyme preparation was used for the measurement. All the values given in the Table or in the diagrams are means of at least four parallel measurements.

RESULTS

ADH from germinating pea seedlings is inactivated by diethyl pyrocarbonate. The inactivation rate is proportional to the molar ratio of concentrations of enzyme and diethyl pyrocarbonate. The plot of inactivation rate expressed by a first order rate constant versus diethyl pyrocarbonate concentration is linear.

Diethyl pyrocarbonate reacts with the imidazole group of histidine thus giving rise to N-ethoxyformylimidazole whose absorption maximum lies at 240 nm. The increase of absorbance at 240 nm is directly proportional to the number of modified histidine residues. The number of the latter calculated from the absorbance difference at 240 nm of the modified and unmodified enzyme, is 3·43 residues per one molecule of pea ADH. The correlation of the absorbance increase at 240 nm with the activity decrease to a zero value shows that 1·22 histidine residue is essential for the activity of the enzyme.

We examined the inactivation of pea ADH by diethyl pyrocarbonate in 0·1 mol/l phosphate buffer at four different pH-values, *i.e.* at pH 5, 6, 7, and 8. The p*K*-value of the modified group, obtained in several experiments, varies over a relatively small pH-range (7·0 – 7·1, Fig. 1). The p*K*-value indicates that the side chain of the histidine residue was modified. The profile of the plot of inactivation rate constant on pH shows that one group only, namely the imidazole of histidine was modified.

The action of hydroxylamine on the ethoxyformylated and completely inactive pea ADH decreased the absorbance of the preparation at 240 nm to a value characteristic of the native enzyme. The absorbance dropped to this value in 30 min (Fig. 2). The activity of the enzyme was restored only after hydroxylamine had been removed by dialysis, since hydroxylamine itself is an inhibitor of pea ADH. The activity of the enzyme was restored by 88% after 40 h dialysis against 0·025 mol/l Tris-acetate buffer pH 6·4 with 0·01 mol/l mercaptoethanol.

We found that NADH shows a protective effect during the modification by diethyl pyrocarbonate; this effect, however, can be observed only if the NADH concentration is comparable to the K_m-value of the given enzyme (Table I), lower NADH concentrations do not protect the enzyme against inactivation. The inactivation rate is influenced by the length of the period of preincubation of the enzyme with

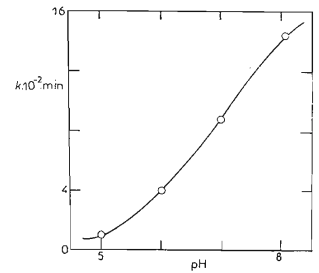

FIG. 1

Rate constant of pea alcohol dehydrogenase inactivation by diethyl pyrocarbonate as function of pH of medium. Experimental conditions: NAD 0·05 μmol; ethanol 10 μmol; diethyl pyrocarbonate 0·008 μmol; reaction medium volume 1 ml

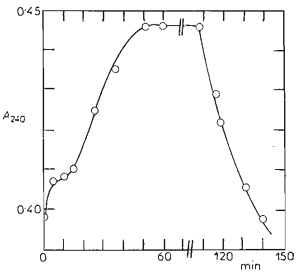

FIG. 2

Modification of histidine residues of pea alcohol dehydrogenase by diethyl pyrocarbonate with subsequent deacylation by hydroxylamine. Abscissa: absorbance at 240 nm; ordinate: time in min. Experimental conditions: hydroxylamine 1 mol/l; molar enzyme to inhibitor ratio 1 : 30

NADH (Table I): the inactivation was slower after longer preincubation periods. The pH-profile of the rate of inactivation of pea ADH by diethyl pyrocarbonate in the absence and presence of NADH is described by the same curves. They correspond to the reaction of diethyl pyrocarbonate with one group: with a group of pK 7·1 in the apoenzyme and with a group of pK 7·2 in the binary ADH–NADH complex.

The effect of diethyl pyrocarbonate on the iodoacetamide modified enzyme and on the demetalized enzyme has also been examined. If the SH-groups of the enzyme are blocked by iodoacetamide[8] and subsequently the enzyme is modified by diethyl pyrocarbonate then the absorbance increase at 240 nm corresponds to the acylation of 3·25 histidine residues per one ADH molecule. It can therefore be stated that the same number of histidine residues as in the native enzyme is modified. When we assayed the effect of diethyl pyrocarbonate on the demetalized enzyme we were able to calculate from the absorbance difference at 240 nm that 4·75 histidine residues per one molecule of the enzyme had been modified.

DISCUSSION

The kinetic measurements of the effect of the reaction medium on the activity of pea ADH have shown that the group which plays a role in the active center of the enzyme during the catalytic process has a pK-value of about 7 (ref.[9]). This pK suggests the participation of the imidazole groups of histidine[10]. ADH from horse liver (LADH)

TABLE I

Rate of pea ADH inactivation by diethyl pyrocarbonate as function of time of preincubation with NADH. Experimental conditions: k_0 inactivation rate constant of 1st order; activity measured after 20 min incubation of enzyme with diethyl pyrocarbonate; NADH 0·1 μmol; ethanol 10 μmol; NAD 0·05 μmol; diethyl pyrocarbonate 0·005 μmol; reaction medium volume 1 ml

Time of preincubation with NADH	% of inactivation	k_0 min
5	41	$2{\cdot}43 \cdot 10^{-2}$
10	37	$2{\cdot}15 \cdot 10^{-2}$
15	36	$2{\cdot}06 \cdot 10^{-2}$
20	34	$1{\cdot}99 \cdot 10^{-2}$
25	29	$1{\cdot}89 \cdot 10^{-2}$
30	26	$1{\cdot}74 \cdot 10^{-2}$
without preincubation with NADH	85	$1{\cdot}21 \cdot 10^{-1}$

and yeast ADH (YADH) contain a histidine residue in the neighborhood of the essential SH-group; the alkylation of this residue brings about a loss of enzymatic activity. This histidine residue can also be modified by diethyl pyrocarbonate[11]. It follows from our measurements of the change of absorbance at 240 nm as a function of time and from measurements of the correlation of the absorbance change with the activity decrease to zero value that ADH contains one reactive histidine residue essential for the activity of the given enzyme.

It is known that of the amino acids present in the active center[12] of LADH Cys 46, Cys 174, and His 67 play the role of zinc ligands. If we compare the number of histidine residues in native and demetalized pea alcohol dehydrogenase it becomes obvious that the number of residues modified in the demetalized enzyme is by roughly one residue higher than in the native enzyme. This phenomenon could be explained by postulating that the demetalization of the enzyme leads to the release of the bond between Zn (ref.[13]) and histidine in the active center of the enzyme which subsequently can react with diethyl pyrocarbonate. This could provide indirect evidence showing that histidine is one of the Zn-atom ligands of the active center of pea ADH, too.

The results of our measurements of the inactivation rate as a function of pH show that one group only of pK 7·1 is modified. The pK-value of this group is not altered even during the protective action of NADH on the enzyme: the value is merely shifted to pK 7·2. Both pK-values show that the imidazole group of histidine is modified.

The presence of NADH partly protects the enzyme against inactivation by diethyl pyrocarbonate, however, only on condition that the NADH concentration is of the order of its K_m-value. The protection of the enzyme by NAD could not the tested since a solution of pyrocarbonate in ethanol, *i.e.* in the substrate of the reaction catalyzed by ADH, was used. The results of the modification experiments carried out with ADH, iodoacetamide, and diethyl pyrocarbonate, where the absorbance increase at 240 nm was the same as in the absence of iodoacetamide, permit us to conclude that the thiol groups are not labeled by diethyl pyrocarbonate under our experimental conditions.

The reactivation of the completely modified and inactive enzyme by hydroxylamine, investigated in terms of absorbance decrease at 240 nm shows that the absorbance drops to the original value corresponding to the unmodified enzyme during 30 min. This indicates a complete removal of the ethoxyformyl groups from the enzyme within a short time; according to Melchior and Fahrney[14], this shows that the ethoxyformylimidazole bonds only are cleaved by hydroxylamine. The hydrolysis of bonds involving other groups, such as amino groups, requires a much longer time. The activity of the enzyme was restored to 88% of the original value by removal of hydroxylamine by dialysis. This is another piece of evidence in favor of the important role of the histidine residue on the molecule of pea alcohol dehydrogenase.

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