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HISTIDINE RESIDUES AT THE ACTIVE SITE OF MAIZE δ-AMINOLEVULINIC ACID DEHYDRATASE

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Key Word Index—*Zea mays*; Gramineae; maize; δ-aminolevulinic acid dehydratase; diethylpyrocarbonate; histidine residues; active site studies.

Abstract—Modification of maize δ-aminolevulinic acid dehydratase (ALAD) by diethylpyrocarbonate (DEP) caused rapid and complete inactivation of the enzyme. The inactivation showed saturation kinetics with a half inactivation time at saturating DEP equal to 0.3 min and $K_{DEP} = 0.3$ mM. Substrate δ-aminolevulinic acid (ALA) and competitive inhibitor levulinic acid protected against inactivation, thereby indicating that DEP modifies the active site. The modified enzyme showed an increase in absorbance at 240 nm which was lost upon treatment with 0.8 M hydroxylamine. Most of the activity lost by DEP treatment could be restored after treatment with 0.8 M hydroxylamine. The results suggest that DEP modifies 7.4 residues/mole of the enzyme. These histidine residues are essential for catalysis by ALAD.

INTRODUCTION

Identification of the amino acid residues which comprise the active site of an enzyme and the assignment of specific role to them are essential for understanding the mechanism of enzymatic reactions. The enzyme δ-aminolevulinic acid dehydratase (5-aminolevulinate hydrolyase, EC 4.2.1.24; ALAD) catalyses the synthesis of the pyrrole, porphobilinogen (PBG), which is then utilized for the formation of heme, porphyrins, chlorophylls and other tetrapyrrole compounds. ALAD has been studied in wide variety of animal [1, 2], plant [3–5] and microbial [6, 7] systems. Although the enzymes from rat and bovine liver have been extensively studied with respect to their properties and mechanism of action, not much information is available on the plant enzyme, especially from C-4 plants like maize. It is noteworthy that the enzymes from plant and microbial sources differ from the animal enzyme in several respects [3, 5, 8]. In the proposed mechanism for PBG synthesis in animal systems a lysine residue at the active site is postulated [9]. Histidine, cysteine and arginine residues [8, 10] have also been implicated in the reaction mechanism of ALAD. There has been no clear-cut evidence about the nature of various amino acid residues involved in the reaction mechanism of the plant enzyme. Inhibition by several sulphydryl blocking agents suggests that ALAD from plant sources may be a sulphydryl enzyme [11]. However, the role of these residues is not clear. A recent report on ALAD from spinach [8] suggest that an arginine residue is essential for substrate conversion.

Using a histidine selective reagent, diethylpyrocarbonate (DEP), we present evidence to show that histidine residues of maize ALAD are essential for activity. The role of these residues in catalysis is postulated based on the mechanism suggested for PBG formation in the animal system.

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RESULTS

DEP at neutral or slightly acidic pH values has been shown to modify histidine residues in proteins with considerable specificity [15]. Maize ALAD rapidly lost its activity when incubated in the presence of DEP at 25° at pH 6.2. The inactivation followed pseudo-first-order reaction kinetics. The time course of inactivation of the enzyme at different DEP concentrations is shown in Fig. 1. The order of reaction with respect to DEP was determined according to the method employed by several workers [16–18]. In this type of plot a straight line is expected with a slope equal to 'n', where 'n' is the number of inhibitor molecules reacting with each active unit to produce an inactive enzyme–inhibitor complex. When the data of Fig. 1 was plotted on this basis, an 'n' value of 0.95 was obtained (Fig. 2a). The 'pre-equilibrium' model predicts that $t = \frac{\ln 2}{k_2} + \frac{\ln 2}{k_2}\frac{K_{DEP}}{[DEP]}$ (19) where t is the half inactivation time. Thus a plot of t (min) vs $\frac{1}{[DEP]}$ should give a straight line with a Y intercept equal to minimum half inactivation time, i.e. the time for half inactivation at saturating concentration of DEP. Such a plot is shown in Fig. 2b with t minimum = 0.3 min and $K_{DEP} = 0.3$ mM. Thus inactivation shows saturation kinetics, as would be predicted if DEP was reversibly binding the active site prior to modification, presumably at the same site.

Protection of the enzyme activity against diethylpyrocarbonate inactivation

Substrate, ALA, and the competitive inhibitor levulinic acid offered substantial protection against DEP inacti-

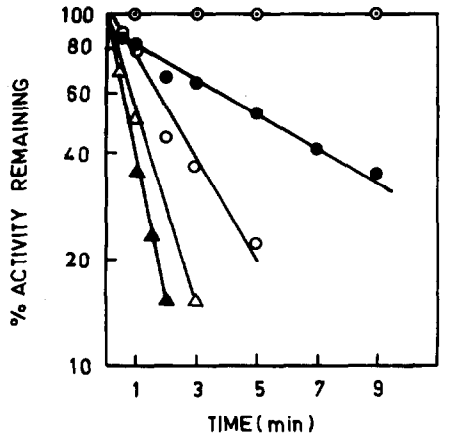

Fig. 1. Time course of inactivation of ALAD at different diethylpyrocarbonate concentrations. The enzyme in HEPES–KOH pH 6.2 was incubated with the indicated concentrations of diethylpyrocarbonate. At the indicated time intervals aliquots were removed and quenched in a buffer system containing 20 mM imidazole. ⊙, Ethanol; ●, 28 μM; ○, 56 μM; △, 112 μM; ▲, 224 μM DEP in ethanol.

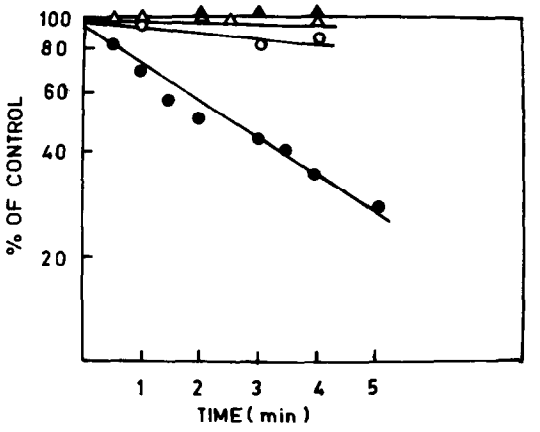

Fig. 3. Protection of ALAD against inactivation by diethylpyrocarbonate. The percent activity remaining was compared with control lacking diethylpyrocarbonate but contained the same amount of protective agent and ethanol. DEP concentration was 56 μM in all the samples. ▲, Ethanol; △, 2 mM levulinic acid + DEP; ○, 2 mM aminolevulinic acid + DEP; ●, only DEP in ethanol. Preincubations with ALA were done at 0° to minimize catalysis during preincubation.

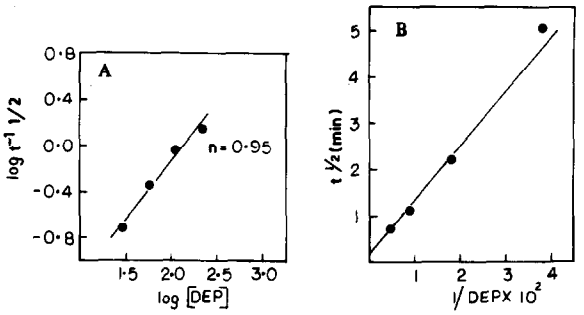

Fig. 2. (A) Log $1/t_{1/2}$ against log [DEP]; plot of the data in Fig. 1. (B) A plot of half inactivation time, t, versus 1/[DEP].

vation (Fig. 3). These two compounds had no effect on the formation of *N*-carbethoxyimidazole in a model system.

Amino acid residues responsible for inactivation

Even though at pH 6.0, DEP is highly specific for modification of histidine residues, other unwanted side reactions with lysyl, tyrosyl and cysteinyl residues have been reported. To rule out the possibility that the inactivation is due to the modification of residues other than histidine, the following checks were made:

(i) Reversal of inhibition by hydroxylamine to rule out the possibility of lysine modification (discussed below).

(ii) No decrease in the difference *A* was observed at 278 nm in the DEP modified enzyme (data not shown).

(iii) The enzyme was protected with hydroxymercuribenzoate and was subsequently modified with DEP. If the inactivation were due to thiol group modification rather than histidine group modification, hydroxymercuribenzoate should offer complete protection against DEP inactivation. The results clearly indicate that hydroxymercuribenzoate did not protect the enzyme against DEP modification, since the DEP inhibition was the same at 91% even after dithiothreitol (DTT) treatment.

Reversal of DEP inhibition by NH_2OH

Hydroxylamine at 0.8 M caused a considerable reversal of DEP inhibition with a concomitant decrease in *A* at 240 nm indicating a nucleophilic attack on *N*-carbethoxyhistidine (Fig. 4). The modified enzyme having 77%, 46% and 31% residual activity was reactivated to 91%, 74% and 63% respectively following 3 hr treatment with 0.8 M NH_2OH at 25°. The DEP modified enzyme in the absence of NH_2OH did not show any increase in residual activity. Even though the reversal of the *A* 240 nm increase was almost complete, following NH_2OH treatment (Fig. 4), a complete reactivation of the enzyme activity was not observed even after 16–18 hr incubation.

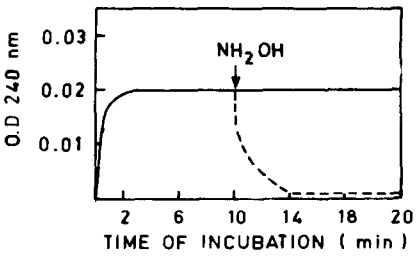

Fig. 4. Difference spectra of diethylpyrocarbonate modified enzyme (0.2 mg/ml) in HEPES–KOH pH 6.2. The spectrum was recorded by addition of 60 μM diethylpyrocarbonate to the sample cuvette. At the end of 10 min (as indicated by the arrow) 0.8 M NH_2OH was added to both reference and the sample cuvettes. The broken line indicates the loss of 240 nm absorbance upon addition of NH_2OH.

A similar observation has also been reported in case of other enzymes [22, 24].

Relation between activity and number of histidine modified

The modification of maize ALAD by DEP was accompanied by a sharp increase in differential *A* at 240 nm (Fig. 4). The difference spectrum of DEP-treated enzyme versus untreated enzyme, in the ultraviolet region, showed an *A* maximum at 240 nm (not shown). This absorption increase corresponded to 7.4 residues modified/mole of enzyme.

DISCUSSION

Histidine residues have been implicated in the catalytic mechanism of severah enzymes [10, 20–24] including ALAD from bovine liver. Our data show that maize ALAD was rapidly inactivated by DEP and the loss of activity was due to modification of histidine residues at or near the active site. The interpretation is supported by following observations: (1) At pH 6.2, modification by DEP is highly selective for histidine residues; (2) decarbethoxylation by NH_2OH showed that amino groups are not modified; (3) substrate, δ ALA, and competitive inhibitor levulinic acid showed almost complete protection of the enzyme activity indicating that DEP was interacting at or near the active site; (4) sharp increase in *A* at 240 nm which corresponded to modification of 7.4 residues/mole of enzyme.

Garrison and Hime [25] have reported that DEP reacts with *N*-acetylcysteine and that this reaction causes an increase in *A* at 230 nm which is also reversed by NH_2OH. However, the reaction occurs only in carboxylate buffer. We do not think that the present DEP modification is due to cysteine residues for the following reasons: (1) Protection of essential sulphydryl groups with *p*-hydroxymercuribenzoate and subsequent modification of the enzyme with DEP has clearly shown that modification of cysteinyl residues is not responsible for DEP inactivation; (2) DEP is not known to react with cysteine in HEPES buffer; (3) the differences in the absorption maxima between *N*-carbethoxyhistidine and the product of reaction between *N*-acetylcysteine and DEP; (4) the product of the reaction between *N*-acetylcysteine and DEP is less stable than *N*-carbethoxyhistidine, 2 hr as compared to 55 hr [24].

In the proposed mechanism for ALAD from animal sources, a lysine residue at the active site is postulated to form a Schiff base with the substrate molecule which gives rise to the A site of PBG, whereas covalent bonding was assigned to the other ALA, the source of P-site. It is known that thiol groups are essential for ALAD [11, 26]. The mechanism presented by Barnard *et al.* [26] involves the initial deprotonation by B^- of the ALA at the A site and the protonation of carbonyl group by BH^+ at the P site. In order to participate throughout the catalysis as proposed by Barnard *et al.* [26], each group (B^- or BH^+) must alternate its acid/base status. Consistent with the suggestion is our observation that histidine residues may be essential for ALAD catalysis making them a better candidate as a basic group involved in proton abstraction.

EXPERIMENTAL

All chemicals were purchased from Sigma. DEP was diluted with EtOH to the required concn which was calculated using an extinction coefficient of 3×10^3/M/cm at 240 nm.

Enzyme preparation. ALAD from field grown maize leaves was purified according to the following procedures. Maize leaves were de-ribbed and cut into small pieces. Leaves (150 g) were homogenized in a Waring blendor with 600 ml of 0.1 M Tris–HCl pH 8 containing 10 mM mercaptoethanol and 5% polyvinylpyrrolidone. The homogenate was filtered through cheesecloth and the filtrate was centrifuged at 7000 rpm for 30 min in GSA rotor of RC-5 centrifuge. The 20–35% $(NH_4)_2SO_4$ ppt contained most of the ALAD activity. This ppt was suspended in 10 mM Tris–HCl pH 8, containing 5 mM mercaptoethanol, 5 mM $MgCl_2$, 5% glycerol and 50 mM NaCl, and was dialysed against the same buffer. The enzyme was further purified through DEAE-cellulose (DE-52) and Blue-Sepharose chromatography. The final preparation showed a single protein band on PAGE at three different concns. The final sp. act. of the enzyme was 12.24 units/mg protein with a final yield of 14%. The details of the purification procedure and properties of maize ALAD will be published subsequently.

Protein determination. Protein concn was estimated by the method of ref. [12] using BSA as standard.

Enzyme activity. The enzyme activity was determined by measuring the amount of PBG formed according to the method of ref. [13]. A standard reaction mixture contained 100 μmol of Tris–HCl (pH 8.2), 1 μmol $MgCl_2$, 1 μmol mercaptoethanol, 3 μmol of ALA in a final vol. of 1 ml. The amount of PBG formed was calculated from a molar extinction coefficient of 6.2×10^4 for the Elrich salt. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of PBG per 60 min. Sp. act. is expressed as units per mg protein.

Chemical modification using DEP. The modification of the enzyme with DEP was done in HEPES–KOH buffer pH 6.2. The enzyme was treated with an appropriate concn of DEP in EtOH to yield final EtOH concn of 2–4%. This concn had no effect on the enzyme activity. The reactions were done at 25° and were stopped by addition of 20 mM imidazole. A small aliquot (50 μl) was assayed in a final vol. of 1 ml.

For studies on protection of the enzyme against DEP modification, the enzyme was preincubated with the substrate or the competitive inhibitor for 2 min. To minimize the catalysis during ALA preincubation, the DEP modification was done at 0°. The extent of modification at 0° or at 25°was not found to be any different.

The total number of histidine residues modified was calculated from the *A* at 240 nm (ε = 240 nm = 3200/M/cm) as described in ref. [14]. The M_r of maize ALAD was taken as 237000, determined using Sephadex G-200 column chromatography.

Reversal of DEP inhibition. The enzyme was modified by DEP at pH 6.2 in HEPES–KOH buffer to a various extent. To one batch of control and DEP treated enzyme was added 0.8 M NH_2OH (final concn) at pH 7.2, 2 mM dithiothreitol and incubated for 3 hr at 25°. All samples were assayed for ALAD activity after suitable dilution. NH_2OH does not inhibit ALAD activity.

Masking of sulphydryl groups prior to DEP modification. The masking of thiol groups was performed by incubation of ALAD with 0.5 mM *p*-hydroxymercuribenzoate for 10 min at 30° in 60 mM HEPES–KOH (pH 7). DEP treatment was carried out by incubation with 224 μM DEP for 3 min at 30°. The enzyme activity was measured before and after treatment with 25 mM dithiothreitol for 30 min.

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