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PRESENCE OF ESSENTIAL HISTIDINE RESIDUES IN NADP-MALIC ENZYME FROM MAIZE

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Key Word Index—*Zea mays*; Gramineae; NADP-malic enzyme; diethyl pyrocarbonate; histidine residues; active site studies.

Abstract—Modification of maize leaf NADP-malic enzyme by diethylpyrocarbonate (DEP) caused rapid and complete inactivation of the enzyme. The inactivation followed pseudo-first-order reaction kinetics. The inactivation of the enzyme showed saturation kinetics with a half inactivation time, at saturating DEP, equal to 0.15 min and K_{DEP} = 20 mM. The rate of inactivation was faster at 25° as compared to 0° ($t_{0.5}$ 0.75 min at 25° as against 5.6 min at 4° at 5 mM DEP). The enzyme was partially protected against DEP inactivation by NADP and complete protection was seen in the presence of NADP + Mg^{2+} + malate or its analogues, 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 0.25 M NH_2OH and almost complete recovery of the enzyme activity was also observed. The results suggest that DEP modifies 3.0 residues per subunit and of these at least two residue per subunit can be modified without loss of activity in the presence of substrate. Modification of about one histidine residue is correlated with the loss of enzyme activity.

INTRODUCTION

The C-4 plants show a unique dimorphic leaf structure as well as compartmentalized photosynthetic machinery [1, 2]. NADP-malic enzyme (EC 1.1.1.82) is the primary decarboxylating enzyme in maize which is responsible for releasing carbon dioxide in bundle sheath chloroplasts. This carbon dioxide is eventually fixed by RuBP carboxylase which is also present in the bundle sheath chloroplasts. Hence this enzyme plays a crucial role in the carbon dioxide concentrating mechanism [3]. Even though this enzyme has been purified to homogeneity from maize leaf [4], the reaction mechanism and the chemistry of the active site are not fully understood. The NADP-malic enzyme from pigeon liver, where it plays a major role in lipogenesis, has been extensively studied. The mechanism of reaction, role of metal ions and the amino acid residues at the active site have been well documented [5–7]. As far as we know, there have been no reports of chemical modification studies on maize NADP-malic enzyme.

The NADP-malic enzyme from maize leaf has a molecular weight of 227 000 and is composed of four identical subunits [4]. The subunit structure of the plant enzyme is comparable to that reported for liver NADP-malic enzyme [5]. However, we do not know whether each subunit of the plant enzyme contains a 'complete' active site and also whether a 'half of the site' mechanism exists in the plant enzyme [8].

To answer some of these questions we have undertaken studies on the maize enzyme. In the present report, the reaction of maize NADP-malic enzyme with the histidine selective reagent, diethylpyrocarbonate, is characterized. The results indicate that about one histidine residue is located at the active site and that this residue may be involved in the catalytic function of the enzyme.

RESULTS

Diethylpyrocarbonate at neutral or slightly acidic conditions has been shown to modify histidine residues in proteins with considerable specificity [9]. Maize NADP-malic enzyme was rapidly inactivated in the presence of DEP at 25° at pH 6.0. The inactivation followed pseudo-first-order reaction kinetics. The time course of inactivaion 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

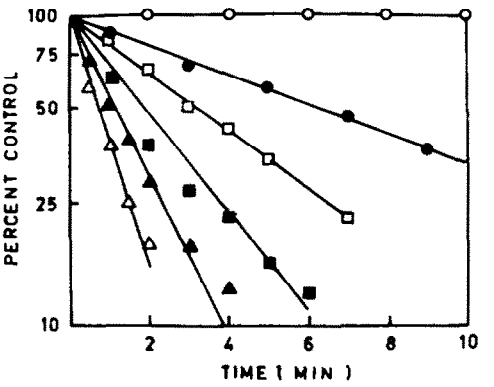

Fig. 1. Time course of inactivation of malic enzyme at different diethylpyrocarbonate (DEP) concentrations. The inactivation was carried out as described in the Experimental. At the indicated time intervals aliquots were removed and quenched in a 10 mM imidazole buffer. DEP in ethanol, ○——○:0.0 mM; ●——●: 0.5 mM; □——□:1.0 mM; ■——■:2.0 mM; ▲——▲:3.0 mM; △——△:5.0 mM.

methods employed by several workers [10–12]. 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 were plotted on this basis, an n value of 0.84 was obtained (Fig. 2a). The pre-equilibrium model predicts that:

$$t_{0.5} = \frac{\ln_2}{K_2} + \frac{\ln_2}{K_2}\frac{K_{DEP}}{(DEP)}$$

where $t_{0.5}$ is the half inactivation time [13]. Thus a plot of $t_{0.5}$ (min) vs 1/(DEP) should give straight line with a Y intercept equal to minimum half inactivation time, i.e. the time for half inactivation at a staturating concentration of DEP. Such a plot is shown in Fig. 2b with $t_{0.5}$ minimum $= 0.15$ min and $K_{DEP} = 20$ mM. Thus the inactivation shows the saturation kinetics that would be predicted if DEP was reversibly binding at the active site prior to modification, presumably at the same site.

Effect of temperature on DEP inactivation

The modification of the enzyme with DEP was faster at 25° as compared to 4°. The data in Fig. 3 show that $t_{0.5}$ for inactivation at 25° was 0.75 min as compared to 5.6 min at 4° at 5 mM DEP. Such a difference in the rates of inactivation was observed for several other enzymes with DEP treatment [14, 15].

Protection of the enzyme activity against DEP inactivation

The data presented in Table 1 show that malate, its analogues or Mg^{2+} alone did not offer any protection against DEP inactivation. NADP alone showed only partial protection. However, NADP along with Mg^{2+} and malate or its analogues strongly protected the enzyme against inactivation. This suggests a sequential ordered binding of the substrates.

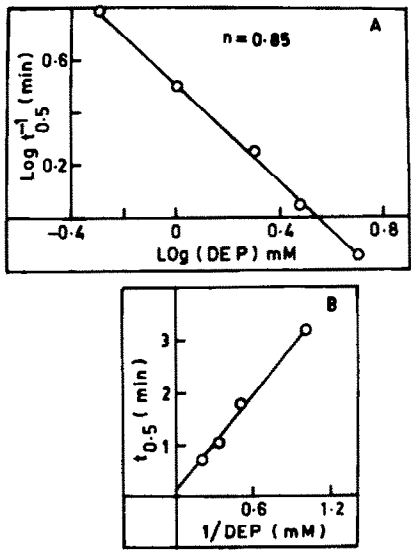

Fig. 2. (A) Plot of the data from Fig. 1. Log $t_{0.5}$ vs log (DEP). (B) Plot of half inactivation time $t_{0.5}$ vs reciprocal of DEP concentration.

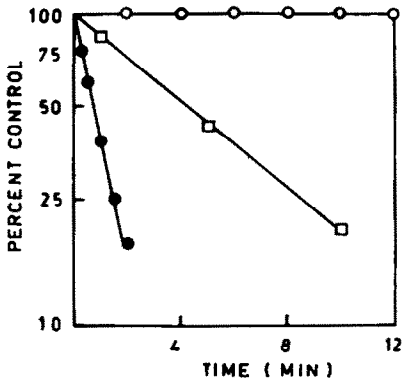

Fig. 3. Time course of inactivation of malic enzyme at different temperatures. The inactivation was carried out with 5 mM DEP as described in the Experimental except that the temperatures during incubations were, 25° (●——●) and 5°(□——□); control: (○——○).

Table 1. Inactivation of malic enzyme by DEP: protection by substrates and their analogues

Ligands	Percentage activity remaining
None	40
Malate or oxalate or tartronate	40
Mg^{2+}	40
(a) $NADP^+$ (0, 1 mM)	50
(b) $NADP^+$ (1.0 mM)	60
(c) $NADP^+$ (10.0 mM)	65
Mg^{2+} plus $NADP^+$ (0, 1 mM) + malate or oxalate or tartronate	100

Diethylpyrocarbonate (2 mM) inactivation was carried out for 3 min as given in the Experimental except that the enzyme was preincubated with the indicated ligands for 15 min before DEP addition. The concentrations of the ligands used in the experiments were 25 mM malate, 10 mM oxalate, 40 mM tartronate, and 20 mM Mg^{2+}. The ligands did not react with DEP in a model system.

Specificity of DEP inactivation

Even though at pH 6.0 DEP is highly selective for modification of histidine residues, other side reactions with lysyl, tyrosyl and cysteinyl residues have been reported [9]. To ascertain that the modification of the maize NADP–malic enzyme was not due the residues, other than histidine, the following experiments were performed.

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

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

(iii) The enzyme was protected with hydroxymercuribenzoate (PHMB) and was subsequently modified with DEP. If the inactivation was due to thiol group modification rather than histidine group modification, the enzyme protected with PHMB should have shown no inhibition with DEP, and complete activity should have been restored following dithiothreitol treatment. The

results clearly indicate that PHMB did not protect the enzyme against DEP modification, since the inhibition by DEP was the same at about 90% after 10 min DEP treatment at 0.5 mM even after dithiotheritol treatment.

Reversal of DEP inhibition by NH_2OH

Hydroxylamine at 0.25 M caused total reversal of DEP inhibition with a concomitant decrease in absorbance at 240 nm indicating a nucleophilic attack on *N*-carbethoxy-histidine (Fig. 4). The enzyme modified with 1 mM DEP for 6 min having a residual activity of 25% could be reactivated by addition of 0.25 M hydroxylamine in a time dependent way, reaching total reactivation by 30 min. Similarly the absorbance at 240 nm also decreased with time, upon addition of hydroxylamine, and at the end of 30 min there was no absorbance at 240 nm.

Relation between activity and number of modified histidine residues

The modification of maize NADP-malic enzyme by DEP was accompanied by a sharp increase in absorbance at 240 nm. This increase in absorbance was used to calculate the number of histidine residues modified using $E_{240}nm = 3.2 \times 10^3/M/cm$ [9].

A plot of percentage of remaining activity against the number of histidine residues modified indicates how many histidine residues could be involved in activity (Fig. 5). With no protecting ligands, one histidine residue could be modified without loss of enzyme activity and activity was lost when two or more histidine residues were modified per subunit. When protected with NADP + Mg + tartronate almost two histidine residues could be modified without loss of activity and the slope indicates that modification of 1.25 residues is correlated with the loss of enzymic activity.

DISCUSSION

Histidine residues have been implicated in the catalytic mechanism of several enzymes including NADP-malic enzyme from pigeon liver [14–19]. In the case of pigeon liver enzyme histidine residues were found to be located at the binding site of pyridine nucleotide [20]. Our data show that maize NADP-malic enzyme was rapidly inactivated by DEP and the loss of activity was due to modification of histidine residues at or near the active site.

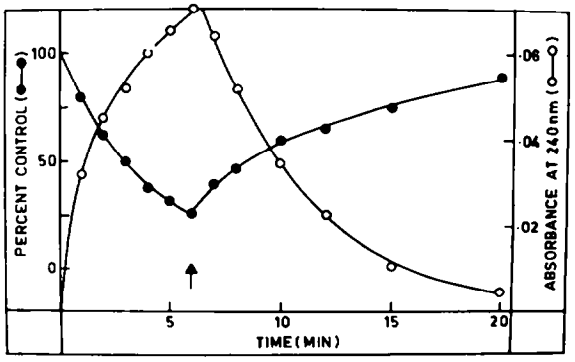

Fig. 4. Reversal of enzyme activity and 240 nm absorption by NH_2OH. Details are as given in the Experimental.

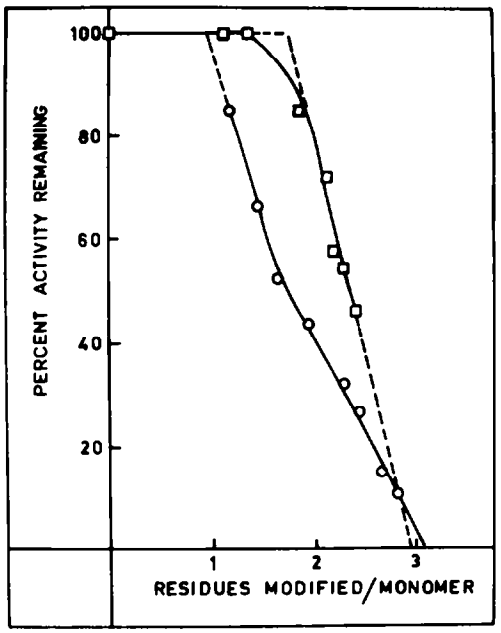

Fig. 5. Plot of the number of residues modified per monomer vs percentage activity remaining. The inactivation of malic enzyme (0.4 mg/ml) in 50 mM HEPES buffer (pH 6.0) with 0.5 mM DEP was carried out in a vol. of 1 ml in the presence or in the absence of protective ligands. Increase in absorbance at 240 nm with time was recorded and the activity at the same time intervals was measured as described in the Experimental. ○—○:None; □—□:NADP (0.2 mM) plus $MgCl_2$ (20 mM) plus tartronate (40 mM).

This conclusion is supported by the following observations: (1) at pH 6.0, modification caused by DEP is highly specific for histidine residues; (2) reversal by NH_2OH indicate that amino groups were not affected; (3) substrates NADP + Mg^{2+} + malate offered complete protection against DEP inactivation, showing the DEP was interacting at or near the active site; (4) increase in absorbance at 240 nm corresponded to the modification of three residues per subunit; (5) inactivation was faster at 25° as compared to 4°.

The reaction of DEP with *N*-acetylcysteine has been reported [21]. The reaction caused an increase in absorbance at 230 nm and it is also reversed by NH_2OH. We do not think the present modification by DEP is due to cysteine residues because the reaction of DEP and *N*-acetylcysteine occurs in carboxylate buffers which were not used in the present studies and also protection of exposed -SH groups with PHMB in the present studies rule out such a possibility.

NADP-malic enzyme isolated from pigeon liver has been shown to follow an orderly sequential mechanism with NADP or Mn^{2+} adding randomly followed by malate binding. The partial protection shown by NADP alone and total protection observed against DEP inactivation by addition of all the three components (NADP + Mg^{2+} + malate) suggest that the enzyme from maize leaf also follows the same orderly sequential mechanism. In view of the partial protection offered by NADP alone it is likely that, as in the case of liver enzyme, the histidine residues in this case may be involved in NADP binding.

Our future work on fluorescence of NADP binding with DEP modified enzyme will be useful in deciding this point.

The total number of histidine residues modified in the presence and absence of the substrates were found to be *ca* 12 residues modified per mole of enzyme. The non-essential histidine residues of maize NADP–malic enzyme react faster than the essential ones at pH 6.0. A similar observation has been reported for horse liver alcohol dehydrogenase [22].

Since NADP + Mg^{2+} + malate significantly reduced the rate of inactivation and the loss of activity in the presence of substrates correlated with the modifications of one histidine per subunit which may be located at the catalytic site.

EXPERIMENTAL

All chemicals were purchased from Sigma Chemical Co. DEP was diluted with absolute ethanol to the required concentration which was calculated, using an extinction coefficient of 3 $\times 10^3$/M/cm at 240 nm for the *N*-carbethoxy imidazole.

Enzyme preparation. NADP malic enzyme from field grown maize leaves was purified according to the method of Asami *et al.* [4]. The final sp. act. of the enzyme was 60–70 units/mg protein.

Protein determination. Protein concn was estimated by measuring the absorbance at 280 nm, and using a ratio of 1.00 D_{280}/ml = 0.87 mg/ml.

Enzyme activity. The standard assay for malic enzyme was carried out spectrophotometrically using an Amino DW2a spectrophotometer. The reaction mixture for measuring malate decarboxylation contained the following components: 50 mM HEPES–NaOH (pH 7.6), 2.5 mM EDTA, 25 mM $MgCl_2$, 0.4 mM NADP, 25 mM L-malate, 5 mM DTT, and enzyme soln in a total vol. of 1.0 ml. The reaction was carried out at 25° and the absorbance increase at 340 nm was measured.

Chemical modification using DEP. The enzyme modification using DEP was done in HEPES–KOH buffer (pH 6.0). The enzyme was treated with an appropriate concn of DEP in EtOH to yield a final concn of EtOH of 2–3%. The reactions were done at 25° and were stopped by adding 25 mM imidazole. A small aliquot (20 μl) was assayed in a final vol. of 1 ml.

For studies on the protection of the enzyme against DEP modification, the enzyme was preincubated with 0.2 mM NADP, 20 mM Mg^{2+} and 40 mM tartronate either separately or together for 15 min.

Reversal of DEP inhibition. The enzyme (414 μg/ml) was modified with 1 mM DEP, as mentioned above. After 6 min the enzyme soln was brought to a concn of 0.25 M hydroxylamine by adding 5 M hydroxylamine soln (pH 6.0). In one set of experiments, the absorbance at 240 nm was monitored, and in an otherset, the enzyme activity was monitored, by taking an aliquot of 10 μl from the incubation medium and assaying directly. The carry over hydroxylamine inhibited the activity by 10%.

Masking of thiol groups prior to DEP modification. The masking of thiol groups was performed by incubation of malic enzyme with 50 μM PHMB for 3 min at 25° and the DEP treatment was carried out by incubation of the enzyme with 5 mM DEP for various times. The enzyme activity was measured before and after DTT treatment (25 mM) for 30 min.

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