Biochimica et Biophysica Acta, 483 (1977) 228—235

BBA 68198

MECHANISM OF PIGEON LIVER MALIC ENZYME MODIFICATION OF HISTIDYL RESIDUES BY ETHOXYFORMIC ANHYDRIDE

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(Received January 29th, 1977)

Summary

Incubation of malic enzyme (L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) with ethoxyformic anhydride caused the time-dependent loss of its ability to catalyze reactions requiring the nucleotide cofactor $NADP^+$ or NADPH, such as the oxidative decarboxylase, the $NADP^+$-stimualted oxalacetate decarboxylase, the pyruvate reductase, and the pyruvate-medium proton exchange activities. Similar loss of oxidative decarboxylase and pyruvate reductase activities was affected by photo-oxidation in the presence of rose bengal. The inactivation of oxidative decarboxylase activity by ethoxyformic anhydride was accompanied by the reaction of $\geqslant 2.3$ histidyl residues per enzyme site and was strongly inhibited by $NADP^+$. Ethoxyformylation also impaired the ability of malic enzyme to bind $NADP^+$ or NADPH. These results support the involvement of histidyl residue(s) at the nucleotide binding site of malic enzyme.

Introduction

Malic enzyme (L-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) from pigeon liver catalyzes the following reactions (refs. 1—5):

Oxidative decarboxylation

$$\text{L-Malate} + NADP^+ \xrightarrow{Mg^{2+} \text{ or } Mn^{2+}} CO_2 + \text{pyruvate} + NADPH \qquad (1)$$

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Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

Decarboxylation

$$\text{Oxalacetate} \xrightarrow[(NADP^+)\ pH\ 4.5]{Mg^{2+}\ or\ Mn^{2+}} CO_2 + \text{pyruvate} \quad (2)$$

Reduction

$$\alpha\text{-Keto acid} + NADPH \xrightarrow{Mg^{2+}\ or\ Mn^{2+}} \alpha\text{-OH acid} + NADP^+ \quad (3)$$

Proton exchange

$$E + CH_3{-}\overset{O}{\overset{\|}{C}}{-}COO^- \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} E{-}CH_2{=}\overset{O^-}{\overset{|}{C}}{-}COO^- \quad (4)$$

Reaction 1, the oxidative decarboxylation of L-malate, functions in the lipogenic pathway by providing reducing equivalents for the synthesis of fatty acids (cf. ref. 1). The physiological importance of this enzyme prompted us to undertake a systematic investigation on the functional residues at the active site. Modification of a cysteinyl group by bulky reagents such as 5,5′-dithiobis(2-nitrobenzoic acid), *N*-ethylmaleimide, *p*-chloromercuribenzoate, or the active site-directed reagent bromopyruvate sterically hinders the C-C bond cleavage step, resulting in the loss of oxidative decarboxylase and oxalacetate decarboxylase activities [6—9]. Bromopyruvate was shown to alkylate malic enzyme with half site stoichiometry [9].

In the present study, we report the effects of photo-oxidation and ethoxyformic anhydride on the catalytic activities of this enzyme. Results of this study indicate the involvement of histidine residue(s) at the binding site of nucleotide cofactors.

Materials and Methods

Chemicals. Rose bengal was obtained from Colemen and Bell Co. and was purified according to Brand et al. [10]. The concentration of this compound was determined from its absorbance at 545 nm using a molar extinction coefficient of $9.5 \cdot 10^4\ M^{-1}\ cm^{-1}$. Ethoxyformic anhydride (diethyl pyrocarbonate) purchased from Eastman Kodak was dissolved in redistilled 95% ethanol immediately before each use. Tartronic acid and DTNB were products of Calbiochem. Tritiated water (100 mCi/g) was purchased from New England Nuclear. Dowex-1X resin was obtianed from Bio-Rad. It was treated with 0.5 N HCl and washed exhaustively with water until the effluent was neutral and free of chloride. Other chemicals used in this study were obtained as described previously [2,6]. Distilled, deionized water was used throughout this work.

Pigeon liver malic enzyme was purified according to Hsu and Lardy [1]. Preparations which showed homogeneity in the analytical ultracentrifuge were dialyzed exhaustively against 50 mM Tris · Cl buffer (pH 7.0) containing 10% glycerol before use. Protein concentration was determined from absorbance at 278 nm [1]. The tetramer and subunit molecular weights of 260000 and 65000, respectively, were used in all calculations [11].

Enzyme assays. Oxidative decarboxylase and pyruvate reductase activities were dtermined according to Hsu and Lardy [1], and Tang and Hsu [6], respec-

tively. The $NADP^+$-independent oxaloacetate decarboxylase activity was measured essentially as described by Kosicki [12] by following the disappearance of oxaloacetate at 260 nm and 24°C. The reaction mixture contained oxaloacetate, 0.76 μmol; $MnCl_2$, 1 μmol; and potassium acetate buffer (pH 4.5), 160 μmol in a total volume of 1.3 ml. Malic enzyme was added to start the reaction. All optical assays were monitored in a Cary Model 16 spectrophotometer equipped with a Cary Model 1626 recorder interface and Hewlett-Packard Model 7101B recorder. The cell compartment was thermoregulated to give the desired temperature. Pyruvate was tritiated in 0.5 ml of ^3H_2O (5 mCi) containing histidine · Cl (Tris) buffer, pH 6.5, 6.25 μmol; sodium pyruvate (neutralized), 5.36 μmol; $MgCl_2$, 0.8 μmol; NADPH, 0.535 μmol; $NaHCO_3$ (pH 7.0), 5 μmol and 0.77 nmol of active or ethoxyformylated enzyme for 5 h at 30°C essentially according to Bratcher [4]. The reaction mixture was quantitatively transferred to a Dowex-1 column (1 × 5 cm), and washed with water until the effluent was free of radioactivity. Trace amounts of enzymatically synthesized L-lactate and L-malate were eluted with stepwise addition of 0.005 N and 0.01 N HCl, respectively. Radioactive pyruvate was eluted by 0.05 N HCl in 3 ml fractions. Pyruvate was quantitated enzymatically essentially according to Hohorst [13], and radioactivity was counted on 0.5 ml aliquots in a liquid scintillation counter.

Photo-oxidation studies. The DTNB-modified enzyme was prepared by mixing malic enzyme (0.946 nmol) and DTNB (4 nmol) in 0.45 ml of 25 mM Tris · Cl buffer (pH 7.5) containing 5% glycerol. The inactivation was completed in 12 h at 24°C. Purified rose bengal (5 nmol) was then added, and the enzyme illuminated in a 5 ml beaker at 0°C by a 200 W incandescent bulb placed at a distance of 32 cm directly above the liquid surface. At the designated time intervals, 0.025 ml aliquots were withdrawn, reduced by dithiothreitol (12.5 mM) for 1 h at 24°C and then assayed for oxidative decarboxylase and reductase activities. In control experiments, enzyme samples incubated without illumination or rose bengal were completely reactivated by the reducing agent.

Ethoxyformylation studies. Ethoxyformylation of malic enzyme was carried out at 24°C by the addition of an ethanolic solution of ethoxyformic anhydride (ETF) to an enzyme solution in 50 mM Tris · Cl buffer (pH 7.0), containing 10% glycerol. The progress of reaction was monitored by 240 nm absorption measurements and the appropriate enzyme assays. The difference spectrum was measured in tandem cells. To each compartment (light path, 0.437 cm), 1 ml reagent mixture in 50 mM Tris · Cl buffer (pH 7.0), 10% glycerol was added according to the following arrangement. Ethoxyformylation was allowed to proceed to completion and the cells were then scanned for ultraviolet absorption.

Control Cell

Enzyme	ETF
+	+
buffer	buffer

Sample Cell

Enzyme	
+	
ETF	buffer
+	
buffer	

Results and Discussion

Photo-oxidation studies. The photo-oxidation experiments were performed on malic enzyme which has been pretreated with DTNB to protect the active-site thiol groups [6,8]. Illumination of the DTNB-modified enzyme in the presence of Rose Bengal resulted in the rapid irreversible loss of oxidative decarboxylase activity and a slower decrease of reductase activity (Fig. 1), suggesting the photo-destruction of functional histidine and possibly of tryptophan and tyrosine residues [14]. Methionine sulfoxide, the photo-oxidation product of methionine was converted to the parent compound by the dithiothreitol treatment in these experiments, thereby precluding the reaction of methionine as the cause of inactivation [15]. In order to improve the specificity of modification, the reagent ethoxyformic anhydride was used in further studies.

Ethoxyformylation of malic enzyme. Incubation of malic enzyme with ethoxyformic anhydride at pH 7.0 affected the loss of oxidative decarboxylase and reductase activities similar to those observed during photo-oxidation (Fig. 2A). At an ethoxyformic anhydride concentration of 0.83 mM and a malic enzyme concentration of 1.30 μM, 75% of the oxidative decarboxylase activity was lost in 17.5 min. The remaining activity was abolished by further addition of this reagent. Figure 2B shows Dowex chromatograms of tritiated pyruvate synthesized by malic enzyme. The incorporation of tritium into pyruvate was $4.59 \cdot 10^4$ dpm/h by the native enzyme and decreased to $5.8 \cdot 10^3$ dpm/h (or 12.6%) by the ethoxyformylated enzyme, indicating inactivation of the pyruvate-medium proton exchange activity. In contrast, the $NADP^+$-independent oxalacetate decarboxylase activity was unaffected by this treatment (Fig. 2A). In other experiments, it was shown that pretreatment of the enzyme with DTNB did not afford protection from ethoxyformic anhydride, indicating that the inactivation resulted from the reaction of non-thiol groups.

The ultraviolet difference spectrum of the ethoxyformylated enzyme versus the native enzyme showed the characteristic absorption of ethoxyformyl

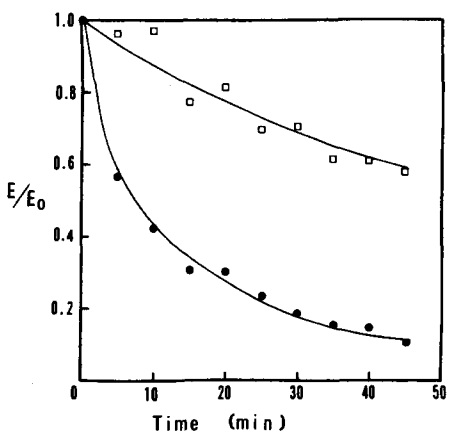


Fig. 1. Inactivation of malic enzyme by photo-oxidation. Malic enzyme was reversibly modified by DTNB and subjected to photo-oxidation at 0°C as described in Methods. Oxidative decarboxylase activity ●———●; reductase activity □———□. E/E_0 in Figs. 1–4 represent fractional enzyme activities.

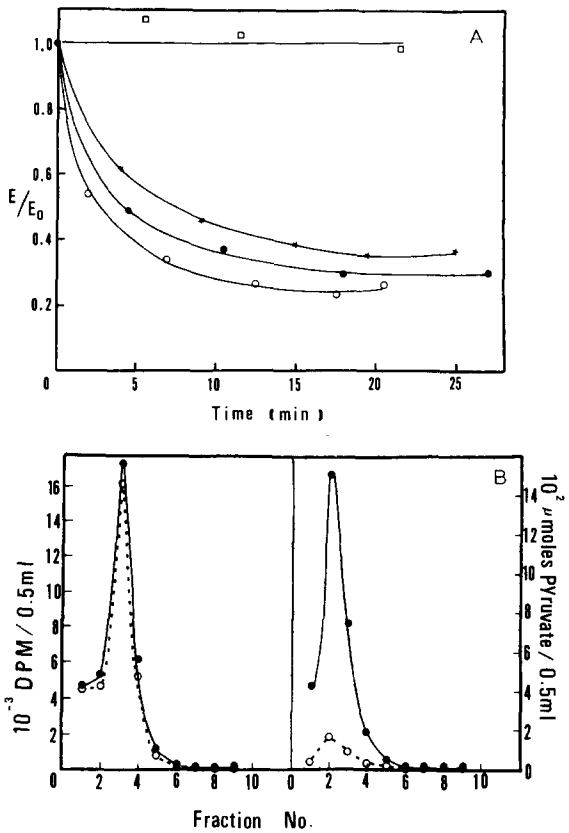
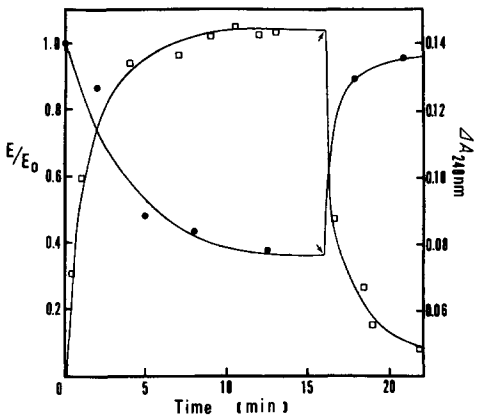


Fig. 2. Inactivation of malic enzyme by ethoxyformic anhydride. A. Time course. The malic enzyme tetramer and ethoxyformic anhydride concentrations were: 1.30 μM and 0.83 mM (★, ○); and 2.61 μM and 1.86 mM (□, ●), respectively. The incubations were carried out in 50 mM Tris · Cl buffer (pH 7.0), 10% glycerol as described in Methods. Oxidative decarboxylase activity (○———○; ●———●); reductase activity (★———★); oxalacetate decarboxylase activity (□———□). In a control experiment, the solvent (i.e. 9.5% ethanol, no ethoxyformic anhydride) had no detectable effect on oxidative decarboxylase activity. B. Inactivation of pyruvate-medium proton exchange activity. The tritiation experiments were carried out as described in Methods. Dowex-1 chromatograms of the pyruvate fractions of incubations with native enzyme (left) and ethoxyformylated enzyme (3.2% residual activity, right) are shown. Pyruvate, ●———●; radioactivity, ○———○.

histidine with a sharp maximum at 240 nm [16,17], indicating the modification of histidyl residues. The tyrosyl residues were unreactive with ethoxyformic anhydride, since no spectral change was observed at 278 nm [18]. The increase in 240 nm absorption during inactivation is shown in Fig. 3. The addition of hydroxylamine to the inactivated enzyme caused the rapid restoration of oxidative decarboxylase activity concomitant with the disappearance of the 240 nm peak. The reversibility of inactivation appears to rule out lysyl and arginyl residues as functional sites of modification, since the corresponding derivatives were resistant to nucleophilic attack [16,19].

Malic enzyme has a total of 46 histidyl residues [11], approximately half of which were reactive with ethoxyformic anhydride. A plot of the residual oxidative decarboxylase activity versus the number of histidyl residues modified by this reagent is shown in Fig. 4. Extrapolation of the linear plot to complete



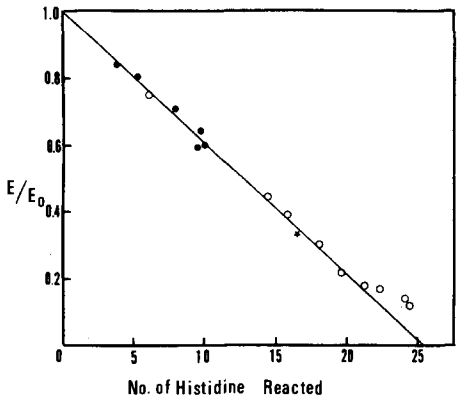


Fig. 3. Reactivation of ethoxyformylated malic enzyme by hydroxylamine. The ethoxyformylation of malic enzyme was performed at 24°C in a tandem cell as described in Methods for the measurement of difference spectrum. Malic enzyme, 3.0 μM; ethoxyformic anhydride, 3.45 mM. At the designated time (arrows), neutralized hydroxylamine (pH 7.0) was added to chamber 1 (left) of both the sample and control cells to a final concentration of 0.5 M. The oxidative decarboxylase activity (●) and 240 nm absorption (□) measurements were corrected for additions and withdrawals. The former was also corrected for inhibition (14.6%) by hydroxylamine.

Fig. 4. Correlation of residual oxidative decarboxylase activity and reaction of histidyl residues with ethoxyformic anhydride. Solutions of malic enzyme (2.54 μM) and ethoxyformic anhydride were placed in the chambers of tandem cells containing 1.0 ml of 50 mM Tris · Cl buffer (pH 7.0), and 10% glycerol as described in Methods. Temperature, 24°C. Aliquots were withdrawn at time intervals for oxidative decarboxylase assays as in Methods. The number of modified histidine molecules was calculated using a 240 nm extinction coefficient of 3200 for ethoxyformyl histidine [17]. The ethoxyformic anhydride concentrations were ●, 0.66 mM; ★, 1.38 mM; and ○, 3.3 mM.

inactivation yielded 25.6 residues per tetramer (or 6.4 residues per enzyme site), which included functional histidines, as well as non-functional histidyl residues having similar reactivity. The addition of tartronate, $NADP^+$, and Mn^{2+} under conditions described in Table I reduced the extent of activity loss to 27%, and the reaction of histidines to 4.1 residues per site. The difference of 2.3 protected residues per site for the partially inactivated enzyme therefore represented the minimum number of histidines essential for oxidative decarboxylase activity.

TABLE I

PROTECTION OF ETHOXYFORMIC ANHYDRIDE INACTIVATION BY SUBSTRATES AND TARTRONATE

The reaction mixtures (0.2 ml) contained malic enzyme (0.25 μM) and ethoxyformic anhydride (1.77 mM) in 25 mM Tris · Cl buffer (pH 7.0) and 5% glycerol. $NADP^+$, $MnCl_2$, L-malate, tartronate were 0.1 mM, 2.0 mM, 2.0 mM, and 40 mM when present. Oxidative decarboxylase activities were determined as described in Methods on aliquots taken before and 8 min after the addition of ethoxyformic anhydride.

Additions	% Inactivation
None	68
L-Malate	72
$MnCl_2$	80
$NADP^+$	38
$NADP^+$, $MnCl_2$, L-malate	31
$NADP^+$, $MnCl_2$, tartronate	30

The apparent Michaelis constants of L-malate, $NADP^+$, and Mn^{2+} (oxidative decarboxylase) and pyruvate (reductase) in the reactions catalyzed by the native enzyme, as determined under standard assay conditions at variable levels of the limiting substrate were 83, 1.2, 17.8 μM, and 7.7 mM, respectively. The corresponding values obtained for the partially ethoxyformylated enzyme with 14% residual oxidative decarboxylase activity were essentially unchanged, indicating that inactivation did not result from the decreased level of substrate saturation in the enzyme assays.

The effects of substrates and the inhibitor tartronate on the inactivation of malic enzyme are shown in Table I. L-Malate and Mn^{2+} slightly enhanced the inactivation, whereas $NADP^+$ afforded strong protection and this effect was increased by Mn^{2+} in combination with either L-malate or tartronate. The protective effect of $NADP^+$, and the selective inactivation of those reactions requiring the presence of $NADP^+$ or NADPH (i.e., oxidative decarboxylase, pyruvate reductase, proton exchange, Fig. 2) and not the $NADP^+$-independent decarboxylation of oxalacetate suggested that chemical modification by ethoxyformic anhydride abolished the ability of malic enzyme to bind the nucleotide cofactors. This suggestion was confirmed by our finding that $NADP^+$, which at 0.17 mM caused approximately 3-fold increase of the oxalacetate decarboxylase activity of the native enzyme [1], failed to stimulate this activity of the ethoxyformylated enzyme having 5% residual oxidative decarboxylase activity. Direct evidence was provided by the fluorescence titration experiments shown in Fig. 5. Titration of the native enzyme with NADPH resulted in

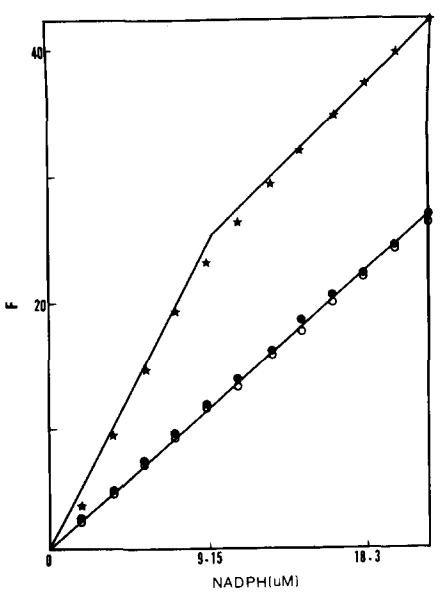


Fig. 5. Titration of malic enzyme with NADPH. All cuvettes contained Tris · Cl buffer (pH 7.0), 100 mM; water, and the following: ●———●, no enzyme; ★———★, native enzyme, 1.79 μM; and ○———○, ethoxyformylated enzyme with 8% residual oxidative decarboxylase activity, 1.68 μM. Titration was carried out with the addition of small aliquots of NADPH essentially as described previously [20]. The nucleotide fluorescence was monitored in an Aminco spectrofluorometer at an excitation wavelength of 350 nm and an emission wavelength of 470 nm. F represents fluorescence in arbitrary units.

binary complex formation and the enhancement of nucleotide fluorescence as was demonstrated previously [20]. In contrast, the ethoxyformylated enzyme did not enhance NADPH fluorescence, indicating the lack of complex formation.

Results of this study provide evidence for the presence of functional histidine(s) at the nucleotide binding site of malic enzyme. A histidine residue was implicated for the binding of NADPH by NADPH-adrenodoxin reductase [21]. The X-ray studies of Adams et al. [22] on lactate dehydrogenase showed that the nicotinamide ring of NAD^+ in the E-NAD^+-pyruvate complex was located proximal to histidine 195. Whether $NADP^+$ (or NADPH) interacts with the histidyl residue(s) of malic enzyme in a similar manner will be the subject of further investigation.

Acknowledgement

This study was supported by grants (AM 13390 and 5 S07 RR05402-15) from the National Institutes of Health.

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