Biochimica et Biophysica Acta, 660 (1981) 341–347
Elsevier/North-Holland Biomedical Press

BBA 69344

MODIFICATION OF ESSENTIAL ARGININE RESIDUES OF PIGEON LIVER MALIC ENZYME

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(Received March 3rd, 1981)

Key words Malic enzyme Substrate binding, Active site, Arginine residue, Chemical modification, (Pigeon liver)

The reaction of pigeon liver malic enzyme (L-malate $NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1 1 1 40) with dicarbonyl compounds (2,3-butanedione, methylglyoxal, 2,4-pentanedione, and phenylglyoxal) resulted in a rapid loss of its enzymatic activity The inactivation showed pseudo-first-order kinetics for all the dicarbonyls studied All the log (pseudo-first-order rate constants) vs log (dicarbonyl concentration) plots had slopes of near one, indicating approx 1 1 reagent-active site complexes Butanedione inactivation was reversible and was buffer-dependent Pentanedione-modified enzyme showed a new absorption peak at 310 nm. NADP could completely protect the enzyme from inactivation Oxaloacetate, ADP, AMP, NMN and adenosine were also effective in protection Complete inactivation of the enzyme was accompanied by a loss of about six arginine residues per enzyme monomer Butanedione-modified enzyme still bound NADPH as shown by fluorescence titration, nor was its binding with NADP impaired as determined by equilibrium gel filtration The arginine residues, therefore, do not function in the coenzyme binding However, the binding between the modified enzyme and [^{14}C]malate was significantly decreased These results led us to conclude that the arginine residues of malic enzyme are involved in the binding of the carboxyl group of substrate malate

Introduction

Pigeon liver malic enzyme (L-malate $NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1 1 1 40) catalyzes the oxidative decarboxylation of malate to CO_2 and pyruvate (M^{2+} = divalent metal ion)

$$\text{Malate} + NADP^+ \overset{M^{2+}}{\rightleftharpoons} CO_2 + \text{pyruvate} + NADPH + H^+ \quad (1)$$

This reaction is resolvable into the decarboxylase and reductase partial reactions

$$\text{Oxaloacetate} \xrightarrow[(\text{pH } 4\ 5)]{M^{2+}} CO_2 + \text{pyruvate} \quad (2)$$

$$\alpha\text{-Keto acid} + NADPH + H^+ \xrightarrow{M^{2+}} \alpha\text{-hydroxy acid} + NADP^+ \quad (3)$$

Chemical modification by ethoxyformic anhydride and photo-oxidation in the presence of rose bengal indicated the involvement of histidine residues in the nucleotide binding of malic enzyme [1] Nitration of the enzyme with tetranitromethane or acetylation with *N*-acetylimidazole led us to propose a role of tyrosine residues in the dicarboxylic acid binding [2] In addition, we found that oxidized NADP is an affinity label for this enzyme [3] Lysine was identified as the only amino acid residue modified by oxidized NADP, and this lysine residue was involved in nucleotide binding (Chang et al, unpublished data)

In our previous tyrosine modification experiments, both carboxyl groups of substrate were required to give protection against acetylation [2] Therefore we postulated that some other amino acid residues may be involved in the substrate binding This paper describes the inactivation of malic enzyme by arginine specific dicarbonyl compounds (for a review see Ref 4) Our results indicate that arginine residues

function in substrate binding for pigeon liver malic enzyme

Materials and Methods

Materials [U-^{14}C]Malic acid (Radiochemical Centre, U K), methylglyoxal, phenylglyoxal, NADP, NADPH, NMN, AMP, ADP, adenosine, adenine, nicotinamide, oxaloacetate, Sephadex G-25 (Sigma, U S A), L-malic acid, pyruvic acid (Calbiochem, U S A), 9,10-phenanthrenequinone (Aldrich, (U S A), 2,3-butanedione (Wako, Japan) and 2 4-pentanedione (Riedel-de Haen, F R G) were purchased from the designated sources All other chemicals were of reagent grade Distilled deionized water was used throughout this work

Pigeon liver malic enzyme was purified according to Hsu and Lardy [5] The purified enzyme was routinely checked for purity by polyacrylamide gel electrophoresis Protein concentration was determined spectrophotometrically at 278 nm, using an extinction coefficient of 0 86 for a 0 1% (w/v) protein solution [5] M_r 260 000 [6] was used for calculation of enzyme concentration

Enzyme assay Malic enzyme activity was assayed at 30°C according to Hsu and Lardy [5] The formation of NADPH was monitored continuously at 340 nm in a Gilford 250, Beckman 24 or Varian 635 spectrophotometer The reductase and decarboxylase partial activities of this enzyme were assayed according to Tang and Hsu [7] and Kosicki [8], respectively

Modification of malic enzyme with dicarbonyl compounds Butanedione, methylglyoxal, pentanedione or phenylglyoxal were dissolved in buffer just before use Modification experiments were performed at 24°C by the addition of the reagent into the enzyme solution. Immediately after the addition, a sample was withdrawn and assayed for the zero-time activity The progress of reaction was monitored by assaying the enzymatic activity on small aliquots withdrawn at convenient time intervals The kinetics of inactivation were examined with semilog plots of residual activity vs time The slopes of the lines represent the observed pseudo-first-order rate constants (k_{obs})

Protection studies Protection experiments were performed essentially as described above except that the enzyme was preincubated with the protective agent before adding the modifying reagent The k_{obs} of the inactivation was obtained from each experimental set and the percentage protection was calculated according to the equation

$$\{[k_{obs}\,(\text{unprotected}) - k_{obs}\,(\text{protected})]/k_{obs}\,(\text{unprotected})\}\times 100$$

Quantitation method for arginine content Malic enzyme was inactivated with butanedione until most of its activity had been lost The modified enzyme and a control sample were dialyzed overnight against water The arginine contents were then determined by the phenanthrenequinone method [9] The reaction between the enzyme and reagent was performed at 60°C for 1 h to get optimum color yield The fluorescence was measured in a Aminco-Bowman spectrofluorimeter The excitation and emission wavelengths were 312 and 395 nm, respectively A standard curve was made for the calculation of arginine content

Fluorescence titration Fluorescence titration of the native enzyme or butanedione-modified enzyme with NADPH was performed according to Hsu and Lardy [10] The nucleotide was excited at 350 nm and the emission fluorescence at 450 nm was measured In the control experiment, all reagent except the enzyme were added for correction of the quenching due to reagents

Binding studies by gel filtration The NADP binding capacity of the enzyme was measured by the method of Hummel and Dryer [11] A column of Sephadex G-25 was equilibrated with 0 1 mM NADP/ 50 mM Tris-HCl buffer (pH 7 0) The absorbance, at 260 nm, of the eluant was monitored When equilibrium was reached, as indicated by a constant baseline, 0 5, 0 25 and 1 ml water were applied to calibrate the column After the emergence of the last trough, the same amount of native or modified enzyme (with 3 5% residual activity) (0 24 mg in 0.6 ml equilibrium solution) was applied to the column and eluted with the equilibrium solution

Binding studies by equilibrium dialysis The malate binding of the native or modified enzyme was investigated by equilibrium dialysis essentially according to Pry and Hsu [12] In a total volume of 0 3 ml, both chambers of each cell contained 0 18 mM NADPH/ 3 3 mM $MnCl_2$/42 mM triethanolamine-HCl buffer (pH 7 0)/0 83 mM dithioerythritol/83 μM EDTA/

0 83 mM [^{14}C]malate (pH 7 0) (10^4 cpm/μmol) The native (15 7 μM or modified malic enzyme (12 6 μM) was added to one chamber Radioactivity in each chamber was determined before and after dialysis overnight at 24°C in a Packard 3320 liquid scintillation spectrometer

Results and Discussion

Modification of malic enzyme with dicarbonyl compounds

Incubation of malic enzyme with arginine-specific dicarbonyl compounds 2,3-butanedione [13], methylglyoxal [14], 2,4-pentanedione [15] or phenylglyoxal [14,16] all caused inactivation of the enzyme The activity loss followed pseudo-first-order kinetics in all cases For phenylglyoxal and butanedione, the pseudo-first-order kinetics were traced up to >97% inactivation The inactivation process was dependent on reagent concentration The inactivation of malic enzyme at different levels of phenylglyoxal or butanedione is shown in Fig 1 and Fig 2, respectively Similar results were obtained with methylglyoxal or pentanedione (data not shown) When k_{obs} for the inactivation was plotted against the reagent concentration, four straight lines go through the origin (Fig 3), indicating that there is no enzyme-reagent complex formed prior to inactivation From the

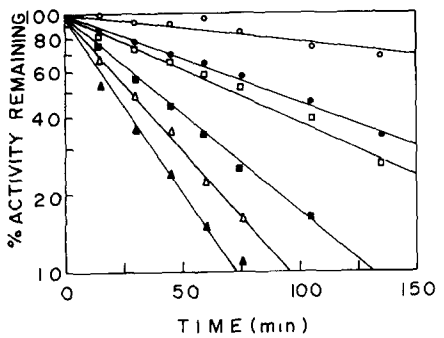

Fig 1 Inactivation of malic enzyme by phenylglyoxal Malic enzyme (0 3 μM) was incubated with different concentrations of phenylglyoxal in 37 mM borate buffer (pH 7 5) at 24°C Aliquots were removed and assayed for enzyme activity The phenylglyoxal concentrations were as follows ○——○, 0 62 mM, ●——●, 0 93 mM, □——□, 1 24 mM, ■——■, 1 86 mM, △——△, 3 11 mM and ▲——▲, 4 97 mM

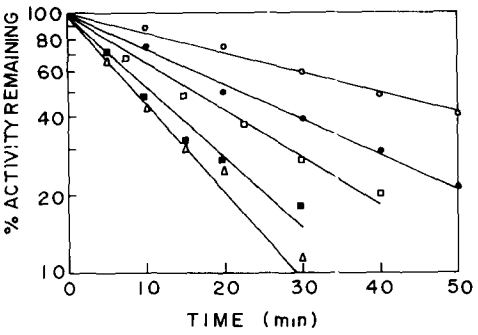

Fig 2 Inactivation of malic enzyme by butanedione Conditions were the same as in Fig 1 execpt that the enzyme and buffer concentrations were 0 69 μM and 48 mM, respectively The butanedione concentrations were ○——○, 2 82 mM, ●——●, 5 63 mM, □——□, 8 45 mM, ■——■, 11 3 mM and △——△, 14 1 mM

slopes of the lines, the second-order rate constants were calculated and are summarized in Table I A plot of log k_{obs} vs log reagent concentration also gave straight lines (data not shown) with the slope approaching 1 (Table I), suggesting that one molecule of reagent is required for the inactivation per molecule of the enzyme [17] Table I also show that α-dicarbonyls reacted with malic enzyme much faster than β-dicarbonyl (2,4-pentanedione) Pentanedione reacted with the enzyme at a slower rate The modi-

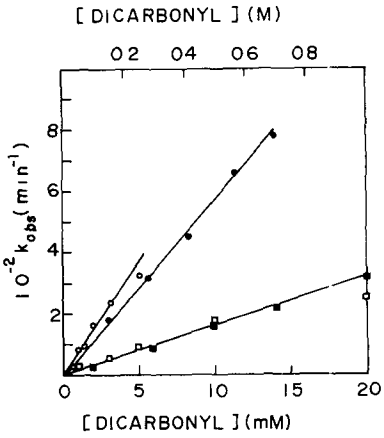

Fig 3 Effect of dicarbonyl concentrations on the rate of inactivation The pseudo-first-order rate constants (K_{obs}) from Figs 1 and 2, and similar plots for methylglyoxal and pentanedione inactivation were plotted against dicarbonyl concentrations ○——○, phenylglyoxal (mM), ●——●, butanedione (mM), □——□, methylglyoxal (mM), ■——■, pentanedione (M)

TABLE I

SECOND-ORDER RATE CONSTANTS FOR THE REACTION BETWEEN MALIC ENZYME AND DICARBONYL COMPOUNDS

The constants were calculated from the slopes of the lines in Fig 3 The last column shows the slopes of the lines in a log-log replot of the data from Fig 3

Reagent	k_{obs} (M^{-1} min^{-1})	Slope
Phenylglyoxal	7 08	0 98
2,3-Butanedione	5 48	1 05
Methylglyoxal	1 69	0 83
2,4-Pentanedione	0 032	1 2

fied enzyme showed an absorption peak at 310 nm, characteristic for the enamine or the pyrimidine formation from the lysine or the arginine modification, respectively [15] The increase of the absorbance was in good correlation with the decrease in enzyme activity (Fig 4) The modification of arginine was

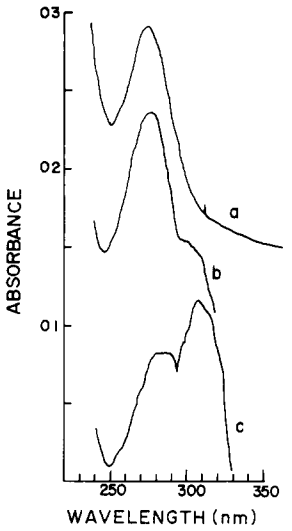

Fig 4 Spectra change of malic enzyme during pentanedione inactivation Malic enzyme (0 66 μM) samples were reacted with 48 mM pentanedione in 0 5 M carbonate buffer (pH 8 5) for 0, 0 5, 1, 2 or 4 h, respectively The enzyme activity remaining was 100, 65, 38, 21 and 0 8%, respectively These samples were then dialyzed overnight against 5 mm borate buffer (pH 7 5) and the ultraviolet spectra monitored Only the samples with 100% (a), 65% (b), and 0 8% (c) activity were shown for clarity

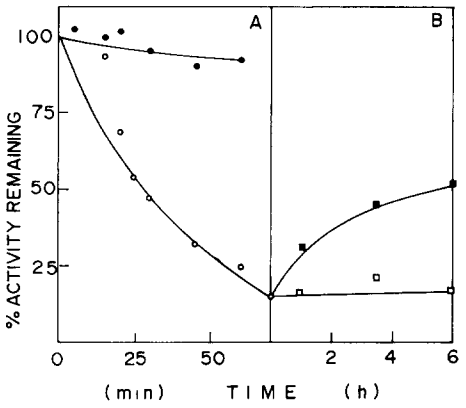

Fig 5 Effect of buffer on the inactivation of malic enzyme by butanedione A Malic enzyme (0 63 μM) was incubated with 6 45 mM butanedione in 45 mM borate buffer (pH 7 5) (○———○), or Tris-HCl buffer (pH 7 5) (●———●) B The solution in borate buffer was diluted 200-fold with 50 mM Tris-HCl buffer (■– – – –■) or borate buffer (□———□) Both dilution buffers contained 10 mg/ml bovine serum albumin

indicated by the irreversibility after hydroxylamine treatment [15]

The inactivation of malic enzyme by butanedione was found to be buffer-dependent Borate buffer specifically enhanced the rate of inactivation The inactivation in Tris-HCl buffer was much slower (Fig 5A) Dilution of the inactivated enzyme in borate buffer with Tris-HCl partially reversed the enzyme activity (Fig 5B), while dilution with borate buffer was without any effect These results indicate arginine modification by butanedione [4,18] The low reversibility could be due to a rearrangement of the intermediate glycol into a non-dissociable product by a pinacol-type rearrangement (cf Scheme I of Ref 4)

The modification of arginine residues was further demonstrated by comparing the arginine contents of the native and modified enzyme by the fluorimetric method Malic enzyme has a total of 102 arginine residues in an enzyme tetramer [6], of which about 72 ± 7 (weighted mean of eight determinations) reacted with phenanthrenequinone We found that a butanedione-modified enzyme with 3 5% residual activity had only 48 ± 6 (in eight determinations) arginine residues, indicating modification of about 24 arginine residues per tetramer (or 6 arginine per

enzyme subunit) Experimental errors make the above number an uncertain one Furthermore, the number of modified arginine residues may be underestimated if some modified groups were reversed after dialysis However, we did not observed reactivation of the enzyme activity after dialysis with water The attempts to determine the arginine content of NADP-protected enzyme were unsuccessful, probably due to the interference of NADP which could not be completely removed by dialysis, as noticed by abnormally high ultraviolet absorption of the dialyzed sample Prolonged dialysis caused protein precipitation

Both the reductase and decarboxylase partial activities of malic enzyme were decreased during the course of butanedione modification but at a slower rate than the overall oxidative decarboxylase inactivation (data not shown)

Protection studies

NADP (2 8 mM) was able to protect 90% of the enzyme activity when 0 7 μM malic enzyme was incubated with 3 11 mM phenylglyoxal in 40 mM borate buffer, at pH 7 5 and 24°C Full protection of malic enzyme against butanedione inactivation was afforded by NADP (Table II) The nucleotide analogues, ADP, AMP, NMN and adenosine, which are inhibitors of malic enzyme (Chang et al , unpublished data), all gave substantial protection However, adenine, nicotinamide, PP_i and P_i did not Substrate L-malate or pyruvate did not give any protection either On the other hand, oxaloacetate, a substrate in the decarboxylase partial activity (K_m = 70 μM) [2] and a competitive inhibitor vs L-malate (K_{is} = 0 32 mM) in oxidative decarboxylase activity [19], gave a substantial protection (Table II) Similar results were also obtained from the phenylglyoxal modification experiments These results suggest that inactivation of the enzyme was due to modification of active-site arginine residues It should be noted that malic enzyme has an ordered kinetic mechanism [20], with NADP as the leading substrate followed by malate The products are released in the order of CO_2, pyruvate, and NADPH The lack of protective effect of L-malate or pyruvate could be attributed to the low affinity between the enzyme and substrate in the absence of nucleotide The decarboxylation of oxaloacetate by malic enzyme is metal-dependent (reaction 2) NADP activates the reaction but it is not required, indicating that oxaloacetate can combine with the enzyme in the absence of nucleotide

Binding studies

The protection by nucleotides or oxaloacetate suggests the involvement of arginine residues in the nucleotide cofactor or the substrate binding site How-

TABLE II

PROTECTION OF MALIC ENZYME FROM BUTANEDIONE INACTIVATION

Malic enzyme (0 7 μM) was incubated with 2 82 mM butanedione in 20 mM borate buffer (pH 7 5), at 24°C, in the presence of NADP, ADP, AMP, NMN (6 34 mM), adenosine (3 7 mM) or oxaloacetate (2 53 mM) as indicated Enzyme assay and the calculation of % protection were as described in Materials and Methods

Additions	% Protection
NADP	100
ADP	59
AMP	34
NMN	52
ADP + NMN	70
AMP + NMN	64
Adenosine	54
Oxaloacetate	71

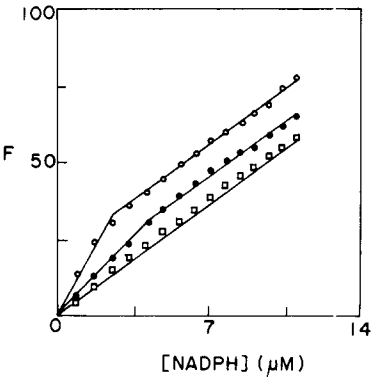

Fig 6 Fluorimetric titration of the native and butanedione-modified enzymes with NADPH All cuvettes contain 100 mM Tris-HCl buffer (pH 7 0) and the following □———□, without enzyme, ○———○, 1 35 μM native enzyme and ●———●, 1 35 μM butanedione-modified enzyme with 4 3% residual activity The quenching due to butanedione was corrected

ever, one can not assign a functional role to any amino acid residue only on the basis of protection experiments [21] Therefore we went on to carry out some binding studies to ascertain whether the modification of arginine residues abolishes the nucleotide or the substrate binding The binding between malic enzyme and NADPH was tested by fluorescence titration The fluorescence of NADPH was enhanced when bound to malic enzyme [10,12] Fig 6 shows the plot of relative fluorescence vs NADPH concentration Both the native and butanedione-modified enzyme gave fluorescence enhancement The reciprocal plot of 1/F against 1/[NADPH] gave straight lines with site dissociation constants for NADPH of 4 7 and 30 μM for the native and modified enzyme, respectively Similar results were obtained with the phenylglyoxal-modified enzyme The site dissociation constants for NADPH were found to be 9 and 43 μM, respectively, for the native and modified enzyme with 9 6% residual activity Thus, NADPH binding was not abolished but the affinity for NADPH appeared to be decreased after arginine modification The above results were unexpected since the involvement of arginine residue in the pyridine nucleotide coenzyme binding has been found in many dehydrogenases [4] and was suggested as a general role in the binding of pyrophoshate group throughout the family of pyridine nucleotide-dependent enzymes [22] To further characterize the nucleotide binding site, the binding between malic enzyme and NADP was examined by equilibrium gel filtration experiment A butanedione-modified enzyme did not lose its binding capacity toward NADP The bound NADP in the modified enzyme (with 3 5% residual activity) was about 80% as compared with the same amount of native enzyme Although the amount of bound NADP was slightly decreased by the modification, but it certainly can not be explained as the residual activity

Another possible function of arginine residues may be the interaction with the substrate carboxyl group Kinetic studies indeed suggested the involvement of positively-charged groups of pigeon liver malic enzyme in the binding of 1-carboxyl group of L-malate [23,24] From the inhibition and alternate substrate studies, Schimerlik and Cleland [23] suggested that binding of the 1-carboxyl group of L-malate was by ion pairing with lysine or arginine residues However, pH studies ruled out lysine residues since binding of oxaloacetate did not decrease at high pH, as it should have done if lysine was involved [24] 'pH' studies on the profile V/K for L-malate [24] showed a dependence of L-malate binding on a group with a pK value of 9 The pK value of arginine is usually much higher than 9 However, decrease of the pK value of arginine residues in the active center is possible as proposed by Patthy and Thèsz [25] to explain the site specificity of the α-dicarbonyls Competitive inhibition by anions vs L-malate [23] also suggests that there is a cationic group at the active site which binds the 1-carboxyl group of L-malate

We have examined the kinetic parameters of the native and the butanedione-modified enzyme (with 18% residual activity) The apparent K_m for pyruvate increased 3-fold from 18 to 56 mM after modification The K_m for L-malate, NADP and NADPH, and the K_i for oxaloacetate were not changed The V value of the oxidative decarboxylation of L-malate by the partially modified enzyme was decreased to 18% of the original These data do not permit the distinction between decreased binding of carboxylic acid substrate and the decreased binding of nucleotide coenzymes Direct evidence for the involvement of arginine residues in the malate binding was provided by equilibrium dialysis (Table III) The binding capacity of the modified enzyme with [^{14}C]malate was decreased The loss of enzyme activity was progressively increased with the decreased binding of [^{14}C]-malate

Results of the present study provide evidence for the presence of arginine residues in the malate binding site of pigeon liver malic enzyme Together with

TABLE III

MALATE BINDING ABILITY OF THE MODIFIED ENZYME

The binding of native and phenylglyoxal-modified enzyme with L-[^{14}C]malate was determined by equilibrium dialysis as described in Materials and Methods

Enzyme activity (%)	L-[^{14}C]Malate bound/subunit
100	0 98
71	0 63
65	0 58
31	0 43
12	0 23

the results from a previous study on the effects of nitration and acetylation on this enzyme [2], we may conclude that both arginine and tyrosine residues are involved in the binding of the carboxyl group of malate to the enzyme-coenzyme complex Arginine has been proposed to be in the substrate binding site of bovine lactate dehydrogenase [26], pig heart cytoplasmic malate dehydrogenase [27] and pig heart isocitrate dehydrogenase [28] The relativeness of these enzymes to malic enzyme implies that they might have a similar reaction mechanism

Acknowledgments

We thank Professor Foo Pan for reading the manuscript This work was supported in part by the National Science Council, Republic of China

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