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Mechanism of Pigeon Liver Malic Enzyme. Kinetics, Specificity, and Half-Site Stoichiometry of the Alkylation of a Cysteinyl Residue by the Substrate-Inhibitor Bromopyruvate†

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ABSTRACT: Malic enzyme from pigeon liver is alkylated by the substrate analogue bromopyruvate, resulting in the concomitant loss of its oxidative decarboxylase and oxalacetate decarboxylase activities, but not its ability to reduce α-keto acids. The inactivation of oxidative decarboxylase activity follows saturation kinetics, indicating the formation of an enzyme-bromopyruvate complex ($K \simeq 8$ mM) prior to alkylation. The inactivation is inhibited by metal ions and pyridine nucleotide cofactors. Protection of malic enzyme by the substrates L-malate and pyruvate and the inhibitors tartronate and oxalate requires the presence of the above cofactors, which tighten the binding of these carboxylic acids in accord with the ordered kinetic scheme (Hsu, R. Y., Lardy, H. A., and Cleland, W. W. (1967), *J. Biol. Chem. 242,* 5315-5322). Bromopyruvate is reduced to L-bromolactate by malic enzyme and is an effective inhibitor of L-malate and pyruvate in the overall reaction. The apparent kinetic constants (90 μM-0.8 mM) are one to two orders of magnitude lower than the half-saturation constant (K) of inactivation, indicating a similar tightening of bromopyruvate binding in the E-$NADP^+$ (NADPH)-Mn^{2+} (Mg^{2+})-BP complexes. During alkylation, bromopyruvate interacts initially at the carboxylic acid substrate pocket of the active site, as indicated by the protective effect of substrates and the ability of this compound to form kinetically viable complexes with malic enzyme, particularly as a competitive inhibitor of pyruvate carboxylation with a K_i (90 μM) in the same order as its apparent Michaelis constant of 98 μM. Subsequent alkylation of a cysteinyl residue blocks the C-C bond cleavage step. The incorporation of radioactivity from [^{14}C]bromopyruvate gives a half-site stoichiometry of two carboxyketomethyl residues per tetramer, indicating strong negative cooperativity between the four subunits of equal size, or alternatively the presence of structurally dissimilar active sites.

Pigeon liver malic enzyme (L-malate:$NADP^+$ oxidoreductase; decarboxylating EC 1.1.1.40) plays a major role in lipogenesis by providing NADPH reducing equivalents for the hepatic biosynthesis of fatty acids (Lardy et al., 1964; Young et al., 1964; Wise and Ball, 1964). The oxidative decarboxylation of L-malate (eq 1) catalyzed by this enzyme involves two mechanistically distinct functions: the $NADP^+$-dependent oxidation of L-malate, followed by decarboxylation of the enzyme-bound α-keto acid to produce CO_2 and pyruvate. These reactions are resolvable by the appropriate activity measurements into the decarboxylase, the reductase, and the pyruvate-medium proton-exchange partial reactions shown in eq 1-4 (Ochoa et al., 1948; Salles and Ochoa, 1950; Hsu and Lardy, 1967c; Tang and Hsu, 1973; Bratcher, 1974).

Oxidative Decarboxylation

$$\text{L-malate} + NADP^+ \overset{Me^{2+}}{\rightleftharpoons} CO_2 + \text{pyruvate} + NADPH \quad (1)$$

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Decarboxylation

$$\text{oxalacetate} + H^+ \xrightarrow[(NADP^+)]{Me^{2+}} CO_2 + \text{pyruvate} \quad (2)$$

pH 4.5

Reduction

$$\alpha\text{-keto acid} + NADPH + H^+ \xrightarrow{Me^{2+}} \alpha\text{-OH acid} + NADP^+ \quad (3)$$

Proton Exchange

$$E + CH_3C(=O)COO^- \overset{NADPH,\ HCO_3^-,\ Me^{2+}}{\rightleftharpoons} \times E\text{-}CH_2{=}C(O^-)COO^- + H^+ \quad (4)$$

Previous studies carried out in this laboratory have demonstrated that a thiol group is associated with the active site of malic enzyme; modification of this group by bulky reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) or *N*-ethylmaleimide sterically hinders the C–C bond cleavage step, resulting in the loss of its ability to catalyze the oxidative decarboxylase and decarboxylase reactions (cf. Tang and Hsu, 1974).

In order to obtain further information on the functon and environment of the active-site thiol group, the reaction of the substrate analogue bromopyruvate with malic enzyme was investigated. The present report shows that bromopyruvate is a substrate and an inhibitor of this enzyme, as well as an efficient affinity label, which binds to the substrate pocket prior to the alkylation of two out of the four presumed equivalent cysteinyl residues per tetramer.

Materials and Methods

Reagents. [2-^{14}C]- and [1-^{14}C]bromopyruvate were gifts of Dr. H. P. Meloche. Bromopyruvic acid, sodium fluoropyruvate, L-malic acid, sodium pyruvate, sodium tartronate, oxalacetic acid, dithiothreitol, cysteine, triethanolamine-HCl, 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2),[1] and EDTA were purchased from Calbiochem; $NADP^+$ and NADPH were from P-L Biochemicals; MalNEt and ClHgBzO were from Sigma; Tris and guanidine·HCl were from Schwarz/Mann; 2-butanol, formic acid, and 2-propanol were from Eastman; *N*-acetylcysteine and KCN were from Baker; ethyl acetate and Dowex-1 were from Mallinckrodt; Aquasol was from New England Nuclear; and crystalline lactate dehydrogenase was from Boehringer. The S-CHE-Cys standard was prepared from *N*-acetylcysteine according to Barnett et al. (1971). Distilled, deionized water was used throughout this work.

Pigeon liver malic enzyme was purified according to Hsu and Lardy (1967a). Highly active (i.e., specific activity of 37–40) preparations showing homogeneity in the analytical ultracentrifuge were used in this study. The purified enzyme was dialyzed exhaustively against 50 mM Tris-Cl buffer (pH 7.0) containing 10% glycerol. The dialyzed enzyme solution was stable at −20 °C for several months. Protein concentration was determined in a Gilford 2000 spectrophotometer at 278 nm, using an extinction coefficient of 0.86 for a 0.1% (w/v) protein solution (Hsu and Lardy, 1967a). A tetramer molecular weight of 260 000 (Nevaldine et al., 1974) was used in all calculations.

Enzyme Assays. All enzyme assays were performed spectrophotometrically in a Cary Model 16 spectrophotometer equipped with a Hewlett Packard recorder, at 30 °C unless otherwise noted. The reduction of $NADP^+$ (or oxidation of NADPH) was monitored at 340 nm. The oxidative decarboxylase activity (Mn^{2+} activated) was assayed according to Hsu and Lardy (1967a); the pyruvate reductase activity (Mg^{2+} activated) was assayed according to Tang and Hsu (1974). The reductive carboxylase activity was assayed in a medium containing $N(EtOH)_3$-Cl buffer (pH 7.0), 50 mM; $MnCl_2$, 4 mM; NADPH, 50 μM; $NaHCO_3$ (neutralized), 50 mM; pyruvate, 13.3 mM; and enzyme. The oxalacetate decarboxylase activity was determined according to Kosicki (1968) by following the disappearance of the substrate at 260 nm and 24 °C in a medium containing potassium acetate buffer (pH 4.5), 123 mM; $MnCl_2$, 0.77 mM; oxalacetate, 0.58 mM; and enzyme. The enzyme-catalyzed decarboxylase activity was obtained after correcting for the metal-depenent reaction.

A unit of enzyme activity is defined as the amount which catalyzed the conversion of 1 μmol of substrate under the conditions of each assay. Specific activity is expressed as enzyme units per milligram of protein.

Time-Dependent Changes of Malic Enzyme Activities during Reaction with Bromopyruvate. Kinetic studies of bromopyruvate modification were carried out by adding bromopyruvate (made up freshly each day in water; the pH was adjusted to ≤6.5 with KOH or NaOH) to the enzyme solution in Tris-Cl buffer at pH 7.0 (or 7.5). At various time intervals of incubation at 0 °C (or 24 °C), small aliquots were withdrawn and assayed for malic enzyme activities. Further reaction of the enzyme with bromopyruvate was negligible after dilution in assay mixtures containing substrate protectors.

The kinetics of inactivation was analyzed by plotting the natural logarithm of residual activity ($\ln E_t/E_0$) against time according to:

$$-\ln(E_t/E_0) = k_{obsd}t \quad (5)$$

where k_{obsd}, the pseudo-first-order rate constant, was obtained from the slope of the plot.

Covalent Labeling of Malic Enzyme with [^{14}C]Bromopyruvate. The lyophilized [2-^{14}C]- (or [1-^{14}C]-) bromopyruvate was kept frozen at −20 °C and purified freshly for each use. A water solution of the lyophilized material was passed through a Dowex-1 column, and eluted stepwise with water and 0.05 N HCl. The purified material in the acidic fraction was used immediately after neutralization as before. The radioactivity was determined in a Beckman scintillation counter. The concentration of bromopyruvate in each preparation was determined enzymatically with lactate dehydrogenase in a reaction mixture containing Tris-Cl buffer (pH 7.0), 0.5 M; NADH, 120 μM; [^{14}C]bromopyruvate; and crystalline lactate dehydrogenase, 50 μg.

In the direct labeling method, malic enzyme (10.2 μM) was incubated with [1-^{14}C]bromopyruvate (4.03 mM, 2.9×10^6 cpm) in Tris-Cl buffer (pH 7.0), 214 mM, and glycerol, at 3.3% at 24 °C. Samples were removed at various time intervals, diluted with dithiothreitol (42 mM) to stop the reaction, and assayed for oxidative decarboxylase activity. The samples were then reduced with $NaBH_4$ (200 mM, 20 min at 24 °C), dialyzed exhaustively against 50 mM Tris-Cl buffer (pH 7.0) to remove unbound radioactivity, and analyzed for protein bound radioactivity. The incorporation of covalent label was

[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); MalNEt, *N*-ethylmaleimide; ClHgBzO, *p*-chloromercuribenzoate; $N(EtOH)_3$, triethanolamine, CKM, carboxyketomethyl; CHE, carboxyhydroxylethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NADP^+$ and NADPH, oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate.

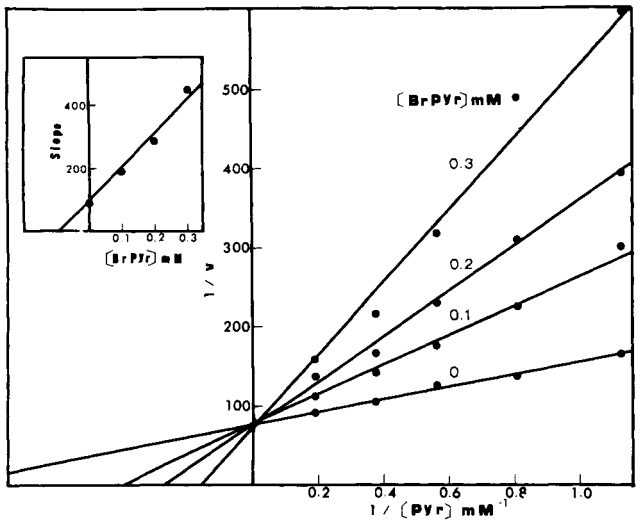

FIGURE 1: Inhibition of reductive carboxylase activity by bromopyruvate. The reductive carboxylase assays were performed as described under Materials and Methods at varying levels of the substrate pyruvate and the inhibitor bromopyruvate. The velocities were corrected for the reduction of bromopyruvate by malic enzyme. The slope replot is shown in the inset.

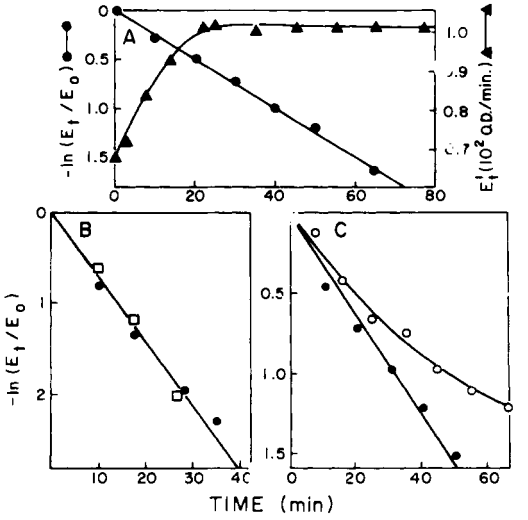

FIGURE 2: Modulation of malic enzyme activities by bromopyruvate. The incubations and enzyme assays were carried out as described under Materials and Methods. (A and C) Malic enzyme (0.38 μM), bromopyruvate (5 mM) in Tris-Cl buffer (pH 7.5, 67 mM); temperature, 0 °C; (B) malic enzyme (3.0 μM), bromopyruvate (3.4 mM) in Tris-Cl buffer (pH 7.0, 91 mM); temperature, 24 °C; oxidative decarboxylase activity (●); pyruvate reductase activity (▲); oxalacetate decarboxylase activity (□). The bromopyruvate reductase activity (○) was determined essentially as that for pyruvate using bromopyruvate (1.25 mM) as the substrate.

expressed as ^{14}C residues per enzyme tetramer. Malic enzyme was differentially labeled by preincubating the enzyme (15.3 μM) with unlabeled bromopyruvate (20.9 mM) in Tris-Cl buffer (pH 7.0), 200 mM; glycerol, 4.3% in the presence of tartronate (28.6 mM); $MnCl_2$ (4.3 mM); and $NADP^+$ (0.18 mM) to protect the essential group. The protected enzyme which retained 99% of oxidative decarboxylase activity after 10 min at 24 °C (as compared to 4.3% residual activity of an unprotected control sample) was precipitated with ammonium sulfate at 90% saturation, and dialyzed against 50 mM Tris-Cl buffer (pH 7.0). The dialyzed enzyme was incubated at 24 °C with [1-^{14}C] bromopyruvate (3.5 mM, 2.9 × 10^6 cpm) in Tris-Cl buffer (pH 7.0), 50 mM; and glycerol, at 10% to label the essential group. Samples were removed at various time intervals, reduced, and analyzed for enzyme activity and radioactivity incorporation as before.

Malic enzyme used for the identification of the S-CHE-Cys residue was reacted similarly with bromopyruvate, except that [2-^{14}C]bromopyruvate was used and after reduction with borohydride the protein was dialyzed exhaustively against water and hydrolyzed with 6 N HCl in a sealed, evacuated tube at 110 °C for 24 h. The hydrolysate was lyophilized and redissolved in water several times to remove traces of HCl, and subjected to descending chromatography on Whatman No. 1 filter paper in three solvent systems: ethyl acetate–pyridine–H_2O (120:50:40, v/v); 2-butanol–88% formic acid–H_2O (15:3:2, v/v); or 2-propanol–pyridine–formic acid–H_2O (30:20:6:24, v/v). Radioactivity was detected by cutting the paper into 1-cm strips which were counted in 10 ml of Aquasol in a scintillation counter. The R_f values of the radioactivity peaks were compared to that of the S-CHE-Cys visualized by ninhydrin spray (0.5% ninhydrin in 1-butanol). The standard S-CHE-Cys was prepared from *N*-acetylcysteine and bromopyruvate essentially according to Barnett et al. (1971), and the purity established by the appearance of a single ninhydrin positive spot in all three solvent systems.

Results

Kinetic Properties of Bromopyruvate. Bromopyruvate, an analogue of the substrate pyruvate, was reduced by malic enzyme according to eq 3. The reaction was linear with time and enzyme concentration and required the presence of a divalent metal, since no detectable rate was observed in a control sample containing EDTA (30 mM) in place of Mg^{2+} to chelate trace amounts of contaminating metal. The reduction product was identified as L-bromolactate by paper chromatography in a solvent system of 1-butanol–acetic acid–H_2O (12:3:5, v/v) using L-bromolactate synthesized by the method of Berghaüser et al. (1971) as the standard. The addition of $NaHCO_3$ had no effect on the rate of NADPH oxidation, indicating that bromopyruvate was not carboxylated to bromomalate.

The kinetic parameters of bromopyruvate and pyruvate were obtained from double reciprocal plots of velocities at varying levels of each substrate. The apparent Michaelis constants and maximum velocities of bromopyruvate and pyruvate were 0.45 mM (V_m = 0.29 μ/mg) and 12.5 mM (V_m = 1.74 μ/mg), respectively, in the 8 mM Mg^{2+}-activated reactions (Figure 6A and B; Table II), or 98 μM (V_m = 2.95 μ/mg) and 1.1 mM (V_m = 2.61 μ/mg) in the 0.4 mM Mn^{2+}-activated reactions (Table II). Bromopyruvate was previously shown as a classical noncompetitive inhibitor of L-malate in oxidative decarboxylation (Mn^{2+} activated, pH 7.5) with identical slope (K_{is}) and intercept (K_{ii}) inhibition constants of 0.8 mM (Table II). This compound also inhibited pyruvate competitively in reductive carboxylation (Mn^{2+} activated, pH 7.0) with a K_{is} value of 90 μM (Figure 1, Table II), indicating binding at the pyruvate site.

Modulation of Malic Enzyme Activities by Bromopyruvate. Incubation of malic enzyme with bromopyruvate at neutral pH led to the irreversible alkylation of the enzyme and the pseudo-first-order loss of overall oxidative decarboxylase activity (Figure 2A–C) up to >95% inactivation. A parallel decrease in oxalacetate decarboxylase activity was also observed (Figure 2B), indicating destruction of a step (or steps) necessary for the decarboxylase function of the enzyme. The reductase activity was changed relative to the structure of the reduction substrate; thus, the activity on bromopyruvate was

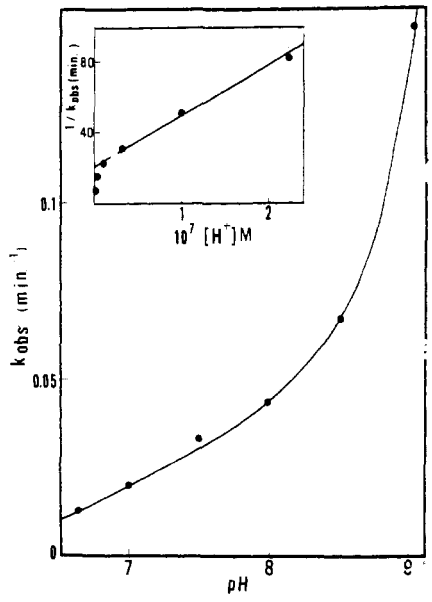

FIGURE 3: The effect of pH on bromopyruvate inactivation. The conditions of this experiment were as described in the legend for Figure 2A, except the Tris-Cl buffer was adjusted to the desired pH values. The k_{obsd} for the inactivation of oxidative decarboxylase activity were obtained according to eq 5. In control experiments without bromopyruvate, the enzyme was fully active in the pH range studied. The $1/k_{obsd}$ vs. [H$^+$] plot is shown in the inset.

decreased (Figure 2C), whereas the activity on pyruvate was enhanced up to a maximum of 1.5-fold (Figure 2A).

Dependence of Inactivation Rate on Bromopyruvate Concentration, pH, and Temperature. The dependence of the loss of oxidative decarboxylase activity on bromopyruvate concentration was studied in a previous paper (Chang and Hsu, 1973). A plot of the first-order rate constant (K_{obsd}) against bromopyruvate concentration was hyperbolic, indicating saturation behavior which was confirmed by the linear double reciprocal plot (Chang and Hsu, 1973; Figure 2). This behavior is in accord with a two-step reaction sequence whereby the alkylation step is preceded by the reversible binding of bromopyruvate to give an E-BP complex:

$$E + BP \overset{K}{\rightleftharpoons} E\text{-}BP \xrightarrow{k_2} E\text{-}Pyr + Br^- \qquad (6)$$

where k_2 is the limiting rate constant at infinite bromopyruvate concentration and K is the apparent half-saturation constant, or, when k_2 is rate limiting as in the present case, the dissociation constant of bromopyruvate (Fan and Plaut, 1974).

The reciprocal equation is:

$$\frac{1}{k_{obsd}} = \left(\frac{K}{k_2}\right)\left(\frac{1}{[BP]}\right) + \frac{1}{k_2} \qquad (7)$$

The values of k_2 at pH 7.0 (0 °C), pH 7.0 (28 °C), and pH 7.5 (0 °C) determined from experimental plots according to eq 7 were 0.028, 0.42, and 0.067 min^{-1}, respectively, indicating that the rate of alkylation was dependent on pH and temperature. In contrast, the corresponding values of dissociation constant K remained relatively constant throughout the experimental range and were 8.45, 7.21, and 8.33 mM, respectively. The effect of pH on inactivation rate was further investigated in order to estimate the pK of the essential residue. A plot of k_{obsd} vs. pH (Figure 3) was analyzed according to eq 8 (Lindley, 1962):

$$\frac{1}{k_{obsd}} = \frac{1}{k_{max}K}[H^+] + \frac{1}{k_{max}} \qquad (8)$$

TABLE I: Protection of Bromopyruvate Inactivation by Substrates and Inhibitors.[a]

Expt	Additions	% Protection: Bromopyruvate	% Protection: Nbs_2
1[b]	L-Malate	0	0
	$NADP^+$	14	17
	Mn^{2+}	60	50
	$NADP^+$ + L-malate	30	
	Mn^{2+} + L-malate	60	
	Mn^{2+} + $NADP^+$	76	
	Mn^{2+} + $NADP^+$ + L-malate	92	50
	Pyruvate	0	0
	NADPH	30	
	Mg^{2+}	60	50
	NADPH + pyruvate	14	
	Mg^{2+} + pyruvate	60	
	Mg^{2+} + NADPH	60	
	Mg^{2+} + NADPH + pyruvate	68	33
	Mg^{2+} + NADPH + pyruvate + HCO_3^-	68	
2[c]	Tartronate	0	
	Mn^{2+} + $NADP^+$ + tartronate	97	
3[d]	Oxalate	0	
	Mg^{2+} + NADPH	39	
	Mg^{2+} + NADPH + oxalate	74	

[a] Malic enzyme was incubated at pH 7.0 with bromopyruvate at 0 °C or with 5,5′-dithiobis(2-nitrobenzoic acid) at 24 °C in the absence and presence of substrates and inhibitors. The k_{obsd} of the inactivation of oxidative decarboxylase activity were obtained from each experimental set. The percent protection was calculated according to the equation: $\{[k_{obsd}$ (unprotected) $- k_{obsd}$ (protected)$]/k_{obsd}$ (unprotected)$\} \times 100$. [b] Malic enzyme, 1.4 μM; Tris-Cl buffer (pH 7.0), 0.5 M; bromopyruvate, 15 mM; Nbs_2, 83 μM; L-malate, 1.5 mM; pyruvate, 40 mM; $NADP^+$, 0.13 mM; NADPH, 27 μM; Mg^{2+}, 4 mM; Mn^{2+}, 2 mM; $NaHCO_3$, 10 mM. [c] Malic enzyme, 0.31 μM; Tris-Cl buffer (pH 7.0), 31 mM; bromopyruvate, 9.3 mM; tartronate, 18.8 mM; Mn^{2+}, 1.25 mM; $NADP^+$, 70 μM. [d] Malic enzyme, 0.83 μM; Tris-Cl buffer, 63 mM; bromopyruvate, 14 mM; oxalate, 0.75 mM; Mg^{2+}, 4 mM; NADPH, 26.9 μM.

where K is the ionization constant of the group involved, k_{max} is the maximum reaction rate, and [H$^+$] is the hydrogen ion concentration. A plot of $1/k_{obsd}$ against [H$^+$] would be linear, allowing for the direct calculation of k_{max} and K. The experimental plot (Figure 3, inset) was linear up to pH 8.0, followed by a significant downward curvature at higher pH values presumably owing to the modification of additional sites. The high rate of inactivation at pH greater than 8 was not caused by hydroxypyruvate, the hydrolysis product of bromopyruvate, since the results of a control experiment showed that the former was only 5% as reactive with malic enzyme as the parent compound. Extrapolation of the linear portion gave a k_{max} value of 0.05 min^{-1} at a bromopyruvate concentration of 5 mM, and an ionization constant (K) of 0.18×10^{-9} M. The latter value corresponds to a pK of 8.3, which is consistent with the known values for thiol and α-amino residues (Cohn and Edsall, 1943).

Protection of Bromopyruvate Inactivation by Substrates and Inhibitors. Table I shows the effects of substrates and inhibitors on inactivation. Protection (60%) was afforded by divalent metal ions Mn^{2+} and Mg^{2+}, and by the nucleotide cofactors $NADP^+$ and NADPH (14–30%). The carboxylic acid substrates L-malate and pyruvate did not afford protection unless both a metal ion and a nucleotide cofactor were present.

TABLE II: Kinetic Parameters of Native and Modified Malic Enzyme.

			Substrate or Inhibitor									
			Bromopyruvate			Pyruvate		L-Malate		Tartronate,	Oxalate	
Enzyme	Reaction	Metal Cofactor	K_m (mM)	K_i (mM)	V_m (μ/mg)	K_m (mM)	V_m (μ/mg)	K_m (mM)	V_m (μ/mg)	K_i (μM)	K_{is} (μM)	K_{ii} (μM)
Native	Oxidative decarboxylase	Mg^{2+}				6.4[a]		0.086[a]				
		Mn^{2+}		0.8[b]				0.042[c]	37–40			
	Reductase	Mg^{2+}	0.45[c]		0.29[c]	12.5[c]	1.74[c]				2.3[g]	18[g]
		Mn^{2+}	0.098[d]		2.95[d]	1.1[d]	2.61[d]			~20[f]		
	Reductive carboxylase	Mn^{2+}		0.09[e]		1.1[e]	15.4[e]				1.8[g]	10.0[g]
Modified	Reductase	Mg^{2+}	2.5[c]		0.29[c]	8.13[c]	1.74[c]					

[a] Data from Hsu et al. (1967d). [b] Data from Chang and Hsu (1973). [c] Calculated from Figure 6. [d] Reductase assays were performed as described in Figure 6 legend, except that $MgCl_2$ was replaced by $MnCl_2$ (0.4 mM). [e] Calculated from Figure 1. [f] Calculated from Figure 4. [g] Data from Hsu et al. (1976).

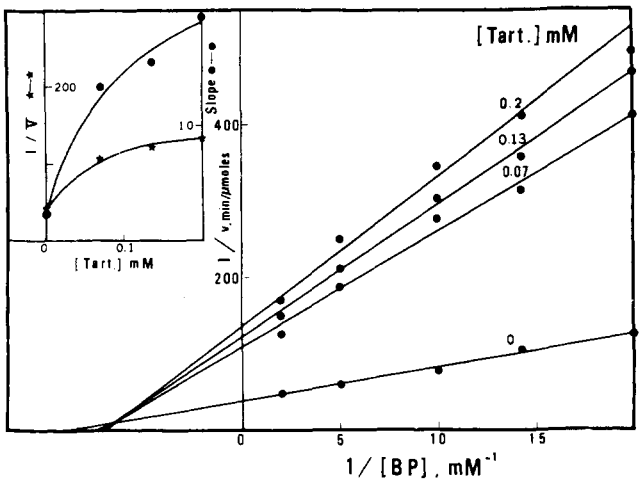

FIGURE 4: Inhibition of bromopyruvate reduction by tartronate. The reductase assays were performed essentially as described in the legend to Figure 2 at varying levels of the substrate bromopyruvate and the inhibitor tartronate with Mn^{2+} (4 mM) as the activating metal. Corrections were not made for the decreases in enzyme concentration due to alkylation by brompyruvate during enzyme assays. The presence of 0.11 mM NADPH effectively inhibited this reaction; the extent of alkylation in the 3-min assay period was estimated to be 0.15% of total enzyme. The slope and intercept replots are shown in the inset.

Hence, maximal substrate protection was obtained in incubations containing Mn^{2+}, $NADP^+$, L-malate (92%), or Mg^{2+}, NADPH ($NaHCO_3$), pyruvate (68%). The weaker protective effect of pyruvate appeared to be due to the lower affinity of the keto acid for the enzyme. Under these conditions, the enzyme-bound substrate precluded the formation of the E–BP complex preliminary to alkylation (eq 6).

Tartronate is a noncompetitive inhibitor of L-malate decarboxylation (Stickland, 1959) and an intersecting hyperbolic noncompetitive inhibitor of bromopyruvate reduction, with K_i values of ~20 μM (Figure 4, Table II). Oxalate is a noncompetitive inhibitor of pyruvate in the reductase and reductive carboxylase reactions with K_{is} values of 1.8–2.3 μM and K_{ii} values of 10–18 μM (Table II). The potent inhibitory effects of tartronate and oxalate prompted an examination of their effects on bromopyruvate inactivation. Like the carboxylic acid substrates, these inhibitors provided significant protection only in the presence of metal ions and a nucleotide cofactor. The protection afforded by Mn^{2+}, $NADP^+$, tartronate was 97% and that by Mg^{2+}, NADPH, oxalate was 74% (Table I). These results imply that tartronate and oxalate are bound to the substrate site of malic enzyme and further indicate that their binding is tightened by metal ions and/or nucleotide similar to the tightening of L-malate binding to the E–Mn^{2+} complex by NADPH (Hsu et al., 1976), yielding dead-end complexes in which the substrate pocket is no longer accessible to bromopyruvate.

Table I also shows strong protection (50%) against Nbs_2 inactivation by divalent metal ions. The carboxylic acid substrates did not afford protection despite the likelihood that the same thiol group is involved (Table IV). This lack of substrate effect on Nbs_2 inactivation indicates that reaction of this aromatic disulfide with malic enzyme occurs without complex formation at the substrate site, as expected from its structural dissimilarity to the substrate and the simple bimolecular kinetics of the reaction (Tang and Hsu, 1974).

The rate of inactivation by bromopyruvate (k_{obsd}) was obtained as a function of NADPH concentration. The linear $1/k_{obsd}$ vs. $1/[BP]$ plots (Figure 5) intersect on the abscissa, indicating that NADPH had no effect on the dissociation constant (K of eq 6) of bromopyruvate in the E–BP complex, but decreased the rate of intramolecular alkylation (k_2). This behavior may be expressed as:

$$\begin{array}{ccccc} E + BP & \overset{K}{\rightleftharpoons} & E\text{-}BP & \xrightarrow{k_2} & E\text{-pyr} + Br^- \\ & & K_p \updownarrow (NADPH) & & \\ & & BP & & \\ & & E & & \\ & & NADPH & & \\ & & K \updownarrow (BP) & & \\ E + NADPH & \overset{K_p}{\rightleftharpoons} & E\text{-}NADPH & & \end{array} \qquad (9)$$

where NADPH binds independently of bromopyruvate with a dissociation constant of K_p and the E–BP–NADPH complex is not susceptible to intramolecular alkylation. The reciprocal rate equation is formally analogous to the equation for noncompetitive inhibition:

$$\frac{1}{k_{obsd}} = \frac{K}{k_2}\left(1 + \frac{[P]}{K_p}\right)\frac{1}{[BP]} + \frac{1}{k_2}\left(1 + \frac{[P]}{K_p}\right) \qquad (10)$$

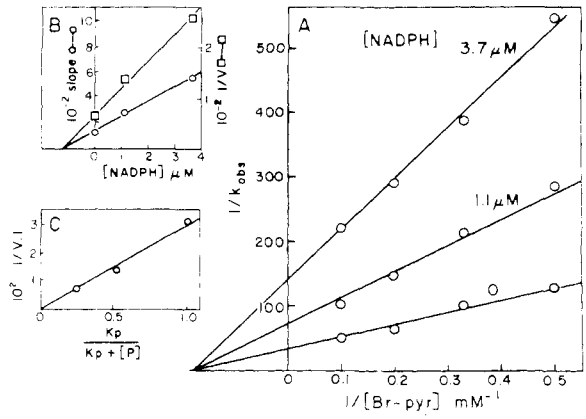

FIGURE 5: Protection of bromopyruvate inactivation by NADPH. Malic enzyme (0.4 μM) was incubated at 0 °C with bromopyruvate in Tris-Cl buffer (pH 7.0, 67 mM) in the absence and presence of NADPH. The concentrations of bromopyruvate and NADPH were varied as indicated. The k_{obsd} values were obtained as in Figure 3 by assaying 25-μl aliquots of the incubation mixture for oxidative decarboxylase activity (volume 3.0 ml) at time intervals. The bromopyruvate and NADPH concentrations in the assay mixtures were $\leq$83 μM and $\leq$31 nM, respectively. The bromopyruvate reductase activity as estimated with a V_m (bromopyruvate reduction) vs. V_m (oxidative decarboxylation) ratio of 1:14 and a K_m of 98 μM for brompyruvate (Table II) was lower than 3.5% of the oxidative decarboxylase activity and was not corrected. The alkylation of malic enzyme by bromopyruvate at concentrations of $\leq$83 μM ($\leq$1% of its k_d), which was negligible, was completely abolished by the presence of saturating amounts of Mn^{2+}, $NADP^+$, and L-malate (Table I). Insets are (B) slope and intercept and (C) 1/V.I. vs. $K_p/(K_p + [P])$ replots.

where [P] is NADPH concentration. A K_p value of 1.2 μM obtained from the replots (Figure 5B) is in reasonable agreement with corresponding values obtained from direct binding studies (0.75 μM; Hsu and Lardy, 1967b), kinetic studies (1–3 μM), and the apparent K_m of NADPH (2.1 μM) in reductive carboxylation (Hsu et al., 1967), indicating that the protective effect of NADPH is due to its binding at the active site. It should be noted that this method allows the determination of the dissociation constant of a protecting ligand at low enzyme concentration as pointed out by Mildvan and Leigh (1964).

The unreactivity of the E-NADPH complex with bromopyruvate may be illustrated by plotting 1/V.I. against $K_p/(K_p + [P])$ according to eq 11. The resulting plot (Figure 5C) was linear extending through the origin, indicating that the inactivation rate (1/V.I.) decreased to zero as the enzyme is fully liganded at infinite NADPH concentration.

$$\frac{1}{\text{V.I.}} = k_2\left(\frac{K_p}{K_p + [P]}\right) \qquad (11)$$

The protective effect of Mn^{2+} was studied by incubating malic enzyme (1.33 μM) with bromopyruvate (3–20 mM) at 0 °C in Tris-Cl buffer (pH 7.0, 67 mM) in the presence of 0–53.2 μM $MnCl_2$. This divalent metal ion was highly effective and at the highest concentration afforded 90% protection against inactivation by 3 mM bromopyruvate. The $1/k_{obsd}$ vs. 1/[BP] plot which was linear in the absence of Mn^{2+} became parabolic with increasing Mn^{2+}. The reason for this complex behavior is not clear, although possibly related to the apparent negative cooperativity of Mn^{2+} binding; under these conditions, malic enzyme binds Mn^{2+} with half-site stoichiometry at two tight binding sites (K_D = 6–10 μM) per tetramer (Hsu et al., 1976).

Properties of the Bromopyruvate Modified Enzyme. The ultraviolet spectra of native (sp act. of 38) and bromopyruvate-modified (sp act. of 0.17) enzymes were determined at pH 7.0 in a Cary Model 16 spectrophotometer. The calculated

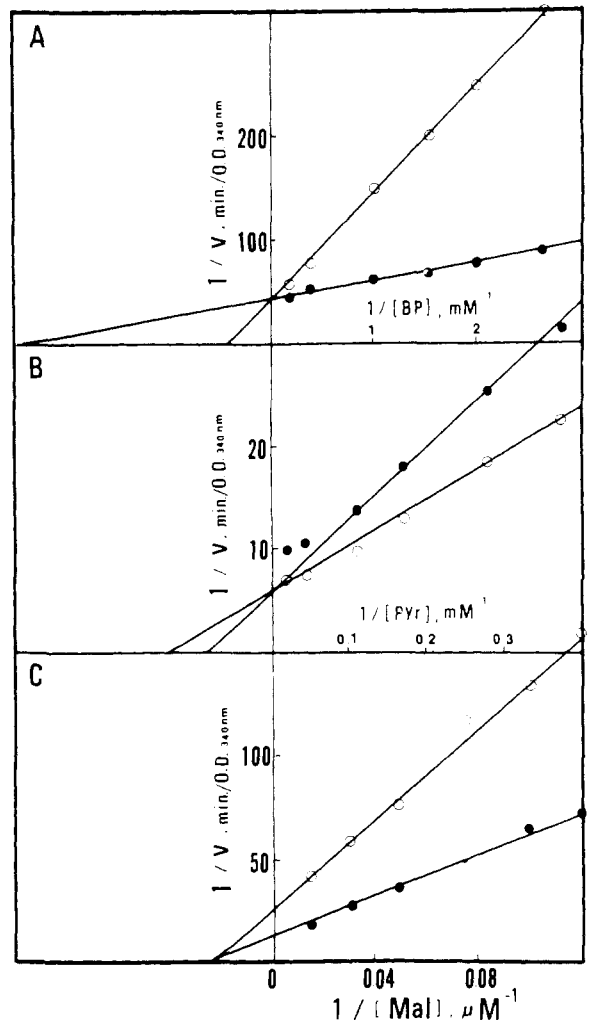

FIGURE 6: Kinetic properties of native and bromopyruvate modified malic enzyme. The modification mixtures contain malic enzyme (28 μM) and bromopyruvate (17.6 mM) in Tris-Cl buffer (pH 7.0, 380 mM) (A and B) and malic enzyme (3.3 μM) and bromopyruvate (1.3 mM) in Tris-Cl buffer (pH 7.0, 89 mM) (C). The reactions were carried out at 24 °C and terminated with dithiothreitol. The residual oxidative decarboxylase activity was 7% (A and B) or 32% (C). The oxidative decarboxylase (C) and reductase activities on pyruvate (B) and bromopyruvate (A) were each assayed with the same amount of native (●) or modified (○) enzyme as described under Materials and Methods or the legend to Figure 2 at varying concentrations of the carboxylic acid substrates.

difference spectrum of native minus modified enzyme gave a large negative peak below 240 nm, consistent with the disappearance of sulfide anions which absorb at 235 nm (Benesch and Benesch, 1955). The modified enzyme gave a homogeneous peak in the Spinco Model E ultracentrifuge with a sedimentation coefficient (10 S) identical with the native enzyme, indicating no major alteration in the quaternary structure.

Figure 6 shows the effect of bromopyruvate modification on the kinetic parameters of malic enzyme. The apparent maximum velocities of the reduction of bromopyruvate (Figure 6A) or pyruvate (Figure 6B) were unchanged, indicating that the reduction site was functional. The apparent Michaelis constant of bromopyruvate increased 5.6-fold from 0.45 to 2.5 mM (Figure 6A; Table II) and that of pyruvate decreased from 12.5 to 8.13 mM (Figure 6B; Table II). These changes would result in reciprocal changes on the level of substrate saturation in enzyme assays, which explains the decreased activity of bromopyruvate and increased activity of pyruvate during

TABLE III: Covalent Labeling of Malic Enzyme by [^{14}C]Bromopyruvate.[a]

Expt	Enzyme	Inactivation (%)	No. Residues Incorporated/Tetramer
1	Native	97	4.04
2	Native	94.5	3.04
3	Denatured[a]		0.68

[a] The Tris-Cl (pH 7.5), malic enzyme, and [2-^{14}C]bromopyruvate concentrations were: 0.6 M, 24 μM, and 2 mM (1.38×10^7 cpm) in experiment 1; 0.71 M, 28 μM, and 0.59 mM (3.46×10^6 cpm) in experiment 2; 0.45 M, 36 μM (denatured enzyme) (see footnote *b*) and 2.1 mM (1.94×10^7 cpm) in experiment 3. Following inactivation at 24 °C, the enzyme solutions were dialyzed to remove unbound bromopyruvate and analyzed for isotope incorporation as described under Materials and Methods. [b] The denatured enzyme was prepared by incubating malic enzyme with guanidine·HCl (6 M) in Tris-Cl buffer (pH 7.0), 50 mM, glycerol 10%, EDTA, 1 mM, at 24 °C for 10 min, and dialyzed against the same buffer to remove guanidine·HCl.

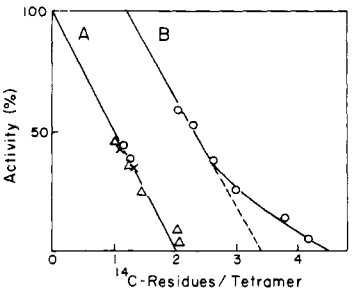

FIGURE 7: Correlation of inactivation and radioactivity incorporation. The residual oxidative decarboxylase activity vs. ^{14}C incorporation plots were obtained by the differential labeling method (A), or direct labeling method (B) as described under Materials and Methods. The symbols represent data from three separate experiments.

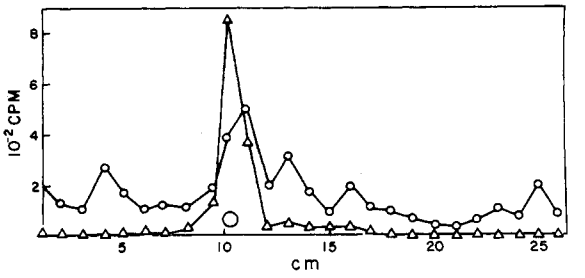

FIGURE 8: Paper chromatograms of the acid hydrolysates of bromopyruvate-modified malic enzyme. The incubation mixture for direct labeling contained malic enzyme (25.5 μM), [2-^{14}C]bromopyruvate (1.0 mM, 2.76×10^6 cpm), and Tris-Cl buffer (pH 7.5, 40 mM). After 165 min at 24 °C, the inactive enzyme (residual activity 3%) was reduced with $NaBH_4$, hydrolyzed, and chromatographed on paper as described under Materials and Methods. The preincubation mixture for differential labeling contained malic enzyme (20.4 μM), tartronate (20 mM), $MnCl_2$ (1.33 mM), $NADP^+$ (74 μM), and unlabeled bromopyruvate (2.5 mM), in Tris-Cl buffer (pH 7.5, 33 mM). After 60 min at 24 °C, the enzyme solution (81% active) was dialyzed against 50 mM Tris-Cl buffer (pH 7.0) and 10% glycerol, and incubated with [2-^{14}C]bromopyruvate (0.77 mM, 2.76×10^6 cpm) for 180 min at 24 °C. The inactive enzyme (residual activity 4%) was reduced, hydrolyzed, and chromatographed as above; solvent, ethyl acetate–pyridine–H_2O; (○) directly labeled sample; (△) differentially labeled sample. The S-CHE-Cys standard is shown as a ninhydrin positive spot (large circle) on the chromatogram.

modification (Figure 2). The apparent V_m of the oxidative decarboxylation of L-malate catalyzed by the partially modified enzyme preparation was decreased to 42% of the original (Figure 6C), in reasonable agreement with the residual activity (32%) of this preparation. This result indicates that a decrease in the number of unmodified enzyme sites was responsible for the inactivation. The kinetic parameters of the native and bromopyruvate modified enzymes are summarized in Table II.

Covalent Labeling of Malic Enzyme by [^{14}C]Bromopyruvate and Identification of the Modified Amino Acid Residue. Table III shows the incorporation of pyruvyl residues from [^{14}C]bromopyruvate by malic enzyme. The incorporations at 2 and 0.59 mM bromopyruvate following complete inactivation were 4.04 and 3.04 residues per enzyme tetramer, respectively. The incorporation by the guanidine·HCl denatured enzyme was much lower (0.68 residue per tetramer), indicating a requirement of active-site structure for optimal alkylation in accord with the two-step reaction of eq 6.

Despite the saturation behavior of inactivation kinetics, bromopyruvate reacted at multiple enzyme sites as indicated by the presence of several radioactive peaks in the paper chromatogram of an acid hydrolysate of [^{14}C]bromopyruvate inactivated enzyme obtained by the direct labeling method (Figure 8). Hence, the stoichiometry of incorporation was reexamined by the differential labeling method. Malic enzyme was incubated with unlabeled bromopyruvate in the presence of the inhibitor tartronate which protected the substrate site, but allowed the modification of other reactive groups. The modified enzyme was then incubated with the radioactive compound to label the essential group after removal of protecting agents by dialysis. The effectiveness of this method was shown by the complete inhibition of inactivation (hence the reaction of the essential group, cf. Materials and Methods) in the initial incubation step. A linear plot of residual activity vs. ^{14}C incorporation (Figure 7A) gave 2.0 residues per tetramer upon extrapolation to complete inactivation. In five separate determinations an average value of 1.95 ± 0.1 was obtained. An aged enzyme preparation which had lost 50% activity incorporated only 0.96 residue per tetramer, indicating parallel loss of reactivity with bromopyruvate presumably due to the oxidation of the essential thiol group during storage. In a separate experiment, the differentially labeled enzyme was hydrolyzed and chromatographed in a solvent system of ethyl acetate–pyridine–H_2O. The resulting chromatogram (Figure 8) gave a single radioactive peak with a R_f of 0.38, which corresponded to that of the S-CHE-Cys standard, indicating specific labeling of cysteine residues by this method. The product of differential labeling was verified by paper chromatography in two other solvent systems: 2-butanol–formic acid–H_2O (R_f 0.20) and 2-propanol–pyridine–acetic acid–H_2O (R_f 0.38). In each case, the acid hydrolysate of the modified, $NaBH_4$-reduced enzyme gave a single radioactive peak corresponding to the cysteine thioether standard.

A plot of the residual activity vs. ^{14}C incorporation from direct labeling data (Figure 7B) was linear up to 63% loss of activity, and upon similar extrapolation yielded an incorporation value of 2.2 residues per tetramer, in good agreement with the value obtained from differential labeling data (Figure 7A). This plot also shows the labeling of faster and slower reacting groups which were not essential for activity. The presence of nonthiol amino acid derivatives was clearly demonstrated in the paper chromatogram of the acid-hydrolyzed sample (Figure 8) as minor radioactive peaks, in addition to the major peak (R_f 0.39) containing the thiol derivative.

Modification of Malic Enzyme by p-Chloromercuribenzoate and the Protection of Bromopyruvate Inactivation by p-Chloromercuribenzoate, 5,5′-Dithiobis(2-nitrobenzoic

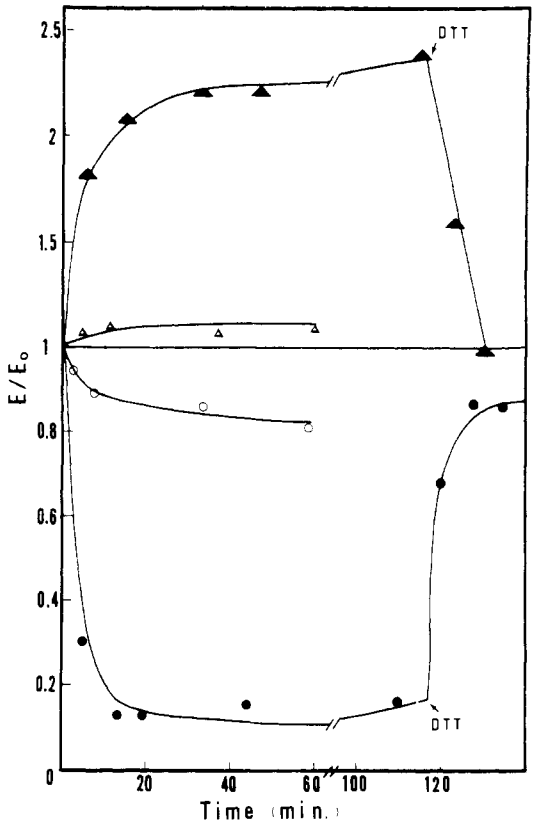

FIGURE 9: Reversible inactivation of malic enzyme by *p*-chloromercuribenzoate. The incubations contained malic enzyme (1.2 μM), ClHgBzO (2.8 μM) in Tris-Cl buffer (pH 7.0, 46 mM), glycerol, 10% in the absence (filled symbols) or presence (tartronate, 5.5 mM; $MnCl_2$, 0.36 mM; $NADP^+$, 20 μM; open symbols) of protecting agents. The oxidative decarboxylase (circles) and pyruvate reductase (triangles) activities were assayed at the indicated time intervals after incubation at 24 °C. Dithiothreitol (10 mM) was added to the unprotected reaction mixture at 117 min.

TABLE IV: Protection of Malic Enzyme from Bromopyruvate Inactivation by Pretreatment with *p*-Chloromercuribenzoate, 5,5′-Dithiobis(2-nitrobenzoic acid) or KCN.[a]

Expt	Treatment	Recovery of Act. (% of Control)
1	None	0
	Nbs_2	83
	ClHgBzO	68
		Act. (% of Original)
2	Nbs_2	7.4
	KCN	59
	Bromopyruvate	44
	Control	7.2

[a] In experiment 1, malic enzyme (0.1 μM) was preincubated with Nbs_2 (80 μM) or ClHgBzO (52 μM) in Tris-Cl buffer (pH 7.5, 95 mM) at 24 °C until the activity on oxidative decarboxylation was completely lost. Bromopyruvate (25 mM) was added and allowed to react for 10 min. The reaction mixture was chilled to 0 °C (see footnote *b*) and reactivated with dithiothreitol (25 mM) until maximum recovery was obtained. The control sample was treated similarly except that dithiothreitol was added immediately before bromopyruvate. In experiment 2, malic enzyme (1.44 μM) was preincubated with Nbs_2 (83 μM) in Tris-Cl buffer (pH 7.0, 50 mM) at 24 °C for 1 h. KCN (50 mM, freshly prepared and neutralized) was added and the incubation continued for 50 min. The enzyme was dialyzed against 50 mM Tris-Cl buffer (pH 7.0) and treated with bromopyruvate (6 mM) for 10 min. The control sample was treated directly with bromopyruvate. The oxidative decarboxylase activity was determined after each treatment. [b] Chilling was necessary to minimize alkylation of the dithiothreitol reactivated enzyme by bromopyruvate.

acid), and KCN. Incubation of malic enzyme with the thiol reagent ClHgBzO caused the loss of oxidative decarboxylase activity and concurrent activation of reductase activity on pyruvate (Figure 9), as was observed with bromopyruvate (Figure 2A), Nbs_2, and MalNEt (Hsu, 1970; Tang and Hsu, 1974). The reaction with ClHgBzO was inhibited by tartronate, Mn^{2+}, and $NADP^+$, and was reversed with dithiothreitol. If we assume the reactions involve the same thiol group as suggested by the similar activity patterns of the resulting enzyme derivatives, the above reagents should react exclusively of each other; this mutually competitive behavior was demonstrated by the protective effects of Nbs_2 or ClHgBzO on bromopyruvate inactivation as shown in Table IV. In this experiment, malic enzyme reversibly modified by either Nbs_2 or ClHgBzO recovered 68 to 83% of enzyme activity after bromopyruvate treatment followed by reactivation with dithiothreitol. Moreover, the catalytically active -SCN enzyme (with 59% original activity), which was prepared by replacing the bulky thionitrobenzene group on the Nbs_2-modified enzyme with the smaller CN^- group, was likewise resistant to bromopyruvate and retained 44% of original activity.

Discussion

Bromopyruvate has been used rather extensively in studies on the structure–function relationship of a number of enzymes (Maldonado et al., 1972; Rashed and Rabin, 1968; Göthe and Nyman, 1972; Heinrikson et al., 1965; cf. Fonda, 1976). In some cases, such as 2-keto-3-deoxy-6-phosphogluconic aldolase (Meloche, 1967, 1970; Meloche et al., 1972), *N*-acetylneuraminic acid aldolase (Barnett et al., 1971), isocitrate lyase (Roche and McFadden, 1969; Roche et al., 1971), aspartate aminotransferase (Morino and Okamoto, 1970; Okamoto and Morino, 1973), lactate dehydrogense (Berghaüser et al., 1971), and 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthetase (Staub and Deñes, 1967), this compound was shown to be specific for the active site.

The present investigation has demonstrated that bromopyruvate is a substrate and inhibitor of malic enzyme as well as an affinity label which alkylates an essential cysteinyl residue under noncatalytic conditions with half-site stoichiometry. This compound cannot be carboxylated to bromomalate, but is catalytically reduced to L-bromolactate according to eq 3. The apparent Michaelis constants of bromopyruvate are lower than pyruvate by more than tenfold (Table II), indicating the former is more tightly bound.

The reduction of pyruvate and bromopyruvate requires either Mn^{2+} or Mg^{2+}. The former is a much better activator, as indicated by the higher apparent V_m's and lower apparent Michaelis constants of both substrates in the Mn^{2+}-activated reactions (Table II). Our finding of identical apparent Michaelis constants of pyruvate (i.e., 1.1 mM, Table II) in the reduction (eq 3) and reductive carboxylation (reverse of eq 1) reactions is consistent with the concept of a common enzyme–pyruvate (NADPH) intermediate as previously postulated (Hsu, 1970).

The reversible binding of bromopyruvate in its reactions with malic enzyme occurs at the carboxylic acid portion of the active site, both in the catalytic reactions and in the formation of a noncovalent E–BP complex prior to alkylation (eq 6). Binding at this site is indicated by the following. (a) Like pyruvate, the

halo acid undergoes reduction to the corresponding hydroxy acid according to eq 3. (b) Bromopyruvate inhibits noncompetitively the oxidative decarboxylation of L-malate (Chang and Hsu, 1973) and competitively against pyruvate in the reductive carboxylation reaction with an inhibition constant of 0.09 mM (Table II) which agrees with the apparent Michaelis constant of the halo acid (0.098 mM, Table II). (c) The inactivation of malic enzyme shows saturation kinetics and is inhibited by pyruvate, or L-malate, in the presence of divalent metal ions and $NADP^+$ (H) (Table I). (d) For optimal alkylation native protein structure is required (Table III).

Protection of malic enzyme by divalent metal ions may be due to a direct competition between the metal activator and bromopyruvate for a thiol ligand, but more likely this was due to a metal-induced conformational change which shields the thiol group from bromopyruvate. The ability of the thiol-substituted enzymes to catalyze metal-dependent reactions, such as the reduction of pyruvate (Tang and Hsu, 1974; Figure 9) or bromopyruvate (Figure 9), and the detritiation of tritiated pyruvate (eq 4; Bratcher, 1974) confirm the latter explanation for the protection. Protection by NADPH is due to a nucleotide-induced conformational change as indicated by the lack of NADPH effect on the half-saturation constant (K in eq 6) of bromopyruvate (Figure 5). Independent evidence for such a conformational change was obtained in an earlier study (Hsu and Lardy, 1967b) which showed that the fluorescence properties of both protein and nucleotide were significantly altered upon combination.

Results of the protection experiments indicate that the thiol group associated with the active site is readily accessible to bromopyruvate only in the unliganded enzyme. These results also allow us to estimate the binding order of substrates and inhibitors as was done by Roche et al. (1971) on the isocitrate lyase reaction. The requirement of a divalent metal ion and a nucleotide for substrate protection (Table I) is in accord with the previously determined kinetic order of reaction (i.e., $NADP^+$ first, L-malate last; Hsu et al., 1967d), and with the 38-fold tightening of L-malate binding to the E-Mn^{2+} complex by NADPH as shown by direct binding studies (Hsu et al., 1976). Similar analysis of the protective behavior of tartronate and oxalate suggests the same binding order for these inhibitors.

Bromopyruvate forms a weak complex with the free enzyme ($K \simeq 8$ mM), which is tightened 10- to 100-fold by divalent metal ions and nucleotide in the E-$NADP^+$ (NADPH)-Mn^{2+} (Mg^{2+})-BP complexes, as indicated by the much lower values (90 μM-0.8 mM, Table II) of the apparent Michaelis constants and inhibition constants of this compound against the carboxylic acid substrates. This behavior is analogous to that observed for lactate dehydrogenase; the K_m of bromopyruvate is 50 times lower than its dissociation constant in the E-BP complex as determined from the kinetics of inactivation (Berghäuser et al., 1971).

Binding of bromopyruvate to the substrate site of the free enzyme orients the β-carbon properly for a subsequent electrophilic attack on a thiol group of a cysteinyl residue to give the inactive S-CKM derivative. Similar two-step binding processes were proposed for the inactivation of glutamate apodecarboxylase (Fonda, 1976), aspartate aminotransferase (Okamoto and Morino, 1973), and 2-keto-3-deoxy-6-phosphogluconic aldolase (Meloche, 1967) by bromopyruvate. This thiol group has a pK of 8.3 (Figure 3) and is probably the same group that reacts with Nbs_2, MalNEt, and other thiol reagents (Hsu, 1970; Tang and Hsu, 1974). This conclusion is based on the specificity of these reagents in disrupting the decarboxylase function of malic enzyme, and that Nbs_2, KCN, and ClHgBzO reversibly protected the enzyme from inactivation by bromopyruvate (Table IV). The possibility of the presence of two (or more) proximally located, mutually exclusive groups similar to the juxtaposed histidine-12 and histidine-119 of ribonuclease (Crestfield et al., 1963) is unlikely, since the small cyanide substitutent which gives minimal steric hindrance is equally protective. The intact SH group is not required for malic enzyme activity, as demonstrated with the preparation of catalytically active SCN enzyme from the inactive enzyme containing a bulky thionitrobenzene substituent (Table IV; Tang and Hsu, 1974). This bulky effect which is also seen with the S-MalNEt, the *S*-mercuribenzoate, and the S-CKM enzymes disrupts the C-C bond cleavage (Tang and Hsu, 1974) step.

The present finding of the ability of the S-CKM enzyme to reduce bromopyruvate (and pyruvate) (Figure 6) is unexpected. Apparently, after initial binding of bromopyruvate at the substrate site, the constraint of alkylation (step 2 of eq 6) causes a significant conformational change and the movement of the S-CKM residue *away* from this site, thus allowing for the admission of another bromopyruvate (or pyruvate) molecule. The interaction between the substrate and the modified enzyme is strengthened by "like" forces (e.g., hydrophobic forces), suggesting possible direct intervention of the thiol substituent with the binding of the ω-carbon to the enzyme. Thus, the rate of pyruvate reduction (but not that of oxalacetate which contains a 4′-carboxyl) is enhanced by a number of thiol reagents (Tang and Hsu, 1974), the magnitude of which generally follows the hydrophobicity of the substituent group. Conversely, the hydrophobic thiol reagent MalNEt increases the rate of hydrogenation of α-ketomonocarboxylic acids, but not α-ketodicarboxylic acids unless the ω-carboxyl group is masked as in 4′-ethyl oxalacetate (Tang and Hsu, 1973).

Malic enzyme is a tetrameric molecule with four indistinguishable subunits (Nevaldine et al., 1974) which has previously been found to contain four essential thiol groups per tetramer associated with oxidative decarboxylase activity (Tang and Hsu, 1974). The half-site stoichiometry of the incorporation of two S-CKM residues per tetramer for the active-site directed inactivation by bromopyruvate (Figure 7) and the half-site stoichiometry of the binding of Mn^{2+} to the enzyme demonstrated in a recent study (Hsu et al., 1976) indicate either strong negative cooperativity between subunits or, alternatively, the presence of two types of structurally dissimilar active sites. Similar half-site stoichiometry was obtained in the acylation of glyceraldehyde-3-phosphate dehydrogenase (McQuire and Bernhard, 1971; Malhotra and Bernhard, 1968). Negative cooperativity was obtained from metal ion kinetic data, which showed non-Michaelian behavior of Mn^{2+} in the oxidative decarboxylation reaction[2] with a Hill coefficient of one at a low level of L-malate, which decreased to 0.1 at a saturating level of L-malate. These results raise the interesting possibility that the malic enzyme tetramer is a double dimer; the monomers of each dimer exhibit strong anticooperative behavior toward each other in the interactions with carboxylic acid substrate as well as the divalent metal ion.

Acknowledgment

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[2] Hsu, R. Y., unpublished results.

these experiments and for his valuable suggestions in some of these studies.

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