Mechanism of Pigeon Liver Malic Enzyme

FORMATION OF L-LACTATE FROM L-MALATE, AND EFFECTS OF MODIFICATION OF PROTEIN THIOL GROUPS ON MALIC ENZYME, OXALACETATE, AND PYRUVATE REDUCTASE ACTIVITIES*

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SUMMARY

Crystalline pigeon liver malic enzyme (malate dehydrogenase (decarboxylating), EC 1.1.1.40) catalyzes the slow, magnesium-dependent reduction of oxalacetate and conversion of L-malate to L-lactate in the presence of TPNH and TPN, respectively, with TPN acting in a catalytic manner. Pyruvate reductase activity associated with this enzyme has a pH optimum of 6.5 to 6.8 and a Michaelis constant of 5.52 mM for pyruvate. The activity ratio of oxidative decarboxylation of L-malate, reduction of pyruvate, and reduction of oxalacetate catalyzed by the enzyme is approximately 7.3:0.31:1. Titration of 4 sulfhydryl groups by 5,5'-dithiobis(2-nitrobenzoic acid) induced a reversible loss of malic enzyme activity and an increase in pyruvate reductase activity, while the oxalacetate reductase activity remained constant. The modified enzyme exhibited a new activity ratio of 0.3:0.6:1. These observations are consistent with both reductases being auxiliary reactions that could be expected from a sequential, ordered mechanism with oxalacetate as the enzyme-bound intermediate in a central complex. It is postulated that alteration of thiol groups led to a reversible blockage of the CO₂ step, which was responsible for the observed changes in the diverse activities of the enzyme.

This paper describes some properties of Reactions 3, 4, and 5. Activities of Reactions 1 and 3 are profoundly affected by alteration of sulfhydryl groups on the enzyme. The significance of these effects is discussed in the context of the postulated kinetic mechanism.

MATERIALS AND METHODS

Chemicals—L-Malic acid, crystalline sodium pyruvate, oxalacetic acid, L-lactic acid lithium salt, dithiothreitol, triethanolamine HCl (all A grade), 5,5'-dithiobis(2-nitrobenzoic acid), and ethylenediaminetetraacetic acid tetrasodium dihydrate (Calbiochem); DPN, DPNH, and 2-mercaptoethanol Type 1 (Sigma); TPN and TPNH (P-L Biochemicals); crystalline heart lactate and malate dehydrogenase (Boehringer and Mannheim); tris(hydroxymethyl)aminomethane and L-histidine-HCl-H₂O, N.R.C. (General Biochemicals) were purchased from the designated sources. Purified pigeon liver malic enzyme was prepared according to the method of Hsu and Lardy (1). protein concentration was determined by absorption at 278 mA in a Beckman DU spectrophotometer. Lactate dehydrogenase activity
of the preparations used in this work ranged from 0.008 to 0.16% of malic enzyme activity.

Enzyme Assays—Malic enzyme activity in the forward direction was assayed according to Hsu and Lardy (1). Malic enzyme assay in the reverse direction was carried out in a reaction mixture consisting of TEA buffer, pH 7.4, 200 μmoles; MgCl₂, 12 μmoles; sodium pyruvate, 40 μmoles; TPNH, 0.32 μmole; sodium bicarbonate, 200 μmoles. In Fig. 6, buffer, pH 6.5, was used to facilitate direct comparison with pyruvate reductase assays. Pyruvate reductase assay contained sodium pyruvate, 20 μmoles; MgCl₂, 12 μmoles. Oxalacetate reductase assays contained oxalacetate, 40 μmoles, and MgCl₂, 6 μmoles. Total volume, 3.0 ml. DTT-activated crystalline malic enzyme was used for these assays.

1 The abbreviations used are: TEA, triethanolamine; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TNB, S-thio(2-nitrobenzoic acid).

2 ME₂, “malic enzyme activity” in the reverse direction.

3 ME₁, “malic enzyme activity” in the forward direction.

Fig. 1 (left). Reduction of oxalacetate by malic enzyme. All cuvettes contained Tris-histidine buffer, pH 6.5, 100 μmoles; oxalacetate, 40 μmoles; enzyme, 25 μg; and water to make 3.0 ml. Other additions: a, MgCl₂, 6 μmoles, and TPNH, 0.32 μmole; b, EDTA, 60 μmoles, and TPNH, 0.32 μmole; c, EDTA, 60 μmoles, and DPNH, 0.4 μmole.

Results

Fig. 1 demonstrates the ability of crystalline pigeon liver malic enzyme to catalyze an oxalacetate reductase activity presumably according to Reaction 4.

These assays were carried out with oxalacetate dissolved and neutralized just before starting the reaction (i.e., within 2 min). In this assay, TPNH must be oxidized directly by the substrate oxalacetate, and not by the decarboxylation product pyruvate, via either the ME₂ or pyruvate reductase pathway for the following reasons. (a) TPNH oxidation by pyruvate requires measurement of the second reaction from two consecutive reactions, and it is not consistent with experimentally observed linearity of reaction and the absence of an initial lag in TPN formation. (b) Although TPNH is provided in sufficient amounts, other substrates required for oxidation of TPNH by pyruvate, such as pyruvate and CO₂ for ME₁, and pyruvate alone for pyruvate reductase, are generated through decarboxylation of oxalacetate. Enzymatic decarboxylation of this compound under unfavorable experimental conditions (pH 6.5 versus optimal pH of 4.5; absence of the activator, TPN) would be negligible. Nonenzymatic decarboxylation of oxalacetate proceeds at a faster rate. If a reasonable rate of 1%/min per min is assumed, the pyruvate (or CO₂) level can reach 0.3 mM during the 2 min required for dissolution of oxalacetate before assay. This concentration is approximately 5% of the Kₐ value for pyruvate and 2% of the Kₐ value for CO₂. Actual CO₂ concentration will be lower as a result of loss from exposure to air. At the above pyruvate concentration, contribution of pyruvate reductase activity to TPNH oxidation is negligible. ME₁ has a much higher velocity than pyruvate reductase but is rendered inoperative by the low concentration to Kₐ ratios of two of its three substrates. (c) Furthermore, titration of protein thiol groups with DTNB (Fig. 6) results in the alteration of ME₁, oxalacetate decarboxylase, and pyruvate reductase activities. Oxalacetate reductase activity, however, is not altered. This observation, which is compatible with oxalacetate reductase activity being a reaction independent of the other reactions catalyzed by malic enzyme, hence implies direct reduction of oxalacetate by TPNH.

Oxalacetate reductase activity was found to be 16.9 milliunits, as compared with 3.5 milliunits after deletion of magnesium ions (Fig. 1, b), and 1.7 milliunits of malate dehydrogenase activity (EC 1.1.1.37, Fig. 1, c). Malate dehydrogenase uses DTNB preferentially. The higher activity observed with TPNH, therefore, is a measure of residual oxalacetate reductase activity under these experimental conditions. Oxalacetate reductase
activity was found in different malic enzyme preparations to range in specific activities between 0.58 and 0.67.

Fig. 2 shows the linear dependence of pyruvate and oxalacetate reductase activities on enzyme concentration. Pyruvate reductase activity was low as a result of the effect of DTN on enzyme (cf. Table II). The pH rate profile was determined from pH 5 to 8 using both histidine-HCl and Tris-histidine-HCl buffers in assays containing sodium pyruvate, 20 μmoles; MgCl₂, 12 μmoles; and TPNH, 0.32 μmole. A bell-shaped curve was obtained, exhibiting a narrow maximum from pH 6.5 to 6.8. The Michaelis constant for pyruvate (Fig. 3) was found to be 6.7, 6.7, 4.5, and 4.2 mM in four separate experiments carried out at pH 6.5, giving an average value of 5.52 mM. In these assays, no measurable activity was found in the absence of MgCl₂, indicating the nonparticipation of lactate dehydrogenase in pyruvate reduction.

Enzymatic Conversion of L-Malate to L-Lactate in Presence of TPN—The ability of malic enzyme to catalyze Reactions 1 and 3 implied that this enzyme carries out Reaction 5 through an enzyme-bound pyruvate intermediate (2). Further characterization of this reaction was performed (Fig. A). At two TPN levels, the reaction was roughly proportional to enzyme concentration (Fig. 4B) and time (Fig. 4A), with no observable lag during the initial period. L-Lactate formation was dependent on TPN concentration. At 0.194 mM TPN, the reaction rate was less than 30% of that at the higher TPN level (2.05 mM). The pyruvate concentration measured at the three designated time intervals was constant and approximately the same as total available TPN, a finding consistent with the high ratio of ME₄ to ME₅ combined ME₄ and pyruvate reductase activities.

Table I shows results of L-lactate synthesis from pyruvate (Reaction 3) and L-malate (Reaction 5) in the presence of TPNH and TPN, respectively. Colorimetric determination of lactic acid in Experiment I yielded results in excellent agreement with enzymatic analyses. A metal requirement was demonstrated with either substrate (I, II, III, IV, I and 4). When L-malate was used as substrate, both pyruvate and TPNH were formed, with the former approaching the limiting amount of TPN present initially (I, II, and 2, III, I and 2, IV, 2 and 3), further confirming the results shown in Fig. A. As would be expected, the relative efficiency of L-lactate formation from L-malate by the three enzyme preparations corresponds closely to their ME₄ and pyruvate reductase activities in the order of B, A, C. The maximal conversion activity of 9.2 μmoles of L-lactate formed per hour per mg of enzyme exhibited by Enzyme B is equal to 0.37% of ME₄ activity. Sensitivity to pyridine nucleotide concentration was more pronounced with L-malate as the substrate (II, 1, 2 versus 4). After an initial period, the TPNH concentration in malate incubations is expected to reach approximately the same level as initially present in the pyruvate incubations because of the fast ME₄ reaction. Thus, pyruvate, rather than TPNH, is probably the rate-limiting factor. Bicarbonate inhibited L-lactate formation from either pyruvate (IV, 5, 6, V, 1, 2) or L-malate (III, 1, 2 and IV, 2, 3). The former is a slow step presumably shared by both reactions. Therefore, it is likely to be the site of inhibition. The primary effect of bicarbonate must be inhibition, rather than lowering of substrate concentrations for pyruvate reductase, since a near maximal level of pyruvate was maintained in the malate experiments despite a shift of equilibrium in favor of L-malate resulting from the noncompetitive inhibition of ME₄ (6) and resulting from promotion of ME₅ by bicarbonate. In accord with Reaction 5, TPN acts in a catalytic manner as shown in Experiment III, Tube 2, where 3.08 μmoles of L-lactate were synthesized in the presence of 1.7 μmoles of TPN.

Mediation of Malic Enzyme, Pyruvate Reductase, and Oxalacetate Reductase Activities by Protein Sulfhydryl Groups—Purified malic enzyme is stable for weeks at -20°C at high protein concentrations (5 to 10 mg per ml). At protein concentrations of 0.2 mg per ml or below, its stability markedly decreases. Decrease in the ability of this enzyme to catalyze the oxidative decarboxylation of L-malate was rapid (15-fold in 19 days). Significantly, the decrease in pyruvate reductase activity was much slower, reaching 50% during the same period (Table II).
### Table I

Malic enzyme-catalyzed formation of L-lactate from L-malate and pyruvate

Experiment I was carried out at pH 6.5 with 75 μmoles of Tris-histidine-HCl buffer. TEA-I-ICI buffer (pH 7.4, 400 μmoles) was used in the other experiments. Incubation times and total volumes for Experiments I through V, respectively, were: 3 hours, 3.0 ml; 1 hour, 3.0 ml; 0.5 min, 2.15 ml; 2 hours, 3.0 ml; and 2 hours, 3.0 ml. All incubations were made in test tubes at 30°C except for Experiment III which was carried out at 33°C in Warburg flasks with shaking. Tube 2 in Experiment III was gassed with N₂ and with CO₂ trapped by 0.2 ml of 2 N NaOH in the center well.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Enzyme</th>
<th>Additions</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>mg</td>
<td>μmoles</td>
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<tr>
<td>Experiment I</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>0.25</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>0.25</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
<td>40</td>
<td>24</td>
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<tr>
<td>Experiment II</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.46</td>
<td>9</td>
<td>24</td>
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<tr>
<td>2</td>
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<td>9</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>0.46</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>0.46</td>
<td>40</td>
<td>24</td>
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<tr>
<td>Experiment III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.67</td>
<td>6</td>
<td>24</td>
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<tr>
<td>Experiment IV</td>
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<td></td>
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<tr>
<td>1</td>
<td>0.51</td>
<td>9</td>
<td>6.25</td>
</tr>
<tr>
<td>2</td>
<td>0.51</td>
<td>9</td>
<td>6.25</td>
</tr>
<tr>
<td>3</td>
<td>0.51</td>
<td>9</td>
<td>6.25</td>
</tr>
<tr>
<td>4</td>
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<td>5</td>
<td>0.51</td>
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<tr>
<td>6</td>
<td>0.51</td>
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<td>5.1</td>
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<tr>
<td>Experiment V</td>
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<tr>
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<tr>
<td>2</td>
<td>0.51</td>
<td>40</td>
<td>6.2</td>
</tr>
</tbody>
</table>

a Enzymes A, B, and C designate three preparations of purified malic enzyme exhibiting different activities resulting from aging. Specific activities for MEr and pyruvate reductase, respectively, were: 20, 0.53; 29, 1.42; and 9.4, 0.41, corresponding to MEr to pyruvate reductase ratios of 38, 20, and 23 for Enzymes A, B, and C, respectively. Enzyme C used in Experiment V was aged for 19 more days after Experiment IV.
b Trace of divalent metal was removed from all tubes containing no added MgCl₂ by the addition of 20 μmoles of EDTA.

### Table II

Changes in malic enzyme and pyruvate reductase activities during aging and reactivation

<table>
<thead>
<tr>
<th>Experimenta</th>
<th>Aged enzymeb</th>
<th>Reactivated enzyme</th>
<th>Activity changec</th>
</tr>
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<tbody>
<tr>
<td>MEf</td>
<td>MEb</td>
<td>Pyruv-</td>
<td>MEf</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reductase</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.94</td>
<td>0.71</td>
<td>0.94</td>
</tr>
<tr>
<td>2</td>
<td>3.03</td>
<td>3.03</td>
<td>1.09</td>
</tr>
</tbody>
</table>

a Purified malic enzyme used for these studies initially had specific activities for MEf and PR of 28.8 and 2.2, respectively.
b En, aged enzyme; Er, reactivated enzyme.
c Aging was carried out at a protein concentration of 0.134 mg per ml at -20°C.
d Aged in 0.02 M TEA buffer, pH 7.4, for 19 days and reactivated for 100 min at 23-25°C in the presence of 5 mM DTT.
e Aged in same buffer as in Experiment 1 for 23 days and reactivated for 100 min at 23-25°C in 5 mM DTT.
Table III

**Synthesis of L-lactate and L-malate from pyruvate by aged and DTT reactivated enzyme**

All incubations were carried out at 30° for 103 min. Reaction was stopped by the addition of trichloracetic acid, and L-malate and L-lactate were analyzed enzymatically as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Tube</th>
<th>Enzyme</th>
<th>L-Malate</th>
<th>L-Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1. Complete</td>
<td>Aged</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>2. Complete + 0.2 μmole DTT.</td>
<td>Aged</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>3. Complete</td>
<td>DTT reactivated</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>4. Complete</td>
<td>Aged</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>5. Complete + 0.2 μmole DTT.</td>
<td>Aged</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>6. Complete</td>
<td>DTT reactivated</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Purified enzyme aged at a protein concentration of 0.134 mg per ml in 0.05 M Tris HCl, 0.02 M magnesium acetate, and 2 mM mercaptoethanol buffer, pH 7.0, for 12 days at -20°. Reactivation was carried out at 23-25° for 1 hour in the presence of 2 mM DTT. In each incubation, 13.4 μg of enzyme were used.

During progressive aging, the MEf to pyruvate reductase ratio decreased from 13.5 for the freshly prepared enzyme to 5.1 after 6 days and 2.1 after 19 days, as would be expected from differential inactivation of these two activities. Reactivation of aged enzyme by DTT has been reported previously (1). In these experiments, incubation of aged enzyme with this compound increased MEf to about 50% of the original activity, corresponding to a change of 8.1- and 2.1-fold from a range of 0.94 to 1.59 to a range of 0.17 to 0.48 in three experiments. As shown in a later experiment (Table III), addition of DTT in an amount equivalent to that present in the DTT-treated enzyme in assays had no effect on either malic enzyme or pyruvate reductase activity. Changes in malic enzyme activity measured in the backward direction (MEb, Experiments 2 and 3) paralleled MEf, as shown by the constant MEf:MEb ratio of 2.4 to 2.7 during aging and reactivation.

Dramatic demonstration of the effect of bicarbonate on the rate of TPNH oxidation by pyruvate is shown in Fig. 5 with DTT-treated enzyme. The initial slope was a measure of pyruvate reductase activity. Addition of bicarbonate enabled MEb to become operative, resulting in a 25-fold increase in TPNH oxidation from an initial specific activity of 0.19 to a final specific activity of 4.7. The ratio of MEb to pyruvate reductase is probably higher than indicated by these values because of the inhibitory effect of bicarbonate on pyruvate reductase. The magnitude of this change is further illustrated by comparison with MEb to pyruvate reductase ratios of approximately 5 and 0.85, respectively, for the fresh and aged enzyme.

The above experiments provide spectrophotometric evidence for the modulation of malic enzyme and pyruvate reductase activities by aging and reactivation. Corroboration evidence based on analyses of L-malate and L-lactate is presented in Table III. This experiment was carried out under conditions similar to MEb and pyruvate reductase assays. Results shown in Table III are in qualitative agreement with previous findings that the time-dependent DTT reactivation of the aged enzyme led to a diminished ability to synthesize L-lactate, both in the
absence (0.50 versus 1.05 μmoles) and presence (0.65 versus 0.25 μmoles) of bicarbonate, and to an enhanced ability to synthesize L-lactate at pH 6.5 in the presence of bicarbonate (0.76 μmole, calculated from a value of 0.25 at pH 7.4, according to Table II) was significantly lower than the corresponding value of 1.05 μmoles found in the absence of bicarbonate, and it further confirms the inhibition of this activity by bicarbonate suggested by early experiments (Table I).

DTT is a potent reagent for reducing disulfide to corresponding sulfhydryl compounds (11). Its ability to reactivate aged malic enzyme (MEa and MEb) suggests that loss of activity is due to formation of disulfides. Alternatively, titration of protein sulfhydryl groups may be accomplished quickly in a controlled manner with DTNB. Crystalline malic enzyme was exhaustively dialyzed against Tris buffer (10% glycerol) to remove DTT. This enzyme solution was titrated with limiting amount of DTNB, then reactivated with excess DTT. In two separate experiments (Fig. 6, A and B), MEa, MEb, pyruvate reductase, and oxalacetate reductase activities were measured on the native, DTNB-modified, and DTT-reactivated enzyme. Results of these experiments showed that titration of approximately four sulfhydryl groups by DTNB led to the loss of malic enzyme activity in both directions (i.e. MEa and MEb), accompanied, however, by a 2-fold enhancement of pyruvate reductase activity. These activities were returned to nearly the initial level after DTT treatment. Moreover, oxalacetate reductase activity remained constant during these treatments.

**DISCUSSION**

In addition to the oxidative decarboxylation of L-malate (Reaction 1), decarboxylation of oxalacetate (Reaction 2), and reduction of pyruvate (Reaction 3), crystalline pigeon liver malic enzyme has been found to catalyze the metal-dependent reduction of oxalacetate (Reaction 4), and conversion of L-malate to L-lactate mediated by TPN (Reaction 5). Reactions 1 and 2 are major activities catalyzed by the enzyme with high efficiency (i.e. specific activity of 20 to 30). Reactions 3 through 5 are minor activities, each accounting for less than 15% of the major activities of the native enzyme. Therefore, the possibility of a contribution from enzymatic contaminants in an otherwise physicochemically homogenous enzyme preparation is real and cannot be overlooked. Experimental evidence is, however, overwhelmingly in support of all five activities residing in the malic enzyme molecule, as shown by the following. (a) All activities have in common requirements for a divalent metal and for TPN in its oxidized (MEa and oxalacetate decarboxylase) or reduced form (pyruvate and oxalacetate reductase). (b) To the author’s knowledge, no other enzyme catalyzes metal-dependent pyruvate or oxalacetate reductase activity. (c) All of these activities involve structurally related substrates and products and are compatible with alternate reaction pathways predictable from the postulated sequential, ordered mechanism for malic enzyme (6). The ability of malic enzyme to use oxalacetate as a substrate in both decarboxylation and reduction reactions suggests that this compound is the enzyme-bound intermediate during catalysis. In an analogous case, oxaloacetate was implicated as the enzyme-bound intermediate on the strength of similar decarboxylation and reduction activities possessed by the mechanistically similar isocitrate dehydrogenase (12). The alternative possibility of a lactate intermediate (13) is untenable according to both chemical and experimental considerations. Therefore, the mechanism of malic enzyme postulated in a previous study (6) can be revised as shown in Scheme 1. In accordance with recent findings of Dalziel and Londesborough (14), CO2 rather than bicarbonate is depicted as the initial product. In this scheme, oxalacetate reductase activity is accounted for by the formation of a central enzyme-TPNH-oxalacetate ternary complex from the required substrates (Step 7), which then isomerizes to an enzyme-TPN-malate complex (Step 3), followed by release of malate and TPN (Steps 2 and 1).

The Michaelis constant for pyruvate was found to be 5.52 mM, in good agreement with the corresponding value of 6.4 mM previously determined for the reductive carboxylation reaction (6). Identical Michaelis constants would be consistent with a single protein catalyzing these two reactions, which share in common Steps 6 and 5 in the reaction mechanism (Scheme I).

For the native enzyme, the rate of reductive carboxylation is approximately 5 times faster than reduction of pyruvate (Fig. 6B), indicating that Step 8 (or possibly Step 9) must be rate-limiting for L-lactate formation. Analogous to lactate dehydrogenase, which has an equilibrium constant of $1.4 \times 10^{-16}$ (15), pyruvate reductase is practically irreversible (i.e. as demonstrated by the failure of 0.25 mg of enzyme to catalyze reduction of TPN at pH 9.3 in the presence of L-lactate and a trapping agent, hydrazine sulfate). TPN-mediated conversion of L-malate to L-lactate occurs only slowly in the presence of relatively large amounts of enzyme (0.15 to 0.67 mg). Lack of an initial lag (Fig. 4) is consistent with formation of L-lactate by enzyme-bound pyruvate from E-TPN-pyruvate complex through sequential Steps 1, 2, 3, 4, 8, and 9. The predominant reaction is the release of free pyruvate through Steps 1 through 5. Preferential formation of free pyruvate (rather than L-lactate) from enzyme-bound pyruvate can be due to the additive effects of low affinity of pyruvate for the enzyme (i.e. a dissociation constant for pyruvate (Kd) of 8 mM is calculated from previous kinetic studies (6)) and to the slow conversion of E-TPN-pyruvate complex to L-lactate (Steps 8 and 9).

Titration of approximately 4 sulfhydryl groups by DTNB on the presumably tetrameric malic enzyme molecule (16, 17) led to profound changes in its activities. Reversibility of this titration was demonstrated by DTT treatment. Aging of this enzyme at low protein concentration resulted in a similar loss of malic enzyme activity. This loss, however, was only partially recoverable by DTT incubation normally sufficient for complete reactivation (Table II). This finding suggests that, in addition
to oxidation of sulfhydryl groups, aging also leads to irreversible denaturation of the enzyme. This denaturation process accounts for the observed loss of pyruvate reductase during aging, rather than an increase expected from results of reversible modification of sulfhydryl groups by DTNB (Fig. 6).

Intracellular enzymes frequently are inactivated by thiol reagents (18). This type of inactivation usually involves disruption of the tertiary or quaternary structures of these proteins as a result of modification of protein monothiols. In only a few cases (19) has there been strong experimental evidence in support of the participation of protein thioesters in the catalytic process. Mercurials can lead to both activation and inactivation. Malate dehydrogenase was activated after titration of 3 to 4 sulfhydryl groups by p-mercuribenzoate and inactivated following more extensive reaction (20). More comparable with the current observation is the finding that O-iodosobenzonate and iodine monochloride induced the concurrent and reversible inactivation of dehydrogenases and activation of acetylphosphatase activities catalyzed by glyceraldehyde 3-phosphate dehydrogenase (21), whereby direct participation of the reactive sulfhydryl group at the active center was implicated.

DTNB-induced inactivation of malic enzyme, activation of pyruvate reductase, and unaltered level of oxalacetate reductase are compatible with Scheme 1 on the basis of blockage of the CO₂ site (Step 4) by limited modification of the sulfhydryl groups. This blockage should lead to the loss of activities involving decarboxylation such as malic enzyme (ME₁ and ME₂) and oxalacetate decarboxylase. Loss of malic enzyme activity was demonstrated in these experiments. Parallel loss of oxalacetate decarboxylase activity was shown in a previous study (1), where the ratio of these two activities remained constant at 1.25, despite a 4-fold change in their absolute values after aging and DTT reactivation. Neither pyruvate nor oxalacetate reduction involves direct participation of CO₂, hence the lack of inactivation. The CO₂ block can occur either at the bond-breaking step (E-oxalacetate → E-CO₂-pyruvate) or the desorption step. Present information, however, cannot resolve these two possibilities.

The specific and reversible effects of DTNB rules out denaturation as a factor. Significant contributions by the attachment of the relatively bulky and negatively charged 5-thio(2-nitrobenzoic acid) molecule to protein monothiols during mixed disulfide formation are unlikely. Under conditions of these titration experiments, DTNB is limiting and forms two TNB equivalents according to Reaction 6.

\[
\text{DTNB} + \text{protein} \rightarrow \text{protein} - S - S - \text{TNB} + \text{TNB} - \text{SH}
\]

\[
\text{protein} - S - S - \text{TNB} + \text{protein} - \text{SH} \rightarrow \text{protein} - S - S - \text{protein} + \text{TNB} - \text{SH}
\]  

(6)

The specific and reversible effects of DTNB rules out denaturation as a factor. Significant contributions by the attachment of the relatively bulky and negatively charged 5-thio(2-nitrobenzoic acid) molecule to protein monothiols during mixed disulfide formation are unlikely. Under conditions of these titration experiments, DTNB is limiting and forms two TNB equivalents according to Reaction 6.

For fatty acid synthesis would then be limited by the greatly diminished malate enzyme activity, as well as by increased oxidation of this coenzyme by the two reductases.

REFERENCES

Mechanism of Pigeon Liver Malic Enzyme: FORMATION OF L-LACTATE FROM L-MALATE, AND EFFECTS OF MODIFICATION OF PROTEIN THIOL GROUPS ON MALIC ENZYME, OXALACETATE, AND PYRUVATE REDUCTASE ACTIVITIES

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