Characterization of the Tetramer–Dimer–Monomer Equilibrium of the Enzymatically Active Subunits of Pigeon Liver Malic Enzyme†

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ABSTRACT: The tetrameric malic enzyme from pigeon liver was reversibly dissociated in the sequence of tetramer–dimer–monomer in an acidic environment (pH 4.5) or when the ionic strength or temperature of the solution was perturbed (0.2 M ammonium sulfate or <10 °C). The dissociated monomer was enzymatically active according to the following criteria: (a) separation and direct activity staining of the monomer in the native gradient polyacrylamide gel, (b) activity staining of the monomer at its p*I* region in the isoelectric focusing gel, and (c) the enzyme showing lower but definite enzyme activity under conditions where only monomer existed in the solution. The catalytic constant (k_{cat}) and specificity constant ($k_{cat}/K_{m_{Mal}}$) for the monomer were found to be 19 ± 6 s^{-1} and 58 × 10^3 $s^{-1}\cdot M^{-1}$, respectively, only one-seventh and one-seventeenth of those for the tetramer. Different types of interactions are involved in the monomer–monomer and dimer–dimer associations: (a) Two dissociation processes showed different pH dependences. The monomer–monomer interactions involve an amino acid with a side chain pK_a value around 5.7, and an amino acid with a side chain pK_a value of 7.2 is involved in the dimer–dimer association. (b) Ammonium sulfate up to 0.2 M only affects the monomer–monomer but not the dimer–dimer interactions. The Gibb's free energy, enthalpy, and entropy all have negative values for the above subunits' dissociations. The overall dissociation is an enthalpy-driven process. Association of the subunits to form dimers and tetramers involves salt-bridge, van der Waals, and hydrogen-bonding interactions. Small amounts of trimer were also detectable under certain experimental conditions. These experimental results are consistent with our previously proposed preexisting asymmetric model for the tetrameric pigeon liver malic enzyme [Lee, H. J., & Chang, G. G. (1990) *FEBS Lett. 277*, 175–179]. The possible role of quaternary structure modulation in regulating the malic enzyme activity in vivo is discussed.

Cytosolic malic enzyme [(*S*)-malate:NADP$^+$ oxidoreductase (oxaloacetate-decarboxylating); EC 1.1.1.40] catalyzes the reversible oxidative decarboxylation of L-malate to give CO_2 and pyruvate, with concomitant reduction of NADP$^+$ to NADPH. In avians, the major physiological function of this enzyme is believed to provide NADPH for fatty acid biosynthesis. It is thus, together with acetyl-CoA carboxylase, fatty acid synthase, and glucose-6-phosphate dehydrogenase, classified as a lipogenic enzyme and is under nutritional and hormonal regulations [for reviews, see Frenkel (1975), Wakil (1983), Goodridge et al. (1989), and Iritani (1992)].

Malic enzyme from pigeon liver is a tetrameric protein composed of identical subunits (Nevaldine et al., 1974; Kam et al., 1987). We have previously demonstrated that, in an acidic environment, the enzyme was reversibly dissociated to its constituted monomers. The dissociated monomer was shown to possess enzymatic activity by an enzyme immobilization technique (Chang et al., 1988).

In this study, we further investigate the factors that influence the dissociation/association equilibrium of the enzyme. The physical interactions involved in the subunit–subunit association are characterized. We provide more evidence to demonstrate that the dissociated monomer is enzymatically active. Furthermore, we present structural data in support of our previously proposed preexisting asymmetric model for the enzyme (Lee & Chang, 1990). The possible role of modulation of the quaternary structure in regulation of malic enzyme activity in vivo is also discussed.

MATERIALS AND METHODS

Materials. Glutaraldehyde, ^2H_2O (99.996% ^2H), NADP$^+$, nitroblue tetrazolium, L-malate, and phenazine methosulfate (Sigma, St. Louis, MO); adenosine 2′,5′-diphosphate–Agarose and precast mini-slab gels (PhastGels); M_r and p*I* markers (Pharmacia-LKB, Uppsala, Sweden); ammonium sulfate, sodium acetate, and sodium chloride (E. Merck, Darmstadt, Germany) were purchased from the designated sources. All other chemicals were of reagent grade purchased from Sigma or E. Merck as described previously (Chang et al., 1988; Lee & Chang, 1990). Distilled deionized water was used throughout this work.

Enzyme Purification. Malic enzyme from pigeon liver was purified according to our published procedure (Chang & Chang, 1982). Homogeneous enzyme was obtained after heat treatment, ammonium sulfate fractionation, DEAE-cellulose chromatography, and adenosine 2′,5′-diphosphate–Agarose affinity chromatography. The purified enzyme was routinely checked for purity by polyacrylamide gel electrophoresis. Only a single band was stained with protein at pH 7.7. The corresponding band was stained positive with enzymatic activity.

Protein concentration was determined spectrophotometrically at 278 nm, using a molar absorption coefficient of 2.236 × 10^5 for the tetramer and 0.559 × 10^5 for the monomer. M_r values of 260 000, 130 000, and 65 000 were used for the

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tetramer, dimer, and monomer, respectively (Nevaldine et al., 1974).

Enzyme Assay. The assay for the malic enzyme activity followed the method described recently (Chang et al., 1992). Since free substrates and metal ion are the true reactants for malic enzyme (Cannellas & Wedding, 1980; Park et al., 1984), the reactant concentrations were corrected for the chelation with Mn^{2+}. A 1-mL reaction mixture contained 66.7 mM triethanolamine hydrochloride buffer (pH 7.4), 0.5 mM L-malate (0.419 mM when corrected for Mn^{2+}–L-malate chelation), 0.23 mM $NADP^+$ (0.177 mM when corrected for Mn^{2+}–$NADP^+$ chelation), 4 mM Mn^{2+} (3.87 mM when corrected for Mn^{2+}–L-malate and Mn^{2+}–$NADP^+$ chelations), and an appropriate amount of enzyme. The formation of NADPH at 30 °C was monitored continuously at 340 nm with a Varian DMS-100 spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzed an initial rate of 1 μmol of NADPH formed per minute under the assay conditions. Specific activity was expressed in units per milligram of protein. Specific activity divided by the molar equivalent gave k_{cat}. Our k_{cat} value calculation thus was based on the active site within the tetrameric complex. On the basis of the same amount of protein, the ratio of the number of molecules for M:T is 4:1; the k_{cat} value for the monomer is thus one-fourth of that for the tetramer. We analyzed the kinetic data with EZ-FIT (Perrella, 1988), a curve-fitting microcomputer program using the Melder–Mead Simplex and Marquardt nonlinear regression algorithms sequentially.

Polyacrylamide Gel Electrophoresis under Native Conditions with a Gradient Gel. Polyacrylamide gel electrophoresis was performed essentially according to our previously reported procedure (Chang et al., 1991). The electrophoresis was performed in duplicate with a Pharmacia PhastSystem in PhastGel 8–25% gradient mini-slab gels and a PhastGel native buffer strip (containing 0.88 M L-alanine, 0.25 M Tris-HCl, pH 8.8). The samples were subjected to electrophoresis at 10 mA, 400 V, and 15 °C for 200 V·h. After electrophoresis, one gel was stained for protein with Coomassie Brilliant Blue R-350 that enabled more sensitive detection than the R-250. The other gel was stained for enzymatic activity.

Enzyme activity staining was performed by immersing the gel into a solution containing 55 mM triethanolamine hydrochloride buffer (pH 7.4), 0.47 mM $NADP^+$, 17.2 mM L-malate, 2.75 mM $MnCl_2$, 0.55 mg/mL nitroblue tetrazolium, and 0.097 mg/mL phenazine methosulfate. After 2 h, the reaction was stopped by replacing the staining solution with 5% acetic acid. The position of active enzyme was stained violet-blue.

Isoelectric Focusing. Isoelectric focusing was performed in duplicate with a PhastGel IEF[1] 3–9 plate (coated with polyacrylamide gel and having a linear pH gradient from 3 to 9 with Pharmalyte) or an IEF 4–6 plate. Electrophoresis was performed at 25 mA, 2000 V, and 15 °C (500 V·h). One plate was subjected to enzyme activity staining. Another plate was stained for protein. Various standards within a wide p*I* range were used as calibrating standards.

Dissociation of the Pigeon Liver Malic Enzyme. The enzyme molecule was dissociated to various degrees in buffers with different pH values or in the presence of various amounts of ammonium sulfate. The associated molecules were cross-linked with glutaraldehyde according to Hermann et al. (1979) with some modifications (Chang et al., 1988). The concentration of glutaraldehyde used was 20 mM, a 104 molar excess over the enzyme concentration. In our experience, glutaraldehyde at concentrations less than 1 mM did not efficiently cross-link the associated subunits. High glutaraldehyde concentrations, however, would cause intermolecular cross-linking. We found that glutaraldehyde concentrations between 5 and 22.5 mM gave satisfactory results.

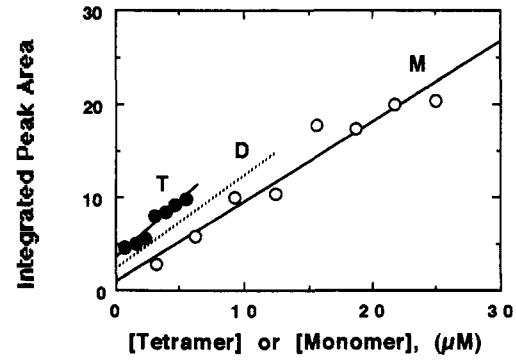

FIGURE 1: Relationship between the protein band integrated area and the enzyme concentration. Various amounts of malic enzyme in borate buffer (pH 8.5) (●) or acetate buffer (pH 3.0) (○) were subjected to cross-linking and SDS–PAGE. The enzyme bands stained with protein were quantitated by densitometric analysis. Only tetramer or monomer was observed at pH 8.5 or 3.0, respectively. The dashed line represented a hypothetical calibration line used in estimating the dimer concentration. Abbreviations: T, tetramer; D, dimer; M, monomer.

After chemical cross-linking (for 2–20 min), the enzyme solution was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with a PhastGel 10–15% gradient mini-slab gel and a PhastGel SDS buffer strip (containing 0.2 M Tricine, 0.2 M Tris, 0.55% SDS, pH 7.5). Electrophoresis was carried out at 10 mA, 250 V, and 15 °C (60 V·h). After completion of the electrophoresis, the protein was stained with Coomassie Brilliant Blue. Various enzyme forms were identified by comparison of the relative mobilities with various M_r markers. This interpretation has been verified with sucrose density gradient ultracentrifugation. The distribution of various enzyme forms was estimated by scanning with a Molecular Dynamics computing laser densitometer and then calculating with the ImageQuant software.

Quantitation of Each Enzyme Form. Malic enzyme existed almost exclusively (>95%) as monomer or tetramer at pH 3.0 and 8.5, respectively. Under these conditions, correlation of the densitometrically determined integrated area and the protein amount applied was obtained. From known enzyme concentrations, calibration lines were constructed for the tetramer and monomer. Reasonably good linearity was obtained for both enzyme forms (Figure 1). We were not able to find conditions under which all enzyme was in the dimeric form. Therefore, the calibration line for the dimer was not experimentally available. For quantitation of the dimer, we thus constructed a hypothetical line between the lines for the tetramer and monomer (Figure 1). In this way, the concentration of each enzyme form was calculated from its integrated peak areas after appropriate correction. In integrating the peak areas, each peak was determined in triplicate to minimize systematic errors. We followed the procedure as recommended in the manual of ImageQuant by drawing a rectangular bar covering ~80% of each protein

[1] Abbreviations: T, tetramer; D, dimer; M, monomer; K_T, dissociation constant for the tetramer giving two dimers; K_D, dissociation constant for the dimer giving two monomers; K_{TD}, overall dissociation constant for the tetramer giving four monomers; eu, entropy unit (cal·mol^{-1}·K^{-1}); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

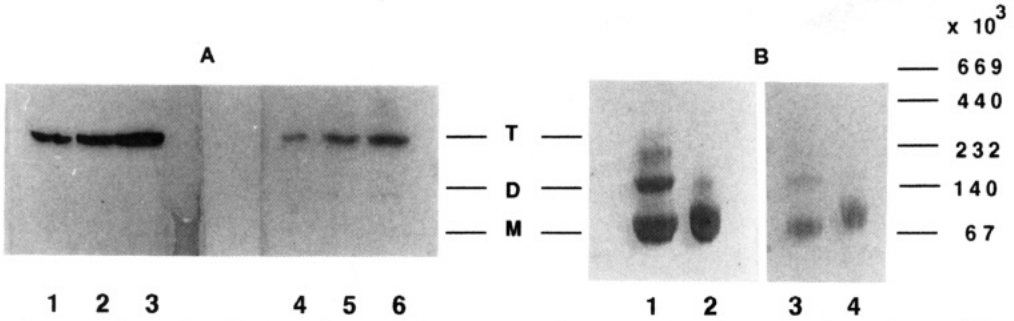

FIGURE 2: Staining of enzymatic activity of various forms of pigeon liver malic enzyme in PAGE gels under native conditions. (A) PAGE was performed with a PhastGel 4–15% gradient gel in duplicate at 15 °C and pH 7.7 for 210 V·h. Lanes 1, 2, and 3 were stained for enzyme activity. Lanes 4, 5, and 6 were stained for protein. Protein amount applied in each lane: (1) 0.032 μg, (2) 0.063 μg, (3) 0.126 μg, (4) 0.105 μg, (5) 0.53 μg, (6) 1.05 μg. (B) A similar experiment was performed with acidic sodium acetate buffer, and the enzyme was cross-linked with glutaraldehyde before being subjected to PAGE at 5 °C and 108 V·h. Lanes 1 and 2 were stained for enzyme activity. Lanes 3 and 4 were stained for protein. Lanes 1 and 3 were cross-linked at pH 5.7. Lanes 2 and 4 were cross-linked at pH 3.9. Abbreviations: T, tetramer; D, dimer; M, monomer. The M_r markers were also labeled (thyroglobulin, M_r 669 000; ferritin, M_r 440 000; catalase, M_r 232 000; lactate dehydrogenase, M_r 140 000; bovine serum albumin, M_r 67 000).

band and calculating its area. This strategy gave acceptable results.

In the working range of our experiments (pH 4.5–7; temperature 15–30 °C; or 1–100 mM $(NH_4)_2SO_4$), reasonable amounts of T, D, and M exist, which make calculation of the K_T and K_D possible. Under certain extreme conditions, calculation of K_T or K_D was difficult and subjected to large error. Only reliable data were used in further analysis.

RESULTS

Demonstration of the Enzymatically Active Subunits of Pigeon Liver Malic Enzyme. Pigeon liver malic enzyme existed exclusively as the tetramer in alkaline pH (Figure 2A). The enzyme was dissociated in an acidic environment (Figure 2B). In pH range 5.5–7.0, various enzyme forms coexisted in solution. The various enzyme forms were well separated in the gradient gel under native conditions that enabled us to directly stain the enzymatic activity in the gel. As shown in Figure 2B, the monomer and dimer possessed definite enzyme activity. In a later section, we will demonstrate that the enzyme also underwent dissociation at a basic pH but at a lower temperature. In the experiment shown in Figure 2B, the associated subunits were chemically cross-linked. The subsequent electrophoresis was performed at 5 °C, and thus the monomeric form did not reassociate to the tetrameric form during the electrophoretic period.

In Figure 2B (lane 1), at pH 5.7, besides the major monomer and dimer and small amount of tetramer forms, we can clearly see the existence of trimer that was also enzymatically active. Since pigeon liver malic enzyme is a half-sited enzyme (Hsu, 1982; Chang et al., 1989), the demonstration of enzymatically active monomer and trimer in solution has profound influence on the interpretation of the origin of the half-of-sites reactivity. We further demonstrated the active subunit by isoelectric focusing electrophoresis. The enzyme was dissociated to various degrees by diluting the enzyme solution with low pH buffers. The enzyme was then subjected to isoelectric focusing under native conditions. After electrophoresis, the slab gel was subjected to enzyme staining. As shown in Figure 3, the p*I* value of the enzyme increased with the lowered pH. At pH 7.4, when most of the enzyme molecule exists as the tetramer, the enzyme has a p*I* value of 6.24 ± 0.10, while at pH 3.9, the subunit of the enzyme has a p*I* value of 6.39 ± 0.17. When the enzyme was dissociated in an acidic environment, the enzyme showed a progressive increase in its p*I* value. Please note that each enzyme band in the gel represents a mixture of T, D, and M of various proportions

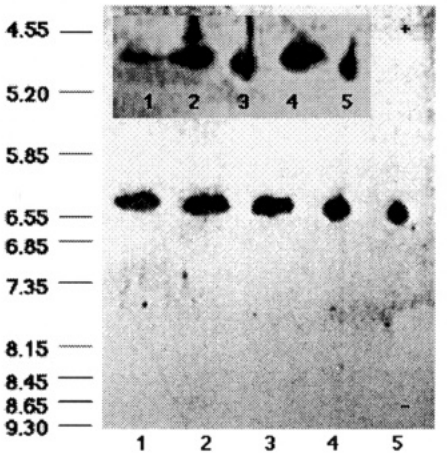

FIGURE 3: Isoelectric focusing of the various forms of pigeon liver malic enzyme. Malic enzyme at various pH values was subjected to IEF under native conditions with a PhastGel IEF 3–9 plate. Lanes 1–5 were malic enzyme in pH 7.44, 7.03, 5.7, 4.56, and 3.9, respectively. After electrophoresis, the plate was subjected to enzymatic activity staining. The inset shows a staggered arrangement of the same samples as above. Lanes 1–5 were the enzyme in pH 5.7, 7.03, 3.9, 7.44, and 4.56, respectively. A video scanning image of the gel was presented. The p*I* markers were also labeled.

in the solution. Probably because various enzyme forms have very close p*I* values, the resolution of the isoelectric focusing is not yet able to separate each enzyme form in the gel.

The above experiment was repeated several times. Reproducible results were obtained. However, to avoid the possible artifact due to the uneven slab gel, we arranged the samples in a staggered manner. As shown in the inset figure of Figure 3, various enzyme forms indeed have different p*I* values. Please also note that all lanes in Figure 3 were stained positive by the enzyme activity staining. The same amount of enzyme was applied to each lane. After densitometric scanning and quantitation, we estimated that the specific activity of the subunit was about half of that for the tetramer. This result is in accordance with our kinetic analysis (see below).

We have examined the enzyme activity at various enzyme concentrations by a kinetic assay. Constant specific activity was obtained in the protein range we employed in this investigation. Thus, the enzyme dilution method of Kurganov (1967) to test the subunit activity of malic enzyme is not applicable in our case.

Effect of pH and p²H on the Dissociation of Pigeon Liver Malic Enzyme. We have previously shown that the enzyme

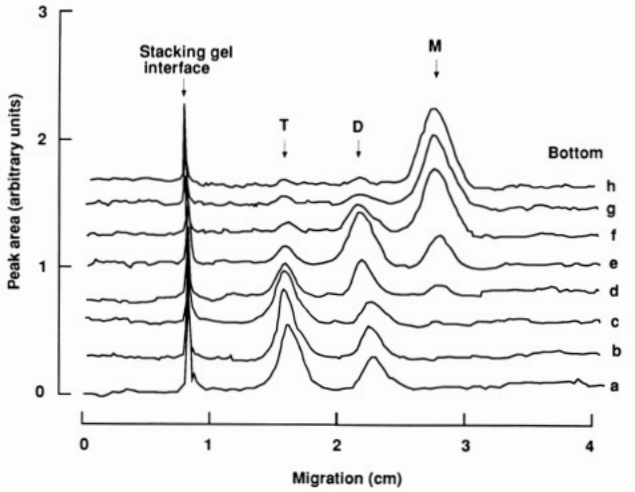

FIGURE 4: Densitometric tracing of the pigeon liver malic enzyme dissociated in an acidic environment. Malic enzyme (1.4 mg/mL) in 0.17 M sodium acetate buffer of various pH was subjected to cross-linking and SDS–PAGE as described under Materials and Methods. The pH values of the solution were (a) 7.44, (b) 7.43, (c) 7.35, (d) 7.03, (e) 6.6, (f) 5.7, (g) 5.1, and (h) 4.56. In this experiment, to minimize other factors that might affect the dissociation, the same buffer was used in at all pH values. The pH values in the first three tracings were very close due to the low buffer capacity of acetate buffer at basic pH. The pH values shown were the actual recorded values.

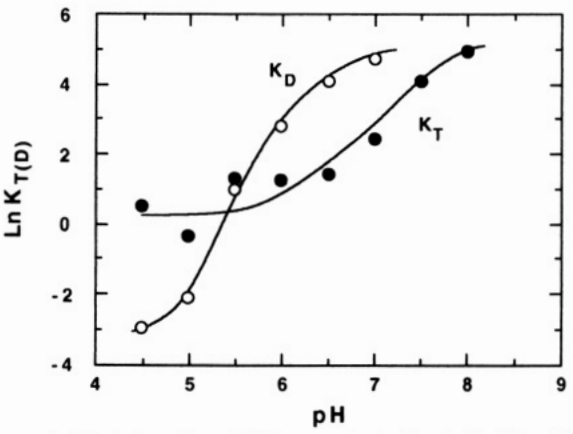

FIGURE 5: Variation of equilibrium constants for the D–M and T–D processes with pH. The natural logarithm of K_D (○) or K_T (●) is plotted versus pH. Borate buffer (pH >7) and acetate buffer (pH <6.5) were used.

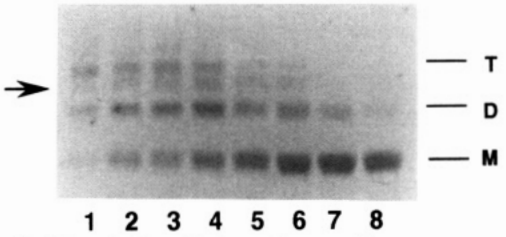

FIGURE 6: Dissociation of pigeon liver malic enzyme at various temperatures. Malic enzyme in sodium acetate buffer (pH 7.5) was cross-linked with 5 mM glutaraldehyde at various temperatures and subjected to SDS–PAGE with a 4–15% gel at 63 V·h. The temperatures during cross-linking and electrophoresis were (1) 30, (2) 25, (3) 22.5, (4) 20, (5) 17.5, (6) 15, (7) 10, and (8) 5 °C.

was reversibly dissociated in an acidic environment (Chang et al., 1988). The progressive dissociation of the enzyme in the sequence of T–D–M is clearly shown in Figure 4. The distribution of enzyme forms was evaluated by densitometric scanning of the gel and calculation of the area of each peak. If we tentatively ignore the minor amount of trimer in the solution, the sequential dissociation of tetramer to dimer and then from dimer to monomer are shown in

$$T \rightleftarrows 2D \quad \text{and} \quad D \rightleftarrows 2M \tag{1}$$

The equilibrium constants for the dissociation of a tetramer to give two dimers (K_T) and a dimer to give two monomers (K_D) are defined as

$$K_T = [D]^2/[T] \tag{2}$$

$$K_D = [M]^2/[D] \tag{3}$$

Although each enzyme band in the gel was diffused due to glutaraldehyde cross-linking, the area under each enzyme form can be estimated with reasonable accuracy by using laser densitometric analysis together with the excellent ImageQuant imaging analysis capability. In this way, we were able to calculate the K_T and K_D under various conditions. The dependence of the K_T and K_D on pH is shown in Figure 5. Amino acid residues with side chain pK_a values of ~5.7 and 7.2 were involved in the M–M and D–D associations, respectively. The pK_a values of these interacting groups are most likely identified with Glu/Asp and His/Cys side chain ionizations.

Hydrophobic interaction may play an important role in maintaining the protein structure. Hydrophobic interaction is stronger in ^2H_2O than in H_2O (Kreshek et al., 1965). If a hydrophobic interaction is involved in the association of malic enzyme, ^2H_2O should enhance that association (Anne et al., 1971). However, we found that dissociation was not detectably different in H_2O and ^2H_2O (data not shown). Thus, the involvement of other physical interactions should be considered.

We then investigated the effects of temperature and salt concentration on the dissociation process.

Effect of Temperature on the Dissociation of Pigeon Liver Malic Enzyme. Malic enzyme was dissociated at a low temperature. Figure 6 shows the progressive dissociation of the enzyme as the temperature was lowered from 30 to 5 °C. No tetramer was found at temperatures below 10 °C. The dissociation also followed the T–D–M sequence as protein bands corresponding to T, D, and M were the major enzyme forms. Again, as indicated by an arrow in Figure 6, trimer was observed. The equilibrium constants K_T and K_D were estimated at each temperature. The overall equilibrium constant (K_{TD}) was also calculated according to

$$K_{TD} = [M]^4/[T] = K_T K_D^2 \tag{4}$$

This information was used to estimate the enthalpy ($\Delta H°$) value in the dissociation process. Figure 7 shows the van't Hoff plot of the dependence of ln K (K_T, K_D, or $K_T K_D^2$) versus the reciprocal of the absolute temperature in Kelvins. Linear plots were obtained in all cases. Both the lines using K_T or K_D data gave almost identical slope values showing a similar enthalpy contribution in the D–D and M–M interactions. The enthalpy values, together with the other thermodynamic parameters the Gibb's free energy ($\Delta G°$) and the entropy contribution ($\Delta S°$) were summarized in Table I. The $\Delta G°$ of dissociation was calculated from the equilibrium constant K_{eq} according to

$$\Delta G° = -RT \ln K_{eq} \tag{5}$$

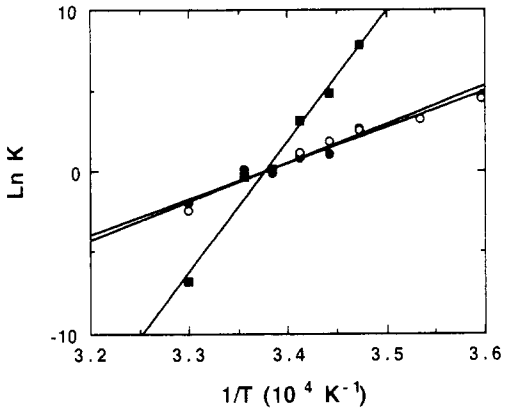

FIGURE 7: van't Hoff plots for the T–D, D–M, and overall T–M processes. The natural logarithm of K_D (○), K_T (●), or K_{DT} ($K_TK_D^2$) (■) is plotted versus the reciprocal of temperature in Kelvin. The slope in these plots was used to estimate the enthalpy contribution to the equilibrium free energy of various processes.

Table I: Thermodynamic Parameters for the T–D, D–M, and Overall T–M Equilibrium Systems of Pigeon Liver Malic Enzyme at 25 °C

process	$\Delta H°$ (kcal·mol^{-1})	$\Delta G°$ (kcal·mol^{-1})	$\Delta S°$ (eu) (cal·mol^{-1}·K^{-1})
T–D	−47.1 ± 0.01[a]	−0.94 ± 0.03	−154.9 ± 1.3
D–M	−42.6 ± 1	−0.29 ± 0.01	−141.9 ± 3.3
T–M	−155 ± 5	−1.5 ± 0.05	−515 ± 17

[a] All values shown are mean ± SEM.

where R is the gas constant and T is the absolute temperature. The $\Delta S°$ of dissociation was estimated according to

$$\Delta S° = (\Delta H° - \Delta G°)/T \quad (6)$$

Our $\Delta H°$ values were derived from the van't Hoff plots. It should be noted that the van't Hoff equation is based upon a two-state model of equilibrium and might not be applicable to a multistate process. As pointed out by Ross and Subramanian (1981), calorimetrically determined enthalpy changes will determine the sum of a multiequilibrium system. However, since no curvature could be reliably detected for the lines shown in Figure 7, there does not appear to be any measurable change in heat capacity, ΔC_p, over the temperature range we employed.

Effect of Salt Concentration on the Dissociation of Pigeon Liver Malic Enzyme. Since dissociation/reassociation of pigeon liver malic enzyme is pH-dependent and electrostatic interactions are proposed to be involved in the process, the effects of ionic strength of the solution were further explored for more information. Figure 8 shows the distribution of various enzyme forms in different concentrations of $(NH_4)_2SO_4$ (Figure 8A) and NaCl (Figure 8B) at constant pH and temperature. The enzyme was dissociated at a high $(NH_4)_2SO_4$ concentration. Further examination of the data indicates that the dissociation of tetrameric malic enzyme in the presence of $(NH_4)_2SO_4$ was mainly due to the increased K_D (Figure 9E). The D–D interactions (K_T) were not influenced even at 200 mM $(NH_4)_2SO_4$. On the other hand, NaCl up to 0.4 M had little effect on the dissociation process. The values of K_T, K_D, and $K_TK_D^2$ varied very little with the NaCl concentrations used (Figure 9B,D,F). These results indicate that ionic strength may not be a major factor affecting the dissociation/association process in vivo.

We have mentioned in previous sections that the monomer of the malic enzyme is enzymatically active. We are interested to know the kinetic properties of the monomer as compared to the tetramer. Now we may conclude that pH and temperature are the major factors governing the quaternary structure of malic enzyme in solution. However, low temperature not only causes enzyme dissociation but also lowers the reaction energy. In a similar manner, low pH not only causes dissociation but also may affect the ionization state of the essential groups involved in the substrate binding or catalysis. The enzyme activity measured under these conditions may not reflect the true kinetic properties. Actually, the activity of malic enzyme at 5 °C or pH 4.5 was too low to be practical in estimating the kinetic parameters for the monomer. As shown in Figure 8A, malic enzyme was almost completely dissociated into monomer in the presence of 0.2 M $(NH_4)_2SO_4$. We believe that enzyme activity measured in the presence of $(NH_4)_2SO_4$ may have fewer other interferences. If we tentatively neglect the possible effect of ionic strength on the ionization of essential groups, it is possible to measure the enzyme activity of the monomer by kinetic assays. The substrates in the assay mixture would only drive the enzyme molecule toward the dissociation direction (Chang et al., 1988). The enzyme activity assayed in the presence of 0.2 M $(NH_4)_2SO_4$ thus represents the activity for the monomer. Table II shows the comparison of various kinetic parameters for the tetramer and monomer. Monomer has higher apparent K_m values for $NADP^+$, L-malate, and Mn^{2+}. The k_{cat} value for the monomer is only one-seventh that for the tetramer. Taking substrate binding affinity into consideration, the specificity constant ($k_{cat}/K_{m_{Mal}}$) for monomer is lowered by 17-fold as compared to that of the tetramer. The monomer also shows substrate inhibition by L-malate with $K_{i_{Mal}}$ of 8.54 ± 4.8 mM.

DISCUSSION

We have observed biphasic changes of fluorescence quenching parameters, and the results were interpreted as dissociation and denaturation of subunits (Lee et al., 1988). The experimental conditions used in the present study dealt with the dissociation/reassociation without denaturation (phase I only). The following criteria suggest that minimum conformational changes occurred after dissociation: (a) The dissociation process was completely reversible. Both the dissociation and reassociation were very fast processes (Chang et al., 1988). (b) Circular dichroism at $[\theta]_{222}$ was not perturbed during dissociation, suggesting minimum secondary structure changes of the enzyme molecule (Lee et al., 1988).

With the assumption that minimum conformational changes occurred, we tried to analyze our thermodynamic data. The pH studies suggest the involvement of ionic (charged neutralization) interactions. As indicated by Aune and Timasheff (1971), the pH-induced dissociation of tetramer could be the result of several factors related to the nature of D–D and M–M interactions: (a) Protonation of an anionic group may eliminate an attractive cationic–anionic interaction; (b) the unfavorable long-range electrostatic repulsion between two macroions with a net negative charge may increase. Our results of the pH-dependence dissociation suggest that amino acids with side chain pK_a values of ~5.7 and 7.2 are involved in the subunit interactions. These results suggest that the dissociation/reassociation process may be analyzed by a stopped-flow spectrophotometer with appropriate indicators (Koren & Hammes, 1976; Kitano et al., 1989). We have delineated the complete amino acid sequence of pigeon liver malic enzyme by cDNA cloning and polymerase chain reaction techniques (Chou et al., unpublished results). However, without the tertiary structure of the enzyme, it is difficult at

Table II: Kinetic Parameters for the Tetrameric and Monomeric Pigeon Liver Malic Enzyme

	sp act. (units/mg)	k_{cat} (s^{-1})	$K_{m_{Mal}}$ (μM)	$K_{i_{Mal}}$ (mM)	$K_{m_{NADP}}$ (μM)	$K_{m_{Mn}}$ (μM)	$k_{cat}/K_{m_{Mal}}$ ($s^{-1}\cdot M^{-1}$)
tetramer	30.8 ± 0.18[a]	134 ± 1	134 ± 22	14.7 ± 3.2	19 ± 5	0.65 ± 0.06	1.0×10^6
monomer[b]	17.5 ± 5.7	19 ± 6	330 ± 50	8.54 ± 4.8	38.6 ± 11	2.62 ± 0.2	58×10^3

[a] All values shown are mean ± SEM. [b] Assayed in the presence of 0.2 M ammonium sulfate.

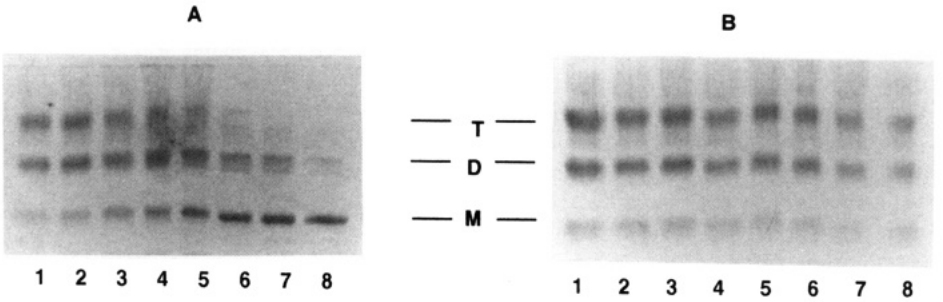

FIGURE 8: Effects of $(NH_4)_2SO_4$ or NaCl concentrations on the dissociation of pigeon liver malic enzyme. Various amounts of NaCl or $(NH_4)_2SO_4$ (pH 7.0) were added into the enzyme solution at pH 7.0 and 25 °C. The enzyme molecule was subjected to cross-linking and SDS–PAGE with a 4–15% gel at 15 °C and 63 V·h. (A) For lanes 1–8, the $(NH_4)_2SO_4$ concentrations were 0.5, 1, 5, 10, 25, 50, 100, and 200 mM, respectively. (B) For lanes 1–8, the NaCl concentrations were 1, 2, 4, 8, 10, 20, 100, and 400 mM, respectively.

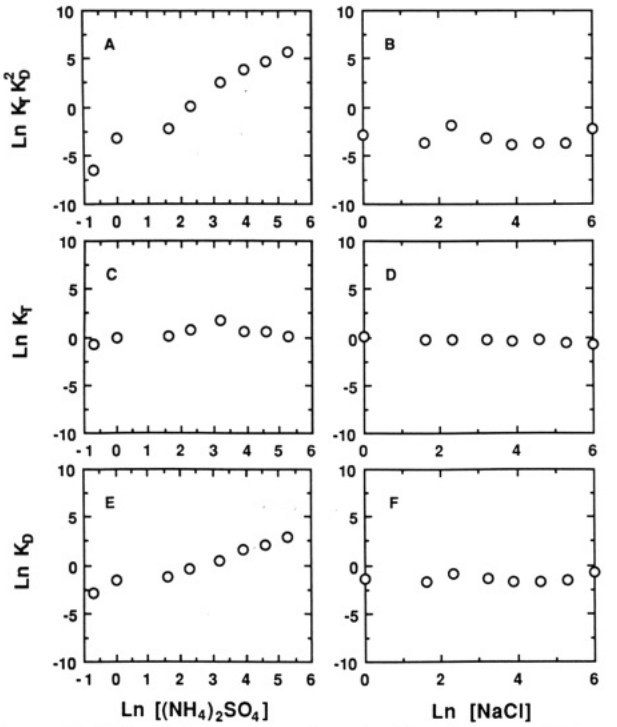

FIGURE 9: Effect of ionic strength on the dissociation equilibrium constants. The natural logarithm of K_{DT} ($K_T K_D^2$) (A, B), K_T (C, D), or K_D (E, F) at pH 7.0 and 25 °C is plotted versus the natural logarithm of $(NH_4)_2SO_4$ concentration (A, C, E) or NaCl concentration (B, D, F).

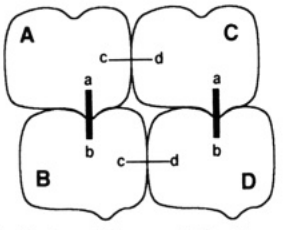

FIGURE 10: Hypothetical working model for the quaternary structure of pigeon liver malic enzyme.

this stage to assign specific amino acid residues involved in the subunits' interactions. We thus interpret our data with physical interactions in general [for reviews, see Burley and Petsko (1988) and Privalov and Gill (1988)].

If only favorable electrostatic salt-bridge-like interactions were involved in association process, increasing the ionic strength up to 0.1 M NaCl should be able to disrupt the subunit–subunit interactions (Mayo & Chen, 1989). Our data showed that malic enzyme subunit interactions were not affected by addition of NaCl up to 0.4 M. This suggests that other forces are also intimately involved in the association process. Hydrophobic interactions have been assumed to play a major role in the formation of a compact structure of globular proteins (Tanford, 1980). Our earlier fluorescence quenching data (Lee et al., 1988) indeed demonstrated the exposure of buried tryptophanyl residues upon dissociation of the tetramer. However, no detectable isotope effect was observed when the dissociation was conducted in ^2H_2O. Furthermore, the hydrophobic interactions resulted primarily from entropy changes. In our case, the overall dissociation is an enthalpy-driven process. Both the negative values for $\Delta H°$ and $\Delta S°$ suggest that the subunit association involves mainly the van der Waals and hydrogen-bonding interactions (Ross & Subramanian, 1981; Chen & Mayo, 1991).

By assuming that immobilization prevents the reassociation of the subunits into the tetramer, we have demonstrated previously that the immobilized subunit at pH 4.5 was enzymatically active when assayed at pH 7.4 (Chang et al., 1988). However, the possibility of conformational restrictions after immobilization mandates caution in reaching such a conclusion. In this paper, we provide conclusive evidence with different approaches in supporting the active subunit of this enzyme: (a) Enzyme activity of the tetramer, dimer, and monomer was stained simultaneously in native gradient gels (Figure 2B); (b) enzymatic activity staining in the isoelectric focusing gel showed that the monomer had a higher pI value albeit with less activity than the tetramer (Figure 3); (c) at 0.2 M $(NH_4)_2SO_4$, most of the enzyme (>95%) existed as the monomer with specific activity of only 17.5 ± 5.7 units/mg, which was about half of that for the tetramer (Figure 8) (Table II). We conclude that the subunit of pigeon liver malic enzyme is active in catalyzing the oxidative decarboxylation reaction.

Our results repeatedly demonstrated the presence of small amounts of trimer (Figures 2B, 6, and 8) which also possessed enzyme activity (Figure 2B). This finding supports our previously proposed preexisting asymmetric model for the enzyme (Lee & Chang, 1990). In summary, this model predicts no symmetry center in the tetramer (Figure 10). The

interactions between subunits are denoted in two types (a–b and c–d). Direct evidence for the different association between AB and AC subunits is shown in Figure 5 where different correlation curves between the ln K_T or K_D versus pH are obtained. The different sensitivity of K_T and K_D toward $(NH_4)_2SO_4$ concentration (Figure 9C,E) provides further evidence. Both the a–b and c–d linkages involve ionic as well as other physical interactions. Binding between A and B subunits may induce conformational changes that prevent further aggregation. However, at high glutaraldehyde and high protein concentrations, we did observe a small amount of higher aggregates (hexamer or octamer), which we interpreted as intermolecular cross-linking (Huang & Chang, 1992). These minor aggregates were not thought to be the major forms of pigeon liver malic enzyme in vivo. It was the asymmetric AB association that caused the half-of-sites reactivity of the enzyme (Lee & Chang, 1990). Due to the asymmetric association, subunits A and B are in different microenvironments. It is thus not surprising to find a trimer in the solution. This model can also explain the atypical modification of the essential sulfhydryl groups of malic enzyme in cyanylation and arylthiolation (Reddy, 1983) and the binding data between the enzyme and $NADP^+$ or etheno-$NADP^+$ (Lee & Chang, 1990). The above asymmetric model can be made directly by X-ray crystallography. To our knowledge, diffraction-quality crystal of malic enzyme has been successfully prepared (Baker et al., 1987). However, no crystal structure of this enzyme is yet available. Thus, the model presented in Figure 10 can only be regarded as a hypothetical working model.

Reversible dissociation was demonstrated for malic enzyme from various sources (Grover & Wedding, 1984; Bartolucci et al., 1987). Regulation of malic enzyme activity in vivo by modulation of quaternary structure has been suggested by Iglesias and Andreo (1990). For pigeon, under the more physiological conditions (pH ~7; [NaCl] = 0.1–0.2 M; body temperature of the bird, 43 °C) (Mayo, 1991; Altman & Dittmer, 1973), malic enzyme highly favors the tetramer. In view of the fast dissociation/reassociation rate, it is possible for this enzyme to regulate its activity through the reversible dissociation process. Malic enzyme is a lipogenic enzyme. We postulate that pH and temperature are the major factors governing the aggregation state of the enzyme in vivo. Among them, temperature might play a more important role. The rectal temperature of the bird varies from ~15 to 47 °C (Altman & Dittmer, 1973). At a low temperature, the bird needs more energy to keep the body warm. Metabolic pathways leading to the biosynthesis of fatty acids should slow down. More substrates (L-malate) would go through the tricarboxylic acid cycle for complete degradation to generate energy.

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