Reversible dissociation of the catalytically active subunits of pigeon liver malic enzyme

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The pH-induced reversible dissociation of pigeon liver malic enzyme (EC 1.1.1.40) was studied by combined use of chemical cross-linking and SDS/polyacrylamide-gel electrophoresis. The tetrameric enzyme showed a pH-dependent dissociation in an acidic environment. At pH values above 8.0 most molecules existed as tetramers. The enzyme was gradually dissociated at lower pH. When the pH was below 5.0 most of the enzyme was present as the monomeric forms. Reassociation of the subunits was accomplished by adjusting the pH to neutrality. The dissociation and reassociation were almost instantaneous. No trimer was detected. The pigeon liver malic enzyme was thus shown to have a double-dimer quaternary structure with D_2 symmetry. In the presence of substrates, the monomer–dimer–tetramer equilibrium favours the direction of dissociation. Tartronate, an L-malate analogue, was found to be more effective than L-malate in this process. When the monomeric forms were immobilized, the enzyme subunits were found to be fully active in catalysis. A possible arrangement of the four identical subunits of the enzyme molecule is proposed to account for the results obtained in this investigation. The origin of the half-of-the-sites reactivity of pigeon liver malic enzyme is also discussed.

INTRODUCTION

Malic enzyme from pigeon liver [malate dehydrogenase (oxaloacetate-decarboxylating) (NADP⁺) EC 1.1.1.40] is a tetrameric protein catalysing the oxidative decarboxylation of L-malate to give CO₂ and pyruvate, with concomitant reduction of NADP⁺ to NADPH (for review see Hsu, 1982). M_r values of the native enzyme and the subunits, as determined by electrophoresis and sedimentation, were shown to be 260 000 and 65 000 respectively (Nevaldine *et al.*, 1974). N-Terminal, C-terminal and peptide-mapping analyses proved that the enzyme is composed of four chemically identical subunits (Kam *et al.*, 1987). Pigeon liver (Hsu & Lardy, 1967a) and rat liver (Baker *et al.*, 1987) malic enzymes have been crystallized. The rat liver enzyme was reported to be an asymmetric unit consisting of tetramers. Electron micrographs of the pigeon enzyme showed that the enzyme has a planar structure with four subunits arranged at the corners of a square (Nevaldine *et al.*, 1974). More recently, the complete amino acid sequence for the rat liver enzyme has been determined through cDNA nucleotide sequence analysis (Magnuson *et al.*, 1986). Half-of-the-sites reactivity for the pigeon enzyme was observed repeatedly through kinetic studies and stoichiometric incorporation of affinity labels into the enzyme active site (Hsu *et al.*, 1976; Chang & Hsu, 1977; Reynolds *et al.*, 1978; Pry & Hsu, 1978, 1980; Hsu & Pry, 1980; Chang *et al.*, 1982). Negative co-operativity between the subunits was used to explain this phenomenon (Pry & Hsu, 1978, 1980; Hsu & Pry, 1980).

In the present paper the reversible dissociation of the enzyme resulting from pH changes is described.

Combined use of cross-linking and SDS/polyacrylamide-gel electrophoresis allowed us to monitor the assembly of this tetrameric enzyme quantitatively. Furthermore, an immobilization technique enabled us to access the subunit activity. A double-dimer quaternary structure model with D_2 symmetry for the subunits is proposed. A preliminary report concerning the reversible dissociation of the enzyme has been published (Chang *et al.*, 1986).

MATERIALS AND METHODS

Materials

Acrylamide (4 × recrystallized) was from Fluka (Buchs, Switzerland). Glutaraldehyde, Tris and CNBr were from E. Merck (Darmstadt, W. Germany). Sodium acetate, cacodylic acid, AADP⁺, NADP⁺, L-malate, tartronate and M_r standards were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Borax was from the Pacific Coast Borax Co. (New York, NY, U.S.A.). Boric acid was from J. T. Baker (Phillipsburg, NJ, U.S.A.). Sepharose 4B-CL and CNBr-activated Sepharose 4B-CL were from Pharmacia (Uppsala, Sweden). Mono-ethanolamine was from Wako (Osaka, Japan). Acetonitrile (h.p.l.c. grade) was from Alps (Taipei, Taiwan, Republic of China). All other chemicals were of reagent grade purchased from Sigma Chemical Co. or E. Merck. Distilled and deionized water was used throughout this work.

Enzyme preparation

Pigeon liver malic enzyme was purified according to our published procedure (Chang & Chang, 1982). The homogeneity of the purified enzyme was routinely

Abbreviations used: AADP⁺, aminopyridine–adenine dinucleotide phosphate; T, tetramer; D, dimer; M, monomer; MB-T, matrix-bound tetramer; MB-M, matrix-bound monomer.

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checked by polyacrylamide-gel electrophoresis. In most cases a homogeneous preparation with a specific activity of about 30 units/mg was obtained. However, sometimes the purified enzyme was contaminated with a small protein with M_r approx. 40000. This protein (usually less than 5%), however, will not interfere with our interpretation of results obtained in the present work. The enzyme was stored at -20°C in 30 mM-Tris/HCl buffer, pH 7.7 at 25°C , containing 10% (v/v) glycerol, 2 mM-2-mercaptoethanol and 50 μM -NADP $^+$.

Protein determination

Protein concentrations for the purified malic enzyme were determined spectrophotometrically at 278 nm, by using an absorption coefficient of 0.86 for a 0.1% (w/v) solution of the enzyme (Hsu & Lardy, 1967a). Protein determination for the immobilized enzyme was performed by the method of Asryants *et al.* (1985) utilizing the protein-dye binding principle. Purified malic enzyme was used as the standard.

Enzyme assay

Malic enzyme activity was assayed at 30°C by the method of Hsu & Lardy (1967a). The formation of NADPH was monitored continuously at 340 nm with a Gilford 2600 spectrophotometer and a 3 ml cuvette. One unit of enzyme activity was defined as an initial rate of 1 μmol of NADPH formed/min under the assay conditions.

Assay of the enzyme activity at different pH values was performed with overlapping buffers, as shown in Fig. 4. The actual pH values of the reaction mixture were recorded as soon as each assay was completed.

Assay of the immobilized enzyme activity was performed by the addition of a small portion (20–200 μl) of a suitably diluted suspension of the immobilized enzyme to the assay mixture. The mixture was maintained at 30°C and continuously stirred in a cuvette as described by Mort *et al.* (1973). At 1–2 min intervals, the cuvette containing the reaction mixture was placed in the spectrophotometer and the absorbance at 340 nm was recorded. Under these conditions the initial rate was linear and the results were reproducible. The contribution of any remaining soluble enzyme to the observed activity was checked by filtration of the assay mixture. The filtrate was without any enzyme activity.

To obtain reproducible amounts of the immobilized enzyme, Sepharose 4B-CL and immobilized enzyme were measured by their packed volumes, determined after centrifugation at 1700 g for 2 min in a graduated centrifuge tube. A stock suspension of the immobilized enzyme was prepared by mixing the gel with an equal volume of 10 mM-sodium phosphate buffer, pH 7.0. Samples could be removed accurately from the suspension by using an automatic pipette with the plastic tips cut to enlarge the opening.

Dissociation and reassociation of the enzyme subunits

Sodium acetate buffers, pH 4.5–8.0, and sodium borate buffers, pH 6.5–11, were used in dissociation and reassociation experiments. Dissociation was performed by diluting the enzyme (19 μM) 10-fold with an acidic buffer and keeping the solution at room temperature for 10 s–1 h. Reassociation was performed by a 2-fold dilution of the dissociated enzyme (1.9 μM) with a high-pH borate buffer (10 s–90 min). The actual pH during

the reassociation and dissociation was recorded accordingly.

In some experiments, the enzyme was first mixed with various combinations of Mn^{2+} (6 mM), L-malate (0.77 mM), tartronate (0.77 mM), NADP $^+$ (0.34 mM) and AADP $^+$ (0.34 mM), and then treated as above to assess the influence of various ligands on the association–dissociation equilibrium.

Determination of the M_r values of the cross-linked enzyme molecules

Chemical cross-linking of the associated subunits with glutaraldehyde was performed by the procedure of Herman *et al.* (1979) with some modifications. The concentration of glutaraldehyde used was 20 mM, which was 104-fold molar excess over the enzyme concentration. After cross-linking (for 2–20 min), the enzyme solution was subjected to SDS/polyacrylamide-gel electrophoresis by the procedure of Weber & Osborn (1969) with minor modifications. Electrophoresis was carried out at 4°C in a Pharmacia GE-2/4 gel-electrophoresis apparatus with a gel concentration of 7.8%. A current of 2 mA/gel was applied for 1 h; the current was then raised to 7 mA/gel and electrophoresis was continued until the dye markers reached the bottom of the tube. Different dyes were used according to the pH used: Bromophenol Blue (pH 3.0–4.6), Methyl Red (pH 4.2–6.3), Phenol Red (pH 6.8–8.2) or phenolphthalein (pH 8.2–10.2). After electrophoresis, the gels were stained for protein by soaking in 0.05% Coomassie Blue R-250 in a solution containing 12% (w/v) trichloroacetic acid, 43% (v/v) methanol and 9% (v/v) acetic acid and diffusion-destained by soaking in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol. After the destaining, the gels were scanned at 560 nm with a Gilford 2600 spectrophotometer equipped with a 2410-S linear-transport gel scanner. Standard proteins with M_r values ranging from 14300 to 280000 were used. Tetramers (T) (M_r 260000 $\pm$ 3000), dimers (D) (M_r 136000 $\pm$ 9000) and monomers (M) (M_r 67000 $\pm$ 5000) of the malic enzyme were quantified from the corresponding peak areas.

Sucrose-density-gradient centrifugation of the enzyme at different pH values

Sucrose solutions were prepared with buffers of different pH. A 5 ml linear sucrose gradient (5–20%) in a centrifuge tube was formed with a Haake–Buchler Auto Densi-Flow IIC gradient former. Malic enzyme in different buffers was layered on top and centrifuged at 35000 r.p.m. (SW 50 L rotor) for 18 h at 4°C in a Beckman L5-65 ultracentrifuge. The centrifuged solution was fractionated into 40 fractions with the same auto-flow apparatus and assayed for enzyme activity. In a separated tube catalase (M_r 232000) and bovine serum albumin (M_r 67000) in buffer of pH 8.0 were run simultaneously as markers for tetramer and monomer of malic enzyme.

Preparation of immobilized enzymes

Procedure A. Immobilized enzymes were prepared from Sepharose 4B-CL or from CNBr-activated Sepharose 4B-CL (Scheme 1). In the former procedure Sepharose 4B-CL particles were activated by CNBr as described by Jurgensen *et al.* (1981) and March *et al.* (1974). The amount of CNBr used was 0.5–10 mg/ml of gel, depending on the protein concentration of each

Scheme 1. Preparation of immobilized enzymes

preparation. After activation, the gel was filtered and washed successively with 5 vol. of ice-cold water and 5 vol. of ice-cold linking buffer (0.1 M-sodium phosphate buffer, pH 8.5, containing 0.5 M-NaCl). A 1:1 slurry of the activated gel was prepared in cold linking buffer containing 0.3–1.5 mg of malic enzyme/ml of the activated gel. Before addition of the enzyme, stoichiometric amounts of L-malate and NADP^+ were added to protect the active site. The slurry was rotated slowly at 4 °C overnight. The gel slurry was then made 0.5 M in ethanolamine/HCl buffer, pH 8.5, to block excess linking sites of the gel, and was allowed to rotate at 4 °C for an additional 12 h. Excess soluble enzyme was removed by washing the gel with ice-cold linking buffer and then with 0.05 M-sodium phosphate buffer, pH 7.5. The immobilized enzyme was stored in a minimum volume of 0.05 M-sodium phosphate buffer, pH 7.5, at 4 °C.

To prepare the matrix-bound monomer (MB-M), the matrix-bound tetramer (MB-T) gel was washed in a Buchner funnel with 200 vol. excess of 0.4 M-sodium acetate buffer, pH 4.5. This treatment removed the three non-covalently bound subunits and resulted in the formation of MB-M.

Procedure B. We found that the CNBr-activated Sepharose 4B-CL from Pharmacia could be used under appropriate conditions to prepare various immobilized enzyme species. MB-T was prepared as described above at pH 8.0. Immobilization was also carried out at pH 4.5 to obtain MB-M directly.

RESULTS AND DISCUSSION

pH-dependent reversible dissociation

Malic enzyme from pigeon liver showed a pH-dependent dissociation in an acidic environment. At pH

values above 8.0 most enzyme molecules existed as tetramers. The enzyme was gradually dissociated at lower pH. When the pH was below 5.0 most of the enzyme was present as the monomeric forms. Selected gel scans are shown in Fig. 1. In the cross-linking reaction, the aldehyde groups of glutaraldehyde reacted with the lysyl ϵ -amino groups of the protein to form a Schiff base. This reaction is assumed to occur within a wide pH range (for review see Peters & Richards, 1977). However, since the cross-linking reaction of glutaraldehyde was usually performed at alkaline pH, a control experiment was performed with sucrose-density-gradient centrifugation in both neutral and acidic pH. As shown in Fig. 1 inset, this direct hydrodynamic method also demonstrated dissociation of the malic enzyme at pH 4.5. Thus our electrophoresis data should be valid.

The above acid-induced dissociation was reversible. Reassociation of the subunits was accomplished by adjusting the pH to neutrality (Fig. 2). No trimer was detectable. Thus the dissociation obviously followed the tetramer $\rightarrow$ dimer $\rightarrow$ monomer sequence. The tetramers started dissociation at pH 8.0 (Fig. 3a). With the disappearance of tetramers, dimers appeared before monomers. The dimer reached its maximum amount at pH 6.0 (Fig. 3b). Further lowering of the pH caused dissociation of the dimers. The dissociation and reassociation seems to follow the same pathway (Fig. 3). The bands due to cross-linked dimers and tetramers on gels are rather diffuse. However, in general, the results are reproducible, as shown in Fig. 3, which is composed from several experiments.

Pry & Hsu (1980) showed that malic enzyme was stable in the pH range 6.1–8.4 during the period of equilibrium dialysis in their binding studies. In our experiments we found that the enzyme was stable at pH 5.5–9.5 in acetate, Tris/HCl or borate buffer, at least

Fig. 1. SDS/polyacrylamide-gel electrophoresis of pigeon liver malic enzyme at different pH values

Gels a, b and c are cross-linked malic enzyme at pH 4.5, 7.5 and 11.0 respectively. Sedimentation of the monomers ($\square$, enzyme at pH 4.5) and tetramers ($\blacksquare$, enzyme at pH 7.5) during sucrose-density-gradient centrifugation is shown in the inset. $\circ$, A_{280} . Catalase (M_r 232 000) and bovine serum albumin (M_r 67 000) were used as markers.

within 1 h at room temperature. The enzyme was unstable in the carbonate buffer. At pH 9.0 only 40% of the enzyme activity remained after 1 h in this buffer. However, the enzyme was still 82% active at pH values up to 11 in Tris/HCl buffer. The enzyme remained as tetramers at alkaline pH in all the buffers tested. Tris/HCl buffer cannot be used in cross-linking experiments because its amino groups may react with glutaraldehyde. Only monomers were observed in all gels when the cross-linking was performed in Tris/HCl buffer. This result gave us a control that the cross-linking reaction was as anticipated in linking the subunits through the lysyl ϵ -amino groups of the protein molecules.

Kinetics of the dissociation and reassociation

In acetate buffer, after 1 h incubation at pH 4.5, the enzyme still remained 70% active when assayed at pH 7.4. This raised the question whether the subunits of

Fig. 2. Scanning of the cross-linked malic enzyme in gels after dissociation and reassociation

Gel a, protein stain for the native enzyme at pH 7.5. Gel b, protein stain for the enzyme when the pH was lowered from 7.5 to 4.5. Gel c, protein stain for the enzyme when the pH was lowered from 7.5 to 4.5 and raised to 7.0 again. The mobility of the M_r markers relative to the dyes is shown in the inset. Order of decreasing mobility: $\circ$, lysozyme (M_r 14 300), β -lactoglobulin (M_r 18 400), trypsinogen (M_r 24 000), pepsin (M_r 34 700), ovalbumin (M_r 45 000) and plasma albumin (M_r 66 000); $\bullet$, carbonic anhydrase (M_r 29 000), ovalbumin (M_r 45 000), plasma albumin (M_r 66 000), phosphorylase b (M_r 97 400), β -galactosidase (M_r 116 000) and myosin (M_r 205 000); $\square$, cross-linked albumin (M_r 66 000, 132 000, 198 000 and 264 000); $\blacksquare$, cross-linked haemocyanin (M_r 70 000, 140 000, 210 000 and 280 000).

malic enzyme were active. Kinetic studies of the dissociation and reassociation were therefore performed. The results showed that the dissociation and reassociation were accomplished almost instantaneously (within 10 s) (Table 1). These results were not unexpected for a protein composed of identical subunits. However, no conclusion can be drawn about the catalytic activity of the subunits, since the enzyme molecules exist only as transient monomers in acidic environment. Under the assay conditions they will spontaneously re-form the tetrameric structure.

Our previous chemical modification experiments have shown a carboxy group with a pK_a of 6.7 to be involved in the catalysis of this enzyme. The low enzymic activity at acidic pH has been interpreted as being due to protonation of this functional group (Chang *et al.*, 1985).

Fig. 3. Distribution of tetramers, dimers, and monomers of the malic enzyme at different pH values during dissociation and reassociation

○, Acetate buffer in dissociation; ●, borate buffer in dissociation; □, reassociation.

When the enzyme was assayed at different pH values the profile for activity loss at acidic pH did not match the dissociation profile (Fig. 4). Thus the decrease in enzyme activity at acidic pH is probably not due to the dissociation of enzyme molecules. In order to ascertain the subunit activity of this enzyme, the technique of enzyme immobilization was utilized (cf. Chan, 1976).

Immobilization of the enzyme

Preparation of MB-T and MB-M was described in the Materials and methods section. The effects of immobilization on the enzyme-catalysed reaction were tested. Both the soluble enzyme and MB-T showed a pH optimum at about 7.2, similar to the reported value (Rutter & Lardy, 1958). Both enzyme preparations showed an optimum reaction temperature at about 60 °C. The immobilized enzyme, however, had a marked decrease in activation energy (E^*). The E^* values calculated according to the Arrhenius equation were 51645 and 30125 J/mol for the soluble enzyme and MB-T respectively. The $K_{m(app.)}$ for L-malate was 63 μ M and 83 μ M for the soluble and immobilized enzymes respectively, whereas the $K_{m(app.)}$ for Mn^{2+} was 12 μ M for both enzyme preparations. The above results suggested that the binding affinity between the enzyme molecule and substrate or metal ion might not be significantly altered. Immobilization thus caused minimum conformational changes of the enzyme molecule. The

Table 1. Rapid dissociation and reassociation of pigeon liver malic enzyme subunits

Expt. I: dissociation from pH 7.4 to 4.5; cross-linking time 2 min. Expt. II: reassociation from pH 4.5 to 7.7 for approx. 10 s, 1, 2, 4, 7, 10, 15, 30, 60 and 90 min; cross-linking time 20 min. Expt. III: reassociation from pH 4.5 to 7.3 for 5, 10, 30 and 60 min; cross-linking time 20 min.

	Time	Amount (%)		
		Tetramer	Dimer	Monomer
Expt. I	0	65	25	10
	~ 10 s → 30 min	0	0	100
Expt. II	0	0	0	100
	~ 10 s → 60 min	66 ± 2	22 ± 1	12 ± 2
	90 min	71	18	11
Expt. III	0	0	6	94
	5 → 60 min	63 ± 3	32 ± 3	5 ± 0.5

system therefore provided us with an instrument for looking into the question whether or not the subunits of malic enzyme are catalytically active.

When the enzyme subunits were fixed by immobilization, which prevented reassociation during the assay period, MB-M was found to be catalytically active. Washing the MB-T prepared by procedure A with acidic buffer removed one-half to three-quarters of the matrix-bound protein. In a typical experiment the MB-T and MB-M were found to have protein contents of 33.2 μ g and 9.2 μ g/0.2 ml of gel respectively. The enzyme specific activity was greatly lowered after immobilization. However, MB-M was fully active compared with MB-T. The MB-M prepared by procedure B was also active. In a typical experiment MB-T and MB-M were found to have specific activities of 0.24 ± 0.02 and 0.25 ± 0.01 unit/mg respectively. The matrix-bound enzymes were very stable. They were stored at 4 °C for over 4 months and no activity was lost. More experiments to compare the properties of MB-T and MB-M with that of the soluble enzyme are needed to delineate the effects of immobilization on enzyme molecules.

Substrate-induced dissociation

In a protein association-dissociation system, high protein concentrations and the presence of substrates usually favour association. In our experiments protein concentrations between 5.5 μ g/ml and 0.5 mg/ml did not show any influence on the distribution among the T, D and M. With the enzyme concentrations used higher- M_r molecules with more than four subunits were not observed, indicating that intermolecular cross-linking did not occur under our experimental conditions.

We also tested the effects of substrate or cofactors on the association-dissociation equilibrium. At pH 4.5, when most of the enzyme was present as monomers, substrates did not show any effect on the association state. However, as shown in Fig. 5, substrate L-malate together with NADP⁺ and Mn^{2+} favour dissociation at pH 5.5. At pH 8.0 the effect of substrates was not evident (Table 2). Substitution of NADP⁺ with AADP⁺ [a competitive inhibitor with respect to NADP⁺ (Lee & Chang, 1987)], to avoid the syn-catalytic reaction, did

Fig. 4. Profiles of dissociation and enzyme activity at different pH values

Amount of tetramer: □, acetate buffer; ■, borate buffer.
Enzyme activity: ○, acetate buffer; ●, cacodylate buffer.

Fig. 5. Substrate-induced dissociation of pigeon liver malic enzyme

The conditions were the same as described in Figs. 1 and 2, except that the cross-linking reaction was performed in the presence of various combinations of ligands. Gel a, protein standards; gel b, with NADP⁺, Mn²⁺ and L-malate (pH 5.5); gel c, with NADP⁺, Mn²⁺ and tartronate (pH 5.5); gel d, control (without any addition) (pH 5.5); gel e, with NADP⁺, Mn²⁺, and tartronate (pH 8.0). The control gel at pH 8.0 is not shown (most of the enzyme molecules were in tetrameric forms; see Fig. 3).

not change the result. L-Malate, NADP⁺ and Mn²⁺, singly or in any combination of two compounds, did not show any significant influence on the dissociation state. Tartronate, an analogue of L-malate without the C-2 methylene group, was found to be more active than L-malate in this process. At pH 8.0, when the enzyme exists as tetramers, only tartronate plus NADP⁺ and Mn²⁺ cause substantial dissociation. Any other combinations

Table 2. Effects of ligands on the association-dissociation equilibrium

The concentrations used were: NADP⁺, 0.34 mM; AADP⁺, 0.34 mM; L-malate, 0.77 mM; tartronate, 0.77 mM; Mn²⁺, 6 mM.

Additions	Amount (%)		
	Tetra-mer	Dimer	Mono-mer
pH 5.5			
None	32 ± 7	10 ± 4	58 ± 6
NADP ⁺			
L-Malate			
Mn ²⁺			
NADP ⁺ + L-malate			
NADP ⁺ + Mn ²⁺			
AADP ⁺ + L-malate	6	17	77
AADP ⁺ + Mn ²⁺			
AADP ⁺ + Mn ²⁺ + L-malate	8	10	82
NADP ⁺ + Mn ²⁺ + L-malate			
NADP ⁺ + Mn ²⁺ + tartronate	92 ± 2	7 ± 1	1 ± 0.4
None			
NADP ⁺			
L-Malate			
Mn ²⁺			
NADP ⁺ + L-malate			
NADP ⁺ + Mn ²⁺	35	11	54
AADP ⁺ + L-malate			
AADP ⁺ + Mn ²⁺	35	11	54
AADP ⁺ + Mn ²⁺ + L-malate			
NADP ⁺ + Mn ²⁺ + tartronate			

failed to influence the dissociation state at this pH. In this respect, it is assumed that pH is the predominant factor affecting the dissociation-association equilibrium. The greater effect of tartronate on dissociation was in agreement with the finding that tartronate binds to all of the enzyme active sites, whereas L-malate binds to only half of them (Vernon & Hsu, 1983).

It should be noted that, under the conditions of overall activity assay or in binding studies, most enzyme molecules are in the tetrameric forms. It is also noteworthy that the overall oxidative decarboxylase activity of malic enzyme can be resolved into decarboxylase and reductase partial activities by appropriate assay systems (Salles & Ochoa, 1950; Hsu & Lardy, 1967b; Tang & Hsu, 1973). The decarboxylase activity was shown to have an optimum pH at 4.5 (Salles & Ochoa, 1950). Under those conditions, the enzyme is in the monomeric form. This result provides more evidence that the subunits are catalytically active.

Double-dimer model for pigeon liver malic enzyme

The above results demonstrate the reversible dissociation of pigeon liver malic enzyme. Since no trimer was detected in any gel, we believe that pigeon liver malic enzyme has a D₂ symmetry in the quaternary structure, i.e. they are composed of two pairs of polypeptide chains (Hucho *et al.*, 1975). From the interspecies hybridization experiments, Li (1972) also demonstrated that the rat liver malic enzyme is an isologous tetramer, consisting of four identical monomers joined by two distinctive symmetrical domains of

Scheme 2. Quaternary structure models proposed for pigeon liver malic enzyme

(a) Symmetrical double-dimer model. Two sets of homologous contacts are considered. The a-a interactions are much stronger than the b-b interactions. There are essentially no c-c interactions. (b) Pre-existing asymmetrical model. The a-a represents heterologous tail-to-head contacts, and b-b represents homologous contact, which is weaker than the a-a contact.

Scheme 3. Reversible dissociation of pigeon liver malic enzyme at different pH values

Dissociation to monomers restored the native conformation or exposed the masked active site. Fully active subunits were obtained.

bonding. Assuming only close subunit interactions, two simple models could be used to describe a tetramer with D_2 symmetry (Cornish-Bowden & Koshland, 1970; Klotz *et al.*, 1970), i.e. a square planar structure with two non-identical isologous contact sites, or a tetrahedral arrangement with three isologous contacts between subunits. The latter can be ruled out in our case because electron-microscopic observation indicated that the enzyme has a planar structure (Nevaldine *et al.*, 1974). The results presented in this paper appear to show that pigeon liver malic enzyme, like rat liver enzyme, has isologous contact sites in a tetrameric structure where only two subunit contact domains could be cross-linked. On the basis of the model of Levitzki & Koshland (1976), Hsu & Pry (1980) have proposed a sequential model for the pigeon liver malic enzyme to explain the kinetic results on half-of-the-sites reactivity.

To account for our results, we propose a double-dimer model for the enzyme as shown in Scheme 2. The molecule is shown in an elliptical shape, as indicated from electron-microscopic observations (Nevaldine

et al., 1974). In the model shown in Scheme 2(a), two sets of homologous interactions are considered. The a-a interactions must be much stronger than the b-b interactions. There are essentially no c-c interactions. Half-of-the-sites reactivity may come from induced conformational changes through a-a interactions, creating asymmetrical dimers. An alternative pre-existing asymmetrical model, as shown in Scheme 2(b), would explain our results as well. In the latter model the a-a binding domains are heterologous in nature, with tail-to-head linkages between subunits, and the b-b interactions are homologous in nature. In the dimeric forms the a-a bindings partially mask the active site of the second subunit, and this results in the formation of two sets of active sites with different affinities towards the substrates. A slightly different picture is that the active site does not need to be masked. The asymmetric association of the subunits might cause uneven conformational changes through neighbouring subunits and create two sets of binding sites. Asymmetrical arrangement of enzyme subunits was also proposed by Degani & Degani (1979, 1980a,b) to account for the abnormal reactivity of 2-nitro-5-thiocyanobenzoate with creatine kinase. Ligand competition experiments in accordance with Henis & Levitzki (1979, 1980) could be used as a diagnostic tool to distinguish between the pre-existing asymmetric model and the sequential model.

The stepwise reversible dissociation of the enzyme tetramers at different pH values is shown in Scheme 3. The weaker b-b contacts must dissociate before the a-a contacts.

There are many other examples where active subunits and half-of-the-sites reactivity have been found (Carvajal *et al.*, 1977; Kochetov & Solovieva, 1978; Jurgensen *et al.*, 1981; Jurgensen & Harrison, 1982; Huang *et al.*, 1982). It is not obvious how these two observations may be compromised. It is well known that mitochondrial malic enzyme is a regulatory enzyme (Frenkel, 1975; Moreadith & Lehninger, 1984a,b). Cytosolic malic enzyme has no known allosteric property and the selection of oligomeric structure is not clearly understood. Jaenicke & Rudolph (1980) have indicated that reversible dissociation may be an important mechanism in the regulation of enzyme function. Pigeon liver cytosolic malic enzyme could be regulated by such a process. The enzyme activity might be regulated by pH and substrate concentrations by shifting the dissociation-association equilibrium.

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REFERENCES

- Asryants, R. A., Duzsenkova, I. V. & Nagradova, N. K. (1985) *Anal. Biochem.* **151**, 571-574
- Baker, P. J., Thomas, D. H., Barton, C. H., Rice, D. W. & Bailey, E. (1987) *J. Mol. Biol.* **193**, 233-235
- Carvajal, N., Martinez, J. & Fernandez, M. (1977) *Biochim. Biophys. Acta* **481**, 177-183
- Chan, W. W.-C. (1976) *Methods Enzymol.* **44**, 491-503
- Chang, G.-G. & Hsu, R. Y. (1977) *Biochemistry* **16**, 311-320

- Chang, G.-G., Chang, T.-C. & Huang, T.-M. (1982) *Int. J. Biochem.* **14**, 621–627
- Chang, G.-G., Huang, T.-M. & Wu, J. A. (1985) *Proc. Natl. Sci. Counc. Repub. China Part B* **9**, 56–66
- Chang, G.-G., Huang, T.-M. & Kam, P. L. (1986) in *Contemporary Themes in Biochemistry (IRCS Short Papers)* (Kon, O. L., Chung, M. C.-M., Hwang, P. L. H., Leong, S.-F., Loke, K. H., Thiyagarajah, P. & Wong, P. T.-H., eds.), vol. 6, pp. 86–87, Cambridge University Press, London
- Chang, J.-T. & Chang, G.-G. (1982) *Anal. Biochem.* **121**, 366–369
- Cornish-Bowden, A. & Koshland, D. E., Jr. (1970) *J. Biol. Chem.* **245**, 6241–6250
- Degani, Y. & Degani, C. (1979) *Biochemistry* **18**, 5917–5923
- Degani, C. & Degani, Y. (1980a) *J. Biol. Chem.* **255**, 8221–8228
- Degani, Y. & Degani, C. (1980b) *Trends Biochem. Sci.* **5**, 337–341
- Frenkel, R. (1975) *Curr. Topics Cell. Regul.* **9**, 157–181
- Henis, Y. I. & Levitzki, A. (1979) *Eur. J. Biochem.* **102**, 449–465
- Henis, Y. I. & Levitzki, A. (1980) *Eur. J. Biochem.* **112**, 59–73
- Hermann, R., Rudolph, R. & Jaenicke, R. (1979) *Nature (London)* **277**, 243–245
- Hsu, R. Y. (1982) *Mol. Cell. Biochem.* **43**, 3–26
- Hsu, R. Y. & Lardy, H. A. (1967a) *J. Biol. Chem.* **242**, 520–526
- Hsu, R. Y. & Lardy, H. A. (1967b) *Acta Biochem. Pol.* **14**, 183–186
- Hsu, R. Y. & Pry, T. A. (1980) *Biochemistry* **19**, 962–968
- Hsu, R. Y., Mildvan, A. S., Chang, G. G. & Fung, C. H. (1976) *J. Biol. Chem.* **251**, 6574–6583
- Huang, C. Y., Rhee, S. G. & Chock, P. B. (1982) *Annu. Rev. Biochem.* **51**, 935–971
- Hucho, F., Müller, H. & Sund, H. (1975) *Eur. J. Biochem.* **59**, 79–87
- Jaenicke, R. & Rudolph, R. (1980) in *Protein Folding* (Jaenicke, R., ed.), pp. 525–548, Elsevier/North-Holland Biomedical Press, Amsterdam
- Jurgensen, S. R. & Harrison, J. H. (1982) *J. Biol. Chem.* **257**, 569–574
- Jurgensen, S. R., Wood, D. C., Mahler, J. C. & Harrison, J. H. (1981) *J. Biol. Chem.* **256**, 2383–2388
- Kam, P. L., Lin, C. C. & Chang, G.-G. (1987) *Int. J. Pept. Protein Res.* **30**, 217–221
- Klotz, I. M., Langerman, N. R. & Darnall, D. W. (1970) *Annu. Rev. Biochem.* **39**, 25–62
- Kochetov, G. A. & Solovieva, O. N. (1978) *Biochem. Biophys. Res. Commun.* **84**, 515–519
- Lee, H. J. & Chang, G.-G. (1987) *Biochem. J.* **245**, 407–414
- Levitzki, A. & Koshland, D. E., Jr. (1976) *Curr. Topics Cell. Regul.* **10**, 1–40
- Li, J. J. (1972) *Arch. Biochem. Biophys.* **150**, 812–814
- Magnuson, M. A., Morioka, H., Teece, M. F. & Nikodem, V. M. (1986) *J. Biol. Chem.* **261**, 1183–1186
- March, S. C., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152
- Moreadith, R. W. & Lehninger, A. L. (1984a) *J. Biol. Chem.* **259**, 6215–6221
- Moreadith, R. W. & Lehninger, A. L. (1984b) *J. Biol. Chem.* **259**, 6222–6227
- Mort, J. S., Chong, D. K. K. & Chan, W. W.-C. (1973) *Anal. Biochem.* **52**, 162–168
- Nevaldine, B. H., Bassel, A. R. & Hsu, R. Y. (1974) *Biochim. Biophys. Acta* **336**, 283–293
- Peters, K. & Richards, F. M. (1977) *Annu. Rev. Biochem.* **46**, 523–551
- Pry, T. A. & Hsu, R. Y. (1978) *Biochemistry* **17**, 4024–4029
- Pry, T. A. & Hsu, R. Y. (1980) *Biochemistry* **19**, 951–962
- Reynolds, C. H., Hsu, R. Y., Matthews, B., Pry, T. A. & Dalziel, K. (1978) *Arch. Biochem. Biophys.* **189**, 309–316
- Rutter, W. J. & Lardy, H. A. (1958) *J. Biol. Chem.* **233**, 374–382
- Salles, J. B. V. & Ochoa, S. (1950) *J. Biol. Chem.* **187**, 849–861
- Tang, C. L. & Hsu, R. Y. (1973) *Biochem. J.* **135**, 287–291
- Vernon, C. M. & Hsu, R. Y. (1983) *Arch. Biochem. Biophys.* **225**, 296–305
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

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