Quaternary structure of pigeon liver malic enzyme

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Pigeon liver malic enzyme (EC 1.1.1.40) has a double dimer quaternary structure. The $NADP^+$ analogs, aminopyridine adenine dinucleotide phosphate and nicotinamide-1,N^6-ethenoadenosine dinucleotide phosphate, bind to the enzyme anti-cooperatively. In the presence of non-cooperative competing ligand $NADP^+$, the binding parameter Hill coefficients of these analogues changed very little. Binding of *L*-malate with enzyme-$AADP^+$ complex first enhanced then reduced the nucleotide fluorescence. Two *L*-malate binding sites, with K_d values of 23–30 and 270–400 μM, respectively, for the tight and weak binding sites were postulated. A hybrid model between the sequential and pre-existing asymmetrical models was proposed for the pigeon liver malic enzyme.

Quaternary structure; Ligand binding; NADP analog; Asymmetric model; Malic enzyme; Pigeon liver

1. INTRODUCTION

Malic enzyme from pigeon liver ((S)-malate: $NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) is a tetrameric protein composed of four chemically identical subunits [1]. Electron micrographs showed that the enzyme has a planar structure with four subunits arranged at the corners of a square [2]. 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 [3–7]. Negative cooperativity between the subunits was used to explain this phenomenon [5,6].

We have demonstrated the pH-induced reversible dissociation of pigeon liver malic enzyme [8]. The enzyme dissociated under acidic environment in the sequence of tetramer→dimer→monomer. Chemical modification experiments showed that 2-nitro-5-thiocyanobenzoate and 2,4-dinitrophenylthiocyanate reacted atypically with essential SH groups of malic enzyme in cyanylation and arylthiolation [9]. One cyano residue and three 2-nitro-5-thiobenzoate residues were found in the resulting enzyme derivative when the enzyme reacted with 2-nitro-5-thiocyanobenzoate, whereas only a single 2,4-dinitrophenyl group was introduced into the enzyme molecule with 2,4-dinitrophenylthiocyanate. Hsu and Pry [6] proposed a sequential model for the pigeon liver malic enzyme to explain the kinetic results on half-of-the-sites reactivity. However, besides the sequential model, an alternative pre-existing asymmetrical model would explain the above results as well. New experimental data on ligand competition binding studies suggest that pigeon liver malic enzyme may have a pre-existing asymmetric quaternary structure.

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Abbreviations: $AADP^+$, 3-aminopyridine adenine dinucleotide phosphate; $\epsilon NADP^+$, nicotinamide,1,N^6-ethenoadenine dinucleotide phosphate

2. EXPERIMENTAL

Pigeon liver malic enzyme was purified to homogeneity as described previously [10]. The purified enzyme had a specific activity of 24 units/mg. Protein concentration for the purified enzyme was determined spectrophotometrically at 278 nm, using an extinction coefficient of 0.86 for a 0.1% (w/v) solution of the enzyme [11]. M_r determination of the cross-linked enzyme molecules was performed according to our published procedure [8].

Fluorescence measurements were performed on a Farrand system 3 spectrofluorimeter equipped with a thermoregulated cell holder at 25°C. Binding measurements based on enhancement in $AADP^+$ or $\epsilon NADP^+$ fluorescence upon binding to the enzyme were performed in the absence or presence of various amounts of $NADP^+$ in a fluorescence cell of 1 cm optical path [12].

Calculation of free and bound ligand concentrations and bound ligand fractions (r) were according to Henis and Levitzki [13]. The binding data were fitted to Eqns 1 and 2 for the sequential model or asymmetrical model, respectively.

$$r = \frac{[L]/K_1 + 2[L]^2/K_1K_2 + 3[L]^3/K_1K_2K_3 + 4[L]^4/K_1K_2K_3K_4}{1 + [L]/K_1 + [L]^2/K_1K_2 + [L]^3/K_1K_2K_3 + [L]^4/K_1K_2K_3K_4} \quad (1)$$

$$r = \frac{2[L]}{K' + [L]} + \frac{2[L]}{K'' + [L]} \quad (2)$$

The binding data were analyzed with a non-linear-regression program (DNRP-53 [14].

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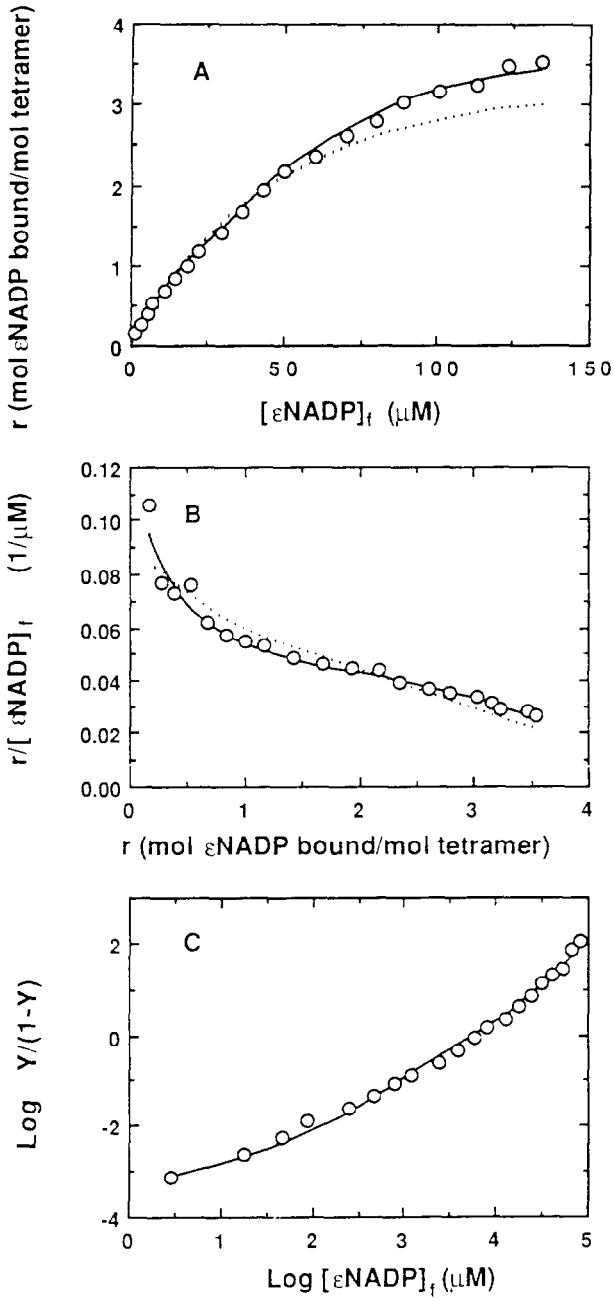

Fig. 1. Binding of $\epsilon NADP^+$ with pigeon liver malic enzyme at pH 7.4. (A) Titration of the enzyme (2.87 mg/ml) with $\epsilon NADP^+$ in 66.7 mM triethanolamine-Cl buffer (pH 7.4). (B) Scatchard plot. (C) Hill plot. Solid line represents the computer generated line fitted to Eqn 1. Dotted line denotes the computer generated line fitted to Eqn 2.

3. RESULTS AND DISCUSSION

3.1. *Binding between $\epsilon NADP^+$ and malic enzyme at pH 7.4*

In the binding studies, $NADP^+$ was found to bind with malic enzyme non-cooperatively [5], whereas the fluorescent $NADP^+$ analogs, $\epsilon NADP^+$ and $AADP^+$ bound to the enzyme anti-cooperatively (Fig. 1) [7]. When excited at 307 nm, $\epsilon NADP^+$ emitted fluorescence at 416 nm at pH 7.4 or 4.5. Binding of the nucleotide to malic enzyme caused an increase of the fluorescence and the maximum peak shifted to 404 nm. $AADP^+$ absorbed 331 nm UV light and emitted fluorescence at 420 nm. Binding of this nucleotide to the enzyme also enhanced the fluorescence intensity but did not change the spectrum. Fig. 1 showed the binding data of $\epsilon NADP^+$ with malic enzyme. Fig. 1A showed the titration of malic enzyme with $\epsilon NADP^+$. The Scatchard and Hill plot of the same data were presented in Fig. 1B and 1C, respectively. Similar biphasic Scatchard plot for the binding between $AADP^+$ and malic enzyme was also observed [7]. The binding data seemed to fit to a sequential model. The intrinsic dissociation constants for $\epsilon NADP^+$ binding to malic enzyme were found to be 0.02 ± 0.012, 0.018 ± 0.014, 0.013 ± 0.005 and 9.4 ± 0.51 μM for K_1, K_2, K_3 and K_4, respectively. These values seemed to represent two groups of binding affinity with at least two orders of magnitude difference. One of the subunits bound with the ligand much more difficult than the other three subunits. This binding phenomenon was consistent with the atypical reaction between malic enzyme and 2-nitro-5-thiocyanobenzoate or 2,4-dinitrophenylthiocyanate [9].

3.2. *Mutual binding of $\epsilon NADP^+$ or $AADP^+$ and $NADP^+$ with malic enzyme at pH 7.4*

Henis and Levitzki [13] developed a new method based on ligand competition experiment to distinguish the above mentioned models. In this approach the way to establish which of the molecular models best describes a certain experimental system is by the use of a non-cooperative competing ligand. The ligand competition experiments were thus performed as a diagnostic to distinguish between the two models for pigeon liver malic enzyme. While anti-cooperativity of the ligand binding was best described by the Scatchard plot, the

Table I

Hill coefficients obtained from the titration of pigeon liver malic enzyme with $AADP^+$ or $\epsilon NADP^+$ in the presence of $NADP^+$

	Hill coefficient (h)				
[$NADP^+$] (μM):	0	0.2	0.4	1	5
$AADP^+$	0.86 ± 0.01	0.77 ± 0.01	0.77 ± 0.01	0.71 ± 0.05	0.77 ± 0.05
$\epsilon NADP^+$	0.98 ± 0.14	0.72 ± 0.17	0.75 ± 0.07	0.97 ± 0.05	1.01 ± 0

Hill coefficient expressed as mean ± S.E.M. from two experiments.

most convenient means to follow the changes in the binding pattern of one ligand by a second competing ligand is by examination of the Hill coefficient at 50% ligand occupancy (h). The h values of ϵNADP$^+$ and AADP$^+$ at different levels of NADP$^+$ were thus estimated. Table I shows the results. Pre-existing model predicted a constant h value in competition ligand binding whereas sequential model predicted the h value to be varied. The results shown in Table I indicated that the Hill coefficient of AADP$^+$ or ϵNADP$^+$, within the experimental errors, did not change very much whether NADP$^+$ was present or not. Therefore, the quaternary

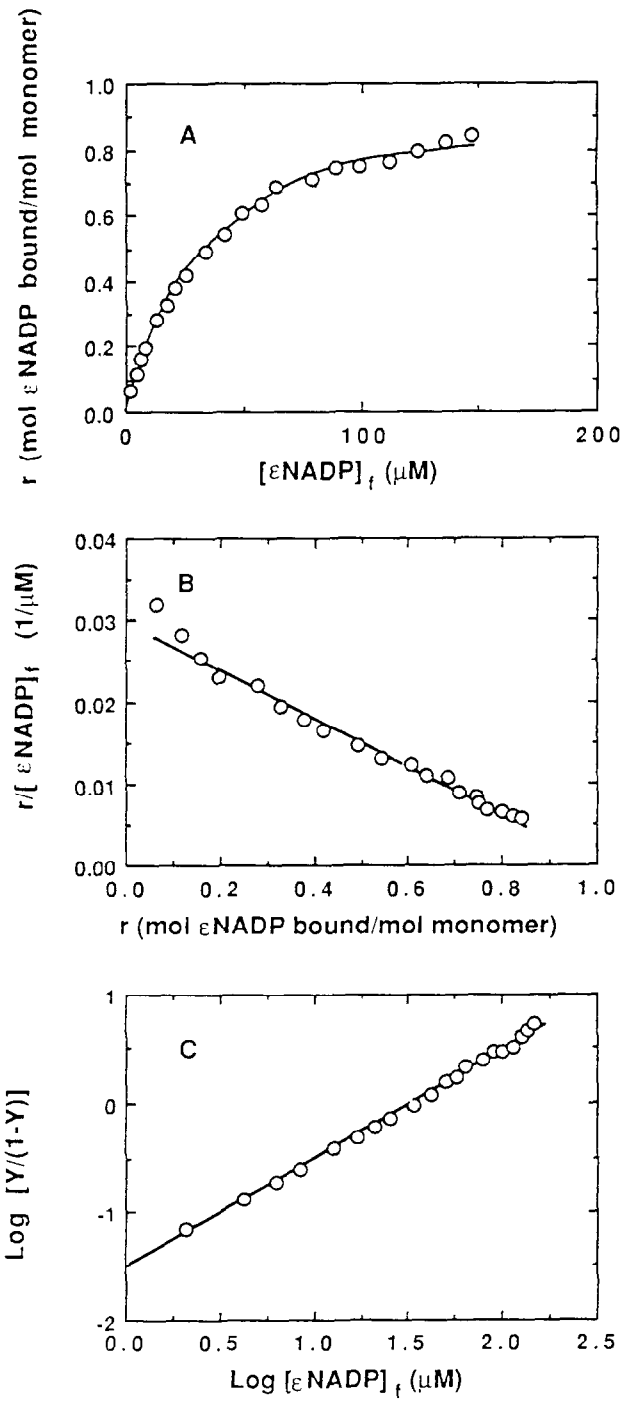

Fig. 2. Binding of ϵNADP$^+$ with pigeon liver malic enzyme at pH 4.5. The conditions were the same as described in Fig. 1, except that sodium acetate buffer (pH 4.5) was used.

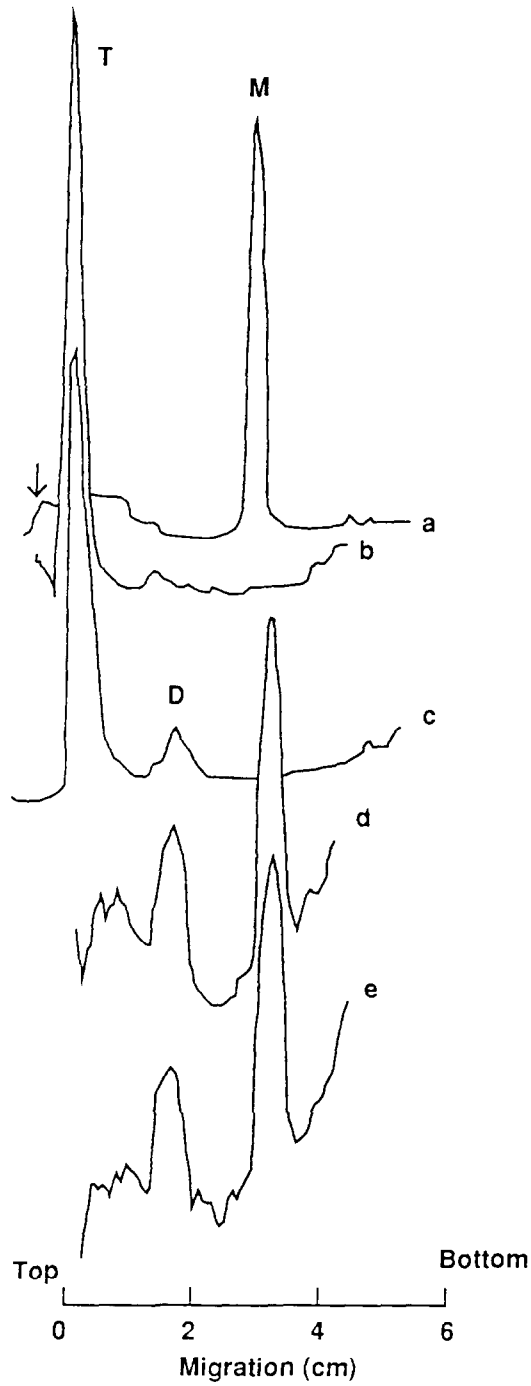

Fig. 3. Sodium dodecyl sulfate electrophoresis gels of malic enzyme in the presence of nucleotide. Gel scan of the enzyme after sodium dodecyl sulfate-electrophoresis: (a) native enzyme at pH 7.4; (b) cross-linked enzyme at pH 7.4; (c) cross-linked enzyme at pH 7.4, in the presence of NADP$^+$ and ϵNADP$^+$; (d) Cross-linked enzyme at pH 4.5; (e) cross-linked enzyme at pH 4.5, in the presence of AADP$^+$ and ϵNADP$^+$. T, tetramers; D, dimers; M, monomers.

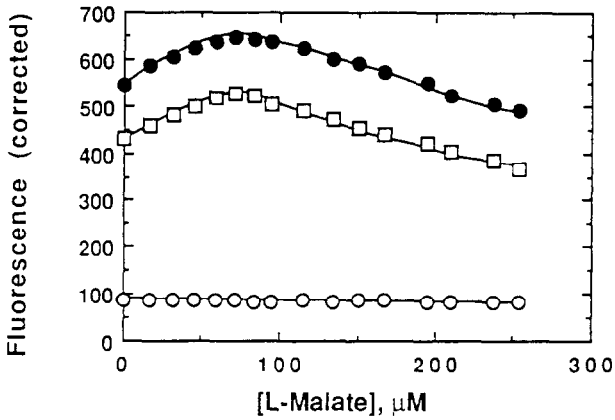

Fig. 4. Titration of malic enzyme/AADP$^+$ complex sith L-malate. $_{331}F_{420}$ of the sample in 66.7 mM triethanolamine-Cl buffer (pH 7.4) was measured. Suitable inner filter effect and volume corrections were made. (○), 2.6 mM AADP$^+$ alone; (•), 2.87 mg/ml malic enzyme plus 2.6 mM AADP$^+$; (□), 2.87 mg/ml malic enzyme plus 2.6 mM AADP$^+$ and 40 mM Mn^{2+}.

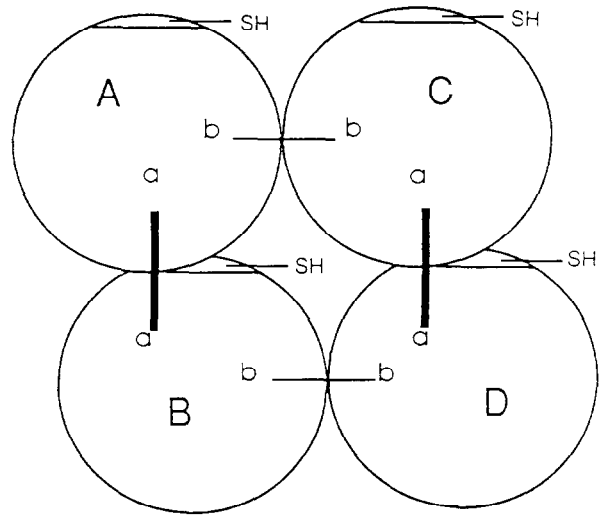

Fig. 5. Proposed quaternary structure for pigeon liver malic enzyme.

structure of malic enzyme seemed to be more fit to a pre-existing asymmetrical model.

3.3. *Binding between εNADP$^+$ and malic enzyme at pH 4.5*

The anti-cooperativity of εNADP$^+$ binding was lost at pH 4.5 (Fig. 2). A linear Scatchard plot was obtained. This was the expected result since the enzyme was dissociated into monomers at this pH. However, since we have demonstrated that substrates caused subunits' dissociation [8], it was necessary to establish the association state of the enzyme during ligand titration experiments. Fig. 3 showed that the enzyme remained as tetramers in the presence of nucleotide ligands used in the ligand competition experiment. Similar results were obtained when the ligands were used singly. Thus the anti-cooperativity was unequivocally due to subunit interactions.

3.4. *Binding between L-malate and malic enzyme*

The addition of L-malate and $MnCl_2$ had no effect on the AADP$^+$ or εNADP$^+$ fluorescence. However, as shown in Fig. 4, the fluorescence intensity of enzyme/AADP$^+$ complex was first enhanced and then decreased by L-malate. Mn^{2+} quenched the fluorescence a little. From the titration curves, we were able to estimate the two binding affinities of L-malate. The tight L-malate site had a K_d value of 23–30 μM, whereas that for the weak site was 270–400 μM.

3.5. *Proposed quaternary structure for malic enzyme*

In accordance with the above experimental results, we propose an asymmetric model for the malic enzyme as shown in Fig. 5. Two sets of interactions are considered. The *a–a* binding domains are heterologous in nature. The *b–b* interactions are homologous which is weaker than the *a–a* contact. In the dimeric forms, the *a–a* binding results in the formation of two sets of active site with different affinities toward L-malate. Formation of tetramer creates another asymmetry between subunits B and D. The local micro-heterogeneity of essential SH group at subunit B explains the atypical reaction of malic enzyme with 2-nitro-5-thiobenzoate or 2-nitro-5-thiocyanobenzoate. This model also explained the much lower binding affinity of subunit B with εNADP$^+$. A slightly different picture is that the active sites need not be masked. The asymmetric association of the subunits might cause uneven conformational changes through neighbour subunits and create two sets of binding sites. Asymmetrical arrangement of enzyme subunits was also proposed by Degani and Degani [15] to account for the abnormal reactivity of 2-nitro-5-thiocyanobenzoate with creatine kinase. Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* crystallized as a symmetrical dimer. However, recent kinetic evidence implies that this enzyme is inherently asymmetrical in solution [16]. It is possible that malic enzyme from pigeon liver is regulated by pH and substrate concentration by shifting the dissociation/association equilibrium and creating enzyme molecules with different specific activities.

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REFERENCES

[1] Kam, P.L., Lin, C.C. and Chang, G.G. (1987) Int. J. Peptide Protein Res. 30, 217–221.
[2] Nevaldine, B.H., Bassel, A.R. and Hsu, R.Y. (1974) Biochim. Biophys. Acta 336, 283–293.
[3] Hsu, R.Y., Mildvan, A.S., Chang, G.G. and Fung, C.H. (1976) J. Biol. Chem. 251, 6574–6583.

[4] Chang, G.G. and Hsu, R.Y. (1977) Biochemistry 16, 311-320.
[5] Pry, T.A. and Hsu, R.Y. (1980) Biochemistry 19, 951-962.
[6] Hsu, R.Y. and Pry, T.A. (1980) Biochemistry 19, 962-968.
[7] Chang, G.G., Shiao, M.S., Liao, J.G. and Lee, H.J. (1989) J. Biol. Chem. 264, 280-287.
[8] Chang, G.G., Huang, T.M. and Chang, T.C. (1988) Biochem. J. 254, 123-130.
[9] Reddy, V.A. (1983) Biochim. Biophys. Acta 743, 268-280.
[10] Chang, J.T. and Chang, G.G. (1982) Anal. Biochem. 121, 366-369.
[11] Hsu, R.Y. and Lardy, H.A. (1967) J. Biol. Chem. 242, 520-526.
[12] Lee, H.J. and Chang, G.G. (1987) Biochem. J. 245, 407-414.
[13] Henis, Y.I. and Levitzki, A. (1980) Eur. J. Biochem. 112, 59-73.
[14] Duggleby, R.G. (1984) Comput. Biol. Med. 14, 447-455.
[15] Degani, Y. and Degani, C. (1980) Trends Biochem. Sci. 5, 337-341.
[16] Ward, W.H.J. and Fersht, A.R. (1988) Biochemistry 27, 1041-1049.