Identification of Asp^{258} as the Metal Coordinate of Pigeon Liver Malic Enzyme by Site-Specific Mutagenesis†

Chien-Hwa Wei, Wei-Yuan Chou,* and Gu-Gang Chang*

Graduate Institutes of Life Sciences and Biochemistry, National Defense Medical Center, Taipei, Taiwan, Republic of China

Received January 18, 1995; Revised Manuscript Received April 4, 1995®

ABSTRACT: Pigeon liver malic enzyme was inactivated by ferrous sulfate in the presence of ascorbate. Manganese and some other divalent metal ions provided complete protection of the enzyme against the Fe^{2+}-induced inactivation. The inactivated enzyme was subsequently cleaved by the Fe^{2+}−ascorbate system at Asp^{258}-Ile^{259}, which was presumably the Mn^{2+}-binding site of the enzyme [Wei, C. H., Chou, W. Y., Huang, S. M., Lin, C. C., & Chang, G. G. (1994) *Biochemistry 33*, 7931−7936]. For identification of Asp^{258} as the putative metal-binding site of the enzyme, we prepared four mutant enzymes substituted at Asp^{258} with glutamate (D258E), asparagine (D258N), lysine (D258K), or alanine (D258A), respectively. These mutant proteins were recombinantly expressed in a bacterial expression system (pET-15b) with a stretch of histidine residues attached at the N-terminus and were successfully purified to apparent homogeneity by a single Ni-chelated affinity column. Among the four mutants, only D258E possessed 0.8% residual activity after purification; all other purified mutants had <0.0001% residual activity in catalyzing the oxidative decarboxylation of L-malate. The D258E mutant was susceptible to inactivation by the Fe^{2+}−ascorbate system, albeit with much slower inactivation rate, and was protected by the Mn^{2+} to a lesser extent as compared to the wild-type enzyme. None of the mutants were cleaved by the Fe^{2+}−ascorbate system under conditions that cleaved the natural or wild-type enzyme at Asp^{258}. The apparent K_{mNADP} and K_{mMal} values of D258E mutant did not change very much as compared with those of the wild-type enzyme; the K_{mMn} and K_{dMn} values, on the other hand, increased approximately 1600-fold and 3000-fold, respectively, which resulted in the specific constant (k_{cat}/K_{mMn}) of the D258E mutant being decreased by approximately 40 000-fold, and the catalytic efficiency [$k_{cat}/(K_{mNADP}K_{mMal}K_{dMn})$] of D258E was only 0.000 52% of that of the WT. These results conclusively indicated the catalytic role of metal ion in the malic enzyme catalyzed reaction and that Asp^{258} is the metal coordinate for pigeon liver malic enzyme. The amino acid sequence around this aspartate residue or in an equivalent site of other malic enzymes is highly conserved. We propose that this aspartate residue is critical for the catalysis in all malic enzymes.

Pigeon liver malic enzyme [(*S*)-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40] catalyzes the Mn^{2+} or Mg^{2+}-dependent reversible oxidative decarboxylation of L-malate to give CO_2 and pyruvate, with concomitant reduction of $NADP^+$ to NADPH (eq 1).

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

A crystallographic three-dimensional structure is not yet available for any malic enzyme. Information about the reaction mechanism of this enzyme mostly comes from kinetic or chemical modification studies, which, in some instances, are ambiguous in interpretation (Hsu, 1982; Chang & Huang, 1980, 1981; Chang et al., 1985, 1989). More recently, we successfully used Fe^{2+}−ascorbate as an affinity cleavage system for the pigeon malic enzyme. The enzyme was very sensitive to the metal-catalyzed oxidation. By using the Fe^{2+}−ascorbate system, we have identified Asp^{258} as a possible metal-binding site of the enzyme (Wei et al., 1994).

In this paper, we report the preparation of various mutants substituted at Asp^{258} with amino acid residues having different charge or side-chain length, or both. We provide conclusive evidence to demonstrate that one of the most important metal-binding ligands of pigeon liver malic enzyme is Asp^{258}.

MATERIALS AND METHODS

Site-Specific Mutagenesis. Site-specific mutagenesis of pigeon liver malic enzyme was carried out according to the procedures of Zoller and Smith (1982). The pET-15b (Novagen) expression vector containing malic enzyme cDNA was digested with *Bgl*II and *Bam*HI and ligated with *Bam*HI-digested pBlueScript vector (Stratagene). All the DNA techniques were performed according to the protocols of Sambrook et al. (1989). This recombinant phagemid was amplified in *ung*⁻ and *dut*⁻ RZ1032 *Escherichia coli* strain with helper phage R408 for uracil-containing single-strand DNA preparation. This DNA, serving as templete, was annealed with phosphorylated mutagenic oligonucleotides

† This work was supported by the National Science Council, Republic of China (Grants NSC 84-2331-B016-063 to W.-Y. C. and NSC 83-0412-B016-001 to G.-G.C.). A preliminary report of this work has been presented at the 16th International Congress of Biochemistry and Molecular Biology held at New Delhi, India, on September 18−22, 1994 (Abstracts, Vol. II, p 290).

* To whom reprint requests should be addressed: Department of Biochemistry, National Defense Medical Center, P.O. Box 90048, Taipei, Taiwan, Republic of China. Fax: +886-2-365-5746.

® Abstract published in *Advance ACS Abstracts,* June 1, 1995.

0006-2960/95/0434-7949$09.00/0

Table 1: Synthetic Oligonucleotides for Site-Specific Mutagenesis of Asp^{258} Residue in Pigeon Liver Malic Enzyme

mutant	synthetic oligonucleotide[a]
D258N	5′ TTTCAATGATAACATTCAAGG 3′
D258K	5′ CTTTCAATGATAAAATTCAAGGAAC 3′
D258E	5′ TTCAATGATGAAATTCAAGGAAC 3′
D258A	5′ TTTCAATGATGCTATTCAAGGAAC 3′

[a] Mutation positions are underlined in the oligonucleotide sequence.

(Table 1) and *in vitro* extented by T4 DNA polymerase. The mutated DNA was screened by the ung^+ and dut^+ system in *E. coli* strain JM109 and further identified by dideoxy chain termination sequencing (Sanger et al., 1977). The entire cDNA was also sequenced to exclude any unexpected mutations resulting from *in vitro* mutagenesis. The mutated *Nde*I−*Bam*HI cDNA fragment containing the full-length open reading frame of malic enzyme was inserted in a linear pET-15b vector for protein expression.

Expression and Purification of Recombinant Pigeon Liver Malic Enzymes. The mutated plasmids were transformed into BL21 bacteria. The transformants were incubated in LB medium and induced by 1 mM isopropyl β-D-thiogalactopyranoside (IPTG).[1] The cells were harvested by centrifugation at 5000*g* for 10 min. Because the expressed recombinant protein fused with a stretch of histidine residues, introduced by the pET-15b vector, after sonication, it was purified on a nickel-containing His-Bind column (Novogene) (Hochuli et al., 1987) according to the protocols supplied by the manufacturer. All purified enzymes were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) to examine the purity.

The recombinant proteins had a Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met- polypeptide tag added at the N-terminus. The wild-type enzyme (WT) thus has a subunit M_r 2293 larger than that of the natural enzyme (M_r 62 061). Subunit M_r values of WT and D258E were 64 354 and 64 368, respectively.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate. The experiments were performed with 12% polyacrylamide slab gels on a Bio-Rad minislab gel apparatus. Electrophoresis was carried out at constant voltage (100 V) for 6 h (Wei et al., 1994). The protein bands were quantified with a laser densitometer (Molecular Dynamics).

Enzyme Assay and Protein Determination. Malic enzyme activity was assayed according to Hsu and Lardy (1967). A 1-mL reaction mixture contained triethanolamine−HCl buffer (66.7 mM, pH 7.4), L-malate (0.419 mM), $NADP^+$ (0.177 mM), Mn^{2+} (3.87 mM), and an appropriate amount of enzyme. Since free manganese and L-malate are the true reactants for the enzyme (Canellas & Wedding, 1980; Parks et al., 1984), the concentrations of substrate or cofactors were corrected for the Mn^{2+}-L-malate and Mn^{2+}-$NADP^+$ chelations. The formation of NADPH at 30 °C was monitored continuously at 340 nm with a Perkin-Elmer Lambda 3B 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. A molar absorption coefficient of 6.22×10^3 M^{-1} cm^{-1} for the NADPH was used in calculations.

Specific activity was expressed as micromoles of NADPH formed per minute per milligram of protein. Protein concentration was determined by the protein dye binding method of Bradford (1976) using purified natural pigeon liver malic enzyme as standard. Natural malic enzyme was purified as described (Chang & Chang, 1982).

Enzyme Inactivation by the Fe^{2+}−Ascorbate System. The inactivation experiments were performed at 0 °C with ferrous sulfate (20 μM) and ascorbate (20 mM) in triethanolamine−HCl buffer (66.7 mM, pH 7.4) as described (Wei et al., 1994). The progress of enzyme inactivation was monitored by assaying the enzyme activity in small aliquots withdrawn at the designated time intervals.

Initial Velocity Experiment. An initial velocity study was performed by varying $[Mn^{2+}]_f$ from 0.096 to 1.935 μM and [L-malate]$_f$ from 12.57 to 83.80 μM for the WT enzyme and by varying $[Mn^{2+}]_f$ from 0.072 to 0.384 and [L-malate]$_f$ from 0.147 to 0.491 mM for the D258E mutant. Concentrations of other components were held constant. The amount of enzyme (3.48 μg/assay for WT and 24.8 μg/assay for the mutant) was selected to give significant reaction rates and yet ensure that the reaction velocity remained linear at least for 2−3 min. The initial slopes of the recorder tracings were taken as initial velocities. The resulting experimental data for $[Mn^{2+}]_f$ from 0.096 to 0.242 μM or from 0.484 to 1.935 μM were fitted separately to an equation derived from the linear sequential ordered bi−bi reaction mechanism (eq 2),

$$v = \frac{V_{max}[Mn^{2+}]_f[Mal]_f}{K_{dMn} \cdot K_{mMal} + K_{mMal}[Mn^{2+}]_f + [Mn^{2+}]_f[Mal]_f} \quad (2)$$

in which v and V_{max} are the observed and maximum velocities, respectively. K_{mMal} and K_{dMn} are the Michaelis constant for free L-malate and the dissociation constant for free Mn^{2+}, respectively. Fitting of experimental data to eq 2 was carried out with the EZ-FIT computer program (Perrella, 1988).

RESULTS

Purification and Apparent Specific Activity of Wild-Type and Various Mutant Pigeon Liver Malic Enzymes. The WT and various Asp^{258} mutants of malic enzyme were successfully expressed and purified to homogeneity by a single nickel column; only one protein band was detected in the SDS−PAGE gel for all proteins (Figure 1). Our preliminary experiments showed that the histidine tag attached to the N-terminus did not appreciably affect the kinetic properties of the WT enzyme (Chou et al., 1994). The mutant proteins were tested for the malic enzyme activity. As shown in Table 2, in the crude cell extract, all mutants possessed detectable but much smaller enzymatic activity as compared to the WT enzyme. The most conservative mutant, D258E, had an activity only 1.3% that of WT enzyme. Other mutants were even less active. After purification, only D258E had detectable activity, which was 0.8% that of the WT enzyme. No detectable enzymatic activity was observed for other mutants. These results confirmed the importance of Asp^{258} and indicated that the mutants might be unstable during the purification step.

[1] Abbreviations: WT, wild-type pigeon liver malic enzyme, that is, with aspartate at position 258; D258X, mutants of pigeon liver malic enzyme with another amino acid residue, X, substituted for aspartate 258; IPTG, isopropyl β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

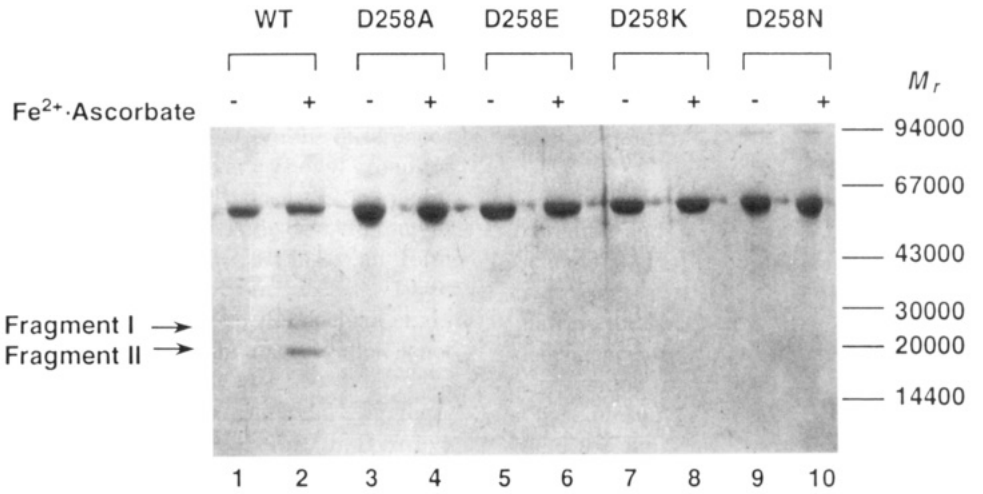

FIGURE 1: SDS−PAGE of the WT and various Asp^{258} mutant pigeon liver malic enzymes. Purified WT (lanes 1 and 2), D258A (lanes 3 and 4), D258E (lanes 5 and 6), D258K (lanes 7 and 8), and D258N (lanes 9 and 10) malic enzymes were subjected to electrophoresis in the presence of sodium dodecyl sulfate with (lanes 2, 4, 6, 8, and 10) or without (lanes 1, 3, 5, 7, and 9) pretreatment with Fe^{2+} (20 μM)−ascorbate (20 mM) at 0 °C for 100 min. The amount of protein applied was the same (1.26 μg) in all lanes.

Table 2: Apparent Specific Activity of Wild-Type and Various Asp^{258} Mutant Pigeon Liver Malic Enzymes[a]

purification steps	WT	D258E	D258N	D258K	D258A
crude extract	6.59 (100%)	8.57×10^{-2} (1.3%)	1.03×10^{-2} (0.156%)	3.30×10^{-3} (0.05%)	1.80×10^{-3} (0.027%)
post Ni column	20 (100%)	0.16 (0.8%)	*b*		

[a] All values shown are expressed in μmol of NADPH formed/min/mg of protein. Numbers in parentheses are percentage to the WT enzyme. [b] Not detectable; the lower limit of our spectrophotometric analysis of the enzyme was ~0.02 nmol/min/mg.

The D258E mutant was more thermolabile than the WT enzyme. Incubation of the proteins at 58 °C for 5 min inactivated 68% and 94% enzyme activity, respectively, for the WT and D258E mutant. Since only the D258E mutant had residual enzymatic activity, we compared the kinetic properties of this mutant with those of the WT enzyme. The following experiments were performed on freshly prepared enzyme solution, collected immediately after affinity chromatography.

Inactivation and Cleavage of WT and D258E Mutant Pigeon Liver Malic Enzymes by the Fe^{2+}−Ascorbate System. Both WT and D258E mutant were sensitive to the Fe^{2+}−ascorbate system, but the latter was inactivated at a much slower rate. After 100 min of incubation, only 5% residual activity was left for the WT enzyme, while the D258E mutant still had 57% activity (Figure 2). Both proteins were protected from the Fe^{2+}-induced inactivation by saturated Mn^{2+} (4 mM). However, the D258E mutant was protected to a smaller extent than WT. After 100 min, Mn^{2+} only partially protected the metal-inactivated activity of D258E mutant, while the WT enzyme, similar to the natural enzyme, was fully protected.

After treatment with Fe^{2+}−ascorbate, all proteins were subjected to SDS−PAGE analysis to examine the peptide bond cleavage. As shown in Figure 1, none of the mutants including D258E, were cleaved under conditions that cleaved the WT or natural enzyme at Asp^{258} to give two fragments.

Initial Velocity Studies of WT and D258E Mutant Pigeon Liver Malic Enzymes. The above experimental results strongly suggest that Asp^{258} is an important ligand for the Mn^{2+} binding of the enzyme. We thus performed a detailed initial velocity experiment to assess the true binding constant between Mn^{2+} and the WT or D258E mutant enzyme. Figure 3 shows the results. The WT enzyme showed a biphasic double-reciprocal plot with two binding constants when [L-malate]$_f$ was plotted as the fixed-concentration substrate and $[Mn^{2+}]_f$ was plotted as the varied-concentration substrate. The biphasic pattern of WT is similar to that found for the natural enzyme, which shows biphasic kinetics at higher L-malate concentrations (Hsu et al., 1976; Hsu & Pry, 1980). The data fitted well to eq 2, which describes an ordered bi−bi mechanism in which Mn^{2+} binding precedes L-malate binding and L-malate binding is promoted by Mn^{2+}. The kinetic parameters extracted from this experiment are summarized in Table 3. When the data were fit to an equation describing a random bi−bi kinetic mechanism, large statistical errors were obtained.

When the tight and loose Mn^{2+}-binding sites were both saturated, the catalytic constant (k_{cat}) decreased from 38 s^{-1} for the WT enzyme to 1.6 s^{-1} for the D258E mutant. The apparent K_{mNADP} values were almost identical in D258E mutant and WT enzyme and were also close to the value for the natural enzyme (10.8 μM). The K_{mMal} was increased by 1.95-fold but was still similar to that of the natural enzyme (150 μM). Dramatic changes were found on Mn^{2+} binding. In WT, the tight-binding site had a $K_{mMn(I)}$ of 0.08 μM and a $K_{dMn(I)}$ of 0.12 μM, while the loose site had a $K_{mMn(II)}$ of 0.45 μM and a $K_{dMn(II)}$ of 1.8 μM. Only one Mn^{2+}-binding site was detected for the D258E mutant, and the binding affinity was drastically reduced. The K_{mMn} and K_{dMn} values were increased approximately 1600- and 3000-fold, respectively, for the D258E mutant; the specific constant (k_{cat}/K_{mMn}) was decreased from 4.75×10^8 M^{-1} s^{-1} for WT to 1.2×10^4 M^{-1} s^{-1} for D258E, which is an approximately 40 000-fold difference, clearly indicating the involvement of Asp^{258} as the Mn^{2+} coordinate. Since the initial velocity pattern of D258E was similar to that of WT at low $[Mn^{2+}]$ with double-reciprocal plot line intercepts at a point above the *x*-axis, the data for D258E were those for the tight Mn^{2+} binding site. Because malic enzyme was inhibited by high ionic

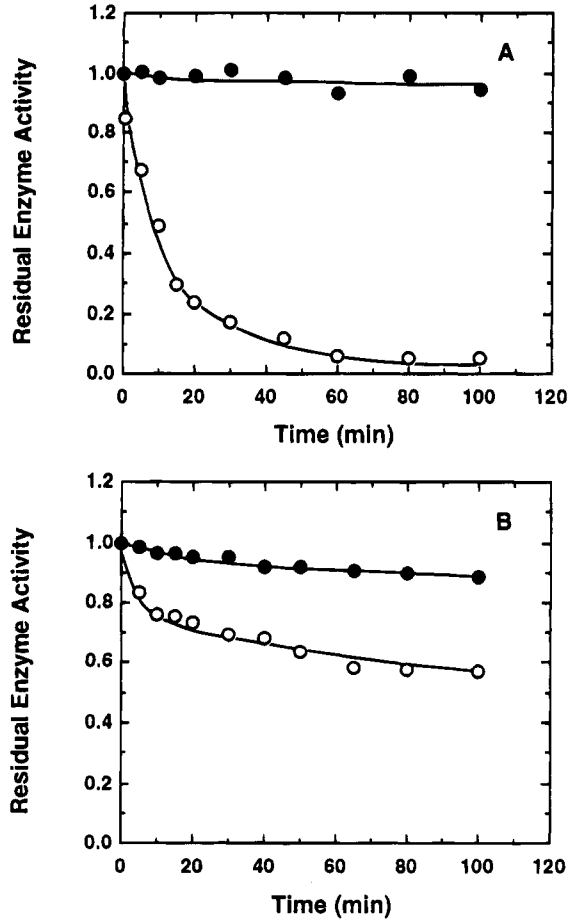

FIGURE 2: Inactivation of WT and D258E mutant pigeon liver malic enzymes by the Fe^{2+}–ascorbate system. (A) WT. (B) D258E mutant. The enzyme was incubated with Fe^{2+} (20 μM)–ascorbate (20 mM) at 0 °C with (●) or without (○) Mn^{2+} (4 mM).

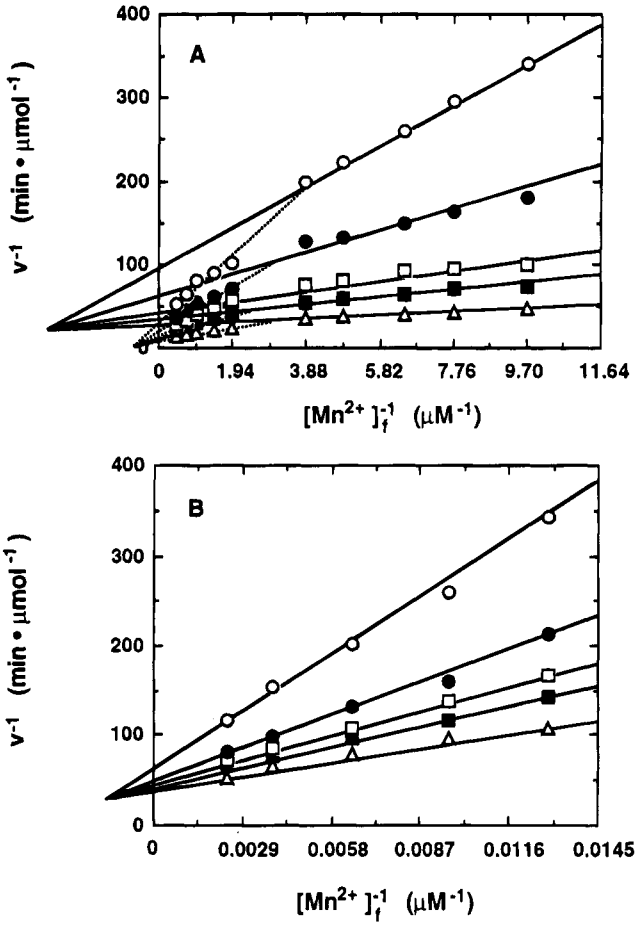

FIGURE 3: Initial velocity patterns for the WT and D258E mutant pigeon liver malic enzymes. (A) WT. (B) D258E mutant. From top to bottom, the L-malate concentrations were (○) 0.015, (●) 0.025, (□) 0.033, (■) 0.05, and (△) 0.1 mM in panel A and (○) 0.15, (●) 0.25, (□) 0.33, (■) 0.4, and (△) 0.5 mM in panel B. The amout of protein used in each assay was 3.48 μg for WT and 24.8 μg for D258E mutant.

strength (Chang et al., 1992), we were not able to use [Mn^{2+}] high enough to detect the possible K_{dMn} and K_{mMn} for the loose binding site of D258E, which should be in the molar range.

DISCUSSION

The histidine and other amino acid residues introduced by the pET-15b vector did not change the kinetic mechanism of the enzyme. A biphasic intercepting initial velocity pattern was obtained for the WT enzyme, which is the same as that of the natural enzyme (Hsu et al., 1976). The kinetic parameters for the WT enzyme were very close to those of the natural enzyme (Chou et al., 1994). Attempts to remove the histidine tag from the purified recombinant proteins by proteolysis without touching the other parts of the molecule were not successful. However, another recombinant WT malic enzyme having a maltose-binding protein at the N-terminus also has kinetic parameters, including K_{mMn} and K_{dMn}, nearly identical with those of the recombinant WT enzyme reported in this study (W.-Y. Chou, S.-M. Huang, and G.-G. Chang, unpublished results). It is reasonable to assume that the active site geometry of the WT recombinant is not perturbed by the N-terminus attachment. On the basis of the above discussion, we are tempted to conclude that whatever the effect of N-terminal attachment on the enzyme molecule is, it affects WT and the mutants equally. Therefore it should be relevant to compare the kinetic parameters of the WT and D258E mutant enzymes.

The Michaelis constant, which is the apparent dissociation constant including all enzyme-bound forms, provides a good indication for the binding affinity with a ligand. The K_{mNADP} values were almost identical for WT and the mutant, indicating that the metal ion is not involved in the nucleotide binding. This result was consistent with the electron paramagnetic resonance (EPR) determinations that the coenzyme $NADP^+$ or NADPH did not interact with the enzyme-bound Mn^{2+} (Hsu et al., 1976). K_{mMal} for the D258E mutant was increased by 1.95-fold compared to that for WT, which was not a large difference. L-Malate was proposed to bind with the enzyme at the active site through a metal bridge, serving as an electrophile in the activation of substrate (Hsu et al., 1976). Our previous data suggested that the 1-carboxyl and 4-carboxyl groups of L-malate were ion-paired and hydrogen-bonded to arginine and tyrosine residues, respectively (Chang & Huang, 1980, 1981). These binding ligands of L-malate were not perturbed by the mutation. The smaller binding affinity of L-malate for D258E than for WT might be a secondary effect due to impaired metal binding.

The most striking observation was the tremendous increase in K_{dMn} and K_{mMn} values, which were at least 3 orders of magnitude larger for D258E than for WT. Unfortunately, the histidine tag at the N-terminus prevented us from

Table 3: Kinetic Parameters of Recombinant Wild-Type and D258E Mutant Pigeon Liver Malic Enzymes[a]

constant	description	value	
		WT	D258E
V_{max} (μmol/min/mg)	maximum velocity at low [Mn^{2+}]	25 ± 2.5	1.5 ± 0.1
V_{max}' (μmol/min/mg)	maximum velocity at high [Mn^{2+}]	36 ± 1.4	
$K_{dMn(I)}$ (μM)	dissociation constant for tight Mn^{2+} binding site	0.12 ± 0.03	386 ± 62
$K_{dMn(II)}$ (μM)	dissociation constant for loose Mn^{2+} binding site	1.8 ± 0.2	
$K_{mMn(I)(app)}$ (μM)	apparent Michaelis constant for tight Mn^{2+} binding site	0.08 ± 0.01	130 ± 5.2
$K_{mMn(II)(app)}$ (μM)	apparent Michaelis constant for loose Mn^{2+} binding site	0.45 ± 0.05	
K_{mMal} (μM)	Michaelis constant for L-malate at low [Mn^{2+}]	133 ± 32	259 ± 56
K_{mMal}' (μM)	Michaelis constant for L-malate at high [Mn^{2+}]	44 ± 5.4	
$K_{mNADP(app)}$ (μM)	apparent Michaelis constant for $NADP^+$	13.4 ± 1.0	16.3 ± 2.5
$k_{cat}/K_{mMn(I)}$ (M^{-1} s^{-1})	specificity constant for Mn^{2+}	4.75×10^8	1.2×10^4

[a] Values shown are expressed as mean ± SE. Only one binding constant for Mn^{2+} was detected for the D258E mutant.

measuring the Mn^{2+} binding with other mutants directly. The 3000-fold increase in dissociation constant of Mn^{2+} with D258E mutant compared to WT confirmed the important role of Asp^{258} as Mn^{2+} coordinate. The slower rate of the D258E mutant in Fe^{2+}-induced inactivation was presumably also due to decreased affinity of the mutant protein for Fe^{2+}.

The catalytic constant (k_{cat}) or specific activity for the recombinant WT was similar to that for the natural enzyme (Hsu & Pry, 1980; Chang & Chang, 1982). The specific activity values shown in Tables 2 and 3 for purified D258E mutant were not consistent with each other. This discrepancy was due to experimental errors in assaying small activities because of weak binding of metal ion with the mutant. The specific activity value for D258E shown in Table 2 was determined at fixed concentrations of substrates and Mn^{2+} and thus is an apparent value. The much smaller specific activities for other mutants must also be due to much weaker metal binding. The strong binding affinity of WT for Mn^{2+} caused the second-order rate constant ($k_{cat}/K_{mMn} = 4.75 \times 10^8$ M^{-1} s^{-1}) to approach that of diffusion limitation for a chemical reaction ($\sim 10^9$ M^{-1} s^{-1}) (Moran et al., 1994; Simopoulous & Jencks, 1994). The recombinant WT malic enzyme thus is an almost perfect enzyme in terms of metal catalysis. On the other hand, the catalytic efficiency [$k_{cat}/(K_{mNADP}K_{mMal}K_{dMn})$] of D258E was only 0.000 52% that of WT.

Malic enzyme requires a divalent metal ion for the catalysis. The metal ion is involved in stabilizing the enolate form of the β-keto acid (Hsu et al., 1976; O'Leary, 1992; Villafranca & Nowak, 1992). We have conclusively demonstrated the importance of Asp^{258} of pigeon liver malic enzyme in the binding of Mn^{2+}. We propose that the previously reported essential carboxyl group, which has a pK_a value of 6.7 and was modifiable by Woodwards reagent (Chang et al., 1985), is that of Asp^{258}. This aspartate residue also may be the kinetically unidentified X-group, which has a pK_a value of 5.4−6 and accepts the transferred proton during the catalysis (Schimerlik & Cleland, 1977). Even the most conservative substitution of this aspartate with glutamate drastically reduces the metal-binding ability. Any other substitutions resulted in abortive mutants. Since D258E mutant still has a k_{cat} value that is 4.1% that of the WT enzyme, we conclude that both the negative charge and the side-chain length are critical for the correct binding. The binding cavity for Mn^{2+} must have a very precise geometry and may involve other ligands. The distance between Mn^{2+} and the carbonyl carbon of pyruvate as determined by EPR is 4.8 Å in the E·Mn^{2+}·pyruvate ternary complex (Hsu et al., 1976). This distance is too great (by 1.9 Å) for direct coordination but is compatible with a second-sphere E·Mn^{2+}·H_2O·pyruvate complex (Fung et al., 1974). An increase of the side-chain length by -CH_2 at Asp^{258}, which extends the carboxyl group by 3.6 Å in length or 25.1 $Å^3$ in volume (Chothia, 1975; Nakano et al., 1994), may perturb the proper alignment of all ligands for Mn^{2+} and thus render its binding affinity much looser.

In conclusion, we demonstrate that metal ion is essential in the catalytic reaction of pigeon liver malic enzyme, and Asp^{258} is an important ligand in the metal binding for the enzyme. Since this essential aspartate is conserved in all malic enzymes with known amino acid sequence (Chou et al., 1994), from human normal (Loeber et al., 1994) and cancer cells (W.-Y. Chou, S.-M. Huang, and G.-G. Chang, unpublished results) to bacteria (Kabayashi et al., 1989), we suggest that this aspartate residue is crucial in the catalytic process for all malic enzymes.

REFERENCES

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Canellas, P. F., & Wedding, R. T. (1980) *Arch. Biochem. Biophys. 199*, 259−264.

Chang, G. G., & Huang, T. M. (1980) *Biochim. Biophys. Acta 611*, 217−226.

Chang, G. G., & Huang, T. M. (1981) *Biochim. Biophys. Acta 660*, 341−347.

Chang, G. G., Huang, T. M., & Wuu, J. A. (1985) *Proc. Natl. Sci. Counc., Repub. China 9B*, 56−66.

Chang, G. G., Shiao, M. S., Liaw, J. G., & Lee, H. J. (1989) *J. Biol. Chem. 264*, 280−287.

Chang, G. G., Huang, T. M., Wang, J. K., Lee, H. J., Chou, W. Y., & Meng, C. L. (1992) *Arch. Biochem. Biophys. 296*, 468−473.

Chang, J. T., & Chang, G. G. (1982) *Anal. Biochem. 121*, 366−369.

Chou, W. Y., Huang, S. M., Liu, Y. H., & Chang, G. G. (1994) *Arch. Biochem. Biophys. 310*, 158−166.

Chothia, C. (1975) *Nature 254*, 304−308.

Fung, C. H., Mildvan, A. S., & Leigh, J. S., Jr. (1974) *Biochemistry 13*, 1160−1169.

Hochuli, E., Dobeli, H., & Schacher, A. (1987) *J. Chromatogr. 411*, 177−184.

Hsu, R. Y. (1982) *Mol. Cell. Biochem. 43*, 3−26.

Hsu, R. Y., & Lardy, H. A. (1967) *J. Biol. Chem. 242*, 520−526.

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.

Kobayashi, K., Doi, S., Negoro, S., Urabe, I., & Okada, H. (1989) *J. Biol. Chem. 264*, 3200−3205.

Loeber, G., Dworkin, M. B., Infante, A., & Ahorn, H. (1994) *FEBS Lett. 344*, 181−186.

Moran, L. A., Scrimgeour, K. G., Horton, H. R., Ochs, R. S., & Rawn, J. D. (1994) *Biochemistry*, 2nd. ed., pp 713−714, Neil Patterson, Englewood Cliffs, NJ.

Nakano, T., Spenser, H. T., Appleman, J. R., & Blakley, R. L. (1994) *Biochemistry 33*, 9945−9952.

O'Leary, M. H. (1992) *The Enzymes, 3rd Ed. 20*, 236−269.

Park, S. H., Kiick, D. M., Harris, B. G., & Cook, P. F. (1984) *Biochemistry 23*, 5446−5453.

Perrella, F. W. (1988) *Anal. Biochem. 174*, 437−447.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., & Conlson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.

Schimerlik, M. I., & Cleland, W. W. (1977) *Biochemistry 16*, 576−583.

Simopoulos, T. T., & Jencks, W. P. (1994) *Biochemistry 33*, 10375−10380.

Villafranca, J. J., & Nowak, T. (1992) *The Enzymes 3rd Ed. 20*, 63−94.

Wei, C. H., Chou, W. Y., Huang, S. M., Lin, C. C., & Chang, G. G. (1994) *Biochemistry 33*, 7931−7936.

Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487−6500.

BI950117D