Affinity Cleavage at the Putative Metal-Binding Site of Pigeon Liver Malic Enzyme by the Fe^{2+}–Ascorbate System†

Chien-Hwa Wei,‡ Wei-Yuan Chou,‡ Shih-Ming Huang,‡ Ching-Chun Lin,§ and Gu-Gang Chang*,‡,§

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

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ABSTRACT: Pigeon liver malic enzyme was rapidly inactivated by micromolar concentrations of ferrous sulfate in the presence of ascorbate at neutral pH and 0 or 25 °C. Omitting the ascorbate or replacing the ferrous ion with manganese ion did not lead to any inactivation. Manganese, magnesium, zinc, cobalt, or calcium ion at 200 molar excess over ferrous ion offered complete protection of the enzyme from Fe^{2+}-induced inactivation. Ni^{2+} provided partial protection, while Ba^{2+} or imidazole was ineffective in protection. Addition of 4 mM Mn^{2+} or 5 mM EDTA into a partially modified enzyme stopped further inactivation of the enzyme. Inclusion of substrates (L-malate or $NADP^+$, singly or in combination) in the incubation mixture did not affect the inactivation rate. The enzyme inactivation was demonstrated to be followed by protein cleavage. Native pigeon liver malic enzyme had a subunit M_r of 65 000. The inactivated enzyme with residual activity of only 0.3% was cleaved into two fragments with M_r of 31 000 and 34 000, respectively. The cleavage site was identified as the peptide bond between Asp^{258} and Ile^{259}. Native pigeon liver malic enzyme was blocked at the N-terminus. Cleavage at the putative metal-binding site exposed a new N-terminus, which was identified to be at the 34-kDa fragment containing the C-terminal half of original sequence 259–557. Our results indicated that Fe^{2+} catalyzed a specific oxidation of pigeon liver malic enzyme at Asp^{258} and/or some other essential amino acid residues that caused enzyme inactivation. The modified enzyme was then affinity cleaved at the Mn^{2+}-binding site.

Malic enzyme [(*S*)-malate:$NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40] catalyzes the divalent metal ion-dependent reversible oxidative decarboxylation of L-malate to form CO_2 and pyruvate, with concomitant reduction of $NADP^+$ to NADPH:

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

Among the divalent cations tested, Mn^{2+} or Mg^{2+} was found to be most active, and Ni^{2+}, Zn^{2+}, or Co^{2+} was weakly active (Rutter & Lardy, 1958). The enzyme was first identified in avian by Ochoa and colleagues (1947). Later, it was found to be widely present in nature, from human to bacteria, and was thought to be a housekeeping enzyme. In avian, its major physiological function is believed to be to provide NADPH for the fatty acid biosynthesis [for reviews, see Goodridge et al. (1989) and Iritani (1992)].

Recently, malic enzyme was found to be involved in many other important biochemical functions, such as detoxification (Ayala et al., 1986) and aging (Fukuda et al., 1990). Malic enzyme activity was demonstrated to be reduced in aged rats (Fukuda et al., 1990; Vitorica et al., 1981). The detailed mechanism of aging is still unclear. Metal-catalyzed oxidation is one of the observed biochemical reactions during aging process [for reviews, see Stadtman (1986, 1990a,b, 1992) and Stadtman and Oliver (1991)]. The oxidizing-tagged amino acids are more susceptible to protease degradation that represents a normal protein turnover *in vivo*. The metal-catalyzed oxidation system generates active species H_2O_2, $OH^•$, or $O_2^{•-}$ radicals through Fenton chemistry (Fenton, 1984). Those reactive species then react with nearby susceptible amino acid residues. Basic amino acid and proline residues were found to be most susceptible to oxidative modification (Amici et al., 1989). Gordillo et al. (1988) proposed the involvement of histidine residues in the loss of rat liver malic enzyme activity during aging. The physiological significance of Fe^{2+}-catalyzed oxidation of enzyme in accumulating less active or inactive enzyme forms as a function of age has been discussed (Stadtman, 1986, 1990a,b, 1992; Stadtman & Oliver, 1991; Szweda & Stadtman, 1992).

In this paper we show that, in the presence of ascorbate, malic enzyme was very sensitive to ferrous ion oxidation. Furthermore, the modified enzyme was found to be cleaved at the metal-binding site. For the first time, Asp^{258} was identified as one of the ligands of the putative Mn^{2+}-binding site of malic enzyme. Using this technique, Farber and Levine (1986) and Jhon et al. (1991) located the metal-binding site in glutamine synthetase. The binding site was subsequently confirmed when the crystal structure was available (Liaw et al., 1993).

MATERIALS AND METHODS

Materials. Ferrous sulfate and ascorbic acid were purchased from E. Merck. Other chemicals used were of reagent grade as described previously (Chang et al., 1989). Distilled water, further purified with a Millipore MilliQ system, was used throughout the present work.

Immobilon-P membrane [poly(vinylidene difluoride)] was purchased from Millipore. Before using, the membranes were

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

‡ National Defense Medical Center.

§ Academia Sinica.

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wetted in methanol for 1–3 s, then immersed in water for 1–2 min to remove the methanol, and finally soaked in "Towbin" transfer buffer (25 mM Tris–192 mM glycine buffer, pH 8.3, containing 20% methanol) for 30 min.

Malic enzyme from pigeon liver was purified to apparent homogeneity according to our published procedure (Chang & Chang, 1982).

Enzyme Assay. Malic enzyme activity was assayed as described previously (Chang et al., 1992). 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 appropriate amount of enzyme (1.26 μg/assay). 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/min under the assay conditions. A molar extinction coefficient of 6.22×10^3 $M^{-1}\cdot cm^{-1}$ for the NADPH was utilized in the calculation.

Enzyme Inactivation. We performed the inactivation experiments at 0 °C by adding freshly prepared solutions of ferrous sulfate (20 μM) and ascorbate (20 mM) or various ligands into the enzyme solution (0.97 μM) in 66.7 mM triethanolamine hydrochloride buffer, pH 7.4. We monitored the progress of enzyme inactivation by assaying the enzyme activity in small aliquots withdrawn at the designated time intervals. The data were fitted to the following equation, assuming a double exponential inactivation process (Dixon & Webb, 1979):

$$E_t/E_0 = (1 - A)e^{-(k_1+k_2)t} + Ae^{-k_2t} \quad (2)$$

where E_0 is the original enzyme activity and E_t is the enzyme activity at the time indicated, A is the fraction of slow-reacting groups, and k_1 and k_2 are the inactivation rate constants for the fast- and slow-reacting groups, respectively. Nonlinear regression fitting of the data was performed with SigmaPlot (Jandel Scientific), a microcomputer program using the Marquardt–Levenberg algorithm.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS/PAGE). The experiments were performed with PhastGel 8–25% gradient gel and PhastGel SDS buffer strip (containing 0.2 M Tricine, 0.2 M Tris, and 0.55% SDS, pH 7.5) on a Pharmacia-LKB Phast-System. Electrophoresis was carried out at 10 mA, 250 V, and 15 °C (60 V·h) (Chang et al., 1991).

Relative molecular masses (M_r) of the subunit and cleaved fragments of malic enzyme were estimated by coelectrophoresis of various M_r standards as shown in the figure legend.

Isolation of Protein Fragments. For sequence analysis, the inactivated enzyme (126.4–252.8 μg; 0.49–0.98 nmol) was mixed with equal volume of 5% SDS, and the solution was incubated for 10 min at 100 °C. An equal volume of sample buffer (60 mM Tris buffer, pH 6.8, 100 mM dithiothreitol, and 30% glycerol) was added, and the intact protein and cleaved fragments were separated on a 12% polyacrylamide slab gel at a constant voltage (100 V) for 6 h with a Bio-Rad mini-slab gel apparatus.

After electrophoresis, the slab gel was soaked in transfer buffer for 30 min. The gel and a presoaked Immobilon-P membrane were inserted between filter papers. The electroblotting was conducted in a blotting cassette (Bio-Rad) at a constant current (150 mA) for 3 h at 4 °C with protein transfer efficiency estimated to be >85%. After transferring, the protein bands were visualized by staining with 0.1% Coomassie brilliant blue R-250 and destained with 30% 2-propanol/10% acetic acid (v/v). The membrane was then rinsed several times in Q-grade water and air-dried. The desired transblot protein bands were cut out, dried with a Speed-Vac, and stored at −70 °C for amino acid sequence analysis.

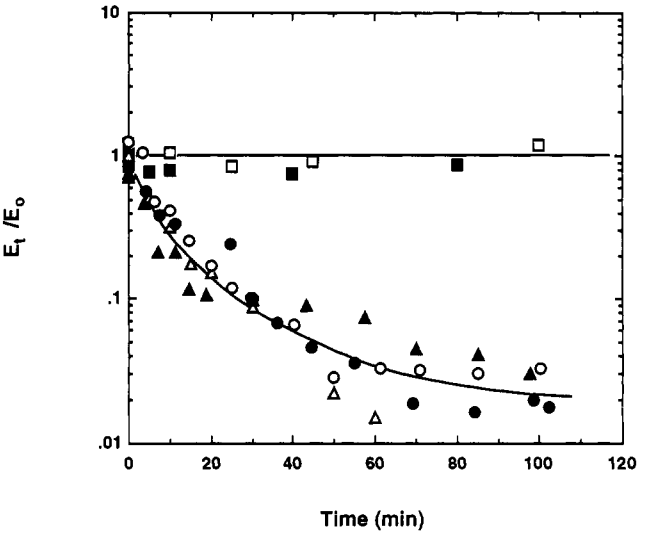


FIGURE 1: Inactivation of pigeon liver malic enzyme by ferrous sulfate and ascorbate. The reaction mixture contained 0.97 μM malic enzyme in 66.7 mM triethanolamine hydrochloride buffer (pH 7.4), 20 μM ferrous sulfate, and 20 mM ascorbate with the following additions or omissions: (○) complete system, (●) plus 0.23 mM $NADP^+$, (△) plus 0.5 mM L-malate, (▲) plus 0.23 mM $NADP^+$ and 0.5 mM L-malate, (□) minus ferrous ion but plus 4 mM Mn^{2+}, and (■) minus ascorbate. Incubation was at 0 °C. Results were from two separate experiments. The semilogarithm of the residual enzyme activity (E_t/E_0) was plotted against incubation time.

N-Terminal Amino Acid Sequencing. The amino acid sequence of the isolated fragments was analyzed with an Applied Biosystem pulsed-liquid protein sequenator (Model 477A), equipped with an on-line phenylthiohydantoin analyzer (Model 120A) and a Macintosh IIsi computer. The transblot papers containing the protein fragments were directly sequenced by Edman degradation for 20–25 cycles.

RESULTS

Inactivation of Pigeon Liver Malic Enzyme by Ferrous Sulfate. In the presence of ascorbate, ferrous sulfate (at $[Fe^{2+}]/[\text{enzyme}] = 20$) caused a rapid time-dependent inactivation of pigeon liver malic enzyme at pH 7.4 and 0 °C with a half-time ($t_{1/2}$) of about 8 min (Figure 1). Replacing ferrous sulfate with manganese sulfate or removing ascorbate from the incubation mixture did not lead to any inactivation. Inclusion of 0.5 mM L-malate or 0.23 mM $NADP^+$ or both in the incubation mixture did not affect the inactivation rate (Figure 1). In our control experiment, under the same conditions but without Fe^{2+} and ascorbate added, the enzyme was found to be completely stable during the experimental period.

The inactivation rate was temperature dependent. Under the otherwise identical conditions described above, the inactivation rate was too fast to follow at room temperature (25 °C). In 5 min, malic enzyme activity was already down to <10% of the original activity.

Dependence of Fe^{2+}-Catalyzed Inactivation of Pigeon Liver Malic Enzyme on Ferrous Sulfate Concentration. The above

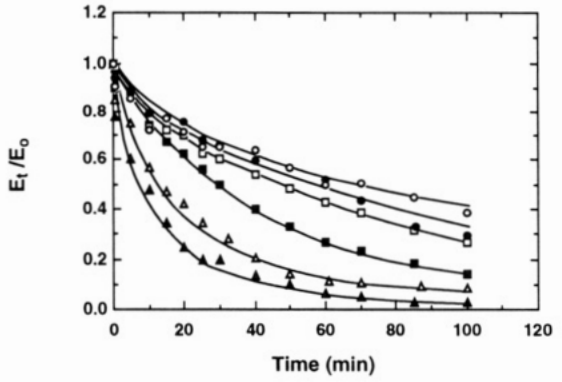


FIGURE 2: Effect of ferrous sulfate concentrations on the Fe^{2+}-induced inactivation rates of pigeon liver malic enzyme. Experimental conditions were the same as the complete system in Figure 1, except that the ferrous sulfate concentration was varied as follows: (○) 0.05, (●) 0.25, (□) 0.75, (■) 2.5, (△) 7.5, and (▲) 15 μM. For clarity, some data were not shown. The curves were computer fittings to eq 2.

inactivation process did not follow a pseudo-first-order kinetics as shown by the nonlinear semilogarithmic plot (Figure 1). This nonlinear semilog plot was generally observed in the oxidative modification of enzymes with Fe^{2+}–ascorbate or other similar systems. We then fitted the data to eq 2. The apparently well-fitted curves shown in Figure 2 seem to suggest that the inactivation process is composed of a fast and a slow process. At $[Fe^{2+}] = 0.05$ μM, the inactivation rates for the fast-reacting group k_1 (0.836 fraction) and the slow-reacting group k_2 (0.164 fraction) were found to be 1.486 ± 0.76 s^{-1} and 0.008 ± 0.0005 s^{-1}, respectively. However, the inactivation data also fit well to an equation composed of three exponential components (not shown). The kinetics of inactivation may be more complex.

Although the detailed kinetic mechanism for the inactivation process is not clear at the present stage, inactivation of the enzyme by the Fe^{2+}–ascorbate system was clearly dependent on Fe^{2+} concentration (Figure 2). The $t_{1/2}$ progressively decreases as the $[Fe^{2+}]$ increases. When $t_{1/2}$ was plotted versus $1/[Fe^{2+}]$, the data turn to a concave-down curve (not shown).

Protection of Pigeon Liver Malic Enzyme against Fe^{2+}-Induced Inactivation by Divalent Cations. Inclusion of 4 mM manganese sulfate, which was 200 molar excess over ferrous sulfate, in an enzyme solution completely protected the enzyme from Fe^{2+}-induced inactivation. Lower concentrations of Mn^{2+} also provided less but substantial protection (Figure 3A). The $[Mn^{2+}]$ giving 50% protection was ~7.5 μM, in excellent agreement to the K_D value of the tight Mn^{2+}-binding site of the enzyme (6–10 μM) (Hsu et al., 1976). Mg^{2+}, Zn^{2+}, Co^{2+}, or Ca^{2+} at 4 mM also afforded 100% protection. Ni^{2+} gave 60% protection, while Ba^{2+} was ineffective. Cu^{2+} increased the Fe^{2+}-induced inactivation rate. Cu^{2+} at 4 mM together with 20 μM Fe^{2+} completely inactivated the enzyme activity in 30 s.

Addition of 4 mM Mn^{2+} to a partially inactivated enzyme solution prevented further inactivation of the remaining enzyme activity (Figure 3B). The metal-chelating agent EDTA also afforded 100% protection. Addition of 5 mM EDTA (pH 7.4) to a partially modified enzyme solution also stopped further inactivation of the remaining enzyme activity.

Addition of 4 mM imidazole (pH 6.8) did not affect the inactivation rate. This implied that either the histidine residue was not modified or the locally generated reactive species in the active center were not reachable by the imidazole in solution.

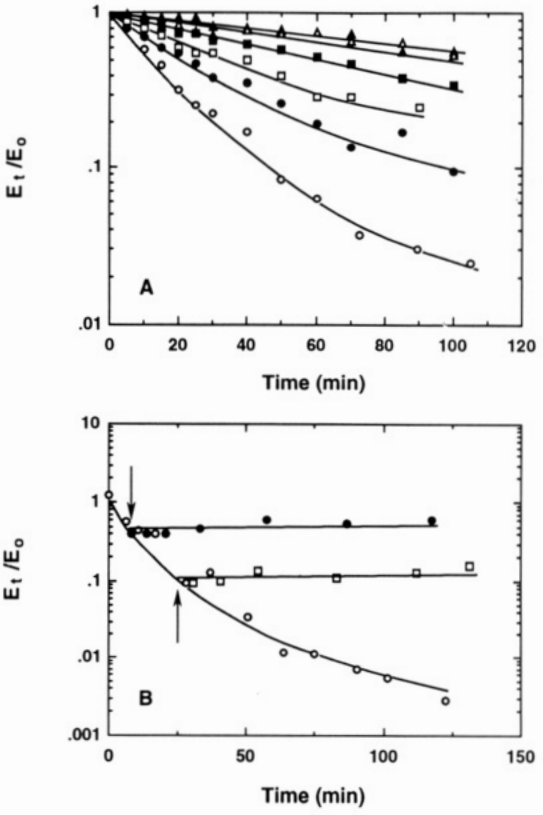


FIGURE 3: Protection of pigeon liver malic enzyme by Mn^{2+} against Fe^{2+}-catalyzed inactivation. (A) Experimental conditions were the same as in Figure 1, except that 80 (▲), 60 (△), 40 (■), 20 (□), 10 (●), or 5 μM (○) Mn^{2+} was added into the reaction mixture of a complete system. (B) Effect of Mn^{2+} on the partially inactivated enzyme. Experimental conditions were the same as in Figure 1, except that, at the designated time intervals (indicated by arrows), aliquots of the reaction mixture were withdrawn and added to a solution containing 4 mM Mn^{2+} (pH 7.4) and the enzyme activity was monitored again.

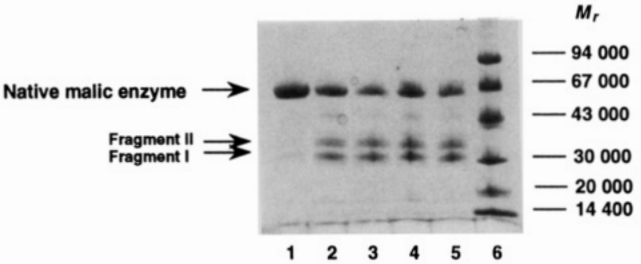


FIGURE 4: SDS/PAGE of pigeon liver malic enzyme before and after the metal-catalyzed modification. Lane 1: Unmodified malic enzyme. Lanes 2–5: Modified malic enzyme (residual activity 0.3%) prepared by the procedure as shown in Figure 1: (lane 2) complete system; (lane 3) plus 0.5 mM L-malate and 0.23 mM $NADP^+$; (lane 4) plus 0.23 mM $NADP^+$; (lane 5) plus 0.5 mM L-malate. (Lane 6) M_r standards: phosphorylase *b*, M_r 94 000; bovine serum albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 000; α-lactalbumin, M_r 14 400.

SDS/PAGE Gel Pattern of the Fe^{2+}-Inactivated Malic Enzyme. Extensively inactivated pigeon liver malic enzyme (with residual activity of only 0.3%) was subjected to polyacrylamide gel electrophoresis under reducing conditions in the presence of SDS to examine the possible oxidative cleavage of the polypeptide chain. The native enzyme has a subunit M_r of 65 000. The inactivated enzyme was clearly demonstrated to be cleaved into two fragments, E'_I and E'_{II}, having M_r of 31 000 and 34 000, respectively (Figure 4). Inclusion of L-malate, $NADP^+$, or both, which did not affect the inactivation rate, gave identical gel patterns as shown in

Table 1: Correlation of Fe^{2+}-Catalyzed Inactivation of Pigeon Liver Malic Enzyme and Cleavage of the Metal-Binding Site[a]

time (min)	enzyme act. remaining (%)	rel amt of uncleaved enzyme (%)	rel amount of E'_{II} fragment (%)	relative amount of E'_{I} fragment (%)
0	100	100	0	0
10	42	95	7	4
20	24	81	10	8
45	8	73	16	11
70	4	60	18	17
105	2	59	20	20

[a] Experimental conditions were the same as in Figure 1. The enzyme samples inactivated to various degrees were subjected to SDS/PAGE separation of the cleaved and uncleaved molecule and quantified with a densitometer. Original enzyme amount was taken as 100%.

Figure 4. The enzyme may have been cleaved at another site, since a small protein band with M_r ~45 000 was also visible. Due to the limited amount available, this fragment was not pursued further in the present study.

The percentage of the cleaved enzyme was estimated by scanning the gel with a Molecular Dynamics computing laser densitometer and then calculating with the ImageQuant software. The relative ratio of fragment I to fragment II was ~0.68, which suggested that the cleavage site was near the center of the original polypeptide chain. The data shown in Figure 4 (lanes 2–5) also indicated that about 42% of the enzyme were still intact when the enzyme activity was almost completely lost. When the enzyme was inactivated to different stages and then analyzed with SDS/PAGE, there was a clear correlation between the decreasing of native protein and the increasing of fragments I and II. There was also a correlation between enzyme inactivation and peptide bond cleavage although the peptide bond cleavage process lagged behind the activity loss in time scale (Table 1).

Identification of the Metal-Binding Site of Pigeon Liver Malic Enzyme. After separation of the cleaved protein fragments by SDS/PAGE, fragments I and II were transblotted electrophoretically onto an Immobilon-P membrane. The N-terminal amino acid sequences of the native pigeon liver malic enzyme and fragments E'_{I} and E'_{II} were analyzed with an automatic protein sequenator. The N-terminus of the native enzyme was found to be blocked. Fragment I was also blocked at the N-terminus, which therefore was the N-terminal half of the enzyme molecule. The sequence of the N-terminal 20 amino acid residues of fragment II was successfully determined as Ile-Gln-Gly-Thr-Ala-Ser-Val-Ala-Val-Ala-Gly-Leu-Leu-Ala-Ala-Leu-Arg-Ile-Thr-Lys, which is identical to residues 259–278 of the native enzyme (Chou et al., 1994). In a duplicate experiment, fragment II from another enzyme preparation was sequenced for 25 cycles and gave identical results for the first 20 cycles as shown above, followed by Asp-Arg-Leu-Ser-Asp, corresponding to residues 279–283 of the native enzyme. We thus conclude that the major cleavage site of the enzyme by the Fe^{2+}–ascorbate system was the peptide bond between Asp^{258} and Ile^{259}, and Asp^{258} was identified as one of the Mn^{2+}-binding ligands of pigeon liver malic enzyme.

After proper alignment, the putative metal-binding site of malic enzyme from various sources was found to be highly conserved (Figure 5) (Bagchi et al., 1987; Börsch & Westhoff, 1990; Chou et al., 1994; Hsu et al., 1992; Kobayashi et al., 1989; Kulkarni et al., 1993; Loeber et al., 1991; Magnuson et al., 1986; Rothermel & Nelson, 1989). From human to bacteria, an aspartate residue was invariable. Except for the bacteria enzyme, the amino acid residues from 255 to 265 were almost identical for all malic enzymes including a human breast cancer cell cytosolic malic enzyme (Chou et al., unpublished results).

DISCUSSION

The results presented in this paper indicated that malic enzyme was specifically inactivated by ferrous ion. A reducing agent such as ascorbate was necessary for this process. Manganese, magnesium, or some other divalent cations provided full protection against the inactivation. The inactivation thus must be due to the modification of an essential amino acid residue at or near the metal-binding site of the enzyme. Mn^{2+} may act as an $OH^{\bullet}$ scavenger (Stadtman & Oliver, 1991) and thus may reduce the reactive free radicals and slow down the inactivation rate. However, since Mg^{2+} and some other divalent cations also afforded full protection, this seems not to be the case in our experiments.

We also observed protein cleavage after oxidative modification (Figure 4). This cleavage was very specific, only one major site being cleaved after extensive inactivation. This site was identified as the bond between Asp^{258} and Ile^{259}. The finding that aspartate is the metal ligand in malic enzyme is in accordance with the general phenomenon that Asp predominates in cocatalytic metal sites where the binding frequency is Asp > His ≫ Glu (Vallee & Auld, 1993). The M_r values of fragments E'_{I} and E'_{II} calculated from the amino acid sequence were 29 521 and 32 559, respectively, in good agreement with the experimental value estimated from SDS/PAGE. E'_{I} and E'_{II} were calculated to have opposite charge at neutral pH (+1.04 for E'_{I} and −4.15 for E'_{II}). The highly conserved region 255–265 was predicted to be in the β-turn (248–260) and random coil (261–262) conformation and to be connected with α-helical regions 237–248 and 263–278. Asp^{258} was predicted to be in a slightly hydrophilic microenvironment.

Barely detectable cleaved fragments were also observed for the native enzyme (Figure 4, lane 1), suggesting that Asp^{258} may also be the most susceptible site *in vivo* and oxidative modification could be responsible for the decreased malic enzyme activity during the aging process. The *in vitro* Fe^{2+}–ascorbate system we employed in this study accelerated the aging process of the enzyme. This site was also susceptible to cleavage in the cloned malic enzyme, which may account for the lower catalytic activity of the cloned enzyme as compared to that of the natural pigeon liver enzyme (Chou et al., 1994). As shown in Figure 4 (lane 2), only 58% of the enzyme molecules were cleaved after extensive inactivation. We thus suggest that the Fe^{2+}-catalyzed oxidation of malic enzyme proceeds in three steps: First, the enzyme forms a reversible complex with the Fe^{2+}. Mn^{2+} and other divalent cations afford protection at this step by competing for the metal-binding site. Then, the Fe^{2+}–ascorbate system generates some reactive species (in this case, may be the H_2O_2, $OH^{\bullet}$ or $O_2^{\bullet-}$ radicals), which react with the nearby Mn^{2+} ligand Asp^{258} or some other essential groups and cause enzyme inactivation. Finally, the modified enzyme is cleaved at the susceptible chemical bond (Scheme 1). This mechanism is consistent with a recently proposed model for the oxidative modification of glutamine synthetase based on crystal structure (Liaw et al., 1993). Since we successfully determined isoleucine259 as the newly generated N-terminal amino acid, the cleavage position of the enzyme must be at the chemical bond between C_α of Asp^{258} and the carbonyl carbon of the peptide bond (Platis et al., 1993). However, in the case of malic enzyme, since the cleavage of peptide bond did not correlate with

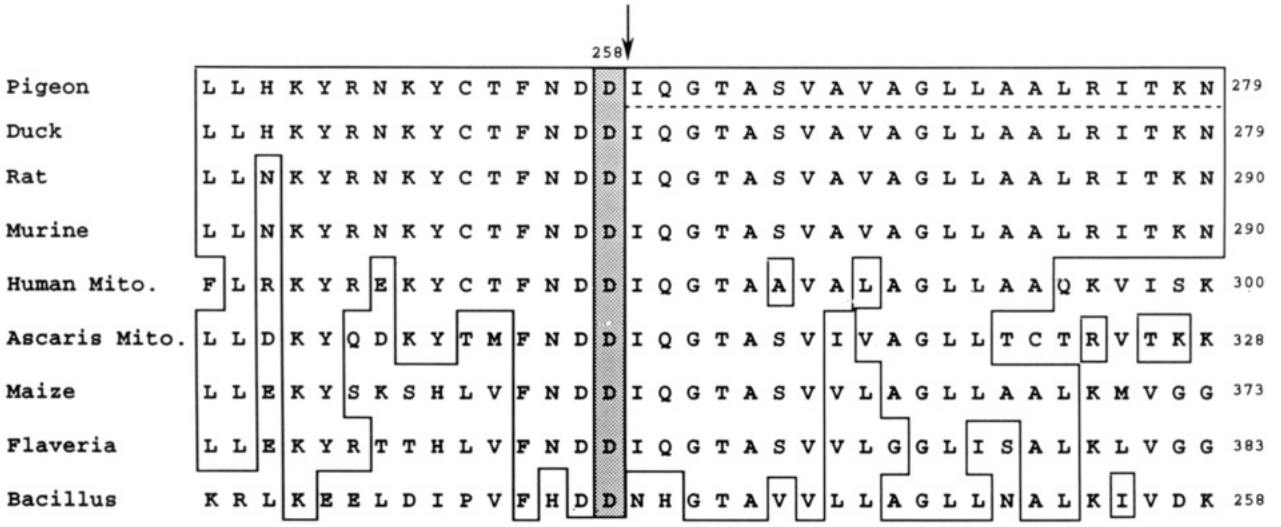
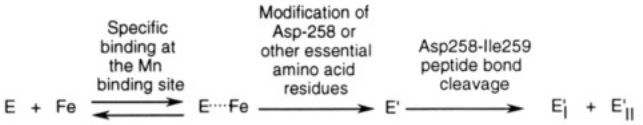


FIGURE 5: Comparison of the putative Mn^{2+}-binding site of malic enzymes. Amino acid sequences around Asp^{258} of pigeon and duck, Asp^{269} of rat and mice, Asp^{279} of human liver mitochondria, Asp^{307} of *Ascaris suum* mitochondria, Asp^{352} of maize chloroplast, Asp^{362} of dicotyledonous C4 plant *Flaveria trinervia*, and Asp^{237} of *Bacillus stearothermophilus* malic enzymes were aligned to show the high conservation in this region. The cleavage site of pigeon liver malic enzyme by ferrous sulfate was indicated by an arrow. The dashed underline sequence was confirmed by amino acid sequence analysis.

Scheme 1: Proposed Reaction Mechanism for the Oxidative Modification of Pigeon Liver Malic Enzyme by Fe^{2+}–Ascorbate System



enzyme inactivation in time scale, oxidation of other essential amino acid residues may induce the inactivation and then the locally generated reactive species may cleave the nearby susceptible peptide bond. Furthermore, since the inactivation was a biphasic process, the actual metal-mediated oxidation could modify more than one essential group and some of the modifications were not followed by peptide bond cleavage. Experiments with various oxidative conditions are in progress in this laboratory, in order to increase the amount of the cleaved peptide from the minor cleavage site shown in Figure 4, which may represent another metal ligand site.

Gordillo et al. (1988) found that modification of young rat malic enzyme by 15 mM ascorbate caused a 36% enzyme activity inactivation and loss of 1.2 histidine residues. The modified enzyme was in full length, as assessed by SDS/PAGE. However, considering that the pigeon liver malic enzyme was inactivated at as low as 0.05 μM iron (Figure 2) and that peptide cleavage was detectable only after 50% inactivation (Table 1), Gordillo's system evidently contained a low concentration of iron, from the trace contaminants. Pigeon liver malic enzyme has an essential histidine residue at the nucleotide binding site (Chang & Hsu, 1977), but we did not observe enzyme inactivation by 20 mM ascorbate alone (Figure 1), and imidazole did not protect the enzyme against Fe^{2+}-induced inactivation. These results seem to suggest that the Fe^{2+}-catalyzed oxidation causes modification of the Mn^{2+} ligand Asp^{258}, which in turn may be responsible for the observed enzyme inactivation. This result is consistent with our earlier chemical modification experiments (Chang et al., 1985), which indicated that modification of carboxyl group(s) with Woodward reagent K (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate) resulted in rapid inactivation of the enzyme. However, at this stage, it would be difficult to attribute the inactivation to the same amino acid residue as that for cleavage. If the present system is a model for aging, one would expect modification of a histidine residue is responsible for inactivation. Further experiments are needed to clarify this point. His^{269} was identified to be liganded to the bound metal in glutamine synthetase, which was susceptible to oxidative modification by the iron/ascorbate system. Mn^{2+} prevented the inactivation but imidazole did not (Levine, 1983; Farber & Levine, 1986).

Pigeon liver malic enzyme belongs to one of the most susceptible enzymes toward metal-catalyzed oxidation, and this system is highly specific for the metal-binding site of the enzyme. Malic enzyme requires divalent metal cation for catalysis. The function of metal ion is to polarize the carbonyl groups, forming a second-sphere complex with the substrate (Hsu et al., 1976). Thus, metal ion plays a central role in transition-state stabilization for the malic enzyme-catalyzed reaction. Our results demonstrated that Fe^{2+}–ascorbate is an excellent affinity cleavage system for this metal-binding site. Using this system, we identified Asp^{258} as one of the Mn^{2+} ligand sites of the enzyme, and this is probably also true for other malic enzymes. Employing the Fe^{2+}–isocitrate–ascorbate system, Soundar and Colman (1993) recently delineated the metal–isocitrate binding site of pig heart isocitrate dehydrogenase that also catalyzes the oxidative decarboxylation reaction as malic enzyme does. The crystal structure of *E. coli* isocitrate dehydrogenase indicated that the metal ion is coordinated to Asp^{307} and $Asp^{283'}$ from the other subunit with Asp^{311} as a neighbor (Hurley et al., 1991). Our results suggest that Asp^{258} in malic enzyme acts in a similar manner to one of the Asp residues in isocitrate dehydrogenase. Studies on site-directed mutagenesis at Asp^{258} and Asp^{257} or other possible metal ligand sites are in progress in this laboratory in order to further elucidate the active site structure of this enzyme.

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