Selective Oxidative Modification and Affinity Cleavage of Pigeon Liver Malic Enzyme by the Cu$^{2+}$-Ascorbate System*

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Pigeon liver malic enzyme was rapidly inactivated by micromolar concentration of Fe$^{2+}$ in the presence of ascorbate at neutral pH. The inactivated enzyme was subsequently cleaved by the Fe$^{2+}$-ascorbate system at the chemical bond between Asp$^{258}$ and Ile$^{259}$ (Wei, C. H., Chou, W. Y., Huang, S. M., Lin, C. C., and Chang, G. G. (1994) Biochemistry, 33, 7931-7936), which was confirmed by site-specific mutagenesis (Wei, C. H., Chou, W. Y., and Chang, G. G. (1995) Biochemistry 34, 7949-7954). In the present study, at neutral pH, Cu$^{2+}$ was found to be more reactive in the oxidative modification of malic enzyme and the enzyme was cleaved in a similar manner as Fe$^{2+}$ did. At acidic pH, however, Fe$^{2+}$ was found to be ineffective in oxidative modification of the enzyme. Nevertheless, Cu$^{2+}$ still caused enzyme inactivation and cleaved the enzyme at Asp$^{258}$-Gly$^{259}$, Asp$^{258}$-Pro$^{259}$, or Asp$^{258}$-Asp$^{259}$-Mn$^{2+}$, Mn$^{2+}$ and L-malate synergistically protect the enzyme from Cu$^{2+}$ inactivation at acidic pH. Cu$^{2+}$ is also a competitive inhibitor versus Mn$^{2+}$ in the malic enzyme-catalyzed reaction with $K_i$ value 70.3 ± 5.8 μM. The above results indicated that, in addition to the previously determined Asp$^{258}$ at neutral pH, Asp$^{141}$, Asp$^{194}$, and Asp$^{664}$ are also the coordinate sites for the metal binding of malic enzyme. We suggest that the mechanism of affinity modification and cleavage of malic enzyme by the Cu$^{2+}$-ascorbate system proceed in the following sequence. First, Cu$^{2+}$ binds with the enzyme at the Mn$^{2+}$ binding site and reduces to Cu$^{+}$ by ascorbate. Next, the local oxygen molecules are reduced by Cu$^+$, thereby generating superoxide or other reactive free radicals. These radicals interact with the susceptible essential amino acid residues at the metal-binding site, ultimately causing enzyme inactivation. Finally, the modified enzyme is cleaved into several peptide fragments, allowing the identification of metal site of the enzyme. The pH-dependent different specificities of metal-catalyzed oxidation system may be generally applicable for other enzymes or proteins.

Cytoplasmic malic enzyme (S-malate:NADP$^+$ oxidoreductase, oxaloacetate-decarboxylating, EC 1.1.1.40) catalyzes the di-valent metal ion-dependent reversible oxidative decarboxyla-

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1 The abbreviations used are: MCO, metal-catalyzed oxidation system; PAGE, polyacrylamide gel electrophoresis.
Metal Site of Malic Enzyme

RESULTS

Selective Inactivation of Pigeon Liver Malic Enzyme—Pigeon liver malic enzyme was highly sensitive to metal-catalyzed oxidation (Wei et al., 1994). The inactivation rate of the enzyme by the MCO system was highly pH-dependent. At pH 7.0 and

\[ \text{pH} = 0.25 \]°C, 20 μM Fe²⁺-20 mM ascorbate caused 98% enzyme activity loss in 1 h (Fig. 1), i.e, in correlation with our previous observation (Wei et al., 1994). On the other hand, under otherwise identical conditions, the enzyme lost only 10% activity in 1 h at pH 5.0. Experimental results indicated that Cu²⁺ caused a faster inactivation at a smaller concentration than Fe²⁺, especially at pH 5.0, in which Cu²⁺ caused substantial inactivation (Fig. 1).

Inactivation of malic enzyme by Cu²⁺ required ascorbate in the system; Cu²⁺ or ascorbate alone did not cause any inactivation. Cu²⁺ was much less effective; Cu⁺ (6 μM)-ascorbate (20 mM) caused only 10% inactivation in 1 h under conditions that caused >95% inactivation by the Cu²⁺-ascorbate system.

Effect of pH on the Cu²⁺-catalyzed Inactivation of Malic Enzyme—The above results indicate that malic enzyme has a different sensitivity toward the metal-catalyzed oxidation system at two different pH values. Next, the inactivation of malic enzyme was investigated by the Cu²⁺-ascorbate system between pH 4.0–9.0 in which the enzyme was stable. There are two optima for the inactivation rate: one at pH 6.0–7.0, and the other at approximately pH 4.0 (Fig. 2). These results would suggest that different modification mechanisms involved in acidic or neutral pH. For obtaining manageable inactivation rates, the inactivation of malic enzyme by Cu²⁺ at pH 5.0 is explored in the following experiments.

Dependence of Cu²⁺-catalyzed Inactivation of Malic Enzyme on Cu²⁺ Concentration—At pH 5.0, the inactivation of malic enzyme activity does not follow a pseudo-first-order kinetics as the natural logarithmic of residual activity versus time does not result in a straight line. The inactivation rate is clearly dependent on Cu²⁺ concentration (Fig. 3), i.e. much smaller in a concentration than Fe²⁺ requiring to cause the same extent inactivation.

Similar to Fe²⁺-induced inactivation, Cu²⁺-induced inactivation could be stopped by EDTA (4 mM), which, however, did not reverse the already inactivated enzyme activity.

Protection of Malic Enzyme against Cu²⁺-induced Inactivation by Substrates—For demonstrating that the inactivation of enzyme activity was due to modification of essential amino acid residues in or near the active site, the inactivation process was examined in the presence of various combinations of substrates. In contrast to Fe²⁺-ascorbate inactivation of malic enzyme, which was completely protected by some divalent cations, Mn²⁺ (4 mM) alone only protected 56% enzyme activity against the Cu²⁺-ascorbate induced inactivation at pH 5.0 (Fig. 4). l-Malate, which did not give any protective effect in the Fe²⁺ system, provided 15% protection in the inactivation in the Cu²⁺ system. l-Malate plus Mn²⁺ yielded synergistic protection (~90%). Nucleotide NADP⁺, on the other hand, did not provide any protection by itself or in combination with Mn²⁺ and l-malate in both Fe²⁺ and Cu²⁺ systems.

Inhibition of Pigeon Liver Malic Enzyme by Cu²⁺—The above results indicate that at pH 5.0 the binding mode between divalent cation and the enzyme is different from that at pH 7.0. Direct kinetic evidence for the binding of Cu²⁺ at the Mn²⁺ binding site of malic enzyme was provided by inhibition studies shown in Fig. 5, where Cu²⁺ was demonstrated to be a competitive inhibitor with respect to Mn²⁺ with Ki value of 70.3 ± 5.8 μM. This result indicates that Cu²⁺ and Mn²⁺ compete for the same binding site. The fitted value of Ki,Mn was 2.7 ± 0.23 μM, which is within the range (1.8–9 μM) as determined previously (Hsu et al., 1976).

For comparison, Cu²⁺ was also tested for inhibition of malic enzyme. Cu²⁺ as high as 160 μM showed no inhibition on the enzyme. At 169 μM, the inhibition was only 7%. These results strongly suggest that only divalent Cu²⁺ has a high affinity with malic enzyme.

Peptide Bond Cleavage Pattern of the Cu²⁺-Inactivated Malic Enzyme—We have demonstrated previously that, at pH 7.0, the Fe²⁺-ascorbate system deaved malic enzyme at Asp²⁵⁸, Ile²⁵⁹ (Wei et al., 1994). In this study, the cleavage pattern of the enzyme inactivated by Cu²⁺-ascorbate system is also ex-
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Effect of Cu²⁺ concentration on the inactivation of pigeon liver malic enzyme. Experimental conditions were the same as in Fig. 1 at pH 5.0, except that the Cu²⁺ concentrations used were: 2 μM; 3 μM; 4 μM; 5 μM; 6 μM.

Protection of pigeon liver malic enzyme from Cu²⁺-catalyzed inactivation by substrates. Experimental conditions were the same as in Fig. 1 with 6 μM Cu²⁺ and pH 5.0, but with the following additions: ○, none; □, 5 mM L-malate; ■, 4 mM Mn²⁺; ●, 5 mM L-malate + 4 mM Mn²⁺; Δ, 5 mM L-malate + 4 mM Mn²⁺ + 0.23 mM NADP⁺.

Competitive inhibition of pigeon liver malic enzyme by Cu²⁺ with respect to Mn²⁺. In the routine assay mixture, the Mn²⁺ concentration was varied from 1 μM to 10 μM with all other components held at fixed concentration. Malic enzyme activity was assayed in the presence of various concentrations of Cu²⁺. ○, none; ●, 100 μM; □, 200 μM. Lines are computer fitted to Equation 1.

Correlation between Enzyme Activity Inactivation and Peptide Bond Cleavage—To correlate the Cu²⁺-induced enzyme inactivation and peptide bond cleavage, the modification was performed in various stages, the reaction was stopped with EDTA, and the protein samples were subjected to SDS-PAGE to examine the peptide bond cleavage. Results shown in Table I clearly indicate that with the increasing of incubation time, a rapid loss in enzyme activity occurred and the peptide bond cleavage increased. However, the enzyme activity lost proceeded much faster than the peptide bond cleavage. When the enzyme activity was down to 27%, 91% of the enzyme molecules were still intact. We can conclude that peptide bond cleavage follows the enzyme inactivation, as for other MCO systems of enzyme inactivation. Site C is a minor cleavage site as compared to sites A and B. Site C seems to have a similar probability of cleavage. However, these sites do not cleave simultaneously in the same enzyme molecule. Similar mutually exclusive cleavages were also observed for pig heart isocitrate dehydrogenase with Fe²⁺-ascorbate system (Soundar and Colman, 1993).

The switch of specificity was examined by monitoring the

Figure 6. SDS-PAGE pattern of the metal-catalyzed oxidized malic enzyme. A, lane 1 is M, standards (phosphorylase b, M, 94,000; bovine serum albumin, M, 67,000; ovalbumin, M, 43,000; carbonic anhydrase, M, 30,000; trypsin inhibitor, M, 20,000; a-lactalbumin, M, 14,400). Lane 2 is the unmodified native enzyme. Lanes 3 and 4 are enzymes modified at pH 7.0. Lanes 5 and 6 are enzymes modified at pH 5.0. Lanes 3 and 5 are enzymes modified with Fe²⁺-ascorbate system. Lanes 4 and 6 are enzymes modified with Cu²⁺-ascorbate system. B, cleavage positions of malic enzyme by the MCO systems identified by amino acid sequence analysis of the peptide fragments.
protein cleavage pattern at various pH values. As the pH decreased from neutral to acidic, fragments I, II, III, VI, VII, and VIII gradually increased, while fragments IV and V decreased (data not shown).

Identification of the Metal-binding Site of Pigeon Liver Malic Enzyme—From Fig. 6, we see that, at pH 5.0, Cu²⁺ cleaved malic enzyme at three sites that are different from the Asp⁵⁸, Ile²⁵⁹ observed at pH 7.0. The SDS-PAGE-separated peptide fragments were electrophoretically transblotted onto an Immobilon-P membrane and each peptide was analyzed via an automatic protein sequencer. The N terminus of fragment II was found to have the following sequence: Gly-Glu-Arg-Ile-Leu-Gly-Asp, which is identified as Pro₁₉₅–Asp₂₁₂ of the cDNA sequence of pigeon liver malic enzyme (Chou et al., 1994). Peptide E⁹ has the N-terminal sequence Pro-Leu-Tyr-Ile-Gly-Leu-Arg-His-Lys-Arg-Ile-Gly-Asp, which is identified as Pro₁⁹⁵–Asp₂₁₀ of the cDNA sequence. Peptide E⁹ contains sequence Met¹–Asp₁⁹⁴ and some other minor peptides. Corresponding to peptide E⁹, we can conclude that the catalytically essential carboxyl group of Asp⁵⁸ is essential for the process; Cu²⁺ is much less effective, suggesting that Cu²⁺ must bind with the enzyme at the divalent metal ion binding site before inactivation takes place. (b) Cu²⁺ is a competitive inhibitor versus Mn²⁺ for the enzyme, indicating that Cu²⁺ and Mn²⁺ compete for the same binding site. (c) Inactivation of the enzyme is prevented by Mn²⁺ plus L-malate, indicating that modification is at the active site.

Based on the above discussion, the reaction sequence of oxidative modification and peptide bond cleavage of pigeon liver malic enzyme by the Cu²⁺-ascorbate system can be summarized in Scheme I. First, Cu²⁺ binds with the enzyme at the Mn²⁺ (step 1). Second, ascorbate reduces Cu²⁺ to Cu⁺ (step 2), which, in the presence of dissolved O₂, generates reactive free radicals (e.g., O₂⁻, OH⁻) that in turn modify the essential amino acid residue(s) nearby and forming the inactivated enzyme (E⁻) (step 3). Finally, depending on pH of the solution, the enzyme molecule is cleaved at four possible sites giving peptide fragments E¹ + E⁻, E¹ + E⁻, E¹ + E⁻, or E¹ + E⁻ at pH 5.0, or E¹ + E⁻ at pH 7.0 (step 4). In this manner, different specificities are achieved by manipulating the reaction conditions. We suggest that this strategy can be generally applied to other enzymes or proteins in elucidating the metal-binding sites. We propose that the catalytically essential carboxyl group of Asp⁵⁸ has a pKₐ value of 6.7, as determined by chemical modification experiments (Chang et al., 1985). At pH 5.0, this carboxyl group is protonated and loss its metal-binding ability and is not reactive toward oxidative modification. Under this circumstance, Fe²⁺ is inactive but other metal ligands Asp⁵⁸, Asp²⁵⁹, or Asp⁶⁴, which might have pKₐ values near 4.7, are modified by the more reactive Cu²⁺.

Interestingly, the metal ligands Asp⁵⁸, Asp²⁵⁹, and Asp⁶⁴ of malic enzyme were all aspartate residue, which has been indicated to be the major metal-binding ligand for many metal-proteins (Higaki et al., 1992; Vallee and Auld, 1993; Traut, 1994). Furthermore, isocitrate dehydrogenase, which catalyzes a similar oxidative decarboxylation reaction as malic

### Table I

**Correlation of Cu²⁺-catalyzed inactivation of pigeon liver malic enzyme activity and cleavage of the metal binding site**

Experimental conditions were the same as in Fig. 6. 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%. The minor fragments I and VIII were not included in the calculation.

| Time (min) | Enzyme activity remaining | Relative amount of intact enzyme (%) | Relative amount of enzyme fragments (%) |
|------------|---------------------------|--------------------------------------|-----------------------------------------|
|            |                          |                                      | II | III | VI | VII | II + VII | III + VI |
| 0          | 100                       | 100                                  | 0  | 0   | 0  | 0   | 0        | 0        |
| 20         | 65                        | 98                                   | 1.5| 0.3 | 0.1| 0.1 | 1.6      | 0.4      |
| 60         | 51                        | 95                                   | 2.8| 1.0 | 0.4| 0.4 | 3.2      | 1.4      |
| 90         | 27                        | 91                                   | 4.4| 2.5 | 2.0| 1.0 | 5.4      | 4.5      |
| 120        | 8                         | 86                                   | 6.3| 4.5 | 1.7| 1.0 | 7.3      | 6.2      |
| 180        | 5                         | 78                                   | 9.9| 6.2 | 3.5| 2.5 | 12.4     | 8.7      |
| 300        | 3                         | 61                                   | 13.6|9.6 |9.6|5.9 |19.5     |19.2     |
| 420        | 1                         | 53                                   | 16.4|13.3|9.2|8.2 |24.6     |22.5     |
enzyme, was demonstrated to have Asp$^{311}$, Asp$^{307}$ from the same subunit, and Asp$^{283}$ from the other subunit as the metal coordinates (Hurley et al., 1990); this enzyme from pig heart was also sensitive to MCO system (Soundar and Coleman, 1993). Examination of these sequences shown in Figs. 7–9 reveals that Asp$^{141}$ and Asp$^{194}$, the major cleavage sites by the Cu$^{2+}$ system, are strictly conserved in all malic enzyme with known amino acid sequences. Asp$^{464}$ is also highly conserved; however, this region has higher variations among malic enzyme of different origins. This site is a minor cleavage site. Maximum alignment of other malic enzyme sequences with Asp$^{464}$ of pigeon enzyme reveals that Glu$^{464}$ of duck, Asn$^{512}$ of ascaris, and Glu$^{557}$ of maize enzymes may be the metal coordinates. Although Asn and Glu are found as metal ligand in many metal-proteins (Villafranca and Nowak, 1992; Higaki et al., 1992; Valle and Auld, 1993; Traut, 1994), an observation of the nearby amino acid residues reveals that Asp$^{465}$ of duck, Asp$^{511}$ or Asp$^{513}$ of ascaris, and Asp$^{558}$ of maize enzyme may be the authentic metal ligands. These results enforce the critical value of aspartate residue as the metal coordinate in proteins. Only bacillus malic enzyme was nonconservatively substituted this Asp with Val$^{456}$. The actual metal ligand, however, may be Glu$^{457}$, which is also a conservative substitution.

Malic enzyme is a bifunctional enzyme. It catalyzes both the oxidoreduction and decarboxylation reactions. The reaction mechanism of the enzyme proceeds in two steps with hydride transfer preceding decarboxylation (Hermes et al., 1982). These two functions can be assessed separately by assaying the par-

**Fig. 7. Comparison of the putative Mn$^{2+}$ binding site Asp$^{141}$ of malic enzymes.** Figure shows amino acid sequences around Asp$^{141}$ of pigeon and duck, Asp$^{152}$ of human, rat and mice, Asp$^{162}$ of human liver mitochondria, Asp$^{190}$ of Ascaris suum mitochondria, Asp$^{235}$ of maize chloroplast, Asp$^{245}$ of dicotyledonous C4 plant Flaveria trinervia, and Asp$^{147}$ of Bacillus stearothermophilus malic enzymes were aligned to show the high conservation in this region. The deavage site of pigeon liver malic enzyme by the Cu$^{2+}$-ascorbate was indicated by an arrow. The dashed underlined sequence was confirmed by amino acid sequence analysis. The gray area showed identical amino acid residues between different malic enzymes. The putative Mn$^{2+}$-binding ligand Asp$^{141}$ was highlighted.

**Fig. 8. Comparison of the putative Mn$^{2+}$ binding site Asp$^{194}$ of malic enzymes.** Figure is same as Fig. 7 but with amino acid sequences around Asp$^{194}$ of pigeon and duck, Asp$^{205}$ of human, rat and mice, Asp$^{215}$ of human liver mitochondria, Asp$^{243}$ of Ascaris suum mitochondria, Asp$^{287}$ of maize chloroplast, Asp$^{298}$ of Flaveria trinervia, and Asp$^{180}$ of Bacillus stearothermophilus malic enzymes.
tial reactions with appropriate substrate (Hsu, 1982). One of the distinguishing features of the decarboxylase activity of malic enzyme is its pH optimum being at 4.5 (Salles and Ochoa, 1950). We propose that the proton released during dehydrogenase reaction provides a favorable local active site environment for the decarboxylation reaction, which involves metal ion-stabilized enolate anion transition state (Hsu et al., 1976; O’Leary, 1992). During the catalytic cycle, the enzyme might undergo an isomerization that favors the decarboxylation reaction. Results presented in this study support the hypothesis that the enzyme exists as different conformational isoforms at neutral or acidic environment. However, another possibility that the observed pH effects were due to changes in the catalytic cleavage rate of different sites rather than to global conformational changes was not ruled out (Kufel and Kirsebom, 1994).

Metal-catalyzed oxidation of proteins has been suggested to be the marker for protein turnover in vivo (Stadtman and Oliver, 1991; Stadtman, 1992). According to this theory, the oxidized protein molecules are unstable and prone to degradation by multicatalytic proteases (proteasomes) in cells (Rivett, 1993). The normal copper concentration in serum is 16–31 μM (Murray et al., 1990); both iron and copper ions are normally presented in the cells, which also contain various reducing compounds. If malic enzyme is sensitive to only a few micromolar concentration of Cu2+ in the cytoplasm, an intriguing question is that how could malic enzyme survive in vivo for a reasonable period of time to perform its metabolic roles? Protection of the enzyme by Mg2+ or other divalent cations and substrate is one of the answers. Furthermore, cells contain other defense mechanisms against oxidative damage. The endogenous antioxidant enzymes (catalase, dismutase) are active in normal young tissues; a rapid physiological response of the translational or transcriptional control of the detoxification genes might play an important role when metal ion concentrations exceed a dangerous threshold (O’Halloran, 1993). These detoxification gene products may play important roles in cell protection, e.g. the high Fe2+ content and oxygen carrier function of red blood cells indicate the high oxidation stress experience by red blood cells, which contains an abundant natural killer enhancing factor that protects red blood cells from oxidation injuries (Shau and Kim, 1994). These protecting proteins are highly homologous with yeast thiol-specific antioxidant, which protects yeast cells from oxidation insults (Chae et al., 1993). It is these natural protecting mechanisms that prevent cell proteins and other cellular components from experiencing oxidative damage.

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FIG. 9. Comparison of the putative Mn2+ binding site Asp464 of malic enzymes. Figure is same as Fig. 7 but with amino acid sequences around Asp464 of pigeon, Glu465 (may be Asp465) of duck, Asp475 of human, rat, and mice, Asp512 of human liver mitochondria, Asn513 (may be Asp511 or Asp513) of Ascaris suum mitochondria, Glu557 (may be Asp558) of maize chloroplast, Asp516 of Flaveria trinervia, and Val456 (may be Glu457) of Bacillus stearothermophilus malic enzymes.

SCHEME I. Proposed reaction mechanism for the oxidative modification and affinity cleavage of pigeon liver malic enzyme by the Cu2+-ascorbate system. The N and C under each peptide fragment denote the N- or C-terminal half before cleavage.
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