Inactivation of maize NADP-malic enzyme by Cu$^{2+}$-ascorbate

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Maize malic enzyme was rapidly inactivated by micromolar concentrations of cupric nitrate in the presence of ascorbate at pH 5.0. Ascorbate or Cu$^{2+}$ alone had no effect on enzyme activity. The substrate L-malate or NADP individually provided almost total protection against Cu$^{2+}$-ascorbate inactivation. The loss of enzyme activity was accompanied by cleavage of the enzyme. The cleaved peptides showed molecular mass of 55 kDa, 48 kDa, 38 kDa, and 14 kDa. Addition of EDTA, histidine and imidazole provided protection. The results of protection experiments with sodium azide, DABCO and catalase suggested that reactive oxygen species were generated resulting in loss of enzyme activity. This was further supported by experiments showing that the rate of enzyme inactivation was higher in D$_2$O than in water. It is suggested that maize malic enzyme is modified by reactive oxygen species like singlet oxygen and H$_2$O$_2$ generated by Cu$^{2+}$-ascorbate system and the modified amino acid residue(s) may be located at or near the substrate-binding site of the enzyme.

Maize NADP malic enzyme [L-malate: NADP oxidoreductase (decarboxylating), EC 1.1.1. 40] catalyzes the oxidative decarboxylation of malate to pyruvate in presence of divalent metal ions. In C4 plants, the enzyme is located in bundle sheath chloroplasts and CO$_2$ released by oxidative decarboxylation of malate is refixed by ribulose-1,5-bisphosphate carboxylase/oxygenase. Malic enzyme has been found in most living organisms, including bacteria, yeast, plants and humans and other animals and has highly conserved amino acid sequences. Chemical modifications of the maize malic enzyme had implicated histidyl$^1$, arginyl$^2$, cysteinyl$^3$, carboxyl$^4$, and tyrosyl$^5$ residues involved in the catalysis by the enzyme. We had earlier reported$^6$ that Asp$^{352}$ was the coordination site for Mg$^{2+}$ binding in maize malic enzyme when Fe$^{2+}$-ascorbate at neutral pH was used as metal catalysed oxidation (MCO). Asp$^{352}$ is a conserved residue in malic enzyme from all sources and its role in metal binding was confirmed from crystallographic data of human mitochondrial malic enzyme$^7$.

A number of MCO systems cause the inactivation of enzymes$^{8,11}$. A non-enzymatic MCO system comprises ascorbate, O$_2$ and Fe (III) / Fe (II) or Cu$^+$ / Cu$^{2+}$. MCO of either Asp, His, Cys, Met, Trp, and Tyr at the catalytic site of a number of enzymes has been reported$^{8,11}$. The inactivation mechanism involves the generation of reactive oxygen species in the presence of reduced transition metals such as Fe$^{2+}$ and Cu$^+$. Oxidation of these metal ions by molecular oxygen yields superoxide radical anion, which undergoes dismutation reaction to form hydrogen peroxide. Hydrogen peroxide, in turn, can react with reduced metal ions via the Fenton reaction, generating hydroxyl radical. Reducing equivalents such as ascorbate and DTT are needed to recycle the oxidized metal ions.

Studies on pigeon liver malic enzyme with different MCO (Fe$^{2+}$ and Cu$^{2+}$) systems at neutral and acidic pH indicated that MCO systems at different pH show different specificities in protein modifications and peptide bond cleavage. In addition to Asp$^{288}$ (Asp$^{352}$ of maize malic enzyme) as metal binding ligand (identified by Fe$^{2+}$-ascorbate) at neutral pH, three additional aspartate (Asp$^{144}$, Asp$^{194}$, and Asp$^{405}$) were identified by Cu$^{2+}$-ascorbate system at acidic pH as the coordination sites for the metal binding in pigeon liver malic enzyme$^{13}$.

In the present study we have compared the inactivation of maize malic enzyme at neutral and acidic pH by Fe$^{2+}$-ascorbate and Cu$^{2+}$-ascorbate systems. We had reported previously the inactivation mechanism of maize malic enzyme and identified Asp$^{352}$ as Mg$^{2+}$ binding site by using Fe$^{2+}$-ascorbate at neutral pH. The present paper attempts to study the mechanism of inactivation of maize malic enzyme by Cu$^{2+}$-ascorbate at acidic pH (pH 5) and also to check for additional coordination sites for metal binding in...
the maize NADP-malic enzyme, as seen for the pigeon liver malic enzyme.

Materials and Methods

Chemicals
Most of the biochemicals like NADP, malate and catalase were obtained from Sigma Chemical Co., USA. SOD was obtained from Boehringer Mannheim India. All other chemicals including cupric nitrate were of analytical reagent grade.

Enzyme preparation, assay and protein estimation

NADP-malic enzyme from maize leaves was purified to apparent homogeneity according to method described by Asami et al. The final sp. activity of the enzyme was 70 units per mg protein per min. One unit was defined as the amount of enzyme that catalyses an initial 1 µmole of NADPH formation per min under assay conditions. A molecular mass of 250 kDa was used for the calculation of enzyme protein concentrations. Malic enzyme was assayed in a 1-ml reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM malate, 0.23 mM NADP, 10 mM MgCl₂ and appropriate amount of enzyme. The formation of NADPH at 25°C was monitored at 340 nm with a UVICON-940 spectrophotometer (Kontron instruments, Japan). Protein concentration was determined according to the method of Asami et al. (1979).

Enzyme inactivation

The enzyme (1-2 µM) in 50 mM sodium-acetate (pH 5.0) or 50 mM Tris-HCl (pH 7.5) treated with the indicated concentrations of freshly prepared cupric nitrate and 20 mM ascorbate. Inactivation was monitored by assaying the enzyme activity in small aliquots withdrawn at different time intervals and quenched with 4 mM EDTA. Controls (with only ascorbate or with copper without ascorbate) were run. Other experimental conditions are provided in the respective Table or Figure legend.

Results and Discussion

Inactivation of maize malic enzyme at neutral and acidic pH by Fe²⁺-ascorbate and Cu²⁺-ascorbate

Fig. 1 (A and B) shows the inactivation of NADP-malic enzyme from maize with Fe²⁺, and Cu²⁺-ascorbate system at neutral pH (Tris-HCl, pH 7.5) and acidic pH (sodium acetate, pH 5.0) respectively. In both systems at neutral pH, the inactivation was faster than at acidic pH. The time dependence of inactivation at acidic pH was almost similar in both systems.

Inactivation at varying concentrations of Cu²⁺ and 20 mM ascorbate at acidic pH

Inactivation of maize malic enzyme with micromolar concentrations of Cu²⁺ in presence of 20 mM ascorbate at acidic pH resulted in a rapid and irreversible loss of enzyme activity (Fig. 2). The time course of inactivation did not follow pseudo-first-order reaction kinetics. The presence of ascorbate, oxygen and Cu²⁺ in the reaction mixture was essential for inactivation as reported in the case of several other enzymes. Presence of ascorbate or Cu²⁺ alone had no effect. EDTA (4 mM) provided complete protection against Cu²⁺-ascorbate inactivation of malic enzyme (Table 1).

Protection of NADP-malic enzyme against inactivation by Cu²⁺-ascorbate

Effect of varying concentrations of substrate and co-factor on the rate of enzyme inactivation by Cu²⁺-ascorbate was examined. Preincubation of enzyme with varying concentrations of either malate or NADP provided substantial protection (Table 2) against the maize NADP-malic enzyme, as seen for the pigeon liver malic enzyme.

Figure 1—Inactivation of maize NADP-malic enzyme by (A): Fe²⁺ or (B) Cu²⁺-ascorbate at neutral and acidic pH (Maize NADP-malic enzyme (1-2 µM) in 50 mM sodium acetate (pH 5.0, ○—○, A—A), or in 50 mM Tris-HCl (pH 7.5, O—O, Δ—Δ); in presence of 20 µM ferrous sulphate plus 20 mM sodium ascorbate, (●, O) or 20 µM cupric nitrate plus 20 mM sodium ascorbate (●, Δ) at 25°C. At the indicated time intervals a small aliquot removed and used for assayng enzyme activity after termination of reaction with 4mM EDTA.
Table 1—Effect of active oxygen scavengers, EDTA, DABCO, imidazole and histidine on the inactivation of malic enzyme by 
Cu²⁺-ascorbate

<table>
<thead>
<tr>
<th>Ligands</th>
<th>% Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100.0</td>
</tr>
<tr>
<td>Cu²⁺ (0.01 mM)</td>
<td>10.0</td>
</tr>
<tr>
<td>Catalase</td>
<td>67.7</td>
</tr>
<tr>
<td>SOD</td>
<td>10.0</td>
</tr>
<tr>
<td>n-propyl gallate (1 mM)</td>
<td>8.0</td>
</tr>
<tr>
<td>Sodium azide (1 mM)</td>
<td>80.0</td>
</tr>
<tr>
<td>DABCO (1 mM)</td>
<td>85.0</td>
</tr>
<tr>
<td>EDTA (4 mM)</td>
<td>100.0</td>
</tr>
<tr>
<td>Cu²⁺ (0.01 mM)</td>
<td>20.0</td>
</tr>
<tr>
<td>Imidazole (2.5 mM)</td>
<td>74.0</td>
</tr>
<tr>
<td>(5.0 mM)</td>
<td>84.0</td>
</tr>
<tr>
<td>(10.0 mM)</td>
<td>87.0</td>
</tr>
<tr>
<td>Histidine (10 mM)</td>
<td>72.0</td>
</tr>
</tbody>
</table>

Cu²⁺-ascorbate induced inactivation. Malate and NADP alone at 0.5 mM and 1 mM respectively, provided maximum protection. Malate (0.5 mM) plus Mg²⁺ (10 mM) resulted in complete protection of malic enzyme activity (Table 2). These results indicated that the residue involved in modification may be located at the active site, probably at the substrate binding site. Earlier, Chou et al. observed only 15% protection by malate (0.5 mM). NADP (0.23 mM) did not provide any protection against Cu²⁺-ascorbate.

SDS-PAGE pattern of Cu²⁺-ascorbate inactivated NADP-malic enzyme

The inactivated malic enzyme (with residual activity 1%) along with native (uninactivated) protein was subjected to SDS-PAGE to study the oxidative cleavage of the protein. The native malic enzyme is a tetramer with a subunit molecular mass of 62 kDa. The inactivated protein (99% loss of enzyme activity) was cleaved into four fragments of 55 kDa, 48 kDa, 38 kDa, and 14 kDa and this resulted in the considerable reduction of 62 kDa band of modified protein by Cu²⁺-ascorbate. Since cleavage fragments gave very diffused bands on PAGE and could not be obtained in sufficient quantity, sequence analysis was not carried out.

Effect of different oxygen scavengers on the inactivation of malic enzyme

Effect of active oxygen scavengers (Table 1) on the loss of malic enzyme activity by Cu²⁺-ascorbate was
studied. Catalase (for hydrogen peroxide), superoxide dismutase (for superoxide anion), n-propyl gallate (for hydroxyl and alkoxyl radicals\textsuperscript{17}), sodium azide and DABCO (for singlet oxygen\textsuperscript{18}) were used as scavengers for various active oxygen species.

Catalase protected 68% enzyme activity, while DABCO and sodium azide (both 1 mM) provided 80% and 85% protection respectively. These results confirmed that the inactivation of malic enzyme by Cu\textsuperscript{2+}-ascorbate was predominantly due to the generation of reactive oxygen species (H\textsubscript{2}O\textsubscript{2} and singlet oxygen) in the reaction mixture.

The effect of different concentrations of catalase on the inactivation of malic enzyme is shown in Fig. 3. Catalase at 20 µg/ml provided 70% protection and there was near total protection above 50 µg/ml. Protection of malic enzyme by catalase confirmed the formation of H\textsubscript{2}O\textsubscript{2} in the reaction mixture during inactivation of enzyme by Cu\textsuperscript{2+}-ascorbate.

Addition of 250 µM H\textsubscript{2}O\textsubscript{2} to the reaction mixture resulted in 83% loss of enzyme activity in 60 min (Table 3). Kim et al\textsuperscript{9} observed that catalase protected yeast glutamine synthetase from inactivation by Fe\textsuperscript{2+}-ascorbate while H\textsubscript{2}O\textsubscript{2} alone, at high concentration (10 mM) for 2 hr, caused no protein cleavage but inactivated the enzyme partially.

As seen in Table 1, DABCO and sodium azide also offered protection against Cu\textsuperscript{2+}-induced inactivation of malic enzyme activity. The rate of singlet oxygen dependent reactions is known to increase in D\textsubscript{2}O as singlet oxygen has longer lifetime in D\textsubscript{2}O\textsuperscript{19,20}. The loss of malic enzyme activity due to Cu\textsuperscript{2+}-ascorbate inactivation was found to be about 20% faster in D\textsubscript{2}O than in water (Fig. 4), confirming the generation of singlet oxygen in the reaction mixture.

Histidine can chelate metal ions\textsuperscript{21} or can act as scavenger for singlet oxygen\textsuperscript{22}. The effects of histidine and imidazole on the inactivation of malic enzyme activity by Cu\textsuperscript{2+}-ascorbate were studied (Table 1). It can be seen that histidine at 10 mM reduced the loss of enzyme activity by about 70%. Addition of varying concentrations of imidazole to the reaction mixture also protected malic enzyme activity against Cu\textsuperscript{2+}-ascorbate inactivation (Table 1). In our earlier studies\textsuperscript{6} with the Fe\textsuperscript{2+}-ascorbate system, protection of maize malic enzyme activity by imidazole was not found.

References
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