Evidence for the Existence of Two Essential and Proximal Cysteinyl Residues in NADP-Malic Enzyme from Maize Leaves

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ABSTRACT

Incubation of maize (Zea mays) leaf NADP-malic enzyme with monofunctional and bifunctional N-substituted maleimides results in an irreversible inactivation of the enzyme. Inactivation by the monofunctional reagents, N-ethylmaleimide (NEM) and N-phenylmaleimide, followed pseudo-first-order kinetics. The maximum inactivation rate constant for phenylmaleimide was 10-fold higher than that for NEM, suggesting a possible hydrophobic microenvironment of the residue(s) involved in the modification of the enzyme. In contrast, the inactivation kinetics with the bifunctional maleimides, ortho-, meta-, and para-phenylenebismaleimide, were biphasic, probably due to different reactivities of the groups reacting with the two heads of these bifunctional reagents, with a possible cross-linking of two sulfhydryl groups. The inactivation by mono- and bifunctional maleimides was partially prevented by Mg²⁺ and L-malate, and NADP prevented the inactivation almost totally. Determination of the number of reactive sulfhydryl groups of the native enzyme with [³H]NEM in the absence or presence of NADP showed that inactivation occurred concomitantly with the modification of two cysteinyl residues per enzyme monomer. The presence of these two essential residues was confirmed by titration of sulfhydryl groups with [³H]NEM in the enzyme previously modified by o-phenylenebismaleimide in the absence or presence of NADP.

NADP-dependent malic enzyme (l-malate: NADP oxido-reductase/decarboxylase; EC 1.1.1.40) acts in a wide range of metabolic pathways in both animals and plants. The enzyme catalyzes the reversible oxidative decarboxylation of l-malate in the presence of NADP and a bivalent metal ion to produce pyruvate, NADPH, and CO₂. In animals, cytosolic malic enzyme generates reducing power for the biosynthesis of fatty acids (9, 28). In plants, two forms of this enzyme are known to occur and have important metabolic roles. The cytosolic form is thought to participate in the regulation of intracellular pH (3, 20) and/or in the provision of reducing power that can be used in processes requiring NADPH (8). The chloroplast stromal form is found specifically in the bundle sheath chloroplasts of NADP-malic enzyme type C₄ plants such as maize. The enzyme plays a key role in C₄ photosynthetic metabolism because it generates reducing power and CO₂ in the bundle sheath chloroplasts where Rubisco and the Calvin cycle operate (7, 8).

Thiol groups play a key role in the activity of the enzyme obtained from different sources. The importance of sulfhydryl residues in pigeon liver malic enzyme has been investigated extensively (10). Studies of the inactivation of this enzyme with reagents selective for sulfhydryl groups, e.g. 5,5'-dithiobis(2-nitrobenzoic acid) or NEM⁺, confirmed the presence of one sulfhydryl group near each substrate site on the enzyme tetramer (24). In contrast, evidence for the presence of two essential sulfhydryl residues per monomer of the enzyme from maize leaves has been recently reported (4).

To obtain further information concerning the function and microenvironment of the active site thiol group(s), the reaction of NADP-malic enzyme from maize leaves with different N-substituted maleimides was studied. Bifunctional maleimides were used to obtain further evidence about the existence of two proximal sulfhydryl groups near the NADP-binding site of the C₄ enzyme (4). Several isomers of phenylenebismaleimide with different estimated cross-linking distances, as well as maleimides that differ in hydrophobic properties, were all found to modify the enzyme in an irreversible fashion.

MATERIALS AND METHODS

Plant Material

Plants of maize (Zea mays L.) were grown outdoors with 14 h of light, at temperatures of 25 to 35°C (day) and 15 to 25°C (night). Mature leaves from approximately 5-week-old plants were used.

Protein Measurement

Protein concentration was determined in each set of experiments by the colorimetric method of Lowry et al. (14) using BSA as the standard.

2 Abbreviations: NEM, N-ethylmaleimide; NPM, N-phenylmaleimide; OPBM, o-phenylenebismaleimide; PPBM, p-phenylenebismaleimide; MPBM, m-phenylenebismaleimide; k⁺, observed rate constant for inactivation; k₃, maximum inactivation rate constant; Kₐ, apparent dissociation constant of the enzyme-inactivator complex; kₑ (kₑ), observed pseudo-first-order rate constant in the absence (presence) of NADP.

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Chemicals

NEM, OPBM, l-malic acid, NADP, and Tris were purchased from Sigma Chemical Co. NPM and PPBM were from Aldrich Chemical Co. MPBM was obtained from K & K Laboratories and was recrystallized from acetone/water mixtures. N-[ethyl-2-3H]Maleimide was purchased from Dupont (specific radioactivity 55.0 Ci/mmole). All other reagents were of analytical grade.

Enzyme Purification and Assay

NADP-malic enzyme was purified from maize leaves as described by Iglesias and Andreo (11). The purified enzyme migrated as a single band in SDS-PAGE, indicating that it was >95% pure. This preparation has high specificity for NADP compared with NAD. With NADP as the cofactor and high concentrations of l-malate, the pH optimum of the maize enzyme is about 8.3 and the activity with NAD is negligible, whereas with NAD as cofactor, the pH optimum is 7.1 and activity is completely inhibited by low levels of NADP (7).

Enzyme activity was determined spectrophotometrically at 30°C by monitoring NADPH production at 340 nm. The standard assay medium contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 0.5 mM NADP, and 4 mM l-malate in a final volume of 1.0 mL. The reaction was initiated by addition of l-malate.

Enzyme Inactivation

Kinetic studies of malic enzyme inactivation were carried out by adding the sulfhydryl modifier to 150 μL (25 μg) of purified enzyme, previously desalted into 50 mM Tris, pH 8.0 (17). NEM was dissolved in 50 mM Tris, pH 8.0, and the hydrophobic maleimides were dissolved in DMSO. After various times of incubation at 0°C, small aliquots containing 2.5 μg of enzyme were withdrawn and assayed for modified enzyme activity. Inactivation was stopped at the desired time by 100-fold dilution of the modifier in the 1-mL assay medium. No significant decrease in activity was found when the enzyme was incubated in the absence of the modifier or in the presence of DMSO at the same concentration (the highest concentration used was 3% [v/v]) added in the experiments.

Titration of Sulphydryl Groups Implicated in the Inactivation of the Enzyme by NEM

Radiochemical titration of sulfhydryl groups was performed by incubation of NADP-malic enzyme (1.0 mg/mL) with 2.5 mM [ethyl-2-3H]NEM (0.83 Ci/mol) in the presence and absence of 10 mM NADP for 70 min at 0°C in a medium (1.5 mL) containing 50 mM Tris-HCl, pH 8.0. Aliquots (0.2 mL) were withdrawn at various times and desalted twice through a Sephadex G-50 column (17) equilibrated with 50 mM Tris-HCl, pH 8.0, to remove unbound [3H]NEM. Activity, protein concentration, and [3H]NEM incorporation were determined in aliquots of the desalted protein. The mol of NEM reacting per mol of enzyme monomer was calculated based on a subunit mol wt of 62,000 (22).

Titration of Sulphydryl Groups Implicated in the Inactivation by OPBM

Radiochemical titration of sulfhydryl groups of malic enzyme preincubated with 0.2 mM OPBM in the presence and absence of 10 mM NADP was performed using the same conditions as described above. When 1.5 mg of purified malic enzyme modified by OPBM in the absence of NADP was inactivated to 20% of initial activity, the reaction mixture was passed twice through a small column of Sephadex G-50 (17), and 2.5 mM [3H]NEM was added. Protein concentration and [3H]NEM incorporation were determined in aliquots of the desalted protein after different times of incubation. The same experiment was performed on enzyme preincubated with 0.2 mM OPBM in the presence of 10 mM NADP (no loss of activity was observed in this case).

RESULTS

Inactivation of NADP-Malic Enzyme by the Monofunctional Reagents NEM and NPM

Incubation of malic enzyme with NEM or NPM at pH 8.0 led to an irreversible inactivation of the enzyme. A plot of the log of percentage activity remaining versus time showed that inactivation followed pseudo-first-order kinetics (Fig. 1). The kobs for inactivation by both reagents was linearly dependent on the concentration of modifier. The k₂ and the Kᵢ were calculated from a double-reciprocal plot of kobs versus modifier concentration (Fig. 1, insets). The calculated value of k₂ for NPM (2.0 min⁻¹) was 10-fold higher than that obtained for NEM (0.17 min⁻¹), whereas the Kᵢ values were essentially identical (5.4 mM for NPM and 5.3 mM for NEM). This different reactivity of the reagents suggests a possible hydrophobic environment of the sulfhydryl groups involved in the inactivation of the enzyme.

The percentage of protection afforded by several effectors of the enzyme is shown in Table I. The results indicate that NADP strongly protected the enzyme against inactivation, whereas Mg²⁺ protected the enzyme only partially. Similarly, l-malate afforded partial protection against inactivation by NPM.

Analysis of protection against inactivation by NEM afforded by different concentrations of NADP is presented in Figure 2. The plot of kᵢ/kᵢ₀ versus (1 − kᵢ₀/[NADP]) based on the model developed by Scrutton and Utter (23), is linear (Fig. 2, inset). Upon extrapolation, the straight line passes through the origin, implying that the enzyme–NADP complex cannot react with the modifier (23).

Inactivation of Enzyme by Bifunctional Maleimides

Incubation of the enzyme with the bifunctional maleimides OPBM (Fig. 3), MPBM, and PPBM caused irreversible inactivation. The kinetics of inactivation by the three reagents were biphasic. The semilog plot of residual activity versus time did not yield a straight line (Fig. 3A). In these experiments, the three modifiers were used in excess (although higher concentrations could not be used because they precipitated in aqueous solution) so that the observed biphasic kinetics cannot be attributed to limiting reagent concentra-
tion. The results must, therefore, represent different reactivities of the residues reacting with the two heads of these bifunctional reagents. A possible cross-linking of two sulfhydryl groups that are important for activity must be considered. Protection against inactivation by OPBM afforded by NADP, Mg²⁺, and L-malate, alone or in combination, followed the same general pattern as in the case of inactivation by NPM (Fig. 3B versus Table I).

A comparison of the rate of inactivation by the three bifunctional reagents at the same concentration (20 μM) is shown in Figure 4. The order of reactivity was OPBM > MPBM > PPBM. Taking into account the estimated cross-linking distances (16) of the three bismaleimides (OPBM, 5–6 Å; MPBM, 8.5–12 Å; PPBM, 12–13 Å), we conclude that the two possible sulfhydryl groups that react with the two heads of each reagent are sufficiently close together to react with the longer cross-linking reagents. A possible steric impediment of the larger bismaleimides must also be considered.

**Titration of Sulfhydryl Groups of NADP-Malic Enzyme with [³H]NEM**

The number of NEM-reactive sulfhydryl groups was determined by incorporation of [³H]NEM into the enzyme in the presence and absence of NADP. The results (Fig. 5A) show that 7.2 residues per 62-kD monomer are susceptible to NEM modification (extrapolating the straight line to 0% activity). In contrast, in the presence of 10 mM NADP, the number of residues modified is 5.1. In this case, no loss of activity was found (see Fig. 2), but the extrapolated line was drawn by taking into account the percentage of inactivation obtained at the same time of incubation in the absence of NADP. These results suggest that there are two essential sulfhydryl groups per monomer of maize NADP-malic enzyme, which are protected by NADP against modification by NEM.

**Titration of Sulfhydryl Groups Involved in Reaction with OPBM**

The number of sulfhydryl groups that react with OPBM was determined by incorporation of [³H]NEM into the native enzyme, the enzyme modified by OPBM to 20% of initial activity, and the protected enzyme incubated with OPBM in the presence of NADP (90% of initial activity remaining). The results (Fig. 5B) show that 7.3 residues per monomer of the native and NADP-protected enzymes are free to react with [³H]NEM. In contrast, the enzyme preincubated with OPBM in the absence of NADP has only 5.2 residues per monomer available for subsequent reaction with [³H]NEM. These results suggest that OPBM reacts with two residues per monomer that are important for activity, because they are protected by NADP.

**DISCUSSION**

Although the deduced primary structure of NADP-malic enzyme from maize leaves has been reported (22), little attention has been devoted to the study of amino acids essential for catalysis. Recent studies of the chemical modification of the enzyme suggested that histidine (13) and arginine (21) residues are required for activity. We have provided evidence for the existence of sulfhydryl groups at or near the NADP-binding site of maize malic enzyme using the relatively selective reagent bromopyruvate (4). The present paper provides new information concerning the microen-

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**Table I. Protection of NADP-Malic Enzyme against NEM or NPM Inactivation**

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<thead>
<tr>
<th>Ligand</th>
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<tr>
<td>% protection</td>
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<tr>
<td>Mg²⁺ (10 mM)</td>
<td>36</td>
<td>21</td>
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<tr>
<td>L-malate (4 mM)</td>
<td>53</td>
<td>13</td>
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<tr>
<td>NADP (1 mM)</td>
<td>78</td>
<td>83</td>
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<tr>
<td>Mg²⁺ + L-malate + NADP</td>
<td>92</td>
<td>88</td>
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Inactivation of the enzyme by different concentrations of NADP was measured using aliquots of enzyme in the presence of 2.5 mM NEM in the absence (○) or presence of 0.05 (◇), 0.1 (▲), 0.25 (□), 0.5 (●), or 1 mM (▲) NADP. Inset, k_e and k_i were calculated from the data of Figure 2 and plotted according to the method of Scrutton and Utter (23). The slope of this plot passes through the origin, indicating that the malic enzyme-NADP complex cannot react with NEM (23).

The rapid and complete inactivation of the enzyme by NEM or NPM suggests that the modified amino acids are essential for catalysis. Moreover, the essentially complete protection afforded by NADP (Fig. 2) indicates that the reacting groups are at or near the NADP-binding site of the enzyme. The kinetics observed for inactivation by the monofunctional maleimides are consistent with a two-step process in which the first is a rapid, noncovalent formation of an enzyme-inhibitor complex, followed by the irreversible inactivation of the enzyme. A comparison of the inactivation of the enzyme by NEM and NPM, which is 10-fold faster with the latter (Fig. 1), allows us to suggest that the group(s) involved in the modification of the enzyme is located in a hydrophobic domain. The only difference between these two reagents is the group substituting the N-position of the maleimide. This conclusion is in agreement with our previous results regarding the modification of malic enzyme by bromopyruvate (4). In this case, the two essential sulfhydryl groups per monomer were exposed only in the presence of SDS (4).

The lack of significant protection by L-malate in the case of NEM (Table I) was also reported for other reagents that react with sulfhydryl groups of NAD(P)-malic enzyme (4, 27). It appears that the group(s) that reacts with NEM is located at a distance from the malate-binding site. On the other hand, L-malate afforded partial protection against inactivation by NPM (Table I) or the three phenylenebismaleimides, e.g., OPBM (Fig. 3B). This may suggest a possible interaction of these reagents with the L-malate-binding site of the enzyme, about which very little is known (7). The partial protection afforded by Mg^{2+} may be due to a direct

Figure 2. Protection by different concentrations of NADP against inactivation by NEM. Enzyme (25 μg) was incubated with 2.5 mM NEM in the absence (○) or presence of 0.05 (◇), 0.1 (▲), 0.25 (□), 0.5 (●), or 1 mM (▲) NADP. Inset, k_e and k_i were calculated from the data of Figure 2 and plotted according to the method of Scrutton and Utter (23). The slope of this plot passes through the origin, indicating that the malic enzyme-NADP complex cannot react with NEM (23).

Figure 3. A, Kinetics of inactivation of malic enzyme by different concentrations of OPBM. Purified enzyme (25 μg) was incubated with 0.05 (◇), 0.068 (▼), 0.147 (▼), and 0.2 mM (●) OPBM. At the indicated times, aliquots containing 2.5 μg of malic enzyme were withdrawn and assayed for activity. As a control, the enzyme was also incubated with the maximum concentration of DMSO used in these experiments (■). B, Protection afforded by different effectors of NADP-malic enzyme against inactivation by OPBM. Enzyme (25 μg) was incubated with 0.2 mM OPBM in the absence (○) or presence of 10 mM Mg^{2+} (▼), 4 mM L-malate (▼), 0.5 mM NADP (□), or Mg^{2+} + L-malate + NADP (■). At different times of incubation, aliquots were withdrawn and assayed for activity.

Figure 4. Comparison of the kinetics of inactivation of malic enzyme by OPBM, MPBM, and PPBM. Purified enzyme (10 μg) was incubated with 0.02 mM OPBM (○), MPBM (▼), or PPBM (▼). At the indicated times, aliquots (2.0 μg of enzyme) were withdrawn and assayed for activity.
competition between the metal cofactor and the maleimides for a thiol ligand. The two proposed binding sites of malic enzyme for the metal (5, 6) or a change in conformation of the enzyme induced by the metal (12, 18) seems to have no effect on the modification by maleimides.

The kinetics of inactivation of the enzyme by the bifunctional maleimides OPBM, MPBM, and PPBM were biphasic (Figs. 3 and 4). These biphasic time courses can be divided in two pseudo-first-order kinetic phases, the rate constant of each depending on the nature and concentration of the reagent used. In each case, the concentration of the inactivating reagent was kept much higher than the enzyme concentration (higher than 50 mol of reagent/mol of enzyme monomer, Fig. 4), although precipitation of these hydrophobic reagents in aqueous solution did not allow us to test even higher concentrations. Because the biphasic kinetics cannot be attributed to limiting reagent concentration, they must represent a different reactivity of the groups reacting with the two heads of these bifunctional reagents. A possible cross-linking of two sulphydryl groups important for activity must be considered. On the other hand, the presence of two sulphydryl groups on NADP-malic enzyme that confers a different reactivity toward the bifunctional reagent cannot be ruled out. These maleimides were previously studied as crosslinkers of the γ subunit of the chloroplast-coupling factor (16, 25) and of the myosin subfragment I (26).

Comparison of the rates of inactivation by the three bifunctional reagents at the same concentration (Fig. 4) shows that the shorter cross-linker, OPBM, appears to be a better inactivator. We conclude that the two possible sulphydryl groups that react with the two heads of these reagents are sufficiently close together to react with the longer cross-linking reagents. As was observed in previous work using these reagents (16, 26), a high degree of flexibility of the protein would allow reaction of the longer molecules.

Modification of cysteiny1 groups by [3H]NEM shows that all of the sulphydryl residues present in the enzyme (seven per monomer [22]) are susceptible to NEM modification. In contrast, in the presence of an excess of NADP, only five residues per monomer are susceptible to modification by NEM. These results are in accordance with our previous report on the groups involved in the modification of the enzyme by bromopyruvate and the protection afforded by NADP (4). In the case of modification of the enzyme by OPBM, it is implied that there are two groups that are both protected against inactivation by NADP. Because reaction of bifunctional reagents with proteins is stabilized if the two heads of the reagent react and a cross-linkage is produced, and taking into account the results obtained concerning the titration of malic enzyme with OPBM, we conclude that the two sulphydryl groups are located at the active site of the enzyme, near enough to react with these reagents. Moreover, the native and OPBM-modified enzymes gave a single band at a molecular mass of 62 kD following SDS-PAGE (not shown), suggesting an intrasubunit cross-linkage of the enzyme.

Examination of the deduced primary structure of the maize enzyme (22) reveals that, of the seven total cysteiny1 residues per monomer, two are at the putative dinucleotide-binding site at positions 192 (adjacent to the proposed NADP-binding site) and 246 (adjacent to the NAD-binding site). The amino acid sequences that bind NAD(P) are highly conserved among different enzymes that use these cofactors. Modification of cysteiny1 residues on NADP-malic enzyme from pigeon liver identified four independent sulphydryl groups on the enzyme tetramer that react with NEM or 5,5'-dithiobis(2-nitrobenzoic acid) in an "all-of-the-sites" manner but that react asymmetrically with bromopyruvate, yielding "half-of-the-sites" reactivity (2, 19). This is not inconsistent with our present and past (4) results, because comparison of the NADP-malic enzyme deduced sequences from mammals (mouse and rat [15]) and C₄ plants (maize [22] and Flaviera
trinervia [1]) reveals many differences between them. Of the seven sulfhydryl residues found in the mature enzyme from maize leaves, four are also found in the mammalian and F. trinervia enzymes, and two of these residues are adjacent to the putative dinucleotide-binding sites. However, the maize enzyme also has a unique cysteinyl residue (at position 231) near the dinucleotide-binding site that could form a single binding site as a result of protein folding (22). Sequencing the peptide(s) modified by $^{3}$HJNEM will be critical in identifying the two residues involved in the inactivation of the maize leaf enzyme.

**LITERATURE CITED**