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Tyrosyl residue at or near the active site of maize NADP-malic enzyme

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Abstract

Incubation of maize NADP-malic enzyme with tetranitromethane (TNM) resulted in a total loss of enzyme activity. The loss of enzyme activity was not observed at pH 6.3 but at pH 8.0. NADP-malic enzyme was inactivated to almost 90 % by incubation with an 80-fold molar excess of TNM for 5 min at 30 °C. The substrate malate or Mg^{2+} alone gave no protection, while NADP provided considerable protection. NADP in the presence of malate and Mg^{2+} totally protected the enzyme activity, suggesting that tyrosine residue may be located at or near the active site of maize NADP-malic enzyme. The spectral analysis of the modified enzyme indicated that modification of at least one tyrosine residue per subunit resulted in complete loss of the enzyme activity. The fluorescence study of unmodified and modified enzymes postulated that essential tyrosine residue at maize NADP-malic enzyme is possibly involved in malate binding.

Additional key words: malate; Mg; pH; tetranitromethane; *Zea*.

Introduction

Maize NADP-malic enzyme [malate: NADP oxidoreductase (decarboxylating), EC 1.1.1.40] catalyzes the reversible oxidative decarboxylation of malate to CO_2 and pyruvate in bundle sheath chloroplasts of C4 plants, and the liberated CO_2 is then utilized in Calvin cycle.

Chemical modification and metal catalyzed oxidation studies on maize NADP-malic enzyme have implicated histidyl, arginyl, cysteinyl, carboxyl, and aspartic acid residues in the catalysis by the enzyme. Rothermal and Nelson (1989) have reported the complete nucleotide sequence of a full-length cDNA for maize NADP-malic enzyme.

Tetranitromethane (TNM) is highly specific for tyrosyl residues (Riordan and Valle 1972). The tyrosyl side chains of enzymes may be involved in either stabilizing the structure of proteins by means of internal hydrogen bonding with side chains such as

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aspartic or glutamic acid, or by affecting the activity by ionization of the phenolic hydroxyl group (Attasi and Habeeb 1969, Menendez and Herskovits 1969, Hjelmgren *et al.* 1976). Tyrosine residues are involved in the substrate binding of pigeon liver malic enzyme (Chang and Huang 1980). In the present communication, we show that the loss of maize NADP-malic enzyme activity by TNM is due to the modification of tyrosine residues, probably involved in the substrate (malate) binding.

Materials and methods

Chemicals: Most of the chemicals including tetranitromethane, NADPH, NADP, and malate were obtained from *Sigma*. All other chemicals used were of reagent grade.

Enzyme preparation and assay, and protein estimation: NADP-malic enzyme from maize leaves was purified according to Asami *et al.* (1979). The final specific activity of the enzyme was 1.0 to 1.2 mol(NADPH) kg^{-1}(protein) s^{-1}, and the enzyme was at least 95 % pure as judged by SDS-PAGE. Malic enzyme was assayed in a 1 cm^3 reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mm malate, 0.40 mM NADP, 10 mM $MgCl_2$, and appropriate amount of the enzyme. The formation of NADPH at 25 °C was monitored at 340 nm with an *UVIKON 940* spectrophotometer. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 0.86 for 0.1 % (m/v) protein solution as suggested by Asami *et al.* (1979). M_r of 230 000 was used for the calculation of enzyme concentration.

Chemical modification: The enzyme (2.5 μM) in 50 mM Tris-HCl, pH 8.0 was treated with 80-100 fold excess of TNM in 95 % ethanol to yield a final ethanol concentration of 1-2 %. This concentration of ethanol had no effect on the enzyme activity. The reaction was carried out at 30 °C and stopped by the addition of 25 mM dithiothreitol (DTT). A small aliquot was used for the activity measurement. A sample without TNM was used as control. The commercial TNM was taken as 8.4 M and further dilutions were made in ethanol.

Sulphydryl group analysis: The method of Ellman (1959) was used to estimate the free and total sulphydryl. The enzyme was completely denatured with 5 % SDS to determine total sulphydryl groups.

Stoichiometry of TNM modification: The enzyme (2.5 μM) in 50 mM Tris-HCl, pH 8.0 was incubated with 80-fold molar excess of TNM at 30 °C for various periods. The reaction was terminated by addition of 25 mM DTT (final concentration) and small amount was used for determining the residual enzyme activity. The samples were dialyzed against 200 mM Tris-HCl, pH 9.5, and absorbance at 428 nm was measured for detecting the number of nitrotyrosine formed. The extinction coefficient of 4200 mol^{-1} cm^{-1} at pH 9.5 (Sokolovsky *et al.* 1966) was used for calculating nitrotyrosine.

Fluorescence titration: Fluorescence titration of NADP-malic enzyme with NADPH was carried out using *Hitachi* spectrofluorimeter according to the method of Rao *et*

al. (1997). The nucleotide was excited at 350 nm and its emission at 465 nm monitored. The emission spectrum for NADPH-enzyme complex was monitored at 450 nm.

Results and discussion

The modification of maize NADP-malic enzyme with different concentrations of TNM resulted in loss of enzyme activity. Almost 90 % enzyme activity was lost by incubation with an 80-fold molar excess of TNM for 5 min at 30 °C (Fig. 1). The enzyme was irreversibly inactivated by treatment with micromolar concentrations of TNM. The controls with ethanol but without TNM retained full enzyme activity over the highest time interval of modification used in this experiment. The kinetics of inactivation of the enzyme by TNM is very complex as observed for other enzymes (Maralihalli and Bhagwat 1992).

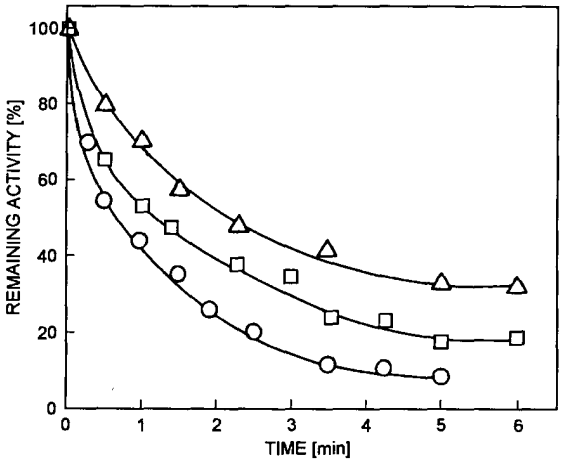

Fig. 1. Inactivation of NADP-malic enzyme (2.5 μm) with 50 (Δ), 100 (□), or 200 (○) μM tetranitromethane (TNM).

The results of the following experiments indicated that the inactivation of NADP-malic enzyme by TNM may be due to the modification of tyrosine residue and not due to cysteine residues. This conclusion is based on the following observations: (*a*) Nitration of tyrosine is dependent on pH, with little or no modification occurring at pH 6.3 (Fig. 2). (*b*) This was further corroborated by reversibly masking –SH groups of the malic enzyme with *p*-hydroxymercuribenzonate before TNM treatment. If the inactivation was due to thiol oxidation rather than modification of tyrosyl residues, full enzyme activity should reappear after regeneration of –SH groups by treatment with excess DTT. The enzyme activity did not reappear in modified enzyme samples after incubation with 25 mM DTT while the control enzyme sample (without TNM) showed 80 % recovery in the enzyme activity after DTT treatment (Table 1). Also after DTT treatment, almost the same number of –SH groups was detected by the method of Ellman (1959), control and TNM modified enzyme samples gave two free –SH groups per subunit while under denaturing condition both these enzyme samples

had seven cysteinyl residues. Thus, it seems that modification of cysteinyl residues is not responsible for TNM inactivation.

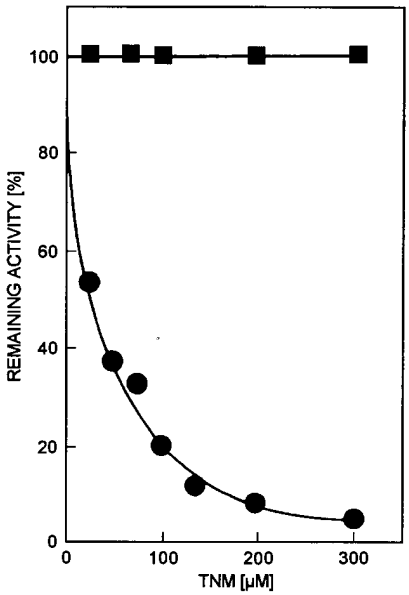

Fig. 2. Effect of pH 6.3 (■) or 8.0 (●) on tetranitromethane (TNM) modification of NADP-malic enzyme. The enzyme was treated with concentrations of TNM for 5 min.

Table 1. The effect of -SH groups on inactivation by tetranitromethane (TNM). The masking of thiol groups was done by incubating the enzyme (2.5 μM) with 200 μM hydroxymercuribenzoate for 10 min in 50 mM Tris-HCl, pH 8.0 at 30 °C. The enzyme (2.5 μM) was incubated with 200 μM TNM for 10 min at 30 °C. The enzyme activity was measured before and after treatment with 25 mM dithiothreitol (DTT).

PHMB	TNM	Activity [%]	
		-DTT	+DTT
–	–	100	100
–	+	10	9
+	–	20	80
+	+	4	7

Modification of NADP-malic enzyme with TNM at pH 8.0 resulted in spectral changes as reported for the nitration of tyrosine (Riordon and Vallee 1972). A difference spectrum of the modified *versus* control enzyme showed a broad peak at about 428 nm (Fig. 3). Using an extinction coefficient for nitrotyrosine of 4200 kmol m^{-3} cm^{-1} at pH 9.5 (Sokolovsky *et al.* 1966), it was calculated that the modification of one tyrosine residue per enzyme subunit caused the complete loss of the enzyme

activity. The enzyme protected by NADP prevented the formation of nitrotyrosine (values not shown).

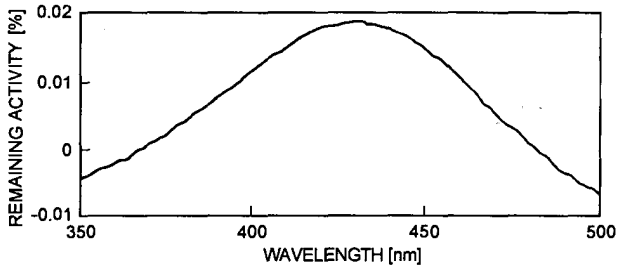

Fig. 3. Difference spectrum between TNM-modified and control NADP-malic enzyme. The enzyme (1.2 μM) was treated with 100-fold molar excess of TNM for 10 min.The samples were dialyzed against 200 mM Tris-HCl (pH 9.5) and the difference spectrum was recorded in *UVIKON 940* spectrophotometer.

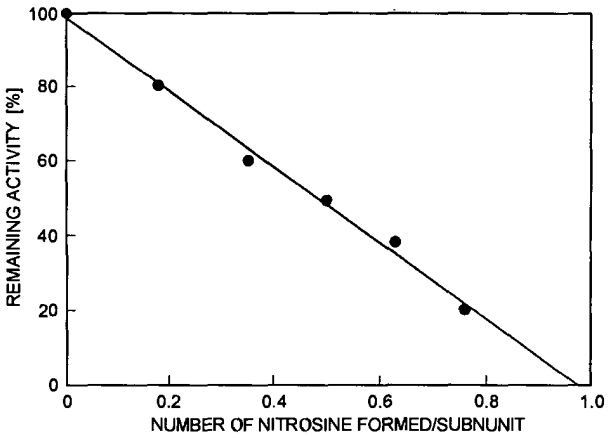

Fig. 4. Stoichiometry of inactivation. Malic enzyme (2.5 μM) was modified with 100-fold molar excess of tetranitromethane (TNM) at various time intervals. The residual enzyme activity was measured in a small aliquot. The samples were dialyzed in 200 mM Tris-HCl buffer, pH 9.5. Absorbance was measured at 428 nm and nitrotyrosine was estimated using an extinction coefficient of 4200 M^{-1} cm^{-1}. The enzyme concentration (2.5 μM) is used for calculating mol(nitrotyrosine incorporated) mol^{-1}(malic enzyme).

Measurement of absorbance at 428 nm along with the estimation of residual activity at various time intervals showed incorporation of one mol of nitrotyrosine per mol of enzyme subunit when extrapolated to 100 % inactivation (Fig. 4). Thus stoichiometry also confirmed that loss of malic enzyme activity was due to the modification of one tyrosine residue.

In order to find out whether the essential tyrosine residues are located at or near the active site of the enzyme, protection experiments with substrates and cofactors were carried out. Malate or Mg^{2+} by itself gave no protection against TNM

inactivation while NADP provided considerable protection (Table 2). The protection by NADP was concentration dependent and $K_{1/2}$ protection value for NADP was 76 μM (values not shown). NADP+malate+$MgCl_2$ resulted in complete protection against TNM inactivation of NADP-malic enzyme activity (Table 2). Hence tyrosine residues may be located at or near the active site of maize malic enzyme.

Table 2. Protection of maize NADP-malic enzyme against tetranitromethane (TNM) inactivation. The enzyme (2.5 μM) was modified with 200 μM TNM for 5 min after preincubation with each effector for 10 min.

Addition	[mM]	[% of control]
None	-	100
TNM	0.2	10
NADP	1.0	75
Malate	10.0	10
$MgCl_2$	10.0	10
NADP+malate+$MgCl_2$	1+10+10	100

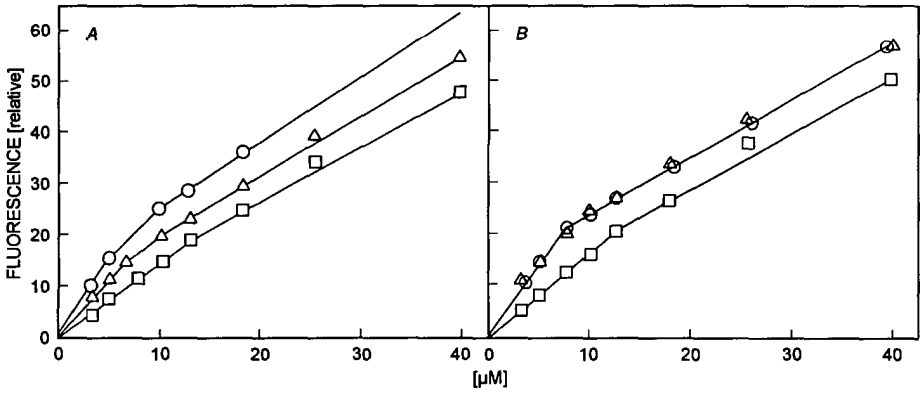

Fig. 5. Fluorimetric titration of unmodified (*A*) and modified (*B*) enzyme samples: free NADPH (□), NADPH+enzyme (Δ), or NADPH+enzyme+malate+Mg^{2+} (○).

Fig. 5*A,B* shows changes in fluorescence intensities of NADPH on binding to native and the modified enzymes. The lines connecting points □ in Fig. 5*A,B* represent the fluorescence emission of free NADPH. Both native and modified enzymes samples after binding of free NADPH showed enhancement in fluorescence (lines Δ in Fig. 5*A,B*) intensity. The addition of malate and Mg^{2+} to the control enzyme+NADPH complex resulted in further enhancement in fluorescence intensity (line ○ in Fig. 5*A*). This enhancement in fluorescence intensity was observed only in the presence of both malate and $MgCl_2$, and not with either one alone. On the contrary, the modified enzyme+NADPH complex did not show this further enhancement in fluorescence intensity after addition of malate and Mg^{2+} (line ○, Fig. 5*B*), indicating that the malate binding site in maize NADP-malic enzyme may

be affected by TNM modification. These results indicated the involvement of tyrosine residue(s) in malate binding of maize NADP-malic enzyme.

The enzyme modified with TNM may sometimes form unwanted side reactions which may alter the properties of the enzyme, *e.g.*, formation of dityrosine (Vincent *et al.* 1970) or reaction with nitrous acid, a decomposition product of TNM (Means and Feeney 1977). These effects may cause polymerization of the enzyme protein. The SDS-PAGE analysis of control and modified enzyme sample showed identical mobility (values not shown) confirming that the inactivation of NADP-malic enzyme by TNM does not involve changes in molecular size or subunit structure. We observed that NADP alone offered considerable protection while NADP+malate+Mg^{2+} offered complete protection against TNM inactivation of maize malic enzyme, indicating that the modification may be at or near the active site. The results of fluorescence study indicated that this tyrosine residue may be involved in malate binding site. It seems likely that nitrotyrosine causes delocalization of the charges at or near the active site either by enhancing the ionization of the phenolic proton or by increasing the charge density due to high electronegativity of the attached group. If it were true, then the phenolic proton could act as a proton donor during catalysis.

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