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Enzyme Regulation in C_4 Photosynthesis[1,2]

PURIFICATION AND PROPERTIES OF THIOREDOXIN-LINKED NADP-MALATE DEHYDROGENASE FROM CORN LEAVES

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ABSTRACT

NADP-malate dehydrogenase, a light-modulated enzyme of C_4 photosynthesis, was purified to homogeneity from leaves of corn. The pure enzyme was activated by thioredoxin *m* that was reduced either photochemically (with ferredoxin and ferredoxin-thioredoxin reductase) or chemically (with dithiothreitol). Unactivated corn leaf NADP-malate dehydrogenase had a molecular weight of 50,000 to 60,000 and was chromophore-free. The enzyme appeared to have a high content of serine and glycine and to contain both S—S and SH groups. Consequently, NADP-malate dehydrogenase seems to be capable of undergoing reversible oxidation/reduction during its photoregulation.

NADP-MDH[5] is a light-modulated chloroplast enzyme that is widely distributed in green plants (1, 9, 13, 21). NADP-MDH is particularly abundant in C_4 plants where it functions photosynthetically in the NADP-dependent reduction of oxaloacetate to malate. Prior to effecting this catalytic reaction, NADP-MDH requires an activation that can be achieved *in vivo* by light (13) or *in vitro* by DTT added in the presence of a soluble protein factor (11, 14, 22, 23) that has been identified as a specific chloroplast thioredoxin (thioredoxin *m*) (12, 19, 24, 25). NADP-MDH can also be activated photochemically *in vitro* by thioredoxin *m* reduced *via* ferredoxin and ferredoxin-thioredoxin reductase (19, 24, 25) or by a soluble protein factor that appears to be independent of the ferredoxin/thioredoxin system (2).

Although NADP-MDH has been partially purified from leaves of several different C_3 and C_4 plants (13, 19, 22–24), a procedure for obtaining homogeneous preparations has not been reported and consequently the enzyme has not been characterized. We have therefore focused our attention on NADP-MDH and now describe a purification procedure that yields homogeneous preparations of the enzyme from corn leaves. Certain properties of corn NADP-MDH are also presented. A preliminary account of these findings has been published (10).

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[5] Abbreviation: NADP-MDH, NADP-malate dehydrogenase.

MATERIALS AND METHODS

Plant Material. Corn plants (*Zea mays* var. Golden Cross Bantam T51, Burpee Seed Co., Riverside, CA) were grown in a greenhouse under normal day to night conditions. Plants were germinated and grown in U. C. mix and watered as needed with half-strength Hoagland solution (17). Spinach plants (*Spinacia oleracea* var. Marathon, Asgrow Seed Co., Tracy, CA) were grown in nutrient solution in a greenhouse as described previously (15).

Reagents. Biochemicals were purchased from Sigma Chemical Co. All other reagents were purchased from commercial sources and were of the highest quality available.

Assay for NADP-MDH Activity. NADP-MDH was activated by DTT-reduced thioredoxin *m* and assayed at 22 C by a modification of previously described methods (12, 25). The activation (preincubation) mixture contained (in a volume of 0.04 ml): enzyme as needed; purified thioredoxin *m*, 10 μg; Tris-HCl buffer (pH 7.9), 10 μmol; and DTT, 0.5 μmol. Unless indicated otherwise, the mixture was incubated for 10 min, and then 0.03 ml was injected into the assay (reaction) mixture. The assay mixture (final volume, 1 ml) contained in addition to the activated enzyme the following (in μmol): Tris-HCl buffer (pH 7.9), 100; oxaloacetic acid, 1.7; NADPH, 0.25; and EDTA, 0.5. The change in *A* at 340 nm was followed with a Gilford model 252 recording spectrophotometer.

Purification of NADP-MDH. All purification steps were carried out at 4 C. Buffers were adjusted to the indicated pH at 22 C. NADP-MDH was purified from corn leaf extracts by fractionation with acid and ammonium sulfate followed by chromatography on DEAE-cellulose, hydroxyapatite, and Sephadex G-100 (see below).

Estimation of Molecular Weight. Sedimentation velocity ultracentrifugation was carried out at 19 C in a Spinco Model E ultracentrifuge (30 mm rotor) with samples added to a 12-mm aluminum single sector cell fitted with quartz windows (6). The sedimentation velocity pattern, visualized with schlieren optics, was measured every 4 min (total time = 40 min) after the rotor reached the full speed of 56,100 rpm. The approximate molecular weight of NADP-MDH was estimated by its elution properties in a Sephadex G-100 column (medium grade, 1.5 × 90 cm) that was equilibrated with a solution containing 100 mM K-phosphate (pH 6.9), and calibrated with BSA (mol wt = 68,000), ovalbumin (mol wt = 45,000), and chymotrypsinogen (mol wt = 25,000) as standards. The column was eluted at a rate of 7 ml/h.

SDS-Polyacrylamide Gel Electrophoresis. Electrophoresis was

carried out with 0.5 × 7 cm disc gels by following the procedure of Laemmli (16). BSA, chymotrypsinogen, and ovalbumin were used as protein standards. Gels were stained with Coomassie Brilliant Blue$_R$, destained with a solution of 7.5% acetic acid and 5% methanol, and scanned in a Gilford M220 densitometer with a full scale of 1.5.

Amino Acid Analysis. Hydrolysis of corn leaf NADP-MDH was carried out in 6 N HCl under vacuum for 24, 48, and 72 h (8). Half-cystine content was determined by using performic acid oxidation also as in Ref. 8. A Biotronic LC 6000 amino acid analyzer was used in these experiments.

Other Methods. Twice washed chloroplast membrane fragments were prepared from chilled greenhouse-grown spinach leaves by blending in sucrose in the absence of EDTA by a method described previously (5, 15). Previously described methods were also used for the estimation of Chl (15) and protein (20), for the isolation from corn leaves of thioredoxin *m* (12, 23, 25) and ferredoxin-thioredoxin reductase (7), and for the preparation of spinach leaf ferredoxin (4).

RESULTS AND DISCUSSION

Purification of NADP-MDH. Table I shows a summary of the purification and yield of NADP-MDH from corn leaves in each of the steps described below. The enzyme was purified 640-fold with a recovery of about 8%. The details for the figures in this section are supplied in the text.

Step I. Preparation of Leaf Extract. Leaves, 2 kg, from 3- to 4-week-old corn plants were harvested, destemmed, washed with distilled H_2O, chilled, and chopped into small pieces with a knife. The leaf fragments were blended in 4 liters buffer solution (30 mM Tris-HCl buffer [pH 7.9], 1 mM EDTA, 14 mM 2-mercaptoethanol, 5 mM $MgSO_4$, 1 g polyvinylpyrollidone [PVP 40 from Sigma Chemical Co.]) in batches of 500 g. Each batch was homogenized by sequential addition, in 50- to 100-g lots, to 1 liter buffer solution in a Waring Blendor (gallon size, CB-6 model 31BL79). Final homogenization was for 40 s at full speed after the last addition of leaves. The homogenate so obtained was filtered through four layers of cheesecloth and the residue was discarded.

Step II. Acid Precipitation. The green filtrate was adjusted to pH 4.5 with 2 N formic acid and centrifuged for 10 min at 13,700*g*. The green precipitate was discarded and the yellow supernatant fraction was used in the next step after adjustment of pH to 6.2 with 0.5 N NaOH.

Step III. Ammonium Sulfate Fractionation. Solid ammonium sulfate was added to the acid supernatant fraction (3.7 liters) to 35% saturation. The suspension was centrifuged (10 min, 13,700*g*) and the precipitate discarded. Solid ammonium sulfate was added to this supernatant solution to 55% saturation, which was stirred for 30 min and then centrifuged (10 min, 13,700*g*): the final supernatant solution was discarded and the pellet resuspended in a minimal amount of a solution containing 50 mM Na-acetate (pH 5.5) and 0.5 mM EDTA (henceforth called buffer A). The resuspended 35 to 55% ammonium sulfate fraction (220 ml) was dialyzed for 12 h against 4 liters buffer A.

Step IV. DEAE-Cellulose Chromatography. The dialyzed sample was applied to a DEAE-cellulose column (2.5 × 18 cm) that had been equilibrated with buffer A. NADP-MDH was eluted batchwise with 200 ml buffer A containing 300 mM NaCl.

Step V. Hydroxyapatite Chromatography. The DEAE-cellulose eluate was applied directly to a hydroxyapatite column (3 × 5 cm) that had been equilibrated with buffer A. The column was eluted batchwise with 150 ml buffer A solution containing 500 mM KH_2PO_4. The eluate was concentrated to a volume of 5 ml in an Amicon cell (7.5 cm diameter) fitted with a PM-10 membrane that was maintained under a nitrogen pressure of 3.5 kg/cm^2.

Step VI. Sephadex G-100 Gel Filtration. The concentrated sample was applied to a Sephadex G-100 column medium grade (2.5 × 100 cm) that had been equilibrated with buffer A. The column was eluted with the same buffer at a flow rate of 20 ml/h. Fractions (6 ml) were collected and assayed for NADP-MDH activity. The activity peak was eluted after a somewhat larger and before a much larger peak absorbing at $A_{280\ nm}$ (Fig. 1). The active fractions were pooled (volume = 40 ml).

Step VII. Second DEAE-Cellulose Chromatography. The pooled Sephadex G-100 fractions were applied to a DEAE-cellulose column (2.5 × 10 cm) that had been equilibrated with buffer A. The column was eluted with 900 ml linear gradient of 0 to 200 mM NaCl in buffer A. Fractions (5 ml) were collected and assayed for NADP-MDH activity. The activity peak was the largest of the several peaks absorbing at 280 nm (Fig. 2). The active fractions were pooled and used in the next step.

Step VIII. Second Hydroxyapatite Chromatography. The pooled DEAE-cellulose fractions (90 ml) were applied to a hydroxyapatite column (2.5 × 4 cm) that had been equilibrated with buffer A. The column was eluted with 900 ml linear gradient 0 to 500 mM KH_2PO_4 in buffer A. Fractions (5 ml) were collected and assayed for NADP-MDH activity. This step separated NADP-MDH from three to four minor peaks of contaminating material absorbing at 280 nm (Fig. 3). The active fractions were pooled and concentrated by ultrafiltration to about 0.5 mg/ml as described in Step V above. The enzyme, which was homogeneous at this stage (see below) was stored at −15 C in a solution containing 37.5 mM Na-acetate (pH 5.5), 0.37 mM EDTA, and 25% glycerol. The enzyme stored in this manner showed no significant loss in activity after 3 months, including occasional thawing and refreezing.

Some Properties of Corn Leaf NADP-MDH. Purified NADP-MDH appeared to be homogeneous: it showed a single component in SDS-polyacrylamide gel electrophoresis (Fig. 4), native gel electrophoresis, and the analytical ultracentrifuge (data not shown). The unactivated enzyme had a mol wt of 50,000 to 60,000 as determined both by filtration on a calibrated G-100 column (Fig. 5, left panel, mol wt = 61,000) and by SDS-polyacrylamide gel electrophoresis (Fig. 5, right panel, mol wt = 53,000). The basis for the difference between these two values, which were obtained with several different enzyme preparations, is not known.

Table I. *Purification of Corn NADP-MDH*

Step	Total Activity	Specific Activity	Recovery
	μmol NADPH oxidized/min	*μmol NADPH oxidized/ min·mg protein*	%
Leaf extract	4,800	0.25	100
pH 4.5 ppt	4,770	0.68	99
Ammonium sulfate ppt	3,664	4.9	77
DEAE-cellulose and hydroxyapatite chromatography	3,319	19.0	70
Sephadex G-100 filtration	2,681	26.5	55
DEAE-cellulose chromatography	806	48.0	17
Hydroxyapatite chromatography	400	160	8

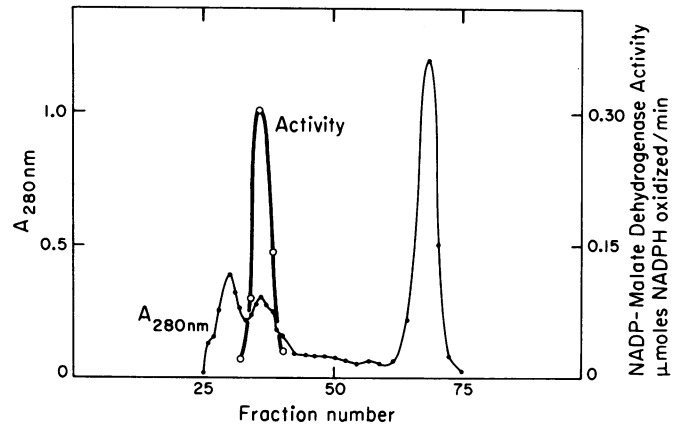

FIG. 1. Sephadex G-100 column profile of corn leaf NADP-MDH preparation.

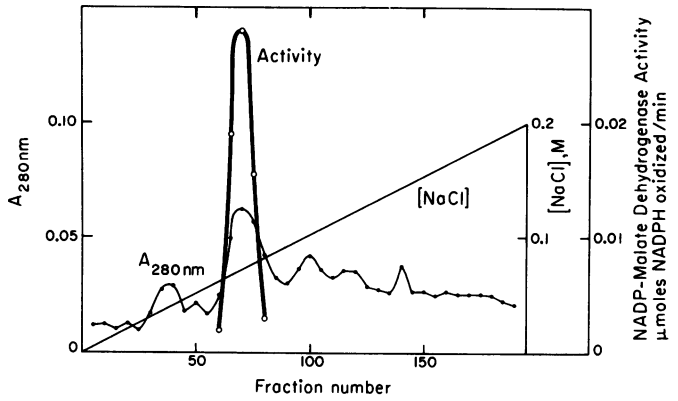

FIG. 2. DEAE-cellulose column profile of corn leaf NADP-MDH preparation.

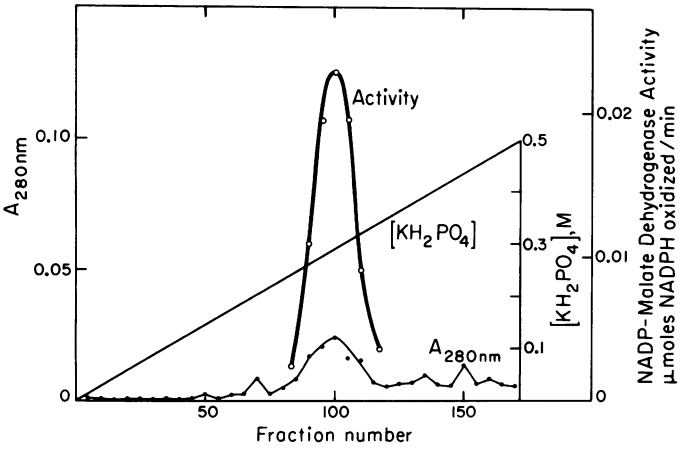

FIG. 3. Hydroxyapatite column profile of corn leaf NADP-MDH preparation.

In sedimentation velocity ultracentrifugation, the enzyme showed a sedimentation coefficient, $s_{20,w}$ of 5.3. No attempt was made in the current study to determine the molecular weight of NADP-MDH following its activation by reduced thioredoxin *m*. The activated enzyme has been reported to show a molecular weight twice that of its unactivated counterpart (14). Multiple molecular weight forms of the activated spinach enzyme have also been described (21).

The purified enzyme was NADP-specific and utilized NAD at only a marginal rate (ratio of utilization of NADH to NADPH in

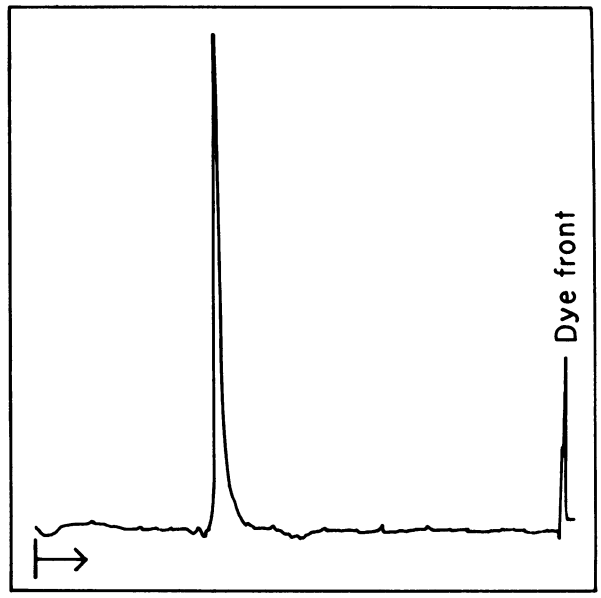

FIG. 4. Densitometric trace of corn leaf NADP-MDH. The trace was made at 560 nm with a SDS-polyacrylamide gel containing 30 μg enzyme.

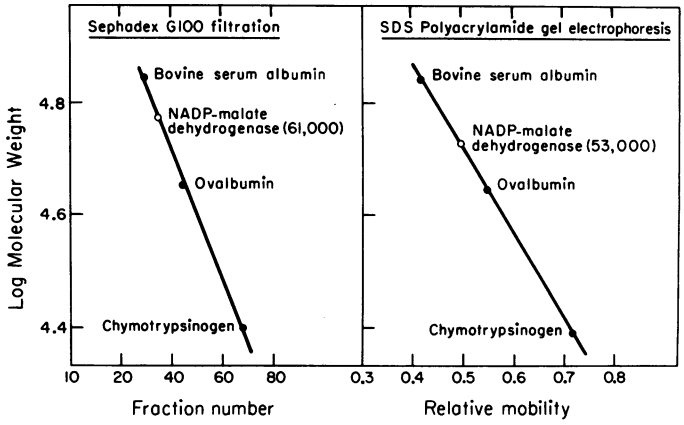

FIG. 5. Molecular weight of corn leaf NADP-MDH estimated by gel filtration and gel electrophoresis.

reduction of oxaloacetate was 1:160). Thus, once activated, NADP-MDH appears to be well suited to make use of the NADPH generated photochemically during photosynthesis.

The absorption spectrum of corn leaf NADP-MDH is shown in Figure 6. The enzyme was characterized by a single protein peak at 280 nm and was devoid of significant absorption in the visible region of the spectrum. Thus, on a spectral basis, NADP-MDH is a classical chromophore-free enzyme.

The amino acid composition of corn leaf NADP-MDH was determined in the current investigation (Table II). In comparison to NAD-linked malate dehydrogenases from various sources corn NADP-MDH was enriched in serine, glycine, and half-cystine residues (3). Data obtained with its carboxymethylated derivative support the presence of both S—S and SH groups in the corn enzyme (*cf.* 21). Based on amino acid content, the minimum mol wt of corn leaf NADP-MDH is 49,300. The basis for the discrepancy in the molecular weight of NADP-MDH determined by gel filtration, SDS-polyacrylamide gel electrophoresis, and amino acid analysis is not understood.

Activation of NADP-MDH by DTT-Reduced Thioredoxin *m*. The rate of activation of NADP-MDH is known to be slow relative to the rate of catalysis, *i.e.* the enzyme shows hysteretic properties (11, 13, 14, 19, 24, 25). Hysteresis is observed when the

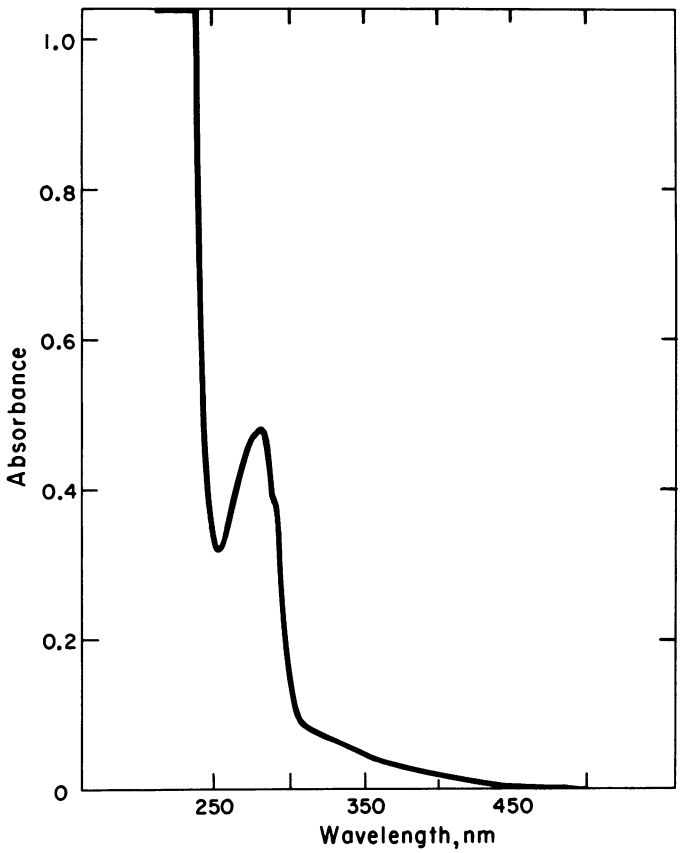

FIG. 6. Absorption spectrum of corn leaf NADP-MDH. The spectrum was measured with a cuvette (1 cm light path) containing 0.5 mg/ml enzyme in 37.5 mM Na-acetate buffer (pH 5.5), supplemented with 0.37 mM EDTA and 25% glycerol. Measurement was made at 22 C in a Cary 219 recording spectrophotometer.

Table II. *Amino Acid Composition of NADP-MDH from Corn Leaves*

Amino Acid Residue	No. Residues per Molecule
Lysine	17
Histidine	19
Arginine	18
Aspartic acid[a]	32
Threonine	18
Serine	54
Glutamic acid[a]	29
Proline	20
Glycine	42
Alanine	32
Half-cystine	13
Valine	29
Methionine	5
Isoleucine	15
Leucine	24
Tyrosine	11
Phenylalanine	20
Tryptophan	nd[b]
Total	398

[a] Values for asparagine and glutamine are included with those for aspartic acid and glutamic acid, respectively.

[b] Not detected.

enzyme is assayed either in crude extracts or in partially purified preparations irrespective of whether activation is effected by photochemically or DTT-reduced thioredoxin *m*. With a saturating concentration of DTT, activation of NADP-MDH depends both

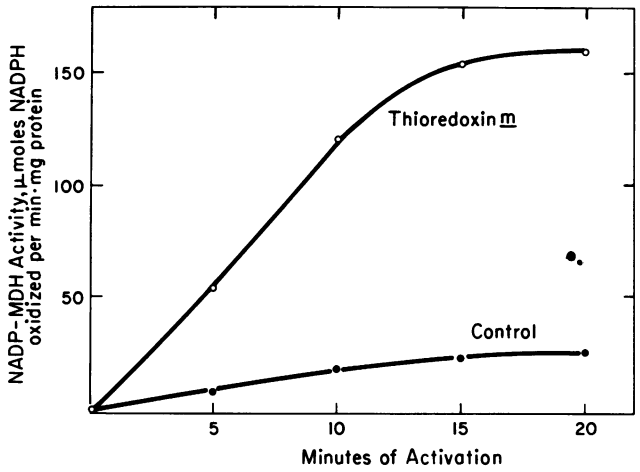

FIG. 7. Effect of time on the activation of corn leaf NADP-MDH by DTT-reduced thioredoxin *m*. Except for changing activation time as indicated, NADP-MDH was assayed as described under "Materials and Methods."

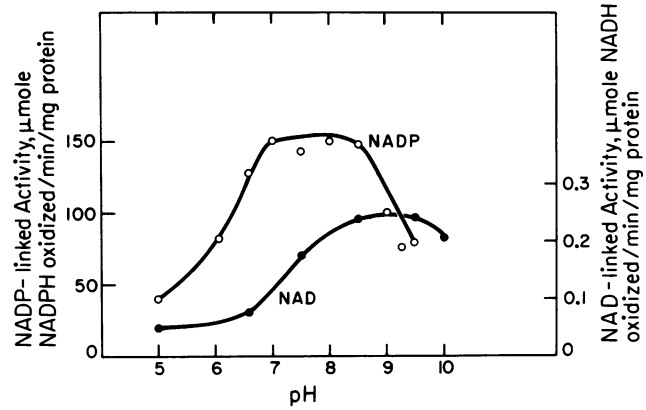

FIG. 8. Effect of pH of reaction mixture on the activity of corn leaf NADP-MDH. Enzyme was activated at pH 7.9 and assayed as described under "Materials and Methods," except for changing the buffer and pH of the reaction mixture as indicated. For pH values greater than 7, Tris-HCl buffer was used. Tris-HCl was replaced by an equivalent concentration of K-phosphate for pH values between 6 and 7 and of Na-acetate for pH values below 6. The pH indicated is the value measured at the end of the reaction.

on time and on the concentration of added thioredoxin *m*. The different parameters involved in the activation of the enzyme have been discussed in detail by Vidal *et al.* (22), who applied a mathematical analysis to verify the need for a protein factor (thioredoxin *m*) for the activation of this enzyme.

The time course of activation of NADP-MDH by the thioredoxin *m*/DTT system used in the current investigation is shown in Figure 7. Full activation of the enzyme, which was observed only in the presence of thioredoxin *m*, required about 15 min preincubation. Following activation under these conditions, the enzyme showed a broad pH optimum (7.0–8.5) for the expression of its NADP-linked catalytic activity (Fig. 8). The pH optimum of the NAD-linked activity of the preparation was more basic (pH = ~9).

Activation of NADP-MDH by Ferredoxin/Thioreduction System. Partially purified NADP-MDH preparations are known to undergo a light-dependent activation when supplied with chloroplast membranes and the components of the ferredoxin/thioredoxin system, *i.e.* ferredoxin, ferredoxin-thioredoxin reductase, and thioredoxin (in this case thioredoxin *m*) (19, 24, 25). Table III

Table III. *Light Activation of Corn Leaf NADP-MDH by Ferredoxin/Thioredoxin System*

Activation was carried out in Warburg-Krippahl flasks that contained (in the center compartment) for the complete system: spinach chloroplast ferredoxin, 75 μg; corn leaf thioredoxin *m*, 6 μg; corn leaf ferredoxin-thioredoxin reductase, 10 μg; thrice washed spinach chloroplast membranes equivalent to 20 μg Chl; Tris-HCl buffer (pH 7.9), 20 μmol; sodium ascorbate, 2 μmol; and 2,6-dichlorophenol indophenol, 0.02 μmol. Corn leaf NADP-malate dehydrogenase, 2 μg, was added to the side arm. Final volume, 0.4 ml. Temperature, 20 C. Flasks were equilibrated with nitrogen for 10 min, NADP-MDH was added from the side arm, and flasks were illuminated 10 min. Of the activation medium, 0.3 ml was then transferred with a micropipette to the assay (reaction) mixture and NADP-MDH activity was measured as described under "Materials and Methods."

Treatment	NADPH Oxidized
	nmol/min
Light	
Complete	34[a]
Minus ferredoxin	0
Minus thioredoxin *m*	2
Minus ferredoxin-thioredoxin reductase	3
Minus NADP-MDH	3
Minus chloroplast membranes	3
Dark	
Complete	2

[a] Values are corrected for endogenous NADH oxidation observed in the absence of oxaloacetate (8 nmol NADH oxidized/min).

shows that a homogeneous preparation of corn leaf NADP-MDH was also activated photochemically by the ferredoxin/thioredoxin system and that, as found previously with less pure preparations, activation was dependent on each of the components of that system. Except for ferredoxin, the protein components used in Table III were derived from corn leaves.

CONCLUDING REMARKS

The present article describes the isolation of a stable homogeneous preparation of thioredoxin-linked NADP-MDH from corn leaves. Corn leaf NADP-MDH is chromophore-free and has a mol wt of 50,000 to 60,000. Significantly, the enzyme appears to contain both S—S and SH groups and, consequently, to be capable of undergoing reversible reduction and oxidation such as might take place during its photoregulation. Evidence that such redox changes occur is provided by the recent observation that NADP-MDH shows a net increase in SH content upon reduction by DTT-reduced thioredoxin *m* (10). It remains to be seen whether a similar reduction occurs when the enzyme is activated by light via enzymically reduced thioredoin *m*.

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