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Structural and Kinetic Properties of NADP-Malic Enzyme from the Inducible Crassulacean Acid Metabolism Plant *Mesembryanthemum crystallinum* L.

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NADP-malic enzyme (EC 1.1.1.40), which is involved in Crassulacean acid metabolism (CAM), was purified to electrophoretic homogeneity from the leaves of the inducible CAM plant *Mesembryanthemum crystallinum*. The NADP-malic enzyme, which was purified 1,146-fold, has a specific activity of 68.8 μmol (mg protein)$^{-1}$ min^{-1}. The molecular weight of the subunits of the enzyme was 64 kDa. The native molecular weight of the enzyme was determined by gel-filtration to be 390 kDa, indicating that the purified NADP-malic enzyme is a hexamer of identical subunits. The optimal pH for activity of the enzyme was around 7.2. Double-reciprocal plots of the enzymatic activity as a function of the concentration of L-malate yielded straight lines both at pH 7.2 and at pH 7.8 and did not reveal any evidence for cooperativity of binding of L-malate. The K_m value for L-malate was 0.35 mM. Hill plots of the activity as a function of the concentration of $NADP^+$ indicated positive cooperativity in the binding of $NADP^+$ to the enzyme with a Hill coefficient (nH) of 2.0. An $S_{0.5}$ value (the concentration giving half-maximal activity) of 9.9 μM for $NADP^+$ was obtained. Oxaloacetate inhibited the activity of the NADP-malic enzyme. Effects of succinate and $NaHCO_3$ on the activity of NADP-malic enzyme were small.

Key words: Crassulacean acid metabolism — Enzyme purification — *Mesembryanthemum crystallinum* L. — NADP-malic enzyme.

NADP-malic enzyme (EC 1.1.1.40) occurs in almost all living organisms, including animals (Frenkel 1975) and higher plants (Asami et al. 1979), but its metabolic functions differ depending on the organism.

NADP-malic enzymes from several plant species and tissues have been shown to exhibit large differences in kinetic and immunochemical properties. These differences have been discussed by Nishikido and Wada (1974), Pupillo and Bossi (1979) and Fathi and Schnarrenberger (1990), and it appears that one type of NADP-malic enzyme exists in C_3 and Crassulacean acid metabolism (CAM) plants and in nongreen tissues of C_4 plants and a second type exists only in green leaves of C_4 plants. The differences in properties are thought to be correlated with the fact that the NADP-malic enzymes of the two types appear to be compartmentalized in different subcellular locations.

C_3 plants do not have a direct requirement for NADP-malic enzyme for the photosynthetic fixation of CO_2. In many CAM plants, both NADP-malic enzyme and NAD-malic enzyme (EC 1.1.1.38) are believed to decarboxylate malic acid during deacidification in the light (Osmond and Holtum 1981, Winter 1985). In the inducible CAM plant *Mesembryanthemum crystallinum* (Winter and von Willert 1972), the significance of NADP- and NAD-malic enzymes in the decarboxylation of malic acid is indicated by the considerable increases in extractable activities during the induction of CAM (Holtum and Winter 1982, Winter et al. 1982), i.e., after exposure of plants to rooting medium of high salinity. However, there are few reports on purified NADP-malic enzymes from CAM plants. The present report describes the structural and kinetic properties of an NADP-malic enzyme that was purified to a high degree of homogeneity from the leaves of the inducible CAM plant *Mesembryanthemum crystallinum*.

Abbreviation: CAM, Crassulacean acid metabolism.

Materials and Methods

Plant materials—Seeds of *Mesembryanthemum crystallinum* L. were germinated in soil and seedlings were transferred to aerated culture solutions (Saitou et al. 1991). All plants were kept in a growth cabinet with a daylight pe-

riod of 12 h, at a photon flux density (400–700 nm) of 250 μmol m^{-2} s^{-1}, an air temperature of 25°C, and a relative humidity of 70%. CAM was induced by the addition of 400 mM NaCl for twenty days (Saitou et al. 1991.). The fully developed leaves were harvested during the late daylight period and stored at −55°C until use.

Purification of the enzyme—The following steps were carried out at 0 to 4°C. Leaves (about 300 g) of *M. crystallinum* in which CAM had been induced were chopped into fine pieces and then ground with a chilled mortar and pestle in 3 volumes of extraction buffer. The buffer consisted of 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 20% (w/v) glycerol, 10 mM 2-mercaptoethanol, and 2% (w/v) insoluble polyvinylpolypyrrolidone. After filtration of the extract through cheesecloth, the filtrate was centrifuged at 10,000 × *g* for 60 min. To the supernatant fraction, crystalline ammonium sulfate was gradually added up to 50% saturation. After centrifugation at 10,000 × *g* for 30 min, the supernatant was brought to 70% saturation with ammonium sulfate. After a second centrifugation, the pellet was resuspended in about 18 ml of buffer A that contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (w/v) glycerol, and 10 mM 2-mercaptoethanol, and centrifuged at 23,000 × *g* for 60 min. The supernatant fraction was passed through a NAP column (Pharmacia LKB, Uppsala, Sweden; 1.5 cm i.d. × 5 cm), which had previously been equilibrated with buffer A.

The eluate was then applied to a column of DE52 (Whatman, Maidstone, England; 1.6 cm i.d. × 22 cm) that had previously been equilibrated with buffer A. The enzyme was not adsorbed onto the column.

The peak fractions with enzymatic activity were pooled and applied to a column (1.6 cm i.d. × 25 cm) of Blue Sepharose CL-6B (Pharmacia LKB) that had previously been equilibrated with buffer A. The enzyme was eluted with a linear 320-ml gradient of NADPH (0–0.8 mM) in buffer B, which contained 50 mM Tris-HCl, pH 7.7, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (w/v) glycerol, and 10 mM 2-mercaptoethanol.

The fractions with maximum enzymatic activity were pooled and applied to a column of hydroxylapatite (Bio-Rad Co., Richmond, California, U.S.A.; 1.6 cm i.d. × 10 cm) that had previously been equilibrated with buffer B. The enzyme was eluted with a linear 200-ml gradient of potassium phosphate (0–200 mM) in buffer C, which contained 50 mM Tris-HCl, pH 7.3, 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (w/v) glycerol, and 10 mM 2-mercaptoethanol. The fractions with maximum enzymatic activity were pooled and concentrated in a cellulose tubing using Aquacide II (Calbiochem Co., San Diego, California, U.S.A.) to about 14 ml.

The concentrated solution was chromatographed on a column of Sepharose 6B (Pharmacia LKB; 1.6 cm i.d. × 86 cm) which had previously been equilibrated with buffer C. The most active fractions were pooled and stored at 4°C.

Assay of NADP-malic enzyme and units of activity—The enzyme was assayed spectrophotometrically at 25°C by following production of NADPH at 340 nm. The standard reaction mixture contained (unless otherwise specified) 50 mM Tris-TES, pH 7.2, 5 mM $MgCl_2$, 0.5 mM $NADP^+$ and 5 mM L-malate in a final volume of 1 ml. One unit of activity is defined as the amount of enzyme that catalyzed the reduction of 1 μmol of $NADP^+$ min^{-1} under the conditions of the reaction.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)—The system developed by Laemmli (1970) for SDS-PAGE was used. Analytical polyacrylamide gels containing SDS were stained with Coomassie brilliant blue R-250.

Molecular weight determination—The molecular weight of the subunits of NADP-malic enzyme was determined by SDS-PAGE linear-gradient slab gel, 5% to 20% acrylamide. The proteins used as molecular weight standards were ovalbumin (45 kDa; Bio-Rad Co.), BSA (67 kDa; Pharmacia LKB), phosphorylase b (94 kDa; Pharmacia LKB), phosphorylase b (97.4 kDa; Bio-Rad Co.), and β-galactosidase (116.25 kDa; Bio-Rad Co.). The native molecular weight of the enzyme was determined by gel-filtration chromatography on a HPLC system (Shimadzu, Kyoto, Japan) with a Shim-pack Diol-300 column (0.79 cm i.d. × 25 cm) at a flow rate of 60 ml h^{-1}. The column was equilibrated with 200 mM Tris-TES buffer, at pH 7.0 or at pH 8.0, that contained 5 mM $MgSO_4$, 0.1 mM EDTA, 10% (w/v) glycerol, and either 10 mM 2-mercaptoethanol or 5 mM dithiothreitol and had been precalibrated with bovine pancreatic ribonuclease A (13.7 kDa), hen egg ovalbumin (43 kDa), BSA (67 kDa), rabbit muscle aldolase (158 kDa), and horse spleen ferritin (440 kDa). The molecular-mass markers were obtained from Pharmacia LKB.

Protein determination—Protein concentrations were determined spectrophotometrically at 595 nm by the method of Bradford (1976), with Coomassie brilliant blue G-250 (Bio-Rad Co.). A standard curve was established using BSA.

Results

The purification of NADP-malic enzyme from the leaves of the inducible CAM plant *Mesembryanthemum crystallinum* is summarized in Table 1. The enzyme was predominantly recovered in the 50–70% ammonium sulfate precipitate. The enzyme was then applied to a column of DE52 and was not adsorbed onto the column. The purification was particularly successful after chromatography on Blue Sepharose CL-6B. The specific activity was 31-fold greater than after chromatography on DE52. NADP-malic enzyme obtained from the column of Blue Sepharose CL-6B was further purified by chromatography on hydrox-

Table 1 Purification of NADP-malic enzyme from the leaves of *Mesembryanthemum crystallinum*

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units mg^{-1})	Purification (fold)
Crude extract	1,025.0	1,029.47	58.24	100.0	0.06	1.0
Ammonium sulfate, 50–70% fraction	35.0	237.88	56.40	96.8	0.24	4.0
DE 52	60.0	59.01	40.73	69.9	0.69	11.5
Blue Sepharose CL-6B	42.5	1.37	29.57	50.8	21.58	359.7
Hydroxylapatite	3.4	0.65	19.84	34.1	30.52	508.7
Sepharose 6B	20.0	0.13	8.94	15.4	68.77	1,146.0

ylapatite. The subsequent gel-filtration step yielded one peak of enzymatic activity which coincided with the peak of protein. The enzyme was purified 1146-fold to a final specific activity of 68.8 units mg^{-1} protein. SDS-PAGE of the final preparation obtained by molecular exclusion chromatography on Sepharose 6B revealed a single band without any contaminating bands (Fig. 1).

The molecular weight of native NADP-malic enzyme purified from the leaves of *M. crystallinum* was estimated to be 390 kDa from chromatography in 200 mM Tris-TES buffer, at pH 7.0 or pH 8.0, that contained 5 mM $MgSO_4$, 0.1 mM EDTA, 10% (w/v) glycerol, and either 10 mM 2-mercaptoethanol or 5 mM dithiothreitol (Fig. 2). When the chromatography was performed in 100 mM potassium phosphate buffer, pH 7.3, that contained 5 mM $MgSO_4$, 0.1 mM EDTA, 10% (w/v) glycerol, and 10 mM 2-mercaptoethanol, no change in the apparent molecular weight was observed. The molecular weight of the subunits of the NADP-malic enzyme was 64 kDa. The native NADP-malic enzyme from the leaves of *M. crystallinum* appears to be a hexamer composed of identical subunits.

The optimal pH for NADP-malic enzyme activity was around 7.2 (Fig. 3). This value is consistent with the values reported for the analogous enzymes from C_3 and CAM plants and from nongreen tissues of C_4 plants (Nishikido and Wada 1974, Pupillo and Bossi 1979).

When the initial velocity of the reaction was studied as a function of the concentration of L-malate, in the presence of saturating concentrations of $NADP^+$ (0.5 mM) and Mg^{2+} ions (5 mM), a hyperbolic response was obtained both at pH 7.1 and at pH 7.8. Double-reciprocal plots of the data were typically linear (Fig. 4), and Hill plots of the data gave values of the Hill coefficient (nH) of 1.0 for L-malate at the two pH values. A K_m value of 0.35 mM for L-malate was obtained from double-reciprocal plots of data obtained at pH 7.1.

When the initial velocity of the reaction was studied as a function of the concentration of $NADP^+$, in the presence

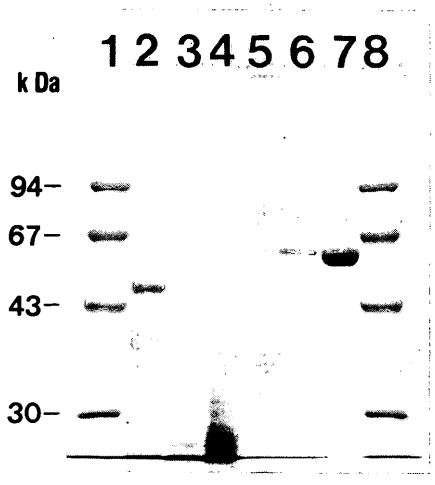

Fig. 1 Results of SDS-PAGE of NADP-malic enzyme at different stages of purification from *M. crystallinum*. 1 and 8, Standard proteins; 2, crude extract; 3, ammonium sulfate fraction; 4, after DEAE-gel ion-exchange chromatography; 5, after affinity chromatography; 6, after hydroxylapatite chromatography; 7, after gel-filtration.

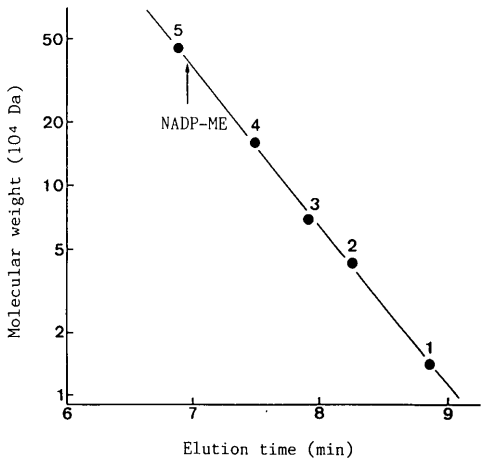

Fig. 2 Determination of the molecular weight of native NADP-malic enzyme from *M. crystallinum* by gel-filtration on a column of Shim-pack Diol-300 (Shimadzu). 1, Ribonuclease A; 2, ovalbumin; 3, BSA; 4, aldolase; 5, ferritin.

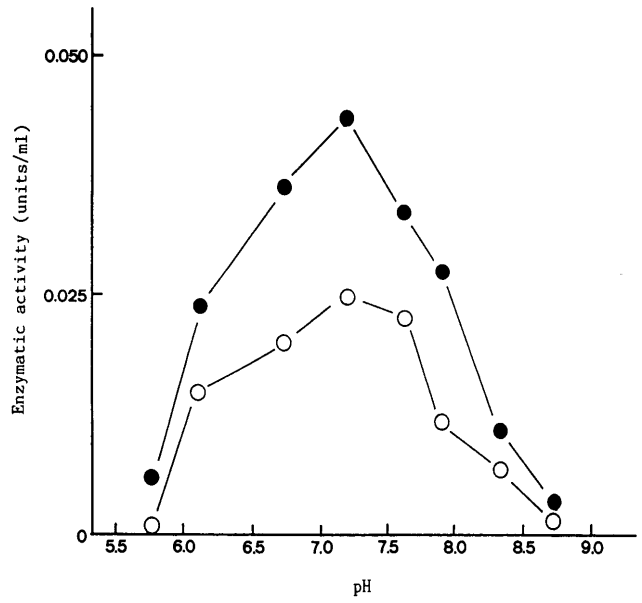

Fig. 3 Effects of pH on the activity of NADP-malic enzyme in the presence of 2 mM oxaloacetate and in the absence of oxaloacetate, at 0.75 mM malate. ●, Control assay; ○, 2 mM oxaloacetate.

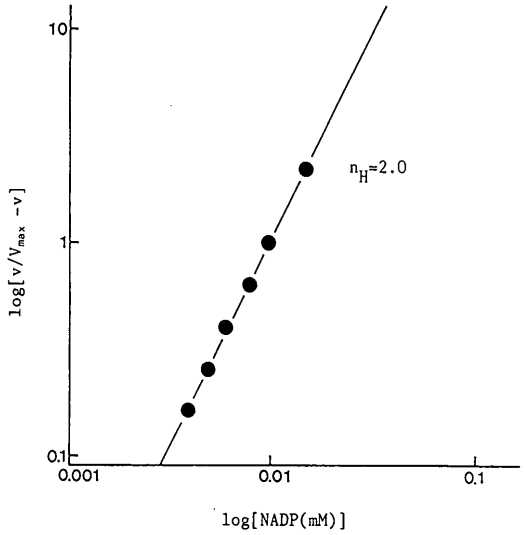

Fig. 5 Hill plots of the activity of NADP-malic enzyme from *M. crystallinum* as a function of the concentration of $NADP^+$.

of saturating concentrations of L-malate (5 mM) and Mg^{2+} ions (5 mM) at pH 7.2, a sigmoidal response was obtained. Double-reciprocal plots of the data were not linear. From Hill plots of the data, an *n*H value of 2.0 was obtained, indicating that the binding of $NADP^+$ to the enzyme exhibited cooperativity (Fig. 5). An $S_{0.5}$ value (the concentration giving half-maximal activity) of 9.9 μM for $NADP^+$ was obtained from the Hill plots.

The purified NADP-malic enzyme showed absolute specificity for $NADP^+$. No activity was observed when $NADP^+$ was replaced by NAD^+ (up to 4 mM) in the assay medium.

The effects of various organic acids on the activity of the purified NADP-malic enzyme were investigated in the presence of 5 mM or 0.1 mM L-malate at pH 7.2. Minimal effects were observed upon inclusion, at 1 mM, of the following compounds in the standard assay mixture: glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, fructose 2,6-bisphosphate, glyceraldehyde 3-

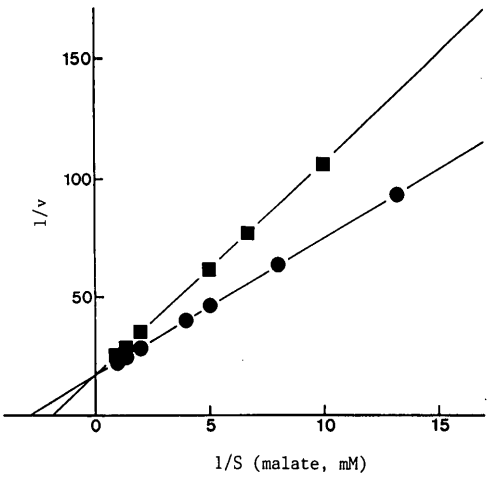

Fig. 4 Double-reciprocal plots of the activity of NADP-malic enzyme from *M. crystallinum* as a function of the concentration of L-malate at pH 7.1 (●) and pH 7.8 (■).

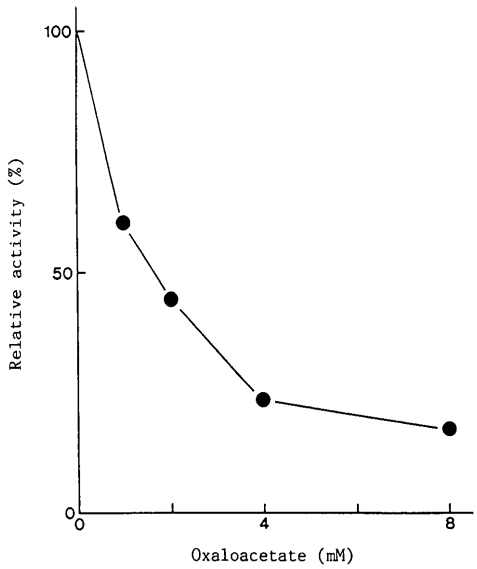

Fig. 6 Effect of the concentration of oxaloacetate on the relative activity of NADP-malic enzyme. The assays were performed at pH 7.2 in the presence of 0.75 mM L-malate.

phosphate, 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, pyruvate, citrate, isocitrate, succinate, fumarate, acetyl-coenzyme A, ATP, AMP, K_2HPO_4, L-alanine, glutarate, and L-aspartate. Dihydroxyacetone phosphate, 2-ketoglutarate, coenzyme A, and ADP caused 20–30% inhibition of the enzymatic activity.

Oxaloacetate effectively inhibited the activity of the NADP-malic enzyme (Fig. 6). The double-reciprocal plots indicated a competitive-type inhibition of NADP-malic enzyme by oxaloacetate since this inhibitor increased the K_m for L-malate with little or no effect on the maximum velocity. Over a wide range of pH values, oxaloacetate inhibited the activity of the NADP-malic enzyme, but it did not cause a shift in the optimal pH (Fig. 3).

$NaHCO_3$ was somewhat effective as an inhibitor of the enzymatic activity: the presence of 40 mM $NaHCO_3$ resulted in 45% inhibition.

Discussion

Purified NADP-malic enzyme from the leaves of the inducible CAM plant *M. crystallinum*, which belongs to the malic enzyme subgroup, migrated as a single band of protein with an apparent molecular weight of 64 kDa during SDS-PAGE. This result, together with the native molecular weight of 390 kDa, indicates that the enzyme from *M. crystallinum* is a hexamer of identical subunits. This conclusion is not consistent with the structure generally accepted for the same enzyme from different sources (Asami et al. 1979, Dubery and Schabort 1981, Fathi and Schnarrenberger 1990, Frenkel 1975, Häusler et al. 1987, Hsu 1982, Iglesias and Andreo 1989).

The existence of different oligomeric states is a well documented feature of the NAD-malic enzyme from plants (Artus and Edwards 1985), as well as of the NADP-dependent enzyme from pigeon liver (Chang et al. 1988). NADP-malic enzymes from maize (Thorniley and Dalziel 1988) and sugarcane (Iglesias and Andreo 1990) were shown to exist in different forms when molecular weights were estimated under different sets of conditions. The molecular weight of the native enzyme from *M. crystallinum* did not change as a function of the buffer used or the pH. This result indicates that NADP-malic enzyme from *M. crystallinum* was present in only one state under our experimental conditions.

Plant NADP-malic enzymes have been categorized as being of the "C_3+CAM type" and the "C_4 type" (Nishikido and Wada 1974, Pupillo and Bossi 1979) on the basis of their respective affinities for L-malate and pH optima. NADP-malic enzymes of the former type have a relatively high K_m for L-malate (0.3–1.0 mM) and an optimal pH close to 7.0. NADP-malic enzymes of the latter type have a K_m of 0.05–0.15 mM and an optimal pH around 8. The purified enzyme from *M. crystallinum* catalyzed the oxidative decarboxylation of L-malate, requiring $NADP^+$ as an essential cofactor. The specific activity of the final preparation was 68.8 units mg^{-1} protein. This value is of the same order as the highest reported values for the purified enzymes from C_3, C_4, and CAM plants (Asami et al. 1979, Brandon and van Boekel-Mol 1973, Fathi and Schnarrenberger 1990, Iglesias and Andreo 1989). The K_m value for L-malate and the $S_{0.5}$ value for $NADP^+$ were determined to be 0.35 mM and 9.9 μM, respectively. These values are similar to those reported for the purified enzymes from C_4 and CAM plants (Asami et al. 1979, Brandon and van Boekel-Mol 1973, Iglesias and Andreo 1989). The optimal pH for activity of the NADP-malic enzyme was around 7.2. This value is consistent with the values reported for enzymes of the C_3+CAM type.

NADP-malic enzymes from a wide range of higher plants have been reported to exhibit cooperativity during binding of malate (Davies et al. 1974, Davies and Patil 1974, Drouet and Hartmann 1977, Possner et al. 1981). However, leaves of *Bryophyllum crenata* and *Bryophyllum tubiflorum*, which are CAM plants, have been reported to contain a malic enzyme that does not exhibit cooperativity for binding of malate (Brandon and van Boekel-Mol 1973, Walker 1960). The purified enzyme from *M. crystallinum* gave a hyperbolic activity curve both at pH 7.1 and at pH 7.8, and we found that the purified enzyme exhibited cooperativity for binding of $NADP^+$.

Davies et al. (1974) reported that partially purified NADP-malic enzymes from *Gramineae*, including sugarcane, did not exhibit allosteric properties, and that the enzymes were inhibited, rather than activated, by succinate. However, Iglesias and Andreo (1989, 1990) reported that NADP-malic enzyme purified from sugarcane leaves, which exhibited no cooperativity for binding of L-malate, was activated by succinate when the activity was assayed at low concentrations of L-malate. The activating effect of succinate on the purified NADP-malic enzyme from the leaves of *M. crystallinum*, which exhibited no cooperativity for binding of L-malate, was small.

Oxaloacetate inhibited the activity of the NADP-malic enzyme effectively over a wide range of pH values. If we assume that the enzyme's properties in vitro reflect the approximate properties of the enzyme in vivo, oxaloacetate, which is produced by the dark fixation of CO_2, reduces the potential for decarboxylation of malic acid via NADP-malic enzyme during the night. The accumulation of oxaloacetate during the night has been reported by Milburn et al. (1968) for *Bryophyllum crenatum* and by Kenyon et al. (1981) for *Kalanchoë daigremontiana*. However, the actual cytoplasmic concentration of oxaloacetate in *M. crystallinum* is unknown.

It has been proposed that CO_2 can inhibit deacidification due to the inhibition of NADP-malic enzyme in the leaves of *Bryophyllum daigremontianum* (Nishida 1977).

However, the inhibitory concentration of CO_2 with respect to NADP-malic enzyme is too high for this inhibition to be of physiological importance in the leaves of *M. crystallinum*.

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