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Expression and characterization of recombinant cytosolic NAD^+-dependent malate dehydrogenase from *Mesembryanthemum crystallinum*

Karina E. J. Trípodi[a,1], María E. Gómez Casati[a,1], Antonio D. Uttaro[b] and Florencio E. Podestá[a,*]

[a]*Centro de Estudios Fotosintéticos y Bioquímicos (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531 2000 Rosario, Argentina*
[b]*IBR, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531 2000 Rosario, Argentina*
**Corresponding author, e-mail: fpodesta@fbioyf.unr.edu.ar*
[1]*These authors contributed equally to the work*

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An NAD^+-dependent cytosolic malate dehydrogenase (MDH, EC 1.1.1.37) from leaves of *Mesembryanthemum crystallinum* in the Crassulacean Acid Metabolism (CAM) mode was cloned, expressed in *E. coli* and characterized. The recombinant enzyme had a subunit molecular mass of 39.5 kDa and was recognized by antibodies raised against the cytosolic MDH from *Ananas comosus*. Its activity showed a maximum in the pH range of 7.5–9.5. The purified MDH is highly but not completely specific for oxaloacetate, as indicated by a low activity using various other α-ketoacids as substrates. The sequence data, subunit mass and immunoreactivity suggest that the MDH that has been cloned and characterized corresponds to the cytosolic isoform. Yet, the biochemistry of this enzyme comparative with the only other CAM plant cytosolic MDH characterized so far (that of pineapple) hints at a distinct isoform being expressed in *M. crystallinum* leaves.

Introduction

Plant cells contain multiple molecular forms of malate dehydrogenases (L-malate-NAD^+-oxidoreductase, MDH, EC 1.1.1.37), present as mitochondrial, microbody, cytosolic and chloroplastic isoforms, which serve different metabolic roles within their subcellular location (Gietl 1992, Berkemeyer et al. 1998). Plant MDHs require NAD^+ as cofactor, except for a chloroplastic form that uses $NADP^+$. MDH action is pivotal in the leaf cells of CAM plants as it drives the nocturnal OAA reduction. CAM plants are characterized by the ability to assimilate CO_2 during the night via PEPCase when stomata are open, generating OAA, which is reduced to malate by MDH (Ting 1985, Cushman and Bohnert 1997).

M. crystallinum, the common ice plant, belongs to the malic enzyme subgroup of CAM plants and uses predominantly starch as a carbon supply to support malic acid production during the night. In facultative CAM plants such as this, the transition from C_3 photosynthesis to CAM may be induced by high-salt treatment and is accompanied by a redirectioning of carbon flow (Cushman and Bohnert 1997). During the C_3-CAM transition, several enzymes directly involved in carbon metabolism are induced (i.e. PEPCase, cytosolic NAD^+ and $NADP^+$-malic enzyme, mitochondrial NAD^+-malic enzyme, chloroplastic pyruvate orthophosphate: dikinase, NAD $(P)^+$-MDH) (Holtum and Winter 1982, Winter et al. 1982, Cushman and Bohnert 1996). A 2-fold increase in the activity of total MDH has also been reported (Winter et al. 1982), although the nature of the isozyme(s) involved is not clear at present.

In spite of the presumably important role of MDH, little effort has been dedicated to elucidating the role of

Abbreviations – CAM, Crassulacean acid metabolism; MDH, malate dehydrogenase; OAA, oxaloacetate; PEPCase, phosphoenolpyruvate carboxylase; IgG, immunoglobulin G.

its different isozymes in the carbon economy in CAM. Ocheretina and Scheibe (1997) isolated and characterized a cDNA clone coding for the cytosolic NAD^+-MDH from *M. crystallinum*. More recently, Cuevas and Podestá (2000) purified and characterized a cytosolic MDH isoform from pineapple (a PEPCK-subtype CAM plant) that seems well suited to drive the nightly reduction of OAA. Still, data are scant as to unambiguously ascribe this task to a single isoform. Actually, cytosolic reduction is just one possibility, since mitochondria could also be involved in OAA/malate metabolism owing to the fact that plant mitochondria possess both the ability to transport these two metabolites (Hanning et al. 1999) and a highly active MDH (Gietl 1992).

As part of an effort to elucidate the role of the various MDH isozymes and their distribution among the different CAM subtypes, we have cloned a cDNA coding for the cytosolic *M. crystallinum* MDH and purified to homogeneity the expressed protein. This paper reports the physical and biochemical properties of the over-expressed cytosolic MDH.

Materials and methods

Reagents

Restriction enzymes, AMV Reverse Transcriptase and Taq DNA Polymerase were purchased from Promega Corp., Madison, Wisconsin, USA. Substrates, coupling enzymes, α-ketoacids, Phenyl Sepharose, DEAE-cellulose, molecular weight markers for gel filtration and SDS-PAGE were from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased from Merck, Buenos Aires, Argentina.

Plant growth conditions

Seeds of *M. crystallinum* were a kind gift of Dr Gerald Edwards, Washington State University, Pullman, USA. After 2–3 weeks germination in Murashige-Skoog medium (Murashige and Skoog 1962), plantlets were transferred to vermiculite and left for 8–13 weeks before being placed in a growth cabinet with a 16-h/8-h day/night cycle at a photon flux density of 300–400 $\mu mol\ m^{-2}\ s^{-1}$ at the leaf level and a day/night temperature of 25°C/15°C. Lamps used were Philips TLD 36 W/79 (the Netherlands). To induce CAM, the NaCl concentration in the watering solution was raised to 100, 200, 300 and 400 m*M* over the first 4 days and was maintained at 400 m*M* thereafter. Induction was complete after 7 days, as judged by a 25-fold increase in PEPCase activity in leaves (i.e. from 0.28 $U\ mg^{-1}$ chlorophyll before salt stress to 6.93 $U\ mg^{-1}$ chlorophyll at day 7), which remained constant in the following weeks.

Molecular cloning and DNA sequencing

Total RNA from CAM mode *M. crystallinum* leaves was extracted following the method described by Lessard et al. (1997). This material was used to obtain cDNA through dT-oligomer priming, which was then used as a template for a PCR reaction. Primers were designed at the 5′- and 3′-end region of the cytosolic MDH from *M. crystallinum* (Ocheretina and Scheibe (1997), accession number X96539), including the natural ATG and stop codons, with the addition of the recognition sites of the restriction enzymes *Nde*I (in the forward primer, 5′GGAATTCCATATGGCCGTTGAACCTC-TTCG) and *Bam*HI (in the reverse primer, 3′CGGG-ATCCTTAAGTCAGGCATGAGTAGGCCAA). With these primers, a product of around 1000 bp was obtained. The product was purified from an agarose gel and ligated with the pGEM-T Easy Vector (Promega Corp. Madison, Wisconsin). The plasmid containing the insert was amplified in *Escherichia coli* XL-1 Blue and purified after alkaline lysis. The fragment was removed from the pGEM-T Easy Vector after incubation with the restriction enzymes *Nde*I and *Bam*HI and subcloned in the expression vector pT7-7.1 (Allert et al. 1991), which is under the control of promoters recognized by T7 RNA polymerase. The new construction was used to transform *E. coli* BL21.

Both strands of the insert were fully sequenced by the method of the fluorescent-ends PRISM (Applied Biosystems). All molecular procedures followed standard protocols described elsewhere.

Expression of heterologous MDH in *E. coli* BL21

For the expression assay, a bacterial colony was inoculated in M9 medium (Sambrook et al. 1989) containing 100 $\mu g\ ml^{-1}$ ampicillin and incubated at 30°C with shaking. The culture was grown until the absorbance at 600 nm reached 0.6. At this time, 0.5 m*M* isopropyl thio-β-D-galactoside was added and the incubation continued for at least 15 h. Expression of the protein was monitored by MDH activity assays and polyacrylamide gel electrophoresis in the presence of SDS using bacterial lysates taken at different time intervals after induction.

MDH purification

The following buffers were used during purification: B, 25 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA; 2 m*M* 2-mercaptoethanol; C, 25 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA; 2 m*M* 2-mercaptoethanol; 40% saturation $(NH_4)_2SO_4$; D, 20 m*M* Tris-HCl pH 7.5, 1 m*M* EDTA, 2 m*M* 2-mercaptoethanol, 10% glycerol; E, 20 m*M* Tris-HCl pH 7.0, 1 m*M* EDTA, 2 m*M* 2-mercaptoethanol 20% glycerol.

The induced culture was centrifuged 15 min at 10 000 *g*. The pellet was washed with a buffer containing 25 m*M* Tris-HCl pH 7.8, 1 m*M* EDTA, 1 m*M* phenylmethylsulphonyl fluoride and 1 m*M* 2-mercaptoethanol. Cells were lysed in a French Press and centrifuged as above to eliminate cellular debris. The supernatant was loaded at 1 ml min^{-1} onto a 4-ml DEAE-cellulose column previously equilibrated with buffer B. The column was washed with buffer B until the absorbance decreased below 0.05 and subsequently eluted by increasing KCl concentration in buffer B stepwise to 0.05, 0.30 and 0.50 *M*. As a control, an extract of non-transformed *E. coli* was

chromatographed in a column of DEAE-cellulose under the same conditions. Fractions with MDH activity were pooled and loaded onto a 4.5-ml Phenyl Sepharose column equilibrated with buffer C. After washing the column, elution of bound proteins was performed by increasing buffer D stepwise to 25% and 50%. Finally, MDH was eluted by applying 100% buffer E. Active fractions were collected and loaded onto a 2.5-ml column of Reactive Red 120 equilibrated in buffer E. Elution was performed by steps of 100, 250 and 500 m*M* KCl in buffer E. Fractions with MDH activity were pooled, desalted by three cycles of concentration through Centricon PM-30 tubes and dilution with buffer E. Finally, the preparation was aliquoted and stored at −80°C until use.

Electrophoretic procedures

SDS-PAGE was carried out according to the method of Laemmli (1970) in 10% (w/v) polyacrylamide gels. The molecular mass of a given polypeptide was estimated by comparing its electrophoretic mobility to the following standards: BSA (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde-3-P dehydrogenase (39.0 kDa), carbonic anhydrase (29.0 kDa) and β-lactalbumin (14.2 kDa).

Immunological detection

Immunodetection by Western blot was done following the method described by Bollag et al. (1996). Crude extracts or purified MDH were subjected to denaturing PAGE, transferred to nitrocellulose membranes and probed with affinity-purified antipineapple leaf cytosolic MDH IgG (Cuevas and Podestá 2000). Antigenic polypeptides were visualized through alkaline phosphatase-tagged secondary antibodies.

Assay of activity

MDH activity was assayed spectrophotometrically at 30°C in the OAA-reducing direction in a medium containing 50 m*M* imidazole-HCl pH 7.5, 0.15 m*M* NADH and 200 μ*M* OAA. The reverse reaction was followed in a coupled assay with 50 m*M* imidazole-HCl pH 7.5, 30 m*M* L-malate, 2 m*M* NAD^+, 50 m*M* L-glutamate and 20 units porcine heart glutamic-oxalacetic transaminase. One unit (U) is defined as the amount of enzyme catalysing the production or consumption of 1 μmol NADH min^{-1}. For the pH-dependence studies 20 m*M* imidazole-HCl, 50 m*M* Bis-Tris Propane or a mix of 50 m*M* Bis-Tris Propane and 50 m*M* MES were used. Reactions were started by the addition of the enzyme preparation. For the determination of the kinetic parameters for OAA, NAD^+ and L-malate, the respective fixed co-substrate concentrations were: 0.15 m*M* NADH, 30 m*M* L-malate, and 2 m*M* NAD^+. Data were analysed using a non-linear, least-square regression computer kinetics program developed by Brooks (1992). K_i values were determined according to Dixon and Webb (1979). To determine the K_m for NADH, a starting NADH concentration of 0.1 m*M* (at pH 6.0) or 0.034 m*M* (at pH 8.2) was used. The reaction was left to proceed until completion and data were analysed using the integrated Michaelis-Menten equation (Dixon and Webb 1979). Under these conditions, the activity in the opposite direction was negligible. Measurements of activity at pH 6.8 were carried out in 20 m*M* imidazole-HCl, and at pH 8.2 in 50 m*M* Bis-Tris propane. K_i values for OAA were calculated from plots of 1/activity versus OAA concentration as described by Dixon and Webb (1979) for enzymes that show substrate inhibition.

Changes in absorbance were linear during the time taken for an assay and the activity was proportional to the amount of enzyme used. During the studies of the pH effects on activity, it was checked that the substrate concentrations were saturating at the different pH values tested. Similar care was taken in the temperature studies, in which the activity was assayed at several points between 10 and 70°C. In all cases, results presented are the average of at least 3 independent measurements.

Results

Molecular cloning, expression and purification of cytosolic MDH from *M. crystallinum*

The MDH cloned in the expression vector was sequenced completely to ascertain that no mutations had been introduced during the amplification reaction. The sequence data showed an identity of 100% with the published sequence of the MDH from *M. crystallinum* (Ocheretina and Scheibe 1997).

Recombinant *E. coli* cells contained high levels of a 39.5-kDa polypeptide, which was recognized by anti-pineapple leaf cytosolic MDH IgG (Fig. 1A). After expression of recombinant MDH in *E. coli*, the enzyme was purified 19-fold with a total yield of 57%. A single MDH activity peak was observed in each of the three chromatographic procedures. As a control, a crude extract of non-transformed *E. coli* was loaded in the DEAE-cellulose column to establish the elution pattern of endogenous MDH. Figure 2 shows that both recombinant and endogenous MDHs are separated in the first chromatographic step, since the *E. coli* enzyme did not interact significantly with the DEAE-cellulose column while the recombinant enzyme elutes at 50 m*M* KCl. The final preparation had a specific activity of 31.7 U mg^{-1}. The high expression levels and total yield of the purification contrast with the relatively low specific activity of the purified protein as compared to other plant MDHs (Gietl 1992, Cuevas and Podestá 2000).

The purified recombinant MDH presented a subunit molecular mass of 39.5 kDa in SDS-PAGE (Fig. 1B). This value corresponds to that observed for the cytosolic but not the mitochondrial or microbody enzymes isolated from other species, which display subunit sizes ranging from 33 to 38 kDa (Walk and Hock 1978, Gietl 1992, Cuevas and Podestá 2000). Antibodies raised against the cytosolic pineapple leaf MDH were used to immunolocalize MDH in Western blots. These antibodies

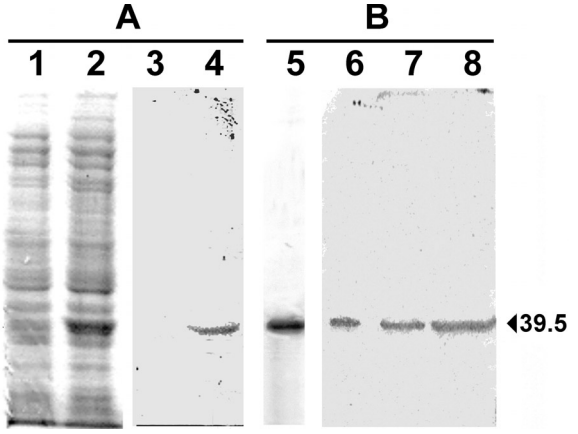

Fig. 1. Panel A: electrophoretic analysis of *M. crystallinum* expression in transformed *E. coli* cells. Twenty micrograms of protein were loaded in each lane. Lanes 1 and 2: Coomassie blue-stained SDS-PAGE of wild-type and transformed *E. coli* crude extracts, respectively. Lanes 3 and 4: Western blots of the same extracts. Panel B: SDS-PAGE and Western blot of purified *M. crystallinum* MDH and controls. Lane 5 contains 5 μg purified recombinant MDH. Lanes 6–8: Western blot of the purified recombinant MDH (1 μg), *M. crystallinum* leaf crude extract (20 μg) and pineapple leaf crude extract obtained under denaturing conditions (10 μg), respectively. Transferred membranes were probed with a 1/100 dilution of affinity-purified antipineapple leaf cytosolic MDH IgG.

showed cross-reaction with the purified recombinant enzyme and a leaf extract of *M. crystallinum* in the CAM mode (Fig. 1B, lanes 6 and 7, respectively), but not with a crude extract from non-transformed *E. coli* (Fig. 1A, lane 3).

Kinetic characterization pH-dependence of MDH activity

The effect of pH on MDH activity was assayed in different buffer systems: 20 m*M* imidazole, 50 m*M* Bis-Tris Propane or a mixture of 50 m*M* MES and 50 m*M* Bis-Tris Propane. The OAA-reducing MDH activity was dependent on assay pH, displaying a maximum in the range of 7.5–9.5 (Fig. 3) with the first two buffers, while

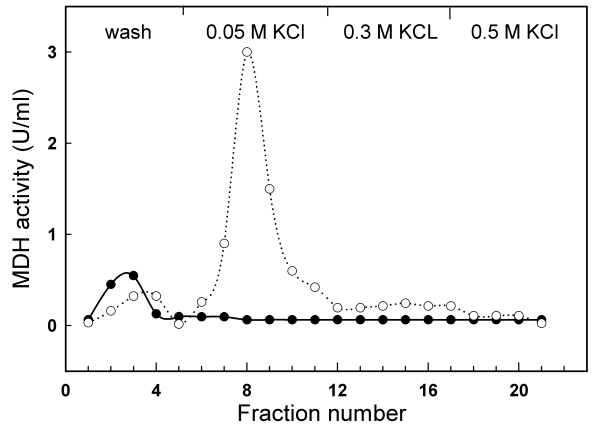

Fig. 2. Elution profile in a DEAE-cellulose column of *E. coli* (●) and recombinant (○) MDH.

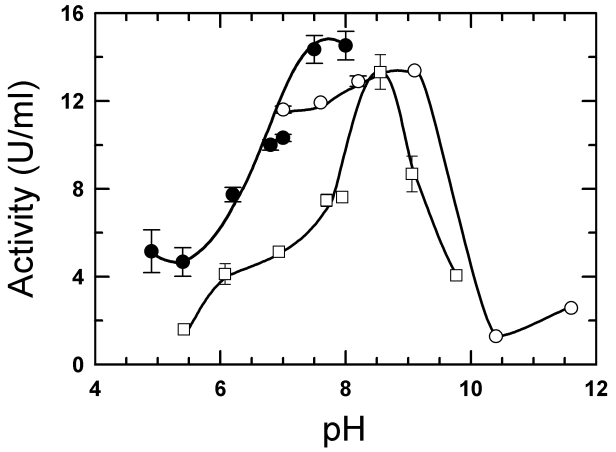

Fig. 3. Dependence of purified MDH activity on pH. Activity was assayed in the OAA-reducing direction using 20 m*M* imidazole (●), 50 m*M* Bis-Tris propane (○) or 50 m*M* Mes/50 m*M* Bis-Tris propane (□) as buffer. Bars indicate standard deviation.

in the presence of MES/Bis-Tris Propane a more defined peak of activity was observed at pH 8.5.

Thermal stability

MDH activity and stability was followed at pH 7.5 in the OAA-reducing direction. MDH activity was maximum at 50°C and the activation energy, calculated from an Arrhenius plot, was of 28.8 kJ mol^{-1} (Fig. 4A, inset). Results demonstrated that the enzyme was stable up to 50°C; higher temperature values resulted in a sharp decay in activity (Fig. 4B).

Kinetic parameters

The kinetic parameters were determined at two different pH values reflecting the changes in cytosolic pH during the day-night cycle of CAM plants. Results are summarized in Table 1. Comparison of OAA reduction with malate oxidation showed that OAA reduction is particularly favoured at pH 6.8, but it is still higher than malate oxidation at pH 8.2. The K_m value for NADH at pH 6.8 was more than twice that observed at pH 8.2, although it persisted in the low micromolar range. The K_m for OAA was significantly (5-fold) lower at pH 6.8. At this pH no activity in the malate oxidation direction could be detected, probably as a consequence of the low overall specific activity. K_m values observed for the *M. crystallinum* MDH resemble those obtained with pineapple MDH for both NAD and NADH (Cuevas and Podestá 2000). On the other hand, *M. crystallinum* MDH shows greater affinity for OAA but lower for malate. Another important difference between pineapple and ice plant cytosolic MDHs is that the *M. crystallinum* MDH showed inhibition by excess OAA, considerably more marked at pH 6.8 than 8.2 (K_i 225 μ*M* versus 775 μ*M*, respectively, see Table 1). The physiological meaning, if any, of this effect is not evident at present.

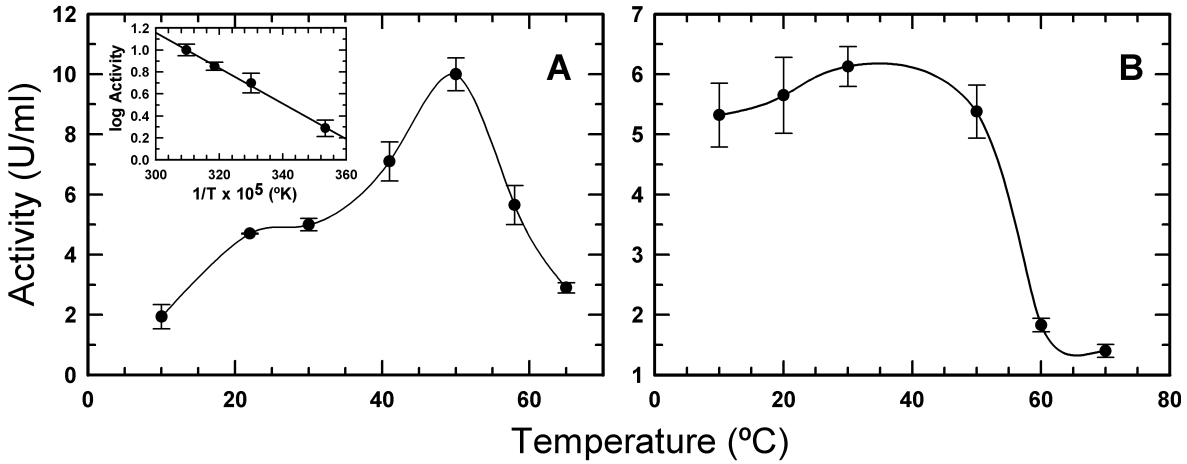

Fig. 4. Dependence of recombinant MDH activity on temperature. (A) Effect of the temperature on recombinant enzyme OAA reduction activity. The determinations were done at the temperatures shown in the graph at pH 7.5. The assay began with the addition of purified MDH. The inset shows an Arrhenius plot of the data. (B) Effect of the temperature on the stability of MDH. The purified enzyme was pre-incubated in assay buffer for 5 min at each temperature and then activity was assayed at 30°C. Bars indicate standard deviation.

Substrate specificity

The substrate specificity of recombinant MDH was investigated testing various other α-ketoacids as substrates at a concentration of 1 m*M* (Fig. 5) or 0.2 m*M* (results not shown). Results were compared with the activity in the presence of similar OAA concentrations. Experiments were performed at pH 6.8 and 8.2. As depicted in Fig. 5, the highest specific activity of MDH was measured with OAA, indicating that recombinant MDH is highly specific for OAA. Except for 3-bromopyruvate, higher relative activities were observed at pH 8.2, notably when pyruvate and phenylpyruvate were tested. Essentially the same results were observed when final concentration of all substrates was 0.2 m*M* (not shown), a concentration at which the OAA inhibitory effect is negligible. The same experiment was repeated with purified pineapple leaf MDH but no activity was recorded with substrates other than OAA (not shown).

Discussion

Induction of CAM in *M. crystallinum* by high salinity in the rooting medium is evidenced by a substantial CO_2 uptake and malic acid synthesis in the dark and a decrease in stomatal conductance during deacidification in the subsequent light period. Studies of the enzymatic content of this species have demonstrated a 2-fold increase in the extractable activity of MDH in response to high salinity in the culture medium (Holtum and Winter 1982, Winter et al. 1982).

Although MDH plays a central role in CAM, there is little information about the function of the different MDH isozymes existing in these plants. As part of an effort to fully understand the role of the various MDH isozymes in CAM, we have cloned and characterized the cytosolic MDH from leaves of *M. crystallinum* performing CAM photosynthesis.

The recombinant MDH was purified 19-fold to a final specific activity of 32 U mg^{-1} protein. This value is very low, taking into account the high levels of extractable MDH activity present in a leaf crude extract (Winter

Table 1. Kinetic constants of the cytosolic MDH from *M. crystallinum*. Experiments were carried out in 20 m*M* imidazole-HCl, pH 6.8, or 50 m*M* Bis-Tris propane, pH 8.2. Values represent the mean of 3 independent replicates ± SD. ND, not detected.

Parameter	pH 6.8	pH 8.2
V_{max} (μmol min^{-1}mg^{-1})	29.3 ± 0.7	35.6 ± 2.9
K_m OAA (μ*M*)	8.8 ± 0.9	44.9 ± 3.9
K_i OAA (μ*M*)	225 ± 7	775 ± 106
K_m NADH (μ*M*)	36.6 ± 0.5	13.6 ± 2.0
V_{max} (μmol min^{-1}mg^{-1})	ND	1.20 ± 0.06
K_m malate (m*M*)	–	34.1 ± 3.3
K_m NAD^+ (μ*M*)	–	319 ± 39
Ratio: OAA reduction/malate oxidation		28.4

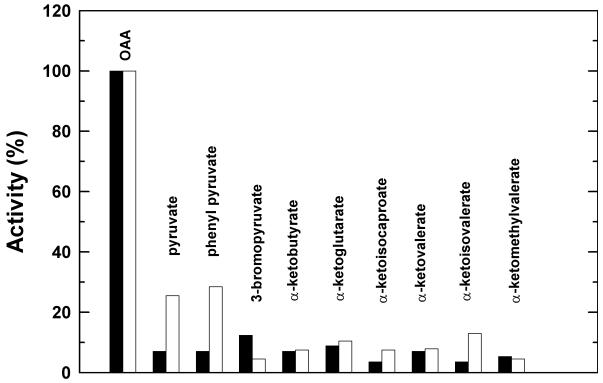

Fig. 5. Substrate specificity of MDH at pH 6.8 (black bars) and pH 8.2 (white bars). The reduction of α-ketoacids was followed spectrophotometrically at 30°C as the decrease in absorbance at 340 nm following the addition of 1 m*M* of each α-ketoacid.

et al. 1982, Cuevas and Podestá 2000), a specific activity of nearly 1600 U mg^{-1} protein for the purified pineapple cytosolic MDH, and the intense band obtained in a denaturing PAGE analysis of the purified recombinant *M. crystallinum* MDH. A non-efficient assembly of the two subunits or improper folding of these (a process in which some chaperone-type protein could be involved) could be among the reasons for the low specific activity. Alternatively (or additionally), an inaccurate polymerization of the subunits (i.e. formation of tetramers instead of dimers) might lead to an enzyme form with decreased specific activity. Previous observations support the idea that both a dimeric structure and tertiary structure are essential for MDH activity (Breiter et al. 1994). Since, aside of V_{max}, K_m values for the substrates of both direct and reverse reactions are within published values for other MDHs (Gietl 1992, Cuevas and Podestá 2000), we conclude that the purified recombinant MDH low specific activity can be ascribed to a poor recovery of active enzyme, rather than the purification of a modified form of the enzyme.

That the MDH that has been cloned and characterized corresponds to the cytosolic isoform is suggested by: (1) sequence data of the cloned cDNA that showed a 100% of homology with the published sequence of the *M. crystallinum* (Ocheretina and Scheibe 1997); (2) a molecular mass consistent with those reported for other cytosolic MDH but not with those of the mitochondrial or microbodies plant MDH (Gietl 1992, Cuevas and Podestá 2000); (3) recognition of the purified enzyme by antibodies raised against the cytosolic MDH from *A. comosus*; and (4) similarity of its kinetic parameters with those observed for other plant cytosolic isozymes (Gietl 1992, Cuevas and Podestá 2000). Whether this isozyme is responsible for the observed increase in total MDH remains to be established. Preliminary isoelectrofocusing experiments (M. Martín, K.E.J. Trípodi, F.E. Podestá, unpublished results) suggest that a single cytosolic isozyme exists in CAM-performing *M. crystallinum*, as has been suggested for the constitutive CAM plant pineapple (Cuevas and Podestá 2000).

As with other MDHs characterized, the recombinant enzyme activity is favoured in the OAA reduction direction as compared to malate oxidation. At pH 6.8 the enzyme did not show any activity with malate and NAD^+ as substrates. However, when the pH was increased to 8.2 activity in the direction of malate oxidation is restored, but it is still about 30 times lower than OAA reduction. These results implicate that maximum activity of *M. crystallinum* MDH coincides with conditions prevailing during the night period in the cytosol of mesophyll cells (Heber et al. 1996), and that its properties are well suited to serve in the reduction of the OAA generated by the action of PEPCase. A comparison with its counterpart purified from pineapple leaf shows some differences, notably that the latter does have some activity with malate as substrate at pH 6.8. The physiological relevance for this difference could lie in the fact that in pineapple an MDH-catalysed step that is necessary during the day involves malate oxidation to OAA, while there is no need for such a metabolic step in *M. crystallinum*, in which the malate released from the vacuole is directly decarboxylated by malic enzyme. The fact that both enzymes, while being cytosolic, show different properties, is also indicated by a dissimilar response to temperature. Thus, while pineapple MDH activity increases up to around 80°C (Cuevas and Podestá 2000), *M. crystallinum* MDH is rapidly inactivated over 50°C. In addition, pineapple cytosolic MDH is not active with α-ketoacids other than OAA and is not inhibited by excess OAA. Hence, it seems possible that different isozymes have been recruited during the emergence of both types of CAM plants, and that these differ not only in their decarboxylating mechanism but also in that one (*M. crystallinum*) is inducible while the other (pineapple) is not. Whether these different isozymes are present in variable amounts in all CAM plants and how their expression levels are regulated during CAM induction is a matter that awaits further experimentation.

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