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Characterization of NADP-malic enzyme from two species of Chenopodiaceae: *Haloxylon persicum* (C_4) and *Chenopodium album* (C_3)

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Abstract

Kinetic and structural properties of NADP-malic enzyme (NADP-ME, E.C. 1.1.1.40) were analyzed from two members of the Chenopodiaceae family, *Haloxylon persicum*, an unusual C_4 NADP-ME type tree species native to Central Asia Deserts, and *Chenopodium album*, a C_3 weedy species. Two isoforms of the enzyme, with molecular masses of 67 and 72 kDa and p*I*s (isoelectric points) of 6.75 and 5.85, respectively, were found in crude extracts of *H. persicum* by western blots using an antibody against the 62 kDa isoform from maize leaves. In *C. album*, a C_3 plant, only one immunoreactive isoform, of 72 kDa was found, with an isoelectric point of 5.85. The 67 kDa isoform was purified from *H. persicum* shoots by precipitation with crystalline ammonium sulfate, anion-exchange, adsorption and affinity chromatographies. The 72 kDa isoform was purified from *C. album* in the same way, except that the adsorption chromatography step was omitted. The 67 kDa polypeptide of *H. persicum* was identified as a C_4 isoform based on its high specific activity, low K_m values for malate and NADP, and characteristics of its pH optima. However, this isoform is very different in molecular mass and isoelectric point from the previously characterized C_4 isoform found in C_4 monocots and C_4 *Flaveria* species. The 72 kDa isoform from *C. album* has kinetic properties which are distinct from the C_4 isoform of *H. persicum*, and characteristic of the low activity constitutive form found in various plants. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Haloxylon persicum*; *Chenopodium album*; Chenopodiaceae; C_4; C_3; NADP-ME

1. Introduction

A major degree of diversity in plant metabolism occurs in photosynthetic carbon assimilation. Different mechanisms of photosynthesis have evolved to enable plants to grow under severe stress, i.e. under drought, saline and mineral-deficient soils, and temperature extremes. C_4 photosynthesis is one such metabolic adaptation, which increases efficiency under limiting CO_2 caused by various stresses, and enables a greater carbon gain than is possible in C_3 plants (Edwards & Walker, 1983). The family Chenopodiaceae has species which are adapted to various habitats including extremely harsh conditions, and the occurrence of C_4 photosynthesis in this family is second only to Poaceae. There is diversity of photosynthetic types in Chenopodiaceae, including C_3 species and different C_4 subgroups, and these differences are related to geographical and climatic distribution (Pyankov, Kuzmin, Demidov & Maslov, 1992; Pyankov, Voznesenkaja, Kondratschuk & Black, 1997). Chenopodiaceae is a family composed of approximately 100 genera and 1500 species, including annuals, perennial herbs, and also, trees or shrubs., with over 200 species possessing Kranz anatomy and performing C_4 photosynthesis. These species have a broad ecological amplitude. For example, they can inhabit lowland deserts, like the

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Sahara, and regions in the high mountains (4000–4500 m in Tibet).

The most tree-like genus of Chenopodiaceae is *Haloxylon*; *H. persicum* reaches a height of 6 m and forms forests in some arid, saline areas where it is an important source of wood. This species is broadly distributed in Central Asia, the Middle East (Karakum and Kuzylkum deserts), Iran, Afganistan, northwest China (Kashgar and Dzhungar regions) and near eastern deserts (Botschantsev, 1953; Butnik, Nigmanova, Paizieva & Saidov, 1991; Iljin, 1936; Netchaeva, Vasilevskaja & Antonova, 1973; Nikitin, 1966). It is very tolerant to environmental extremes in temperature, light and water availability. The main photosynthetic organ of *Haloxylon* species is the cylindrical shoot which performs C_4 photosynthesis; the cotyledons perform C_3 photosynthesis (Pyankov et al., in press). The photosynthetic shoots have a Salsoloid type Kranz anatomy (Voznesenskaja & Gamaley, 1986) with a $[^{13}C]/[^{12}C]$ ratio similar to C_4 plants (Akhani, Trimborn & Ziegler, 1997; Winter, 1981; Zalensky & Glagoleva, 1981), primary $[^{14}C]CO_2$ fixation in C_4 acids, mainly malate (Glagoleva, Zalensky & Mokronosov, 1978; Pyankov, 1984, in press; Pyankov & Vakhrusheva, 1989; Zalensky & Glagoleva, 1981), and a high activity of C_4 photosynthetic enzymes with NADP-ME type C_4 photosynthesis (Pyankov et al., 1992, in press). Due to the unique features of this species, it is of interest to characterize the biochemical and molecular properties of its C_4 pathway enzymes. One of the critical steps in the evolution of C_4 photosynthesis is the development of high activity C_4 acid decarboxylases, one of which is NADP-malic enzyme. NADP-malic enzyme (E.C. 1.1.1.40), which is ubiquitous in plants, catalyses the decarboxylation of L-malate to pyruvate and CO_2. A constitutive form may fulfil diverse housekeeping roles because of its universal presence in plants. These functions may be anaplerotic in some tissues, providing NADPH and pyruvate for biosynthesis, or catabolic in other tissues, participating in respiration of these products (Edwards & Andreo, 1992). The purpose of the current study was to purify and compare the physical, immunological and kinetic properties of NADP-ME from the C_4 chenopod *H. persicum* versus the C_3 chenopod *Chenopodium album*. *C. album* is a widely occurring agricultural weed which has typical C_3 type photosynthetic characteristics, including a lower temperature optimum for photosynthesis compared to C_4 plants (Sage & Pearcy, 1987).

2. Results

Crude protein extracts from *H. persicum* shoots and *C. album* leaves were prepared and assayed for NADP-ME activity. For *H. persicum*, the activity was 2.24 nkat mg^{-1} soluble protein, while the activity for *C. album* was only 0.21 nkat mg^{-1}; thus the activity in the C_4 plant was about 10 fold higher. In comparison, in the genus *Flaveria* of the Asteraceae family, *F. bidentis* (C_4) leaves had an activity of 14.14 nkat mg^{-1} compared to 0.19 nkat mg^{-1} for *F. pringlei* (C_3).

Total protein was extracted from *H. persicum* shoots and *C. album* leaves using phenol, and separated by electrophoresis on denaturing gels in order to perform western blots for NADP-ME. These show that extracts from *H. persicum* shoots have two immunoreactive forms corresponding to 72 and 67 kDa (Fig. 1A, lane C). The 67 kDa band, which is more intense than the 72 kDa band, has a larger molecular mass than the 62 kDa photosynthetic form from maize leaves (Fig. 1A, lane A). Extracts from the C_3 plant, *C. album*, had one

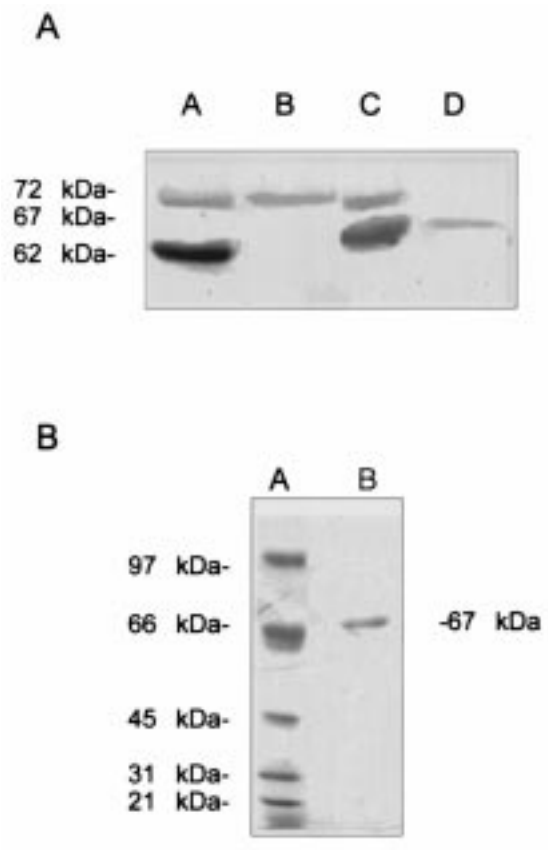

Fig. 1. (A) Immunoblot analysis of crude extracts from *Zea mays* (lane A) and *C. album* leaves (lane B), *H. persicum* shoots (lane C) and NADP-ME purified from *H. persicum* shoots (lane D). Total protein from *H. persicum* and *C. album* (30 μg) and *Zea mays* (10 μg) and purified NADP-ME from *H. persicum* shoots (3 μg) was analysed by western blot probed with purified anti-maize 62 kDa NADP-ME IgG. The lines indicate the position of the 72, 67 and 62 kDa immunoreactive bands. (B) Silver stained SDS-PAGE with the purified NADP-ME from *H. persicum* shoots (lane B) (3 μg). Lane A was loaded with standard molecular weight markers as identified in the figure.

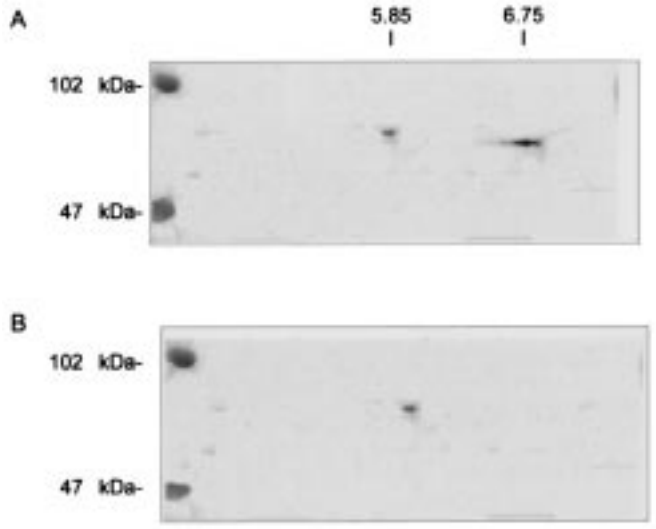

Fig. 2. Two dimensional western blots of total protein extracts (150 μg) from *H. persicum* shoots (A) and *C. album* leaves (B). The membranes were treated with purified anti-maize 62 kDa NADP-ME. The estimated isoelectric points for the proteins are shown in the figure. The molecular weight of the protein standards are also shown on the left side of the gel.

reactive band which corresponds to the higher mass form of *H. persicum* (72 kDa) (Fig. 1A, lane B).

Two-dimensional PAGE coupled with western immunodetection was performed with total protein extracted from *H. persicum* shoots and *C. album* leaves (Fig. 2). Again, the total protein extracted from *H. persicum* shoots exhibited two immunoreactive proteins: the 72 kDa polypeptide, with an apparent p*I* of 5.85, and the 67 kDa polypeptide, with an apparent p*I* of 6.75 (Fig. 2A). In contrast, the 2-D gels with proteins from *C. album* leaves showed only one immunoreactive band corresponding to a 72 kDa polypeptide with an apparent p*I* of 5.85 (Fig. 2B). These results show that the NADP-ME in *C. album* leaves has the same mobility as the 72 kDa form from *H. persicum* as demonstrated on the 2-D gel with respect to isoelectric point and molecular mass.

The enzyme from *H. persicum* shoots was purified about 440-fold with a recovery of 13% by conventional techniques to a final activity of 985 nkat mg^{-1} protein. Fig. 1B shows a silver stained SDS-PAGE with the purified enzyme; the western blot analysis showed only the 67 kDa form (Fig. 1A, lane D). Apparently, the less abundant 72 kDa form does not copurify with the 67 kDa form; the reason for its absence has not been determined. For comparison with the enzyme from *H. persicum*, the enzyme from *C. album* was purified 24 times (final activity of 5 nkat mg^{-1}, recovery 17%) and kinetic analyses were performed.

When the enzyme from *H. persicum* was assayed in the presence of 0.04 mM L-malate, 0.5 mM NADP and 10 mM Mg^{2+}, it exhibited a pH/activity profile with a maximum occurring at about pH 7.0 and half maximal activity at pH 6.5 and 8.1. At 4 mM L-malate (100 fold higher concentration), the maximum was shifted to a more basic pH value, around 8.2, with half maximal activity at pH 7.1 and 8.9. In contrast, when the same assays were performed with the enzyme from *C. album*, the pH optimum was 7.8 when the concentration of L-malate was 4 mM (half maximal activity at pH 6.0 and 8.5), and showed very little shift to a more acidic pH optimum value when the concentration of L-malate was 0.04 mM (7.55, half maximal activity at pH 7.25 and 7.85).

As in the case of other sources of NADP-ME (Drincovich, Iglesias & Andreo, 1991; Edwards & Andreo, 1992), both enzymes required a divalent cation, Mg^{2+} or Mn^{2+}, for activity. The saturation curves of the enzymes for Mg^{2+} or Mn^{2+} in the presence of saturating concentrations of NADP (0.5 mM) and L-malate (4 mM), showed a nonhyperbolic response with biphasic Lineweaver–Burk plots, suggesting the existence of at least two different binding sites on the enzyme. From these results, two V_{max} and K_m values could be calculated for each cation, with Mn^{2+} having lower K_m values and higher V_{max}/K_m ratios than Mg^{+2} (Table 1), indicating that both of these sources of NADP-ME have a preference for Mn^{2+} over Mg^{2+}. However, the physiological role for these metal ions remains uncertain in the absence of precise data on their relative concentration around the enzyme in vivo. The V_{max}/K_m ratios for the C_4 plant were considerably higher than the values obtained from the C_3 enzyme when both Mg^{2+} and Mn^{2+} were used as cofactors, which is in agreement with the previous data for NADP-ME from C_3 and C_4 plants (Edwards & Andreo, 1992).

Table 1
Kinetic parameters of NADP-ME from *H.* persicum and *C. album* using Mg^{2+} and Mn^{2+} as cofactors. V_{max} is in nkat mg^{-1}[a]

	Cation	V_{max}	K_m (mM)	V_{max}/K_m
H. persicum	Mg^{2+}	192 ± 10	$1.1 \times 10^{-2} \pm 1.2 \times 10^{-3}$	1.8×10^{4}
		807 ± 8	$3.5 \pm 2.3 \times 10^{-2}$	2.3×10^{2}
	Mn^{2+}	133 ± 5	$4.5 \times 10^{-5} \pm 5.8 \times 10^{-6}$	2.9×10^{6}
		713 ± 12	$2.1 \times 10^{-2} \pm 4.7 \times 10^{-3}$	3.4×10^{4}
C. album	Mg^{2+}	1.1 ± 0.2	$4.6 \times 10^{-3} \pm 3.2 \times 10^{-4}$	2.4×10^{2}
		3.0 ± 0.3	$2.8 \times 10^{-1} \pm 1.5 \times 10^{-2}$	1.1×10^{1}
	Mn^{2+}	1.1 ± 0.1	$1.9 \times 10^{-4} \pm 3.6 \times 10^{-5}$	5.8×10^{3}
		5.2 ± 0.3	$5.0 \times 10^{-2} \pm 8.1 \times 10^{-3}$	1.0×10^{2}

[a] The range of concentrations used in assays for Mg^{2+} and Mn^{2+} were 4×10^{-3} to 10 mM and 10^{-3} to 1 mM, respectively. The kinetic assays were performed at saturating NADP (0.5 mM) and L-malate (4 mM) concentrations at pH 8.0. Each experiment was performed in duplicate.

The saturation curves for reaction rates, as a function of free NADP in the presence of saturating concentration of L-malate (4 mM) and Mg^{2+} (10 mM), were hyperbolic for both the 67 kDa enzyme from *H. persicum* and the 72 kDa enzyme from *C. album*. From these data, a K_m value of 3.15 ± 0.05 μM was calculated for the C_4 enzyme and 28.4 ± 0.2 μM for the C_3 plant enzyme. When NAD was used as a cofactor no activity was measured.

For NADP-ME from *H. persicum*, when the activity was assayed as a function of free L-malate at pH 8.0, an hyperbolic response was obtained, with a K_m value of 80 ± 8 μM (Fig. 3). However, when the assays were performed at pH 7.0, inhibition was observed at high concentrations of L-malate (Fig. 3). At the lower pH, the K_m value for malate was 35 ± 2 μM. For the enzyme from *C. album*, when the experiments were performed at pH 8.0 the K_m value for malate was 0.88 ± 0.01 mM, one order of magnitude higher than the value obtained under the same conditions for the

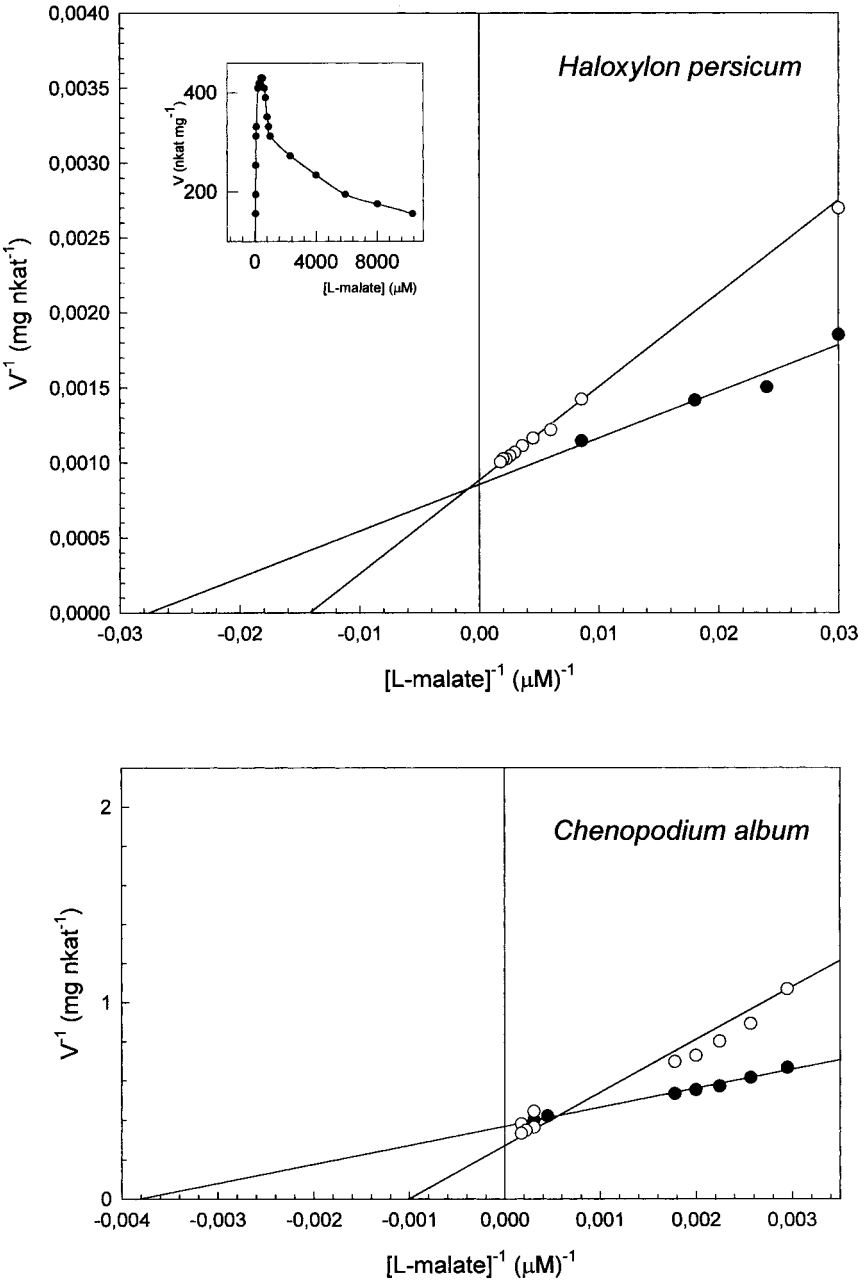

Fig. 3. Lineweaver-Burk plots of NADP-ME activity as a function of L-malate concentration. Enzyme activity from *H. persicum* and *C. album* was determined at different L-malate concentrations at pH 8.0 (○) or pH 7.0 (●). The figure inset shows the plot of v vs. [L-malate] at pH 7.0 for *H. persicum* NADP-ME, showing that substrate inhibition occurs at concentrations higher than 250 μM. The double reciprocal plot in this case was done at concentrations of L-malate lower than 250 μM.

enzyme from *H. persicum* (Fig. 3). At pH 7.0, no inhibition was observed and the K_m of malate was 0.30 ± 0.04 mM (Fig. 3). For both enzymes assayed, when 2.5 mM succinate was added to the assay medium at the two pH values there was no effect on activity (not shown), which is contrary to the activating effect observed for NADP-ME from some other sources including potato tuber, sugarcane leaves and various fruit (Edwards & Andreo, 1992). Nevertheless, this activating effect was not observed for the enzyme from other C_3 or C_4 monocots (Edwards & Andreo, 1992), this being coincident with our results.

3. Discussion

While the biochemical and physical properties of the C_4 isoform of NADP-ME have been studied in sugarcane and maize (Edwards & Andreo, 1992; Maurino et al., 1997; Spampinato, Casati & Andreo, 1998), the enzyme has not been studied in the family Chenopodiaceae which is second in importance to the Poaceae for the occurrence of C_4 photosynthesis. In this paper, we studied the structure and kinetic properties of NADP-ME from two members of Chenopodiaceae, *H. persicum*, an important C_4 species of the Central Asian deserts, and *C. album*, a weedy C_3 species. The crude leaf extract from *C. album* has low NADP-ME activity which is attributed to a higher molecular mass form (72 kDa) of the enzyme as demonstrated on western blots following one and two dimensional electrophoresis (Figs. 1A and 2). This is the only form found in leaves of wheat (C_3) (Casati, Spampinato & Andreo, 1997) and C_3 *Flaveria* species (Drincovich et al., 1998). This form is also found in various organs in C_4 plants, including roots, etiolated leaves, and, as a minor form, in green leaves of maize (Maurino et al., 1997) and in roots, stems, and as a minor form in leaves of C_4 *Flaveria* (Drincovich et al., 1998). The 72 kDa form in *C. album* has a p*I* of 5.85, which is similar to the form of the protein found in maize roots and etiolated leaves (Maurino et al., 1997). Thus, the form in *C. album* has physical properties expected of the constitutive form.

In contrast, *H. persicum* has high activity of NADP-ME in the photosynthetic shoots. Western blots show that the major isoform in shoots is 67 kDa, with a p*I* of 6.75. This differs greatly from the C_4 isoform found in bundle sheath chloroplasts in maize, which is 62 kDa with a p*I* of 4.6 (Maurino et al., 1997). Also, the C_4 form in C_4 species of *Flaveria* (*F. bidentis* and *F. trinervia*) of the family Asteraccae is 62 kDa. Yet, as discussed below, analysis of the kinetic properties of the 67 kDa enzyme from *H. persicum* indicates it has properties similar to the 62 kDa form found in maize. This, along with a predominant labelling of bundle sheath chloroplasts of *H. persicum* by immunolocalization with antibody to NADP-ME (Vosnesenskaya, Franceschi & Edwards, unpublished), indicates that the 67 kDa protein is the C_4 isoform in this species. It is of interest that some intermediate and C_4-like species of *Flaveria* have, in addition to the 62 and 72 kDa forms, a third form of NADP-ME which, although not yet characterized, is similar in size to the major C_4 form in *Haloxylon* (Drincovich et al., 1998).

The specific activity of the purified 67 kDa enzyme from *H. persicum* was about 200 times higher than the enzyme purified from *C. album*. While this difference may be due to less than complete purification of the *C. album* enzyme, it could, at least in part, be due to the C_4 form having a higher specific activity than the C_3 form. There is some previous evidence that the C_4 isoform has a higher specific activity; i.e. the activity of the 62 kDa form purified from maize leaves is about 30 fold higher than that of the 72 kDa form purified from etiolated maize leaves (Maurino, Drincovich & Andreo, 1996), and about 30 fold higher than the 72 kDa form purified from wheat (Casati et al., 1997).

When the 67 kDa form from *H. persicum* was studied as a function of pH, it exhibited a behavior similar to the C_4 form from maize and sugarcane (Edwards & Andreo, 1992). The *Haloxylon* enzyme had a higher pH optimum, it was inhibited by high concentrations of L-malate at lower pH values, showing a shift to a more acidic pH optimum when the assay was done using subsaturating concentration of the substrate. In contrast, when the same experiment was done using the enzyme from *C. album*, the pH optimum was more acidic than the value obtained for the C_4 enzyme, and it was not inhibited by L-malate, showing very little shift to a more acidic pH optimum when decreasing the L-malate concentration. It has been suggested previously that the C_4 isoform may have a group, with a p*Ka* value of 8.4, which controls malate binding and catalysis, maintaining a proper conformation of the enzyme and which would be responsible for the high activity at high pH (Edwards & Andreo, 1992).

With respect to L-malate, kinetics vary from hyperbolic, to negative cooperative, to sigmoidal, depending on the source of the enzyme and the pH of the assay (Edwards & Andreo, 1992). For NADP-ME from *H. persicum* and *C. album*, at pH 8.0 an hyperbolic response was obtained (Fig. 3), with $K_m s$ of 80 ± 8 and 880 ± 10 μM, respectively. These values differed by an order of magnitude, in agreement with previous results with the enzyme from C_3 versus C_4 plants (Edwards & Andreo, 1992). When the assays were performed at pH 7.0, saturating levels of malate inhibited catalysis of the enzyme from *H. persicum* (as in sugarcane and maize; Edwards & Andreo, 1992), while the enzyme from *C. album* was not inhibited by the substrate, a

finding also reported for the enzyme from wheat (Casati et al., 1997). The K_ms for L-malate at this pH value were 35 ± 2 μM for NADP-ME from *Haloxylon* persicum and 300 ± 40 μM from *C. album*, differing by an order of magnitude and being lower than the values at pH 8.0. Thus, both enzymes are characterized by a strong decrease in affinity for L-malate with increasing pH. In the case of the C_4 enzyme, there is a release of inhibition by malate at high pH which may be associated with the increased pH optimum that is observed with increasing malate concentration. In some plant NADP-ME, several organic acids, like succinate, cause a marked increase in activity at limiting or saturating malate (Edwards & Andreo, 1992). This was not the case in the present study where succinate had no effect on activity of the enzyme from the two chenopod species. For NADP-NE from *H. persicum*, the enzyme is predominantly located in the chloroplasts of bundle sheath cells (Vosnesenskaya et al., unpublished). In this way, the activation by succinate of this enzyme would not have a significant physiological function, as succinate is predominantly metabolized in other compartments of the cell. Finally, succinate activation was not observed with the enzyme from some Gramineae like sugarcane (Edwards & Andreo, 1992), so the importance of such physiological effect remains to be determined.

Because of differences in degree of purification, assay conditions and questions of oligomerization in earlier studies, it was not clear whether there were any difference in the K_m for NADP between NADP-ME isolated from different sources (Edwards & Andreo, 1992). In the present study, the K_m for NADP for the enzyme from. *H. persicum* was clearly different, being one order of magnitude lower than that for the enzyme from *C. album*. Some other recent results show a similar trend, in that the K_m for NADP for the C_4 form from maize is about 4-fold lower than that of the enzyme from wheat leaves (C_3) (Casati et al., 1997), and the higher mass form of the enzyme in C_3 *Flaveria* appears to have a higher K_m for NADP than the lower mass form in C_4 *Flaveria* (Drincovich et al., 1998).

The above results indicate that increased affinities for both substrates, malate and NADP, are features of the evolution of the C_4 form of NADP-ME. The 72 kDa protein, present in both plants, seems to be constitutive as it has been described in photosynthetic and nonphotosynthetic tissues of C_3, C_3-C_4 intermediate and C_4 plants (Drincovich et al., 1998; Maurino et al., 1997; Spampinato et al., 1998) and may be involved in nonautotrophic function, for example, providing NADPH and pyruvate for lipid biosynthesis. On the other hand, the 67 kDa isoform present in *H. persicum* shoots, although different in size and p*I* comparing with the enzyme described for other C_4 plants, has a higher specific activity and increased affinities for both substrates. This may facilitate maintenance of high rates of decarboxylation of malate and delivery of CO_2 to the C_3 cycle in bundle sheath cells at relatively low substrate concentrations. NADP-ME from both the C_3 and the C_4 plants required either Mg^{2+} or Mn^{2+} as a cofactor, although the affinity for Mn^{2+} was higher for both enzymes according to the K_m values (Table 1). In our experiments, negative cooperativity, resulting in biphasic double reciprocal plots of enzyme activity versus metal ion concentration (not shown), was observed with respect to binding of Mg^{2+} and Mn^{2+} as cofactors. These results were also observed for other C_4 forms, e.g. maize (Edwards & Andreo, 1992), and C_3 forms, e,g. wheat (Casati et al., 1997), although in a number of studies, hyperbolic saturation kinetics have been reported for other plant NADP-ME (Edwards & Andreo, 1992). Mg^{2+} promotes oligomerization of the enzyme from wheat, maize and sugarcane (Iglesias & Andreo, 1990; Spampinato et al., 1998), so the biphasic kinetics could be related either to changes in the quaternary structure, or to the presence of sites with different affinity to the substrates (Edwards & Andreo, 1992). This effect was proposed for NADP-ME from pigeon liver (Hsu, 1982), and may occur through an asymmetric quaternary structure with two sets of active sites with different affinities for the cations.

To conclude, this is the first description of the biochemistry and physical properties of a C_4 enzyme in *Haloxylon*, a species with an unusual photosynthetic apparatus. Further biochemical studies and molecular analyses will be required to determine why the molecular mass of the C_4 form of NADP-ME of *H. persicum* is greater by about 5 kDa than that of the C_4 form studied in other species such as maize, sugarcane and species of *Flaveria*. The present analysis indicates that NADP-ME from *H. persicum* has similar kinetic properties at 30°C to other C_4 forms, despite different physical properties with respect to molecular mass and p*I*. Whether this difference in physical properties is of functional significance is uncertain, but it might increase its stability or improve its kinetic properties under some extreme environmental conditions experienced by this species.

4. Experimental

4.1. Plant material and enzyme purification

Plants were grown in a greenhouse, and the temperatures were maintained at 25/20°C day/night, with a maximum midday photosynthetic radiation of 1500 μmol quanta m^{-2} s^{-1} on sunny days and a photoperiod of 14/10 h under supplemental sodium vapor lighting. For the purification of NADP-ME from

Haloxylon persicum shoots, 10 g of tissue was homogenized in a blender in 150 mL of extraction buffer (buffer A) containing 100 mM Tris–HCl (pH 7.3), 10 mM $MgCl_2$, 2 mM Na_2HPO_4, 1 mM EDTA, 20% (v/v) glycerol, 10 mM 2-mercaptoethanol, 1 mM PMSF (phenyl methyl sulfonide fluoride), and 20 g PVPP (polyvinylpolypyrrolidone). The homogenate was filtered through cheesecloth and centrifuged at 9000 × *g* for 15 min. Crystalline ammonium sulfate was gradually added to the supernatant up to 40% saturation. After centrifugation at 9000 × *g* for 30 min, the supernatant was brought to 70% saturation with ammonium sulfate and centrifuged. The precipitate was dissolved in 7 mL of purification buffer (buffer B) containing 50 mM Tris–HCl (pH 7.3), 5 mM MgO_2, 0.1 mM EDTA, 10% (v/v) glycerol, 10 mM 2-mercaptoethanol, and passed through a column of Sephadex G-50 equilibrated previously with buffer B. Then eluate was applied to a column of DEAE Sepharose CL-6B equilibrated previously with the above buffer. After washing with 3 volumes of buffer B, the enzyme was eluted with a linear gradient of NaCl (0–200 mM). The fractions containing NADP-ME activity were eluted at 120 mM NaCl and were precipitated with solid ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation, dissolved in 5 ml of buffer containing 5 mM $MgCl_2$, 0.1 mM EDTA, 10% (v/v) glycerol and 10 mM 2-mercaptoethanol (buffer C) with 10 mM Na_2HPO_4, and dialyzed overnight against 500 mL of buffer C with 10 mM Na_2HP0_4. The fraction was then applied to a Hydroxylapatite column, and after washing with three volumes of buffer C plus 10 mM Na_2HPO_4, the enzyme was eluted with a nonlinear gradient of 15, 25, 40 and 50 mM Na_2HPO_4. The fractions containing the enzyme eluted at 25 mM Na_2HPO_4 and were precipitated with solid ammonium sulfate at 70% saturation. The precipitate was centrifuged and dissolved with 5 mL of buffer B, and applied to an Affi-Gel blue column. The column was washed with 3 volumes of buffer B and NADP-ME was eluted with a linear gradient of NaCl (0–400 mM). Purified NADP-ME was eluted at 300 mM NaCl and was stored at −20°C until use.

For the partial purification of NADP-ME from *Chenopodium album* leaves, 20 g of leaves were homogenized with 250 mL of buffer A and processed similar to that as described for NADP-ME from *H. persicum* shoots, except that the Hydroxylapatite column was omitted as it resulted in no further increase in purity.

4.2. Protein extraction

For assay of enzyme in crude extracts total soluble protein was extracted with a buffer containing: 0.1 M Tris–HCl pH 7.3, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10 μM leupeptin, 1 mM PMSF and 20% (v/v) glycerol. The samples (0.5 mL buffer/0.2 g tissue) were ground in a mortar using liquid nitrogen and centrifuged at 10,000*g* for 10 min.

For western blots, total protein from *H. persicum* shoots and *C. album* leaves was extracted according to Van Etten, Freer and McCune (1979).

4.3. Protein measurement

Total soluble protein concentration was measured using the Sedmak and Grossberg (1977) method with BSA as a standard.

4.4. Activity measurement

NADP-ME activity was determined spectrophotometrically at 30°C by following NADPH production by measuring the absorbance change at 340 nm in a spectrophotometer. The standard assay medium contained (unless otherwise stated) 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 0.5 mM NADP, and 4 mM L-malate in a final volume of 1 mL. For enzyme activity assays, the enzyme was desalted in a Sephadex G-50 column, previously equilibrated with Tris–HCl, 50 mM pH 8.0, according to Penefsky (1977), prior to activity measurement.

Since metal–ligand chelating complexes are not reactants for the NADP-ME reaction (Iglesias & Andreo, 1990), the concentration of each substrate or cofactor was corrected for the concentration of the chelate complexes (Grover, Canellas & Wedding, 1981). The following dissociation constants were used in the correction: Mg-malate, 28.2 mM, Mg-NADP, 19.1 mM, Mn-malate, 5.75 mM, Mn-NADP, 1.1 mM (Grover et al., 1981).

The experimental data were fitted to the Michaelis–Menten equation by a nonlinear least squares regression computer kinetics program.

4.5. Polyacrylamide gel electrophoresis and western blot

SDS-PAGE were carried out using a 10% (w/v) acrylamide separating gel and a 5% (w/v) acrylamide stacking gel according to Laemmli (1970). All samples were incubated in the presence of 2% SDS and 0.5% 2-mercaptoethanol for 2 min at 100°C prior to being loaded onto the gel.

For two-dimensional PAGE, the pH gradient used for isoelectrofocusing was from 4.2 to 7.5, and a gradient polyacrylamide gel (7.5–15% w/v) containing SDS was used for separating proteins by size in the second dimension. MW markers were used, and the p*I*'s of the reactive bands were calculated from a pH calibration curve.

After electrophoresis, the proteins were either silver

stained to detect total proteins according to the method described by Rabilloud, Carpentier and Tarroux (1988) or, alternatively, electroblotted to a nitrocellulose membrane for western blot analysis using an antibody raised and purified against the 62 kDa subunit of malic enzyme (NADP-ME) from maize green leaves (photosynthetic form) (Hsu, 1982). Bound antibodies were visualized by linking to alkaline phosphatase-conjugated goat anti-rabbit IgG. The molecular masses of the proteins were estimated from a plot of log molecular weight of marker standards versus migration distance. The markers and the samples were run on the same gel.

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