Three Different and Tissue-specific NAD-Malic Enzymes Generated by Alternative Subunit Association in Arabidopsis thaliana*§

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The Arabidopsis thaliana genome contains two genes encoding the mitochondrial NAD-malic enzyme (NAD-ME), NAD-ME1 (At2g13560) and NAD-ME2 (At4g00570). The characterization of recombinant NAD-ME1 and -2 indicated that both enzymes assemble as active homodimers; however, a heterodimeric enzyme (NAD-MEH) can also be detected by electrophoretic studies. To analyze the metabolic contribution of each enzymatic entity, NAD-MEH was obtained by a co-expression-based recombinant approach, and its kinetic and regulatory properties were analyzed. The three NAD-MEs show similar kinetic properties, although they differ in the regulation by several metabolic effectors. In this regard, whereas fumarate activates NAD-ME1 and CoA activates NAD-ME2, both compounds act synergistically on NAD-MEH activity. The characterization of two chimeric enzymes between NAD-ME1 and -2 allowed specific domains of the primary structure, which are involved in the differential allosteric regulation, to be identified. NAD-ME1 and -2 subunits showed a distinct pattern of accumulation in the separate components of the floral organ. In sepals, the NAD-ME1 subunit is present at a slightly higher proportion than the NAD-ME2 subunit, and thus, NAD-MEH and NAD-ME1 act in concert in this tissue. On the other hand, NAD-ME2 is the only isoform present in anthers. In view of the different properties of NAD-ME1, -2, and -H, we suggest that mitochondrial NAD-ME activity may be regulated by varying native association in vivo, rendering enzymatic entities with distinct allosteric regulation to fulfill specific roles. The presence of three different NAD-ME entities, which originate by alternative associations of two subunits, is suggested to be a novel phenomenon unique to plant mitochondria.

Malic enzyme (ME)2 decarboxylates malate to pyruvate and CO2 in the presence of a divalent metal ion using NAD or NADP as cofactor. MEs can be classified in three different groups. The first group includes NADP-MEs (NADP-ME, EC 1.1.1.40), which are widely distributed in animals, plants, and microorganisms; this type uses NADP as cofactor and can decarboxylate oxaloacetate (OAA). The second group (NAD-ME, EC 1.1.1.38) is composed of enzymes found in Ascaris suum and bacteria, which use NAD and can decarboxylate OAA in addition to malate. Third category is plant NAD-MEs, which are not able to decarboxylate OAA (NAD-ME, EC 1.1.1.39).

MEs are widely distributed in nature, and the products of their reaction participate in many biosynthetic pathways and in respiratory metabolism. In plants NADP-MEs are localized to both plastids and cytosol (1, 2), whereas NAD-MEs are found in mitochondria (3). Apart from being involved in C4-photosynthesis and Crassulacean acid metabolism, non-photonsynthetic roles have been proposed for NADP- and NAD-ME, including plant defense responses (4–6), tolerance to osmotic stress (7), lipid and lignin biosynthesis (8–10), control of cytosolic pH (11), and malate respiration (12).

NADP-MEs, and non-plant NAD-MEs are homooligomeric proteins. The tetramer is the most common form, but higher and lower structural conformations have also been reported (2, 13–16). On the other hand, plant mitochondrial NAD-MEs are composed of two dissimilar subunits (α and β) at a 1:1 molar ratio (17). Depending on the source of the enzyme, pH, and 1-malate concentration, plant NAD-MEs assemble as heterodimers, heterotetramers, or heterooctamers (17–19). However, NAD-MEs purified from some C4 plants, e.g. Eleusine coracana, Panicum dichotomiflorum, and Amaranthus tricolor, are octamers composed of identical subunits (20).

In Arabidopsis thaliana, two genes encoding NAD-MEs, At2g13560 (NAD-ME1) and At4g00570 (NAD-ME2), show expression in all mature organs (12). The products of both genes, NAD-ME1 and NAD-ME2, share about 65% sequence identity and are immunological different (12). Recombinant NAD-ME1 and -2 are active homodimers with similar kinetic parameters (12). Arabidopsis insertion mutants defective in each NAD-ME gene exhibit residual NAD-ME activity, indicating that NAD-ME1 and -2 are functional as homodimers in vivo (12). Moreover, electrophoretic studies revealed that NAD-ME1 and -2 can also associate to form a heterodimeric enzyme both in vivo and in vitro (12).

Previous kinetic analyses of plant NAD-ME have been limited to enzymes that were purified from plant tissues, where the α and β subunits always co-purified (17–19). Currently, the

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§ The abbreviations used are: ME, malic enzyme; NAD-ME, NAD-dependent ME; FBP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; Mes, 4-morpholineethanesulfonic acid; TCA, tricarboxylic acid; CoA, coenzyme A.
available resources and characteristics of Arabidopsis as a model plant organism allow us to achieve a more complete understanding of the different properties of the separated NAD-ME oligomers. Here, we present the first biochemical characterization of a plant heteromeric NAD-ME (NAD-MEH) obtained by the recombinant technology and a co-expression-based approach. The metabolic regulation of the purified NAD-ME was investigated and compared with that obtained with the isolated NAD-ME1 and -2 homodimers. In addition, the analysis of chimeric proteins between NAD-ME1 and -2 allowed assignment of the differential regulatory properties to specific regions of the primary structure. Finally, differential relative protein accumulation patterns suggest that NAD-ME activity may also be modulated by alternative associations of the subunits in some organs of Arabidopsis.

EXPERIMENTAL PROCEDURES

Heterologous Expression and Purification of Arabidopsis NAD-ME1, -2, and -H—For the co-expression of NAD-ME1 and -2, the cDNA fragment corresponding to the mature NAD-ME2 (12) was cloned in the pET29a vector using the BamHI and SalI sites. The pET29-NAD-ME2 construction expresses the ME2 (12) was cloned in the pET29a vector using the BamHI and HindIII and SalI sites. The pET29-NAD-ME2 construction expresses the mature NAD-ME1, -2, and -H fragments of the primary structure. Finally, differential relative protein accumulation patterns suggest that NAD-ME activity may also be modulated by alternative associations of the subunits in some organs of Arabidopsis.

Construction and Purification of Chimeric NAD-ME1q and -2q—For the generation of NAD-ME chimeric protein NAD-ME1q and NAD-ME1q, a PCR reaction was conducted using as template the NAD-ME2 full-length cDNA and the following primer pairs: NAD-ME2F (5’-GGATCCTGCATGCAGGCTCTAAGTC-3’) and NAD-ME2PstI-HindIII (5’-CTGCAACAGCGACATCATGACAAGCCTTCCAA-3’). The cloned product was treated with the restriction enzymes BamHI and HindIII, and the purified fragment was ligated to the fragment pET32-NAD-ME1 treated with the same endonucleases (Fig. 4B). For the generation of the chimeric protein NAD-ME2q, the amplification product obtained as indicated above was treated with the restriction enzymes BamHI and PstI, and the purified fragment was ligated to the fragment pET29-NAD-ME2 treated with the same endonucleases. The ligation product obtained has a cDNA of NAD-ME2 with a HindIII site at the position 530, not present in NAD-ME2 sequence. This construction was treated with HindIII and Sall, and the fragment obtained was purified and ligated to the vector pET32-NAD-ME1 digested with HindIII and Sall (Fig. 4B). The inserts of the chimeric constructs (pET32-NAD-ME1q and pET32-NAD-ME2q) were sequenced to verify correct swapping of the corresponding fragments and assume that no mistakes were introduced during the subcloning procedures. BL21(DE3) E. coli was transformed with the pET32 plasmid containing the chimeric NAD-MEs. The induction and purification of NAD-ME1q and NAD-ME2q were performed as previously described (12). The amino-terminal sequences encoded by the expression vectors were removed using enterokinase, and the mature proteins were stored as previously described (12).

Gel Filtration Chromatography—The molecular mass of NAD-MEH was evaluated by gel filtration chromatography on a fast protein liquid chromatography system using a Superdex 200 10/300 GL column (Amersham Biosciences). The column was equilibrated with 25 mM Tris-HCl, pH 7.5, or with 50 mM Mes-NaOH, pH 6.5, and calibrated using molecular mass standards. The sample, and the standards were applied separately in a final volume of 50 µl at a constant flow rate of 0.5 ml/min.

Enzyme Activity Measurements—The enzymatic activity was determined spectrophotometrically using a standard reaction mixture containing 50 mM Heps (pH 6.4 or 6.6 depending on the enzyme), 10 mM MnCl2, 4 mM NAD, and 10 mM L-malate in a final volume of 50 µl. The reaction was started by the addition of L-malate. Initial velocity studies were performed by varying the concentration of one of the substrates around its K_m value while keeping the other substrate concentration at saturating levels. All kinetic parameters were calculated at least in triplicate using free concentrations of all substrates (21). Data were fitted to the Michaelis-Menten equation. In the case of sigmoidal kinetics, initial rates were fitted to the Hill equation by nonlinear regression. When testing different compounds as possible inhibitors or activators of the enzymatic activity, NAD-ME activity was measured in the presence of 0.5 or 2 mM concentrations of each effector and non-saturating concentrations of malate (K_m L-malate value of each enzyme, Table 1).

The apparent activation constant (A_{50}) values were obtained by varying the concentration of activator while keeping the NAD concentrations at saturating levels and the L-malate at non-saturating concentrations. Data were fitted, by nonlinear regression to Equation 1,
where \( V_0 \) is the rate in absence of activator; \( V_s \) is the maximum activated rate, \( A \) is the concentration of activator, and \( A_{50} \) is the concentration of activator that gives the 50% \( V_{50} \).

In the case of the activation by CoA for NAD-ME2 and NAD-MEH, the studies involved varying of concentration of a substrate while keeping the other substrate concentration fixed and at saturating levels and with varying concentrations of CoA. For NAD-ME2, the resulting data were fitted to Equation 2,

\[
    V = \frac{V_{\text{max}} \times \left(\frac{1 + \Phi A / K_A}{1 + A / K_A}\right) \times S}{K_m \times \left(\frac{1 + A / K_A}{1 + A / K_A}\right) + S} \quad \text{(Eq. 2)}
\]

where \( V \) is the activity measured at a specific l-malate concentration (S), \( V_{\text{max}} \) is the maximum activity, \( K_A \) and \( K_A \) are the dissociation constants of the activator (A) for the enzyme free and for the enzyme plus malate, respectively, and \( \Phi \) is the ratio between \( k_2 \) and \( k_1 \). This last value (\( \Phi \)) should be higher than 1, as the activity when the allosteric site is occupied is higher than when it is empty.

The model of activation that fit best to the kinetic data obtained is shown as Scheme 1, where the activator (CoA) can bind to the free enzyme and to the enzyme complexed with the substrates.

The decarboxylation of OAA was monitored by measuring the disappearance of OAA at 260 nm (\( \epsilon_{260 \text{nm}} = 850 \text{ M}^{-1} \text{cm}^{-1} \)) in an assay medium containing 50 mM Mes-NaOH, pH 5.5, 1 mM OAA, and 10 mM MnCl\(_2\) in a final volume of 0.25 ml. The reported velocities were corrected for the background rate.

Native PAGE was performed using a 6% (w/v) acrylamide separating gel. Electrophoresis was run at 150 V at 10 °C. Gels were electroblotted onto a nitrocellulose membrane and subjected to Western blot analysis.

Circular Dichroism (CD) Spectra—CD spectra were made with a Jasco J-810 spectropolarimeter using 0.1-cm path length cell and averaging 10 repetitive scans between 250 and 200 nm. Typically, 50 µg of the wild type or chimeric NAD-ME in phosphate buffer (20 mM NaPi, pH 6.5) were used for each assay. Mean residue ellipticity (\( [\Phi] \)) was obtained by the equation.

\[
    [\Phi] = \Phi \times M_{\text{MRW}} / 10 \times d \times c \quad \text{(Eq. 3)}
\]

in which 111.42 was used as \( M_{\text{MRW}} \) (the mean amino acid residue weight), \( d \) is the cell path in cm, and \( c \) is the concentration of the protein in mg/ml.

RESULTS

Heterologous Co-expression of Arabidopsis NAD-ME1 and -2 and Purification of NAD-MEH—To obtain the heteromeric Arabidopsis NAD-ME (NAD-MEH), NAD-ME1 fused to a His tag and NAD-ME2 without the His tag were co-expressed in E. coli. After induction of the expression, the bacterial extracts showed the presence of both proteins with the expected molecular masses of 80 kDa in the case of NAD-ME1 fusion protein and 58 kDa in the case of NAD-ME2 (Fig. 1A, lane 1). NAD-ME1 and -2 co-eluted after elution with imidazole (Fig. 1A, lane 3). To rule out the possibility of a nonspecific interaction of NAD-ME2 with the affinity column, E. coli expressing only NAD-ME2 without the His tag was similarly treated (Fig. 1A, lanes 5–7). In this case, NAD-ME2 mature protein was detected in bacterial extracts (Fig. 1A, lane 5) and the washing fractions (Fig. 1A, lane 6), but it was absent in the fraction eluted from the column after imidazole treatment (Fig. 1A, lane 7), indicating its failure to bind to the column due to the lack of the His tag.

Moreover, specific antibodies against NAD-ME1 or -2 reacted with the respective proteins, indicating that the lower band that co-eluted with NAD-ME1 corresponds to NAD-ME2 and was not a partial proteolysis of NAD-ME1 (Fig. 1B). Densitometric analysis of the bands obtained after SDS-PAGE of the eluted fraction indicated that NAD-ME1 and -2 co-purify in an equimolar ratio (Fig. 1B, lane 1). These results indicate that the co-elution of NAD-ME1 and -2 is due to specific protein-pro-
tein interactions resulting in an heteromeric assembly (1:1) of NAD-ME1 and -2 (NAD-MEH).

Structural Properties of NAD-MEH—The purified recombinant NAD-MEH was analyzed by Western blot after native PAGE using a mixture of antibodies against NAD-ME1 and -2. NAD-MEH showed an immunoreactive band with similar mobility to the major immunoreactive band observed in Arabidopsis leaf mitochondrial extracts (Fig. 1C). Previous work demonstrated that this band is composed of both NAD-ME1 and -2, and it reacts with antibodies against NAD-ME1 or -2 used separately (12). Apart from this band, mitochondrial extracts also show bands corresponding to NAD-ME1 and -2 homodimers (Fig. 1C).

The native molecular mass of NAD-MEH determined by size exclusion chromatography was $125 \pm 10$ kDa. Thus, NAD-MEH is a dimer composed of NAD-ME1 and -2 in a 1:1 ratio. By the same technique, the native molecular mass of NAD-ME1 and -2 was previously estimated (120.0 and 117.5 kDa, respectively (12)).

Kinetic Properties of Arabidopsis NAD-ME1, NAD-ME2, and NAD-MEH—A kinetic characterization of the recombinant NAD-MEH was performed, and the results were compared with those obtained with NAD-ME1 and -2 homodimers. NAD-MEH has a pH activity optimum of 6.5 (Table 1), a value similar to those of NAD-ME1 and -2 (12). The $k_{\text{cat}}$ and the $K_m$ values for NAD of NAD-MEH were also similar to those reported for the homodimers (Table 1). On the other hand, NAD-MEH displayed a non-hyperbolic kinetic behavior with respect to malate, showing sigmoidal kinetics ($n_{\text{Hill}} = 2$). This kinetic response was also observed for NAD-ME1 but not for NAD-ME2 (Table 1 (12)). Despite the different kinetics that were observed with respect to malate, the three enzymes showed similar affinity toward this substrate (Table 1). Hence, the three Arabidopsis NAD-MEs display similar catalytic efficiency ($k_{\text{cat}}/K_m$) for both NAD and malate (Table 1). Finally, as in the case of NAD-ME1 and -2, NAD-MEH was unable to decarboxylate OAA.

Regulatory Properties of Arabidopsis NAD-ME1, NAD-ME2, and NAD-MEH—Several intermediates of glycolysis and the tricarboxylic acid (TCA) cycle were tested as possible effectors of the individual NAD-ME activities. The results indicated that each NAD-ME responds differentially to the effectors tested (Fig. 2). Interestingly, succinate and fumarate showed opposite effects on the activity of the homodimers (Fig. 2). Although NAD-ME1 was strongly activated by these organic acids, NAD-ME2 was inhibited. The activation effect of succinate and fumarate was also observed in the case of NAD-MEH (Fig. 2). On the other hand, although PEP and FBP were the strongest activators of NAD-ME2 and -H (nearly 400% activation with 2 mM PEP or FBP), they did not modify the activity of NAD-ME1 (Fig. 2). The three NAD-MEs were activated by 2-ketogluutarate, whereas OAA activated NAD-ME1 and -H but did not modify the activity of NAD-ME2 (Fig. 2). NAD-ME2 and -H were activated more than 2-fold in the presence of CoA or acetyl-CoA, whereas the activity of the NAD-ME1 was not significantly modified (Fig. 2). Finally, phosphorylated nucleosides (AMP and ATP) did not significantly modify the activity of the NAD-MEs even at high concentrations (Fig. 2).

The apparent activation constant ($A_{50}$, activator concentration that gives 50% of activation) was estimated in the case of FBP, PEP, and fumarate (Table 2). The results showed that NAD-MEH is more sensitive with respect to FBP and PEP than NAD-ME2, exhibiting a 5.2- and 4.8-fold decrease in the $A_{50}$.

**TABLE 1**

| Kinetic properties of recombinant Arabidopsis NAD-ME1, -2, and -H |
|-----------------|---------|---------|---------|-----------|-----------|
|                  | pH optimum | $k_{\text{cat}}$ | $K_m$ NAD | $k_{\text{cat}}/K_m$ NAD | $k_{\text{cat}}/K_m$ 1-malate |
| NAD-ME1*         | 6.4      | 31.1    | 0.50    | 60.2      | 3.0* (1.9) |
| NAD-ME2*         | 6.6      | 44.1    | 0.50    | 88.2      | 3.0       |
| NAD-MEH          | 6.5      | 39.0    | 0.55    | 67.0      | 2.7* (2.0) |
| NAD-ME1q         | 6.6      | 32.1    | 0.52    | 61.7      | 0.2       |
| NAD-ME2q         | 6.4      | 11.4    | 0.58    | 19.6      | 4.0* (2.1) |

* Values previously obtained (12) are included for comparison.

$^*$ $n_{\text{Hill}}$; the calculated Hill coefficient ($n_{\text{Hill}}$) is indicated in parentheses.
value, respectively (Table 2). NAD-MEH displays a similar apparent affinity ($A_{50}$ values) for fumarate as NAD-ME1 (Table 2).

**CoA Activation of NAD-ME2 and NAD-MEH**—CoA is a typical activator of plant NAD-MEs, and it is present at high levels in mitochondria (24). Thus, the activation of NAD-ME2 and NAD-MEH by CoA was further analyzed. In the presence of 50 μM CoA, NAD-ME2 and NAD-MEH optimum pH was shifted to a more alkaline value (pH 6.8; Tables 1 and 3). In addition, in the presence of CoA, the NAD saturation curves of NAD-ME2 and NAD-MEH did not significantly differ from the ones obtained in the absence of CoA (not shown), but the kinetic behavior of both enzymes with L-malate was dependent on the CoA concentration (Fig. 3). The CoA activation effect was examined by varying L-malate concentration at several fixed concentrations of the activator. L-Malate concentration was kept at the $K_m$ value for each enzyme (Table 1). The results represent the % of activity in the presence of each effector in relation to the activity measured in the absence of the metabolites (100%). Assays were done at least by triplicate, and error bars indicate S.D. Bars with dark gray and parallel lines, significant inhibition (less than 70% residual activity). Bars with light gray and crossed lines, significant activation (more than 140%).

**TABLE 2**

| Effector | FBP | PEP | Fumarate | CoA |
|----------|-----|-----|----------|-----|
| NAD-ME1  | 1.1 | 0.48| 15.0     | 22.0|
| NAD-ME2  | 115 | 0.48| 15.0     | 22.0|
| NAD-MEH  | 22.0| 0.10| 0.84     | 16.1|

Notes: Effector values are the average of the values obtained from at least three different data sets with no more than 5% S.D. among them. --, no activation was observed (Fig. 2).

**TABLE 3**

| pH | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (mM) |
|----|------------|---------------------|-------------------|
| 6.8| 0.20       | 69.1                | 345               |
| 6.8| 0.80*      | 40.6                | 51                |
| 6.8| 0.06       | 45.0                | 750               |

Notes: $K_m$, $K_m$ for L-malate; $K_a$, $K_a$ for CoA; $S_0.5$, $S_0.5$ for NAD-ME2.

$S_0.5$, $S_0.5$ $= 1.3$.
of 17.2 and 2.2 μM, respectively. The apparent activation constant ($A_{50}$) for CoA was 15.0 μM (Table 2).

In the case of NAD-MEH, the increase in CoA concentration resulted in a decrease in the $n_H$ value, with a concomitant decrease in the $S_{0.5}$ for L-malate and no significant modification of the $k_{cat}$ (Fig. 3, B and D). Thus, the catalytic efficiency of NAD-MEH was increased by 3.6-fold in the presence of 50 μM CoA (Table 1 and Table 3). The $A_{50}$ value for CoA was similar to that of NAD-ME2 (16.1 μM, Table 2). No further variations in the kinetic parameters of NAD-ME2 and NAD-MEH were observed with CoA concentration higher than 50 μM (Fig. 3, C and D).

**Synergistic Activation of NAD-MEH by Fumarate and CoA—**

The analysis presented above indicated that the individual NAD-MEs showed differential activation by several metabolites (Fig. 2). Among the different metabolites tested, fumarate was the strongest activator of NAD-ME1, whereas low concentrations of CoA activated NAD-ME2 (Fig. 2, Table 2). As fumarate and CoA increased the activity of NAD-MEH (Fig. 2, Table 2), it was important to analyze if these compounds are able to simultaneously modify the activity of NAD-MEH. The results obtained showed that the $A_{50}$ for CoA decreases at increasing fumarate concentration, e.g. in the presence of 5 mM fumarate, the $A_{50}$ for CoA was 4.5 μM, a value more than 3-fold lower than that obtained in the absence of fumarate (16.1 μM, Table 2). The converse experiment in which the $A_{50}$ for fumarate was estimated in the presence of different CoA concentrations also demonstrated the synergistic effect of both activators on the NAD-MEH activity (not shown). In the presence of CoA (0.1 mM), the $A_{50}$ for fumarate was 0.30 mM, a value nearly 3-fold lower than that obtained without CoA (0.84 mM, Table 2).

**Construction, Expression, and Characterization of Chimeric NAD-MEs—**

At the amino acid level, *Arabidopsis* NAD-ME1 and -2 share 65% identity, exhibiting the most sequence divergence at the amino-terminal end (Fig. 4A). To determine whether this segment of the primary structure is responsible for the differences in regulatory properties, two chimeras (NAD-ME1q and NAD-ME2q) were constructed by interchanging the first 176 amino residues between NAD-ME1 and -2 (Fig. 4B). Both chimeric proteins were expressed in *E. coli* and purified to homogeneity. NAD-ME1q and -2q showed enzymatic activity and, thus, were kinetically and structurally characterized. The comparison of CD spectra of all chimeric and parental proteins did not indicate substantial changes (not shown), suggesting that the construction of the chimeric proteins did not result in a loss of overall structural integrity.

After native electrophoresis, purified NAD-ME1q presented a similar mobility to that of NAD-ME2, whereas NAD-ME2q mobility was similar to that of NAD-ME1 (supplemental Fig. 1). These results indicate that, as in the case of the parental proteins, the chimeric proteins assemble as dimers, and the amino-terminal segment of each NAD-ME homodimer is responsible for the differential mobility of NAD-ME1 and -2.

The kinetic characterization of NAD-ME1q and -2q indicated that the $k_{cat}$ value of NAD-ME1q was similar to the one obtained for NAD-ME1 (Table 1). However, the $k_{cat}$ value of NAD-ME2q was 4-fold lower than that of NAD-ME2 (Table 1). Both chimeric proteins showed $K_m$ NAD values similar to those of the parental enzymes (Table 1). Nevertheless, although NAD-ME2q affinity for L-malate was slightly lower than that of NAD-ME1 and -2, NAD-ME1q affinity for L-malate was 15-fold higher than that of the parental enzymes (Table 1). Moreover, the kinetic behavior of NAD-ME1q with respect to malate was hyperbolic, whereas NAD-ME2q presented sigmoidicity ($n_H = 2.1$, Table 1).

The effect of CoA, acetyl CoA, fumarate, and succinate on the enzymatic activity of the chimeras was analyzed and compared with the results obtained with the parental enzymes. As in the case of NAD-ME2, NAD-ME1q was activated by CoA and acetyl-CoA (Fig. 4B). On the other hand, NAD-ME2q activity was not modified by these effectors, as in the case of NAD-ME1 (Fig. 4B). Moreover, although NAD-ME2q was activated by fumarate and succinate, NAD-ME1q activity was not modified by these compounds, which were strong activators of NAD-ME1 and inhibitors of NAD-ME2 (Figs. 4B and 2). The
Arabidopsis Mitochondrial Malic Enzyme

A.

|    | NAD-ME1  | NAD-ME2  |
|----|----------|----------|
|    | PTIVHKQGLDILHDHPFNKGTA 20 | PCTIVHKRADIILHDHPFNKDGT 20 |
|    | FTMTERNRLDLRGLPVPVMDSEQQFIRFMTDKLRLQEEQARDQPDSPNLAackersNLRLH 80 | FPLTERDLRGIRGLPPVVMTCQQCDRFZSFSNLRENWXTGEPENYVALKWRMLNLRLH 80 |

B. HindIII PstI

|    | CoA (100 μM) | Acetyl-CoA (100 μM) | Fumarate (2 mM) | Succinate (2 mM) |
|----|--------------|---------------------|-----------------|------------------|
| NAD-ME1 | N | N | A | A |
| NAD-ME2 | A | A | I | I |
| NAD-ME1q | A | A | N | N |
| NAD-ME2q | N | N | A | A |

C.

Fumarate/Succinate

NAD-ME1

CoA/acyl CoA

NAD-ME2

estimated $A_{50}$ value for fumarate of NAD-ME2q (0.88 mM) as well as the $A_{50}$ value for CoA of NAD-ME1q (24.5 μM) was similar to those of the respective parental enzymes (Table 2), indicating that the binding sites for these metabolites were conserved in the chimeric enzymes.

The CoA activation effect on NAD-ME1q was further analyzed, and the kinetic parameters were estimated in the presence of $50\mu M$ CoA (Table 3). The results obtained show that, in the presence of CoA, the apparent $K_{m}$ 1-malate value of NAD-ME1q decreased 3-fold, whereas the $k_{cat}$ value increased 1.4-fold (Tables 1 and 3). Finally, as in the case of NAD-ME2 and -H, the pH optimum of NAD-ME1q moved to a more alkaline value in the presence of CoA (Tables 1 and 3). Relative Organ-specific Accumulation of NAD-ME1 and -2—Previous work indicated that the transcripts of NAD-ME1 and -2 can be detected in leaf, stem, flower, and root (12). Moreover, the comparison of the abundance of each transcript was very similar in all mature organs (12). However, as the level of protein accumulation might not directly parallel the level of transcript, Western blot analysis of crude extracts from different organs was performed using a mixture of specific antibodies against NAD-ME1 and -2. To quantify the amount of immunoreactive protein detected in each organ, NAD-ME1 was used as control, as it is composed of NAD-ME1 and -2 in a 1:1 protein:protein ratio (Fig. 1). The relative expression of NAD-ME1 and -2 was determined by densitometric analysis of several gels using different crude extracts (Fig. 5A). The results indicated that the relative abundance of NAD-ME2 to -1 in inflorescences differ significantly from the control (1:1) in that NAD-ME2 protein accumulation was higher than that of NAD-ME1 (Fig. 5A). In leaf, stem, and root, the relative abundance of NAD-ME1 and -2 was not significantly different from the control (Fig. 5A).

To further analyze NAD-ME1 and -2 protein accumulation in inflorescences, Western blot analysis was performed in the separated parts of the flowers (gynoecium, sepal, filaments, and anthers). The densitometric analysis of the immunoreactive bands obtained indicated that the relative level of NAD-ME2 to -1 in gynoecium and filaments did not differ from the one estimated in leaves (not shown). However, NAD-ME1 was prevalent in sepal, whereas only NAD-ME2 was found in anthers (Fig. 5B).

In addition, crude extracts from sepal, gynoecium, filament, and anthers were analyzed by Western blot after native PAGE (Fig. 5C). Whole flowers, gynoecium, and filament showed three immunoreactive bands with mobilities corresponding to NAD-ME1, -2, and -H (Figs. 5C and 1C). On the other hand, in sepal, two immuneactive bands corresponding to NAD-ME1 and -2 was not significantly different from the control (Fig. 5A). The Transgenic Arabidopsis lines expressing the GUS reporter gene (12) were used to analyze the activity of NAD-ME1 and -2 promoters in inflorescences. In mature flowers, NAD-ME1 is expressed in the filament, vasculature of sepal, and apical part of the gynoecium (supplemental Fig. 2). In developing flowers, a weak activity could be observed in the sepals. GUS expression driven by the NAD-ME2 promoter was found in the filaments, apical part of the gynoecium, and mature pollen grains (supplemental Fig. 2). It should be noted that the differential expression pattern observed is consistent with AtGenExpress?
Arabidopsis Mitochondrial Malic Enzyme

NAD-ME (NAD-MEH) was successfully obtained by a recombinant approach (Fig. 1). NAD-MEH was active as a dimer composed of NAD-ME1 and -2 in a 1:1 ratio. The maximum catalytic rate and the affinity toward the substrates were very similar among the three native associations (Table 1). Native molecular masses assessed by gel filtration chromatography were very similar and in all cases compatible with a dimer. Nevertheless, differential migration in native gels was observed for the three NAD-MEs (Fig. 1C), which may indicate a different net charge of the oligomeric forms.

NAD-ME1, -2, and -H Are Differentially Regulated by Key Metabolites—The very distinct regulatory patterns obtained for NAD-ME1, -2, and -H (Fig. 2) suggest different metabolic contribution of each NAD-ME in Arabidopsis. Fumarate, succinate and OAA behaved as activators of NAD-ME1 and NAD-MEH (Fig. 2). Because of the structural similarity of these organic acids, the activation observed could be a result of the existence of a unique allosteric site in the NAD-ME1 subunit. The low $A_{50}$ values of fumarate of NAD-ME1 and -H (nearly 1 mM, Table 2) and the high levels of this compound found in Arabidopsis leaves (26) suggest that the regulation of NAD-ME activity by this organic acid should have physiological significance in vivo. Fumarate and l-malate can be used to replenish the TCA cycle pool and as a carbon source for mitochondrial respiration. During the day, Arabidopsis leaves accumulate great amounts of these organic acids, which are metabolized during the following night (26). Total NAD-ME activity is enhanced at the end of the night period due to a higher transcriptional activity of NAD-ME1 and -2 genes (12). By the onset of darkness, when the fumarate levels are high, the allosteric activation of NAD-ME1 and -H by this organic acid would further increase the NAD-ME activity in addition to the transcriptional up-regulation. In addition, fumarate, succinate and OAA levels increase by cold stress (27). Low temperatures reduce the availability of $P_i$ and adenylates, and thus, a lower supply of pyruvate to the mitochondria via the pyruvate kinase reaction is expected (28). However, a respiratory homeostasis has been reported in plants stressed by low temperature (29). Thus, in these situations increased concentrations of organic acids would stimulate the supply of pyruvate by increasing mitochondrial NAD-ME activity. Interestingly, fumarate and succinate behave as inhibitors of NAD-ME2 (Fig. 2). These compounds could inhibit the activity of this enzyme by competing with the substrate l-malate in the active site as a result of structural homology.

The strong effect of the products of the two irreversible reactions of glycolysis, FBP and PEP, on the enzymatic activity of NAD-ME2 and -H (Fig. 2) is consistent with the hypothesis that NAD-ME would supply pyruvate when pyruvate kinase is inhibited. NAD-MEH showed the highest sensitivity to FBP and PEP (Table 2), suggesting that this enzyme could be involved in such function in vivo. In this regard, plant mitochondria can import PEP from the cytosol by a PEP/H$_{sym}$-porter or a PEP/ATP antiporter (30). Moreover, the PEP concentration in plant cells is in the micromolar range (31), and it increases by conditions that limit the pyruvate kinase reaction, e.g. by cold stress or low phosphate (28).

**DISCUSSION**

Reconstitution of an Active NAD-MEH through a Co-expression-based Approach—Previous studies indicated a heteromeric association between NAD-ME1 and -2 in Arabidopsis mitochondria (12). However, the homodimeric forms of NAD-ME1 and -2 were also active in vitro and in vivo (12). To compare the properties of the different native associations between Arabidopsis NAD-ME1 and -2, the heteromeric Arabidopsis

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**FIGURE 5. SDS- and native-PAGE of extracts of Arabidopsis organs analyzed by Western blot.** A, shown is a Western blot of crude extracts of different Arabidopsis organs after SDS-PAGE. To assess the relative NAD-ME1 and -2 immunoreactivity toward the antibodies, 50 ng of NAD-MEH, which has a 1:1 protein:protein relationship between NAD-ME1 and -2, was loaded in the first lane (NAD-MEH). Fifty µg of total soluble protein from leaf (L), stem (S), flower (F), and root (R) were loaded. Molecular masses of the immunoreactive bands are shown on the right (kDa). The assay was performed using a mixture of specific antibodies against NAD-ME1 and -2. The relative quantification of the immunoreactive bands of NAD-ME1 and -2 for each line is shown in the upper graph. Standard deviations of the densitometric analysis among at least three different Western blots are shown, and the asterisk indicates a significant different relative level of expression ($p < 0.05$). B, shown is a Western blot of the separated components of Arabidopsis flowers after SDS-PAGE. Fifty µg of total soluble protein from leaf (L), flower (F), gynoecium (G), sepal (Sp), filament (Fi), and anther (A) were loaded. Molecular masses of the immunoreactive bands are shown on the right (kDa). The assay was performed using a mixture of specific antibodies against NAD-ME1 and -2. The relative quantification of the immunoreactive bands of NAD-ME1 and -2 for each line is shown in the upper graph. Standard deviations of the densitometric analysis among at least three different Western blots are shown, and the asterisk indicates a significant different relative level of expression ($p < 0.05$). C, shown is a Western blot of the separated components of Arabidopsis flowers after Native-PAGE. Fifty µg of total soluble protein from flower (F), gynoecium (G), sepal (Sp), filament (Fi), and anther (A) were loaded. The assay was performed using a mixture of specific antibodies against NAD-ME1 and -2. The relative quantification of the immunoreactive bands of NAD-ME1 and -2 for each line is shown in the upper graph. Standard deviations of the densitometric analysis among at least three different Western blots are shown, and the asterisk indicates a significant different relative level of expression ($p < 0.05$).
CoA is a typical activator of plant NAD-ME that has been shown to activate all enzymes characterized so far (24). In contrast to this, the results of this study clearly show that the activity of NAD-ME1 is not modulated by CoA, whereas the catalytic efficiency of NAD-ME2 was drastically increased by this compound (Tables 1 and 3). The kinetic data suggest a mixed mechanism of activation of NAD-ME2 with modification of the maximal catalytic activity and the affinity toward L-malate (Fig. 6A), suggesting a concerted function with phosphoenolpyruvate carboxylase under situations where the pyruvate supply to the TCA cycle by the pyruvate kinase reaction is diminished. Finally, NAD-MEH is a form that can integrate the several forms of regulation and respond synergistically to activators of NAD-ME1 or NAD-ME2.

The Amino-terminal Region of NAD-ME1 and -2 Is Critical for Activation by C4 Organic Acids and CoA—NAD-ME1 and -2 significantly diverge at the amino-terminal end where the differences mostly reflect changes of charge (Fig. 4A). The analysis of the recombinant chimeras NAD-ME1q and -2q indicated that the amino-terminal regions of NAD-ME1 and -2 are associated with differences in the regulatory properties observed in these proteins; that is, activation by CoA and the C4-organic acids fumarate and succinate (Fig. 4, B and C).

As fumarate activates both NAD-ME2q and NAD-ME1 (Fig. 4B), it is concluded that an allosteric site responsible for such activation is present in the amino-terminal region of NAD-ME1 (Fig. 4C). This segment of NAD-ME1 possesses homologous residues to those of the human NAD(P)-ME that are involved in fumarate binding at an allosteric site: Arg-67, Arg-91, and Asp-102 (Fig. 4A) (33). However, these residues are also present in NAD-ME2, which is inhibited by this metabolite. Thus, there may be additional factors controlling the binding capacity and response to fumarate in the plant enzyme. In addition, the activation of NAD-ME2q by succinate further supports the hypothesis of an allosteric site for organic acids at the amino-terminal region of NAD-ME1 (Fig. 4C). However, further studies are necessary to establish the existence of this site.

On the other hand, NAD-ME1q was activated by CoA and acetyl CoA but not by the C4-organic acids fumarate and succinate (Fig. 4B). Thus, residues of the amino-terminal region of NAD-ME2 are involved in the regulation by CoA and acetyl-CoA (Fig. 4, B and 4C), probably by binding at the same allosteric site. Notable is the high affinity for malate of this chimeric enzyme, which displays a nearly 15-fold decrease in the $K_m$ value for malate with respect to the parental enzymes (Table 1). This increase in affinity may be due to a particular arrangement of the amino acid residues in the malate binding site of the parental enzymes (Table 1). This increase in affinity may be due to a particular arrangement of the amino acid residues in the malate binding site of the parental enzymes (Table 1).
quently evolved unique regulatory features. Although NAD-ME1 and -2 genes show a similar pattern of expression in mature organs of *Arabidopsis* (12), it cannot be ruled out that the proteins accumulate at different levels due to differences in stability or because the proteins are produced at different rates. Such changes in the protein ratio can influence the formation of the different NAD-ME native associations. In this regard, in mid-vein tissues of *Arabidopsis*, NAD-ME functions as a heterodimer, and NAD-ME2 can form an active homodimer, at least when NAD-ME1 is not present (34). In contrast, NAD-ME1 gene product is unable to form functional homodimers in cells of the mid-vein (34).

In this work, we show that NAD-ME1 and -2 proteins accumulate at different levels in the separate parts of *Arabidopsis* inflorescences (Fig. 5B). In this respect, NAD-ME1 is present at higher proportions than NAD-ME2 in sepals (Fig. 5B), and thus, NAD-MEH and NAD-ME1 can act in concert in this tissue (Fig. 5C). On the other hand, NAD-ME2 homodimer is dominant in anthers (Fig. 5, B and C). As NAD-ME2 responds mostly to CoA and intermediates of the glycolytic pathway but not to intermediates of the TCA cycle (Fig. 6), it is possible that in anthers, where mitochondrial respiration is highly active, NAD-ME activity could be linked to the rate of glycolysis.

In plants, multisubunit composition and changes in oligomeric assembly, depending on the tissue and the metabolic situation, are properties of important glycolytic and TCA cycle enzymes such as pyruvate kinase, NAD-isocitrate dehydrogenase, and PP-dependent phosphofructokinase and phosphoenolpyruvate carboxylase (35–39). These characteristics represent a mechanism of allosteric regulation of enzymes of central metabolic pathways (40). The presence of three different NAD-MEs originating by alternative associations of NAD-ME1 and -2 may be a novel phenomenon unique to plant mitochondria.

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