Review Letter

NAD-malic enzyme from plants

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NAD-malic enzyme (NAD-ME) is a primary regulatory enzyme for the metabolism of malate in plant mitochondria. NAD-ME serves an anaplerotic function for the production of pyruvate, and provides CO_2 for refixation in the Calvin cycle in certain C_4 and Crassulacean acid metabolism plants. Clues regarding the mechanism of control of NAD-ME in vivo come from numerous studies on the physical and kinetic properties of the enzyme. The kinetics are complex and are altered by the pH of the assay medium as well as by serveral effectors, including divalent cations. CoA, sulfate, acetyl CoA, and fructose 1,6-bisphosphate (activators) and chloride, citrate, and bicarbonate (inhibitors). The enzyme is functional as a dimer, tetramer and octamer and the variation in kinetics is at least in part due to its association/dissociation.

NAD-malic enzyme *Plant* *Mitochondria* *Respiration* *Photosynthesis* *Polymerizing enzyme*

1. INTRODUCTION

NAD-malic enzyme (NAD-ME) decarboxylates malate to yield pyruvate in the mitochondria. The enzyme is of interest in plants due to its central role in several metabolic functions. Along with NAD-malate dehydrogenase it serves as a branch point for metabolism of malate in plant mitochondria. The NAD-malic enzyme allows for the continual turnover of the tricarboxylic acid (TCA) cycle when pyruvate is limiting and provides a means for the oxidation of reserves of TCA cycle intermediates. In certain C_4 and Crassulacean acid metabolism species, NAD-ME plays a key role in photosynthesis by providing CO_2 for the Calvin cycle. Since NAD-ME was first isolated from a plant source 13 years ago [1], there has been a growing recognition of its importance in plant metabolism. It is likely to be highly regulated in vivo and recent studies indicate that it has complex properties. The enzyme is still poorly understood due to the variable reports on its physical and kinetic properties. This is the first review which deals with NAD-ME from plants and its purpose is to discuss its role in plant metabolism and means by which it may be regulated.

2. CATALYTIC FUNCTION, OCCURRENCE, AND ROLE

The NAD-dependent malic enzyme from plants and animals (L-malate:NAD oxidoreductase (decarboxylating), EC 1.1.1.39), catalyzes the oxidative decarboxylation of malate in the presence of a divalent cation:

$$\text{Malate} + \text{NAD}^+ \xrightarrow{\text{Me}^{2+}} \text{pyruvate} + \text{CO}_2 + \text{NADH}$$

It is distinguished from the bacterial NAD-malic enzyme (EC 1.1.1.38) by the inability to carry out oxaloacetate decarboxylation, which is possible by malic enzyme 1.1.1.38 [2]. Another malic enzyme (EC 1.1.1.40) preferentially utilizes $NADP^+$ [3], distinguishing it from 1.1.1.38 and 1.1.1.39, which can use $NADP^+$ to varying extents but prefer NAD^+. The NAD-ME reaction is reversible at high pyruvate and CO_2 concentrations [1,4,5].

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With few exceptions [6–8] NAD-ME has an absolute requirement for a divalent cation. It is likely that Mn^{2+} and/or Mg^{2+} fill this requirement in vivo. In vitro Co^{2+} and Ni^{2+} have substituted for Mn^{2+} or Mg^{2+} to varying extents [1,9].

The occurrence of NAD-ME is widespread. In plants it has been found in cauliflower buds [1,5,9–14], potato tubers [14–16], Jerusalem artichoke tubers [17], sweet potato roots [18], citrus fruits [19], and leaves of C_3 [20,21], C_4 [4,22–24], and CAM [25–29] plants. In animals, NAD-ME has been reported from canine small intestine mucosa [30], rat and calf adrenal cortex, rat liver, rat kidney [31], rabbit heart [32], and muscle tissue from the tapeworm *Ascaris suum* [33] and humans [34]. It is also found in bacteria [2,35–37] and yeast [8]. In plant and animal cells it is localized in the mitochondria [17,22,25,31,34,38].

Several roles have been proposed for NAD-ME in plants. It may function anaplerotically for the oxidation of pools of TCA cycle intermediates (e.g., in fruit ripening) [19]; for the oxidation of excess carbon fed into the TCA cycle from sources other than pyruvate [5]; or to provide carbon to the mitochondria when pyruvate uptake by plant mitochondria is limited [39] or when glycolysis is inhibited [1]. In addition to these functions, it has been suggested that the coordinated operation of NAD-ME and a mitochondrial isozyme of PEP carboxylase (if one exists), serves as a 'pH stat' in the mitochondria [5].

In the leaves of certain C_4 and CAM plants, NAD-ME serves to release CO_2 for fixation in the Calvin cycle. In C_4 photosynthesis, C_4 acids (malic and aspartic) are synthesized in the mesophyll cells and transported to the bundle sheath cells. The decarboxylation of malate in the bundle sheath cells is believed to result in the concentration of CO_2 at the site of its refixation in the Calvin cycle. NAD-ME is the major or sole C_4 acid decarboxylating enzyme in one group of C_4 plants, designated NAD-malic enzyme type C_4 plants. In the CAM pathway, a C_4 acid (malic acid) is synthesized at night as the result of the fixation of atmospheric CO_2, and stored until the following day. In malic enzyme-type CAM plants, mitochondrial NAD-malic enzyme and the cytoplasmic NADP-malic enzyme together serve to decarboxylate the malate during the day in order to provide CO_2 for the Calvin cycle (see [28,40–42]).

3. PHYSICAL AND KINETIC PROPERTIES

The plant NAD-ME is apparently composed of two dissimilar 58 and 62 kDa subunits that occur in the enzyme at a molar ratio of 1:1 [16,29]. It is conceivable, however, that the 58 kDa polypeptide is a proteolytic product of the 62 kDa subunit since animal and bacterial NAD-MEs are composed of identical subunits [30,35]. The enzyme is active as a dimer, tetramer and octamer, and appears to readily interconvert between these three states [16]. Literature reports on the molecular mass of NAD-ME from plant sources are compiled in table 1. The size of the enzyme is more a function of the conditions employed during isolation than of the source of the enzyme, though both of these factors may be important. Values range from 115 (dimer) to 514 kDa (octamer) with several values in between, suggesting that certain conditions may favor an equilibrium between two aggregation states. Factors that influence association and dissociation include enzyme concentration, pH, concentration of dithiol reducing agent, ionic strength, the presence of malate or citrate, and storage.

It is possible that NAD-ME from CAM is more aggregated than the enzyme from non-photosynthetic sources. Wedding and Black [29] analyzed NAD-ME from *Crassula* and from potato at the same time on the same electrophoretic gels and found that 90% of the activity of the CAM enzyme was in the octamer form, while only 5% of the activity of the potato enzyme corresponded to the octamer form, and over 60% corresponded to the dimeric form. This may be the result of an elevated level of the enzyme in CAM, since enzyme concentration is known to influence aggregation equilibria in proteins that undergo associations and dissociations [43].

Grover and Wedding [16] determined the kinetic parameters for the enzyme from potato in the different states of aggregation. The tetramer is the most active form with the highest V_{max} and a low K_m (malate). The dimer exhibits the lowest activity with the smallest V_{max} and highest K_m. The octamer is characterized by intermediate kinetic properties, with a V_{max} between that of the dimer and tetramer (but closer to that of the dimer), and a K_m similar to that of the tetramer.

If one considers the variable reports on the M_r values (table 1) and the kinetic results obtained by

Table 1

Literature reports on the molecular mass of NAD-ME from various plant sources

Source	Method	Conditions	Variable factor	M_r ($\times 10^{-3}$)	Reference	Probable state of aggregation[a]
Cauliflower buds	Sepharose 6B gel filtration	20 mM Tris–HCl (pH 7.5), 10 mM malate, 1 mM $MnCl_2$, 0.1 mM NAD^+, 100 mM NaCl	0.2 mM DTT	200	10	tetramer
			5 mM DTT	400		octamer
Cauliflower buds	Sepharose 6B gel filtration	10 mM malate, 1 mM $MnCl_2$, 0.1 mM NAD, 100 mM NaCl, 5 mM DTE	pH 6.5–8	250–400	10	tetramers to octamers
Cauliflower buds	Sephadex G-200 gel filtration	50 mM Tris–HCl (pH 7.2), 25 mM mercapto-ethanol	–	400	5	octamer
Potato tuber	polyacrylamide gel electro-phoresis	–	9 μg enzyme	170	14	dimer-tetramer equilibrium
			45 μg enzyme	170 (major)		dimer-tetramer equilibrium
				492 (minor)		octamer
		9 μg protein	$-Mg^{2+}$, $-$DTT	170		dimer
			$+Mg^{2+}$, $+$DTT	170 (64%) 490 (36%)		dimer octamer
Potato tuber	Bio-Gel gel filtration	100 mM Tes (pH 7.0), 2 mM MgO, 5 mM DTE	storage at 70°C	132	16	dimer
			50 mM L- or D-malate	264		tetramer
			65 mM citrate	–		tetramer
		2 mM MgO	50 mM Tes (pH 7.0)	514		octamer
		5 mM DTT	150 mM Tes (pH 7.0)	115		dimer
		50 mM free malate, 6 mM free NAD^+, 8 mM free Mg^{2+}, 5 mM DTT, 50 mM Tes (pH 7.0)	–	279		tetramer
Atriplex spongiosa	Sephadex G-200 gel filtration	25 mM Hepes-KOH (pH 7.5), 1.5 mM $MnCl_2$, 50 mM mercaptoethanol	–	279	4	tetramer
Crassula argentea	polyacrylamide gel electro-phoresis	–	–	490 (90%) 230 120	29	octamer tetramer dimer

[a] Estimated based on subunit weights determined by Grover et al. [14]

Table 2

Literature reports on the kinetic parameters of NAD-ME from various sources[a]

Source	Assays without CoA								Assays with CoA						
	Me^{2+}	pH	V_{max}[b]	$S_{0.5}$				H/S[c]	V_{max}[b]	$S_{0.5}$				H/S[c]	Ref.
				mal (mM)	NAD (mM)	NADP (mM)	Me^{2+} (µM)			mal (mM)	NAD (mM)	NADP (mM)	Me^{2+} (µM)		
Cauliflower buds	Mn	6.6		1.4	0.17			H							5
		6.8					20								1
		7.0		1.8–2.4	0.22			S							5
		7.2	25.7				4[d,f]								9
		7.5		2.0	1.38	12.2		S		0.35	0.50	4.3		H	11
	Mg	6.5	37.5	7.9	0.75		160	H	40.6	1.8	0.36		160	H	13
		6.5	27.2	4.2[d]	0.55[d]			H							9
		6.5	31.7		0.83[d]		40[d]								9
		6.6		4.5–5.3	0.36			H							5
		6.8					500								1
		7.0		8.7–10.0	0.51			S							5
	Co	7.0	26.3	10.2[d]	0.19[d]		18[d]	H							9
Potato tuber	Mn	6.5	13.7	0.76[d]	0.5[d]	0.3[d]	5[d]	H							15
	Mg	6.5	24.9	14.0[d]	0.9[d]	1.7[d]	300[d]	H							15
Orange fruit	Mn	6.9			0.67	3.45	65	S							19
	Mg	6.9					5000								19
Mesembryanthemum crystallinum (C_3)	Mn	7.2		5.9				S		0.39				H	44
C_4 leaves:															
Atriplex spongiosa	Mn	7.4								0.8	0.25		800	S	4
Amaranthus edulis	Mn	7.4								1.5	0.28		200	S	4
Panicum miliaceum	Mn	7.4								1.1	0.55		550	H	4
CAM leaves:															
Crassula argentea	Mn[e]	6.5	16.6	0.80[d]	0.59[d]		240[d]	H							29
	Mn	7.0	9.3	1.6[d]	0.7[d]		60[d]	S							28
	Mg[e]	6.5	23.3	6.1[d]	0.77[d]		950[d]	S							29
	Mg	7.0	8.9	8.3[d]	1.1[d]	3.3[d]	1400[d]	S							28
Kalanchoe diagremontiana	Mn	7.5		7.0				S		1.4			310	S	25
M. crystallinum	Mn	7.2		6.0				S		0.42				H	44

[a] All enzyme preparations were purified or partially purified
[b] Activity as $\mu mol \cdot mg^{-1}$ protein $\cdot min^{-1}$
[c] H = hyperbolic malate saturation curve; S = sigmoidal malate saturation curve
[d] $S_{0.5}$ values are expressed as free, noncomplexed species
[e] Mn^{2+} assays contained 10 mM Cl^- whereas corresponding Mg^{2+} assays did not
[f] Mn^{2+} isotherms at subsaturating levels of malate were biphasic

Grover and Wedding, it is no surprise that there are widely diverse reports in the literature concerning the kinetic behaviour of NAD-ME. Conditions employed during isolation and assay are likely to influence the state of aggregation and hence the kinetics. Table 2 is a compilation of kinetic parameters for NAD-ME from various sources. The table is subdivided according to pH of the assay, the divalent cation used (Mn^{2+} or Mg^{2+}), and whether or not CoA (an activator) is present, since these three factors have a major influence on the results.

The pH of the assay largely influences the shape of the malate saturation curve. With the exception of the enzymes from *Panicum miliaceum* [4], *Mesembryanthemum crystallinum* [44], and cauliflower (when Co^{2+} is present) [9] cooperativity with malate occurred when the pH was greater than, or equal to, 7 [4,5,11,25,28]. Lowering the pH below 7, with either Mn^{2+} or Mg^{2+}, in the absence of CoA decreases the concentrations of malate and NAD^+ that give half-maximal velocity [5], and increases the V_{max} [14,24]. In the presence of Mn^{2+} and CoA, the V_{max} is greatest above 7 [14], whereas with Mg^{2+} plus CoA, it is greatest slightly below pH 7 [45]. The lower pH optimum with Mg^{2+} plus/minus CoA or with Mn^{2+} minus CoA may be due to inhibition by HCO_3^- at pH > 7. Together, Mn^{2+} and CoA may overcome this inhibition [20].

Substituting Mg^{2+} for Mn^{2+} increases the concentration yielding half-maximal velocity ($S_{0.5}$) for malate, NAD^{2+}, $NADP^{2+}$, and the divalent cation, and in some reports reduces the V_{max} [5,15,19,28,29]. Canellas and Wedding [9] have determined that only the free ionic forms of malate, NAD^+, and metal ion are used by NAD-ME. Thus, chelation of malate and NAD by metal ions can result in underestimates of the affinity of the enzyme for its substrates. Studies in which the $S_{0.5}$ values are expressed as free, noncomplexed species (where indicated in table 2) represent the true affinity of the enzyme for its substrates. Otherwise the $S_{0.5}$ values indicate the amount of substrate required to give half-maximum activity under the given assay conditions without correcting for chelation of substrates. In addition to the effect on $S_{0.5}$ values and V_{max}, Mg^{2+} increases the sigmoidicity of the saturation curves for malate and metal ion [5,28,29]. A number of researchers have reported little or no activity of the C_4 and CAM enzymes with Mg^{2+} [4,22,25,27], although only 5 mM malate was used in their assays, which is approx. the $S_{0.5}$ value for malate when Mg^{2+} is used (see table 2). With NAD-ME from some sources, cauliflower buds, potato tuber, and *Crassula argentea* leaves, the V_{max} is as high or higher with Mg^{2+} than with Mn^{2+} (table 2). In one case where the V_{max} was reported to be higher with Mg^{2+} [29], chloride (an inhibitor) was included in the Mn^{2+} assays but not the Mg^{2+} assays. This may account for the lower V_{max} these investigators observed with Mn^{2+}.

CoA is a potent activator of NAD-ME from plants. Most reports claim that it is both a 'K' and a 'V' type activator, reducing substrate K_m values and stimulating V_{max} [4,20,28,29,44]. It has also been described as only a 'K'-type activator [13]. Hatch et al. [4] found that whether it acts more as a 'K' or 'V' type activator depends on the species. With Mn^{2+}, CoA does not activate at pH 6.5 and has its maximum effect between pH 7.2 and 7.5 [14,20,44]. With Mg^{2+}, CoA activates at pH 6.5–7.5 [45]. There are reports of CoA both reducing [5,11,19,44] and increasing [1,25] the sigmoidicity of the malate saturation curve. There is no direct evidence on the nature of CoA activation. It may influence the molecular mass of the enzyme or it may act as an allosteric effector.

Sulfate is another good activator of NAD-ME. Sulfate is competitive with CoA but less effective than CoA. In a study with the cauliflower enzyme, sulfate only activated at less than saturating malate and had no effect on the K_m for NAD^+ or Mg^{2+}, but reduced the K_m for malate [12]. Hatch and Kagawa [22] found that the response to sulfate is widely variable between species.

Several intermediates of the TCA cycle and glycolysis, as well as some other metabolites, have been reported to activate NAD-ME from plants. They include: *cis*-aconitate [5,19], isocitrate [5,19,23], 2-oxoglutarate [19], succinate [5,19], fumarate [13,19,29,43], oxaloacetate [19], glucose 1,6-bisphosphate [5], fructose 1,6-bisphosphate (FBP) [5,23,29], PEP [29], acetyl CoA [11,22,25], and AMP [29]. Hirai [19] found that the effectiveness of fumarate, succinate, oxaloacetate, and 2-oxoglutarate depends on the enzyme concentration, which may explain why there are two conflicting reports on the ability of these compounds to activate NAD-ME [1,25]. The best substantiated of these claims on activators of NAD-ME are those for fumarate, FBP and acetyl CoA.

Known inhibitors of NAD-ME include Cl^- [12,13,23], NO^{3-} [23], citrate [5,29], HCO_3^- [20,27], NADH [1,19] and NADPH [11]. Inhibition by Cl^- and NO^{3-} is competitive with malate. Malate, FBP, CoA, and sulfate partially reverse this inhibition [23]. In addition to its effect on the steady-state activity, Cl^- increases the time required for the *Crassula* enzyme to attain maximal activity [28]. Citrate inhibition is also competitive with malate [29]. As products of the NAD-ME reaction, HCO_3^- and NADH probably inhibit by affecting the mass action ratio.

N-Ethylmaleimide and KCNO have recently been shown to irreversibly inhibit NAD-ME from cauliflower [9]. A partial protection from *N*-ethylmaleimide inhibition was achieved by incubation with saturating levels of malate or Mg^{2+} which is indicative of the presence of essential sulfhydryl groups at the active site of the enzyme. KCNO, which may have its effect by modifying lysine residues, inactivates NAD-ME by 50%. This is consistent with the proposal that NAD-ME from C_3 plants is composed of two, nonidentical, active subunits [15].

As mentioned earlier, NAD-ME can use $NADP^+$ to a limited extent. The ratio of $NAD^+:NADP^+$ activity depends on pH [4], choice of divalent cation [15], the presence of an activator [4], and the species [22]. The pH optimum in the presence of Mn^{2+} and CoA for the enzyme from *Atriplex spongiosa* shifts from 7.3 with NAD^+ to 6.9 with $NADP^+$ [4]. With $NADP^+$at pH 6.5, the potato enzyme exhibits a V_{max} 62 or 18% of the NAD^+ V_{max} with Mn^{2+} or Mg^{2+}, respectively. In addition to a lower V_{max}, the K_m values for malate, Mn^{2+}, and Mg^{2+} are substantially higher with $NADP^+$ [15]. Among NAD-ME type C_4 species, the $NADP^+$ activity with Mn^{2+} and CoA ranged from 8% (*Amaranthus edulis*) to 34% (*Portulaca oleracea*) of the corresponding NAD^+ activity [22].

Wedding et al. [46] remarked that the CAM enzyme may have an intrinsically lower affinity for $NADP^+$ than the C_3 plant enzyme. The mitochondrial malic enzyme of *Crassula argentea*, with Mg^{2+} and without CoA, exhibited $NADP^+$ activity of 14–22% of the NAD^+ activity at pH 7.0 [28,29], and 7% at pH 7.2 [46]. The *Sedum praealtum* enzyme showed little response to $NADP^+$ at pH 7.2 with Mn^{2+} and CoA [27], and no response was observed for the *Kalanchoe daigremontiana* enzyme at pH 7.5 with Mn^{2+} and CoA [25].

NAD-ME from the CAM species that have been investigated display hysteretic behavior. That is, they undergo very slow changes (in the order of seconds or minutes) in kinetic properties after the addition or removal of ligand [43]. The hysteresis reported for NAD-ME has usually been in the form of a lag, changing from a lower rate to a higher steady-state rate, though hysteretic enzymes can also display a burst, i.e., a change from a higher rate to a lower steady-state rate.

The lag exhibited by NAD-ME from *Crassula argentea* is influenced by several factors. The duration of the lag is inversely proportional to the enzyme concentration and malate concentration. It is more pronounced with a buffer than without, or with Mg^{2+} than with Mn^{2+}. The inhibitor Cl^- and the products pyruvate and CO_2 enhance the lag, while the activators CoA and sulfate, as well as the product NADH, reduce the lag. No treatment will completely eliminate the lag of freshly purified enzyme, however it diminishes with storage at $-70°C$ [28,29,46]. The lag displayed by the nonpurified enzyme from *M. crystallinum* was eliminated by CoA when Mn^{2+} was used to fill the divalent cation requirement, but not when Mg^{2+} was used. This enzyme was also reported to display a burst in activity when malate was limiting and CoA was omitted from the assay mixture [44]. Wedding [28] has speculated that the lag is due to a slow aggregation of the enzyme. He has proposed that NADH binds to the enzyme to form a ternary complex, $E \cdot Me^{2+} \cdot$ malate $\cdot$ NADH, after which a conformational change is induced, preparing the enzyme for aggregation. The effect of activators on the lag may therefore be secondary, since increasing the rate of the reaction increases the concentration of NADH.

4. STABILITY

The stability of purified NAD-ME depends on the enzyme concentration [1,5,16,28], ionic strength [1,16] and the presence of a sulfhydryl reducing agent [1,25]. There is also one report of BSA contributing to the stability of the purified cauliflower enzyme [5]. Diluting the enzyme, increasing the ionic strength, and omitting a reducing agent increase the rate of deactivation, probably by causing dissociation of tetramers and/or octamers to dimers. The stability of NAD-ME in desalted crude extracts is influenced by temperature [4,22,24], exposure to oxygen [4,22], and the presence of a sulfhydryl reducing agent [4,22], $MnCl_2$ [4,22,25], and polyvinylpolypyrrolidone [25].

5. REGULATION

If there are as many roles for NAD-ME as have been suggested (see section 2) then one might expect multiple means for its regulation. In C_3 plants, the NAD/NADH ratio [1], levels of sulfate [12], fumarate, CoA [5,14], and TCA cycle intermediates [5], the Mg^{2+}/Mn^{2+} ratio [15], and pH [14] have all been proposed to play a part in NAD-ME regulation. Control of NAD-ME activity by the NAD/NADH ratio would permit mitochondrial respiration to continue by the anaplerotic functioning of NAD-ME when glycolysis has ceased. Sulfate is present in *Brassica oleracea* tissue at 6–15 mM [47], and because a transport system exists for sulfate uptake into the mitochondria [48], it is possible that even higher levels occur in this organelle. Canellas et al. [12] have therefore proposed that sulfate may have a role in the in vivo regulation of NAD-ME. Recently Day et al. [14] demonstrated that CoA was taken up by isolated plant mitochondria and, at higher pH, shifts malate metabolism from oxidation through malate dehydrogenase to decarboxylation through malic enzyme. This is the first evidence that CoA may function physiologically to regulate malic enzyme activity in vivo.

The associating/dissociating properties of NAD-ME have led Wedding [28] to suggest that malate mediated aggregation of relatively inactive dimers to active tetramers may serve as a crude means of regulation, while more subtle regulation (i.e., allosteric) may be accomplished by fumarate or CoA. Grover et al. [15] suggested that the Mg^{2+}/Mn^{2+} ratio may control the aggregation state and hence the kinetic parameters of NAD-ME.

In C_4 plants HCO_3^- or CO_2 may impose a fine control between decarboxylation via NAD-ME and carboxylation by RuBP carboxylase during the day (high CO_2 inhibiting malic enzyme and favoring RuBP carboxylase), and FBP (activator) may regulate NAD-ME on a day/night basis, since pools of this sugar phosphate form during Calvin cycle activity [23]. Chapman and Hatch [23] showed that FBP stimulates C_4 acid decarboxylation in bundle sheath mitochondria from NAD-ME type species. In addition, mitochondria from C_4 plants having high NAD-ME activity decarboxylate malate via the alternative, cyanide insensitive, pathway. This allows CO_2 to be provided from malate to the Calvin cycle without any constraints which might occur by coupling malate oxidation to the oxidative phosphorylation [49].

Wedding has proposed that, in CAM plants, malic acid released from the vacuole during the light period activates NAD-ME by inducing the aggregation of dimers to tetramers. At night, when malic acid is actively transported into the vacuole, NAD-ME would become inactivated by dissociation. Evidence for this mechanism comes primarily from physical and kinetic studies on the C_3 enzyme [16]. Studies on the CAM enzyme, however, do not support this hypothesis. Comparisons of the kinetic behavior of NAD-ME from CAM tissue, after rapid extractions of less than 2 min during the light period vs the dark period, did not reveal any difference in the extractable form of the enzyme [44]. Additionally, Wedding and Black [29] recently discovered that 90% of the enzyme isolated from *C. argentea* was in the octamer form. Hence, if the enzyme is regulated by the association and dissociation of subunits, then it is likely that the interconversions involve the octamer and tetramer forms rather than the dimer and tetramer. It is possible that metabolite levels, particularly malate concentration, control the activity of NAD-ME in CAM on a diurnal basis without altering the aggregation state of the enzyme [44]. The low V_{max} and intermediate K_m of the octamer (as described for the potato enzyme, [16]) could serve to inhibit activity during the night when malate is severely limiting, and buffer the rate of decarboxylation during the day when cytoplasmic malate is presumably abundant. In addition to diurnal regulation by malate, it is possible that CO_2 may be involved in feedback inhibition (as proposed for C_4 metabolism) to control the rate at which NAD-ME decarboxylates the available malate during the day [27].

In summary, NAD-ME is an oligomeric protein in plants which can be regulated in vitro by a number of effectors. Studies with the isolated enzyme indicate certain ligands influence the state of its polymerization. It is not yet clear how some effectors regulate the enzyme (i.e., whether CoA is an allosteric effector or whether it influences polymerization). There are sufficient data with the isolated enzyme and with plant mitochondria to suggest factors which may regulate the enzyme in vivo in plant metabolism, but further work is needed to determine how (by polymerization or allosteric changes) the enzyme is regulated in vivo.

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