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# Decarboxylation of Malate in the Crassulacean Acid Metabolism Plant *Bryophyllum (Kalanchoë) fedtschenkoi*[1]

## Role of NAD-Malic Enzyme

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**The role of NAD-malic enzyme (NAD-ME) in the Crassulacean acid metabolism plant *Bryophyllum (Kalanchoë) fedtschenkoi* was investigated using preparations of intact and solubilized mitochondria from fully expanded leaves. Intact, coupled mitochondria isolated during the day or night did not differ in their ability to take up [$^{14}$C]malic acid from the surrounding medium or to respire using malate or succinate as substrate. However, intact mitochondria isolated from plants during the day decarboxylated added malate to pyruvate significantly faster than mitochondria isolated from plants at night. NAD-ME activity in solubilized mitochondrial extracts showed hysteretic kinetics and was stimulated by a number of activators, including acetyl-coenzyme A, fructose-1,6-bisphosphate, and sulfate ions. In the absence of these effectors, reaction progress curves were nonlinear, with a pronounced acceleration phase. The lag period before a steady-state rate was reached in assays of mitochondrial extracts decreased during the photoperiod and increased slowly during the period of darkness. However, these changes in the kinetic properties of the enzyme could not account for the changes in the rate of decarboxylation of malate by intact mitochondria. Gel-filtration experiments showed that mitochondrial extracts contained three forms of NAD-ME with different molecular weights. The relative proportions of the three forms varied somewhat throughout the light/dark cycle, but this did not account for the changes in the kinetics behavior of the enzyme during the diurnal cycle.**

CAM is an adaptation to arid environments. In plants exhibiting CAM, stomata remain closed during the day to reduce transpirational water loss from the leaves and open at night for the fixation of $CO_2$ into malate through the sequential action of PEPC (EC 4.1.1.31) and malate dehydrogenase (EC 1.1.1.37). Malic acid synthesized in the dark accumulates in the cell vacuole and is then decarboxylated in the following light period. Previous work has shown that the activity of PEPC in the CAM plant *Bryophyllum (Kalanchoë) fedtschenkoi* is regulated by reversible phosphorylation (Nimmo et al., 1984, 1986; Carter et al., 1991). Phosphorylation of PEPC at night results in a decrease in the sensitivity of the enzyme to the feedback inhibitor L-malate and hence allows $CO_2$ fixation to be maintained during malate accumulation. Less is known about the regulation of other steps in CAM. Recent evidence suggests that malate can move passively into and out of the cell vacuole via specific voltage-dependent channels in the tonoplast (Iwasaki et al., 1992). Specific $H^+$-ATPases for the energized transport of malate across the tonoplast have also been identified (Bremberger and Lüttge, 1992). However, very little is known about the regulation of malate uptake into and release from the vacuole.

The decarboxylation of malate in *B. fedtschenkoi* during the day is catalyzed by cytoplasmic NADP-malic enzyme (EC 1.1.1.40) and/or mitochondrial NAD-ME (EC 1.1.1.39). Although these enzymes differ in their cellular locations and cation and nucleotide requirements (Wedding, 1982), they both catalyze the oxidative conversion of malate into pyruvate and $CO_2$, the latter of which is refixed during the day via the Calvin cycle. Flux through these malic enzymes must be controlled at night to prevent the recycling of $CO_2$ through the action of PEPC. The relative contributions of the two enzymes to the decarboxylation of malate are unknown; however, NMR studies of the [$^{13}$C]malate resulting from the nocturnal fixation of $^{13}CO_2$ have shown that little of the malate synthesized at night is decarboxylated, even though it can pass into the mitochondrial matrix and is therefore exposed to NAD-ME activity (Osmond et al., 1988; Kalt et al., 1990). This suggests that the activity of NAD-ME must be reduced in some way at night. In this paper we report results showing that this is indeed the case and indicate a number of possible mechanisms by which this regulation may be achieved.

## MATERIALS AND METHODS

### Materials

*Bryophyllum (Kalanchoë) fedtschenkoi* plants were propagated and adapted to an 8-h photoperiod (light, 28°C, 200 $\mu$mol photons $m^{-2}$ $s^{-1}$ PPFD; dark, 16°C) as described previously (Nimmo et al., 1984). [U-$^{14}$C]Suc and L-[U-$^{14}$C]malic acid were from Amersham. Percoll and silicone oil (density = 1.05) were from Sigma. Other materials

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Abbreviations: NAD-ME, NAD-dependent malic enzyme; PEPC, PEP carboxylase.

were obtained as described previously (Nimmo et al., 1984, 1986; Carter et al., 1991).

### Preparation of Intact Mitochondria

Mitochondria were isolated from mature, fully expanded leaves taken from 3- to 4-month-old plants using adaptations to methods described by Arron et al. (1979) and Day (1980). Mitochondria isolated during the light and dark phases are referred to as day and night mitochondria, respectively; the times at which mitochondria were isolated in each experiment are stated in the text. Approximately 40 to 50 g of leaves were removed from plants, and the petiole and midribs were excised. The remaining tissues were sliced into 0.5-cm-thick strips and incubated in 200 mL of ice-cold extraction buffer (200 mM Tricine-KOH, pH 7.6, 100 mM sorbitol, 2% [w/v] PVP-40, 20 mM isoascorbate, 5 mM $MnCl_2$, 2 mM DTT, 2 mM EGTA, 0.5% [w/v] fatty acid-free BSA) for 30 min with gentle stirring. The tissues were then transferred to a Waring blender and homogenized by four 0.5-s pulses. The homogenate was squeezed through four layers of muslin and centrifuged for 10 min at 1,200*g* to pellet chloroplasts and cell debris. The supernatant fraction was transferred to chilled Corex (Corning, Corning, NY) tubes and centrifuged for 20 min at 9,000*g* to pellet mitochondria. The supernatant fluid was decanted, and the pellet was resuspended in 10 mL of ice-cold wash buffer (50 mM Hepes-KOH, pH 7.0, 400 mM sorbitol, 1 mM DTT, 1 mM EGTA) before being repelleted by centrifugation for 10 min at 9,000*g*. The supernatant fraction was again removed, and the mitochondria were resuspended in 0.5 mL of wash buffer. Mitochondria were then purified using a discontinuous Percoll gradient (52/42/31/19% Percoll in wash buffer) centrifuged for 45 min at 37,000 rpm (82,000*g*) using a Beckman 70.1 Ti rotor. Mitochondria, which formed a distinct white band at the 42/52% interface, were carefully removed into 40 mL of wash buffer and pelleted by centrifugation for 15 min at 20,000*g* before being resuspended in wash buffer to a final protein concentration of 5 mg $mL^{-1}$. Yields were typically 50 to 100 mg of mitochondrial protein from 50 g fresh weight of tissue.

### Mitochondrial Respiration

Mitochondria were centrifuged and resuspended in a solution of 50 mM Hepes-KOH, pH 7.0, 400 mM sorbitol, 0.1% (w/v) BSA (fatty acid free), 5 mM $MgCl_2$, and 5 mM $KH_2PO_4$. Respiration was measured polarographically in this buffer using a Rank $O_2$ electrode (Rank Brothers, Bottisham, Cambridge, UK) at 25°C. The $O_2$ concentration of the air-saturated medium was taken as 260 $\mu$M (Estabrook, 1967). Respiration rates were measured in a final volume of 1 to 2 mL of solution containing 5 mg $mL^{-1}$ mitochondrial protein. ADP/O ratios were determined according to the method of Chance and Williams (1955).

### Decarboxylation of Exogenous Malate

Malate decarboxylation was measured at 25°C as pyruvate formed in a reaction medium of 50 mM Hepes-KOH, pH 7.0, 400 mM sorbitol, 0.1% (w/v) BSA (fatty acid free), 5 mM ADP, 5 mM $MgCl_2$, and 5 mM $KH_2PO_4$. The final concentration of mitochondrial protein was 5 mg $mL^{-1}$, and the reaction was started by the addition of malate to a final concentration of 30 mM. Samples (0.2 mL) were removed from the incubation at various times, and protein was denatured by the addition of HCl to a final concentration of 0.5 N. The acidified samples were left to stand for 10 min before precipitated protein was pelleted by centrifugation for 10 min at 20,000*g* at room temperature. The resulting supernatant fraction was neutralized by the addition of KOH and made up to 1 mL with phosphate buffer (50 mM $KH_2PO_4$, 50 mM $K_2HPO_4$). Samples were then assayed for pyruvate using exogenous lactate dehydrogenase according to the method of Czok and Lamprecht (1974).

### Uptake of [$^{14}$C]Malic Acid

The uptake of [$^{14}$C]malic acid into mitochondria was monitored using silicone oil centrifugation techniques adapted from methods described by Harris and Van Dam (1968), Van Dam and Tsou (1968), and Kraaijenhof et al. (1969).

Final mitochondrial preparations were suspended in standard wash buffer supplemented with 50 mM KCl and 5 mM $MgCl_2$ to a final concentration of 5 mg $mL^{-1}$ mitochondrial protein. Portions of the final suspension (2–3 mL) were then incubated with 5 mM L-[U-$^{14}$C]malate (0.1 Ci $mol^{-1}$), or [U-$^{14}$C]Suc as a nonpenetrating control, at 0°C with slow stirring in the $O_2$ electrode. Samples (usually 0.5 mL) were removed and carefully pipetted into microcentrifuge tubes containing 300 $\mu$L of silicone oil (density = 1.05) layered on top of 75 $\mu$L of 1.5 N $HClO_4$. The tubes were centrifuged immediately for 10 min at 13,000*g* to pellet the mitochondria into the acid layer and then left to stand for a further 10 min at room temperature. Samples were taken from the remaining supernatant fraction above the oil layer and from the acid layer and counted in Hi-Safe (Pharmacia) scintillation cocktail.

### Solubilization of Intact Mitochondria and Assay of NAD-ME Activity

Mitochondria were solubilized by incubation in standard wash buffer supplemented with 0.1% (v/v) Triton X-100 for 30 min on ice. Mitochondrial debris was removed by centrifugation and the supernatant fluid was desalted through a Sephadex G-25 column (10-mL bed volume). NAD-ME activity was assayed at 25°C in a mixture of 50 mM Hepes-KOH, pH 7.0, 1 mM EDTA, and 1 mM DTT containing 5 mM L-malate and 5 mM $NAD^+$ unless stated otherwise. In all cases, the enzyme preparation was incubated with the assay cocktail for 2 min before the assay was initiated by the addition of 5 mM $MnCl_2$. All assays were performed using a Philips (Cambridge, UK) PU8720 spectrophotometer, and data were expressed relative to citrate synthase activity in the extracts, assayed according to the method of Ochoa (1955). One unit of activity catalyzes the conversion of 1 $\mu$mol of substrate to product per min.

### Superose 6 Gel Filtration of Mitochondrial Extracts

The $M_r$ value of the NAD-ME activity in solubilized extracts was estimated by gel filtration on a Superose 6HR (10/30) column linked to a Pharmacia fast-protein liquid chromatography system. Experiments were carried out at room temperature with a column buffer containing 50 mM Hepes-KOH, pH 7.1, 5 mM $MnCl_2$, 1 mM DTT, and 1 mM EDTA. The column was calibrated using thyroglobulin ($M_r$ 669,000), ferritin (440,000), aldolase (157,000), lactate dehydrogenase (144,000), and ovalbumin (45,000) as molecular weight standards. Solubilized mitochondrial extract (300 $\mu$g of protein in 200 $\mu$L) was injected onto the column and eluted at 0.4 mL $min^{-1}$ in column buffer. Fractions (0.2 mL) were collected and assayed for NAD-ME activity in the presence of 10 mM malate, 5 mM $NAD^+$, and 50 $\mu$M $MgSO_4$ to give maximum rates of activity.

### Other Assays

Mitochondrial protein was measured in solubilized extracts according to the method of Lowry et al. (1951). To determine mitochondrial malate content, intact mitochondria were resuspended in standard wash buffer and lysed by the addition of $HClO_4$ to a final concentration of 0.5 N. Mitochondrial debris was removed by centrifugation and the supernatant fraction neutralized with 5 N KOH. Tubes were left to stand for 10 min before centrifugation to remove $KClO_4$. The supernatant fluid was assayed for malate using exogenous malate dehydrogenase and glutamate-oxaloacetate transaminase according to the method of Bergmeyer (1974).

## RESULTS AND DISCUSSION

### Respiration, Uptake, and Decarboxylation of Malate by Intact Mitochondria

To investigate whether there are any stable diurnal changes in the functional properties of mitochondria from *B. fedtschenkoi* leaves, we developed a procedure that allows the isolation of coupled mitochondria from plants in both the day and the night phase. These preparations consistently exhibited similar respiration rates and ADP/O ratios with malate, malate plus Glu, or succinate as substrate (Table I). Somewhat higher rates of respiration, 36 to 39 nmol $min^{-1}$ $mg^{-1}$ protein, were obtained with malate plus Glu in the presence of 5 mM ADP (not shown). These rates are comparable to, or somewhat lower than, the rates obtained with mitochondria from other CAM species (Arron et al., 1979; Day, 1980).

There was no detectable difference in malate uptake between the preparations of day and night mitochondria. An initial burst of uptake was followed by a phase in which uptake was linear with time (approximately 0.2 nmol $min^{-1}$ $mg^{-1}$ protein) during the period 0.5 to 3 min after addition of malate. Uptake continued for up to 10 min at a slightly reduced rate. The linear rate of uptake was stimulated 2-fold by 10 mM Pi, suggesting the presence of a malate-Pi translocase activity on the inner mitochondrial membrane (data not shown). The rate of malate uptake in the presence of Pi was approximately 30-fold less than the rate of metabolism of malate by intact mitochondria (Table I), but it should be noted that the measurements were made at 0 and 25°C with malate concentrations of 5 and 30 mM, respectively.

**Table I.** *Respiratory properties of B. fedtschenkoi mitochondria*

State 3 rates represent $O_2$ uptake in the presence of 1 mM ADP and state 4 when added ADP had been phosphorylated. RCR, Respiratory control ratio (state 3:state 4). Substrate concentrations were: malate and succinate, 30 mM; Glu, 10 mM. Values for $O_2$ consumption are given as nmol $min^{-1}$ $mg^{-1}$ protein and, in parentheses, nmol $min^{-1}$ $unit^{-1}$ citrate synthase activity.

| Mitochondria/Substrate | $O_2$ Consumption | | RCR | ADP/O |
|---|---|---|---|---|
| | State 3 | State 4 | | |
| Isolated 4 h into light phase | | | | |
| Malate | 12.8 (1.1) | 3.2 (0.3) | 4.0 | 2.2 |
| Malate + Glu | 14.6 (1.2) | 4.2 (0.4) | 3.5 | 2.4 |
| Succinate | 14.8 (1.3) | 3.2 (0.3) | 4.6 | 2.0 |
| Isolated 8 h into dark phase | | | | |
| Malate | 11.2 (0.9) | 3.3 (0.3) | 3.4 | 2.2 |
| Malate + Glu | 20.0 (1.7) | 6.0 (0.5) | 3.3 | 2.1 |
| Succinate | 12.0 (1.0) | 4.6 (0.4) | 2.6 | 1.8 |

Coupled mitochondria isolated from plants 4 h into the light period decarboxylated malate to pyruvate significantly faster than mitochondria isolated from plants in the middle and at the end of the dark period when the results were expressed per unit of citrate synthase activity in corresponding solubilized mitochondrial extracts (Fig. 1) or per milligram of mitochondrial protein (not shown). There was also a lag in the decarboxylation of malate by intact mitochondria isolated at the end of the dark period, with no significant accumulation of pyruvate detected until a few minutes after the addition of malate (Fig. 1). The increased malate decarboxylation activity in coupled mitochondria in the light in the absence of any light/dark regulation of malate uptake suggests that the activity of NAD-ME is increased in plants in the light.

### NAD-ME Activity in Solubilized Mitochondrial Extracts

Assays of NAD-ME activity in solubilized mitochondrial extracts were complicated by slow changes in enzyme activity during the time course of the assay itself. There was generally a lag period of 1 to 2 min at the start of each assay before activity began to increase. The extent of this lag period was dependent not only on the amount of enzyme present in each assay but also on the concentrations of malate and $NAD^+$ and the time of incubation of the extract with the assay mixture prior to initiation with $MnCl_2$. It was therefore necessary to standardize assay conditions and to devise an arbitrary scale for measuring the increase in enzyme activity during the first few minutes of each assay. The solubilized mitochondrial extract was therefore incubated with the assay mixture for 2 min at room temperature prior to initiation by the addition of $MnCl_2$.

As shown in Figure 2, extracts prepared from mitochondria isolated 6 h into the light phase gave rates that accelerated rapidly, reaching a steady state of activity in 4 to 8

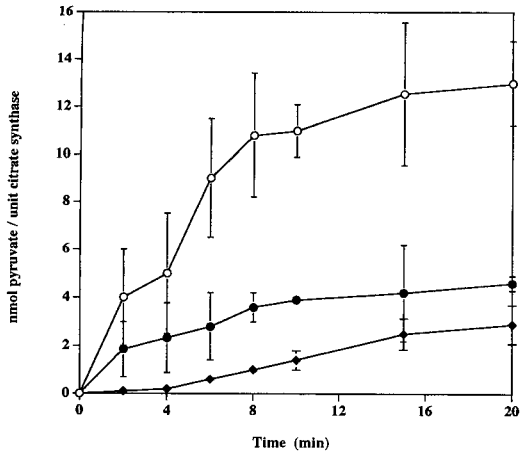


**Figure 1.** Conversion of malate to pyruvate by intact *B. fedtschenkoi* mitochondria. Mitochondria were isolated from leaves 4 h into the light period (day mitochondria; ○), 8 h into the dark period (night mitochondria; ●), and 16 h into the dark period (end of the night; ◆). Results are expressed as nmol pyruvate formed $unit^{-1}$ of solubilized mitochondrial citrate synthase activity. Error bars denote SE, $n = 5$.

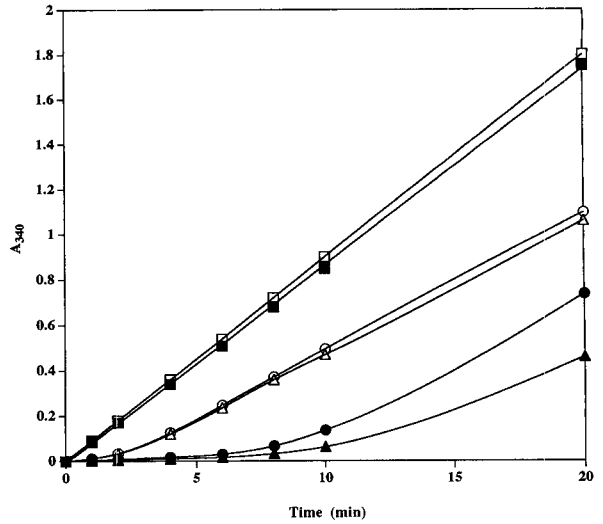


**Figure 2.** Progress curves of NAD-ME assays of extracts of solubilized *B. fedtschenkoi* mitochondria. Extracts of day (6 h into the light, open symbols) or night (10 h into the dark, filled symbols) mitochondria corresponding to 1 unit of citrate synthase activity were assayed for NAD-ME activity with 5 mM malate and 5 mM $NAD^+$, with no additions (○, ●), 10 μM acetyl-CoA (□, ■), or 10 mM pyruvate (△, ▲). Progress curves for assays containing $MgSO_4$ or Fru-1,6-$P_2$ (10 μM) were essentially identical with those with acetyl-CoA (not shown).

min. Extracts from mitochondria isolated 10 h into the dark phase showed more pronounced lags in activity, with the rates accelerating much more slowly over 20 min to achieve a similar final steady state. This would again suggest that NAD-ME is in a potentially more active state in plants in the light compared to plants in the dark. In general, lags in enzyme activity were found to be more pronounced in mitochondrial extracts from plants in the dark and became increasingly less evident in extracts made from plants harvested at increasing times into the period of illumination. As shown in Figure 2, addition of 10 mM pyruvate, the enzyme product, greatly enhanced the lag effect in extracts from mitochondria isolated at night but had no effect on extracts made from mitochondria isolated during the day. Addition of any of acetyl-CoA, $MgSO_4$, or Fru-1,6-$P_2$ to 10 μM after initiation of assays by $MnCl_2$ caused immediate abolition of any lag in activity, resulting in linear rates that were not significantly different between the two preparations of mitochondria. These rates were slightly higher than the final linear rates established in the absence of activators (Fig. 2). The NAD-ME activity of solubilized mitochondria exhibited a broad pH optimum between pH 6.7 and 7.5, irrespective of the time of isolation of the mitochondria (not shown).

The ratio of the rate 4 min after initiation of the assay to that 1 min after initiation was used as an indicator of the activity state of NAD-ME, considering the data in Figure 2. As shown in Figure 3, when this ratio was monitored throughout a daily cycle, the activity state of NAD-ME increased slowly up to 5-fold during the period of illumination and then decreased slowly during the period of darkness. In plants maintained in continuous darkness at 15°C, the leaf malate content is stable for 60 h (Nimmo et al., 1987), showing that there is no net decarboxylation. In these conditions the activity state of NAD-ME increased only slightly during the period in which the plants would normally be illuminated (Fig. 3). This suggests that several hours of illumination are required to increase the activity state of the enzyme in the mitochondria significantly and also that the diurnal rhythm in the potential activity state of the NAD-ME may be of considerable importance in terms of the regulation of decarboxylation of malate in

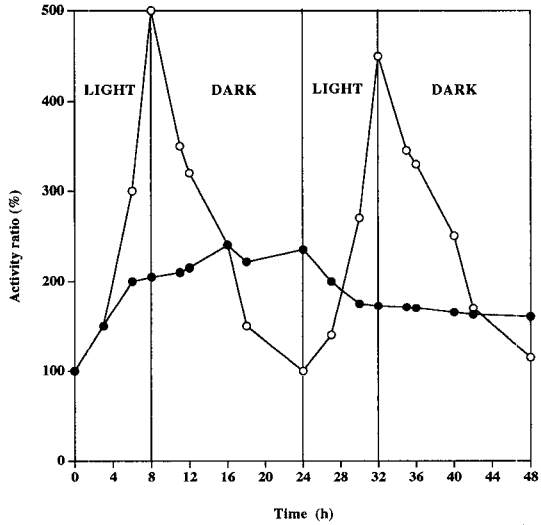


**Figure 3.** Diurnal changes in the activity state of NAD-ME. Mitochondria were isolated from *B. fedtschenkoi* plants maintained under an 8-h light/16-h dark photoperiod (○) or from plants placed in continuous darkness (●) at the start of a normal day. Extracts corresponding to 1 unit of citrate synthase activity were assayed for NAD-ME activity with no additions. The NAD-ME activity ratio is defined as the ratio of the rate 4 min after the initiation of the assay to that after 1 min (see text and Fig. 2).

CAM plants. However, mitochondria isolated in the middle of the day and night exhibited similar activity states of NAD-ME (Fig. 3), although they decarboxylated malate at different rates (Fig. 1). In addition, the activity state of the enzyme remained high during the first part of the night, whereas the rate of decarboxylation of malate in intact leaves at this stage is low. Thus, the change in kinetic properties of NAD-ME cannot be the only factor that determines the rate of decarboxylation of malate.

### Possible Regulation of NAD-ME by Malate and Changes in Aggregation State

Hysteretic shifts similar to those observed during assay of *B. fedtschenkoi* NAD-ME have also been reported for NAD-ME activity in other plant species. The enzyme from *Solanum tuberosum* has been reported to exist in both a high-$K_m$ (malate), low-activity dimeric state and low-$K_m$, high-activity tetrameric and octameric states, with a change in favor of the higher activity forms induced by millimolar concentrations of malate (Grover and Wedding, 1982, 1984). The hysteretic activity of *Crassula* NAD-ME may also result from changes in the aggregation state of the enzyme. Malate (50 mM) induced the conversion of octameric and dimeric forms of NAD-ME to a tetrameric form. The three forms exhibited different progress curves but similar final steady-state rates (Wedding and Black, 1983; Willeford and Wedding, 1986). It is therefore possible that the slow activation of *B. fedtschenkoi* NAD-ME activity with increasing time of illumination (Fig. 3) may be due to a slow change in the $M_r$ value, of the enzyme, possibly related to changes in intramitochondrial malate concentration. Indeed, we found that the acceleration of rates of NAD-ME activity in *B. fedtschenkoi* mitochondrial extracts was generally greater with higher (5–10 mM) rather than lower (1–2 mM) malate concentrations in the assay medium (not shown). However, incubation of extracts of mitochondria isolated at night with a range of malate concentrations from 5 to 50 mM prior to assay did not result in any activation of NAD-ME (not shown). In addition, we measured the malate content of mitochondria isolated throughout the light/dark cycle. Changes in mitochondrial malate did not correlate with changes in activity of NAD-ME (not shown). Although the malate content of isolated mitochondria probably does not reflect the situation in leaf tissue, there is no evidence to suggest that the observed changes in NAD-ME activity in isolated mitochondria are directly related to the malate content of the mitochondria.

In view of the potential importance of changes in the aggregation state of NAD-ME to its regulation, we also tested whether the observed difference in the activity of the enzyme between day and night mitochondria correlated with a change in aggregation state. The apparent $M_r$ values of NAD-ME in mitochondrial extracts were estimated by gel filtration on Superose 6. Extracts prepared from mitochondria isolated at the end of the day or in the middle of the night could be resolved into three separate activity peaks corresponding to $M_r$ values of 480,000, 240,000, and 120,000 (Fig. 4). In both cases the largest single peak of NAD-ME activity corresponded to the $M_r$ 480,000 species.

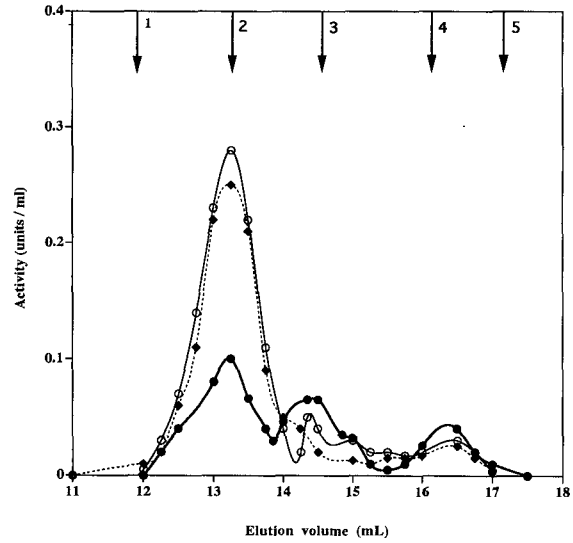


**Figure 4.** Gel filtration of NAD-ME in solubilized mitochondrial extracts. Mitochondria were isolated 8 h into the light period (○) and 8 h into the dark period (●). An extract of night mitochondria was also incubated with 20 μM acetyl-CoA (◆) prior to loading onto the column. Arrows indicate the following $M_r$ marker proteins: 1, thyroglobulin; 2, ferritin; 3, aldolase; 4, lactate dehydrogenase; 5, ovalbumin.

However, extracts of the mitochondria isolated at night contained slightly more low-$M_r$ activity and appreciably less high-$M_r$ activity than those from the mitochondria isolated at the end of the day. These differences could indicate that a higher proportion of NAD-ME is in the $M_r$ 480,000 form in the latter extracts than the former. However, since there is no significant difference between the NAD-ME activities of the two extracts when these are assayed in the presence of activators, the results in Figure 4 could also simply result from different recoveries of the activity of the various species from the column between the two extracts.

Fractions corresponding to the $M_r$ 480,000, 240,000, and 120,000 peaks were pooled separately. To investigate the kinetic properties of these activity peaks, equal amounts of NAD-ME (as judged by assays in the presence of saturating concentrations of activator) were assayed in the absence of activator (Fig. 5). The $M_r$ 120,000 and 240,000 activities from both preparations of mitochondria were similar, giving rates that accelerated slowly over 20 min to reach maximum activity. However, the properties of the $M_r$ 480,000 peak from the two preparations of mitochondria were different. The high-$M_r$ form of NAD-ME from mitochondria isolated at the end of the day showed a minimal lag in activity typical of that observed with unfractionated extracts of these mitochondria, with rates becoming linear 2 to 3 min after initiation of assays. Equivalent amounts of $M_r$ 480,000 activity from extracts of mitochondria isolated in the middle of the night gave prolonged lags on assay, with rates still increasing 20 min after initiation. Addition of 10 μM acetyl-CoA to these assays again abolished any lag, giving linear rates (not shown).

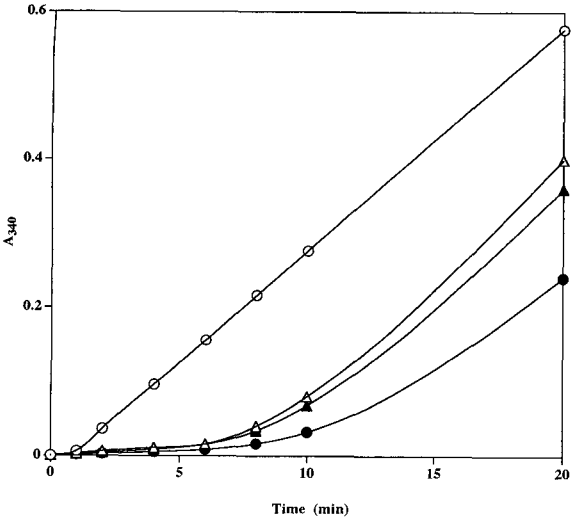


**Figure 5.** Progress curves of NAD-ME assays after gel filtration of mitochondrial extracts. Mitochondrial extracts were resolved into different $M_r$ forms as shown in Figure 4. Fractions were assayed in the presence of 50 $\mu$M acetyl-CoA to determine their maximal activities. Equivalent amounts of activity were then assayed in the absence of activator. Progress curves are shown for the $M_r$ 480,000 (○, ●) and 120,000 (△, ▲) forms. Open and filled symbols represent day (8 h into the light) and night (8 h into the dark) mitochondria, respectively. Progress curves for the $M_r$ 240,000 form were similar to those of the $M_r$ 120,000 form (not shown).

Incubation of the night mitochondrial extracts with 20 $\mu$M acetyl-CoA prior to gel filtration increased the amount of $M_r$ 480,000 activity recovered from the column (Fig. 4) and slightly decreased the amount of the low-$M_r$ forms detected. The kinetics of the $M_r$ 480,000 form eluted from the column were characteristic of the $M_r$ 480,000 form of the enzyme from the mitochondria isolated at the end of the day, suggesting that binding of acetyl-CoA may be a mechanism by which the high-$M_r$ form of the enzyme is activated or stabilized in the light.

## CONCLUSIONS

It is evident from previous work (Osmond et al., 1988; Kalt et al., 1990) that malate can enter mitochondria of CAM plants at night without being decarboxylated. Although the nature of the mechanism that reduces NAD-ME activity at night remains unclear, the work reported here makes two significant contributions toward resolution of this enigma. First, intact mitochondria isolated at night decarboxylate malate to pyruvate at a significantly slower rate than mitochondria isolated during the day, even though they have similar rates of malate uptake and respiration. Hence, there seems to be a diurnal change in the properties of CAM mitochondria that is stable to isolation and whose nature can therefore be investigated. Inactivation of the leaf mitochondrial pyruvate dehydrogenase complex in the light (Budde and Randall, 1990) may contribute to the effect. However, the potential activity of mitochondrial NAD-ME is much greater than that of the pyruvate dehydrogenase complex, so there must also be a significant reduction in NAD-ME activity at night.

Second, in attempts to identify the nature of the mechanism that regulates NAD-ME activity, we noted a difference in the kinetic properties of the enzyme between mitochondria isolated during the day and at night. During in vitro assays there are clear differences in the progress curves of the two forms of the enzyme, the day form accelerating to reach a linear final rate more rapidly than the night form. The results presented here are at variance with the work of Artus and Edwards (1985), who assayed NAD-ME activity in rapidly prepared and desalted extracts of *Mesembryanthemum crystallinum*, an inducible CAM species. These workers detected no differences in the progress curves obtained with extracts prepared during the day or at night. However, the extraction and assay conditions that they used differed from those used in this work, as illustrated by the fact that they observed bursts rather than lags in their assays. This difference may have been caused by the fact that Artus and Edwards (1985) desalted tissue extracts in a buffer containing $MnCl_2$, whereas in our experiments mitochondria were isolated in a buffer without divalent metal ions; alternatively, species differences may be involved.

The kinetic difference in NAD-ME between day and night mitochondria observed in our work may contribute to the difference between the rates of decarboxylation of malate by these preparations. The fact that only a small change in activity occurs during continuous darkness (Fig. 3), when malate concentration is stable, supports this view. However, as noted above, the change in kinetic properties of NAD-ME cannot be the only factor that determines the rate of decarboxylation. The kinetic changes in NAD-ME occur relatively slowly, and the activity of the enzyme in mitochondria isolated in the middle of the light and dark periods is similar (Fig. 3), whereas the rates of decarboxylation of malate by such mitochondria are different (Fig. 1). This suggests that an additional factor, perhaps the mitochondrial content of an effector of NAD-ME (e.g. acetyl-CoA), plays a role in determining the rate of decarboxylation of malate.

The mechanism that underlies the changes in the kinetic properties of NAD-ME is not clear. Our results are consistent with much previous work in showing that the enzyme can exist in different aggregation states and also indicate that acetyl-CoA, an activator shown to increase NAD-ME affinity for malate and $NAD^+$ (Wedding, 1982; Willeford and Wedding, 1986), can stabilize the highest-$M_r$ form. However, it seems unlikely that aggregation-disaggregation is solely responsible for the kinetic changes. The kinetic properties of the predominant aggregation state ($M_r$ 480,000) differ between day and night mitochondria (Fig. 5). Since this difference is stable to gel filtration, it is probably caused by very tight noncovalent binding of an effector or perhaps by a covalent modification. Although further work is required to define the mechanism(s) involved, it seems likely that the observations recorded in this paper are of physiological importance for the reduc-

tion of flux through NAD-ME at night and hence for the regulation of decarboxylation in CAM plants.

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