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Review

Malic Enzymes of Higher Plants

Characteristics, Regulation, and Physiological Function

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ABSTRACT

The characteristics and distribution of the malic enzyme in plants is discussed as well as those features which appear to be limited to the plant NAD malic enzyme. Regulation of the malic enzyme as it relates to the physiological roles of this enzyme is also discussed.

CHARACTERISTICS

Enzyme Forms

The malic enzyme is a MDH[1] that also decarboxylates malate using NAD(P) yielding pyruvate, CO_2, and NAD(P)H. A divalent metal ion is required for the reaction. As presently classified by the International Union of Biochemistry, the malic enzyme exists in three different forms. One, MDH (oxaloacetate-decarboxylating, EC 1.1.1.38), uses NAD and can decarboxylate oxaloacetate in addition to malate. This enzyme is predominantly microbial in distribution. Another, MDH (oxaloacetate-decarboxylating, NADP, EC 1.1.1.40), is widely distributed in the animal and plant worlds and is the standard malic enzyme discovered by Ochoa (18). This enzyme primarily uses NADP and can decarboxylate OAA. The third category, MDH (decarboxylating, EC 1.1.1.39), uses NAD by preference and cannot decarboxylate oxaloacetate. Earlier, this category had included the NAD malic enzyme from round worms, but recent information (22) indicates that the round worm enzyme can decarboxylate OAA, so that all the known members of this group are from the plant kingdom. This review deals primarily with enzymes of this category, together with plant enzymes which may fit in EC 1.1.1.40 on the basis that they are reported to prefer NADP or that they were studied only with NADP.

Occurrence and Cellular Localization

Although Ochoa and his colleagues (18) found the malic enzyme in plants, those studied all used NADP. While it is probable that at least some of the enzymes in question were what is today called the NAD malic enzyme (EC 1.1.1.39), no evidence for this is available. The first report of a plant malic enzyme operating preferentially with NAD was that of Macrae (16), who purified the enzyme from cauliflower florets. The malic enzyme appears to have been present in most plants where it has been looked for, and in one or more forms, it may be universal in plants.

The distribution of the malic enzyme in plant tissues is also very wide. It has been found in flowers, fruits, seed, roots, and special storage organs as well as in leaves, where it is thought to have some special functions (2, 14, 15, 19). Dittrich (7), surveying the occurrence of the NAD and NADP malic enzymes, found that the NADP enzyme appeared in excess of the NAD enzyme in many plants, but it should be noted that these assays were of crude extracts and that the NAD malic enzyme was assayed by allowing the MDH activity to come to equilibrium with NAD, then adding Mn^{2+}. The NADH produced in this procedure is a potent inhibitor of the activity of the NAD malic enzyme (22) and in addition, the assay with NADP would include about 60% of the NAD enzyme activity (10). The relative activity of the enzyme varies considerably depending on the tissue and the species. Outlaw (21) found that the malic enzyme activity in *Vicia faba* guard cells was much higher than in leaf parenchyma cells. The highest reported activity in fresh tissue was 0.24 U[2]/g fresh tissue in the spadix of *Arum* compared with 0.098 U/g in *Crassula* leaves and 0.026 U/g in potato tubers (25).

Within the cell the malic enzyme has been reported from the cytosol and chloroplasts (2, 14, 15), but it is principally found in mitochondria (16, 17). In isolating the enzyme from potato tubers, we found (10) that one malic enzyme that preferred NADP occurred in the soluble fraction, and that the mitochondrial fraction contained predominantly the NAD malic enzyme, but including a small amount of an NADP form with a low K_m for malate which disappeared before all steps of purification were complete. Similar results were obtained with purification from *Crassula* leaves, but the amount of the soluble NADP form was a much smaller fraction of the total.

Reversibility

The NAD malic enzyme of plants can be run in the reverse, *i.e.* reductive carboxylation, direction, although with some

[1] Abbreviations: MDH, malate dehydrogenase; OAA, oxaloacetate.

[2] One unit, U, is defined as an activity which produces 1 μmol of NADH/min·mg protein^{-1} at 25°.

difficulty. Macrae (16), using Mn^{2+}, 20 mM pyruvate, 20 mM HCO_3^-, and NADH with the cauliflower NAD malic enzyme found that the rate was one-twenty-fifth of that of oxidative decarboxylation. The most complete study is with the *Crassula* enzyme (23). It appears to require CO_2 rather than HCO_3^-, and because of problems with the solubility of $MgCO_3$, it was run in the reverse direction only with Mn^{2+} as the metal ion. The K_m for pyruvate is much higher in the reverse direction than that for malate in the oxidative direction (15.03 mM compared with 0.8 mM) and the K_m for NADH is fivefold lower than that for NAD in the forward direction, 0.24 mM. The maximal activity in the oxidative direction is 66 times greater than in the reverse and the equilibrium constant, K_{eq}, is 1.1 mM (23). There is, thus, little reason to think that the reverse reaction of the NAD malic enzyme has a significant role in plant metabolism.

Metal Ion Requirement

The malic enzyme requires a divalent cation to decarboxylate malate. Mg^{2+}, Mn^{2+}, and Co^{2+} are effective (4, 16, 18, 23). The affinity of the NAD malic enzyme for malate and NAD varies with different cations. The most interesting of these differences is that the $K_{m(malate)}$ is strikingly lower with Mn^{2+} than with Mg^{2+}. However, the maximal velocity attainable is reduced about 45% when Mn^{2+} is used rather than Mg^{2+}. In addition, the presence of Mn^{2+} produces a complex malate saturation curve suggesting changes in aggregation state rather than the normal hyperbola found with Mg^{2+} (4).

The malic enzyme binds only free cations as well as free malate^{2-} and NAD (3, 4). The kinetic mechanism is random (4, 23) and either the metal ion or malate may bind first, but the K_ms for both Mg^{2+} and malate^{2-} are smaller than the respective K_is, so it appears that when either metal ion or malate is present the other is bound more tightly than to the free enzyme.

Davies and Patil (5) determined the levels of magnesium and manganese in cauliflower bud tissue and measured rates with similar proportions of these cations. They suggested that both cations may be used *in vivo* by the enzyme. We have proposed (24), on the other hand, that the preferred metal ion *in vivo* may be Mg^{2+} on the basis that only the free cation can bind and that the generally higher affinity of Mn^{2+} for ligands occurring in the cell as well as the lower concentration of manganese usually present in plant cells make it likely that little free Mn^{2+} can be found under most circumstances.

Much of the apparently conflicting information concerning the malic enzyme has been produced by ignoring the requirement of the enzyme for a free metal ion and free substrate ion. Additions have been made to assays which altered the level of either or both, and the resulting change in activity has been attributed directly to the ligand added. We have developed and use a computer program to produce assays for the malic enzyme taking into account the dissociation constants of the ligands present with the metal ions used and write protocols which maintain the level of uncomplexed ligands at specified levels. The problem of the effect of the anion supplied with the metal ion has been minimized by the use of MgAces made by reacting MgO with Aces buffer (3, 4).

Chemical Mechanism

The distinction between EC 1.1.1.39 and EC 1.1.1.40 is partially based on nucleotide preference, but it particularly relates to the inability of the 39 form to decarboxylate OAA. Since the chemical mechanism which produces OAA as an intermediate is the simple one of hydride transfer and decarboxylation of the resulting OAA to pyruvate, the fact that the plant enzymes cannot attack OAA provided as such, raised questions concerning the possibility that these enzymes operate by some completely different mechanism. Studies of the isotope effect kinetics of the *Crassula* enzyme (9) showed that this enzyme also uses successive steps of hydride transfer and decarboxylation, indicating that the distinction between 39 and 40 is rather that the plant enzyme cannot bind OAA from solution, even though, when it is produced *in situ* from malate, it is readily decarboxylated. This difference probably is related to conformational divergence between the two forms and may derive from the distinctive characteristic of the plant enzyme noted below.

Nonidentical Subunits

All reported cases of malic enzyme structure from microorganisms or animals have indicated that it is an aggregate of a single subunit, usually one of about 60 kD. Early in our study of the NAD malic enzyme from cauliflower, we observed that the enzyme, which appeared to be homogeneous on the basis of native gels, showed two protein stained bands of approximately equal density when run on denaturing SDS gels (4). It was first thought that this might represent clipping of the enzyme during purification. However, when the enzyme was purified from potato tubers, it also showed two bands of slightly different size (10). Purification using several gentle procedures and including a variety of proteinase inhibitors (10) also produced an enzyme with the same two bands. When the enzyme was purified from *Crassula* two bands of slightly different size (M_r = 55 and 61 kD) were again produced (23), and we came to believe in the existence of the nonidentical subunits as a feature of the plant NAD malic enzyme. This has been confirmed (27) by preparative separation with anion exchange HPLC, using 8 M urea, of the two subunits from both the potato and CAM enzymes, showing that the two subunits have different isoelectric points, different amino acid composition, and different structures based on peptide mapping by limited proteolysis. The separated subunits are inactive, but can be recombined as α-β and will regain activity. The same methods of regenerating activity were ineffective with α-α or β-β preparations.

It is tempting, in the absence of evidence, to attribute some of the other characteristics peculiar to the plant NAD malic enzyme to conformational or other differences arising from the nonidentical subunits. Several such differences exist and some are mentioned in the appropriate categories below.

Kinetic Parameters

The literature on the malic enzyme abounds with erroneous or misleading reports on the values of the kinetic constants of this enzyme. In part, this reflects the fact that early workers

with the plant enzyme assumed that it was likely similar to the more widely studied malic enzyme (18) and set assay conditions with too low a level of malate or, being unaware of the requirement for free malate^{2-}, often provided too high levels of metal ion, thus reducing the availability of the anionic substrate. The metal ion was usually provided as the sulfate or chloride salt, which complicated the determination because sulfate is a potent activator of the enzyme and chloride is a specific inhibitor (4) as well as inhibiting by virtue of increased ionic strength (11, 12). Where manganese was used as the metal ion, the distorting effect of the anion given with the metal ion was minimized except when considerable excesses were used. Some reports that the enzyme can use only manganese as the required metal ion apparently resulted from the assumption that both metals should be effective in the same concentration range.

In part, the problem arises because the plant NAD malic enzyme apparently exists in several different forms with respect to affinity for malate and metal ion (11, 12). The effect of metal ion on the affinity for malate has already been mentioned. Other differences are due to the nucleotide being used, and in at least some cases, the aggregational state of the enzyme. This rather complex situation was summarized in Table 2 of an earlier review (2). More recently, it has been shown that some of the complexity is due, in the case of the *Crassula* enzyme (23, 26), to extremely slow hysteretic shifts during assay, which apparently result from changes in aggregation. It now appears that the very high K_m and low activity of the dimeric enzyme and the low K_m and high activity of the tetramer and octamer as shown for the potato enzyme (11, 12) probably exist for the enzyme from CAM and other species as well, although slow hysteresis makes it difficult to demonstrate (24, 26). The K_m for the native enzyme thus is a mean value of the several forms present and represents the existing aggregational equilibrium. As an example, we find that fresh *Crassula* enzyme with a specific activity of 50 U/mg usually has a $K_{m(malate)}$ of about 4 mM. On the basis of a K_m of 1 mM for the tetramer and one of 35 mM for the dimer (12) this could represent a mixture of about 20% dimer and 80% tetramer. Such a simplistic calculation ignores the presence of octamer but agrees reasonably well with the proportions that we find by column chromatography of freshly prepared enzyme. On storage at −70°C, the specific activity decreases, and the K_m increases (23) stabilizing at about 20 U/mg and 8 mM, respectively, which would represent nearly equal proportions of dimer and tetramer.

There is reason to think that many of the variations in the affinity of the NAD malic enzyme for malate such as the lower $K_{m(malate)}$ with Mn^{2+} (23, 24), the decrease in K_m with CoA, fumarate, and other activators (4, 23, 24), and the increased K_m induced by the presence of Cl^- or high ionic strength (11, 12, 23) may reflect the effects of these ligands on the aggregational equilibrium of the malic enzyme. Demonstration of these relationships has proven quite difficult. The equilibrium between various forms may shift quite rapidly and is influenced by the conditions of measurement. For example, size-exclusion HPLC separation of the malic enzyme, even at very low pressures, results in the presence of only the octamer. Column chromatographic separation at atmospheric pressure shows that the equilibrium is shifting in a matter of minutes (26) and that this may be equivalent to the half time for overcoming the lag of activity during assay. Thus the lag may represent the time scale for shifting from dimer to the highly active tetramer/octamer form. Support for this suggestion is provided by the fact that all the treatments mentioned, when added to ongoing assays, produce an increased activity after a variable lag (23, 24, 26). The time required ranges from <1 min for the cauliflower enzyme to >30 min for the *Crassula* enzyme (24) at assay levels of enzyme concentration that probably makes the process slower than would be found with *in vivo* concentrations of enzyme. One environmental effect where activity has been shown to be closely associated with aggregation is the rapid drop in activity correlated with a shift to the dimeric enzyme under the influence of an increase in pH (28).

The recent development of highly sensitive and stable laser light scattering instruments may provide the means by which the manifold uncertainties surrounding the question of the role of aggregation/disaggregation in regulation of the NAD malic enzyme can be resolved.

PHYSIOLOGICAL FUNCTION AND REGULATION

Anaplerotic Role

Although the malic enzyme plays crucial roles in certain types of specialized plant metabolism such as CAM and C_4, the apparently universal presence of the malic enzyme in plants suggests that it has a function broader than these specialized purposes. It seems likely that this broad function is an anaplerotic one (2, 14, 15). The dual paths for the metabolism of malate, using either MDH or the malic enzyme, reduces the dependence of the plant cell on glycolysis for production of energy and carbon skeletons and opens the process to ready use of the large reservoirs of stored carboxylic acids (especially malate and citrate) found in many plants. It also provides a greater metabolic volume in cases where environmental or developmental stresses require large amounts of energy or metabolites to meet emergencies. Looked at in this way, the specialized adaptations such as CAM using the malic enzyme represent continuous utilization of an existing enzyme to meet emergencies on a permanent rather than a transient basis and thus permit adaptation to environments otherwise impossible to tolerate.

A crucial aspect of the regulation of the two paths for malate metabolism derives from the characteristics of MDH. The equilibrium of this enzyme at neutral pHs is strongly in the reverse direction so that a small buildup of OAA will drive the MDH reaction toward malate and slow or stop the tricarboxylic acid cycle. When it occurs, and especially when acid accumulation results in a drop in the pH of the mitochondria (17, 24, 25, 28), the malic enzyme forms pyruvate from the malate thus permitting the production of acetyl CoA and condensation with OAA to form citrate, allowing the repeated cycling of carbon skeletons through the tricarboxylic acid cycle as well as production of NADH and ATP. In cases where OAA concentration is too low to maintain the cycle but glycolytic input is prevented, OAA can be supplied from pyruvate by a loop through pyruvate kinase and phosphoen-

olpyruvate carboxylase. In this way the combined action of malate dehydrogenase and the malic enzyme balances the intramitochondrial levels of pyruvate and OAA so that malate or other tricarboxylic acid cycle intermediates can be readily oxidized.

Regulation by pH

The means by which the malic enzyme part of this dual path is regulated is by no means completely established. However, it seems clear that one means is by changing pH. The NAD malic enzyme activity drops precipitously as the pH increases from 7.0 (28) probably by virtue of shifting to the low activity dimer (12). Since this response to pH is unique to the plant form of the malic enzyme, it may derive from the nonidentical subunits of this enzyme. The balance between MDH and NAD malic enzyme activity controlled by pH postulated on the response of purified enzymes is confirmed by the relative activity of the two enzymes in intact mitochondria. As an example, in *Arum* mitochondria the activity of the malic enzyme is 3.7-fold larger than that of MDH at pH 6.5, whereas at pH 7.7 it is 0.5 of the MDH rate (25).

Activation by CoA

A major activator of the plant malic enzyme is CoA (16). The microbial enzyme (EC 1.1.1.38) by contrast is inhibited by CoA (18), perhaps another manifestation of the unique subunit structure of the plant enzyme. If the malic enzyme were activated by CoA but not by CoA derivatives, a neat control method would be available depending on the accumulation of free CoA to turn on the malic enzyme. Unfortunately, the derivatives of CoA are equally or more effective in activating the malic enzyme (4). CoA greatly reduces the hysteretic lag of the NAD malic enzyme (24), its presence was found essential for recombination of malic enzyme subunits separated by 8 M urea treatment (27) and it increases the affinity of the enzyme for malate and for NAD (24, 26). The effectiveness of CoA in boosting the activity of the malic enzyme, $K_a = 4$ μM (8), and the increased velocity from 50 to 400% that it produces (4, 26) make it seem unlikely that CoA does not play a role in regulating the malic enzyme. One can only postulate that it could occur by sequestering the CoA (and perhaps its derivatives) in such a way that some signal, perhaps pH change, indicating need for malic enzyme activation can bring the two together. This is made more feasible by the demonstration that CoA can enter intact mitochondria (6). The question of whether and how CoA regulates the malic enzyme *in vivo* constitutes one of the major mysteries surrounding this enzyme.

Regulation by Other Activators

Other activators of the malic enzyme present less difficulty. Fumarate is effective in increasing activity (8) although the concentration required ($K_a = 1.4$ mM fumarate^{2-}) is somewhat higher than the amount usually thought to represent a steady state concentration during tricarboxylic acid cycle operation. However, if the tricarboxylic acid cycle is blocked, it is reasonable to assume that fumarate would accumulate to levels capable of activating the malic enzyme.

Malate is also an activator for the malic enzyme. This comes about because malate concentrations in the 5 to 10 mM range induce aggregation of the dimer to the more active tetramer or ocatamer forms (11, 12). This means of regulation would be useful in maintaining the balance between the activity of MDH and the malic enzyme for all types of plants, but might be most useful in the special case of CAM metabolism.

Fructose-1,6-*bis*P is a potent activator of the malic enzyme (23) with a $K_a = 20$ μM. This ligand is particularly effective in shortening the lag of the CAM malic enzyme (23). This feature would be especially beneficial in starting the turnover of the malic enzyme when blocking of the tricaraboxylic acid cycle or of glycolysis had resulted in accumulation of fructose-1,6-*bis*P.

AMP is an activator of the malic enzyme with a $K_a = 20$ μM (23) and ATP is an inhibitor, although not a particularly effective one ($K_i = 5$ mM) (23). It seems possible that under some circumstances energy charge regulation of the malic enzyme may occur.

Sulfate activates competitively with malate, *i.e.* it is effective only at low malate concentrations and in addition, as the concentration of sulfate increases, it deactivates the enzyme reaction. So, although the $K_a = 0.4$ mM and some possible means for sulfate accumulation in mitochondria exist (24), it is not clear that sulfate is an important *in vivo* regulator of the malic enzyme.

Although a wide range of ligands, mostly anions, are capable of activating the malic enzyme, relatively few inhibitors which might participate in regulation of its activity are known. Citrate, the K_i for which is 4.9 mM (24), is perhaps the one which exists in concentrations likely to make it an effective inhibitor, but it is difficult to rationalize shutting down the malic enzyme when citrate is high since using excess stored carboxylic acids seems to be one of the malic enzyme's useful functions.

Another potent inhibitor of the malic enzyme is Cl^-. It again is competitive with malate—in this case there are some indications that the competitive nature is due to opposing effects of the two ions on aggregation (24). It is difficult to visualize a productive type of malic enzyme regulation using variations in Cl^- concentration, and it may be that Cl^- effects on the malic enzyme are simply among the burdens which plants growing in saline environments must bear.

CAM Metabolism

Although other means are known (19, 20), the NAD malic enzyme and phospho*enol*pyruvate carboxylase constitute the principal enzymes on which CAM metabolism in a large number of plants depends. The carboxylation (phospho*enol*-pyruvate carboxylase)/decarboxylation (malic enzyme) processes that constitute the chief components of CAM are ideally suited to become a futile cycle, accomplishing only the expenditure of energy, if the two enzymes are not tightly regulated. How this regulation is accomplished is by no means certain at this point, but focusing on the malic enzyme component only, it seems probable that one or more of the potential effectors discussed above are responsible for turning the malic enzyme on in the morning to carry out its responsibility for decarboxylating the malate accumulated overnight. Various possibilities can be sketched and many are reasonable

within the present limits of knowledge, but our favorite (24, 26) is a combination of changes in malate concentration and pH. Malate reaching the mitochondria (by means which can be guessed, but for which there is little evidence) perhaps causes a drop in the mitochondrial pH or directly induces the malic enzyme to aggregate to tetramer or octamer and change the K_m for malate from about 35 mM to about 1 mM and also significantly increase the intrinsic velocity of the reaction. So long as the malate concentration around the enzyme remains at mM or higher levels, the malic enzyme will continue to decarboxylate the malate, providing CO_2 for the Calvin cycle. When the malate concentration drops and/or the pH rises, the malic enzyme will disaggregate to the dimer with characteristics which leave the enzyme essentially inactive (12, 26). Diurnal changes in malate concentration in CAM plants are consistent with what is needed to operate a system like that postulated here, and although some workers have found that their experimental procedures did not reveal the required changes in aggregation with a facultative CAM plant (1), we believe that the indirect evidence (which is the only form obtainable with the *Crassula* enzyme) strongly supports the sequence of events listed above (26). Perhaps sensitive light scattering determinations will resolve the question.

C_4 Metabolism

The physical separation of the carboxylation and decarboxylation events which comprise the crux of C_4 metabolism make the need for strict regulation less pressing than in CAM plants. However, there is no benefit to be gained from decarboxylation of malate in darkness, when the resulting CO_2 will not be fixed, so that control is desirable. The variety of C_4 systems (2, 13) means that a variety of regulatory methods may be used, but the malic enzyme in both the NADP and NAD forms is widespread in C_4 plants, and it appears (13) that the methods found to regulate the C_3 and CAM malic enzymes are at work here. The two major activators found effective in *Atriplex, Amaranthus,* and *Panicum* (13) were CoA and fructose-1,6-*bis*P, each of which increased malic enzyme activity from 4- to 16-fold. Smaller amounts of activation were obtained with glycerate-3-P and isocitrate. No activation response was found when CoA or fructose-1,6-*bis*P was added to intact mitochondria from these plants.

Inhibitory ligands were similar to those found with other types of malic enzyme. Chloride was quite effective as was nitrate and high levels of bicarbonate also gave some inhibition (13).

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