Short Communication

Regulation of the NAD Malic Enzyme from Crassula

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ABSTRACT

Using size exclusion chromatography, the nicotinamide adenine dinucleotide malic enzyme purified to near homogeneity from leaves of Crassula argentea was found to exist in at least three aggregational states (dimer, tetramer, and octamer). These forms differ in their apparent kinetic characteristics in initial rate assays, but all display similar characteristics at the steady state. The presence of 50 micromolar malate during chromatography causes a shift in the forms with the tetramer predominating. The native enzyme, when diluted 1/1000 and incubated 18 hours in buffer of high ionic strength, changes its steady state kinetic parameters to ones which indicate a low activity and low affinity for malate. When 50 micromolar malate or 50 micromolar coenzyme A are present the loss of activity and increase in $K_m$ is reduced. When both malate and coenzyme A are present the effects in minimizing the change in kinetic characteristics are additive.

A diurnal cycle of carboxylation/decarboxylation using phosphoenolpyruvate carboxylase and the NAD malic enzyme has been identified in many plants with Crassulacean acid metabolism (7). The obvious need for strong regulation in such a cycle to avoid futile use of energy has made the question of how one and obtaining a very interesting one.

Using enzyme purified from potato tubers, we showed (5) that the NAD malic enzyme exists in an equilibrium of aggregation states with the dimeric form possessing a low affinity for the substrate malate, and a low intrinsic activity, so that under conditions likely to be found in the cell, it would be essentially inactive. The tetramer and octamer had lower $K_{m\text{malate}}$ and higher $V_{\text{max}}$ so that the enzyme could be turned on by aggregation. It was also shown (5, 6) that malate in concentrations in the mM range was capable of causing such aggregation, and that the equilibrium constant for this effect of malate is approximately the same as the $K_m$ observed in native mixtures of the several oligomers (6). A number of other factors such as pH, ionic strength, and enzyme concentration were found to be involved in the aggregation equilibrium, and the monomeric form was shown to be inactive (6).

These observations have not been repeated with NAD malic enzyme from a CAM plant, although several studies (8-10) have shown the appearance of similar regulation with that enzyme. The reason for this lack of direct evidence for the existence of different oligomers of the CAM enzyme with differing activities is the pronounced and prolonged lag displayed by the NAD malic enzyme from Crassula (8) which makes it virtually impossible to determine the initial kinetic parameters of the enzyme.

Recent reports of failure to find different oligomers of this enzyme from a plant operating in the CAM mode (1) and the application of the applicability of the regulation found with the potato enzyme to CAM plants (2) has led us to undertake a study of this question with the Crassula NAD malic enzyme.

MATERIALS AND METHODS

Enzyme. The NAD malic enzyme (EC 1.1.1.39) was prepared from leaves of Crassula argentea collected from plants growing in the field. The methods used were those previously described (9) and the final enzyme preparation had a specific activity of 30 IU/mg protein. The enzyme was stored in 50 mM Aces$^2$ buffer at pH 7 with 5 mM Mg$^{2+}$ and 5 mM DTT at -70°C and thawed and diluted as required.

Chemicals. Chemicals used in enzyme preparation, assay and column chromatography were as described earlier (4, 5, 9) and were of the highest purity commercially available.

Assay. Enzyme activity was routinely determined in 50 mM Aces buffer at pH 7.0 with a 1.0 ml assay containing 50 mM malate$^{-2}$, 5 mM Mg$^{2+}$, 8 mM NAD$^+$, and 2 mM DTT. For these standard assays, and for those in which malate$^{-2}$ concentration was varied, the total amount of constituents required to maintain the desired level of free-ligands was calculated with a BASIC program described earlier (3, 4). Assays followed the increase in absorbance of NADH at 340 nm in a spectrophotometer cell compartment maintained at 25°C.

Gel Filtration Chromatography. Column chromatography was carried out with a 1 x 61 cm column of Fractogel TSK HW-55 (F) size exclusion resin, from E. M. Science, equilibrated with solutions as described below. The column was calibrated both before and after the malic enzyme runs with standards including apoferritin ($M_r = 443$ kDa), catalase ($M_r = 240$ kDa), $\beta$-amylase ($M_r = 200$ kDa), alcohol dehydrogenase ($M_r = 150$ kDa), BSA ($M_r = 66$ kDa), carboxic anhydrase ($M_r = 29$ kDa) and myoglobin ($M_r = 17$ kDa). The void volume was determined with Blue Dextran and the log $M_r$ of the standard proteins versus $V/V_0$ fitted to a linear regression (correlation coefficient $r = 0.992$). The slope of that line was used for calculating the size of location of major enzyme oligomers. With enzyme chromatograms, fractions of 0.4 ml were collected and immediately assayed for malic enzyme using the standard assay plus 50 $\mu$m CoA to minimize the lag (8). The column was run at a pump speed of 0.4 ml/min and elution was followed with an Altex model 150 monitor reading protein A at 280 nm.

RESULTS

Chromatography. The elution of NAD malic enzyme from a size exclusion chromatography column is shown in Figure 1.

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1 Supported in part by NSF grant PCM 82-0271.

2 Abbreviations: Aces, 2-[2-(amino-2-oxoethyl)amino]ethanesulfonic acid; $V_e$, elution volume; $V_0$, void volume.
REGULATION OF CAM MALIC ENZYME

FIG. 1. Column chromatography on fractogel TSK HW-55 (F) of Crassula NAD malic enzyme. The enzyme was diluted 1/25 and preincubated 1 h in 50 mM Aces buffer (pH 7.0) or buffer plus 50 mM malate before being chromatographed on a column equilibrated with the same solution. See text for details. (---), control, buffer only; (- - - -), buffer + malate. Fractions collected as indicated for determination of kinetic parameters of different oligomeric forms. O, octamer; T, tetramer; D, dimer.

Table 1. Kinetic Parameters of Different Oligomeric Forms of the NAD Malic Enzyme Separated by Size Exclusion Chromatography

<table>
<thead>
<tr>
<th>Enzyme Form</th>
<th>$V_{\text{max}}$ (μmol/min/mg protein)</th>
<th>$K_m$ (mM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial rates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octamer</td>
<td>18.9 ± 0.1</td>
<td>16.8 ± 0.1</td>
<td>9.5 ± 0.2</td>
</tr>
<tr>
<td>Tetramer</td>
<td>22.3 ± 0.1</td>
<td>21.3 ± 0.1</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Dimer</td>
<td>7.6 ± 0.1</td>
<td>32.9 ± 0.6</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>Steady state rates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octamer</td>
<td>30.6 ± 0.1</td>
<td>6.8 ± 0.1</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>Tetramer</td>
<td>28.1 ± 0.2</td>
<td>6.7 ± 0.1</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Dimer</td>
<td>23.0 ± 0.2</td>
<td>9.0 ± 0.2</td>
<td>3.1 ± 0.4</td>
</tr>
</tbody>
</table>

Stock enzyme containing 200 μg of protein was diluted 1/25 and preincubated 1 h in 100 mM Aces buffer (pH 7.0), with 2 mM Mg²⁺ and 5 mM DTT before loading on the column. At this point the activity was 2.2 IU. Elution used the same buffer and fractions were assayed immediately. The activity profile of the native enzyme (----) shows that this freshly prepared enzyme exists in an equilibrium consisting mostly of octamer and tetramer. Fractions indicated were used for determination of kinetic parameters as shown in Table 1.

This activity profile coincides completely with the protein profile in the regions of octamer to dimer of the malic enzyme, but the protein shows a small peak at $V_f$ - $V_o$ < 1 which is without activity. This probably represents a contaminant, but might be a more highly aggregated form of the malic enzyme which is inactive as SDS gels run on this preparation and silver stained show very low levels of contamination.

The second (- - - -) line of Figure 1 shows the results obtained when the same enzyme is preincubated 1 h with 100 mM Aces buffer (pH 7.0) with 2 mM Mg²⁺ and 5 mM DTT plus 50 mM malate and run in the column equilibrated with the same solution. It is apparent that the effect of this concentration of malate is to alter the average size of the enzyme, with the bulk now centered around the position of the tetramer. Also note that while the same amount of enzyme was used for each column, the overall activity of the malate profile is greater than that of the control. This activity profile also coincided quite well with the A at 280 nm. The activity profile with malate shows somewhat more of the enzyme as dimer than the control, but this may represent an effect of the added ionic strength due to malate ($\mu = 54$ mM), since high ionic strength can disaggregate the enzyme (7) and the control line ($\mu = 63$ mM) was run without added ions to minimize external effects on the equilibrium of the native enzyme. A third line, not shown, run with 160 mM NaCl ($\mu = 160$ mM) in addition to the Aces buffer, showed a more nearly equal distribution of enzyme over the size range of all three oligomeric forms than in the control or plus malate runs, but all three forms were present and substantial amounts of monomer also appeared.

Kinetic Parameters of Oligomeric Forms. In the case of an enzyme which has an extremely long hysteretic lag in assay it is impossible to identify accurately the kinetic characteristics possessed by the enzyme prior to the assay. The form which produces the steady state rate is almost certainly different from that which existed before initiation of the reaction. There seems a high probability that with the Crassula NAD malic enzyme the most active form when the steady state rate is reached is the same as the most active form of the enzyme because factors known to influence the conversion of the potato malic enzyme to tetramer or to dimer such as high malate concentration and high ionic strength respectively (5, 6) also shorten or lengthen the lag period of the CAM enzyme (8-10).

Since our interest is to identify the characteristics of the differing oligomers of the enzyme eluted from the column, it seems clear that this cannot be done unequivocally by use of assays which themselves change the characteristics of the enzyme. We have therefore attempted to identify as much of the initial characteristics as possible by measuring rates during the first minute of the reaction and then allowing the reaction to proceed to a steady state condition. The results of such studies are summarized in Table 1.

In the upper portion of the table are shown the kinetic characteristics of the three different oligomeric forms of the native enzyme, selected as shown in Figure 1, determined at the initial stage of the reaction. The very large values of the cooperativity coefficient indicate that only the highest concentrations of malate have initially produced rates which approximate those eventually reached. The $V_{\text{max}}$ values thus may approach those of the initial enzyme forms. $V_{\text{max}}$ values for the three different forms of the enzyme are like those found with the potato enzyme (5), with tetramer having the highest intrinsic activity, dimer the lowest, and the octamer intermediate, but closer to the tetramer rate. The $K_m$ values, although they probably reflect factors other than simple affinity for the substrate, are like those found with the potato enzyme, in relative terms, with dimer having a lower affinity for malate.

In the lower portion of Table 1 are shown the kinetic parameters determined after the reaction had reached the steady state. In this case, the fractions indicated in Figure 1 were collected from the enzyme experiment with malate because the malate treated enzyme had significantly shorter lags. Here it may be seen that all three oligomers of the enzyme have reached essentially the same condition as expressed by their kinetic parameters. The $K_m$ values are now in the range expected with the native Crassula enzyme at steady state (10), and the specific activities of the tetramer and dimer are almost identical, although the dimer still is somewhat lower. The Hill numbers are in the range expected of the native equilibrium of enzyme forms (9, 10) and, with the exception of the dimer, probably represent cooperativity relating to the number of dimeric active units comprising the
oligomers, e.g. 4 for the octamer and 2 for the tetramer. The high value for the dimer probably indicates that the true steady state had not yet been reached with this form.

The differences in kinetic parameters seen in initial and steady state rates as in Table I give further support to the postulate that at least part of the lag of the Crassula enzyme is due to the slow conversion of low activity forms of the enzyme to ones with higher affinity for the substrate and higher intrinsic activity during assay. Some further support is given by the half-times (T) of the lags for the assays with saturating levels of malate determined for each of the three forms separated from the native enzyme. These values, octamer = 2.9 ± 0.1 min; tetramer = 2.1 ± 0.06 min; and dimer = 4.6 ± 0.14 min, are consistent with the concept that the tetramer is the form most nearly at the condition arrived at in the steady state. The failure of the tetramer to show a zero lag probably is due to cross-contamination of the fractions used with other oligomers, perhaps by equilibration during separation. This may also indicate a need for some additional conformational change in the tetramer to reach optimal activity.

Dilution and Preincubation Effects. Earlier work (8, 10) had shown that the change in enzyme state manifested by the hysteric lag is less rapidly accomplished when the enzyme is diluted or when it has been exposed to prolonged preincubation with ligands which affect the lag. We have made use of this phenomenon to demonstrate the effect of malate and CoA in alleviating the dissociation of the enzyme which accompanies extensive dilution and prolonged preincubation at high ionic strength. Because the enzyme was diluted 1/1000 in these experiments, the large sample size made it impossible to run size exclusion columns on the variously treated enzyme and the effects of the treatments were monitored by determination of kinetic parameters of the treated enzymes. Because of the dilution and low activity after extended preincubation, few of these treatments showed any activity on being first introduced into the assay, and the results of these experiments, shown in Table II, are steady state rates attained after 40 min assays.

From Table II it is apparent that dilution 1/1000 and 18 h preincubation at the high ionic strength contributed by 400 mM Aces (μ = 253 mM) causes the loss of more than two-thirds of the original activity (compare specific activity in Table I). The presence of 50 mM malate during preincubation results in an enzyme with slightly higher Vmax and a substantially lower Km and the lag is significantly shortened. CoA at 50 μM, a potent activator of the malic enzyme and very effective in reducing the lag when present at 50 μM in assays (8, 10) has also lowered the Km and reduced the lag even further (only 1 μM CoA carried over into the assay). The effects of malate and CoA appear to be additive in that together they have produced the maximal specific activity, the lowest Km and the shortest lag. It is unfortunate that it has not been possible to determine the effects of these treatments on aggregation state of the enzyme, but this question will be investigated using other methods.

Table II. Effect of 18 Hours Preincubation at 1/1000 Dilution on Kinetic Parameters of the NAD Malic Enzyme

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vmax (μmol/mg protein min)</th>
<th>Km (mM)</th>
<th>pH</th>
<th>T (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 mM Aces</td>
<td>6.5 ± 0.4</td>
<td>38.0 ± 0.2</td>
<td>8.1 ± 0.2</td>
<td>11.4 ± 1.7</td>
</tr>
<tr>
<td>Malate (50 mM)</td>
<td>7.7 ± 0.2</td>
<td>11.7 ± 0.1</td>
<td>3.4 ± 0.3</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>CoA (50 μM)</td>
<td>10.1 ± 0.2</td>
<td>16.2 ± 0.1</td>
<td>4.2 ± 0.3</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>Malate (50 mM) + CoA (50 μM)</td>
<td>9.9 ± 0.2</td>
<td>9.8 ± 0.2</td>
<td>4.7 ± 0.3</td>
<td>4.6 ± 1.1</td>
</tr>
</tbody>
</table>

DISCUSSION

Since regulation of the NAD malic enzyme by malate concentration is more obviously pertinent to the metabolism of a CAM plant than to potato tubers, it is unfortunate that the best evidence for such regulation can be obtained with enzyme prepared from potatoes (5, 6). We have felt, however, that the probability of a completely different mode of regulation of the same enzyme in Solanum and Crassula is rather low. Many indirect bits of evidence have been compiled which reinforce this belief (8–10). The hysteric lag of the CAM enzyme, during which a change to a more active form occurs, is influenced by many of the same factors which have been shown to alter the aggregation state of the potato enzyme and those influences are consistent with changes like those found in the potato enzyme.

The change in activity of the CAM enzyme on storage is consistent with the similar change in the potato enzyme which is correlated with changes in oligomeric state (5). Perhaps most compelling is the attractiveness of the concept of the large diurnal changes in malate in CAM plants as a means of controlling the malic enzyme/PEP carboxylase system. We have found that malate may also be a major factor in regulation of PEP carboxylase in Crassula (11, 12).

The evidence reported here, still mostly indirect, adds to the confidence with which the hypothesis of malate regulation of CAM malic enzyme can be put forward. We have shown that the native CAM enzyme can be separated by gel filtration into forms which correspond to at least three oligomeric sizes. Malate moves the equilibrium toward the tetrameric size, which appears to have—like the potato enzyme—a higher affinity for malate and a higher intrinsic activity.

When the Crassula enzyme is diluted 1/1000 and stored overnight on ice in a buffer of high ionic strength, it loses much of its initial activity. The residual activity shows a Kma at steady state much higher than the native, un-preincubated, enzyme at the steady state. The presence of malate results in more residual activity and a 3-fold lower Kma. CoA, which activates and also preserves more activity on diluted preincubation, it has somewhat less effect on the Kma, but the lag to reaching steady state is even shorter than with malate. When malate and CoA are combined, their effects appear additive, the activity is higher than with malate alone, and the Kma is lower than with either ligand alone, as is the lag.

The additive effects of malate and CoA suggest that these ligands bind at different sites even though CoA as an activator is competitive with the substrate malate (9). The fact that malate has a greater effect on Kma and CoA on Vmax also suggests that they exert their effects in different ways. There is no direct evidence that CoA affects the aggregation state of the malic enzyme, and it is possible that both the activation by CoA and the effect on the lag as seen here comes about through some more complex conformational change induced by CoA.

While the results obtained in this study favor the hypothesis of an aggregational change as a major factor in regulation of the Crassula malic enzyme, the persistence of a lag in all treatments strongly supports the suggestion that some further change in the enzyme must occur after the aggregational equilibrium is shifted to produce an enzyme form with optimal activity. Identification of this change presents a strong challenge.

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LITERATURE CITED


REGULATION OF CAM MALIC ENZYME


