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Kinetic Ramifications of the Association-Dissociation Behavior of NAD Malic Enzyme[1]

A POSSIBLE REGULATORY MECHANISM

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SCOTT D. GROVER AND RANDOLPH T. WEDDING
Department of Biochemistry, University of California, Riverside, California 92521

ABSTRACT

NAD malic enzyme can exist in dimer, tetramer, or octamer form. Freshly prepared enzyme from *Solanum tuberosum* var. Chieftan exists predominantly as the octamer and during storage is progressively converted into lower molecular weight forms. High ionic strength favors dimer formation, whereas high concentrations of malate or citrate favor tetramer formation. The tetramer is the most active form, having a low K_m for malate and a high V_{max}. The dimer, with its high K_m and low V_{max}, is the least active form. Malate may regulate NAD malic enzyme by controlling its state of oligomerization.

NAD malic enzyme plays a key role in the organic acid metabolism of plant mitochondria. When stored reserves of malate or other acids which the tricarboxylic acid cycle converts to malate are mobilized, NAD malic enzyme allows their complete oxidation via conversion of the malate to pyruvate. Pyruvate is then converted to acetyl CoA, which in turn can be completely oxidized in the normal reactions of the tricarboxylic acid cycle. Thus, malic enzyme allows the conversion of C_4 acids into acetyl CoA, the normal respiratory substrate. Furthermore, in some C_4 and CAM plants, the mitochondrial NAD malic enzyme also serves a major photosynthetic role by providing CO_2 for fixation in the Calvin cycle (5, 9).

Our studies on the aggregation state of NAD malic enzyme arose from observations of complex substrate saturation curves having two or more distinct phases. These results suggested that multiple forms of the enzyme, each with its own kinetic properties, might be present under assay conditions. In addition, the kinetic parameters of the enzyme were found to change with enzyme concentration, suggesting that the multiple forms may simply be different oligomeric states of the enzyme. Oligomerization of enzymes, like conformational change, can be an important regulatory mechanism (6). We have pursued the question of oligomeric changes using enzyme which we have purified to homogeneity from potato tuber mitochondria.

MATERIALS AND METHODS

Enzyme. NAD malic enzyme was purified from red potato (*Solanum tuberosum* var. Chieftan) tubers as previously described (8).

Enzyme Assays. NAD malic enzyme activity was assayed spectrophotometrically by following the reduction of NAD (or NADP, as indicated) at 340 nm in 1.0 ml of assay medium at 25°C. For routine assays of column fractions, the following assay mix was used: 4 mM NAD, 100 mM malate, 5 mM DTT, 20 mM $MgCl_2$, 50 mM Tes (pH 7.0). Because the enzyme does not utilize metal-substrate complexes, detailed kinetic evaluations were performed using assays in which malate^{2-}, Mg^{2+}, and NAD^+ concentrations were all expressed in terms of free, uncomplexed species, as previously described (3, 8). Assays for malate saturation series contained 6 mM free NAD, 8 mM free Mg^{2+} and 50 mM Tes (pH 6.8). Assays were run for a period of time ranging from 8 to 30 min in order to ensure that the reaction velocity had obtained a constant value. Kinetic parameters were obtained by fitting the data from a substrate saturation curve (usually comprising data from 16 individual assays) to the equation

$$v_{obs} = \frac{V_m \times S^n}{K_m^n + S^n}$$

where n is the Hill coefficient and V_m is the maximal velocity under the given experimental conditions. The data were fitted to this model by means of an iterative nonlinear fitting program, based on the Marquart algorithm, as previously described (3). This program was written in BASIC, and was used with a TRS-80 Model II computer.

Gel Filtration. Molecular weights were estimated by gel filtration according to the method of Andrews (1) using a 1.6 × 75 cm or 1.6 × 79 cm column of Bio-Gel A-0.5m at 5°C. As the mol wt calibration of the column is sensitive to the ionic strength of the eluting buffer, mol wt standards were run in each buffer system used with malic enzyme. The standard column buffer comprised 100 mM Tes (pH 7.0), 2 mM Mg^{2+}, and 5 mM DTT. The Mg^{2+} was supplied as MgO rather than as a salt to avoid complications caused by most of the common anions such as Cl^- (which causes progressive irreversible inactivation of malic enzyme with prolonged exposures) and SO_4 (which activates the enzyme). When additional ligands were added to the elution buffer, the Mg^{2+} and ligand levels were corrected for any depletion caused by metal complexation and all concentrations expressed in terms of free, uncomplexed species, as with the assay medium. Filtration was carried out in an upward direction at 15 ml/h and 0.75 ml fractions were collected. The loading of samples onto the column was followed by the application of 1 to 2 ml of 10% sucrose in column buffer to insure a sharp sample boundary. Elution profiles were standardized by dividing the elution volumes by the void volume for each column. The void volume was taken to be the elution peak of thyroglobulin (mol wt = 669,000) or that of the ferritin dimer (mol wt = 880,000). Samples of concentrated malic enzyme, containing 94 μg protein, were diluted into 0.5 ml of column buffer and preincubated for 1 h at 0°C prior to loading. The elution of the mol wt standards was followed with a UV monitor

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and the elution of malic enzyme was followed by assaying 10 to 20 μl samples of the collected fractions. The following were used as mol wt standards; ferritin (440,000), catalase (232,000), alcohol dehydrogenase (141,000), hexokinase (102,000), BSA (68,000) and ovalbumin (45,000).

Protein Determinations. Protein was determined by the Bio-Rad protein assay based on the dye binding method of Bradford (2).

RESULTS

These studies have focused on two features of NAD malic enzyme; the change of mol wt of the enzyme in response to various factors and the kinetic consequences of this aggregation change. Unlike the malic enzymes isolated from animal and bacterial sources, which contain a single type of subunit, the NAD malic enzyme from potato appears to have two subunits, with mol wt of 61,000 and 58,000, in roughly equal proportions (8). The states of oligomerization discussed below are based on an average subunit mol wt of 60,000, without addressing the question of the relative proportions of the two subunits in a given form of the enzyme.

Aggregation State. Gel filtration revealed that the mol wt of malic enzyme depends on the nature of the eluting buffer. Using a standard buffer (100 mM Tes [pH 7.0], 2 mM Mg^{2+}, 5 mM DTT) which is based on the buffer system used for storage of the enzyme, two peaks of enzyme activity appear; one with an average mol wt of 477,000 (probably an octamer) and a smaller form whose mol wt ranges from 132,000 to 201,000 (Fig. 1). The smaller form may represent a mixture of dimer and tetramer. The relative proportions of these two observed peaks change with the length of time that the enzyme has been stored. With freshly prepared enzyme, most of the enzyme elutes as an octamer. However, with increasing time in storage at −70°C, more of the enzyme appears in the low mol wt peak, which eventually becomes the predominant form of the enzyme. In older samples the mol wt of the smaller form (which we believe to be a mixture of two forms) appears to shift toward that expected of dimer. Thus, with increasing time in storage, the enzyme appears to disaggregate from octamer to dimer on the basis of gel filtration in this standard buffer system.

A second factor which controls the mol wt of malic enzyme is ionic strength. The standard column buffer has an ionic strength of 27 mM (this value is less than 100 mM because the zwitterionic form of the buffer does not contribute to the ionic strength). When the ionic strength is increased to 38.7 mM by increasing the buffer strength to 150 mM, most of the octamer disaggregates into a form which appears to be a dimer, having a mol wt of 115,000 (Fig. 2). Conversely, if the ionic strength is reduced to 15.6 mM by reducing the buffer strength to 50 mM, the enzyme exists mainly in a form which appears to be an octamer, with a mol wt of 514,000 (Fig. 2). This high mol wt form predominates at low ionic strength, even with samples which have been stored for considerable periods of time.

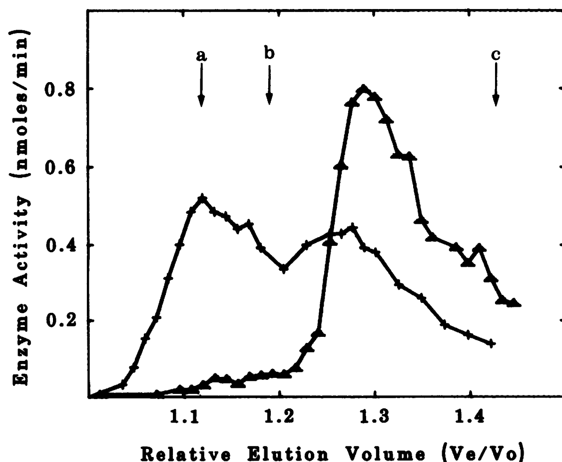

FIG. 1. Elution profile of: (+), freshly prepared malic enzyme; (△), enzyme stored for 4 months at −70°C. Both samples eluted in standard buffer. Activity in each fraction determined by assaying 15 μl aliquots. The relative elution volumes of mol wt standards are indicated by arrows. a, ferritin (440,000); b, catalase (232,000); c, BSA (68,000).

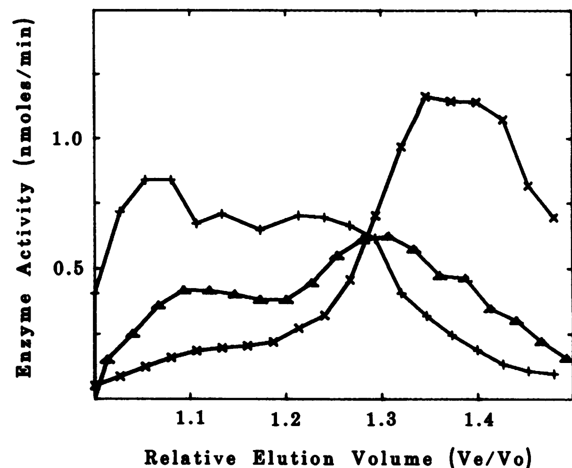

FIG. 2. Effect of buffer strength on the gel filtration behavior of malic enzyme. (+), 50 mM Tes; (△), 100 mM Tes; (×), 150 mM Tes. Other conditions as in standard buffer. Activity in each fraction determined by assaying 15 μl aliquots.

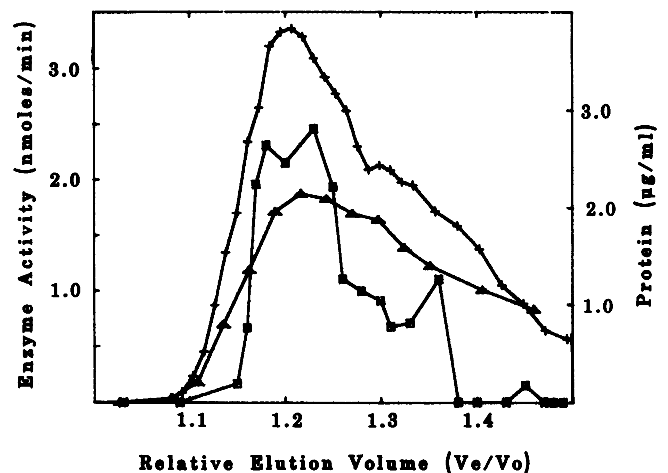

FIG. 3. Elution profile of: (+), enzyme in standard buffer supplemented with 100 mM malate; (△), enzyme in complete assay medium (50 mM $malate^{2-}$, 8 mM Mg^{2+}, 6 mM NAD, and 5 mM DTT). Activity in each fraction determined by assaying 15 μl samples. (□), Protein elution profile for enzyme run in 100 mM malate.

The addition of various ligands to the standard buffer can cause the aggregation equilibrium to shift toward dimer. These include the activator fumarate (6 mM), the substrate NAD (6 mM), the product pyruvate (50 mM) and KCl (20 mM). However, when consideration is given to the ionic strength contribution of these additional ligands, it appears that their effects are probably nonspecific, and due solely to ionic strength. CoA (30 μM), which activates the enzyme, had no effect on the elution profile of freshly prepared enzyme, which ran as an octamer in the absence of CoA.

Despite the importance of ionic strength, certain ligands can specifically alter the aggregation state of malic enzyme in a way clearly distinct from a simple ionic strength effect. One of these is the substrate, $malate^{2-}$. At concentrations of malate sufficient to saturate a kinetic assay (50–100 mM), malic enzyme exists in a

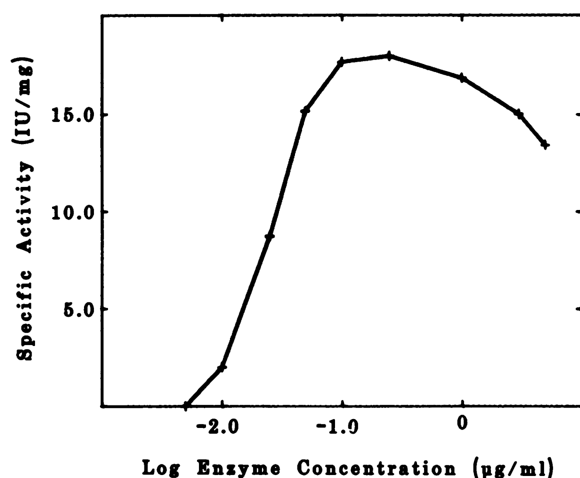

FIG. 4. Effect of enzyme concentration on the specific activity of malic enzyme. Enzyme was diluted from a stock concentration of 59 µg/ml into 1 or 3 ml assays lacking NAD and incubated at 25°C for 2 to 4 min in a water bath. The reaction was started by the addition of NAD. The complete assay mix contained 100 mM malate^{2-}, 8 mM Mg^{2+}, 6 mM NAD, and 50 mM Tes (pH 6.8).

Table I. *Kinetic Parameters of Malic Enzyme in Different States of Aggregation*

The K_m for malate, the maximal velocity (V_m) and the Hill coefficient (n_H) were determined from a malate saturation series as described in "Materials and Methods," using 15-µl samples of enzyme from the peak fraction of each form of the enzyme immediately after elution. Final enzyme concentrations ranged from 0.01 to 0.04 µg/ml in the assays. The values of these three parameters are the means of three to ten determinations.

Aggregation State	$K_{m(malate)}$	V_m	n_H	V_{NADP}:V_{NAD}	Decrease in K_m^{mal} Due to 6 mM Fumarate
	mM	*IU/mg*		*ratio*	*%*
Dimer	26.4	25.9	1.51	0.12	23.0
Tetramer	4.8	80.2	1.38	0.27	60.0
Octamer	4.3	34.7	1.35	0.26	40.0

single form (Fig. 3), with a mol wt of 264,000. Based on a subunit mol wt of approximately 60,000, this form of the enzyme is probably a tetramer. Low levels of malate (4 mM) fail to cause tetramer formation. The other ligands which we have found to convert the enzyme into tetramer are D-malate and citrate. Citrate is not completely ionized at pH 7.0, and thus both citrate^{3-} (50 mM) and citrate^{2-} (12 mM) are present in our column buffer. At present we have no further information on the effect of citrate and thus do not know which form of citrate is responsible for the effect.

Gel filtration of the enzyme in the presence of complete reaction medium (50 mM free malate^{2-}, 6 mM free NAD^+, 8 mM free Mg^{2+}, 5 mM DTT, and 50 mM Tes [pH 7.0]) yields enzyme with a mol wt of 279,000 (Fig. 3). Under catalytic conditions the enzyme thus appears to be in the same tetrameric state that is produced by high levels of malate alone.

Protein elution profiles were not routinely evaluated in all experiments because the protein levels were below the detection limit of the UV monitor. In those instances where protein elution profiles were determined by colorometric assay, the protein levels always peaked at or near the peak of activity (Fig. 3). Protein yields averaged 29% in control runs, 61% in runs with malate, and 39% in runs where the enzyme eluted in the dimer form. The average yield of activity in these cases was 27%, 65%, and 38%, respectively. Thus, the specific activity of the enzyme did not change significantly during gel filtration, and in each case was established by the end of the preincubation period. As low levels (less than 0.1 mg) of enzyme were loaded onto the column, the incomplete recovery of enzyme is not surprising and may simply reflect dilution-induced loss of activity (see below) and the creation of protein concentrations too low to measure with the colorimetric assay.

Kinetic Properties of the Multiple Enzyme Forms. One way to displace the equilibrium in a self-associating system of oligomers is by dilution, which will favor the formation of lower states of aggregation. Alteration of the aggregation state of malic enzyme in this way has dramatic kinetic consequences. Evidence of this is given (Fig. 4), which shows that the specific activity of the enzyme is not constant, but varies with the extent to which the enzyme is diluted.

In order to assess the kinetic properties of the enzyme in each state of aggregation, we have analyzed the peak fractions for each form immediately after elution from the gel filtration column and determined $K_{m(malate)}$, V_{max}, the Hill number (n_H), the fractional activity with NADP, and the decrease in the $K_{m(malate)}$ in response to the addition of the activator fumarate to the assays. The differing characteristics of the enzyme forms found in gel filtration are summarized in Table I. Of the three forms, the dimer is the least active. The $K_{m(malate)}$ is high, and the V_{max} is less than one-third that of the more active form. Of the three enzyme forms, the dimer is also the least able to utilize NADP in place of NAD, and shows the least activation in response to the addition of fumarate to the assay.

Its low $K_{m(malate)}$ and high V_{max} make the tetramer the most active of the three forms. The tetramer is also the most active of the three forms with respect to NADP and furthermore shows the greatest percent activation in response to fumarate. The fumarate activated tetramer has a $K_{m(malate)}$ of 1.66 mM, which is the highest affinity for malate which we have observed for this enzyme under any circumstance.

The octamer seems to have intermediate kinetic properties. Like the tetramer, it has a high affinity for malate, but the V_{max} is less than one-half that of the tetramer. With respect to NADP-linked activity it is similar to the tetramer, but the response of the octamer to fumarate is somewhat lower than that of the tetramer.

DISCUSSION

We have found that NAD malic enzyme is capable of existing in three different mol wt forms that, on the basis of a subunit mol wt of 60,000, appear to be dimer, tetramer, and octamer. Although the shapes of the elution profiles suggest that complete separation of discrete oligomers is not achieved on the column, it is possible to enrich for a given form of the enzyme. Each of these three forms has distinctly different kinetic properties. The variation that we observe among the various forms with respect to their kinetic parameters may explain some of the variation in the published data on this enzyme and may also explain the multiphasic saturation curves and the kinetic variations we observe with changes in enzyme concentration.

The 264,000 D form of the enzyme, which we take to be a tetramer, is the most active form of the enzyme, having a low K_m for substrate and the highest V_{max}. It is also the form of the enzyme which appears in gel filtration under assay conditions, when the enzyme is turning over during the elution process. The mol wt of this form is in close accord with the reported mol wt of NAD malic enzyme isolated from animal sources (7, 12). Mol wt of approximately 300,000 to 400,000 have been reported for impure preparations of NAD malic enzyme from plant tissues (4, 10, 14). As we also find that the purified NAD malic enzyme from potato can exist in a higher mol wt form (477,000 D) it would seem that the plant NAD malic enzymes are capable of existing

in higher states of aggregation than the enzyme from animal sources.

The octamer and tetramer are kinetically similar, differing chiefly in their V_{max}. The most significant kinetic difference between the three different oligomers is the more than 5-fold increase in the K_m for malate when higher aggregates are dissociated into dimer. Its high K_m should render the dimer inactive under most conditions in the cell. In fact, a concentration of malate sufficient to produce significant activity with the dimer is probably adequate to stabilize the enzyme in tetramer form.

Because a mixture of enzyme forms may be present even in peak fractions from the column, and because dilution of enzyme during the assay process will favor dissaggregation, substrate effects on the aggregation equilibrium are likely to be significant in assays and may lead to Hill coefficients greater than one. Even when no classical cooperativity exists between the subunits of an enzyme, if different oligomeric forms of the enzyme have different affinities for substrate, apparent cooperativity can be generated by substrate-induced changes in aggregation state, with Hill coefficients of up to 1.6 for monomer-tetramer transitions and up to 1.33 for monomer-dimer transitions being possible (6). Such an effect may be the basis of the weak cooperativity we observe (Table I), although our data provide no direct evidence of malate-induced aggregation of dimers to tetramer, as required by this model. Any substrate-induced shift in the enzyme equilibrium must be rapid, as lags are not observed with this enzyme except when extremely low levels of enzyme are used.

The low activity dimer is likely to be a prevalent form *in vivo*, as it is the major form of the enzyme at ionic strengths above 40 mM. Although other factors which we have not yet evaluated in detail, such as the concentration of enzyme or the concentration of reduced thiol (8), may modify this interpretation, it nevertheless appears that the activity of malic enzyme within the mitochondrion is likely to be controlled in large part by the concentration of malate or other specific ligands capable of stabilizing the enzyme in its most active form. At this point we have only limited information on the malate dependency of this effect. We know that concentrations of 50 mM or greater are sufficient, whereas 4 mM malate does not lead to tetramer formation. Given the fact that plant cells often accumulate large quantities of malate (13), it seems probable that this effect of malate on the aggregation state of malic enzyme has regulatory significance. *In vitro*, the stabilization of tetramer by malate may cause the complexity which we have observed in malate saturation curves under various circumstances and the variety of K_m values which have been reported in the literature. Any given mixture of dimer and tetramer would, for example, yield a complex malate saturation curve appearing to have either an intermediate K_m (8), or two distinct phases (14). In addition, this equilibrium between kinetically distinct forms can be influenced by the concentration of enzyme. The K_m for malate, for example, has been reported to vary with enzyme concentration (11).

That the NAD malic enzyme should be mainly in the inactive dimer form at physiological ionic strength is not surprising, as the enzyme allows mitochondria to completely oxidize their normal supply of tricarboxylic acid cycle intermediates, which are necessary to keep the cycle in operation. Thus, it is to be expected that malic enzyme should tightly controlled unless high levels of organic acids are present, as when stored acids are mobilized. Indeed, the $K_{m(\text{malate})}$ for malic enzyme in intact sweet potato mitochondria is quite high (42 mM) (15). We suggest that high levels of malate and citrate act to maintain malic enzyme in its active, tetrameric form. The most potent activator of the malic enzyme, CoA, does not appear to alter the state of aggregation. Fumarate, the other activator of malic enzyme we examined, does favor dimer formation when tested in the absence of substrates but this is probably a simple ionic strength effect. Disaggregation could not be the basis of fumarate activation as all forms of the enzyme, including the dimer, can be stimulated by fumarate and furthermore the dimer is the least active form. Thus, fumarate must activate the enzyme through some more subtle mechanism, such as conformational change, which is not discernible in gel filtration. As neither fumarate nor CoA appear to activate through changes of the aggregation state, it seems that alteration of the enzyme's size provides a relatively crude level of regulation which is distinct from the regulation caused by effectors such as CoA and fumarate. At present the relative effects of malate, reduced thiol, enzyme concentration and ionic strength on the enzyme aggregation equilibrium are only partially understood and remain to be examined in greater detail.

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