Malate Dehydrogenase and NAD Malic Enzyme in the Oxidation of Malate by Sweet Potato Mitochondria

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ABSTRACT

Over a range of concentrations from less than 0.1 mM to more than 70 mM, sweet potato root mitochondria display a bimodal substrate saturation isotherm for malate. The high affinity portion of the isotherm has an apparent Km for malate of 0.85 mM and fits a rectangular hyperbolic function. The low affinity portion of the isotherm is sigmoid in character and gives an apparent $S_{0.5}$ of 40.6 mM and a Hill number of 3.7.

Extracts of sweet potato mitochondria contain both malate dehydrogenase and NAD malic enzyme. The malate dehydrogenase, assayed in the forward direction at pH 7.2, shows typical Michaelis-Menten kinetics with a Km for malate of 0.38 mM. The NAD malic enzyme shows pronounced sigmoidicity in response to malate with a Hill number of 3.5 and an $S_{0.5}$ of 41.6 mM.

On the basis of the normal kinetics, the Km, and the fact that oxaloacetate production from malate by mitochondria appears most active at low malate concentrations, the high affinity portion of the malate isotherm with mitochondria is attributed to malate dehydrogenase. The low affinity portion of the malate isotherm with mitochondria is thought, on the basis of the similarity of $S_{0.5}$ values, the Hill numbers, and the greater production of pyruvate from malate at high malate concentrations, to represent the activity of the NAD malic enzyme.

The response of plant mitochondria to malate is often variable and erratic (6, 10, 12, 13, 15). Saturation with the substrate is difficult to achieve, coupling with phosphorylation is sometimes less than with other tricarboxylic acid cycle substrates using NAD as a cofactor (16) and the response to exogenously added cofactors such as NAD may be at variance with that of other substrates (3, 6, 15). In part, these unexpected responses may arise from the characteristics of the enzyme, malate dehydrogenase, usually assumed to be responsible for the oxidation of malate in the tricarboxylic acid cycle. Malate dehydrogenase is strongly inhibited by its product, oxaloacetate, at pH values in the range likely to be found in the mitochondria (10, 17, 18), and is subject to allosteric regulation by a number of effectors (1).

We have observed in mitochondria from a variety of plant tissues, including sweet potato, beet roots, cauliflower florets, and *Arum* spadix, a failure of O_2 uptake rates to be saturated by levels of malate greatly in excess of the concentrations which would be expected to elicit no further response on the basis of the observed Km for malate of malate dehydrogenase in mitochondrial extracts. In addition, it is frequently difficult to obtain smooth curves in the O_2 uptake response of plant mitochondria to malate, and where sufficient numbers of careful determinations are made, the malate response curve appears to be inflected. Similar results have been obtained by others (3, 13).

This report details the characteristics of O_2 uptake by sweet potato mitochondria in response to malate, and shows that the saturation isotherm for malate is bimodal, with an intermediary plateau, and provides evidence that this response is indicative of dual pathways of malate metabolism in these mitochondria, one through malate dehydrogenase to oxaloacetate, the other through NAD malic enzyme to pyruvate.

MATERIALS AND METHODS

Plant Materials and Preparation of Mitochondria. The roots of sweet potato (*Ipomea Batatas*, cv. Garnet) were obtained from a local market and used immediately. Mitochondria were prepared by grinding 200 g of diced sweet potato roots with 300 ml of a grinding medium consisting of 0.5 M mannitol, 0.07 M TES, 1 mM EDTA, 4 mM cysteine, and 1 mg ml^{-1} BSA at pH 7.5. A basket centrifuge-type vegetable juicer (Sanyo model SJ 5400) was used for grinding, with plant tissue and grinding medium being introduced into the grinder simultaneously. The tissue extract was filtered through finely woven polyester cloth and then centrifuged for 10 min at 8,000g. The supernatant liquid was discarded, and the sedimented material resuspended in 60 ml of a washing medium consisting of 0.3 M mannitol, 0.05 M TES, and 1 mg ml^{-1} BSA at pH 7.2. A slow speed centrifugation at 250g for 10 min removed starch, nuclei, cell walls, and other debris. The supernatant liquid was then centrifuged at 12,000g for 5 min, the sediment resuspended in 60 ml of the washing medium, and again centrifuged 5 min at 12,000g. The packed organelles were suspended in 3 ml of a medium consisting of 0.25 M mannitol, 0.05 M TES, 8 mM K-phosphate, 4 mM MgCl$_2$, and 1 mg ml^{-1} BSA, pH 7.2. All operations were carried out at 0 to 4 C. Protein determinations were by the method of Lowry, using BSA as a protein standard.

Oxygen Uptake Measurements. Oxygen uptake was monitored at 25 C using a Clark-type O_2 electrode (YSI, Yellow Springs, Ohio) in a 1.2-ml volume of reaction medium containing 0.25 M mannitol, 0.05 M TES, 8 mM Pi, 4 mM Mg^{2+}, 1 mM ADP, and 1 mg ml^{-1} BSA at pH 7.2. No endogenous rates were observed, and the reaction was initiated by adding malate with a microsyringe.

Assay of Metabolic Products. Products of the metabolism of malate by mitochondria were assayed after O_2 uptake was initiated by the addition of malate. The reaction was stopped in all cases when 10% of the O_2 in the cell had been depleted. At this point, 1 ml was quickly removed from the O_2 electrode cell and added to 0.8 ml of cold 6% HClO$_4$ containing 0.1 mM EDTA. The samples were quickly neutralized with KOH and centrifuged 3 min at 500g to remove KClO$_4$. Two types of assay were carried out on aliquots from this neutralized extract. For pyruvate determination (4), 0.4 ml of the supernatant liquid was transferred to a cuvette containing 0.05 M TES, 0.01 M Mg^{2+}, 0.1 mM NADH in a total volume of 1 ml at pH 7.2. Lactate dehydrogenase (1.4 IU) was added and the reaction followed at 340 nm in a 1-cm light path in a cell compartment maintained at 25 C. When there

was no further change in A at 340 nm, 10 μl (1.2 IU) of citrate lyase were added and citrate estimated by the additional amount of NADH oxidized.

After treatment with $HClO_4$, considerable malate dehydrogenase activity remains in the extracts, making oxaloacetate determination with this enzyme unsatisfactory. Oxaloacetate was, therefore, determined in a second aliquot of the centrifuged extract by removing 1 ml and incubating with 0.01 M $NiCl_2$ for 5 min at 43 C (11). Pyruvate was then determined in the sample in which oxaloacetate had been decarboxylated by Ni^{2+} by the method described above, and the amount of pyruvate found in the first aliquot subtracted from that found after Ni^{2+} treatment to give an estimate of oxaloacetate concentration. Preliminary tests in which known amounts of oxaloacetate were added to mitochondrial extracts indicated that decarboxylation was complete under the conditions used and recovery as pyruvate was quantitative. The enzymes used were analytical grade products from Böehringer Mannheim and were diluted according to the manufacturer's recommendations.

Enzyme Extraction and Assay. Freeze-thaw extracts of mitochondria were prepared by freezing at -20 C for 24 hr, followed by thawing at room temperature. This procedure was repeated twice and the thawed mitochondrial suspension was centrifuged at 37,000g for 30 min. The clear supernatant liquid provided the enzyme extract from which assays for malate dehydrogenase (L-malate-NAD oxidoreductase, EC 1.1.1.37), NAD malic enzyme (L-malate-NAD oxidoreductase [decarboxylating], EC 1.1.1.38), and NADP malic enzyme (L-malate-NADP oxidoreductase [decarboxylating], EC 1.1.1.40) were carried out. Malate dehydrogenase assays contained 0.05 M TES, 1 mM NAD, and varying levels of malate, pH 7.2, in a total volume of 1 ml. The NAD malic enzyme was assayed with 0.05 M TES, 1 mM NAD, 10 mM Mg^{2+}, and appropriate levels of malate, pH 7.2, in 1 ml total volume. The NADP malic enzyme was assayed with the same mixture as the NAD malic enzyme except that 1 mM NADP was substituted for NAD. For the NAD malic enzyme, the reaction was started by the addition of Mg^{2+} after equilibration of malate dehydrogenase, which occurred in less than 1 min. All assays used a 1-cm light path and were carried out in the cell compartment of a Gilford model 2400 spectrophotometer maintained at 25 C. The recorder was set for 0.25 A full scale for malate dehydrogenase and 1.0 A full scale for the NAD and NADP malic enzyme assays.

Data Processing. Rates of O_2 uptake were calculated from O_2 electrode measurements assuming an O_2 concentration of 254 μM at 25 C. Rates of reaction of isolated enzymes were calculated as μmol NADH or NADPH produced/min.

Malate saturation curves were fitted either by the method of Cleland (5) using a program written for a Wang model 720 computer, or by the gradient search least squares fitting procedure using the Fortran program of Bevington (2) translated into Wang Basic and using a Wang model 2200 computer. Lines shown in all figures except Figure 2 are computer-drawn plots of the equation of best fit to the indicated points.

RESULTS

Malate Oxidation by Mitochondria. When sweet potato mitochondria are permitted to take up O_2 in the presence of a wide range of malate concentrations (from 0.02 to 73 mM), a bimodal saturation curve is produced as illustrated in Figure 1. This set of data was chosen because it shows the inflection in the 10 mM malate concentration range more clearly than in some cases. However, with sweet potato mitochondria prepared in a variety of ways and regardless of whether the mitochondria are coupled or uncoupled, we always obtain indications of a bimodal malate saturation isotherm. Although the data of Figure 1 are not typical in the sense that they represent an average response, they
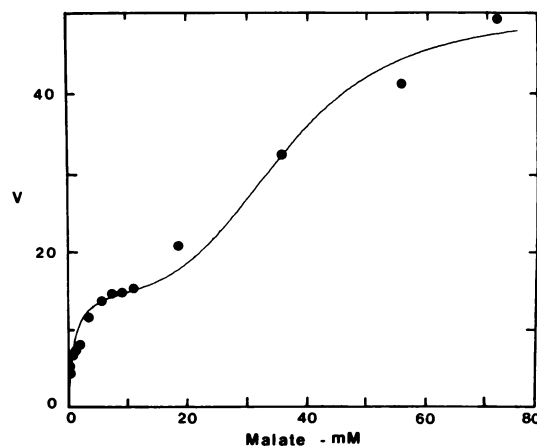

FIG. 1. Uptake of O_2 by sweet potato mitochondria as a function of malate concentration. The line is the best fit of the experimental data to equation 1.

do provide a vivid illustration of a situation which we believe occurs in some degree in all sweet potato mitochondria oxidizing malate, and perhaps with mitochondria from most other plant sources as well. In the case of the data summarized in Figure 1, the mitochondria were provided only with malate, Mg^{2+}, Pi, and an excess of ADP, so that the velocities given are state 3 rates using only endogenous cofactors. We have, however, found that in some mitochondrial preparations, added NAD does produce an increased rate and a more pronounced bimodality of the malate saturation curve.

Curves with the general shape of that in Figure 1 are observed in phenomena as diverse as ion uptake by plant roots and the activity of highly purified enzymes. Explanations of mechanisms which might produce such responses to substrates fall generally into two categories. Either two different systems with differing affinities for the substrate are involved, or the response is due to "negative cooperativity" or half-site reactivity of a single enzyme. In part because of the complex sequences of reactions involved in malate oxidation by intact mitochondria and in part for reasons which will be elucidated below, we do not believe that this case of bimodal response to malate is indicative of half-site reactivity for a single enzyme, but rather that it represents the summation of the activities of two different enzymes, both of which oxidize malate.

Our preference as candidates for these two enzymes is malate dehydrogenase for the high affinity or left half portion of the curve of Figure 1 and NAD malic enzyme for the low affinity or right hand portion of the curve. The reasons for this assessment are developed below, but the general fit of two different enzyme activities to this type of curve may be obtained by assuming that the high affinity enzyme has normal Michaelis-Menten kinetics and that the low affinity enzyme displays sigmoid kinetics. We can sum the equations describing two such enzymes and obtain:

$$v = \frac{V_1 S}{K_1 + S} + \frac{V_2 S^n}{K_2{}^n + S^n} \tag{1}$$

Where v = total rate at any substrate concentration S; $V_1 = V_{max}$ for the high affinity enzyme; $K_1 = Km$ for the high affinity enzyme; $V_2 = V_{max}$ for the low affinity enzyme; $K_2 = S_{0.5}$ for the low affinity enzyme; and n = the Hill coefficient for the low affinity enzyme.

The experimental data of Figure 1 are fitted to equation 1 by the gradient search least squares method (2), and the line shown in Figure 1 is the line of best fit to equation 1. From the fitting process, estimates of the kinetic parameters of the two enzymes are obtained. These are found to be: $K_1 = 0.85$ mM; $K_2 = 40.6$

mM, and $n = 3.67$. These results demonstrate that the data of Figure 1 can be produced by the summed activities of an enzyme with a low Km and another with a large $S_{0.5}$ and a Hill number in the region of 4.

Products of Malate Metabolism. The identification of the two enzymes oxidizing malate in the sweet potato root mitochondria as malate dehydrogenase and NAD malic enzyme is supported by studies of the accumulated products of malate metabolism in the mitochondria (Fig. 2). At low malate concentrations, the fraction of total O_2 uptake which can be accounted for by accumulated oxaloacetate is high, while as the concentration of malate increases, the fraction of O_2 uptake appearing as oxaloacetate diminishes. Conversely, the fraction which appears as pyruvate is low at low malate concentrations and increases with increased malate concentration. The third line shows that citrate, which should increase as larger amounts of pyruvate are made available to produce acetyl CoA, does increase as malate concentration is increased.

Enzymes in Organelle-free Extracts. While Figure 2 indicates that one product of malate, oxaloacetate, does diminish relatively with increasing malate concentration, while another, pyruvate, is relatively increasing, there still remains the question of whether the two enzymes postulated as being responsible for this phenomenon are, in fact, present in sweet potato mitochondria. Evidence on this question was sought by preparing a freeze-thaw extract of sweet potato mitochondria. The mitochondrial suspension was subjected to two cycles of freezing and thawing, and the clear extract was used without further purification to assay for malate dehydrogenase and malic enzymes.

A malate saturation curve for malate dehydrogenase is shown in Figure 3. This enzyme was assayed at pH 7.2, which is unfavorable for malate dehydrogenase activity in the forward reaction, but this pH was chosen to be comparable with the conditions used in the mitochondrial O_2 uptake experiments. The response of malate dehydrogenase to malate shown in Figure 3 fits a "normal" Michaelis-Menten saturation isotherm and indicates a Km of 0.38 mM for malate. Assays at malate concentrations higher than those shown in Figure 3 did not produce a significant increase in reaction rate. Comparative values of the Km for malate are difficult to find, but the value found here falls in the range usually found for malate at higher pH values (1). The most nearly comparable values were determined at pH 7.5 for various malate dehydrogenase isoenzymes from maize where the reported range is from 4.5 to 27 mM (18). Both the nature of the response to malate and the indicated Km (0.38 mM as compared with 0.85 mM) support the identification of malate dehydrogenase as the high affinity component of the two-enzyme malate-oxidizing system postulated as an explanation of the results shown in Figure 1.

The extract of sweet potato mitochondria also contained an

NAD malic enzyme as shown in Figure 4, where the response of this enzyme to malate is seen to be sigmoidal in nature as postulated for the low affinity component of the malate-oxidizing system. The line of Figure 4 is a gradient least squares fit to:

$$v = \frac{V_2 S^n}{K_2^n + S^n} \qquad (2)$$

where the symbols have the same meaning as in equation 1.

The process of fitting the data to this second half of equation 1 produces estimates for this NAD malic enzyme of an $S_{0.5}$ for malate of 41.6 mM and $n = 3.53$. Both of these figures are in good agreement with those obtained for the low affinity enzyme in the O_2 electrode assays of intact mitochondria.

In addition to the NAD malic enzyme, enzymes which produce pyruvate from malate using NADP as a cofactor have been reported from plant sources (7, 8, 15). We have found that the oxidation of malate by extracts of sweet potato mitochondria using NADP as a cofactor is somewhat variable, but in several different preparations, the activity with NADP was found to be $16.4 \pm 1.5\%$ of the activity with NAD. Under the assay conditions used, this NADP malic enzyme appears to have a normal rectangular hyperbolic response to malate (Fig. 5). The indicated Km is 0.98 mM, and both the low Km and the lack of sigmoidicity support the idea that this enzyme is an unlikely candidate for the low affinity, pyruvate-producing enzyme responsible for the right hand portion of the curve shown in Figure 1. To the extent that it is actually operating in intact mitochondria, the activity of the NADP malic enzyme may contribute to the high affinity portion of the curve or may tend to mask the inflection between the two phases seen in Figure 1.

DISCUSSION

Although malate saturation curves for O_2 uptake by mitochondria which do not completely fit a Michaelis-Menten iso-
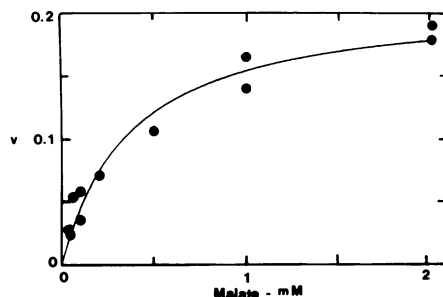

FIG. 3. Activity of malate dehydrogenase in freeze-thaw extract of sweet potato mitochondria as a function of malate concentration. The line is the rectangular hyperbola of best fit to the experimental data.
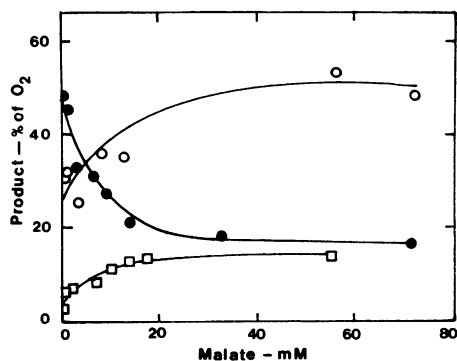

FIG. 2. Production of pyruvate (O), oxaloacetate (●), and citrate (□) during malate oxidation by sweet potato mitochondria as a function of malate concentration.
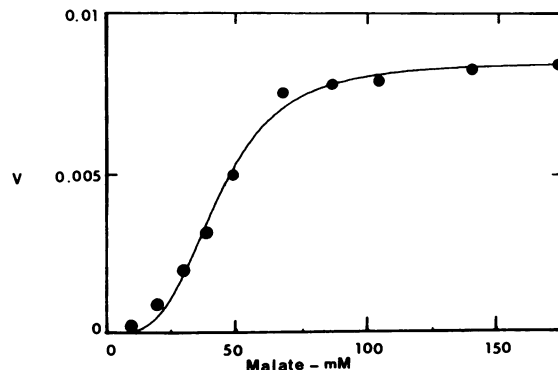

FIG. 4. Activity of NAD malic enzyme in freeze-thaw extract of sweet potato mitochondria as a function of malate concentration. The line is the best fit of experimental data to equation 2.
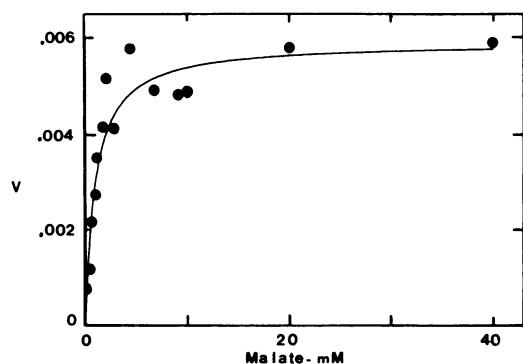
FIG. 5. Activity of NADP malic enzyme in freeze-thaw extract of sweet potato mitochondria as a function of malate concentration. The line is the rectangular hyperbola of best fit to the experimental data.

therm have been published, e.g. (3, 13), the first report of a clearly bimodal malate saturation curve for plant mitochondria of which we are aware is that of Brunton and Palmer (3) which showed, with wheat mitochondria, a definite inflection in the range of 30 to 40 mM malate. These workers also concluded that the bimodal curve was the result of the activity of two enzymes but that, "—it is reasonable to assume that the enzyme saturating at 15 mM is the malic enzyme while the other saturating at 60 mM is the malate dehydrogenase." The basis for this assumption appears to have been the earlier finding (6) that with 100 mM malate, Jerusalem artichoke mitochondria produce more pyruvate when supplied with exogenous NAD, Ca^{2+}, rotenone, and/ or 50 mM thiamine pyrophosphate than in the endogenous reaction. Coleman and Palmer (6) concluded that one pathway for malate oxidation is via a route which is sensitive to the inhibitors rotenone and piericidin A, and which uses malate dehydrogenase as the first step. The other pathway, in their view, is "catalyzed by an exogenous NAD^+-linked malic enzyme and associated with the exogenous NADH pathway of oxidation, which is resistant to piericidin A and rotenone inhibition." The interpretation that piericidin A is a specific inhibitor for the enzyme-oxidizing malate at high malate concentrations (in their view, malate dehydrogenase) is made somewhat problematical by the data of Brunton and Palmer, which show in one case (3, Fig. 8) more than 50% inhibition of malate oxidation by 25 ng piericidin A at 70 mM malate, but in another (3, Fig. 9) relatively little effect of 25 ng piericidin A on malate oxidation at 100 mM malate.

In our view, the evidence cited by these workers in support of malate dehydrogenase as the enzyme responsible for the low affinity portion of the bimodal malate saturation curve and their identification of the NAD malic enzyme as that producing the high affinity portion of the curve is subject to alternative interpretations. For example, the observation that exogenous NAD and rotenone result in increased pyruvate production at 100 mM malate is indicative of a situation in which the NAD malic enzyme is likely to respond more to the added NAD than malate dehydrogenase does. The concomitant inhibition of malate dehydrogenase activity by rotenone operating on the endogenous NADH oxidase would also tend to produce a relatively increased activity of the NAD malic enzyme.

Although a number of workers (3, 13, 15, 16) have shown that pyruvate is produced from malate in plant mitochondria, we are not aware of any report of comparative changes in pyruvate and oxaloacetate production as a function of malate concentration over the range shown in Figure 2. The changing balance

between the two products of malate metabolism seen in that figure seems most compatible with our interpretation that malate dehydrogenase is the enzyme responsible for the high affinity portion of the malate saturation curve of Figure 1 and NAD malic enzyme for the low affinity portion.

The NAD malic enzyme has been isolated from a number of plant mitochondria (3, 8, 9, 14, 15). The reported Km values for malate are generally much lower than the $S_{0.5}$ we have found with the sweet potato enzyme, but these seem to have been determined under conditions in which the enzyme did not display sigmoid kinetics. Our choice of pH (7.2) and divalent metal ion cofactor (Mg^{2+}) was conditioned by the desire to duplicate as nearly as possible conditions under which mitochondrial O_2 uptake measurements were made. It is clear from preliminary studies in our laboratory that the NAD malic enzyme isolated from sweet potato mitochondria is similar to that from other plants (8, 9, 14) in altering the nature of its malate saturation curve in response to changes in both pH and in the metal ion cofactor.

The study of the NAD malic enzyme in plants appears to be potentially very rewarding in better understanding of the organic acid metabolism of plants. In addition to its likely role in contributing to the accumulation of CO_2 in the cell, it also provides a means by which plants can oxidize Kreb's cycle intermediates without the necessity of supplying pyruvate from glycolysis (16), especially under conditions where quantities of malate or citrate may be stored. The exceptional degree to which the NAD malic enzyme appears to be subject to controls of various types (8, 9) also seems to indicate the probable importance of its role as part of a crucial switching point in plant metabolism.

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