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NADP-LINKED MALIC ENZYME AND MALATE METABOLISM IN AGEING TOMATO FRUIT

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Key Word Index—*Lycopersicon esculentum; Solanaceae*; tomato; metabolism; malate.

Abstract—Malate dehydrogenase (oxaloacetate-decarboxylating) ($NADP^+$) EC 1.1.1.40, malic enzyme, has been purified 40-fold to a homogeneous state using affinity chromatography and gel permeation chromatography. The M_r is 260–265 K with four subunits each of 64–65 K. The enzyme has some competitive or non-competitive inhibitors, particularly some of the Krebs cycle acids and exhibits a rapid rise in activity at the same time as activity of the enzymes of the Krebs cycle are decreasing in the tomato mitochrondrion. The malic enzyme is restricted to the cytosol. The relevance of this information to malate metabolism in plants is discussed.

INTRODUCTION

In a recent communication [1] we have shown that the characteristic switch in malate metabolism, from malate accumulation to malate utilization [2], during tomato fruit ripening was accompanied by a radical alteration in the enzymes of the citric acid cycle. The specific activity of both malate dehydrogenase (MDH) and citrate synthase (CS) fell by 60% during a 96 hr period of ripening. At the end of this time period the fruit were just beginning to accumulate lycopene and produce polygalacturonase. Later investigation [Jeffery, D. personal communication] has revealed that only 20% of the MDH is mitochondrial whereas all the CS is within the mitochondria. However, there is a coordinated fall in the activities of these two enzymes, as well as in the exclusively mitochondrial NAD-linked isocitrate dehydrogenase and NAD-linked malic enzyme. This would be expected if all these mitochondrial enzymes are nuclear coded, possibly on a single operon. However the concentration of the enzyme protein in each specific case can only be determined by a technique such as immunoassay. Although both NAD- and NADP-linked malic enzymes are found in tomato fruit, neither are part of the citric acid cycle itself, but both can decarboxylate malate to give pyruvate. The NAD-linked malic enzyme is generally thought to be found only in the matrix of plant mitochondria and this allows malate to be utilized in the presence of oxaloacetate [3]. It has been shown that the NAD-linked malic enzyme is connected to the cyanide insensitive electron transport chain by a specific rotenone insensitive NADH dehydrogenase located on the inner face of the inner membrane [4]. However, the mitochondrial MDH and another pool of NAD^+/NADH are connected to the cyanide (and antimycin) sensitive pathway by a rotenone-sensitive NADH dehydrogenase located on the inner face of the inner membrane.

The presence of an NAD(P)H specific dehydrogenase on the outer membrane of plant mitochondria allows passage of electrons from NADP-linked malic enzyme or cytosolic NAD-linked MDH direct to ubiquinone [5]. The electrons bypass the first site of ATP synthesis and this oxidation of NADPH is rotenone insensitive. Recently it has been shown that NAD can be accumulated by mitochondria [6] and so it is quite feasible that electron transport can still occur without a dependence on citric acid cycle reactions.

In the case of many 'climacteric' fruit there is a characteristic burst of respiration at the onset of colour change or other signs of senescence/ripening. The production of CO_2 has often been associated with increased NADP-linked malic enzyme activity [7]. However, it has generally been assumed that mitochondrial respiration accounted for most of observed oxygen uptake. The increase in NADP malic enzyme has only been briefly mentioned in tomato fruit [8] and is assumed to coincide with the most mature state of the fruit. Our earlier results indicate a dramatic decrease in the capacity of the citric acid enzymes to process malate at the same time as malate decreases and respiration reaches a peak. We therefore investigated the properties of pure NADP malic enzyme and its tissue activity to answer the question whether the increased CO_2 evolution and O_2 consumption was due to decarboxylation of malate in the cytosol and dehydrogenation of the NADPH formed by a mitochondrial system. This latter system would lead to O_2 uptake by a rotenone insensitive oxidation with an ADP/O ratio of two.

RESULTS

Purification

In previous reports the enzyme from other sources (grapes) has been found to bind very tightly to 2'5'ADP-Sepharose [9]. However, the degree of purification reported in the previous work was very high (700-fold).

In another report using the same enzyme [10], a 50-fold purification was achieved but without the use of the affinity column method. The enzyme from tomato fruit proved to be somewhat less stable than the enzyme from grapes in that dialysis led to rapid inactivation of the protein. Also it was found that binding to the affinity substrate was not as strong in this case as in some others. Table 1 indicates that the maximum purification achieved

Table 1. Purification scheme of NADP-linked malic enzyme showing MDH separation at each step

Stage	Protein (mg)	Volume (ml)	Total activity (μmoles NADP reduced min^{-1})	Specific activity (μmoles/mg)	Purification	Yield	%MDH
Crude homogenate	1968.8	1840.0	1.178	0.06	1.0	100.0	100.0
0–30% $(NH_4)_2SO_4$ supernatant	903.2	1930.0	1.302	0.14	2.4	111.0	79.0
30–55% $(NH_4)_2SO_4$ pellet	815.3	13.0	0.950	0.12	2.3	81.3	6.3
G-25 Sephadex	247.8	150.0	0.634	0.26	4.3	54.3	2.9
2'5' ADP Sepharose peak activity	22.4	63.1	0.114	0.51	8.5	9.8	0.9
Lyophilized S-300 peak tube	0.348	0.5	0.009	2.6	43.1	0.8	<0.1

in the case of the tomato enzyme was 40-fold. This represents the peak of the activity recovered from the S-300 column and Fig. 1 shows that the protein was a homogeneous preparation of malic enzyme. Several attempts to improve the purification of the enzyme using isoelectric focusing, amongst other methods, did not succeed in changing the final purification figure. However, it was found that MDH could be mainly removed by differential precipitation with ammonium sulphate and was not tightly bound to malic enzyme as in some other system [9].

Previous work on the enzyme from grapes [9], did not obtain a homogeneous protein and used a method of protein estimation [11] which would give a greater value in the earlier stages of purification than in the later stages, due to thiol reagents.

The M_r of the enzyme from tomato fruit, as revealed by denaturing gel electrophoresis, was in the range of 64–65 K (Fig. 1). Separation of the enzyme on gel permeation chromatography and gradient gel electrophoresis gave a native M_r of 260–265 K. Both crude and ammonium sulphate samples of the enzyme were subjected to non-dissociating PAGE and gave three bands of enzyme activity. The relative mobilities of these bands were 0.14 and 0.24 (main bands) and 0.12 (weak band) relative to bromophenol blue. Only one band at 0.24

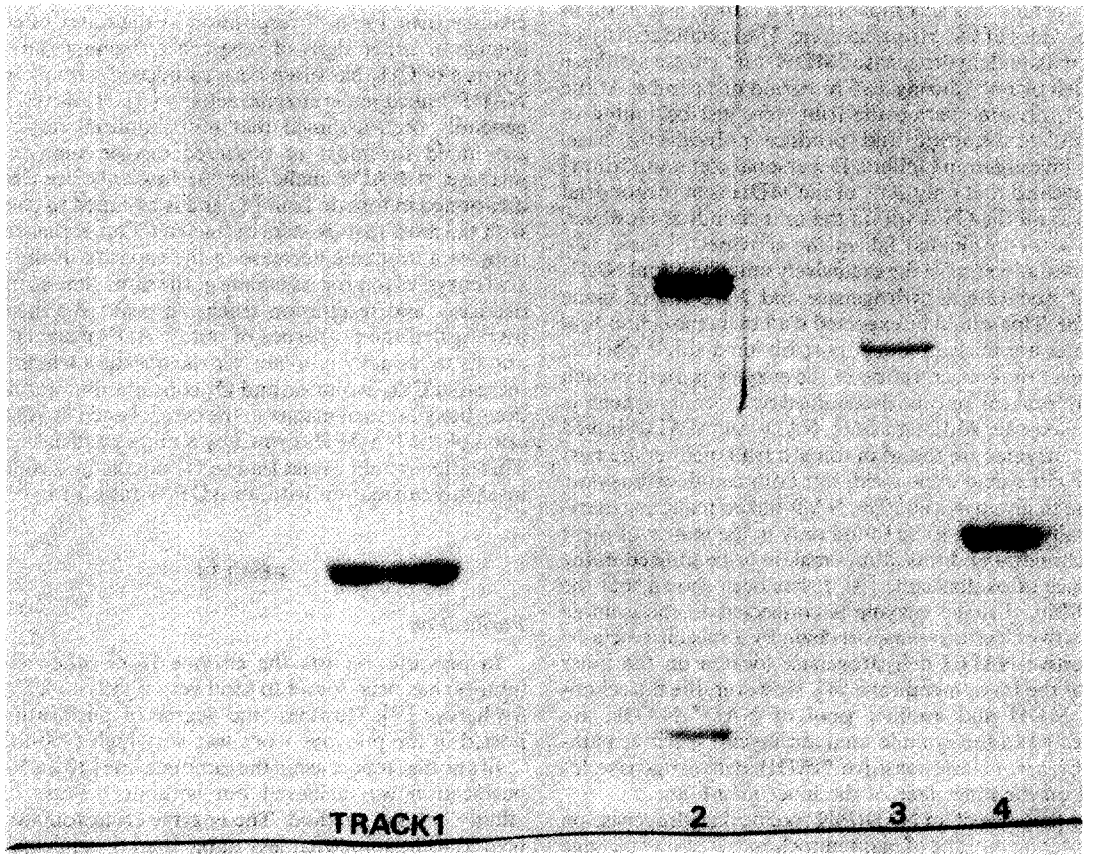


Fig. 1. SDS–PAGE (7.5%). Track 2: RNA polymerase $\beta_1\beta_{11}$ subunits at 165 and 155 K M_r; and α subunit at 39 K M_r; track 3 phosphorylase 97 K M_r and track 4 bovine serum albumin at 66 K M_r. The band in track 1 is NADP-linked malic enzyme subunits at 64 K M_r. Separation was towards the anode.

coincided with a protein band and may be the main component of that band.

Cellular localization of malic enzyme

It was found that the mitochondria could be isolated intact and gave a very good state 3 and state 4 oxidation. Although differential centrifugation gave mitochondria apparently containing 4% of the malic enzyme activity the Percoll step produced organelles totally free of NADP malic enzyme, indicating that the enzyme was cytosolic only.

Enzyme kinetics and isoelectric focusing

In a previous report on the malic enzyme from pear fruit [7], it was shown that, at pH values other than the optimum, sigmoid kinetics were displayed. In Fig. 2 the kinetics of malic enzyme from crude extracts, at a number of pH values, was compared with the kinetics of the same enzyme from a partly purified extract, again at a range of pH values. The apparent sigmoidicity of the Michaelis–Menten plots at pH 8 was missing in partly purified extracts. The enzyme showed an absolute requirement for a divalent cation, maximum activity being achieved with Mn^{2+} rather than with Mg^{2+}. The apparent constants for malate, NADP and manganese as well as the pI are shown in Table 2.

Citric acid cycle inhibitors of the enzyme

The activity of crude preparations of the malic enzyme were strongly inhibited by oxaloacetate (OAA) and ketoglutarate (KG) (Fig. 3). The activity was inhibited by 50% at 5 mM malate by 10 mM OAA and 30 mM KG. Purified NADP-malic enzyme was also inhibited, competitively, by these compounds (Fig. 3). Fumarate caused a slight activation using 5 mM malate up to a concentration of 2.5 mM fumarate and thereafter there was uncompetitive inhibition. Unlike the pear fruit [7] there was no stimulation at 1 mM malate by fumarate and a slight inhibition at higher values. Succinate caused slight activation up to 10.0 mM and slight inhibition at high concentrations; again the activation was not as high as found in other fruits [7] particularly at lower (1 mM) concentrations of malate. Pyruvate inhibited malic enzyme non-competitively.

Table 2. Kinetic parameters for purified malic enzyme

Effector	K_m^{app} (mM)	Correlation coefficient
Malate	0.530	0.999
$NADP^+$	0.038	0.998
Mg^{2+}	0.315	0.999
Mn^{2+}	0.210	0.998
pI	5.1–5.5	

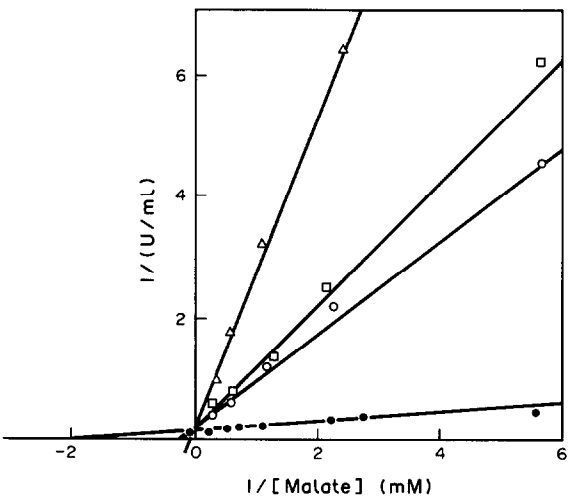


Fig. 3. Lineweaver–Burk plots of purified NADP malic enzyme activity at the following concentrations of oxaloacetate, and all at saturating concentrations of NADP and manganese: △—△, 8.33 mM; □—□, 2.17 mM; ○—○, 0.87 mM; ●—●, 0 mM.

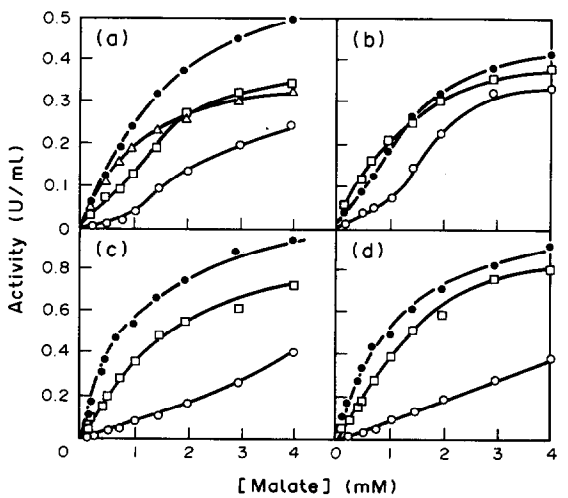


Fig. 2. Michaelis–Menten plot of NADP-malic enzyme activity from crude extracts or 30–55% $(NH_4)_2SO_4$ precipitated extracts. The extracts are from mature-green fruit a and c or red fruit b and d. The pH ranges used are: △—△, 6.5; ●—●, 7.0; □—□, 7.5; ○—○, 8.0.

Quantification of specific activity of enzyme in maturing fruits

When the activity of malic enzyme was estimated in either fruits of different ages direct from the vine or in a sample of fruits as they aged in the laboratory, it was found that the activity increased up to the mature-green stage and then decreased rapidly. This latter result was found if activity was expressed as units/g, units/mg of protein or as units alone. In the work of Jeffery *et al.* [1], mature-green fruit were treated with 27 μl/l of ethylene or left untreated. Figure 4 shows the results of estimating the activity/g of fresh weight of fruit either treated or not treated with ethylene. The specific activity at the greatest value obtained was 0.96 μmole/min/mg protein and the activity fell rapidly as the fruit ripened (Fig. 4). The first

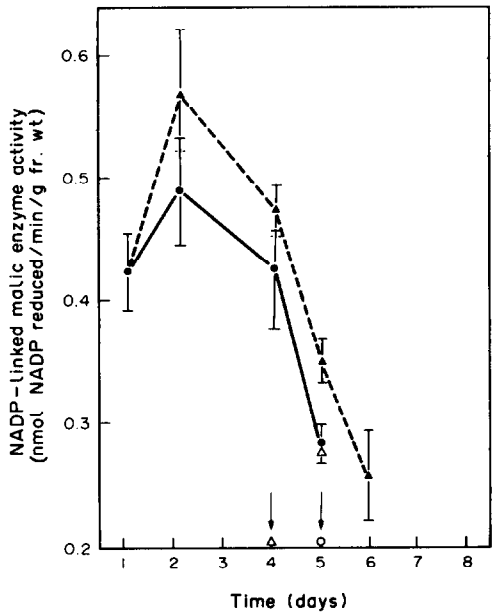


Fig. 4. Concentration of NADP-linked malic enzyme in mature-green fruit as they ripen. The fruits were in glass tanks either in the presence of 27 $\mu l\,l^{-1}$ ethylene (— —) or in air alone (—). The temperature was 22°. Also shown is the time at which polygalacturonase started to be detectable in fruit and ethylene (Δ) or in air alone (○). This latter data is from ref. [1] (Fig. 10) and relates to the same fruit.

appearance of lycopene was at day 4 in the fruit treated with ethylene and at day 5 in the control fruit. This latter data on colour formation was initially published as Fig. 10 of Jeffery *et al.* [1] but related to the same experiment.

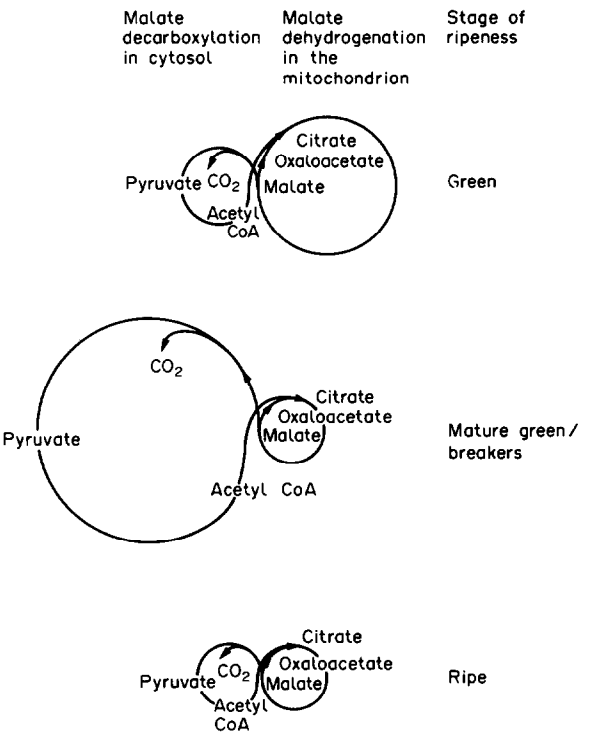
DISCUSSION

In an early communication [12] the timing of the peak of respiration, the 'climacteric rise', was studied in tomato fruit in relation to the ripeness index. Ripeness was defined by visual colour, the average stage of visual colour when the climacteric had peaked being just after the "breaker" (first appearance of colour) stage but before any of the fruit had reached the half-red half-green stage. However, the addition of 1000 $\mu l/l$ of ethylene appeared to advance the average stage of colour at the climacteric to just before the "breaker" stage. Despite using physiologically high concentrations of ethylene the conclusion was drawn that ethylene enhanced the final maximum rate of respiration and speeded up the process compared with the control. The effect of maturity on the sensitivity of tomato fruit to ethylene was studied [13], and the conclusions from the experiments being that even fruit that was only 38% mature still responded to ethylene by increased respiration.

Mitochondria can be isolated in a high state of integrity from tomato fruit of all stages of ripeness [14]. The isolated mitochondria are highly coupled at all ripeness stages but the number of mitochondria fall steadily with age [17]. Efficiency in terms of ADP/O ratios reached a peak at the mature-green–orange–green stage as did integrity [14].

In other fruits, particularly pomé fruits, the increased respiration at ripening has been attributed to large *de novo* synthesis of NADP-linked malic enzyme [7]. However, the discovery of an NAD-linked malic enzyme in plant mitochondria further complicates the situation in relation to the respiratory climacteric.

There has only been one report concerning the concentration of NADP-linked malic enzyme in green or red tomato fruit [8]. The data presented here does not agree with the latter report and coupled with the data presented by Jeffery *et al.* [1], particularly that in Fig. 2 of ref. [1], we conclude that NADP malic enzyme rises in specific activity at the same time as the specific activity of enzymes of the Krebs cycle and NAD mitochondrial malic enzyme fall. The evidence provided by Jeffery *et al.* [1] relates to a total homogenate but in a subsequent paper we will indicate that mitochondrial separation gives similar results for specific activities. In trying to equate the data from previous work with the findings reported here, it seemed appropriate to express the simplest case diagrammatically (Scheme 1). The diagram is a stylized way of representing the change that takes place in the Krebs cycle and the interconnected cytosolic malate decarboxylation loop. For the purposes of the diagram the existence of



Scheme 1. Stylized representation of the increase and subsequent decrease in the activity of the enzyme of the malate decarboxylation loop during tomato fruit ripening. The decrease in MDH activity in the mitochondrion is also shown. The radius of the malate decarboxylation loop in the green fruit is equivalent to production of 1 mole of CO_2/turn of the cycle and all other radii represent proportionate amounts of CO_2 as described in the text. The 'penny-farthing' situation in mature green/breaker fruit represents 3 moles CO_2/turn of the malate decarboxylation loop but a maximum of 4 moles has been determined.

competitive or non-competitive inhibition is ignored and the maximum flux through the pathways is the only parameter represented. At the immature green stage the maximum flux is deemed to be that for each turn of the Krebs cycle, 2 moles of CO_2 are produced and 1 mole of CO_2 for each turn of the malate decarboxylation loop. At the mature-green/breaker stage the Krebs cycle can only produce 40% of the original CO_2 (0.8 moles of CO_2) whereas the malate decarboxylation loop can produce a maximum of 4 moles of CO_2. When the red (table ripe) stage is reached the Krebs cycle still produces 0.8 moles of CO_2 but the malate decarboxylation step also only produces 0.8 moles of CO_2. Thus the amount of CO_2 produced rises to a maximum of 160% of the amount at the immature stage and falls to a low of 50–53% at the red stage. These results closely parallel the observed CO_2 production figures for the various ripening stages.

The maximum figure of 4-fold increase is taken from Fig. 2 in Jeffery *et al.* [1] where the use of retarded ripening conditions allowed the maximum rate of malic enzyme to be measured. Pyruvate rises in concentration during ripening [15] and may play an important role in modulating NADP malic enzyme activity as well as malate oxidation by the Krebs cycle.

This data does not therefore agree with previous attempts to link the peak of malate decarboxylation with development of red colour [8]. This, and the previous paper [1], are the first pieces of evidence to indicate that control of respiration in developing plant organs may be attributed to differences in the specific activities of enzymes associated with respiration. Although it has been shown that mitochondrial efficiency and integrity are greatest at the mature-green stage the individual organelles may be reduced in number and/or contain a lowered specific activity of Krebs cycle enzymes.

It will be most important to determine whether the changes in specific activities which have been measured represent a change in the number of actual enzyme molecules present or an alteration in the ability of each molecule to catalyse its substrate. The gross effect is a partial loss or irreversible inactivation of at least three enzymes of the Krebs cycle, together with an accumulation of the cytosolic malic enzyme. Subsequent loss of malic enzyme and steady state of the cycle enzymes leads to the final stage in fruit ripening. The "climacteric" burst of respiration exactly mirrors these changes, with an initial increase of respiration followed by a decline.

The NAD(P)H released by malic enzyme could be utilized by the NADPH dehydrogenase on the exterior of the mitochondria, bypassing the first ATP synthesis site. If the electrons were passed along the cyanide insensitive pathway of respiration, then there would be no net synthesis of ATP whereas the normal route would yield two-thirds of the normal amount of ATP. The evolutionary significance of the cross-over in malate utilization may be to rapidly reduce the malate concentration in the tissue without producing large amounts of ATP which would bring into play a large number of allosteric effects.

It has to be pointed out that previous results on ethylene stimulation of respiration in this fruit has not been borne out in the case of the respiratory related enzymes [1] in this study. Further careful work will be needed to establish whether physiological amounts of ethylene actually enhances and brings forward the respiratory peak, certainly the present evidence suggests that the cross-over in malate utilization is a group 1 activity as suggested by Jeffery *et al.* [1] and not stimulated by ethylene.

EXPERIMENTAL

Materials. Fruits were obtained at different stages of growth from plants of cultivar Sonatine grown in glasshouses in Long Ashton Research Station. Fruits were marked with a tag at anthesis and harvested at different stages of development. Chemicals were obtained from Sigma Chemical Company. G-25 Sephadex, S-300 Sephacryl and 2′-5′ ADP Sepharose were obtained from Pharmacia. Ampholytes pH 2–9 were from LKB.

Extraction of enzyme. The fruit contained a considerable amount of free organic acids and were extracted with a buffer containing 0.1 M of both MOPS and BICINE to hold the final pH at 8.5. The soln also contained 50 mM mercaptoethanol, 5 mM EDTA and 1% PVP was added post maceration to complex phenolics. The mixture was filtered through three layers of muslin and centrifuged for 15 min at 2° and 17000 *g* (rav. 7.0 cm). The clear supernatant was stirred at 2° and $(NH_4)_2SO_4$ added to a final concentration of 30% and a pH of 8.0. The resulting recipitate was discarded after a 30 min centrifugation at 2° and 17000 *g* (rav. 7.0 cm). The remaining supernatant was made 55% saturated with solid $(NH_4)_2SO_4$ and the precipitate collected by centrifugation as before. It was found that most of the MDH and the CS was recoverable in the final supernatant and the NADP malic enzyme in the second pellet. This latter pellet was resuspended in the extraction buffer without polyclar.

The clear suspension was passed through a column of Sephadex G-25 (290 × 56 mm) swollen in 10 mM MOPS. The desalted enzyme was then passed through a 8.5 × 2.6 mm column of 2′-5′ ADP Sepharose equilibrated with 10 mM MOPS at pH 7.0. After washing with buffer until the A280 was < 0.1 a linear gradient of NAD from 0 to 5 mM was used to elute MDH and this was followed by a linear gradient from 0 to 10 mM of NADP. The soln from this step was lyophilized and redissolved in 10 mM MOPS at pH 7.0. A glass column (680 × 16 mm) was packed with S-300 Sephracyl and calibrated with ferritin, catalase, bovine serum albumin, rabbit serum albumin and chymotrypsinogen. The enzyme was added in MOPS at pH 7.0 and pumped through the column with a peristaltic pump, absorbance was recorded at 280 nm using a Unicord S recorder and associated chopper bar recorder. All operations were at 0°. This final step gave an approximate value for the M_r.

Protein estimation. Protein was estimated using the method of Bradford [16].

Extraction of mitochondria. The mitochondrial extraction buffer contained 100 mM MOPS, 100 mM BICINE, pH 8.3, 300 mM sucrose, 2 mM $MgSO_4$, 3 mM EDTA, and BSA at 0.75 mg/ml. The soln had ascorbate added just before mitochondrial extraction, final concentration 30 mM. The fruit placenta was removed and the pericarp cut into sections before being immersed in the medium. It was found that the hand-operated grinding method of Hobson [17] was the best way of obtaining intact, well coupled mitochondria. The resulting slurry was poured through four layers of muslin before being centrifuged at 3700 *g* (rav. 7.0 cm) for 10 min. Chloroplasts are mainly pelleted at this stage. The supernatant was then centrifuged at 8000 *g* (rav. 7.0 cm) for 30 min and the pellet was resuspended in a large excess of the extraction medium. The resuspended mixture was centrifuged at 1800 *g* for 5 min and the supernatant was then centrifuged at 8000 *g* for 30 min (both rav. 7.0 cm). The pellet was resuspended in 4 ml of a buffer containing 239 mM sucrose, 5 g PEG 8000/l, 0.75 mg/ml BSA and 10 mM MOPS, pH 7.5. 3.5 ml of the resuspended mixture was loaded on to a discontinuous Percoll gradient consisting of three steps, 13.5–26–55%. The

mitochondria banded at the interface between the 26 and the 55% steps after centrifuging for 60 min at 6000 *g* (rav. 7.1 cm).

Enzyme assay. The NADP malic enzyme was assayed in 3 ml of a reaction medium containing 100 mM MOPS, 0.5 mM NADP, 5 mM $MnSO_4$ and 10 mM malate at pH 7.0 and 25°. The formation of NADPH was monitored at 340 nm using a 1.0 cm light path. MDH was assayed in 3 ml of a reaction medium containing 100 mM MOPS, 0.5 mM NAD and 10.0 mM malate at pH 7.0.

Mitochondria were tested for state 3 and 4 coupling in 2 ml of a reaction medium containing 350 mM sucrose, 10 mM KPi, 10 mM Tris–HCl, 5 mM $MgCl_2$, 0.5 mM sodium EDTA at pH 7.3. 100 μl of resuspended mitochondria were used for testing the coupling state in a Clark electrode at 25°. It was found that the mitochondria could be lysed by using 100 μl Tween.

PAGE. Both 'native' and 'denaturing' gel electrophoresis was used in this investigation. Slabs of gel were 14 × 16 × 1.5 cm with 10 wells, each capable of holding 300 μl. Native gels were 7.5% acrylamide, (pH 8.9) separation gel, overlaid with a 2.5% stacking gel (pH 6.7). Electrophoresis was at 3 mA/well for 6 hr at 5° using Tris–glycine buffer, pH 8.5. Gradient native gels (4–30%) were also prepared, either with or without a stacking gel. Denaturing gels (10%) were prepared using Nadodecyl sulphate. The gels were stained for proteins using 0.05% Coomassie Blue R-250 in propan-2-ol and 10% HOAc. The gels were destained in 10% HOAc. The activities of the enzymes on gels was detected as described previously [18].

M_rs of constituents was determined by SDS treatment as described by Weber and Osborne [19]. Standards for denaturing gels were RNA polymerase from *E. coli* α and β_1, β_{11} subunits, bovine serum albumin, phosphorylase.

Isoelectric focusing. Isoelectric focusing was performed in a 110 ml column in a linear 2–50% (w/v) sucrose gradient containing 1.5% (v/v) ampholytes with a pH range of 3.5–10. The enzyme was suspended in 28% sucrose and layered on to the middle of the sucrose gradient (29%) before the completion of the remainder of the gradient (27–2% sucrose). Electrofocusing was conducted at 4° and 600*v*. Fractions were collected in 2 ml aliquots and pH determinations were made. Active fractions were analysed by denaturing PAGE.

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