NADP-linked malic enzyme

Purification from maize leaves, $M_r$ and subunit composition

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INTRODUCTION

Malic enzyme (EC 1.1.1.40) was first isolated from avian liver by Ochoa et al. (1947), and was shown to catalyze the oxidative decarboxylation of L-malate by NADP$^+$ in the presence of bivalent metal ions (Veiga-Salles & Ochoa, 1950) to yield CO$_2$, pyruvate and NADPH:

$$\text{L-Malate} + \text{NADP}^+ \rightleftharpoons \text{CO}_2 + \text{NADPH} + \text{pyruvate}$$

In plants malic enzyme activity has been located in mitochondria, chloroplasts and the cytosol (Murkerji & Ting, 1968; Pupillo & Bossi, 1979).

In C$_4$ plants both the dicarboxylic acid pathway and the reductive pentose phosphate pathway of photosynthesis operate (Slack & Hatch, 1967), and malic enzyme has been reported to be localized in the bundle-sheath chloroplasts (Rathnam & Edwards, 1975), and has a pH optimum near 8, a low $K_m$ for malate and a high specific activity (Pupillo & Bossi, 1979). These observations are consistent with the postulate that in C$_4$ plants malic enzyme decarboxylates malate formed by the action of phosphoenolpyruvate carboxylase and NADP-linked malate dehydrogenase, and thus provides CO$_2$ for reaction with ribulose-bisphosphate carboxylase.

The metabolic importance of the enzyme in C$_4$ plants prompted us to make a detailed study of its physical, chemical and catalytic properties. There have been a few methods reported for its purification. The most significant procedure yielded only 6% of the enzyme in maize leaves as material of high specific activity (Asami et al., 1979a). There have been several estimates of the $M_r$ of native maize malic enzyme, including that by Pupillo & Bossi (1979), who reported an $M_r$ value of 280000 from gel-filtration experiments, slightly greater than the estimate of 226000 obtained by Asami et al. (1979a) from sedimentation-equilibrium and sedimentation-velocity experiments. The $M_r$ determined by gel-filtration experiments was 330000 in the presence of metal ions, whereas in the presence of EDTA oligomers of different $M_r$ values became apparent (Ziegler, 1974). A subunit $M_r$ of 63000 was estimated by Asami et al. (1979a) and by Collins & Hague (1983) by the use of electrophoresis in the presence of SDS.

The main objective of the work described in the present paper was to purify the enzyme from maize, to homogeneity if possible, and by a procedure that is simple, rapid and reproducible, and to perform a detailed study of the $M_r$ of the enzyme in order to clarify the disparities reported by earlier workers.

MATERIALS AND METHODS

Maize plants (Golden Bantam variety) were grown in the Department of Botany, University of Oxford, Oxford, U.K. L-Malate (sodium salt) and dithiothreitol were from Sigma Chemical Co. (Poole, Dorset, U.K.). NADP$^+$ (disodium salt) and NADPH (tetrasodium salt) were from Boehringer Corp. (Lewes, East Sussex, U.K.). (ADP–agarose type 2) N$^\alpha$-Aminohexyl-2',5'-bisphosphoadenosine–agarose was from PLP Biochemicals (London, U.K.). Sephadex G-100, Sephadex G-200 (superfine grade) and DEAE–Sephadex A-50 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Other chemicals were from BDH Chemicals (Poole, Dorset, U.K.) and were of Analar grade where available.

Enzyme assays

The malic enzyme was assayed spectrophotometrically at 25°C, as described by Caldes et al. (1979). The 3 ml reaction mixture consisted of 66 mm-triethanolamine buffer, pH 8.0, 4 mm-L-malate, 6.6 mm-MgCl$_2$, and 0.23 mm-NADP$^+$. Reaction was initiated by the addition of 10 μl or 20 μl of enzyme solution and the increase at 340 nm was monitored with a Zeiss PMQ11 spectrophotometer. The unit of enzyme activity is defined as

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Table 1. Protein standards used in the estimation of the $M_r$ and subunit $M_r$ by the methods of gel filtration (GF), gel electrophoresis (GE) and gel electrophoresis in the presence of SDS (SDS)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Method</th>
<th>$M_r$</th>
<th>Subunit $M_r$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ferritin</td>
<td>Horse spleen</td>
<td>GF</td>
<td>440000</td>
<td></td>
<td>Ornstein (1964); Davis (1964); Andrews (1970)</td>
</tr>
<tr>
<td>2. Glutamate dehydrogenase</td>
<td>Bovine liver</td>
<td>GF</td>
<td>336000</td>
<td></td>
<td>Smith et al. (1970); Cassman &amp; Schachman (1971)</td>
</tr>
<tr>
<td>4. Catalase</td>
<td>Bovine liver</td>
<td>GF</td>
<td>195000</td>
<td></td>
<td>Andrews (1965); Schroeder et al. (1969)</td>
</tr>
<tr>
<td>5. Fumarase</td>
<td>Pig heart</td>
<td>GF</td>
<td>195000</td>
<td></td>
<td>Kanarek et al. (1964); Weber &amp; Osborn (1969)</td>
</tr>
<tr>
<td>6. Alcohol dehydrogenase</td>
<td>Yeast</td>
<td>GF</td>
<td>150000</td>
<td></td>
<td>Hayes &amp; Velick (1954)</td>
</tr>
<tr>
<td>7. Glucose-6-phosphate dehydrogenase</td>
<td>Yeast</td>
<td>GE</td>
<td>131000</td>
<td></td>
<td>Ornstein (1964); Davis (1964)</td>
</tr>
<tr>
<td>8. 6-Phosphogluconate dehydrogenase</td>
<td>Sheep liver</td>
<td>GF</td>
<td>940000</td>
<td></td>
<td>Silverberg &amp; Dalziel (1973)</td>
</tr>
<tr>
<td>9. Hexokinase</td>
<td>Yeast</td>
<td>GE</td>
<td>102000</td>
<td></td>
<td>Ornstein (1964); Davis (1964)</td>
</tr>
<tr>
<td>10. Malate dehydrogenase</td>
<td>Pig heart</td>
<td>GF</td>
<td>68000</td>
<td></td>
<td>Murphey et al. (1967)</td>
</tr>
</tbody>
</table>

an initial rate at 25°C of 1 $\mu$M-NADPH formed/min and the specific activity as units/mg of protein. NADP-linked and NAD-linked malate dehydrogenases were assayed as described by Johnson & Hatch (1970).

Protein concentration

In the early stages of the purification procedure the protein concentrations were determined by the method of Bradford (1976), with bovine plasma $\gamma$-globulin as the standard. In the final stage protein concentration was determined by light-absorption measurements at 278 nm, assuming an absorption coefficient of 0.87 for a 0.1% (w/v) protein solution (Asami et al., 1979a).

Electrophoresis

Electrophoresis in 7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS was carried out as described by Weber & Osborn (1975). The protein standards used in this and other estimations of the $M_r$ of the enzyme are given in Table 1.

Disc gel electrophoresis was carried out by the method of Hedrick & Smith (1968), which is a modified version of that described by Ornstein (1964) and Davis (1964).

Gel filtration

The $M_r$ of the native enzyme was estimated by gel filtration on Sephadex G-200 (superfine grade) prepared as described by Andrews (1965) in columns of length 54 cm and diameter 2.5 cm (volume 270 cm$^3$). A constant flow rate of 4 ml/h was monitored by a peristaltic Pharmacia P-1 pump and 2 ml fractions were collected. The enzymes used as internal standards were loaded in small amounts (0.1–0.5 mg) and were detected by standard activity tests (Bergmeyer, 1974). The sources of the enzymes and their $M_r$ values are given in Table 1.

Sedimentation-equilibrium and sedimentation-velocity studies

Sedimentation-equilibrium studies were made by the meniscus-depletion method of Yphantis (1964) by using a Spinco model E ultracentrifuge with a double-sector cell, rotor AN-D and interference optics. The $M_r$ was calculated as described by Chervenka (1969). All enzyme solutions were dialysed for 24 h at 4°C against the buffer used in the experiment and centrifuged before use at 18000 rev./min for 15 min. In most cases the specific activity of the enzyme was re-determined after the experiments and found to be unchanged.

The sedimentation coefficient of malic enzyme was obtained from sedimentation-velocity experiments by using the automatic scanning absorption method at 280 nm (Schachman et al., 1962; Schachman, 1963). Determination of the sedimentation coefficient was performed as described by Chervenka (1969).
The frictional ratio \( f/f_0 \) was calculated according to the following equation (O'Ney, 1941):

\[
f/f_0 = \frac{1.19 \times 10^{-15}}{s_{20,w} \cdot v^3} \cdot M_v^{1/2} \cdot (1 - \bar{v} \cdot \rho)
\]

where \( f \) is the frictional coefficient (molar) of the sample, \( f_0 \) the coefficient expected for an anhydrous sphere and \( \rho \) the density of the aqueous solution. The values used for the \( M_v, s_{20,w} \) and \( \bar{v} \) are given in the text.

**RESULTS AND DISCUSSION**

**Enzyme purification**

All steps in the purification of malic enzyme were carried out at 0–5°C in buffer solutions containing 1 mM-EDTA and 5 mM-dithiothreitol.

A 125 g batch of 4-week-old maize leaves was put in 300 ml of 25 mM-phosphate buffer, pH 7.5, containing 5 mM-dithiothreitol, 1 mM-EDTA, 2.5% (w/v) polyvinylpyrrolidone and 0.5% (w/v) ascorbate. The mixture was blended for approx. 1 min, filtered through four layers of cheesecloth and centrifuged at 27000 g for 20 min. To the precipitate were added 100 ml of the extraction buffer and 25% (w/v) sand. The mixture was ground by using a pestle and mortar for 20 min at 4 °C, and then filtered and centrifuged as before. The supernatants were pooled and assayed for malic enzyme activity and for protein content.

The extract (350 ml; 2.9 units/ml; specific activity 0.5 unit/mg of protein) was applied to 5 ml of \( N^a \)-aminohexyl-2,5-bisphosphoadenosine-agarose gel (10 cm × 5 cm) that had been equilibrated with 25 mM-phosphate buffer, pH 7.0. The flow rate was 10 ml/h. The column was washed with the same buffer until the \( A_{280}/A_{260} \) ratio was equal to that of the equilibration buffer. Malic enzyme was eluted with 0.1 M-phosphate buffer, pH 8.0, containing 5 mM-MgCl₂ in addition to the usual components of 5 mM-dithiothreitol and 1 mM-EDTA. The flow rate was 15 ml/h: this resulted in 797 units of malic enzyme of specific activity 50.7 units/mg of protein in a total volume of 29 ml. The affinity gel was regenerated by washing with 6 M-urea and 2 M-KCl, and may be used up to five times.

The pooled fractions were loaded onto a Sephadex G-100 column (70 cm × 5 cm) that had been equilibrated with 50 mM-triethanolamine buffer, pH 7.5, and malic enzyme was eluted at a flow rate of 20 ml/h. This gave 616 units of enzyme of specific activity 56.7 units/mg. This product was applied to a DEAE-Sephadex A-50 column of dimensions 30 cm × 2.5 cm equilibrated with 50 mM-triethanolamine buffer, pH 7.5, containing 5 mM-MgCl₂. A linear gradient of 0.1–0.4 M-NaCl in the equilibration buffer was then applied at a flow rate of 20 ml/h. The elution profile is shown in Fig. 1. The specific activity of the enzyme was reasonably constant over the peak. The results of the purification procedure are summarized in Table 2.

The purification procedure was repeated with 900 g of leaves and 20 ml of affinity gel, and gave 20 mg of enzyme of specific activity 70.3 units/mg of protein, a value close to that reported by Asami et al. (1979b), although the yield by the present method is much greater.

It was found that the specific activity of the final product increases after dialysis followed by centrifugation, to a maximum value of approx. 100 units/mg of protein, suggesting that a proportion of the enzyme as isolated has been inactivated. It was observed that if the extraction procedure was performed in the presence of phenylmethanesulphonyl fluoride there was no increase in specific activity or in stability or behaviour on SDS/polyacrylamide-gel electrophoresis. The product appears to be homogeneous from sedimentation-equilibrium and sedimentation-velocity studies described below. The specific activity of the enzyme remains constant between 70 and 75 units/mg of protein for 6–8 weeks. However, after 3 months more concentrated samples (> 1 mg/ml) lose up to 50% of their activity.
Table 2. Purification of malic enzyme from 125 g of 28-day-old maize leaves

For details see the text.

<table>
<thead>
<tr>
<th>Step</th>
<th>Elution volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme solution</td>
<td>350</td>
<td>2030</td>
<td>1015</td>
<td>0.5</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>29</td>
<td>15.7</td>
<td>797</td>
<td>50.7</td>
<td>78.5</td>
<td>104</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>57</td>
<td>10.9</td>
<td>616</td>
<td>56.7</td>
<td>60.6</td>
<td>114</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>44</td>
<td>6.1</td>
<td>492</td>
<td>80.6</td>
<td>48.5</td>
<td>161</td>
</tr>
<tr>
<td>Ultrafiltration concentration</td>
<td>6</td>
<td>4.2</td>
<td>308</td>
<td>73.3</td>
<td>30.3</td>
<td>146</td>
</tr>
</tbody>
</table>

$M_r$ and subunit composition

The $M_r$ was first estimated by gel filtration of the purified enzyme with Sephadex G-200. The $M_r$ values of the marker proteins are summarized in Table 1. From four experiments in 50 mM-triethanolamine buffer, pH 7.5, containing 1 mM-dithiothreitol and either 1 mM-EDTA, 10 mM-EDTA or 10 mM-MgCl₂, the $M_r$ was 330000 ± 10000. In the presence of 10 mM-EDTA the value obtained in phosphate buffer was 280000, although control experiments showed that the $M_r$ values of the marker proteins were the same as in 1 mM-EDTA. A similar effect of EDTA was noted by Ziegler (1974), and has not been investigated further.

In polyacrylamide-gel electrophoresis with several different gel concentrations the purified enzyme ran as two bands. This result can be interpreted in either of two ways (Hedrick & Smith, 1968). The results suggest either two isoenzymes of $M_r$ 360000 and of different net charges, or a mixture of two species with $M_r$ values of 245000 and 460000 (Fig. 2). With the evidence obtained for determination of the $M_r$ by gel-filtration experiments and by sedimentation-equilibrium studies, the evidence would suggest that different $M_r$ forms of the enzyme were more probable than isoenzymes. It had been observed that the addition of phenylmethanesulphonyl fluoride did not alter the pattern obtained on electrophoresis. It is unlikely that the different bands are due to proteolysis to a degree too small to be revealed by other procedures as the two bands were of equivalent proportions.

From electrophoresis in 7.5% (w/v) and in 5.0% polyacrylamide gels in the presence of SDS the mean value for the $M_r$ of the enzyme subunit was 60000 ± 2000 from six experiments (Some enzyme samples when large amounts were used (greater than 100 μg), showed two faint bands in addition to the main band, suggesting the presence of a species of higher subunit $M_r$, although samples prepared from very green plants approx. 6 weeks old did not, and the specific activity of the product was always essentially the same; these bands may be attributed to the presence of a proportion of a precursor protein (Collins & Hague, 1983).

Sedimentation-equilibrium experiments were performed with the use of the meniscus-depletion method in 0.1 m-phosphate buffer, pH 7.0. Linear plots were obtained and the results are consistent with homogeneity of the preparation. The $M_r$ estimated from the slope of this plot, using an assumed value of 0.74 ml/g for the partial specific volume, is 120000. The results of two experiments in 50 mM-triethanolamine buffer, pH 7.5, again gave linear plots but the $M_r$ value estimated from the slopes was 230000 ± 10000 (Fig. 3, curve A). The protein concentration was 0.32–0.36 mg/ml in all these experiments, and 5 mM-MgCl₂ and 5 mM-dithiothreitol were present. In the experiments in triethanolamine buffer 5 mM-malate was also present.

However, experiments performed in the absence of dithiothreitol showed two linear portions (Fig. 3, curve B), corresponding to apparent $M_r$ values of 113000 and 206000, which suggests that dithiothreitol is needed to maintain the quaternary structure. Non-linear plots were also obtained from some experiments with a larger protein concentration of 0.63 mg/ml, both in the presence and in the absence of 0.2 M-NaCl. Dithiothreitol has also been reported to promote higher-$M_r$ conformers for potato malic enzyme (Grover et al., 1981).

Sedimentation-velocity experiments were also made with various additions to the buffers. In 50 mM-triethanolamine buffer, pH 7.5, with protein concen-
NADP-linked malic enzyme

Fig. 3. Equilibrium sedimentation studies of malic enzyme in 50 mM-triethanolamine/HCl buffer, pH 7.5, in the presence (curve A) and in the absence (curve B) of 5 mM-dithiothreitol

The logarithm of the fringe displacement (d) at radial distance r is plotted against r^2. A value of 0.74 ml/g was used for a partial specific volume. Curve A, centrifugation was performed at 6°C and at 11000 rev./min. The protein concentration was 0.32 mg/ml in 50 mM-triethanolamine buffer, pH 7.5, containing 5 mM-dithiothreitol. A value of 230000 was obtained for the Mr. Curve B, centrifugation of a 0.34 mg/ml protein solution was performed at 9°C and at 9000 rev./min. The two linear portions of the graph corresponded to Mr values of 113000 and 206000.

In summary, the results show that the purification procedure consistently gives high yields of enzyme that is apparently homogeneous in the ultracentrifuge. The subunit Mr is 61000±2000, in agreement with the values reported by Asami et al. (1979a) and Collins & Hague (1983), and in triethanolamine buffer in the presence of dithiothreitol the enzyme molecule is tetrameric with an Mr of 230000, in agreement with those authors and with Pupillo & Bossi (1979). In phosphate buffer, however, the enzyme is clearly dimeric with an Mr of 120000. The value of 330000 obtained for the Mr in both triethanolamine buffer and phosphate buffer by the empirical gel-filtration method confirms the observation made by Ziegler (1974), and may be caused by a marked change of shape or hydration of the enzyme molecule at the low protein concentrations used.

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