NADP-linked malic enzyme

Purification from maize leaves, M_r and subunit composition

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1. The isolation of NADP-linked malic enzyme (EC 1.1.1.40) from maize leaves is described, together with studies of its M_r and subunit composition. 2. The enzyme was purified to apparent homogeneity by affinity chromatography on N^6 -aminoethyl-2',5'-bisphosphoadenosine-agarose, gel filtration with Sephadex G-100 and ion-exchange chromatography on DEAE-Sephadex A-50. A purification of 140-fold with a 30% yield was obtained. 3. A detailed study of the M_r by several methods revealed the existence of different M_r forms in solution. 4. In the presence of dithiothreitol the enzyme appears to be present in triethanolamine buffer, pH 7.5, as a tetramer with a subunit M_r of 60000 and an $s_{20,w}$ of 10.75 S. 5. In phosphate buffer, pH 7.0, it seems to be a dimer of M_r 120000 with an $s_{20,w}$ of 7.95 S. 6. In the absence of dithiothreitol, lower- M_r forms were detected by sedimentation-equilibrium and sedimentation-velocity studies in triethanolamine buffer. 7. Results from gel filtration gave M_r values of about 340000 in both buffers.

INTRODUCTION

Malic enzyme (EC 1.1.1.40) was first isolated from avian liver by Ochoa *et al.* (1947), and was shown to catalyse the oxidative decarboxylation of L-malate by NADP⁺ in the presence of bivalent metal ions (Veiga-Salles & Ochoa, 1950) to yield CO₂, pyruvate and NADPH:

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

In C₄ 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 C₄ plants malic enzyme decarboxylates malate formed by the action of phosphoenolpyruvate carboxylase and NADP-linked malate dehydrogenase, and thus provides CO₂ for reaction with ribulose-bisphosphate carboxylase.

The metabolic importance of the enzyme in C₄ 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 M_r 226000 obtained by Asami *et al.* (1979a) from sedimentation-equilibrium and sedimentation-velocity experiments. The M_r determined by gel-filtration experi-

ments 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^6 -Aminoethyl-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-MgCl₂ 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)

Protein	Source	Method	M_r	Subunit M_r	References
1. Ferritin	Horse spleen	GF	440 000		Ornstein (1964); Davis (1964); Andrews (1970)
		GE	440 000		
2. Glutamate dehydrogenase	Bovine liver	GF	336 000		Smith <i>et al.</i> (1970); Cassman & Schachman (1971)
		GE	326 000		
		SDS		56 000	
3. Xanthine oxidase	Cow milk	GE	288 000		Andrews (1970)
4. Catalase	Bovine liver	GF	195 000		Andrews (1965); Schroeder <i>et al.</i> (1969)
		GE	230 000		
		SDS		58 000	
5. Fumarase	Pig heart	GF	195 000		Kanarek <i>et al.</i> (1964); Weber & Osborn (1969)
		GE	195 000		
		SDS		49 000	
6. Alcohol dehydrogenase	Yeast	GF	150 000		Hayes & Velick (1954)
		SDS		37 000	
7. Glucose-6-phosphate dehydrogenase	Yeast	GE	131 000		Ornstein (1964); Davis (1964)
8. 6-Phosphogluconate dehydrogenase	Sheep liver	GF	94 000		Silverberg & Dalziel (1973)
9. Hexokinase	Yeast	GE	102 000		Ornstein (1964); Davis (1964)
10. Malate dehydrogenase	Pig heart	GF	68 000		Murphey <i>et al.</i> (1967)
11. Albumin	Bovine serum	SDS		68 000	Weber & Osborn (1969)
12. Ovalbumin	Hen	GF	45 000		Weber & Osborn (1969)
		GE	45 000		
		SDS		45 000	
13. Chymotrypsinogen	Bovine pancreas	SDS		25 700	Andrews (1970)

an initial rate at 25 °C of 1 μ 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 γ -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 cm³). 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 18 000 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).

Fig. 1. Chromatography of partially purified malic enzyme on DEAE-Sephadex A-50

The ion-exchanger was equilibrated with 50 mM-triethanolamine buffer, pH 7.5, containing 5 mM-dithiothreitol, 1 mM-EDTA and 5 mM-MgCl₂. Elution was effected with a linear gradient of 0.1–0.4 M-NaCl in the equilibration buffer. The column dimensions were 30 cm × 2.5 cm diam. and the flow rate was 20 ml/h. —, NaCl gradient; ●, protein; ○, enzyme activity; ×, enzyme specific activity.

The frictional ratio (f/f_0) was calculated according to the following equation (Onclay, 1941):

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

where f is the frictional coefficient (molar) of the sample, f_0 the coefficient expected for an anhydrous sphere and ρ the density of the aqueous solution. The values used for the M_r , $s_{20,w}$ 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) isoascorbate. 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*⁶-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 on to 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 5–6 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.

Step	Elution volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude enzyme solution	350	2030	1015	0.5	100	1.0
Affinity chromatography	29	15.7	797	50.7	78.5	101
Sephadex G-100	57	10.9	616	56.7	60.6	114
DEAE-Sephadex A-50	44	6.1	492	80.6	48.5	161
Ultrafiltration concentration	6	4.2	308	73.3	30.3	146

 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 $330\,000 \pm 10\,000$. In the presence of 10 mM-EDTA the value obtained in phosphate buffer was 280 000, 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 360 000 and of different net charges, or a mixture of two species with M_r values of 245 000 and 460 000 (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 $60\,000 \pm 2\,000$ from six experiments. Some enzyme samples, when large amounts were used (greater than 100 μ g), 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 120 000. The results of two experiments in 50 mM-triethanolamine buffer, pH 7.5, again gave

Fig. 2. Estimation of the M_r of malic enzyme by polyacrylamide-gel electrophoresis

The logarithm of the relative migration distances (R_r) for malic enzyme was plotted against the acrylamide concentration, as described by Hedrick & Smith (1968), and the slopes were determined. A similar plot was also obtained for the marker proteins (numbered and identified in Table 1) and the slopes were plotted against their respective M_r values. The slopes for the two equivalent bands obtained with malic enzyme correspond to M_r values of 245 000 and 460 000 or to two enzymes of M_r 360 000, as indicated by the arrows.

linear plots but the M_r value estimated from the slopes was $230\,000 \pm 10\,000$ (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 113 000 and 206 000, 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-

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 11 000 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 230 000 was obtained for the M_r . 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 M_r values of 113 000 and 206 000.

trations of 0.32–0.66 mg/ml and in the presence of 5 mM-MgCl₂ and 1 mM-dithiothreitol, with and without the addition of 5 mM-malate and 0.2 M-NaCl, $s_{20,w}$ was 10.7 ± 1.1 S in eight experiments; with the M_r value of 230 000 estimated by sedimentation-equilibrium experiments this gives 1.19 for the frictional ratio. The boundaries were smooth and symmetrical. In the absence of malate and dithiothreitol $s_{20,w}$ was 8.6 S, consistent with the lower M_r indicated by sedimentation-equilibrium measurements in the absence of dithiothreitol. Finally, in 0.1 M-phosphate buffer, pH 7.0, with protein concentrations of 0.36–0.72 mg/ml, 5 mM-MgCl₂ and 1 mM-dithiothreitol, $s_{20,w}$ was 7.95 ± 0.17 S in four experiments, giving a frictional ratio of 1.03 on the basis of an M_r of 120 000. Again the boundaries were smooth and symmetrical.

In summary, the results show that the purification procedure consistently gives high yields of enzyme that is apparently homogeneous in the ultracentrifuge. The subunit M_r is $61\,000 \pm 2000$, 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 M_r of 230 000, in agreement with those authors and with Pupillo & Bossi (1979). In phosphate buffer, however, the enzyme is clearly dimeric with an M_r of 120 000. The value of 330 000 obtained for the M_r 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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