Purification and Immunochemical Characterization of Malate Synthase from *Euglena gracilis*

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Malate synthase (EC 4.1.3.2) from dark-grown *Euglena gracilis* was purified to homogeneity by the criterion of polyacrylamide-gel electrophoresis. The enzyme was released from acetate-grown cells by treatment with ultrasonic waves and purified from broken-cell suspensions by high-speed centrifugation and $(\text{NH}_4)_2\text{SO}_4$ fractionation, followed by gel-filtration on Sepharose 6B. The final enzyme preparation was purified 190-fold compared with the crude extract. The mol.wt. of the enzyme was about 350000 as determined by gel filtration on Sepharose 6B. Treatment with sodium dodecyl sulphate and urea dissociated the enzyme into subunits of mol.wt. 175000. The pH optimum for the enzyme was 8.0 and the K_m values for glyoxylate and acetyl-CoA were 50 and 80 μM respectively. Antibodies raised to the purified enzyme were shown to be monospecific by radiochemical immunoassay. *Euglena* anti-(malate synthase) tested on Ouchterlony double-diffusion gels gave a sharp precipitation band against acetate-grown *Escherichia coli*, but no immunological correspondence was observed with acetate-grown *Chlorella fusca*, *Zea mays* (maize) scutella or purified malate synthase from *Ricinus communis*.

Malate synthase (EC 4.1.3.2), the second key enzyme of the glyoxylate cycle, catalyses the condensation of acetyl-CoA with glyoxylate to form malate (Wong & Aji, 1956). This enzyme is widely distributed in micro-organisms, and in division-synchronized cultures of *Euglena gracilis* the rate of malate synthase induction is constant for much of the cell cycle, but doubles in a single step during the period of DNA replication (Woodward & Merrett, 1975). To determine the expression of the structural gene for malate synthase during the cell cycle of *Euglena* it was necessary to purify and characterize this enzyme immunochemically. Moreover, although malate synthase is present in acetate-grown bacteria (Kornberg, 1959; Falmagne *et al.*, 1965), protozoa (Hogg & Kornberg, 1963), yeast (Dixon *et al.*, 1960; Duntze *et al.*, 1969) and algae (Syrett *et al.*, 1963; Harrop & Kornberg, 1966) the quaternary structure of the enzyme from micro-organisms has not been investigated. In the present paper we report on the quaternary structure of *Euglena* malate synthase and its immunological relationship to the enzyme from other organisms.

Materials and Methods

Growth of cells

Euglena gracilis Klebs strain z was grown at 25°C in the dark in the medium of Cramer & Myers (1952)

Abbreviation used: SDS, sodium dodecyl sulphate.

supplemented with 35mM-sodium acetate at pH 6.7. The initial inoculum added to 24 litres of medium was 500ml of an exponential-phase culture growing photoautotrophically (Collins & Merrett, 1975a) containing approx. 10^5 cells/ml.

Escherichia coli (N.C.T.C. 8007) was grown in the medium of Dixon *et al.* (1960).

Chlorella fusca strain 211/8P (Cambridge Culture Collection) was grown as described by Goulding & Merrett (1966).

Zea mays (maize) seeds were soaked overnight in running tap water, then germinated in moist vermiculite at 25°C and used after 4 days germination.

Labelling *Euglena* proteins with ^{35}S

A photoautotrophically growing *Euglena* culture in mid-exponential phase was harvested aseptically and resuspended in 50ml of Cramer & Myers (1952) medium to give a density of 5×10^4 cells/ml. Sterile sodium acetate was added to give a final concentration of 35mM and the culture incubated in the dark at 25°C for 4h. A pulse of 50 μCi of L- ^{35}S -methionine (555Ci/mmol) was added to the culture, and after 2h the culture was harvested by centrifuging for 10min at 4000g.

Preparation of cell extracts

Cultures of *Euglena* or *E. coli* were harvested by centrifuging for 10min at 4000g, washed once and

resuspended in extraction buffer (50mM-Tris/HCl, pH7.5, containing 10mM-MgCl₂ and 1mM-dithiothreitol) and the cells disrupted at 0°C by three 15s bursts of ultrasonic waves, with 1min intervals between the bursts (MSE ultrasonic disintegrator, 1.5A). *Chlorella* cells were suspended in the same buffer but disrupted by passage through a French Pressure cell at 82.7MPa (12000lb/in²). Seeds of *Zea mays* were ground with sand in the extraction buffer. Broken-cell suspensions were centrifuged for 30min at 4°C at 100000g_{av.} in a 40.3 rotor (Beckman L5-40 ultracentrifuge) and the supernatant was used for enzyme assays and immunological studies.

Purification of malate synthase from Euglena gracilis

Cells were harvested by centrifugation in an MSE continuous-action rotor, weighed, washed and resuspended in extraction buffer to give 0.5g fresh wt. of cells/ml of buffer. Cells were disrupted by passage through a chilled French pressure cell at 82.7MPa (12000lb/in²) and the broken-cell suspension was centrifuged at 4000g for 10min at 4°C to remove intact cells. The resultant pellet was re-extracted with half the original volume of extraction buffer, centrifuged again, and the supernatants were combined and adjusted to pH7.5 when necessary. The extract was then centrifuged at 53000g_{av.} at 4°C for 16h in an SW27 rotor (Beckman L5-40 ultracentrifuge).

Solid (NH₄)₂SO₄ was added slowly with stirring to the high-speed supernatant at 0°C to give 30% saturation (Dawson *et al.*, 1969). During the addition of (NH₄)₂SO₄ the pH was measured with a glass electrode and maintained at 7.5 by the addition of 1M-NaOH or 1M-HCl as necessary. The solution was left without stirring for 30min, then centrifuged at 18000g for 20min and the precipitate retained for enzyme assay. More (NH₄)₂SO₄ was added to the supernatant to give 40% saturation and the above procedure repeated. Finally, more (NH₄)₂SO₄ was added to the supernatant to give 50% saturation, and after standing for 30min, the precipitate was collected by centrifugation as above and dissolved in 10ml of extraction buffer. This enzyme preparation was fractionated at 4°C on a column (2cm×100cm) of Sepharose 6B which had previously been equilibrated with extraction buffer. The same buffer was used to elute the sample and 3ml fractions were collected.

Enzyme purity and molecular weight

Fractions from the Sepharose 6B column showing peak malate synthase activity were tested for purity by SDS/polyacrylamide-gel electrophoresis by the method of Weber & Osborn (1969).

Samples containing 30μg of protein were made 1% (w/v) in SDS, 1% (w/v) in β-mercaptoethanol and 8M in urea, then heated at 90°C for 3min to dissociate the enzyme into subunits. After cooling to 20°C the samples were applied to 5% (w/v) polyacrylamide gels and electrophoresis was carried out at a constant current of 8mA/gel.

Gel electrophoresis for the non-dissociated enzyme was the same as above, except that SDS was omitted from the gel and reservoir buffer and the protein was not pretreated with 1% SDS and 8M-urea. Gels were stained for protein with Coomassie Blue.

The molecular weight of the whole enzyme was determined on a column (2cm×100cm) of Sepharose 6B by conventional methods (Andrews, 1965) with *Euglena* ribulose 1,5-bisphosphate carboxylase (mol.wt. 520000), catalase (mol.wt. 240000), yeast alcohol dehydrogenase (mol.wt. 150000), bovine serum albumin (mol.wt. 68000) and cytochrome *c* (mol.wt. 12400) as the standards.

The molecular weight of the subunits of the dissociated enzyme was determined by SDS/polyacrylamide-gel electrophoresis on 5% gels (Weber & Osborn, 1969). Soya-bean trypsin inhibitor (mol.wt. 21500), bovine serum albumin (mol.wt. 68000), *E. coli* RNA polymerase α-subunit (mol.wt. 39000), β-subunit (mol.wt. 155000) and β'-subunit (mol.wt. 165000) were used as standard proteins.

Stain for glycoprotein

Gels of purified malate synthase run as above were stained for glycoprotein with the Schiff reagent by the method of Segrest & Jackson (1972).

Enzyme assays

Malate synthase was assayed by the method of Cook (1970). The reaction mixture contained, in a final volume of 3.0ml, 200μmol of Tris/HCl buffer, pH8.0, 20μmol of MgCl₂, 0.4μmol of acetyl-CoA, 1μmol of glyoxylate and 0.5ml of enzyme extract. The reaction was started by the addition of glyoxylate and the disappearance of acetyl-CoA followed at 232nm on a Gilford recording spectrophotometer.

Citrate synthase (EC 4.1.3.7) was assayed by the method of Weitzman (1969); the increase in A₄₁₂ was followed after the addition of 0.1μmol of oxaloacetate to a cuvette containing 9.5μmol of Tris/HCl buffer, pH8.0, 0.1μmol of 5,5'-dithiobis-(2-nitrobenzoic acid), 0.15μmol of acetyl-CoA and cell extract to a total volume of 1ml.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951) by using a standard curve prepared for

crystalline bovine albumin. In column-chromatography experiments the protein elution profile was monitored by recording the A_{280} .

Preparation and partial purification of antiserum

Peak fractions from the Sepharose 6B column that were homogeneous for malate synthase by the criterion of polyacrylamide-gel electrophoresis were dialysed against dry Sephadex G-25 at 4°C until the volume was decreased to approx. 0.7 ml. Complete Freund's adjuvant (1 ml) was emulsified with 1 mg of enzyme protein and injected intramuscularly into a New Zealand white rabbit. Injections were repeated at 3-week intervals until a total of four injections were given. Blood was collected by cardiac puncture 3 weeks after the last injection and allowed to clot overnight at 4°C. The serum was collected by centrifugation at 40000g for 15 min at 4°C, then $(\text{NH}_4)_2\text{SO}_4$ was added to give 50% saturation. The precipitate was collected by centrifugation at 18000g for 20 min at 4°C and redissolved in 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl, so that the final volume was half the original serum volume. The immunoglobulin G preparation was dialysed overnight at 4°C against the same buffer before being divided into small batches and stored at -20°C.

Quantitative precipitation reaction of the antiserum to malate synthase

Quantitative precipitation reactions were carried out as described by Kabat & Mayer (1961). Antiserum or control (preimmunization) serum (0.1 ml) was incubated with purified malate synthase (up to 0.22 mg of protein) in a volume of 0.6 ml for 30 min at 37°C, followed by storage at 4°C for 16 h to ensure complete precipitation. Immunoprecipitates were collected by centrifuging at 1500g for 10 min, washed three times with 3 ml of 0.15 M-NaCl, then dissolved in 2 ml of 0.1 M-NaOH and the A_{280} was measured or protein determined by the method of Lowry *et al.* (1951).

Residual enzyme activity was measured by incubating 50 μ l samples of purified malate synthase with increasing amounts of antiserum in a final volume of 150 μ l for 30 min at 37°C, followed by 16 h at 4°C. The mixture was centrifuged at 1500g for 10 min, and 50 μ l samples of the supernatants were used for enzyme assay.

Analysis of malate synthase immunoprecipitate from Euglena cell extracts

Cell extract (200 μ l) from the [^{35}S]methionine-labelled cells was incubated with 100 μ l of anti-(malate synthase) serum for 30 min at 37°C, followed

by 16 h at 4°C. After centrifugation at 1500g for 10 min, the immunoprecipitate was washed three times with 3 ml of 0.1 M-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂, 50 mM-KCl, 5 mM- β -mercaptoethanol and 0.5% (w/v) Triton X-100, and then again in the above buffer without Triton X-100.

The immunoprecipitate was dissolved in sample buffer [10 mM-sodium phosphate, pH 7.2, containing 1% (w/v) SDS, 8 M-urea and 1% (w/v) β -mercaptoethanol], heated at 90°C for 3 min and analysed on 5% SDS/polyacrylamide gels (Weber & Osborn, 1969). Gels were stained for protein with Coomassie Blue, scanned at 600 nm with a Gilford Gel Scanner, frozen with powdered solid CO₂, and cut into 1 mm-thick slices with a Mickle gel slicer. Gel slices were dissolved in 200 μ l of H₂O₂ (30%) by heating at 70°C for 2 h, then added to 5 ml of Bray's (1960) solvent/scintillator and counted for radioactivity at 70% efficiency in a Packard Tri-Carb liquid-scintillation counter.

Immunodiffusion studies

Antibody specificity was determined for anti-(*Euglena* malate synthase) by the Ouchterlony (1968) double-diffusion technique on slides containing 0.8% (w/v) agarose prepared in 0.1 M-sodium phosphate buffer (pH 7.5). Precipitation bands were allowed to develop at 20°C for 48 h. Control gels were run by using control (preimmunization) serum in place of anti-(malate synthase). Gels were stained for the presence of minor bands by flooding with 1.7% (w/v) NaCl for 24 h, soaking for 24 h in several changes of water, staining with Coomassie Blue, then destaining with aq. 5% (v/v) methanol containing 7.5% (v/v) acetic acid.

Cell extracts from *Zea mays*, *Ricinus communis*, *E. coli*, *Chlorella fusca* and phototrophic- and photoheterotrophic-grown *Euglena gracilis* were investigated by this method. All cell extracts contained at least 18 mg of protein/ml.

Other materials

Dithiothreitol, acetyl-CoA, glyoxylic acid, β -mercaptoethanol and agarose were from Sigma (London) Chemical Co., London S.W.6, U.K. Sephadex G-25 and Sepharose 6B were from Pharmacia, Ealing, London W.5, U.K. Freund's complete adjuvant was from Difco Laboratories, Detroit, MI, U.S.A. L-[^{35}S]Methionine was from The Radiochemical Centre, Amersham, Bucks., U.K. Calibration proteins for SDS/polyacrylamide-gel electrophoresis were from Boehringer-Mannheim, London W5 2TZ, U.K. All other reagents were from BDH Chemicals, Poole, Dorset, U.K., and were of the highest purity available.

Table 1. *Purification of malate synthase from dark-grown Euglena gracilis*
For details see the text.

Step	Protein (mg/ml)	Total protein (mg)	Specific activity ($\mu\text{mol/min per mg of protein}$)	Total activity ($\mu\text{mol/min}$)	Yield of activity (%)	Purification (fold)
Homogenates after low-speed centrifugation	135.00	5670	0.003	15.30	100	—
53 000g supernatant	15.20	502	0.026	13.04	85	8.7
(NH_4) ₂ SO ₄ fractionation (40–50% saturation)	11.20	112	0.086	9.64	63	29
Sephacrose 6B peak fraction	0.80	2.4	0.570	1.37	9	190
All fractions with activity	0.66	15.8	0.290	4.58	30	97

Results and Discussion

Enzyme purification

The final procedure used for malate synthase purification had the advantage of simplicity combined with good yield (Table 1). An important step in the procedure was the use of dark-grown *Euglena*; the absence of chloroplast proteins facilitated purification and enhanced the amount of malate synthase in the cell. The molecular weight of the intact enzyme was such that Sepharose 6B gave a good separation from other cellular proteins remaining at that stage of purification. In contrast, attempts to purify the enzyme by centrifugation on linear sucrose gradients were unsuccessful, as the molecular weight of the enzyme was not great enough to facilitate separation from other proteins, as occurs with *Euglena* ribulose 1,5-bisphosphate carboxylase (Brown *et al.*, 1976). Preparative polyacrylamide-gel electrophoresis gave a good separation of malate synthase from other proteins, but had the disadvantage of a major loss of enzymic activity. Inclusion of ion-exchange chromatography in the purification procedure was unnecessary; malate synthase failed to bind to either CM- or DEAE-cellulose at all stages of purification, indicating the absence of high ionic charge on the intact enzyme. After partial purification yeast malate synthase was contaminated with citrate synthase (Dixon *et al.*, 1960), and as malate synthase is present with the glyoxylate-cycle enzymes in *Euglena* glyoxysomes (Collins & Merrett, 1975b) possible contamination by these enzymes was investigated. However, assays for citrate synthase, malate dehydrogenase, aconitase or isocitrate lyase failed to show activity with purified *Euglena* malate synthase (Collins & Merrett, 1975b). Some organelle proteins are glycoproteins (Campbell & Blobel, 1976), but purified *Euglena* malate synthase did not give a positive reaction with the Schiff reagent when tested by the method of Segrest & Jackson (1972).

Molecular-weight determinations

The whole enzyme was found to have a mol.wt. of about 350 000 from its elution profile on the

Sephacrose 6B column. For quaternary-structure determination SDS/polyacrylamide-gel electrophoresis was used. Attempts to dissociate the enzyme into its constituent subunits by using only SDS were unsuccessful, complete dissociation only being obtained when 1% SDS was used in conjunction with 8M-urea. Peak fractions from the Sepharose 6B column treated in this way showed a single protein band. SDS/polyacrylamide-gel electrophoresis of the dissociated enzyme and comparison of electrophoretic mobility with protein monomers of known

Fig. 1. *Effect of pH on enzymic activity of malate synthase* Standard assay conditions were used as described in the Materials and Methods section, except that Tris/HCl buffer was replaced by 33 mM-Tes (2-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulphonic acid) over the pH range 5.0–7.0 and by 33 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] over the pH range 7.5–9.5.

Fig. 2. Analysis of immunoprecipitate resulting from the reaction of anti-(malate synthase) with extract of ${}^{35}\text{S}$ -labelled cells by SDS/polyacrylamide-gel electrophoresis

Cells were labelled, disrupted, made to react with anti-(malate synthase) and immunoprecipitate was examined electrophoretically as described in the Materials and Methods section. (a) Densitometer trace of immunoprecipitate and histogram showing radioactivity of 1 mm gel slices; (b) densitometer trace of pure enzyme for comparison.

molecular weight established the mol.wt. of the subunit as 175000. This indicates that the whole enzyme is composed of two subunits.

pH optimum and kinetic properties of purified enzyme

The optimum pH for enzyme activity was 8.0 (Fig. 1), which differs from the value of 7.0 reported by Cook (1970) for the partially purified enzyme. The purified enzyme had a K_m for glyoxylate of $50\mu\text{M}$ and for acetyl-CoA of $80\mu\text{M}$. As reported previously for malate synthase from yeast (Dixon *et al.*, 1960) and higher plants (Servettaz *et al.*, 1973), glycollate was a potent competitive inhibitor of the enzyme, the K_m for glyoxylate increasing to 0.8 mM in the presence of 0.5 mM-glycollate.

Immunochemical studies

Purified malate synthase gave a single sharp precipitin band on Ouchterlony double-diffusion gels

against anti-(malate synthase), but gave no reaction with control (preimmunization) serum. The antiserum specifically precipitated malate synthase from a mixture of proteins (Fig. 2a). When anti-(malate synthase) was added to an extract of ${}^{35}\text{S}$ -labelled cells, analysis of the resultant immunoprecipitate by SDS/polyacrylamide-gel electrophoresis produced a single peak of radioactive protein with mobility closely corresponding to that of the enzyme subunit (Fig. 2). Negligible radioactivity was precipitated by control serum. The two major non-radioactive protein peaks of the immunoprecipitate, with mol.wts. of approx. 30000 and 60000, correspond to the light and heavy chains respectively of immunoglobulin G. These observations show that the antiserum is mono-specific for malate synthase in *Euglena* cell extracts.

Reaction of anti-(malate synthase) with purified enzyme gave a good titre (Fig. 3) and measurement of catalytic activity demonstrated that the enzyme was completely immunoprecipitated (Fig. 4).

Fig. 3. Immunotitration of purified *Euglena* malate synthase. Antiserum (0.1 ml) was incubated with malate synthase in a volume of 0.6 ml for 30 min at 37°C followed by storage at 4°C for 16 h. Immunoprecipitates were collected, washed and protein determined as described in the Materials and Methods section.

Fig. 4. Effect of malate synthase antiserum on enzyme activity

Samples (50 μl) of malate synthase were incubated with increasing amounts of antiserum in a final volume of 150 μl for 30 min at 37°C followed by 16 h at 4°C. Immunoprecipitates were removed and remaining enzyme activity was determined as described in the Materials and Methods section.

The reaction of anti-(malate synthase) with extracts from cells grown phototrophically on CO₂ and cells grown heterotrophically on acetate showed the presence of malate synthase protein only in the latter. Thus the development of malate synthase activity observed when acetate is added and cultures darkened (Woodward & Merrett, 1975) results from the synthesis of enzyme protein rather than enzyme activation.

Euglena anti-(malate synthase) was tested by Ouchterlony double-diffusion gels against extracts from higher plants and micro-organisms grown on acetate as carbon source. A positive precipitin reaction was observed between the antiserum and extracts of acetate-grown *E. coli*, the presence of the precipitin band being confirmed by staining for protein with Coomassie Blue. Cells of *Chlorella fusca* grown heterotrophically on acetate contain malate synthase (Syrett *et al.*, 1963; Goulding & Merrett, 1966), but cell extracts failed to give a precipitin reaction with *Euglena* anti-(malate synthase). During the germination of the fat-storing seed the glyoxylate cycle functions in the conversion of fat into carbohydrate, but cell extracts of *Zea mays* scutella and malate synthase from *Ricinus communis* cotyledons failed to give a positive reaction with *Euglena* anti-(malate synthase). Thus, although *Euglena* malate synthase is immunologically related to the *E. coli* enzyme there is no immunological correspondence between this enzyme and malate synthase from *Chlorella* or higher plants. This lack of immunological correspondence may reflect the difference in quaternary structure between the *Euglena* and the higher-plant malate synthase. Recent work has shown that malate synthase from cucumber cotyledons has a mol.wt. of 590 000, with a subunit mol.wt. of 63 000 (Köller & Kindl, 1977), and malate synthase from *Ricinus communis* cotyledons has a mol.wt. of 575 000 (Bowden & Lord, 1978).

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