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THERMOSTABLE, AMMONIUM-ACTIVATED MALIC ENZYME OF *CLOSTRIDIUM THERMOCELLUM*

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'Malic' enzyme (L-malate NADP$^+$ oxidoreductase (oxaloacetate-decarboxylating, EC 1 1 1 40) was purified from *Clostridium thermocellum* by DEAE-cellulose, agarose-NADP and Sephadex G-200 column chromatography The 117-fold purified 'malic' enzyme displayed a maximum activity of 135 units/mg at 40°C and represented 0 8% of the total cell protein Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of the protein suggested 90% purity and an approximate tetrameric subunit molecular weight of 40 000. The enzyme absolutely required both bivalent and monovalent cations for catalysis Mn^{2+} and NH_4^+ were the most effective cationic activators examined Increasing NH_4^+ concentration increased both enzyme activity and affinity toward L-malate The apparent K_m for L-malate was 3 10^{-4} M at 0 4 mM NH_4Cl Enzyme activity increased linearly when temperature was raised between 22–60°C and a Q_{10} of 2 1 was calculated from an Arrhenius plot The enzyme was stable to heating at 60°C but was denatured at higher temperatures The enzyme half-life was 10 min at 72°C The enzyme displayed a broad pH optimum (7 2–8 2 for Tris-HCl buffer) but was inactivated by *p*-chloromercuribenzoate The high thermal stability, low apparent molecular weight and NH_4^+ activation are properties not common to all previously described 'malic' enzymes

Introduction

'Malic' enzyme (L-malate NADP$^+$ oxidoreductase (oxaloacetate-decarboxylating, EC 1 1 1 40) catalyzes the oxidative decarboxylation of L-malate to pyruvate and CO_2 (L-malate + NADP $\rightleftharpoons$ pyruvate + CO_2 + NADPH) The existence of 'malic' enzyme has been demonstrated in a variety of plant, animal and microbial tissues, its properties and proposed metabolic functions have been reviewed [1] The presence of high levels of 'malic' enzyme activity in crude cell extracts of *Clostridium thermocellum* was recently reported [2] Anabolic NADPH synthesis was suggested as the physiological role of the enzyme, since the oxidative hexose monophosphate shunt pathway was not detectable and extracts contained a unidirectional, NAD-linked oxaloacetate reductase activity [2] Initial attempts to demonstrate 'malic' enzyme in *Cl thermocellum* by standard spectrophotometric methods failed to show significant activity, however, high activity was observed when pyruvate accumulation was quantified with a commercial lactate dehydrogenase This discrepancy was shown to be a dependence of the activity on $(NH_4)_2SO_4$ contained in the commercial lactic dehydrogenase used

Biotechnological applications of enzymes often depend on the development of stable catalysts Enzymes from thermophilic bacteria are by and large more stable to temperature and certain chemical effectors than similar catalysts from mesophiles [3,4] Thermophilic anaerobic bacteria are considered

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Abbreviation Mops, 4-morpholinepropanesulfonic acid

promising for industrial fermentation of biomass to chemical feedstocks and fuels [4,5] *Cl thermocellum* is employed in processes for the direct fermentation of cellulosic biomass to ethanol [5] Very little is known about the catalytic features or potential applications of enzymes from thermophilic anaerobes that may represent future commercial sources of inexpensive enzymes We report here on the catalytic properties of the first 'malic' enzyme partially purified and characterized from a thermophilic bacterium

Materials and Methods

Chemicals All chemicals were reagent grade N_2 gas was purchased from Matheson (Chicago, IL) Nicotinamide dinucleotides were obtained from Sigma (St Louis, MO) Agarose-NADP type 3 (NADP attached to agarose through C-8) was purchased from P-L Biochemicals (Milwaukee, WI) Molecular weight calibration proteins were obtained from Pharmacia (Piscataway, NJ), Boehringer (Mannheim, F R G) and as a gift from W J Brill, University of Wisconsin

Organisms and cultivation methods Cl thermocellum strain AS39 [6,2] was used as the source of cells for 'malic' enzyme purification *Cl thermocellum* was grown on complex medium [6] in 5 l New Brunswick fermenters that contained the following per l of distilled water 1 5 g KH_2PO_4/2 9 g K_2HPO_4/0 5 g MgCl 6 H_2O/2 14 g urea/0 05 g $CaCl_2$ 2 H_2O/1 25 mg $FeSO_4$ 6 H_2O/10 g Mops buffer/8 g cellobiose/6 g yeast extract/1 g cysteine-HCl/2 mg resazurin Fermenter cultures were constantly stirred at 100 rev /min and continuously gassed with N_2 at 20 cc/min and incubated at 60°C Cells were harvested at the late exponential growth phase (13 h) and were collected by centrifugation at 35 000 × *g* in a Sorvall RC-5 centrifuge (DuPont Instruments) equipped with a KSB continuous-flow system

Preparation of cell extract Cells were suspended in a 20 mM Tris-acetate buffer (pH 7 7)/3 mM dithiothreitol solution (1 g cells/5 ml) that contained 1 μg/ml DNAase The suspension was passed through a French pressure cell at 1 400 kg/cm^2 The lysate was centrifuged at 20 000 × *g* for 30 min Extracts were stored at −20°C The protein content of the cell extract and other fractions was determined by the method of Bradford [7] using Bio-rad (Rockville, NY) reagents

SDS-polyacrylamide gel electrophoresis Standard method [8] used 0 3 × 10 cm gel (7 5%) slabs The following proteins were used to calibrate the molecular weight creatine kinase, 40 000, hen egg albumin, 43 000, *E coli* glutamate dehydrogenase, 53 000 and bovine serum albumin, 67 000

'Malic' enzyme assays The standard assay mixture (1 ml total volume) contained 0 1 M Tris-HCl buffer (pH 7 8, adjusted at 40°C)/2 mM L-malate/0 5 mM NADP/5 mM $MnCl_2$/20 mM NH_4CL The reaction was followed at 334 nm ($\epsilon_{334} = 6\ 1\ mM^{-1}\ cm^{-1}$) using an Eppendorf recording photometer 1 unit was defined as the amount of enzyme catalyzing the reduction of 1 μmol/min of L-malate at 40°C Assays under other conditions are as described in the text The same assay conditions were used to determine the influence of temperature on activity except for replacing Tris-HCl buffer with 50 mM potassium phosphate (pH 7 1) and $MnCl_2$ by $MgCl_2$

Results

Enzyme purification

Cell extract (12 ml, 58 mg protein) was applied to

TABLE I

PURIFICATION OF 'MALIC' ENZYME FROM *Cl THERMOCELLUM*

Activity was measured by the standard 'malic' enzyme assay at 40°C

Purification step		Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (μmol/min per mg)	Yield (%)	Purification (-fold)
I	Crude extract	12	58 0	67	1 15	100	1
II	DEAE-cellulose	5	8 5	74	8 7	100	8
III	Agarose-NADP	1	0 8	63	79	94	67
IV	Sephadex G-200	6	0 4	54	135	81	117

a 1 5 X 3 0 cm DEAE-cellulose column (Whatman 52) that was equilibrated in 20 mM Tris-acetate buffer (pH 7 7) After washing the column with 10 ml of the same equilibration buffer, the activity was desorbed stepwise by the addition of 0.1 M NaCl in 20 mM Tris-acetate buffer (pH 7 7) The activity fractions were pooled (5 ml total volume) and diluted 2-fold in equilibration buffer The diluted enzyme was applied to a 1 5 X 1 2 cm agarose-NADP column previously washed with equilibration buffer The activity was eluted sharply in a 1 0 ml fraction by the addition of a 2 mM NADP in 20 mM Tris-acetate buffer, pH 7 7 The protein solution (1 0 ml) was applied to a 1 0 X 40 cm Sephadex G-200 column equilibrated in 0 05 M Tris-HCl, pH 8 0 The activity eluted as a symmetrical peak (6 ml) centered at $K_{av} = 0\ 39$ ($K_{av} = V - V_0/V_t - V_0$, Blue dextran and dinitrophenol were used for measurement of V_0, the void volume, and V_t, the total solute volume, respectively) On the basis of these results and a calibration curve that included catalase (M_r 190 000), fructose biphosphate aldolase (158 00) and albumin (68 000), the enzyme molecular weight was roughly estimated to be 170 000 The purification procedure is summarized in Table I An overall purification of 117-fold was achieved with a yield of 81% The final specific enzyme activity was 135 U/mg at 40°C The purified enzyme did not recognize NAD as cofactor during activity measurements at a concentration range of 0 05–10 0 mM

Analysis of 'malic' enzyme (purification, Step IV) on SDS-polyacrylamide gel electrophoresis demonstrated one major band that migrated as a 40 000 molecular weight peptide and a much weaker peptide, 30 000 (<10% of total protein) The band pattern exhibited for the enzyme preparation in Step III included an additional 45 000 molecular weight band and a significantly higher amount of the 30 000 molecular weight band

Bivalent and monovalent cation activity requirements

Fig 1 illustrates the absolute monovalent cation requirements for 'malic' enzyme activity Enzyme activity was 2 5-times higher with NH_4^+ than K^+ as monovalent cation Half-maximal activation values were observed at 0 8 mM NH_4^+ and 5 0 mM K^+ Na^+ or Li^+ did not serve as cationic activators of 'malic' enzyme The dependence of 'malic' enzyme activity on NH_4^+ was studied at pH values between 6 3–7 8 Maximal enzyme activity was pH dependent, but enzyme affinity to NH_4^+ was not pH dependent Half-maximal activation values for NH_4^+ were 0 7–0 8 mM for each of the pH values examined The same results

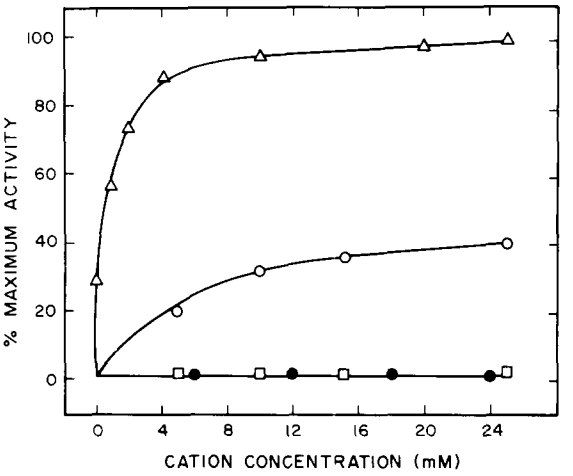

Fig 1 Dependence of 'malic' enzyme activity on monovalent cations Assay mixtures (1 ml) contained 0 1 M Tris-HCl (pH 7 0 adjusted at 40°C)/2 mM L-malate/0 5 mM NADP/5 mM $MnCl_2$/1 μg purified enzyme (Step IV), and the monovalent cation concentration indicated 100% activity represents 135 μmol/min per mg protein at 40°C △———△, NH_4, ○———○, K, ●———●, Na and □———□, Li

TABLE II

EFFECT OF BIVALENT METAL CATIONS ON 'MALIC' ENZYME ACTIVITY

Assay mixtures contained 0 1 M Tris-HCl (pH 7 0)/2 mM L-malate/20 mM NH_2Cl/0 5 mM NADP/0 05 mM EDTA, the indicated bivalent metal chloride(s), and 1 μg purified 'malic' enzyme (Step III) 100% activity represents 79 μmol/min per mg at 40°C

Cation	Maximum activity (%)	
	0 25 mM	5 mM
Mn^{2+}	100	99
Mg^{2+}	62	77
Zn^{2+}	78	19
Co^{2+}	61	61
Cu^{2+}	25	2
Ca^{2+}	0	0

were observed when either Mn^{2+} or Mg^{2+} were present as divalent activators in reaction assay mixtures

'Malic' enzyme activity also had an absolute requirement for divalent cations as shown with various metal chlorides in Table II These experiments used two different metal concentrations, and were performed in assay mixtures that contained 0 05 mM EDTA in order to lower the background activity to zero in the absence of bivalent metal additions Mn^{2+} was the most effective bivalent cationic activator examined Mg^{2+}, Zn^{2+} and Co^{2+} were more effective activators than Cu^{2+} Ca^{2+} did not activate 'malic' enzyme at the concentrations examined

pH, temperature and general activity requirements

The dependence of 'malic' enzyme activity on L-malate concentrations was shown to vary with the $[NH_4^+]$ An apparent K_m for L-malate of 3 10^{-4} M at saturating NH_4^+ (20 mM) and 1 0 10^{-3} M at limiting NH_4^+ (0 4 mM) Raising the NH_4^+ concentration from 0 4 to 20 mM increased both enzyme activity and affinity toward malate

Analysis of the pH dependence of 'malic' enzyme with Tris-HCl buffers demonstrated that enzyme activity increased sharply from 6 2 to 7 2, was nearly constant between 7 2–8 2, and then rapidly declined at higher pH values Mg^{2+} was used as bivalent cation in these experiments because the reaction mixtures with Mn^{2+} precipitated at pH values above 8 2 The pH profile observed with $MnCl_2$ (5 mM) as bivalent cation was identical for pH values up to 8 2

The temperature stability of 'malic' enzyme was examined at 60, 65, 72 and 78°C (Fig 2) The enzyme remained completely stable (i e , no loss of activity) after heating for 2 h at 60°C A 20% loss of activity was observed after heating for 1 h at 65°C Enzyme stability decreased dramatically at higher temperatures and enzyme half-life was only 10 min at 72°C Detailed studies were not performed to examine the effect of chemical additions on thermal stability The relation of temperature to specific activity of 'malic' enzyme was examined in Arrhenius plots Activity increased linearly as the temperature was raised from 20 to 62°C and calculated Q_{10} and energy of activation values obtained were 2 1 and 14 Kcal mol, respectively

The influence of sulfhydryl group inhibitors (i e , *p*-chloromercuribenzoate) on 'malic' enzyme activity was examined The addition of 0 2 mM *p*-chloromercuribenzoate to a standard assay mixture caused complete loss of enzyme activity in less than 1 min Immediate addition of dithiothreitol (4 mM) reserved the observed *p*-chloromercuribenzoate inhibition by greater than 80%

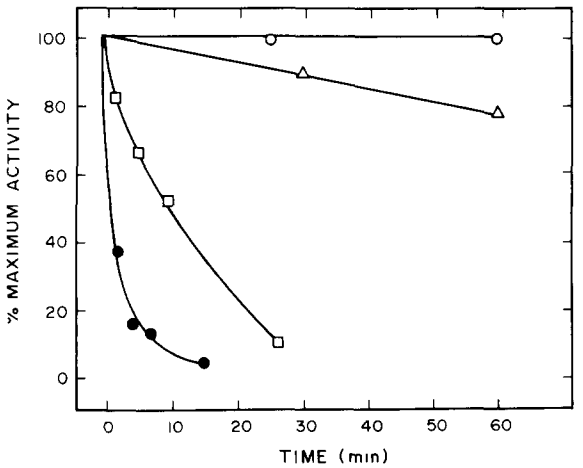

Fig 2 Temperature stability of 'malic' enzyme Incubation conditions the mixture (1 ml) contained 0 05 M potassium phosphate (pH 7 1)/2 mM EDTA/0 1 mM NADP/5 μg purified 'malic' enzyme (Step III) The enzyme was heated in stoppered tubes at the temperature indicated and coded prior to activity measurement at 40°C, 100% activity represents 79 μmol/min per mg protein ○———○, 60°C, △———△, 65°C, □———□, 72°C and ●———●, 78°C

Discussion

'Malic' enzyme was easily isolated from *Cl thermocellum* by simple procedures that included an effective affinity chromatography step with agarose-NADP Analysis of the enzyme by Sephadex G-200 chromatography and SDS-polyacrylamide gel electrophoresis suggested that the enzyme exists as a tetramer with an approximate molecular weight of 170 000 The purification procedures employed yielded a protein that was approx 90% pure This degree of purity was sufficient for analysis of the general enzyme features described here, and for analysis of its practical applications in the future

In general, the physical features of *Cl thermocellum* 'malic' enzyme differ significantly from other 'malic' enzymes reported in the literature The apparent 40 000 subunit molecular weight of this en-

zyme was significantly lower than that reported for 'malic' enzymes (60 000–70 000) from pigeon heart [9], pigeon liver [10] or *E coli* [11] The apparent molecular weight of native 'malic' enzyme from *Cl thermocellum* is therefore considerably lower than the 550 000 molecular weight reported for the *E coli* enzyme[11] The thermal stability of the *Cl thermocellum* enzyme, as expected, was far greater than for 'malic' enzymes isolated from mesophilic organisms [9] The maximal thermal stability of 'malic' enzymes displayed a striking correlation to the organisms' growth temperature optimum of 62°C [12] The temperature-activity relationship from 20–62°C as expressed in Arrhenius plots for *Cl thermocellum* 'malic' enzyme was a straight, non-broken line, and this is not characteristic of some thermophilic pyridine nucleotide-linked oxidoreductases For example, broken Arrhenius plots at 45–50°C have been described for lactate dehydrogenases from thermophilic *Bacillus* species [13] and alcohol dehydrogenases from thermophilic anaerobic species [17] Zuber [13] has suggested that thermophilic enzymes are less efficient catalysts than mesophilic enzymes because of calculated lower values for ΔH and more negative values for ΔS above 45°C than below In contrast, the Q_{10} value of *Cl thermocellum* 'malic' enzyme was 2 1 and the enzyme did not display a lower catalytic efficiency at mesophilic temperatures

The general catalytic features of *Cl thermocellum* 'malic' enzyme appear quite similar to many 'malic' enzymes described [1,14,15] The enzyme was absolutely dependent on NADP as cofactor and bivalent cations as activators ($Mn > Mg > Zn > Co > Ca$) Several 'malic' enzymes described [1,14] are activated by monovalent cations in addition to requiring bivalent cations *E coli* 'malic' enzyme displayed high affinity and absolutely required K^+ or $NH^{\backslash +}$ [11] The pH activity curve for *Cl thermocellum* enzymes was somewhat broader and more alkaline when compared to known 'malic' enzymes [1,14] Also, the *p*-chloromercuribenzoate inhibition studies suggest that the thermophilic enzyme is similar to other 'malic' enzyme activities which are sensitive to cysteine blocking agents [16]

NH_4^+ activation may have a special physiological function for *Cl thermocellum* 'malic' enzyme Enzyme activation appeared specific, the dependence was absolute and saturation occurred at very low concentration Also, the NH_4^+ concentration affected both the apparent V and K_m for L-malate Although K^+ substituted for NH_4^+ and activated 'malic' enzyme, higher substrate concentrations were required and considerably lower activation was observed It is tempting to speculate that NH_4^+ activation of 'malic' enzyme is a physiological signal in *Cl thermocellum* which turns on NADPH generation when enough NH_4^+ is available for biosynthesis

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