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Purification and Properties of L-Malic Enzyme from *Escherichia coli**

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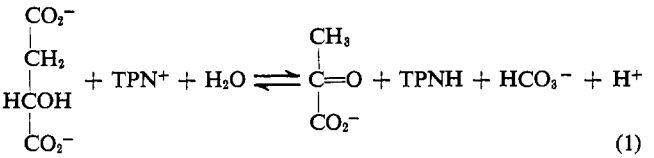
ABSTRACT: A purification procedure for L-malic enzyme (EC 1.1.1.40) from *Escherichia coli*, which gives electrophoretically homogeneous enzyme, is described. The molecular weight of the native enzyme as determined by sedimentation equilibrium measurements was 550,000 g/mole. An $s_{20,w}$ value of 17.5 S was determined by a modified zone centrifugation technique. The subunit molecular weight, determined by sedimentation equilibrium measurements in 6.0 M guanidine hydrochloride and 0.01 M dithiothreitol, was 67,000 g/mole. This result, together with the tryptophan content from amino acid analysis, is consistent with an octameric structure for the native enzyme. The value of ϵ_{279} was $2.65 \times 10^5\ M^{-1}\ cm^{-1}$ and the maximum molar turnover number at 25° and pH 7.4 was about 1000 sec^{-1}. Electron paramagnetic resonance measurements showed that the inhibition of the enzyme by acetyl coenzyme A cannot be due to complexation of the essential activator Mn^{2+}, but rather to interaction of acetyl coenzyme A with the enzyme in a presumably allosteric fashion. There was an obligatory monovalent cation requirement for catalytic activity, which can be satisfied by K^+ or NH_4^+, and acetyl coenzyme A was a noncompetitive inhibitor with respect to K^+. At increasing concentrations in excess of its K_m value (8×10^{-3} M), K^+ progressively inhibited the enzyme and caused the malate saturation curve to become sigmoid. Since the allosteric properties of acetyl coenzyme A and other inhibitors appear to be consistent with a two-state enzyme conformational model (Sanwal, B. D., and Smando, R. (1969b), *J. Biol. Chem. 224*, 1824) in which malate binds preferentially to the conformational state which predominates in the absence of allosteric ligands, this behavior of K^+ at high concentrations may result from the fact that it has a greater affinity for that state of the enzyme which binds malate poorly.

L-Malic enzyme [L-malate:TPN oxidoreductase (decarboxylating), EC 1.1.1.40] catalyzes the oxidative decarboxylation of L-malate (eq 1). The enzyme appears to function in



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both mammals and bacteria to provide pyruvate and TPNH for lipid synthesis, and not as a component of gluconeogenesis (Wise and Ball, 1964; Young *et al.*, 1964; Ashworth *et al.*, 1965; Jacobson *et al.*, 1966). In the case of *Escherichia coli*, the enzyme is induced by growth on L-malate as the sole carbon source (Stern and Hegre, 1966).

Sanwal and his colleagues have recently reported that L-malic enzyme from *E. coli* is inhibited by acetyl coenzyme A (Sanwal *et al.*, 1968), by oxalacetate, TPNH, DPNH (Sanwal and Smando, 1969a), and by adenosine 3′,5′-cyclic phosphate (Sanwal and Smando, 1969c). Sanwal and Smando (1969b) performed a steady-state kinetic analysis of the reaction, using partially (100-fold) purified enzyme, and concluded that an ordered mechanism, with isomerization of free enzyme, was consistent with their data. In the presence of certain inhibitors, such as acetyl coenzyme A, oxalacetate, and DPNH, the steady-state velocity patterns for malate became nonhyperbolic (sigmoidal) and, in the cases of acetyl coenzyme A and oxalacetate, the inhibition was competitive with respect to malate. The conclusion that acetyl coenzyme A, oxalacetate and DPNH are allosteric inhibitors was supported by the finding that the enzyme was desensitized to the action of these ligands by high concentrations of glycine. The allosteric effects were analyzed in terms of a two-state model in which malate has a high affinity for state R, whereas the inhibitors stabilize state T, with which malate binds poorly or not at all. Sanwal and Smando (1969a)

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suggested that the complex regulation of L-malic enzyme from *E. coli* might be necessary because of the absence of cellular compartmentation in bacteria.

We have isolated L-malic enzyme from malate-grown *E. coli* (Stern and Hegre, 1966; Katsuki *et al.*, 1967; Sanwal and Smando, 1969a) in an electrophoretically homogeneous state and we describe here some properties of the pure enzyme.

Experimental Section

Materials

The sources of materials were as follows: Sephadex G-25 and G-200, Pharmacia; Whatman DE23 DEAE-cellulose, H. Reeve Angel Inc.; Bio-Gel HTP hydroxylapatite, Bio-Rad Laboratories; streptomycin sulfate, dithiothreitol, TPN, DPNH, L-malic acid, and acetyl coenzyme A, Calbiochem; lactic dehydrogenase, Boehringer Mannheim Corp.; guanidine hydrochloride and tetramethylammonium hydroxide, Eastman Kodak; standard amino acid mixtures, Beckman Instruments; standard Mn^{2+} solution, Hartman-Leddon Co.; and bovine plasma albumin, Armour Pharmaceutical Co.

Methods

Enzyme Assays and Kinetic Measurements. The reaction was monitored spectrophotometrically by measuring the reduction of TPN at 340 nm in a quartz cuvet of 1.0-cm light path and 1.0-ml volume using a Cary 15 spectrophotometer with the solution temperature maintained at 25°. The stoichiometry of the reaction was verified by pyruvate determination according to Bücher *et al.* (1963) and by measurement of TPNH at 340 nm. Additions to the cuvet of reagents which were to be varied in a single series of measurements were made with a Hamilton microliter syringe. All initial velocity measurements are averages of duplicate measurements which differed by less than ±5%. The standard assay mixture contained 0.1 M Tris-HCl (pH 7.4), 0.1 M KCl, 0.01 M tetramethylammonium L-malate, 1 mM $MnCl_2$, and 1 mM TPN. One enzyme unit is defined as that amount of enzyme which causes the formation of 1 μmole of TPNH/min under the standard assay conditions, and specific activity is expressed as units per mg of protein. Protein concentrations were determined by the method of Lowry *et al.* (1951) unless otherwise indicated, using desiccated bovine plasma albumin as protein standard.

Growth of E. coli and Purification of L-Malic Enzyme. STEP 1. GROWTH OF *E. coli*, STRAIN *K10* (*HfrC*). The bacteria were grown on minimal salts medium with malate as the only carbon source. The yield was typically 4.5 g of packed cells/l. of culture medium. The composition of the medium was as follows: KH_2PO_4, 13.6 g/l.; $(NH_4)_2SO_4$, 2.0 g/l.; $FeSO_4 \cdot 7H_2O$, 0.5 mg/l.; $MgSO_4 \cdot 7H_2O$, 0.2 g/l.; 1% potassium DL-malate. The solution was adjusted to pH 7.0 with solid KOH. Growth and harvesting were carried out at the New England Enzyme Centre, Tufts University, Boston, Mass. The cells were washed with ten volumes of 20 mM potassium phosphate (pH 6.8), containing 1 mM EDTA and 1 mM dithiothreitol, and then frozen, and stored at −10°.

STEP 2. PREPARATION OF CELL EXTRACT. To 1617 g of thawed cell suspension (containing the equivalent of 354 g of wet packed cells) was added 300 ml of 20 mM potassium phosphate (pH 6.8), containing 1 mM dithiothreitol and 1 mM EDTA. The cells were then homogenized in a Manton-Gaulin homogenizer. During this procedure the temperature of the homogenate was maintained near 10°, the final volume being 1882 ml. All subsequent steps were carried out at 4° unless otherwise noted.

STEP 3. STREPTOMYCIN TREATMENT. While maintaining the pH at 6.8 by the addition of KOH, 209 ml of a 20% streptomycin sulfate solution was added, with constant stirring, to the homogenate from step 2 to make the final concentration 2% with respect to streptomycin. This suspension was stirred for 24 hr, after which it was centrifuged for 30 min at 10,000*g*. The precipitate, containing the bulk of the nucleic acids, was discarded and 1706 ml of supernatant solution recovered.

STEP 4. AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate (290 g) was added, with constant stirring, to the supernatant solution at 25°, and the pH maintained at 6.8 by the addition of 1.0 M KOH. This suspension was stirred for 24 hr at 25°. By centrifugation of the suspension at 25° for 30 min at 10,000*g*, 1680 ml of supernatant solution was recovered. Solid ammonium sulfate (218 g) was then added to this supernatant solution, maintaining conditions identical with those noted above for the first ammonium sulfate fractionation. The suspension was stirred for 24 hr at 25°, and was then centrifuged at 25° for 30 min at 10,000*g*. The resulting supernatant solution was discarded. The precipitate was dissolved in 100 ml of 20 mM potassium phosphate (pH 6.8) containing 1 mM dithiothreitol and 1 mM EDTA. The ammonium sulfate was removed by gel filtration on a column (5 × 90 cm) of Sephadex G-25 which had been equilibrated with the same buffer.

STEP 5. HEAT STEP. The pooled fractions (182 ml) from the Sephadex G-25 column in step 4 were made 0.1 M in magnesium acetate and acidified to pH 5.5 with 1 N acetic acid (Hsu and Lardy, 1967) while maintaining the temperature at 4°. This suspension was divided into 25-ml aliquots (in 50-ml erlenmeyer flasks) which were then shaken for 5 min in a bath maintained at 58°. The aliquots were then rapidly cooled to 4°, pooled, and centrifuged at 4° for 20 min at 25,000*g*. The resulting precipitate was discarded. The supernatant solution (170 ml) was desalted and reequilibrated with 10 mM potassium phosphate (pH 6.5), containing 1 mM dithiothreitol by gel filtration on a column (5 × 90 cm) of Sephadex G-25.

STEP 6. HYDROXYLAPATITE COLUMN CHROMATOGRAPHY. A column (2.5 × 40 cm) of Bio-Gel HTP was prepared and washed with 10 mM potassium phosphate (pH 6.5), containing 1 mM dithiothreitol. The pooled fractions (210 ml) from the Sephadex G-25 column in step 5 were placed on the column, and the column was washed with the same buffer. The enzyme was eluted with a linear gradient of between 0.0 and 1.0 M ammonium sulfate. The enzyme appeared after 500 ml of the gradient solution had passed through the column. The fractions having enzyme activity were pooled (280-ml total) and freed from ammonium sulfate and reequilibrated with 20 mM potassium phosphate (pH 7.4), containing 1 mM dithiothreitol and 1 mM EDTA by gel filtration (in two 140-ml aliquots) on a column (5 × 90 cm) of Sephadex G-25. The total volume after this buffer change was 370 ml.

STEP 7. DEAE-CELLULOSE CHROMATOGRAPHY. A column (2.5 × 40 cm) of Whatman DE23 DEAE-cellulose was prepared and washed with 20 mM potassium phosphate (pH 7.4), containing 1 mM dithiothreitol and 1 mM EDTA. The

TABLE I: Purification of L-Malic Enzyme from *E. coli*.

Fraction	Vol (ml)	Protein (mg)	Act.		Sp Act. (units/mg of Protein)
			Enzyme Units	Yield (%)	
Cell extract	1882	33,688	4366	100	0.13
Streptomycin sulfate treated extract	1706	10,065	3617	82.8	0.36
Ammonium sulfate treated extract	182	4,146	2036	46.6	0.49
Heated extract	210	2,058	1783	40.8	0.87
Pooled hydroxylapatite fractions	370	1,073	1783	40.8	1.7
Pooled DEAE-cellulose fractions	126	290	1556	35.6	5.4
Pooled Sephadex G-200 fractions	46	73.9	1242	28.4	16.9
Pooled Sephadex G-200 fractions	32	23	790	18.1	34.3
Pooled DEAE-cellulose fractions (after concentration)	4.8	10.9	611	13.9	56.1

pooled fractions from step 6 were placed on the column and washed with the same buffer. The enzyme was eluted with a linear gradient of between 0.0 and 0.6 M KCl in the same buffer. The enzyme appeared after 500 ml of the gradient solution had passed through the column, and those fractions having a specific activity greater than 2 units/mg were pooled to give a volume of 126 ml.

STEP 8. SEPHADEX G-200 CHROMATOGRAPHY. A column (2.5 × 90 cm) of Sephadex G-200 was prepared and washed with 20 mM potassium phosphate (pH 7.4), containing 1 mM dithiothreitol and 1 mM EDTA. The pooled fractions from step 7 were fractionated twice on this column. Preceding each of the two passes through the column the enzyme was concentrated, the first time by ammonium sulfate (70 g added to the 126 ml of enzyme solution from step 7) precipitation and the second time by means of a Schleicher & Schuell collodion bag apparatus. The enzyme appeared each time in a peak which immediately followed the void volume. Fractions having a specific activity greater than 7 units/mg were pooled (46-ml total) after the first passage, while fractions having a specific activity greater than 20 units/mg were pooled after the second passage (32-ml total).

STEP 9. DEAE-CELLULOSE CHROMATOGRAPHY. This step was performed in a manner identical with step 7 with the exception of column size and eluent volume. In this case, the column was 1.5 × 25 cm and the enzyme appeared after 250 ml of eluent had passed through the column. Fractions of 2-ml volume were collected, and the fractions with a specific activity of between 55 and 60 units per mg were pooled and concentrated to a volume of 4.8 ml by means of a Schleicher & Schuell collodion bag apparatus. The enzyme was then precipitated by the addition of 2.69 g of ammonium sulfate and stored as a suspension at 4°. Under these conditions, the enzyme was completely stable for at least 6 months. Prior to all measurements involving the enzyme, an aliquot of the suspension was dissolved in a suitable volume of buffer (50 mM Tris-HCl (pH 7.4), containing 1 mM dithiothreitol unless otherwise stated) and either extensively dialyzed against this buffer or desalted by gel filtration on a small Sephadex G-25 column (0.9 × 6 cm) which had been equilibrated with this buffer. The 450-fold purification of L-malic enzyme is summarized in Table I. Most of the loss incurred during purification was accounted for by fractions on the leading and trailing edges of column peaks which were discarded.

Polyacrylamide Gel Electrophoresis. The polyacrylamide disc gel electrophoresis technique was used (Davis, 1964). Malic enzyme activity in the gels was detected by the method of Dewey and Conklin (1960). In all experiments described in this paper, unless otherwise specified, the enzyme samples were electrophoretically homogeneous with specific activities varying between 55 and 60 units per mg.

Zone Centrifugation Experiments. The sedimentation coefficient of L-malic enzyme was determined with a modified type of zone centrifugation whereby the sedimentation of a band of enzyme is observed through its catalytic properties (Cohen and Hahn, 1965; Rosenbloom, 1965; Hsiang and Bright, 1967). These experiments were performed in the Spinco Model E analytical ultracentrifuge using the AN-D rotor at 40,000 rpm. The monochromatic light system of the centrifuge (Rosenbloom, 1967) was set at 340 nm and the sedimentation of the enzyme was observed through the formation of TPNH. Photographs (Kodak commercial film) of the absorption patterns were analyzed on a Beckman Analytrol densitometer and the midpoint of the leading edge of the boundary was utilized to calculate the sedimentation coefficient. The sedimentation coefficient was corrected to 20° and water (Schachman, 1959).

Sedimentation Equilibrium Experiments. L-Malic enzyme at concentrations of between 0.5 and 1.0 mg per ml was centri-

fuged to equilibrium in a charcoal-filled Epon six-chambered centerpiece or an aluminum-filled Epon double-sector synthetic boundary centerpiece. The meniscus depletion technique of Yphantis (1964) was used with rotor speeds of between 4000 and 10,000 rpm for the native enzyme, and 18,000–26,000 rpm for the enzyme in guanidine hydrochloride. These experiments were done in the Spinco Model E analytical ultracentrifuge using an AN-H rotor, a 546-nm interference filter with the Rayleigh interference optical system, and Kodak G spectroscopic plates. Rotor temperature was maintained at 20°. The photographic plates were analyzed on a Bausch and Lomb microcomparator. Weight-average molecular weights were calculated from plots of the logarithm of the fringe displacement *vs.* r^2.

Amino Acid Analysis. Amino acid analyses were performed on a Beckman Model 116 automatic analyzer with a scale expander as described by Moore and Stein (1963). A sample of enzyme (1.2 ml containing 1 mg of protein) was dialyzed extensively against 10 mM Tris-HCl (pH 7.4). An equal volume of 12.0 N HCl was then added. Aliquots of the acidified sample were placed in vials, the vials were sealed under nitrogen, and the enzyme was hydrolyzed at 108° for 24, 48, and 72 hr. After hydrolysis, the solvent was removed *in vacuo*, the hydrolysates were redissolved in distilled water, and aliquots of hydrolysates equivalent to between 20 and 40 μg of protein were analyzed in duplicate. The analyzer was calibrated by a standard mixture of amino acids.

Cystine and Cysteine Content. Cystine and cysteine were converted to cysteic acid by performic acid oxidation as described by Hirs (1956). After oxidation, the samples were hydrolyzed and amino acid analysis performed as above.

Tryptophan Content. Tyrosine and tryptophan were determined by measuring the absorbance at 280 and 294.4 nm of an enzyme solution made 0.1 N in NaOH as described by Goodwin and Morton (1946) and by Beaven and Holiday (1952). The ratio of tyrosine to tryptophan could then be used to calculate the content of tryptophan, since tyrosine content had already been determined by amino acid analysis.

Measurements of Free Manganese. These experiments were performed at 25° on a Varian E-3 electron paramagnetic resonance spectrometer system. Concentrations of free Mn^{2+} were obtained by comparing the peak-to-peak heights of the derivative spectra of sample and standard solutions (Cohn and Townsend, 1954).

Results

Purity. L-Malic enzyme, from step 9 of the purification procedure, with a specific activity of 56 units/mg, migrated as a single protein band in polyacrylamide disc gel electrophoresis when 0.1-ml samples containing 0.1 mg of protein were used. Furthermore, L-malic enzyme activity was coincident with this protein band. By this criterion, therefore, our purification procedure yields homogeneous enzyme, and all the experiments described subsequently were carried out with enzyme having a specific activity in the range from 55 to 60 units per mg.

Molecular Weight Determination. The molecular weight of the native enzyme in the presence and absence of acetyl coenzyme A, and of enzyme in the presence of 6.0 M guanidine hydrochloride containing 0.01 M dithiothreitol, was determined by sedimentation equilibrium experiments. In all cases the plot of the logarithm of the fringe displacement *vs.* r^2 was linear. An example of the sedimentation equilibrium data is given in Figure 1. The value of $\bar{v}$ (0.74 ml/g) was computed from the amino acid analysis. The average molecular weight of the native enzyme from three separate experiments was 550,000 ± 15,000 g per mole. The enzyme was fully active and inhibitable at the termination of these experiments. In the presence of 0.5 mM acetyl coenzyme A (a concentration which would inhibit catalytic activity almost completely under most assay conditions) the average molecular weight from three separate experiments was 546,000 ± 15,000 g per mole. The rotor speed was varied from 4000 to 10,000 rpm during each of the six experiments just described.

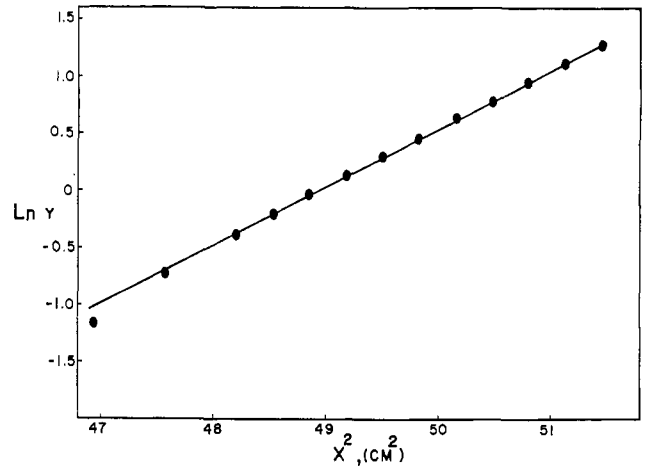

FIGURE 1: Equilibrium centrifugation of L-malic enzyme (0.5 mg per ml). The buffer consisted of 0.1 M Tris-HCl, pH 7.4, containing 1 mM dithiothreitol and 1 mM EDTA. For other details see Methods.

In modified zone centrifugation experiments in which the catalytic formation of TPNH in the standard assay solution was monitored spectrophotometrically at 340 nm, the value of $s_{20,w}$ was determined to be 17.5 S. This corresponds to the largest enzymatically active species of the enzyme and probably differs little from $s^0_{20,w}$ because of the small enzyme concentrations used.

In the presence of 6.0 M guanidine hydrochloride and 0.01 M dithiothreitol, which should fully dissociate the protein into its component polypeptide chains, the molecular weight was 67,000 ± 2000 g per mole. This value was the average from three separate experiments during each of which the rotor speed was varied from 18,000 to 26,000 rpm. A corrected value (0.725 ml/g) for $\bar{v}$ was used for these experiments (Hade and Tanford, 1967).

Amino Acid Composition. The amino acid composition of L-malic enzyme is shown in Table II, and is based on a molecular weight of 550,000 g/mole.

Extinction Coefficient. Our preparations of pure L-malic enzyme have no absorbance except in the ultraviolet region and the absorption maximum is at 279 nm. In order to estimate the molar extinction coefficient of the enzyme, the molarity of tryptophan and tyrosine in an enzyme solution (0.05 M Tris-HCl, pH 7.4) with a known absorbance at 279 nm was determined by the method of Goodwin and Morton (1946). From the tyrosine and tryptophan content of the en-

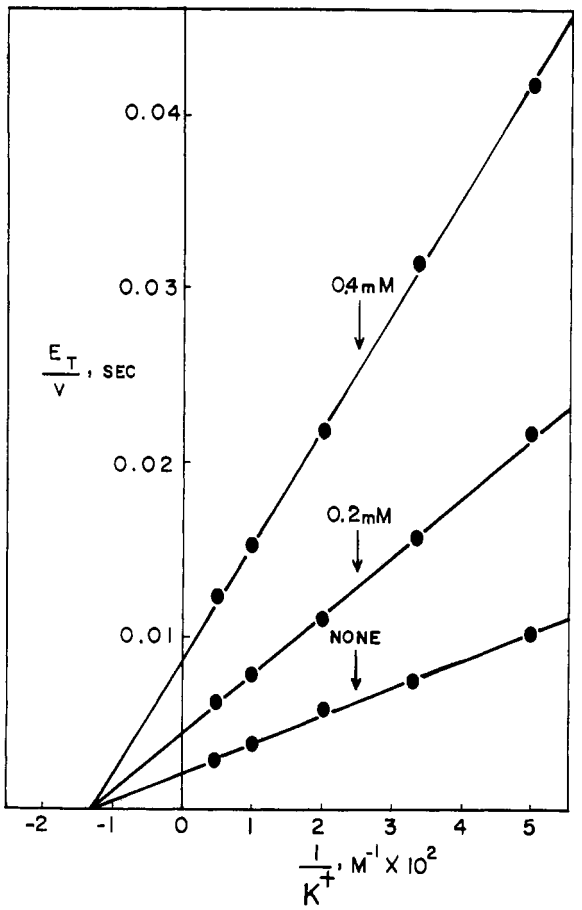

FIGURE 2: Effect of acetyl coenzyme A on K^+ kinetics. The concentration of acetyl coenzyme A is indicated by each line, and K^+ was varied as indicated. The solutions contained in addition 0.1 M Tris-HCl, pH 7.4, 0.01 M tetramethylammonium L-malate, 1.0 mM TPN, and 1.0 mM $MnCl_2$. Ionic strength was maintained at 0.14 using tetramethylammonium chloride.

TABLE II: Amino Acid Composition of L-Malic Enzyme.[a]

Amino Acid	Residues/Molecule of Enzyme
Lys	311
His	96
Arg	224
Asp	371
Thr	165[b]
Ser	219[b]
Glu	465
Pro	264
Gly	312
Ala	475
Val	377
Met	126
Ile	274
Leu	357
Tyr	87
Phe	140
Half-Cys	61[c]
Trp	8[d]

[a] The values of all residues (except for Thr, Ser, half-Cys, and Trp) represent the closest integer to the average of duplicate measurements after 24-, 48-, and 72-hr hydrolysis. [b] Values obtained by extrapolation of results from 24-, 48-, and 72-hr hydrolyses to zero time. [c] Estimated as cysteic acid after performic acid oxidation and hydrolysis of the enzyme. [d] From spectrophotometric determination as described in text.

zyme (Table II), ϵ_{279} was computed to be 2.65×10^5 M^{-1} cm^{-1}. The value of ϵ_{279} determined by the method of Lowry *et al.* (1951), using bovine plasma albumin as protein standard, was 2.81×10^5 M^{-1} cm^{-1}. This is very similar to the value determined by the more direct spectrophotometric method described above, even though the aromatic residue contents of L-malic enzyme and of bovine plasma albumin (Spahr and Edsall, 1964) are not very similar.

Kinetic Properties. Many kinetic properties of L-malic enzyme from *E. coli* have already been described by Sanwal and his coworkers (Sanwal *et al.*, 1968; Sanwal and Smando, 1969a–c). We wish to report here two aspects of the kinetic behavior of the pure enzyme in preliminary form, namely, the role of acetyl coenzyme A as an inhibitor, and the dependence of the reaction kinetics on Mn^{2+} and K^+. Our preparations of L-malic enzyme show an absolute requirement for divalent and monovalent metal ions. Double-reciprocal plots with Mn^{2+} as variable activator were linear, and gave a K_m value (in terms of total Mn^{2+}) of 3.6×10^{-6} M under standard assay conditions at 25°. Acetyl coenzyme A was noncompetitive with respect to Mn^{2+}, and caused a parallel decrease in the turnover number at infinite Mn^{2+} and in the value of $1/K_m$ for Mn^{2+}. The question arises as to whether the marked inhibition of the enzyme by acetyl coenzyme A is due to a trivial effect of complexation of Mn^{2+} by acetyl coenzyme A, or whether it is a more complex, possibly allosteric, phenomenon as has been suggested by Sanwal and Smando (1969b). Table III shows that the addition of 10^{-4} M acetyl coenzyme A to the standard assay solution inhibited the enzyme approximately 63%, but caused negligible changes in the concentrations of free Mn^{2+}, Mn^{2+}–malate, and Mn^{2+}–TPN as determined by electron paramagnetic resonance measurements. Under these experimental conditions, the dissociation constants for Mn^{2+}–acetyl coenzyme A, Mn^{2+}–TPN, and Mn^{2+}–malate were 10^{-3}, 2×10^{-3}, and 10^{-2} M, respectively. The last value agrees quite well with published values (Sillen and Martell, 1964).

Figure 2 shows that acetyl coenzyme A is a noncompetitive inhibitor with respect to the activator K^+. The enzyme has an unusually high affinity for NH_4^+. Therefore, in order to achieve a complete dependence of the velocity on added K^+ in these experiments it is necessary either to take only the initial fraction of the enzyme peak from the small Sephadex G-25 column used to free the enzyme from NH_4^+ (see Methods), or to dialyze the enzyme extensively against buffer solutions free from K^+ and NH_4^+. The experiments of Figure 2 were carried out at a constant ionic strength of 0.14, and give a K_m for K^+ of 8×10^{-3} M. Concentrations of K^+ greater than about 5×10^{-2} M progressively inhibit

TABLE III: Comparison of the Effect of Acetyl Coenzyme A on the Turnover Number and on the Concentrations of Free and Complexed Manganese.[a]

	Turnover No. (sec^{-1})	Free Mn^{2+} ($\times 10^{-4}$ M)	Mn^{2+}–Malate ($\times 10^{-4}$ M)	Mn^{2+}–TPN ($\times 10^{-4}$ M)	Mn^{2+}–Acetyl Coenzyme A ($\times 10^{-4}$ M)
Standard assay solution	286	4.8	3.8	2.0	
Standard assay solution plus 10^{-4} M acetyl coenzyme A	105	4.7	3.8	1.9	0.3

[a] The dissociation constants of Mn^{2+}–malate, Mn^{2+}–TPN, and Mn^{2+}–acetyl coenzyme A (in 0.1 M Tris-HCl, pH 7.4, containing 0.1 M KCl) were first determined in separate experiments by electron paramagnetic resonance measurements of free Mn^{2+}, and were found to be 10^{-2}, 2×10^{-3}, and 10^{-3} M, respectively. The concentrations of free Mn^{2+}, Mn^{2+}–malate, Mn^{2+}–TPN, and Mn^{2+}–acetyl coenzyme A in the standard assay solution (see Methods) were then computed from electron paramagnetic resonance measurements of free Mn^{2+} in the presence and absence of 10^{-4} M acetyl coenzyme A. All experiments were carried out at 25°.

the reaction (*i.e.*, cause positive deviations from the line of Figure 2). The activator NH_4^+ also markedly inhibits the enzyme at concentrations above its K_m value.

Finally, we show in Figure 3 that the initial velocity pattern for L-malate in the presence of 0.1 M K^+ tends to be sigmoid under the conditions of these experiments, rather than hyperbolic. Since, in the absence of allosteric inhibitors, the malate saturation curves obtained by Sanwal and Smando (1969b) in initial velocity experiments were hyperbolic with all TPN concentrations tested, we tested the effect of K^+ on malate kinetics. With 2.5×10^{-4} M K^+, which may correspond to the level of K^+ in the experiments of Sanwal and Smando (1969b), the malate saturation curve was hyperbolic, with TPN concentrations of either 7.5×10^{-5} or 10^{-3} M. It is therefore apparent that high K^+ concentrations, in addition to being inhibitory, cause the malate saturation curve to become sigmoidal. The addition of acetyl coenzyme A causes the curve to become markedly sigmoid as noted by Sanwal and his coworkers (Sanwal *et al.*, 1968; Sanwal and Smando, 1969b). The action of acetyl coenzyme A with respect to malate is competitive, as noted by Sanwal and Smando (1969b), to the extent that acetyl coenzyme A has little effect on the initial velocities at high malate concentrations. The apparent competition between malate and acetyl coenzyme A was shown to be completely reversible by the finding that the reaction rate in the presence of 5×10^{-3} M malate and 2×10^{-4} M acetyl coenzyme A was increased, by the addition of 45×10^{-3} M malate to the same cuvet, to a value which was identical with that observed in a separate experiment with 50×10^{-3} M malate and 2×10^{-4} M acetyl coenzyme A. The data of Figure 3 (in double-reciprocal form) suggest that the maximum molar turnover number of the enzyme (infinite concentrations of malate, TPN, Mn^{2+}, and K^+) at pH 7.4 is at least twice as great as the value of 500 sec^{-1} obtained from Figure 2 (infinite K^+, saturating Mn^{2+} and TPN, and 10^{-2} M malate).

Discussion

In order to study the stoichiometry and thermodynamics of enzyme–ligand interaction, as well as the kinetics and mechanism of action and regulation, it is necessary that the enzyme be in as homogeneous a state as possible. Although the yield (14%) of enzyme from our purification procedure is rather low (owing mainly to the necessity of discarding column fractions of lower specific activity), the final preparations, with a specific activity of about 56 units/mg, appear to be homogeneous. Thus, polyacrylamide disc gel electrophoresis (with relatively large amounts of protein applied) showed only a single protein band, the position of which was coincident with L-malic enzyme activity. Other circumstantial evidence suggesting that the enzyme is homogeneous includes the constant specific activity across most of the enzyme peak in the final DEAE-cellulose fractionation and the fact that the plots of the sedimentation equilibrium data were linear.

The molecular weight of 550,000 g/mole that we have obtained for native L-malic enzyme from *E. coli* in sedimentation equilibrium experiments is considerably larger than the

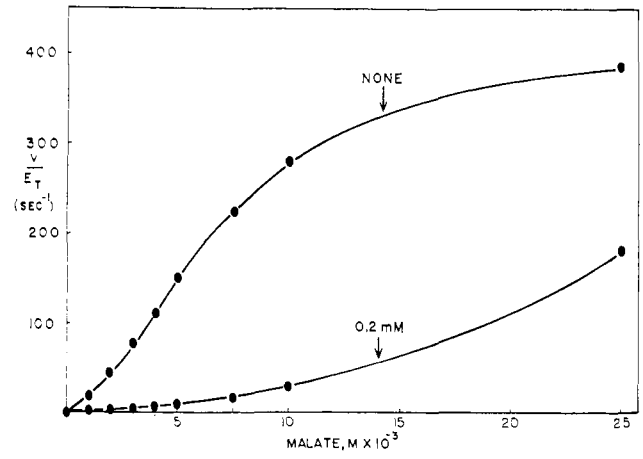

FIGURE 3: Effect of acetyl coenzyme A on malate kinetics. The concentration of acetyl coenzyme A is indicated by each curve, and tetramethylammonium L-malate was varied as indicated. The solutions contained in addition 0.1 M Tris-HCl, pH 7.4, 1.0 mM TPN, 1.0 mM $MnCl_2$, and 0.1 M KCl.

value of 345,200 g/mole obtained by Sanwal and Smando (1969a) from sucrose density gradient centrifugation, and is almost exactly twice as large as the molecular weight of pigeon liver malic enzyme (Hsu and Lardy, 1967). Since the presence of acetyl coenzyme A, at a concentration which would inhibit catalytic activity almost completely, caused no significant alteration of the molecular weight determined by sedimentation equilibrium, it is unlikely that acetyl coenzyme A inhibits L-malic enzyme by dissociating it into subunits.

The sedimentation equilibrium plots for the enzyme in the presence of 6.0 M guanidine hydrochloride and 0.01 M dithiothreitol were linear and gave a molecular weight of 67,000 g/mole for the completely dissociated polypeptide chains. This is almost exactly one-eighth of the molecular weight of the native enzyme and suggests that the enzyme is composed of eight subunits. The amino acid analysis (Table II) supports this contention if it is assumed that each amino acid residue must occur at least once in each subunit. Thus, the tryptophan analysis predicts a minimum molecular weight of 64,000 g/mole, and, therefore, eight units of this size per native enzyme molecule.

The fact that the pure enzyme has an absolute requirement for divalent and monovalent metal cations has enabled us to rule out a trivial mechanism for inhibition by acetyl coenzyme A, namely, complexation of the divalent metal cation. It is clear from the data of Table III that neither the level of free Mn^{2+} nor that of Mn^{2+}–malate (either one of which, or both, may be directly involved as ligands in the catalytic mechanism) is sufficiently altered to account for the 63% inhibition caused by 10^{-4} M acetyl coenzyme A in terms of a complexation mechanism, particularly since there is no evidence for interactions of Mn^{2+} (or Mn^{2+}–malate) with the enzyme of very high order. Sanwal *et al.* (1968) pointed out that the inhibition by acetyl coenzyme A is probably not caused by complexation of Mn^{2+} because 2.5×10^{-4} M acetyl coenzyme A gave 50% inhibition in experiments where the concentration of total Mn^{2+} was 100 times the observed K_m value for Mn^{2+}. However, Mn^{2+} is complexed by several chemical species (chiefly malate) in such experiments. Thus the addition of acetyl coenzyme A might sufficiently deplete the concentration of free Mn^{2+} to account for the observed inhibition if the enzyme binds Mn^{2+}, for example, rather than Mn^{2+}–malate. The data of Table III clearly eliminate this possibility, and acetyl coenzyme A must function through an allosteric interaction with the enzyme.

The K^+ activation data of Figure 2 corresponds to simple hyperbolic functions in the presence and absence of acetyl coenzyme A. The occurrence of noncompetitive inhibition by acetyl coenzyme A strongly suggests that the observed K_m for K^+ (8×10^{-3} M) represents the dissociation constant governing the interaction of K^+ with the various forms of the enzyme under these experimental conditions. The monovalent cation activation data of Sanwal and Smando (1969a) are somewhat different from ours, particularly with respect to the fact that K^+ or NH_4^+ served only to stimulate the enzyme activity in their experiments, rather than being required as obligatory activators of the enzyme. Thus the majority of the kinetic experiments described by Sanwal and Smando do not appear to involve the deliberate addition of K^+ or NH_4^+.

The finding that the initial velocity saturation curves for malate are hyperbolic at low concentrations of the monovalent cation activator (Sanwal and Smando, 1969b) but are sigmoid at 0.1 M K^+ (Figure 3) appears to correlate with other aspects of the interaction of the monovalent cation with the enzyme. Thus, we have found that high concentrations of K^+ and NH_4^+ are inhibitory, and the inhibition by acetyl coenzyme A is greater in the presence of 20×10^{-3} M NH_4^+ than in its absence (Sanwal and Smando, 1969a). Sanwal and Smando (1969b) analyzed the antagonistic effects between malate and acetyl coenzyme A in terms of a multisite two-state model (Monod *et al.*, 1965) in which malate has a high affinity for state R and the inhibitors (including acetyl coenzyme A) have a high affinity for state T, with the conformational equilibrium greatly in favor of state R in the absence of allosteric inhibitors. The various effects of the essential monovalent cation activator which we have noted can be rationalized if K^+, for example, has a greater affinity for the T state than for the R state.

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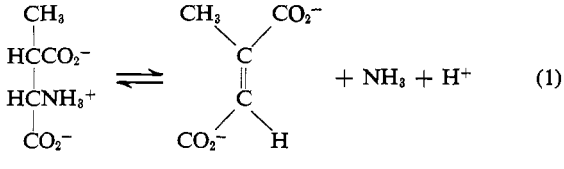
Magnetic Resonance and Kinetic Studies of the Activation of β-Methylaspartase by Manganese*

Gary A. Fields† and Harold J. Bright‡

ABSTRACT: Electron paramagnetic resonance studies of the interaction of Mn^{2+} with β-methylaspartase, which requires both monovalent and divalent metal cations for catalysis, show that the enzyme molecule has two Mn^{2+} sites. The Mn^{2+} sites act independently and the dissociation constant, K_s, for EMn^{2+} is 9×10^{-7} M. The binding of Mn^{2+} depends on an ionization with a pK_a value of 7.4. At pH 5.1, the binding of β-methylaspartate to EMn^{2+} tightens the binding of Mn^{2+} to the enzyme. Analysis of the affinity of Mn^{2+} for the enzyme as a function of the concentration of β-methylaspartate shows that the data are consistent only with a random-order addition of β-methylaspartate and Mn^{2+} to the enzyme. The values of the four dissociation constants for the random-order mechanism obtained with Mn^{2+} and β-methylaspartate from the electron paramagnetic resonance experiments (in the absence of K^+) are in excellent agreement with the corresponding values determined from steady-state turnover experiments in the presence of 0.15 M K^+.

This result, together with the fact that the K_m for K^+ is independent of the concentrations of β-methylaspartate and divalent metal activator at a given pH value, suggests that the binding of β-methylaspartate and divalent metal activator to the enzyme is independent of K^+. This is verified directly with Mn^{2+} in the case of K_s. However, the binding of Mn^{2+} to E–mesaconate is enhanced by K^+. Measurements of the effect of enzyme-bound Mn^{2+} on the longitudinal relaxation rate of water protons show that EMn^{2+} and K^+EMn^{2+} enhance the relaxation rate about 14-fold. The enhancement factor for EMn^{2+}–β-methylaspartate and EMn^{2+}–mesaconate is about 10-fold, and is 4-fold for K^+EMn^{2+}–mesaconate. These results are consistent with, but do not prove, a coordination structure in which Mn^{2+} acts as a bridge between substrate and enzyme.

β-Methylaspartase (*threo*-3-methyl-L-aspartate ammonia-lyase, EC 4.3.1.2) from *Clostridium tetanomorphum*, which requires both a monovalent and a divalent metal cation for reactivity, catalyzes the reversible conversion of *threo*-β-methyl-L-aspartate into mesaconate and ammonia (Barker *et al.*, 1959) according to eq 1. The enzyme has a molecular weight of 100,000 g/mole over a wide range of experimental conditions (Hsiang and Bright, 1967a) and can be dissociated in Gd·HCl[1] into subunits having a molecular weight of 50,000 g/mole (Hsiang, 1967; Hsiang and Bright, 1967b; Hsiang and Bright, 1969). The twenty cysteine residues in the molecule react with *p*MB at very different rates, all catalytic activity being lost after the first two cysteine residues have reacted with the mercurial (Hsiang and Bright, 1967a).



The existence and properties of an enzyme-catalyzed β-deuterium-exchange reaction with solvent protons (Bright *et al.*, 1964; Bright, 1964) led to the formulation of a mechanism which involves the formation of an enzyme-bound β-carbanion intermediate from which ammonia eliminates or is displaced in the rate-determining step of the overall reaction. Indirect evidence indicating that the sulfhydryl might be the base responsible for β-proton extraction from the substrate has been obtained both from studies of the β-deuterium-exchange reaction (Bright, 1964) and from photo-

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[1] The abbreviations used are: Gd·HCl, guanidine hydrochloride; E, active site of β-methylaspartase; βMA, *threo*-β-methyl-L-aspartate; MES, mesaconate; M^{2+}, divalent metal; *p*MB, *p*-mercuribenzoate.