Role of Metal Cofactors in Enzyme Regulation. Differences in the Regulatory Properties of the *Escherichia coli* Nicotinamide Adenine Dinucleotide Phosphate Specific Malic Enzyme, Depending on Whether Magnesium Ion or Manganese Ion Serves as Divalent Cation†

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ABSTRACT: A number of differences in the kinetic and physical properties of the *Escherichia coli* nicotinamide adenine dinucleotide phosphate ($NADP^+$) dependent malic enzyme have been found, depending upon whether Mg^{2+} or Mn^{2+} served to fulfill the divalent cation requirement. The velocity–$NADP^+$ and velocity–cation saturation curves exhibit a simple hyperbolic response in the presence of either metal cofactor, but the affinity for $NADP^+$ (and malate) as well as the V_{max} is increased in the presence of Mn^{2+}. The high affinity of the enzyme for Mn^{2+} coupled with the increased affinity for substrates indicates that Mn^{2+} is the preferred cofactor in vitro. With either Mg^{2+} or Mn^{2+} as cation, the velocity–malate saturation curves in the absence of effectors are complex at pH 7.45, indicating varying combinations of apparent positive and negative cooperative behavior. Greater initial positive cooperative behavior between malate binding sites is observed with Mg^{2+} as cation. The enzyme appears to be equally sensitive to inhibition by the allosteric inhibitors reduced nicotinamide adenine dinucleotide (NADH) and oxaloacetic acid (OAA) in the presence of either cation, but the interaction between malate binding sites, in the presence of effectors, varies significantly with the choice of metal cofactor. The inhibitor NADH increases the interaction between malate binding sites in the presence of Mn^{2+} but has little effect on subunit interaction in the presence of Mg^{2+}. The inhibitor OAA increases the interaction between malate binding sites in the presence of both cations, with increased positive cooperativity observed with Mn^{2+} but increased negative cooperativity with Mg^{2+}. The kinetic data can be explained by a model involving sequential ligand-induced conformational changes of the enzyme, resulting in a mixture of apparent positive and negative cooperative behavior. Alternate explanations involving different classes of noninteracting binding sites or different enzyme forms are also considered. The metal cofactors, Mg^{2+} and Mn^{2+}, appear to stabilize two distinct conformational states of the enzyme which differ in response to varying substrate and effector concentrations. Altered conformational states of the enzyme in the presence of the two cations are further substantiated by proteolytic digestion studies with the homogeneous enzyme. The results are strikingly similar to previous results reported on the nicotinamide adenine dinucleotide (NAD^+) dependent malic enzyme and the NAD^+-dependent isocitrate dehydrogenase, supporting the suggestion that metal cofactors function as regulatory entities.

Recent studies on the role of metal cofactors in enzyme regulation from this laboratory have revealed a number of significant differences in the catalytic and regulatory properties of the nicotinamide adenine dinucleotide (NAD^+)[1] specific malic enzyme of *Escherichia coli* (Milne & Cook, 1979) and the NAD^+-specific isocitrate dehydrogenase of *Neurospora crassa* (Barratt & Cook, 1978), depending upon whether Mg^{2+} or Mn^{2+} served to fulfill the divalent metal requirement. In both cases, the metal cofactors appeared to stabilize two distinct forms of enzyme which differed in response to varying substrate and effector concentrations. In general, the Mg^{2+}–enzyme forms were shown to exhibit complex subunit interactions involving mixed positive and negative cooperative behavior while the Mn^{2+}–enzyme forms exhibited simple hyperbolic behavior. In view of the results reported with the NAD^+-specific malic enzyme (Milne & Cook, 1979), it was of interest to examine the role of the metal cofactors in the functionally related $NADP^+$-specific malic enzyme of *E. coli*.

The $NADP^+$-specific malic enzyme (EC 1.1.1.40) of *Escherichia coli* has been reported to exhibit characteristics of a modulator-dependent cooperative (MDC) system (Sanwal, 1970); i.e., the substrates, malate and $NADP^+$, exhibit normal hyperbolic behavior in the absence of effectors, but cooperative behavior in the presence of the allosteric inhibitors, NADH, NADPH, oxaloacetate, and acetyl-CoA (Sanwal & Smando, 1969a,b; Spina et al., 1970). The catalytic and regulatory properties of the enzyme have been examined exclusively in the presence of Mn^{2+} as the obligatory cofactor, although the divalent cation requirement can apparently be fulfilled by Mg^{2+} (Sanwal & Smando, 1969a; Iwakura et al., 1979a,b). In this paper, we report the effect of substituting Mg^{2+} for Mn^{2+} on the catalytic and regulatory properties of the enzyme. Since the substrates and effectors utilized in the present study are capable of chelating Mg^{2+} and Mn^{2+} to different extents, the experimental data were routinely subjected to computer analysis to determine the concentration of the various free and chelated forms of all ligands. From the kinetic and proteolysis studies reported here, it is concluded that Mg^{2+} and Mn^{2+} stabilize two distinct conformational states of the enzyme which differ in response to varying malate and effector concentrations. In addition, the detailed initial velocity kinetic

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[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; $NADP^+$, NAD^+ phosphate; NADPH, reduced $NADP^+$; OAA, oxaloacetic acid; n_H, Hill coefficient or interaction coefficient; V_{max}, maximum velocity; $S_{0.5}$, half-saturating concentration of substrate; $P_{0.5}$, concentration of ligand giving 50% protection against proteolysis; CoA, coenzyme A; ADP, adenosine 5′-diphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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studies indicate that the $NADP^+$-specific malic enzyme does not exhibit simple hyperbolic kinetics in the presence of either Mg^{2+} or Mn^{2+} but a mixture of positive and negative cooperativity. As a result of this complexity, the previously proposed mechanism for the enzyme (Sanwal & Smando, 1969b) in terms of the two-state model of Monod et al. (1965) must be extensively modified or, alternately, discarded. Although the results are suggestive of possible mixed cooperativity, alternate explanations involving distinct enzyme forms or ligand-induced association–dissociation cannot be eliminated. A more rapid procedure to obtain homogeneous enzyme, utilizing affinity chromatography, is described. A detailed initial velocity kinetic study of the effect of the monovalent cation K^+ and various anions on the catalytic and regulatory properties of the enzyme will be reported in a subsequent paper.

Materials and Methods

Preparation and Characterization of Enzyme. The *E. coli* strain K12 (HfrH) was obtained from Dr. B. D. Sanwal and grown on LB media composed of 1% bactotryptone, 0.5% yeast extract, and 1% NaCl. The media was adjusted to pH 7.0 with solid KOH. Following a 1% inoculum, the cells were grown for approximately 7 h, resulting in 4.5 g of cells per liter of media. The cells were wet packed by centrifugation and stored at −20 °C.

Two purification procedures were employed to obtain homogeneous enzyme. Initially, the enzyme was purified essentially as described by Spina et al. (1970). Because a minimum of 2 weeks was required for this procedure, one of a shorter duration was sought to eliminate the possibility of enzyme denaturation or modification due to the time and adverse conditions during the purification. The enzyme was therefore purified by a modification of the procedure described by Yamaguchi et al. (1973) for the purification of the NAD^+-specific malic enzyme of *E. coli*. The cells were thus disrupted in the French pressure cell and subjected to streptomycin treatment, ammonium sulfate fractionation, heat and acid treatment, calcium phosphate gel adsorption, and DEAE-Sephadex A-50 column chromatography as described (Yamaguchi et al., 1973). The fractions collected from the DEAE-Sephadex A-50 column containing $NADP^+$–malic enzyme activity were pooled, and the enzyme was precipitated by the slow addition of ammonium sulfate to 50% saturation. The precipitate was collected by centrifugation and dissolved in a small volume of 20 mM potassium phosphate buffer, pH 7.45, containing 0.1 mM EDTA and 1.0 mM dithiothreitol. This sample was applied to a Sephadex G-200 column and eluted with the same buffer. Tubes containing activity were pooled, and the enzyme was precipitated with ammonium sulfate. The precipitate was dissolved in as small a volume as possible of 30 mM Tris-HCl buffer, pH 7.7, containing 1 mM $MnCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol and dialyzed against the same buffer overnight at 4 °C. The dialyzed solution was applied to a Sepharose 4B–2′,5′-ADP affinity column (1 × 19 cm) equilibrated with the same buffer (Yeung & Carrico, 1976). Two bed volumes of this buffer were passed through the column to remove unbound protein, and the $NADP^+$-specific malic enzyme was then eluted with a linear gradient of 0.0–0.2 mM NADP in the same buffer. Fractions which were colorless and containing enzyme activity were immediately pooled and made 80% in ammonium sulfate, and the resultant precipitate was collected by centrifugation. The pellets were brought up in a minimum volume of 50 mM Tris-HCl buffer, pH 7.45, containing 0.1 mM EDTA and 1 mM dithiothreitol. The enzyme was routinely stored at 4 °C, the stability being dependent upon enzyme concentration. The enzyme was stable for 3–4 months under these conditions. This procedure required only 7 days and gave a 31% yield of enzyme with a specific activity of 120 units/mg of protein.

All kinetic and proteolysis studies reported here were conducted with homogeneous enzyme. The results obtained with enzyme prepared by the two techniques described, or with impure enzyme, were identical, indicating that the unusual kinetic effects observed were not due to the trivial explanation of partially desensitized enzyme.

The homogeneity of the enzyme from the affinity column was tested at two gel concentrations by polyacrylamide gel electrophoresis following the method of Davis (1964). Only one protein band was observed when the gels were stained with Coomassie blue or Amido black 10B (50–100 μg of protein applied). A corresponding single band was observed when the gels were stained specifically for $NADP^+$–malic enzyme activity (Fine & Costello, 1963).

Assay Procedures. All kinetic studies were carried out at 24–25 °C on a Gilford Model 2400 recording spectrophotometer equipped with dual thermoplates. The temperature was maintained with a Haake thermostat. The enzyme to be used was dialyzed overnight against 50 mM Tris-HCl buffer, pH 7.45, containing 0.1 mM EDTA and 1 mM dithiothreitol at 4 °C. To insure that no denaturation occurred during the course of the experiment, the enzyme activity was checked periodically by using the standard assay mixture, containing 100 mM Tris-HCl buffer, pH 7.45, 50 mM KCl, 24 mM L-malate (pH 7.45 with concentrated Tris), 1 mM $MnCl_2$, and 0.5 mM $NADP^+$, in a total volume of 1.0 mL. The reaction velocity was measured by monitoring the production of NADPH at 340 nm in a quartz cuvette of 1-cm light path and 1.4-mL volume. Addition of all reactants to the cuvette was made by λ pipets and/or Hamilton syringes as warranted. One enzyme unit is defined as that amount of enzyme causing the formation of 1 μmol of NADPH per minute under standard assay conditions. Protein concentration was determined by the method of Lowry et al. (1951), using serum albumin as the reference standard. Specific activity is expressed as units per milligram of protein.

The kinetic behavior of the enzyme was studied in detail; i.e., 20–30 experimental points were routinely attempted per individual curve, with duplicates and overlapping points in areas necessitating dilutions. Temperature equilibration was attained by allowing the cuvettes to stand in the thermostated cuvette holder for 10 min. The reaction was routinely started by the addition of metal cofactor. Control experiments revealed essentially identical resuls when the reaction was initiated with enzyme, $NADP^+$, or malate.

L-Malate, OAA, NADP, and NADH were purchased from Sigma Chemical Co. and Boehringer-Mannheim. The $MnCl_2$ and $MgCl_2$ were analytical grade and purchased from J. T. Baker Chemical Co. Trypsin (twice recyrstallized) was purchased from Worthington Biochemical Corp.

Metal Chelation Analysis. All the data obtained in the present study have been routinely subjected to compuer analysis, utilizing a slightly modified version of the program developed by Perrin & Sayce (1967) to determine the free metal ion concentration and the various free and metal-chelated forms of all ionic species of ligand present. In addition, the binding of metals to the buffer species, free base 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), chloride ion, and EDTA (5 μM in all assays), was considered. However, at the concentrations of these species which were present, the inclusion of these complexes in the calculations influences the

concentrations of free metal and all ionic forms of ligand by less than 1%. Therefore, the formation of these complexes is routinely neglected in the calculations presented here. The following literature values (O'Sullivan, 1969; O'Sullivan & Perrin, 1964; Coleman, 1972; Apps, 1973) for the log stability constants have been used in this study: Mn–NADP, 2.96; Mn–NADH, 1.90; Mn–malate, 2.20; Mn–HOAA, 15.9 (β); Mn–NADP, 1.72; Mg–NADH, 1.73; Mg–malate, 1.60; Mg–HOAA, 15.1 (β). The term β indicates a complex additive stability constant (O'Sullivan, 1969).

Tryptic Digestion Studies. Tryptic digestions were carried out on solutions of 10 μg/mL homogeneous $NADP^+$-specific malic enzyme in 100 mM Tris-HCl buffer, pH 7.45, containing 0.1 mM EDTA and 1 mM dithiothreitol at 23 °C. The final concentration of trypsin in the experiments was 0.1 μg/mL. Samples (50 μL) were removed from the digestion mixture at 5-min intervals, and the enzyme activity was determined by using the standard assay procedure previously described. The results have been expressed in terms of the percent activity remaining after digestion, a 100% activity referring to the enzyme activity prior to the addition of trypsin at zero time. Measurements to exclude direct activation or inhibition of trypsin by the various ligands were carried out by digesting succinylated protamine in the presence and absence of these ligands according to the method of Kessner & Troll (1976). In no case was there any effect of the ligands on trypsin activity.

Results

The results of previous kinetic studies with the $NADP^+$-specific malic enzyme of *E. coli* have been contradictory in regard to the requirement of the monovalent cations, K^+ or NH_4^+, for enzyme activity. The only detailed initial velocity kinetic study of the enzyme was conducted in the apparent absence of monovalent cations, although the monovalent cations were shown to stimulate enzyme activity (Sanwal & Smando, 1969a,b). The results from other less detailed studies, however, have indicated an absolute requirement for K^+ or NH_4^+ (Takeo, 1969; Spina et al., 1970; Iwakura et al., 1979a). Contradictory results have also been reported in regard to the effect of higher concentrations of K^+ on enzyme activity. Inhibition of enzyme activity has been reported at K^+ concentrations greater than 50 mM (Spina et al., 1970) while no inhibition was observed at K^+ concentrations as high as 150 mM (Takeo, 1969).

In view of these anomalies, it was necessary in the present study to determine the optimum K^+ concentration to be used in subsequent studies. The effect of KCl on the initial velocity of the reaction was examined in the presence of saturating concentrations of malate (24 mM), $NADP^+$ (0.5 mM), and either Mg^{2+} (2.0 mM) or Mn^{2+} (0.9 mM) (Figure 1). It is clear from the data presented in Figure 1 that (i) the enzyme is active in the absence of K^+ ions, (ii) the enzyme activity is stimulated approximately 2-fold by the addition of KCl, maximal activation occurring at 30–50 mM KCl, and (iii) concentrations of KCl greater than 50 mM are inhibitory. The inhibition observed with excess KCl could be mimicked by substituting NaCl for KCl, indicating that the inhibition is not due specifically to excess K^+ ions. It is also evident that substituting Mg^{2+} for Mn^{2+} as the required divalent cation lowers the enzyme activity approximately 50%, indicating that Mn^{2+} is the preferred cofactor in vitro. All subsequent kinetic data reported here were obtained in the presence of 40–50 mM KCl, the optimum concentration for enzyme activity. All experiments were conducted with pure $NADP^+$-specific malic enzyme in 100 mM Tris-HCl buffer, pH 7.45, the optimum

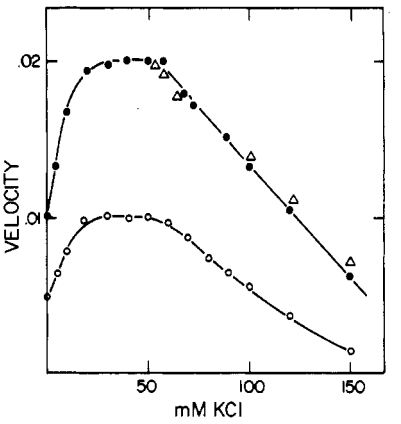

FIGURE 1: Effect of KCl concentration on initial velocity kinetic studies at fixed, saturating concentrations of malate (24 mM), $NADP^+$ (0.5 mM), and divalent cation. Conditions: 100 mM Tris-HCl buffer, pH 7.45, 25 °C. Velocity is expressed as μmol of NADPH formed/min. (○) In the presence of a fixed, saturating concentration of Mg^{2+} (2.0 mM); (●) in the presence of a fixed, saturating concentration of Mn^{2+} (0.9 mM); (△) effect of substituting NaCl for KCl. The KCl concentration is constant at 50 mM with NaCl concentration varied to obtain the total salt concentration indicated on the axis.

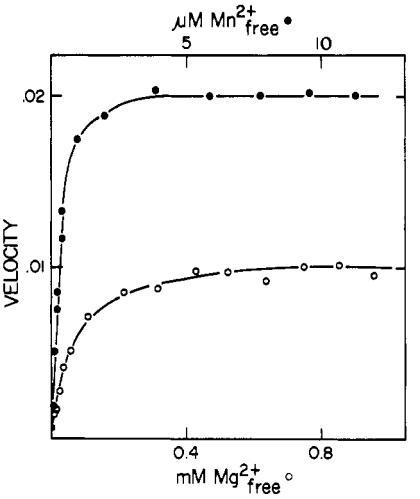

FIGURE 2: Initial velocity kinetic studies with varying divalent cation concentration and a fixed, saturating concentration of malate (25 mM) and $NADP^+$ (0.5 mM). The data are presented in terms of the calculated free cation concentration. The resultant kinetic constants are summarized in Table I. Conditions: 100 mM Tris-HCl buffer, pH 7.45, and 40 mM KCl, 24 °C. Velocity is expressed as μmol of NADPH formed/min. (○) Varying Mg^{2+} concentration (the *total* Mg^{2+} concentration has been varied from 0.005 to 2.0 mM); (●) varying Mn^{2+} concentration (the *total* Mn^{2+} concentration has been varied from 0.2 to 60 μM).

pH for enzyme activity. The concentration of all variable substrates is expressed in terms of their calculated free concentrations as described previously.

Initial Velocity Kinetic Studies in the Absence of Allosteric Inhibitors. The results of previous kinetic studies with the enzyme have indicated that all substrates exhibit hyperbolic rate concentration behavior in the absence of allosteric effectors. Such studies were conducted, however, in the absence of K^+ ions and in the presence of Mn^{2+} as the obligatory divalent cation (Sanwal & Smando, 1969a,b). In the present study, when the velocity of the reaction is studied as a function of the free cation concentration at saturating concentrations of malate (25 mM) and $NADP^+$ (0.5 mM) at pH 7.45, hyperbolic responses are obtained with both Mn^{2+} and Mg^{2+} (Figure 2). Double-reciprocal plots of the data are typically linear. The data have been presented in terms of the calculated

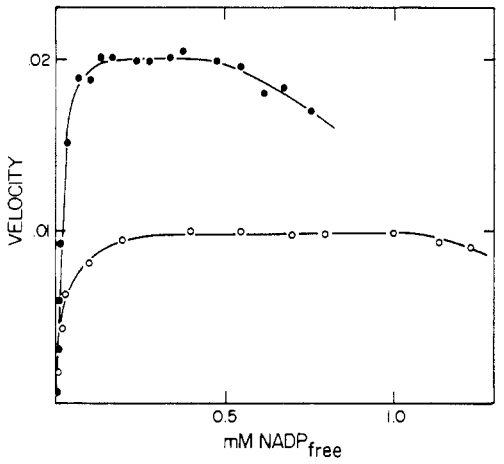

FIGURE 3: Initial velocity kinetic studies with varying $NADP^+$ concentration and a fixed, saturating concentration of malate (25 mM) and divalent cation. The data are presented in terms of the calculated free $NADP^+$ concentration. The resultant kinetic constants are summarized in Table I. Conditions: 100 mM Tris-HCl buffer, pH 7.45, and 40 mM KCl, 24 °C. Velocity is expressed as μmol of NADPH formed/min. (○) Presence of saturating Mg^{2+} (2.5 mM) (the *total* $NADP^+$ concentration was varied from 0.002 to 1.3 mM); (●) presence of saturating Mn^{2+} (1.0 mM) (the *total* $NADP^+$ concentration was varied from 0.008 to 1.0 mM).

Table I: Kinetic Constants for Malic Enzyme[a]

variable substrate	cation	$S_{0.5}$ (mM)	n_H[b]
Mn^{2+} (free)		0.00036	1.0
Mg^{2+} (free)		0.054	1.06
malate (free)	Mn^{2+}	5.6[c]	1.1[d]
	Mg^{2+}	12.4[c]	2.0[d]
$NADP^+$ (free)	Mn^{2+}	0.015	1.1
	Mg^{2+}	0.024	0.97
KCl (total)	Mn^{2+}	6.6	1.0
	Mg^{2+}	8.3	1.0

[a] Kinetic constants for the variable substrate have been determined at saturating concentrations of all other fixed ligands. [b] Interaction coefficients determined from the slopes of the Hill plots. [c] The $S_{0.5}$ values are not equivalent to the K_m values in view of the complex nature of the saturation curves, but are included here for comparative purposes. [d] Minimum values for n_H determined from tangents drawn to the curved Hill plots.

free divalent cation concentration. The V_{max} is decreased approximately 50% in the presence of Mg^{2+} as estimated from double-reciprocal plots of the data. Hill plots of the data indicated n_H values of approximately one for both metal cofactors. The resultant kinetic constants are summarized in Table I.

Many enzymes which utilize Mn^{2+} as a cofactor exhibit inhibition of enzyme activity at high Mn^{2+} concentrations. In the present study, when the velocity of the reaction is studied as a function of a broader range of free Mn^{2+} concentrations (0–2.5 mM), inhibition is observed at free Mn^{2+} concentrations greater than 900 μM (3.2 mM Mn^{2+} total). Such studies were conducted under conditions identical with those described for Figure 2 at both saturating concentrations of malate (25 mM) and subsaturating concentrations (8 mM). Such control studies were required in view of the fact that the free Mn^{2+} concentration varies from a maximum of 650 μM to 90 μM during the course of a malate-saturation study. Since free Mn^{2+} concentrations never exceed inhibitory levels in any of the subsequent studies reported here, we are confident that the variation in free Mn^{2+} concentration has no effect on the substrate-saturation behavior reported in Figures 3–7.

When the velocity of the reaction is studied as a function of the free $NADP^+$ concentration at saturating malate concentration (25 mM) and either Mn^{2+} (1.0 mM) or Mg^{2+} (2.5 mM) at pH 7.45, hyperbolic responses are again observed (Figure 3). The cause of the inhibition observed at higher $NADP^+$ concentrations remains to be determined. It is clear from the chelation analyses that the free Mn^{2+} concentration remains saturating, but the NADP–Mn complex concentration increases significantly as the total $NADP^+$ concentration is increased. We speculate that the presence of increasing concentrations of the NADP–Mn complex may interfere with the binding of free $NADP^+$ to the enzyme, resulting in inhibition of enzyme activity. With the exclusion of the inhibited portions of the saturation curves, double-reciprocal plots are typically linear. Similarly, Hill plots of the data indicated n_H values of approximately one for $NADP^+$ in the presence of either divalent cation. The resultant kinetic constants for $NADP^+$ are summarized in Table I.

When the velocity of the reaction is studied as a function of the free malate concentration at saturating $NADP^+$ (0.5

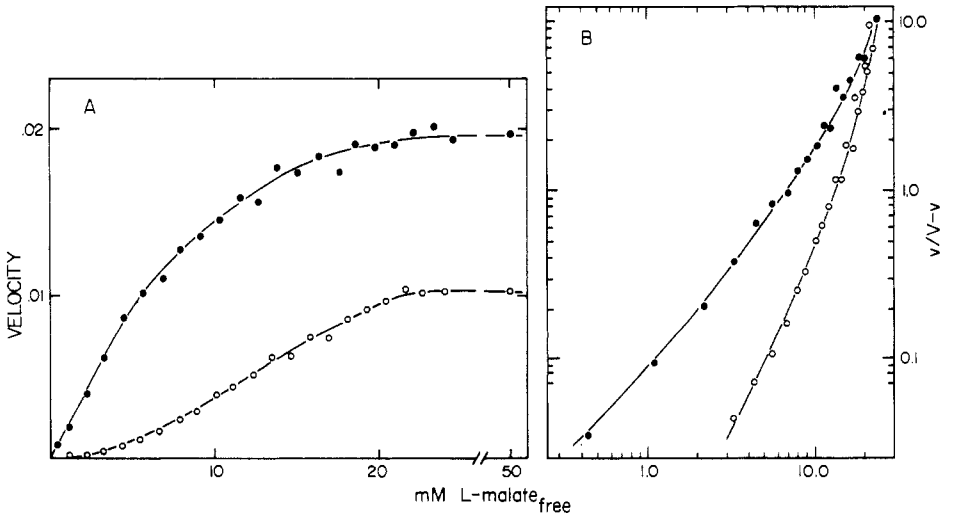

FIGURE 4: Initial velocity kinetic studies with varying malate concentration and a fixed, saturating concentration of $NADP^+$ (0.5 mM) and divalent cation. The data are presented in terms of the calculated free malate concentration. (The *total* malate concentration was varied from 0.24 to 55 mM.) The resultant kinetic constants for malate are summarized in Table I. Conditions: 100 mM Tris-HCl buffer and 40 mM KCl, pH 7.45, 24 °C. Velocity is expressed as μmol of NADPH formed/min. (○) In the presence of saturating Mg^{2+} (2.5 mM); (●) in the presence of saturating Mn^{2+} (1.0 mM). (A) Saturation curves; (B) Hill plots. The V_{max} values required for the Hill plots have been estimated from double-reciprocal plots. The V_{max} values used are 0.0115 and 0.021 μmol of NADPH formed/min in the presence of Mg^{2+} and Mn^{2+}, respectively.

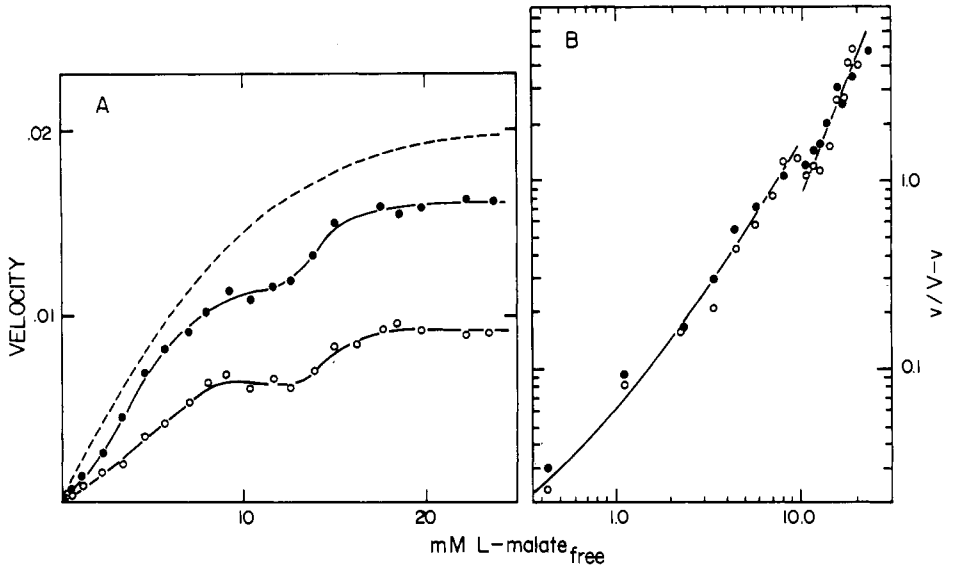

FIGURE 5: Effect of the inhibitor NADH on initial velocity kinetic studies with varying malate concentration and a fixed, saturating concentration of $NADP^+$ (0.5 mM) and Mn^{2+} (1.0 mM). The data are presented in terms of the calculated free malate concentration. (The *total* malate concentration has been varied from 0.24 to 24 mM.) Conditions: 100 mM Tris-HCl buffer, pH 7.45, and 40 mM KCl, 24 °C. Velocity is expressed as μmol of NADPH formed/min. The dashed line represents the control, i.e., in the absence of NADH. (●) In the presence of 0.1 mM NADH; (○) in the presence of 0.2 mM NADH. (A) Saturation curves; (B) Hill plots. The V_{max} values required for the Hill plots have been estimated from double-reciprocal plots. The V_{max} values used are 0.0175 and 0.0102 μmol of NADPH formed/min in the presence of 0.1 and 0.2 mM NADH, respectively.

mM) at pH 7.45, the saturation curves obtained in the presence of Mg^{2+} and Mn^{2+} are significantly different (Figure 4A). In the presence of Mg^{2+} (2.5 mM), an apparent sigmoid hyperbolic response is observed. Double-reciprocal plots of the data are, however, nonlinear in the presence of both Mg^{2+} and Mn^{2+}, indicating a greater complexity to the malate-saturation curve in the presence of Mn^{2+}. The values for V_{max} have been estimated from the intercepts of the double-reciprocal plots to be 0.0115 and 0.021 μmol of NADPH formed/min in the presence of Mg^{2+} and Mn^{2+}, respectively. Estimation of the V_{max} values from the curved plots is difficult, and this problem is discussed in detail under Discussion. When the data are replotted in the form of the Hill plot by using the above V_{max} values (Figure 4B), curves approaching a vertical asymptote can be drawn through the data points. Tangents drawn to the curved Hill plots indicate minimum n_H values of 1.1 and 2.0 for malate in the presence of Mn^{2+} and Mg^{2+}, respectively. The resultant kinetic constants for malate are summarized in Table I.

Initial Velocity Kinetic Studies in the Presence of Effectors. In view of the differences in kinetic properties of the enzyme with Mg^{2+} and Mn^{2+}, it was of interest to examine the effect of the known inhibitors, NADH and OAA, on the activity of the enzyme in the presence of either metal cofactor. A detailed kinetic study was therefore undertaken to determine the effect of NADH on the complex kinetic response obtained in the malate-saturation curves. A 340-nm absorbance vs. NADH concentration plot was linear up to 0.35 mM NADH at 25 °C in 1-cm path-length cuvettes. Therefore, NADH concentrations of 0.1 and 0.2 mM were chosen for inhibition studies, these being high enough to cause measurable inhibition, yet low enough to exclude nonlinear absorbance and aberrations due to stray light in the spectrophotometer (Cavalieri & Sable, 1973). The absence of artifactual inhibition was confirmed by the use of 4-mm quartz cuvettes in control experiments.

The effect of the allosteric inhibitor NADH on initial velocity kinetic studies with varying malate concentration at a saturating concentration of $NADP^+$ and Mn^{2+} is presented in Figure 5. In the absence of NADH, the rate–concentration curve was essentially analogous to Figure 4A. In the presence of increasing concentrations of NADH, there is a corresponding decrease in the maximum velocity and an appearance of a distinct, intermediary plateau region (Figure 5A). Hill plots of the data (Figure 5B) remain curved with a minimum n_H value of 1.1 (determined from tangents to the curve), but a distinct break is evident, corresponding to the plateau region in the saturation curve. The Hill plots at both NADH concentrations are essentially identical and can be compared to the uninhibited curve in Figure 4B.

The effect of NADH on initial velocity kinetic studies with varying malate concentration at a saturating concentration of $NADP^+$ and Mg^{2+} is presented in Figure 6. In the absence of NADH, the rate–concentration curve was essentially analogous to Figure 4A. In the presence of increasing concentrations of NADH, there is a corresponding decrease in enzyme activity but no indication of the appearance of a plateau region. Hill plots of the data (Figure 6B) are superimposable at both concentrations of NADH as well as in the absence of inhibitor. It is concluded, therefore, that the binding of NADH to the enzyme (in the presence of Mg^{2+}) has no effect on the interaction between malate-binding sites.

The double-reciprocal plots of the NADH inhibition data obtained with either Mg^{2+} or Mn^{2+} (not shown), although complex, indicate noncompetitive inhibition with malate; i.e., the lines do not intersect on the vertical axis. Similarly, NADH inhibition studies conducted with $NADP^+$ as the variable substrate indicate noncompetitive inhibition. It is clear, therefore, that NADH is acting as an allosteric inhibitor, confirming the suggestion of Sanwal & Smando (1969a).

The allosteric inhibitor OAA has been reported to bind to a different allosteric site than NADH in previous studies (Sanwal & Smando, 1969a). It was of interest, therefore, to determine if OAA had effects similar to NADH on the enzyme. The effect of OAA on initial velocity kinetic studies with varying malate concentration at saturating concentrations of $NADP^+$ (0.5 mM) and either Mn^{2+} (1.0 mM) or Mg^{2+} (2.0 mM) is presented in Figure 7. The double-reciprocal plot obtained for malate in the presence of Mn^{2+} and OAA (Figure 7A) appears to be a simple parabola containing no interme-

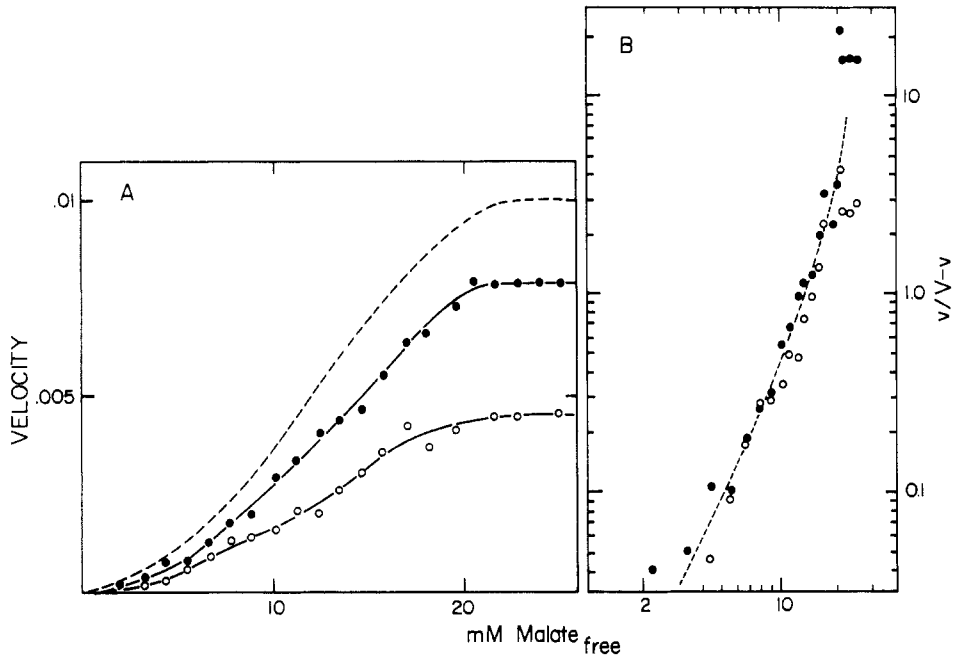

FIGURE 6: Effect of the inhibitor NADH on initial velocity kinetic studies with varying malate concentration and a fixed, saturating concentration of $NADP^+$ (0.88 mM) and Mg^{2+} (2.5 mM). The data are presented in terms of the calculated free malate concentration. (The *total* malate concentration has been varied from 0.24 to 25 mM.) Conditions: 100 mM Tris-HCl buffer, pH 7.45, and 50 mM KCl, 24 °C. Velocity is expressed as μmol of NADPH formed/min. The dashed lines represent the data obtained in the absence of NADH (for experimental data, see Figure 4). (●) In the presence of 0.1 mM NADH; (○) in the presence of 0.2 mM NADH. (A) Saturation curves; (B) Hill plots. The V_{max} values required for the Hill plots have been estimated from double-reciprocal plots. The V_{max} values used are 0.0083 and 0.0052 μmol of NADPH formed/min in the presence of 0.1 and 0.2 mM NADH, respectively.

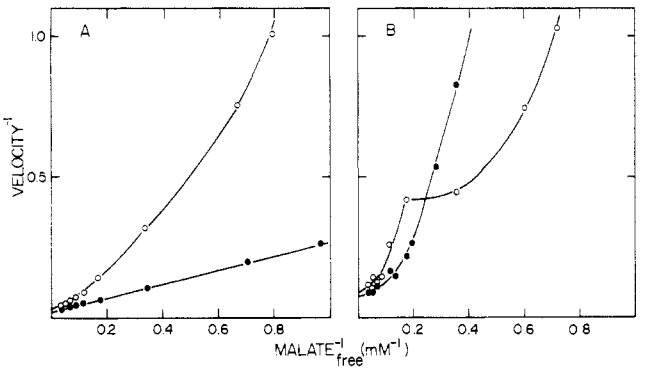

FIGURE 7: Effect of the inhibitor OAA on initial velocity kinetic studies with varying malate concentration at a fixed, saturating concentration of $NADP^+$ (0.5 mM) and divalent cation, presented in the double-reciprocal form. The data are presented in terms of the calculated free malate concentration. (The *total* malate concentration has been varied from 0.24 to 25 mM.) Conditions: 100 mM Tris-HCl buffer, pH 7.45, and 40 mM KCl, 24 °C. Velocity is expressed as (μmol of NADPH formed/min) × 10^3. (●) In the absence of OAA; (○) in the presence of 0.3 mM OAA. (A) In the presence of saturating Mn^{2+} (1.0 mM); (B) in the presence of saturating Mg^{2+} (2.5 mM).

diary plateau regions. In the absence of OAA, the double-reciprocal plot for malate appears to be linear but is more complex when plotted on an expanded scale. It is concluded, therefore, that the binding of OAA to the enzyme in the presence of Mn^{2+} results in increasing positive cooperativity between malate-binding sites. The double-reciprocal plots obtained for malate in the presence of Mg^{2+} are parabolic in the absence of inhibitors (Figure 7B). In the presence of OAA, however, the double-reciprocal plot is biphasic, indicative of an intermediary plateau region in the malate-saturation curve. It is concluded, therefore, that the binding of OAA to the enzyme in the presence of Mg^{2+} results in complex interaction between malate binding sites, possibly involving increased negative cooperativity. Several concentrations of OAA have been tested in the present study, but only the results obtained with 0.30 mM OAA have been presented in Figure 7 for the sake of clarity. The results not shown are consistent with the above interpretation.

Tryptic Digestion Studies. In view of the kinetic results obtained, the question arises whether Mg^{2+} and Mn^{2+} stabilize two distinct conformational states of the enzyme. To test this hypothesis, we have examined the effects of substrates and effectors on the trypsin inactivation of the enzyme. The enzyme by itself is inactivated by trypsin, and control experiments were conducted to determine conditions whereby 50% inactivation occurred in approximately 20–30 min. The effect of the substrates, malate, and $NADP^+$, and the metal cofactors, Mg^{2+} and Mn^{2+}, on trypsin inactivation of the enzyme is presented in Figure 8. The metal cofactor Mg^{2+}, and to a lesser extent Mn^{2+}, stimulates the rate off inactivation of the enzyme by trypsin (Figure 8A). When both metal cofactors are tested together, the stimulation of the rate of inactivation is slightly greater, but not additive for the two cofactors. The degree of protection (or increased susceptibility) of the enzyme to tryptic digestion by the various ligands tested is summarized in Table II.

In the absence of metal cofactors, the substrate malate decreases the rate of inactivation of the enzyme approximately 3-fold (see Table II). A significant difference in the degree of protection of the enzyme by malate is observed, however, when Mg^{2+} or Mn^{2+} is present (Figure 8B). In the presence of Mn^{2+}, the protection by malate is increased, while in the presence of Mg^{2+}, protection is decreased, indicating a possible difference in enzyme conformation in the presence of the two metal cofactors. The second substrate, $NADP^+$, affords the best protection of the enzyme against trypsin inactivation (Figure 8C). Total protection of the enzyme by $NADP^+$ is observed in the presence of Mn^{2+}, while an unusual activation of enzyme activity by $NADP^+$ occurs in the presence of Mg^{2+}.

The protection of the enzyme by the substrates, $NADP^+$ and malate, was further examined to determine if the degree of protection was proportional to substrate concentration. The

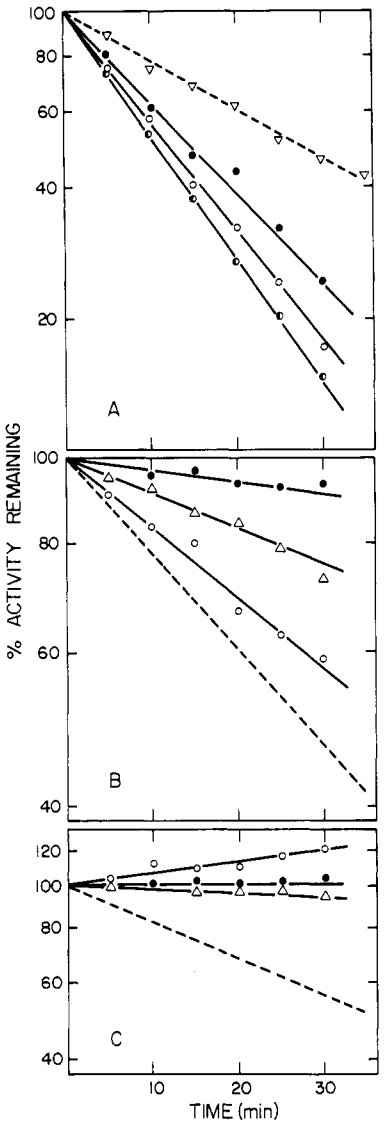

FIGURE 8: Effect of the substrates, $NADP^+$ and malate, and metal cofactors on the tryptic digestibility of the $NADP^+$-specific malic enzyme. Conditions: 10 μg/mL $NADP^+$-specific malic enzyme in 100 mM Tris-HCl buffer, pH 7.45, digested at 23 °C by 0.1 μg/mL trypsin. The results are expressed in terms of the percent of original enzyme activity remaining after varying times of digestion. (A) Effect of divalent cations: (△) in the absence of metal cofactors; (●) in the presence of saturating Mn^{2+} (0.5 mM); (○) in the presence of saturating Mg^{2+} (2.0 mM); (◐) in the presence of saturating concentrations of Mn^{2+} (0.5 mM) and Mg^{2+} (2.0 mM). (B) Effect of malate: (---) control in the absence of malate; (△) in the presence of saturating malate (24 mM); (○) in the presence of saturating malate (24 mM) and Mg^{2+} (2.0 mM); (●) in the presence of saturating malate (24 mM) and Mn^{2+} (0.5 mM). (C) Effect of $NADP^+$: (---) control in the absence of $NADP^+$; (△) in the presence of saturating $NADP^+$ (0.5 mM); (●) in the presence of saturating $NADP^+$ (0.5 mM) and Mn^{2+} (0.5 MM); (○) in the presence of saturating $NADP^+$ (0.5 mM) and Mg^{2+} (2.0 mM).

enzyme was digested for 15 min with trypsin in the presence of varying concentrations of each substrate in the absence and presence of Mg^{2+} or Mn^{2+}. From such studies, the concentration of substrate resulting in half-maximal protection ($P_{0.5}$) of the enzyme was determined (see Table II). The $P_{0.5}$ values thus determined are in reasonable agreement with the $S_{0.5}$ values determined in the kinetic studies (see Table I), indicating that the degree of protection corresponds to the degree of substrate saturation of the enzyme.

The effect of the allosteric inhibitors, OAA and NADH, on trypsin inactivation of the enzyme is presented in Figure 9. In the absence of metal cofactors, both OAA (Figure 9A)

Table II: Effect of Substrates and Effectors on Tryptic Digestibility of Malic Enzyme

ligand(s) tested	protection factor[a]	$P_{0.5}$[b] (mM)
none	1.0	
Mn^{2+} (0.5 mM)	0.54	
Mg^{2+} (2.0 mM)	0.44	
Mn^{2+} (0.5 mM) + Mg^{2+} (2.0 mM)	0.40	
malate (24 mM)	2.8	12
malate (24 mM) + Mn^{2+} (0.5 mM)	8.2	7
malate (24 mM) + Mg^{2+} (2.0 mM)	1.4	14
$NADP^+$ (0.5 mM)	8.6	0.030
$NADP^+$ (0.5 mM) + Mn^{2+} (0.5 mM)	∞[c]	0.010
$NADP^+$ (0.5 mM) + Mg^{2+} (2.0 mM)	*d*	0.015
OAA (0.15 mM)	1.9	
OAA (0.15 mM) + Mg^{2+} (2.0 mM)	0.6	
OAA (0.15 mM) + Mn^{2+} (0.5 mM)	0.6	
NADH (0.2 mM)	1.5	
NADH (0.2 mM) + Mg^{2+} (2.0 mM)	0.7	
NADH (0.2 mM) + Mn^{2+} (0.5 mM)	0.7	

[a] The protection factor is defined as the ratio of the $T_{1/2}$ in the presence of the ligand to the $T_{1/2}$ in the absence of ligand. $T_{1/2}$ is the time required to reduce the original enzyme activity by 50%. [b] $P_{0.5}$ is defined as the concentration of $\text{ligand}_{\text{free}}$ giving 50% of the maximal protection observed. [c] Total protection of the enzyme against proteolysis was observed. [d] Activation of enzyme activity was observed during proteolytic digestion.

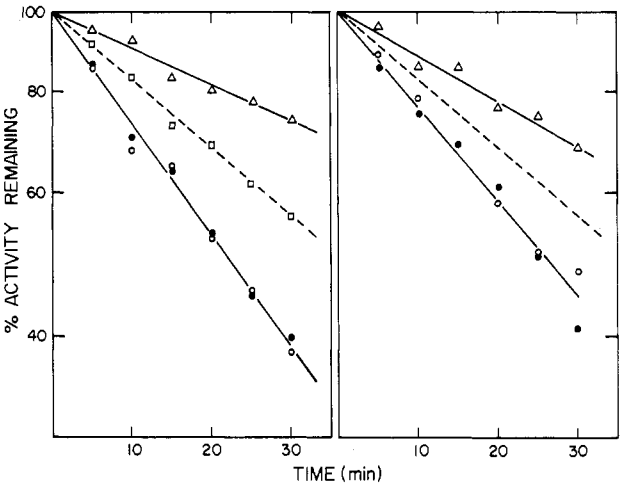

FIGURE 9: Effect of the inhibitors, OAA and NADH, on the tryptic digestibility of the $NADP^+$-specific malic enzyme. Conditions: 10 μg/mL $NADP^+$-specific malic enzyme in 100 mM Tris-HCl buffer, pH 7.45, digested at 23 °C by 0.1 μg/mL trypsin. The results are expressed in terms of the percent of original enzyme activity remaining after various times of digestion. (A) Effect of OAA: (□) control in the absence of OAA; (△) in the presence of OAA (0.15 mM); (○) in the presence of OAA (0.15 mM) and saturating Mg^{2+} (2.0 mM); (●) in the presence of OAA (0.15 mM) and saturating Mn^{2+} (0.5 mM). (B) Effect of NADH: (---) control in the absence of NADH; (△) in the presence of NADH (0.2 mM); (○) in the presence of NADH (0.2 mM) and saturating Mg^{2+} (2.0 mM); (●) in the presence of NADH (0.2 mM) and saturating Mn^{2+} (0.5 mM).

and NADH (Figure 9B) partially protect the enzyme against trypsin inactivation. In the presence of Mg^{2+} or Mn^{2+}, however, the protection by both inhibitors is abolished, the rate of inactivation approaching that observed in the presence of metal cofactors alone (see Figure 8A).

Discussion

In the present study, we have compared the effect of substituting Mg^{2+} for Mn^{2+} on the catalytic and regulatory properties of the $NADP^+$-specific malic enzyme of *Escherichia coli*. On the basis of the kinetic and proteolytic studies reported here, we have concluded that the metal cofactors, Mg^{2+}

and Mn^{2+}, stabilize two distinct forms of the enzyme, presumably by inducing different changes in conformation or structure of the enzyme. The Mn^{2+} form of the enzyme appears to be more active catalytically and exhibits a higher affinity for substrates than the Mg^{2+} form. In contrast to our previous studies on the role of metal cofactors in enzyme regulation (Barratt & Cook, 1978; Milne & Cook, 1979), both the Mg^{2+}–enzyme and Mn^{2+}–enzyme appear to be equally sensitive to inhibition by allosteric effectors. What does differ in the two enzyme forms, however, is the observed interaction between malate binding sites in the presence of allosteric inhibitors.

Complex interaction between malate binding sites was observed in the absence of allosteric effectors with both the Mn^{2+} and Mg^{2+} forms of the enzyme as revealed by the curvature of the Hill plots of the kinetic data. Before attempting to explain the complex interaction observed, it is necessary to discuss the validity of the Hill plot representation of the data. We have chosen to present the data in the form of the Hill plot due to the usefulness of this representation in the interpretation of complex subunit interaction (Cook & Koshland, 1970; Cornish-Bowden & Koshland, 1975). This representation of the data does, however, require a reasonably accurate value for V_{max} which is often difficult to obtain from enzyme systems exhibiting positive or negative cooperativity. The obvious question arises as to whether the curvature of the Hill plots presented in Figures 4–6 is a true property of the enzyme system or is due to an error in the estimation of the V_{max} value. Values for V_{max} have been estimated from double-reciprocal plots of the data which are curved in all cases, resulting in some ambiguity in the estimated value. As a result, we have analyzed all of our data by using several V_{max} values to determine if the curvature of the Hill plots could be abolished. Such analyses indicate that the curvature can be abolished by using V_{max} values approximately 50% higher than the values estimated from double-reciprocal plots. We have been unable to approach such a theoretical V_{max} even at an extremely high malate concentration in control studies. In addition, the V_{max} values estimated from the linear double-reciprocal plots obtained with $NADP^+$ and metal cofactors, even at extremely high, fixed malate concentrations, are in excellent agreement with those obtained from the malate-saturation curves. We are therefore confident that our graphically determined V_{max} values are a reasonable estimate of the true value and not in error by 50%. Since the absolute n_H values are not critical to the interpretation of the data, we are confident that any errors in the determination of V_{max} do not negate the results presented in this study.

Curved Hill plots such as those reported in this study have been normally associated with enzyme systems exhibiting distinct intermediary plateau regions indicative of mixed-cooperative systems (Teipel & Koshland, 1969; Rock & Cook, 1974; Barratt & Cook, 1978; Milne & Cook, 1979). Curved Hill plots have, however, been observed in enzyme systems exhibiting no obvious intermediary plateau regions, demonstrating that the overall shape of the saturation curve is the important criterion (Cook & Koshland, 1970). By analogy to these previous studies, we would conclude from our present results that mixed cooperativity between malate binding sites occurs in both the Mg^{2+}–enzyme and the Mn^{2+}–enzyme complexes. We have attempted to fit the data presented in Figures 4–7 by nonlinear regression analysis (Cornish-Bowden & Koshland, 1970) to a model involving binding and/or catalysis at six sites (the number of subunits in the enzyme). When such analyses are applied to steady-state kinetic data, the basic assumption is made that the rate of equilibration between substrate and enzyme is rapid relative to the rate of catalysis (Teipel & Koshland, 1969; Bardsley & Childs, 1975). The velocity–malate concentration data could not be adequately described by such computer analysis, providing presumptive evidence that rate-determining conformational changes or rate-determining binding steps are also involved in the overall enzymatic process (Teipel & Koshland, 1969). A similar conclusion was reached from the analysis of steady-state kinetic data previously obtained with yeast glyceraldehyde-3-phosphate dehydrogenase, which exhibits strikingly similar Hill plots approaching a vertical asymptote (Rock & Cook, 1974). Although the overall fit of the experimental data to the theoretical curves is poor, it is obvious from the computer analyses that the binding and/or catalytic constants must first increase, than decrease, and finally increase to give rise to the type of saturation curve exhibited by malate. This conclusion is supported by theoretical studies of the binding and catalysis of multisite enzymes, where hypothetical saturation curves comparable to those reported in this study have been generated (Teipel & Koshland, 1969; Bardsley & Childs, 1975).

It is clear from the present study that complex interaction occurs between malate binding sites in the presence of either metal cofactor. The initial positive cooperativity observed between malate binding sites in the presence of Mg^{2+}, but not Mn^{2+} (Figures 4–6), suggests that the metal cofactors stabilize two distinct conformational states of the enzyme. This hypothesis is supported by the varied response of the two enzyme forms to the inhibitors, NADH and OAA, presented in Figures 5–7. The inhibition by NADH and OAA appears to be allosteric in nature, in agreement with the results of previous studies (Sanwal & Smando, 1969a,b). In the case of the Mg^{2+}–enzyme, the assumed conformational change caused by NADH binding causes inhibition but does not affect the interaction between malate binding sites (Figure 6). In the case of the Mn^{2+}–enzyme, however, NADH binding does increase the interaction between malate binding sites, apparently resulting in increased negative cooperative behavior (Figure 5). In contrast to the results obtained with NADH, the binding of the inhibitor OAA to the enzyme causes increased positive cooperativity between malate binding sites in the Mn^{2+}–enzyme, but increased negative cooperativity in the Mg^{2+}–enzyme. These observations substantiate the hypothesis that the structure of the enzyme differs in the presence of the two metal cofactors.

In contrast to the results obtained with malate, no cooperativity between $NADP^+$ binding sites or metal cofactor binding sites is apparent in the absence of allosteric inhibitors (Figures 2 and 3), indicating that the binding sites for $NADP^+$ (and metal cofactors) are most likely equivalent and independent in both the Mn^{2+} and Mg^{2+} forms of enzyme. Allosteric effectors appear to have no effect (other than inhibition) on the interaction between $NADP^+$ binding sites or metal cofactor binding sites.

The results of the proteolysis studies further substantiate the hypothesis that Mg^{2+} and Mn^{2+} stabilze two distinct conformational states of the enzyme. The most significant difference observed in the proteolysis studies is the degree of protection of the enzyme by malate in the presence of the two metal cofactors (Figure 8B). Thus, the enzyme–Mg^{2+}–malate complex and the enzyme–Mn^{2+}–malate complex exhibit a 6-fold difference in susceptibility to proteolysis (see Table II), indicating a significant difference in the conformation of the two complexes. The differences observed in the degree of

protection (or increased susceptibility) of the enzyme by metal cofactors, either alone (Figure 8A) or in the presence of $NADP^+$ (Figure 8C), are consistent with a difference in conformation of the Mg^{2+} and Mn^{2+} forms of the enzyme. The unusual activation of enzyme activity observed with the enzyme–$NADP^+$–Mg^{2+} complex has not been further examined in the present study.

Several general points regarding ligand-induced conformational changes in the enzyme can be made from the proteolysis studies. It is clear than the metal cofactors are capable of binding directly to the enzyme with resultant changes in enzyme conformation. This observation confirms the recent findings of Iwakura et al. (1979b) that Mg^{2+} is capable of altering the conformation of the enzyme. The conformational states induced by metal cofactor binding can obviously be altered by the additional binding of the substrates, $NADP^+$ and malate. The varying degrees of protection of the enzyme to proteolysis by the substrates, even in the presence of metal cofactors, indicate that the substrates have a greater effect on the final enzyme conformation than do the metal cofactors. The correlation observed between the degree of saturation and protection of the enzyme with substrates (see $S_{0.5}$ and $P_{0.5}$ values in Tables I and II) indicates that conformational changes accompany the binding of substrates and that these effects are modified by the presence of the two metal cofactors. In contrast, the allosteric inhibitors, while capable of altering the conformation of the enzyme when tested alone, do not significantly alter the enzyme conformation stabilized by metal cofactors (see Figure 9).

The complex interaction between malate binding sites reported here has not been observed in previous studies on the $NADP^+$-specific malic enzyme. Thus, hyperbolic malate-saturation curves have been reported in the presence of Mn^{2+} (Sanwal & Smando, 1969a) and in the presence of Mg^{2+} (Iwakura et al., 1979a). The previously published studies, however, have been conducted in less detail, at different K^+ (and Cl^-) concentrations, and the resultant data are not corrected for metal chelation, making it difficult to directly compare our present results. We have recently completed a systematic study of the effect of K^+ and various anions on the mechanism of the malic enzyme, and the results substantiate the conclusions of the present study. The results of that study, to be reported in a subsequent paper, further indicate that the complex interaction between malate binding sites can be abolished by acetate, which apparently acts as a specific activator of the enzyme.

The results of previous studies with the $NADP^+$-specific malic enzyme (Sanwal & Smando, 1969b) performed in the presence of Mn^{2+}, but the absence of K^+, have been interpreted in terms of the two-state model of Monod et al. (1965). The complex interaction between malate binding sites observed in the present study cannot be accommodated by the simple two-state model. We have suggested a model for the enzyme involving sequential ligand-induced changes in enzyme conformation (Koshland et al., 1966), resulting in sites of differing affinity and/or catalytic rate. Such a model is consistent with the computer analyses of the kinetic data and the proteolysis studies. Such an explanation assumes that the binding sites for each ligand are originally equivalent in the enzyme. While we favor such an explanation, alternate mechanisms can be proposed to explain the observed results. These alternate explanations would include (i) the presence of nonidentical subunits in the enzyme, resulting in independent sites with different affinities or different rates of catalysis, (ii) the presence of more than one form of the $NADP^+$-specific malic enzyme, i.e., isozymes, and (iii) the possibility of ligand-induced association–dissociation, resulting in different molecular weight forms of enzyme with different affinities or different rates of catalysis. Results from this laboratory as well as the recent studies by Iwakura et al. (1979b) indicate that the enzyme is composed of six subunits of identical size, but the complete identity has not yet been established. No evidence is available to indicate that more than one form of malic enzyme exists, disc gel electrophoresis revealing only one protein band when stained for protein or activity. While sedimentation equilibrium studies indicate no protein concentration dependent association (D. A. Brown and R. A. Cook, unpublished experiments), the effect of the various ligands on the molecular weight of the enzyme has yet to be examined to rule out a possible ligand-induced association–dissociation mechanism.

In conclusion, our results indicate that Mg^{2+} and Mn^{2+} may stabilize two distinct conformational states of the $NADP^+$-specific malic enzyme which differ in response to varying substrate and effector concentrations. In view of the complex malate-saturation curves observed, we have suggested a model for the enzyme involving sequential ligand-induced changes in enzyme conformation, resulting in mixed positive and negative cooperative behavior. A similar explanation has been suggested for the binding of Mn^{2+} and malate to the $NADP^+$-specific malic enzyme of pigeon liver (Hsu & Lardy, 1967; Hsu et al., 1976; Hsu & Pry, 1980; Schimerlik et al., 1977). Although the experimental data obtained in the present study are similar to those reported with the pigeon enzyme, it is hazardous to propose a model for the enzyme based solely on steady-state kinetic data. Whether the altered affinity or catalysis of individual sites is due to cooperativity between subunits or ligand-induced association–dissociation phenomena requires detailed physical studies and equilibrium binding studies with the homogeneous enzyme. By utilization of the purification procedure described herein, the means of obtaining sufficient enzyme is now available to undertake such studies.

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Regulation of Bovine Kidney α-Ketoglutarate Dehydrogenase Complex by Calcium Ion and Adenine Nucleotides. Effects on $S_{0.5}$ for α-Ketoglutarate†

V. Bryan Lawlis and Thomas E. Roche*

ABSTRACT: Regulation of bovine kidney α-ketoglutarate dehydrogenase complex by energy-linked metabolites was investigated. Ca^{2+}, ADP, or inorganic phosphate markedly enhanced the activity of the complex, and ATP or, to a lesser extent, GTP decreased the activity of the complex. Initial velocity studies with α-ketoglutarate as the varied substrate demonstrated that these modulators induced large changes in $S_{0.5}$ for α-ketoglutarate (based on analysis in Hill plots) with no change in the maximum velocity (as determined by double-reciprocal plots). For all conditions studied, the Hill coefficients were significantly less than 1.0 with slopes that were linear over wide ranges of α-ketoglutarate concentrations, indicating negative cooperativity that probably resulted from multiple site–site interactions. Ca^{2+} (maintained at 10 μM by a Ca^{2+} buffer) decreased the $S_{0.5}$ for α-ketoglutarate 63-fold (from 25 to 0.40 mM); even in the presence of a positive effector, ADP or phosphate, Ca^{2+} decreased the $S_{0.5}$ for α-ketoglutarate 7.8- or 28-fold, respectively. Consistent with a mechanism of action independent of Ca^{2+}, ADP (1.60 mM) or phosphate (20 mM) reduced the $S_{0.5}$ for α-ketoglutarate in the presence of Ca^{2+} (i.e., 4.5- or 1.67-fold, respectively); however, these effectors elicited larger decreases in $S_{0.5}$ in the absence of Ca^{2+} (i.e., 37- or 3.7-fold, respectively). ATP (1.6 mM) increased the $S_{0.5}$ for α-ketoglutarate, and Ca^{2+} appreciably reduced the effect, lowering the $S_{0.5}$ 98-fold from 66 to 0.67 mM. Thus the activity of the kidney α-ketoglutarate dehydrogenase complex is poised to increase as the energy potential in mitochondria declines, and Ca^{2+} has a pronounced modulatory effect. Comparative studies on bovine heart α-ketoglutarate dehydrogenase complex and the effects of varying the ADP/ATP ratio in the presence or absence of Ca^{2+} or phosphate are also described.

In aerobic cells, the citric acid cycle serves a major catabolic energy-generating function, and the net flux through the cycle must respond to the energy needs of the cell. At the same time, the levels of intermediates of the cycle must be controlled independently of cycle flux for utilization in biosynthetic processes and also in the integrated shuttling of anions in and out of mitochondria. Thus an intricate system of regulation that reflects the phosphate potential and responds to other requirements of this amphibolic pathway would be anticipated. α-Ketoglutarate is a branch point metabolite that is generated as an intermediate in the citric acid cycle during the oxidation of carbohydrates and fatty acids and by glutamate dehydrogenase during the oxidative deamination of amino acids. In addition, α-ketoglutarate is produced by transamination of glutamate as part of the malate–aspartate shuttle which functions to transfer reducing equivalents from the cytoplasm into mitochondria. The flux through the α-ketoglutarate dehydrogenase complex would be expected to be tightly coupled to the energy state of mitochondria and to reflect the generation of α-ketoglutarate from glutamate. Thus, modulation of the α-ketoglutarate dehydrogenase complex in a manner independent of the preceding reactions in the citric acid cycle is anticipated.

Previous studies (Garland, 1964; Smith et al., 1974) have suggested that the α-ketoglutarate dehydrogenase complex from mammalian tissues is regulated by product inhibition and therefore by the NAD^+/NADH and CoA/succinyl-CoA ratios. Inhibition by GTP has also been suggested (Olson & Allgyer, 1973). Recently, McCormack & Denton (1979) have presented evidence that porcine heart α-ketoglutarate dehydrogenase complex is regulated by the level of Ca^{2+} and adenine nucleotides. They observed that ADP and micromolar levels of Ca^{2+} lowered the K_m for α-ketoglutarate and that ATP increased the K_m for α-ketoglutarate. Because Ca^{2+} serves unique roles in muscle tissue, we initiated studies to test whether similar effects were observable with the bovine kidney complex. We found similar effects with some qualitative differences. In addition, we have extended the studies of

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