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KINETIC STUDIES OF ACETYL COENZYME A ACTIVATED PHOSPHOENOLPYRUVATE CARBOXYLASE; REVERSE EFFECTS WITH A FATTY ACID

R. SILVERSTEIN

Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kan. 66103 (U.S.A.)

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SUMMARY

1. Phosphoenol α-ketobutyrate was discovered to be a highly effective inhibitor of phosphoenolpyruvate (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31) (PEP) carboxylase of *Escherichia coli* for the acetyl coenzyme A activated reaction. Inhibition was overcome by high PEP concentration.

2. Activation by fructose 1,6-diphosphate was found at least additive to that produced by laurate or acetyl coenzyme A. The effect of laurate, in contrast, was found dependent on acetyl coenzyme A concentration, being reversed from activation to inhibition as acetyl coenzyme A concentration was increased.

3. Ca^{2+} inhibited PEP carboxylase action, the acetyl coenzyme A activated reaction being inhibited to a greater degree. High Mg^{2+} concentration overcame the inhibition. Lineweaver–Burk plots suggested that acetyl coenzyme A and Mg^{2+} comprise a K activation system.

INTRODUCTION

Phosphoenolpyruvate (PEP) carboxylase (orthophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31) in Enterobacteriaceae is activated by several metabolites including, among others, acetyl coenzyme A[1], fructose 1,6-diphosphate[2], cytidine 5′-diphosphate[3], laurate, oleate, and their coenzyme A derivatives[4]. These activation effects as well as potent inhibition by aspartate[5–7] are consistent with a function of fine metabolic control of oxaloacetate concentration[8,9]. The enzyme is found in several higher plants[10–13], as well as bacteria, but is absent in animals. *Escherichia coli* mutants lacking the enzyme do not grow on glucose or other PEP precursors as carbon source[14]. PEP carboxylase, PEP carboxykinase (GTP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32), and PEP carboxy-

Abbreviation: PEP, phosphoenolpyruvate.

transphosphorylase (pyrophosphate:oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.38) catalyze the conversion of PEP to oxaloacetate, and differ in phosphate group acceptor. Each appears to involve bivalent metal bridging of PEP to the enzyme[15–17], with carboxyl addition occuring from the *si* side of the enzyme-bound PEP plane[18].

Kinetic and binding studies with PEP carboxykinase led to the formulation of a catalytic mechanism for that enzyme, wherein PEP and bivalent metal (Mn^{2+}) binding is mutually random, with each needing to bind before either bicarbonate or phosphate group acceptor (IDP) for catalysis to occur[19].

Phosphoenol α-ketobutyrate and Ca^{2+} are now reported as inhibitors of PEP carboxylase and the effects related to acetyl coenzyme A activation of the reaction. Also, the relationship of acetyl coenzyme A activation to that by fructose 1,6-diphosphate and laurate was investigated.

MATERIALS AND METHODS

Fructose 1,6-diphosphate, lauric acid, malonyl coenzyme A, α-ketobutyric acid (sodium salt), protamine sulfate (Grade I), and Tris (Trizma base) were purchased from Sigma Chemical Company. Coenzyme A, malate dehydrogenase (L-malate: NAD^+ oxidoreductase, EC 1.1.1.37), and PEP (monopotassium salt) were purchased from Boehringer-Mannheim, and sodium D-pantothenate from Mann Laboratories. L-Aspartic acid was purchased from Eastman Organic Chemicals. *E. coli* cells, strain W, late log phase, were obtained as a paste from General Biochemicals, with reproducibility in PEP carboxylase content. All other chemicals were obtained from the usual chemical reagent sources.

Acetyl coenzyme A was prepared and assayed as described by STADTMAN[20]. *N,S*-Diacetylcysteamine was prepared according to GERSTEIN AND JENCKS[21]. The compound showed strong infrared absorption bands at 1688 cm^{-1} and 1650 cm^{-1}. PEP and phosphoenol α-ketobutyrate, monocyclohexylammonium salts, were prepared from their parent α-keto acids by the Perkow reaction, as modified by CLARK AND KIRBY[22] and WOODS *et al.*[23]. Recrystallized PEP (monocyclohexylammonium) and commercial PEP (monopotassium) gave equivalent substrate profile curves. The monocyclohexylammonium salt was the form of PEP used in this study, except as otherwise indicated.

The phosphoenol α-ketobutyrate of BONDINELL AND SPRINSON[24] was 80% *cis* and 20% *trans*. Relative proportions of *cis–trans* isomers in our phosphoenol α-ketobutyrate were not determined. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn.

Purification of PEP carboxylase from *E. coli* was conducted through sonic cell rupture, centrifugation, and protamine sulfate precipitation according to CANOVAS AND KORNBERG[25]. This was followed by 50–55% $(NH_4)_2SO_4$ fractionation at 0° to give 6–8-fold purified enzyme with 30–40% recovery. 1.5 ml of the concentrated $(NH_4)_2SO_4$ extract was placed on a 2.5 cm × 19.5 cm Sephadex G-200 column equilibrated and eluted with 0.2 M Tris–HCl, pH 7.5, flow rate 10–12 ml/h. The eluted enzyme was 30–40 fold purified (compared with sonicate) with overall recovery 15–25% with a final specific activity of 0.9–1.1 $\mu moles \cdot ml^{-1} \cdot min^{-1}$ per mg of protein. It was found free of contaminating activities when assayed for NADH oxidase, PEP car-

boxykinase, PEP carboxytransphorphorylase, malate dehydrogenase, and lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27).

Protein was assayed according to LOWRY *et al.*[26]. PEP carboxylase activity was assayed in a Beckman DU recording spectrophotometer equipped with Gilford automatic sample changer. Temperature was maintained at 30 ± 0.02° with a circulating water bath. In the assay mixture of 1 ml was contained: 100 μmoles Tris–HCl, pH 8.5, 10 μmoles $MgCl_2$, 10 μmoles $KHCO_3$, 10 μmoles PEP, 0.45 μmole acetyl coenzyme A, 0.1–0.15 μmole NADH and excess malate dehydrogenase. Reaction was initiated by the addition of PEP carboxylase, and NADH disappearance monitored at 340 nm. Control cuvettes lacked PEP.

For each velocity measurement in this study, the coupled reaction was shown not rate limiting by repeating the determination at one-fifth (3 units) the activity of malate dehydrogenase with equivalent results. Reaction velocities remained linear for 10–15 min provided of course NADH was not yet depleted.

RESULTS

Phosphoenol α-ketobutyrate, a methyl-substituted analog of PEP, was found to inhibit PEP carboxylase, being particularly potent in the presence of acetyl coenzyme A. This is evident from experiments with PEP in much higher concentration than that of its analog. At 0.5 mM and 1 mM phosphoenol α-ketobutyrate, acetyl coenzyme A activated reactions were markedly inhibited, even though the PEP concentration range was 5–40 mM. This is in contrast to the relatively weak inhibition of those reactions in which acetyl coenzyme A was excluded (Table I, Exp. 1). Aspartate inhibition data is also included for comparison purposes. Here, inhibition was more pronounced in the absence of acetyl coenzyme A (Table I, Expt. 2). Lineweaver–Burk plots of the phosphoenol α-ketobutyrate inhibition data indicate that inhibition is overcome by high PEP (Fig. 1).

Activation by either laurate or fructose 1,6-diphosphate is similar to that by acetyl coenzyme A insofar as PEP carboxylase is rendered much more susceptible in each instance to phosphoenol α-ketobutyrate inhibition. At concentrations equimolar

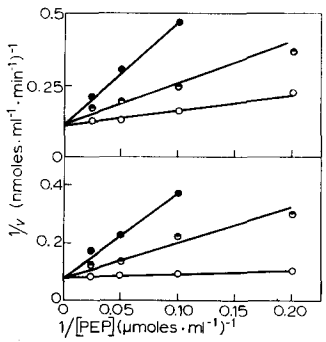

Fig. 1. Double reciprocal plots of the initial velocity of PEP carboxylase catalysis as a function of PEP concentrations at different phosphoenol α-ketobutyrate levels. Top: 0.027 mM acetyl coenzyme A; ○, ◓, and ● correspond to 0, 0.5, and 1.0 mM phosphoenol α-ketobutyrate, respectively. Bottom: 0.135 mM acetyl coenzyme A; ○, ◓, and ● as above. Reactions are those in Table I.

TABLE I

PHOSPHOENOL α-KETOBUTYRATE INHIBITION AT VARIED PEP AND ACETYLCOENZYME A CONCENTRATIONS. COMPARISON WITH ASPARTATE INHIBITION

Reaction conditions, except as indicated, were those of the PEP carboxylase assay, see MATERIALS AND METHODS.

Expt. 1		*Velocity (nmoles·ml^{-1}·min^{-1})*		
PEP (mM)	*Phosphoenol α-ketobutyrate (mM)*	*No acetyl-CoA*	*0.027 mM acetyl-CoA*	*0.135 mM acetyl-CoA*
5	—	1.2	4.5	9.6
10	—	2.4	6.4	11.2
20	—	4.5	7.9	12.5
40	—	5.4	8.1	11.6
5	0.5	1.0	2.8	3.3
10	0.5	2.2	4.1	4.7
20	0.5	3.7	5.3	7.2
40	0.5	4.9	6.0	7.5
5	1.0	1.0	1.5	2.1
10	1.0	1.6	2.2	2.7
20	1.0	2.3	3.3	4.6
40	1.0	4.0	4.9	6.0
Expt. 2		*Velocity (nmoles·ml^{-1}·min^{-1})*		
PEP (mM)	*Aspartate (mM)*	*No acetyl-CoA*	*0.030 mM acetyl-CoA*	*0.150 mM acetyl-CoA*
3	—	0.6	5.8	12.7
5	—	1.6	9.2	15.2
10	—	4.0	11.6	17.3
20	—	7.1	13.9	17.1
3	1.0	0.0	2.1	8.1
5	1.0	0.4	4.3	12.1
10	1.0	1.6	7.2	13.5
20	1.0	4.9	11.1	15.8

with PEP, phosphoenol α-ketobutyrate is seen to effect about a 10-fold drop in rate for each activated reaction, while the PEP carboxylase activity of those reaction mixtures lacking one of the above three activators are little affected under the same conditions (Table II).

Activation by acetyl coenzyme A, fructose 1,6-diphosphate and laurate were then examined separately and in experiments where more than one activator was added to a given reaction mixture. The reactions were conducted at pH 7.5, such that catalysis in the absence of activator was relatively insignificant. Where acetyl coenzyme A and fructose 1,6-diphosphate were both present, velocity was at least as great as the sum of the rates recorded from separate reaction mixtures of each activator. Similar results were obtained from reaction mixtures containing laurate and fructose 1,6-diphosphate. Yet, for reaction mixtures containing both acetyl coenzyme A and laurate the resulting velocity was even less than that with laurate omitted (Table III). The reversal of laurate from activator to inhibitor as acetyl coenzyme A concentration is increased is further demonstrated in Fig. 2.

TABLE II

EFFECT OF ACETYL COENZYME A, FRUCTOSE 1,6-DIPHOSPHATE AND LAURATE ACTIVATORS ON PHOSPHOENOL α-KETOBUTYRATE INHIBITION AT FIXED PEP

Reaction conditions, except as indicated, were those of the PEP carboxylase assay, see MATERIALS AND METHODS.

PEP (mM) (cyclohexyl-amine salt)	*Phosphoenol α-ketobutyrate (mM)*	*Velocity (nmoles·ml^{-1}·min^{-1})*			
		No activator	*0.45 mM acetyl-CoA*	*10 mM Fru-1,6-P_2*	*0.20 mM laurate*
5	—	3.6	38.7	14.1	15.5
5	1	5.2	18.3	7.7	11.8
5	2.5	4.5	11.3	4.7	7.3
5	5	3.4	4.0	1.2	2.1
5	7.5	2.1	2.4		
5	10.0	1.7	1.9		
Controls:					
5 mM PEP (cyclohexylamine salt), 1 mM cyclohexylamine		4.1	39.4		
5 mM PEP (cyclohexylamine salt), 10 mM cyclohexylamine		5.5	38.2	15.6	18.5
5 mM PEP (potassium salt)		3.4	42.2	13.8	16.7
No PEP		0.0	0.0	0.0	0.0

TABLE III

ACTIVATION STUDIES WITH ACETYL COENZYME A, FRUCTOSE 1,6-DIPHOSPHATE AND LAURATE, SEPARATELY AND IN COMBINATION

Reaction conditions: 100 mM Tris–HCl, pH 7.5, 10 mM $KHCO_3$, 5 mM PEP, 10 mM $MgCl_2$, PEP carboxylase, coupled assay reagents as in MATERIALS AND METHODS, temp. 30°.

Acetyl-CoA (mM)	*Fru-1,6-P_2 (mM)*	*Laurate (mM)*	*Velocity (nmoles·ml^{-1}·min^{-1})*	
			Exp.	*Calcd.**
—	—	—	0.3	
0.15	—	—	14.1	
0.45	—	—	18.1	
—	10	—	4.8	
—	20	—	5.5	
—	—	0.2	4.8	
—	—	0.4	6.1	
0.15	10	—	21.3	18.9
0.15	—	0.2	10.0	18.9
0.15	—	0.4	9.2	20.2
—	10	0.2	12.3	9.6
0.15	10	0.2	15.1	23.7

* Sum of the velocities for the corresponding individually activated reactions.

Studies with malonyl coenzyme A were conducted. It is seen that malonyl coenzyme A exerts little catalytic effect, compared to that observed with acetyl coenzyme A. Moreover, no interference with acetyl coenzyme A activation is apparent (Table IV). Similar experiments were conducted with compounds analogous to parts of the coenzyme A structure, namely sodium D-pantothenate, adenosine 3′-phosphate, adenosine 5′-phosphate, and *N*,*S*-diacetylcysteamine, which contains the thioester

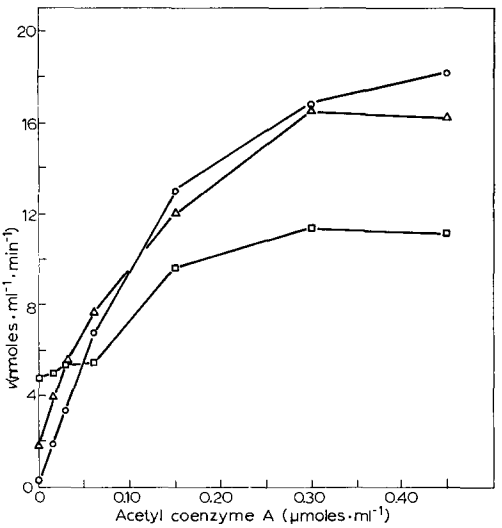

Fig. 2. Initial rates of PEP carboxylase catalysis as a function of acetyl coenzyme A concentration at fixed levels of laurate. Reaction conditions: 100 mM Tris–HCl, pH 7.5, 10 mM $KHCO_3$, 5 mM PEP, 10 mM $MgCl_2$, acetyl coenzyme A as shown, PEP carboxylase, coupled assay reagent as in MATERIALS AND METHODS, temp. 30°. ○, no laurate; △, 0.05 mM laurate; □, 0.20 mM laurate.

TABLE IV

TEST FOR MALONYL COENZYME A INTERACTION AT ACETYL COENZYME A BINDINGS SITE(S)

Reaction conditions, except as indicated, were those of the PEP carboxylase assay, see MATERIALS AND METHODS.

Acetyl-CoA (mM)*	*Malonyl-CoA** (mM)*	*Velocity (nmoles·ml⁻¹·min⁻¹)*
—	—	3.7
0.03	—	11.3
0.15	—	21.0
—	0.03	4.2
—	0.15	5.8
0.03	0.03	12.6
0.15	0.15	21.7

* The K_m for acetyl coenzyme A at pH 8.5 is 0.14 mM[25].

** Malonyl coenzyme A concentration, determined by weight, was confirmed by hydroxamate assay[20].

function. Each compound, and the several combinations tried, neither activated nor interfered with acetyl coenzyme A activation. Analog concentrations were 10 mM as compared with 0.05 mM for acetyl coenzyme A.

The relationship of acetyl coenzyme A activation to the catalytic requirement for a bivalent metal was probed by examining effects of Ca^{2+} on the reaction. Inasmuch as there is strong evidence indicating a bivalent metal bridged intermediate for this reaction[15], it was expected that Ca^{2+} would inhibit rather than substitute for Mg^{2+} during catalysis[16,27]. Ca^{2+} inhibition was confirmed, and examined in the presence and absence of acetyl coenzyme A, and at several concentrations of $MgCl_2$. The degree of inhibition was observed to be greater in the presence of acetyl coenzyme A (Table

TABLE V

Ca^{2+} INHIBITION AT VARIED Mg^{2+} AND ACETYL COENZYME A CONCENTRATIONS

Reaction conditions, except as indicated, were those of the PEP carboxylase assay, see MATERIALS AND METHODS.

$MgCl_2$ (mM)	*$CaCl_2$ (mM)*	*Velocity (nmoles·ml^{-1}·min^{-1})*		
		No acetyl-CoA	*0.030 mM acetyl-CoA*	*0.150 mM acetyl-CoA*
0.4	—	0.7	3.5	8.2
1.0	—	2.3	7.3	13.9
2.0	—	3.6	10.6	16.6
4.0	—	6.0	13.8	21.3
0.4	0.4	1.1	2.3	3.1
1.0	0.4	2.3	4.4	5.5
2.0	0.4	4.2	7.3	9.9
4.0	0.4	5.9	12.0	14.8
0.4	1.0	0.9	1.5	1.9
1.0	1.0	1.8	2.9	3.5
2.0	1.0	3.2	5.2	5.8
4.0	1.0	5.0	8.6	10.2

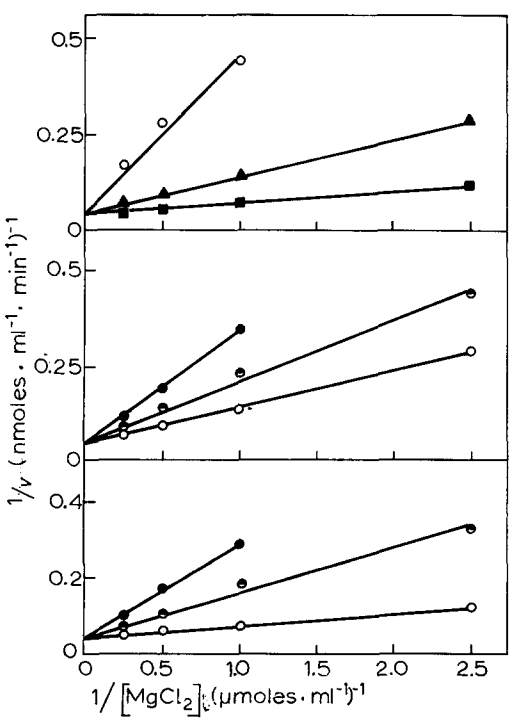

Fig. 3. Double reciprocal plots of the initial velocity of PEP carboxylase catalysis as a function of $MgCl_2$ concentration at different acetyl coenzyme A activator levels (top) or at different $CaCl_2$ inhibitor levels (middle, bottom). Top: ○, ▲, and ■ correspond to 0, 0.030 and 0.150 mM acetyl coenzyme A, respectively. Middle: 0.030 mM acetyl coenzyme A; ○, ◓, and ● correspond to 0, 0.4 and 1.0 mM $CaCl_2$, respectively. Bottom: 0.150 mM acetyl coenzyme A; ○, ◓, and ● as above. Reactions are those of Table V.

V). Lineweaver–Burk plots indicate that inhibition is overcome by high $MgCl_2$ at each of the two acetyl coenzyme A levels studied (Fig. 3, middle and bottom). Moreover, high $MgCl_2$ is seen to diminish the activating effects of acetyl coenzyme A. No activation by acetyl coenzyme A is apparent upon extrapolation to infinite $MgCl_2$ concentration (Fig. 3, top). Acetyl coenzyme A and Mg^{2+} therefore appear to constitute a K system of activation, according to the terminology of MONOD *et al.*[28].

DISCUSSION

Phosphoenol α-ketobutyrate might be anticipated as a particularly selective inhibitor of enzymes that act on PEP, being similar to it in structure. WOODS *et al.*[23], in reporting its chemical synthesis, showed inhibition of pyruvate kinase that was competitive with PEP. BONDINELL AND SPRINSON[24] demonstrated phosphoenol α-ketobutyrate to be also a substrate for pyruvate kinase, with conversion to α-ketobutyrate. The properties of phosphoenol α-ketobutyrate toward a PEP carboxylation enzyme, however, have not previously been reported.

The location of the methyl group in the structure might conceivably result in either more or less potent inhibition depending on the binding sites for both PEP and the particular carboxylating species, be it bicarbonate or CO_2. The potent inhibition now reported for phosphoenol α-ketobutyrate toward PEP carboxylase when activated by acetyl coenzyme A, fructose 1,6-diphosphate, or laurate suggests positive binding interactions of the inhibitor at both PEP and carboxylating species sites (Table II). Phosphoenol α-ketobutyrate was also found a comparably potent inhibitor of PEP carboxykinase, partially purified from pig liver cytosol (M. SAMMER AND R. SILVERSTEIN, unpublished). A comparison of K_m/K_i for phosphoenol α-ketobutyrate inhibition of pyruvate kinase and of PEP carboxylase, based on Lineweaver–Burk reciprocal plots of $1/v$ *vs.* $1/[PEP]$, gave a ratio of approx. 1 for pyruvate kinase[23], and of 22 and 30 for PEP carboxylase, when the enzyme was activated by 0.027 mM and 0.135 mM acetyl coenzyme A, respectively (Fig. 1). Should inhibition of PEP carboxylase arise, indeed, from binding partially at the carboxylating species site, then it may be further suggested from data presented in Table II that activation of PEP carboxylase by acetyl coenzyme A, fructose 1,6-diphosphate, or laurate each relates to carboxylation aspects of the catalytic reaction. The possibility that a methyl group may occupy part of a carbon dioxide binding site has been put forward previously by NOWAK AND MILDVAN[29] with regard to the preferential inhibition of PEP carboxykinase by the L isomer of phospholactate. A hydrophobic environment for PEP carboxykinase, in this regard, is in accord with radiochemical and spectrophotometric studies which support CO_2 rather than bicarbonate as the carboxylating agent for the reaction that enzyme catalyzes[30]. Exchange studies with [^{18}O]bicarbonate, however, support bicarbonate as the carboxylating species for PEP carboxylase[11], albeit decomposition to CO_2 and OH^- on the enzyme is possible[30]. In that event the binding site would be expected to have some hydrophobic character to accommodate the non-polar CO_2.

Inasmuch as activation by fructose 1,6-diphosphate, laurate, or acetyl coenzyme A in each instance induced increased susceptibility of PEP carboxylase to inhibition by phosphoenol α-ketobutyrate (see above), the possibility was considered that these activators might, in essence, bring about fundamentally equivalent changes

insofar as the catalytic reaction is concerned, and that one activator might therefore substitute for another. Accordingly, studies were conducted where more than one of the three activators were present in the same reaction mixture (Table III). With fructose 1,6-diphosphate and laurate both present, the resulting velocity was approximately the sum of the velocities for the individually activated reactions. The same held true for reactions containing both fructose 1,6-diphosphate and acetyl coenzyme A. Doubling or tripling the activator concentration individually produced only slight rate increases at these levels. It would therefore appear that fructose 1,6-diphosphate occupies a binding site distinct from that of acetyl coenzyme A or laurate, and that its ability to facilitate catalysis under the conditions of these experiments complements rather than substitutes for effects produced by acetyl coenzyme A or laurate. A possibility consistent with both phosphoenol α-ketobutyrate inhibition and the complementary nature of fructose 1,6-diphosphate/laurate and fructose 1,6-diphosphate/acetyl coenzyme A activation is that activating effects here are directed toward the carboxylation transformation with orientation of the groups participating being improved in kinetically distinct and complementary respects by fructose 1,6-diphosphate and either of the other two above activators.

It is likely that there is overlap in effects produced by acetyl coenzyme A and laurate, however. Where acetyl coenzyme A and laurate were both present in the same reaction mixture, not only were the effects on reaction velocity less than additive (Table III), but as acetyl coenzyme A concentration was increased at fixed laurate, reaction velocity became even less than that observed for the corresponding reaction mixtures from which laurate was excluded (Fig. 2). Hence, the effect of laurate was reversed from overall activation to inhibition with rise in acetyl coenzyme A concentration.

For *E. coli*, there conceivably exist critically interrelated concentrations of acetyl coenzyme A, laurate, and presumably other fatty acids which together affect some control on the direction of flow of fatty acids metabolism by modulating PEP carboxylase activity. If so, a previous suggestion that high levels of fatty acids channel metabolic flow of PEP toward oxaloacetate[4] might then prove true only up to a given concentration of acetyl coenzyme A. Any further buildup in concentration could result in PEP carboxylase still being activated by acetyl coenzyme A, but the effect of fatty acids now reversed. Such reversal might perhaps be beneficial in maintaining proper balance between fatty acid oxidation and elongation, so that certain fatty acids do not accumulate.

The participation of Mg^{2+} in PEP carboxylase action may be considered as an integral part of the events constituting the catalytic reaction, and also as part of the modulating parameters that affect these events. Bivalent metal regulation of enzyme catalysis, as an extension of the energy-charge hypothesis[31], has been previously considered by STADTMAN[9]. That high Mg^{2+} results in loss of acetyl coenzyme A activation of PEP carboxylase (Fig. 3, top) strongly suggests that Mg^{2+} participates both at catalytic and regulatory sites, and that acetyl coenzyme A and Mg^{2+} may bring about similar changes at the catalytic site. From plots of $1/v$ *vs.* $1/[\text{free } Mg^{2+}]$, SMITH[5] has partitioned the apparent K_m for Mg^{2+} into two K_m values of 0.056 mM and 0.273 mM for free Mg^{2+}. The data here in Fig. 3, top are for 0.4 to 4 mM $MgCl_2$, and correspond with 0.27 mM to 1.55 mM free Mg^{2+}, based on SMITH's[5] constant of $4.76 \cdot 10^{-3}$ M for PEP–Mg^{2+} dissociation.

The more potent inhibition by Ca^{2+} in the presence of acetyl coenzyme A (Table V), when viewed in terms of a dual function for Mg^{2+}, is understandable, assuming inhibition results largely from competition at the catalytic site. In the presence of increasing acetyl coenzyme A concentration, the importance of Mg^{2+} concentration to reaction rate should increasingly reflect Mg^{2+} binding properties at the catalytic rather than regulatory site(s), and hence a greater degree of Ca^{2+} inhibition from blockage at the catalytic site. Were Ca^{2+} affecting the rate largely by inhibiting modulation by Mg^{2+}, then increased acetyl coenzyme A concentration should have been expected to diminish rather than enhance the degree of Ca^{2+} inhibition.

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