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Activation of Yeast Pyruvate Carboxylase: Interactions between Acyl Coenzyme A Compounds, Aspartate, and Substrates of the Reaction†

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ABSTRACT: Chicken liver pyruvate carboxylase has an absolute requirement for short-chain acyl coenzyme A (CoA), whereas the same enzyme from yeast has less stringent requirements. The yeast enzyme has now been studied in an effort to elucidate the mechanism by which acyl-CoA stimulates pyruvate carboxylase activity. Yeast pyruvate carboxylase has an apparent basal level of activity above which CoA and acyl-CoAs of 2–20 carbons activate; the concentration of acyl-CoA required for half-maximum activation ($K_{0.5}$) decreases as the chain length of the acyl moiety increases to 16 carbons. Activation of yeast pyruvate carboxylase by acyl-CoA is brought about in part by increasing the affinity of pyruvate carboxylase for two substrates, bicarbonate and pyruvate. The affinity of pyruvate carboxylase for bicarbonate is also increased by potassium ions. The observation of only low levels of activity in the absence of acyl-CoA or potassium ion leads to the conclusion that the basal activity so frequently referred to is probably due to the presence of activating monovalent cations. Pyruvate carboxylase from yeast probably has an absolute requirement for monovalent cations or acyl-CoA with a combination of the two being required for optimum conditions for maximal activity. Stimulation by acyl-CoA and inhibition by aspartate are mutually antagonistic with each affecting the activation or inhibition constant and the degree of cooperativity brought about by the other. The enzyme from liver is unaffected by aspartate.

Pyruvate carboxylase [pyruvate:CO_2 ligase (ADP), EC 6.4.1.1], first isolated from avian liver by Utter & Keech (1963), catalyzes the direct carboxylation of pyruvate to oxalacetate according to the following reaction:

$$\text{pyruvate} + MgATP^{2-} + HCO_3^- \xrightarrow[\text{(acyl-CoA)}]{M^+,\ M^{2+}} \text{oxalacetate} + MgADP^- + P_i^{2-}$$

This enzyme has an anaplerotic function, replacing TCA cycle intermediates, as well as being implicated in glycerogenesis in addition to gluconeogenesis from three-carbon compounds. Several pyruvate carboxylases from different sources have been described and their properties reviewed by Utter et al. (1975). Their activities are not affected in the same way by acyl-CoA[1] compounds. The avian liver enzyme, as well as that from *Bacillus licheniformis*, absolutely requires acetyl-CoA (Utter & Keech, 1963; Renner & Bernlohr, 1972); the enzymes from photosynthetic bacteria (Fuller et al., 1961), *Arthrobacter globiformis* (Gurr & Jones, 1977), a thermophilic bacillus (Libor et al., 1978), rat liver (Henning & Seubert, 1964) and bakers' yeast (Ruiź-Amil et al., 1965; Cooper & Benedict, 1966) are activated by acetyl-CoA; the enzymes from *Aspergillus niger* (Bloom & Johnson, 1962) and *Pseudomonas citronellolis* (Seubert & Remberger, 1961) are not affected by acetyl-CoA. Except for the inducible pyruvate carboxylases from *P. citronellolis* and *Azotobacter vinelandii* (Scrutton & Taylor, 1974), all these enzymes have been characterized as large proteins of M_r ~500 000 and are composed of four apparently identical polypeptide chains. Electron microscopy has revealed that the quaternary structure is that of a rhombus (Cohen et al., 1979; Valentine, 1968). The enzymes that have been studied in detail show a striking degree of resemblance with respect to the properties of the catalytic site such as metal ion specificity, nucleotide specificity, presence of biotinyl residues, and, in most instances, apparent K_m values for all three substrates. Further, although modification of the nucleotide portion of the acyl-CoA molecule causes loss of the allosteric activation potential in all cases so far examined, the enzymes exhibit unique patterns of specificity for activation by acyl analogues of acetyl-CoA (Fung, 1972; Tolbert, 1970; Scrutton, 1974).

Phosphoenolpyruvate carboxylases from *Salmonella typhimurium* (Theodore & Englesberg, 1964) and *Escherichia coli* (Amarasingham, 1959; Ashworth et al., 1965) have a function equivalent to that of pyruvate carboxylase in the synthesis of oxalacetate and are also activated by acetyl-CoA (Cañovas & Kornberg, 1965; Maeba & Sanwal, 1965). The inhibition of phosphoenolpyruvate carboxylase from *Salmonella* by L-aspartate has been described by Maeba & Sanwal (1965). Inhibition of yeast pyruvate carboxylase by L-aspartate has been shown by Palaciañ et al. (1966) and Cazzulo & Stoppani (1968). The present paper describes some additional aspects of the interaction of yeast pyruvate carboxylase with its acyl-CoA activators (acetyl- and palmitoyl-CoA), substrates of the reaction, and the inhibitor, aspartate. In particular, the results indicate that the activation of palmitoyl-CoA follows

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[1] Abbreviations: CoA, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TCA, tricarboxylic acid cycle; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.

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the same patterns as does the response elicited by acetyl-CoA and cannot be due to any nonspecific, detergent-like effect of the long-chain acyl moiety.

Experimental Procedures

Materials

Coenzyme A (CoASH) (ChromatoPure, Li salt) was obtained from P-L Biochemicals, Inc. Acetyl-CoA, propionyl-CoA, *n*-butyryl-CoA, hexanoyl-CoA, and palmitoleoyl-CoA were prepared as described by Fung (1972) by using CoASH and the corresponding anhydride or acyl chloride. The CoA thio esters were purified by anion-exchange chromatography on DEAE-Sephadex A-50 by using a linear 0.12–0.24 M LiCl gradient. After concentration, they were desalted on a Sephadex G-10 column. All other acyl-CoAs were purchased from Sigma Chemical Co. or P-L Biochemicals, Inc., and were 75–90% pure.

The concentration of acetyl-CoA was determined with citrate synthase and DTNB (Srere, 1969). Concentrations of other acyl-CoA compounds were determined on diluted samples by using the absorbance at 260 nm (E = 15.4 $mM^{-1}\cdot cm^{-1}$) and by measuring the release of inorganic phosphate following treatment with 3′-nucleotidase (Fiske & Subbarow, 1952).

Methods

Enzyme Purification. The basic procedures used for growing cells of bakers' yeast (*Saccharomyces cerevisiae*, Harden & Young strain) and for purifying pyruvate carboxylase were described by Young et al. (1969) although certain modifications in the purification procedure have been introduced. The current scheme differs from that described earlier in that chromatographic steps utilizing Sephadex G-25 and cellulose phosphate, which formerly preceded chromatography on Sagavac 8F, have now been omitted. It has been found that better yields of enzyme of comparable specific activity (15–20 units/mg of protein) can be obtained with the omission of these steps. Material with the highest specific activity was pooled, precipitated with ammonium sulfate, and frozen in aliquots in Tris–sucrose buffer at −70 °C.

Electrophoretic analysis of the purified enzyme on Na-DodSO_4–polyacrylamide gels revealed only one major band with contaminants amounting to less than 15%.

Enzyme Assay. Pyruvate carboxylase was assayed spectrophotometrically in the direction of oxalacetate formation as described by Young et al. (1969). In the earlier studies, little attention was paid to the anion, e.g., sulfate, or its concentration present in the assay system. Subsequently, sulfate and the bovine serum albumin (BSA) used to dilute the malate dehydrogenase in the coupled assay were identified as factors responsible for variability in the observed $K_{0.5}$ for acyl-CoA activation (Scrutton & Fung, 1972; Tolbert, 1970). In the current assay conditions, BSA was omitted as diluent and chloride was used as the buffer anion. Differences in $K_{0.5}$ values may be attributed to differences in the assay conditions.

Protein was determined in most cases by the biuret method with BSA as a standard. In some instances the spectrophotometric method of Warburg & Christian (1941) was used. This method yields lower values and requires a correction factor of approximately 1.4 (Barden et al., 1975).

Results

Activation by Acyl-CoA Compounds. The range of CoA compounds, capable of activating pyruvate carboxylase from yeast, is very broad with respect to the acyl moiety. In contrast, a limited range of activators is effective for pyruvate carboxylase isolated from animal sources (Utter et al., 1975).

Table I: Activators of Pyruvate Carboxylase from Yeast[a]

compound	apparent V_{max} (μmol/min)	$K_{0.5}$ (μM)[b]	n_H (Hill coefficient)
CoASH	5.2	33.5	1.25
acetyl-CoA	6.7	6.6	1.4
propionyl-CoA	4.9	3.6	1.25
n-butyryl-CoA	5.1	2.0	1.27
n-hexanoyl-CoA	5.0	0.17	1.5
palmitoyl-CoA	5.2	0.022	2.3
stearoyl-CoA	6.7	0.062	2.07
arachidoyl-CoA	4.2	0.03	2.0
oleoyl-CoA	4.7	0.035	1.11
palmitoleoyl-CoA	4.3	0.044	2.14
benzoyl-CoA	4.5	0.49	1.2
phenylacetyl-CoA	5.2	2.3	1.25

[a] Spectrophotometric assays according to Scrutton & Fung (1972) using 0.1 unit of enzyme. [b] $K_{0.5}$ is the concentration required for half-maximal activation (calculated from Hill plots). V_{max} values were determined from Lineweaver–Burk plots after the basal activity was subtracted.

Table I shows the results of an analysis of various activators of the yeast enzyme. These data are presented in terms of their respective activator constants ($K_{0.5}$) and Hill coefficients (n_H). The spectrophotometric assays were carried out in the presence of chloride, as the buffer anion, and with no potassium ion (K^+) or BSA present in the assay mixture. Concentrations of other assay components were 5 mM pyruvate, 2 mM ATP, and 5 mM HCO_3^-. Previous experiments (Tolbert, 1970) had shown that the $K_{0.5}$ of any individual activator could vary markedly depending on the anion present in the assay buffer. However, the overall pattern of a decrease in $K_{0.5}$ with an increase in chain length of the acyl moiety was not affected by the assay conditions. Long-chain acyl-CoAs are known to bind to BSA (Taketa & Pogell, 1966); when albumin was included in the assay mix as a diluent of malate dehydrogenase enzyme, the $K_{0.5}$ for palmitoyl-CoA was about 5-fold higher than when BSA was omitted from the mix. Previous studies had also suggested that the lower the $KHCO_3$ concentration, the larger the Hill coefficient and the greater the activation ratios of acetyl-CoA (Tolbert, 1970). The apparent $K_{0.5}$ for acetyl-CoA seemed to be relatively independent of bicarbonate concentration. Under the conditions of this assay (5 mM $NaHCO_3$, NaATP, and sodium pyruvate) it can be seen that the degree of positive cooperativity increases to values greater than 2 for the long-chain saturated acyl-CoAs, arachidoyl-, stearoyl-, and palmitoyl-CoA; the monounsaturated derivatives, oleoyl-CoA and palmitoleoyl-CoA, gave values between 1 and 2. The activation constants differ 1000-fold, with reduced CoASH and palmitoyl-CoA being representative of the least and most effective activators, respectively. The relative effectiveness in terms of $K_{0.5}$ may be related to the hydrophobic or steric nature of the acyl moiety. Aromatic CoAs appear to be less effective than their alkyl-CoA counter parts. The finding that palmitoyl-CoA is a very effective activator of yeast pyruvate carboxylase has possible implications for the control of the metabolism of the yeast cell. Lynen and his colleagues have demonstrated that the most frequent end product of fatty acid synthesis in yeast is palmitoyl-CoA (Lynen et al., 1964), and it would appear that the accumulation of this product could cause an increase in the rate of gluconeogenesis if the pyruvate carboxylase reaction were rate limiting.

The general idea of the specificity requirement of the acyl moiety of the CoA activators was of interest, but a more important consideration was the need to assess the nature of the activation by the extremely effective long-chain fatty

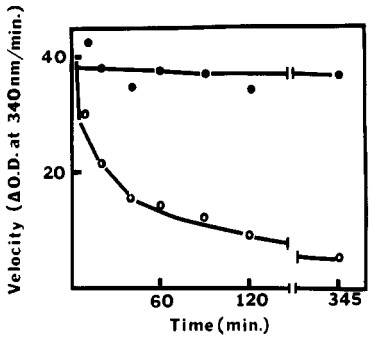

FIGURE 1: Inactivation of yeast pyruvate carboxylase by palmitoyl-CoA. Enzyme was diluted to 1.1 mg/mL in 50 mM Tris-HCl containing 1 mM EDTA, with (○) or without (●) 0.9 mM palmitoyl-CoA. Aliquots were then periodically assayed (Young et al., 1969) with acetyl-CoA instead of palmitoyl-CoA.

acyl-CoAs (for example, palmitoyl-CoA). Palmitoyl-CoA is widely accepted as an inactivator of many enzymes (Taketa & Pogell, 1966; Dorsey & Porter, 1968), and in at least one other case [microsomal glucose-6-phosphatase (Nordlie et al., 1967)] palmitoyl-CoA has been shown to be an activator of its phosphotransferase activity. In most of these instances it is strongly suspected that the mechanism of action is nonspecific and due to the detergent-like action of the acyl moiety. This hypothesis is strengthened by the known tendency for palmitoyl-CoA to form micelles at the concentrations generally effective (3–4 μM).

The possibility was considered that palmitoyl-CoA activation differed from acetyl-CoA activation in the pyruvate carboxylase reaction and, in reality, exerted its effect because of the detergent-like character of the hydrophobic acyl group. One argument against this hypothesis is that activation of pyruvate carboxylase by palmitoyl-CoA is accomplished at concentrations much lower than those required to inactivate other enzymes or activate glucose-6-phosphatase. In the latter cases, concentrations required were often in the range where micellar formation could be expected (10^{-3}–10^{-5} M) while micelle formation would not be expected for concentrations in the range 10^{-7}–10^{-8} M (the $K_{0.5}$ for palmitoyl-CoA, with yeast pyruvate carboxylase). At higher concentrations of palmitoyl-CoA, such as those which inhibit some other enzymes, pyruvate carboxylase was inactivated and dissociated (Figure 1; Tolbert, 1970). The inactivation showed a typical detergent-like time dependency.

Further evidence against a nonspecific mechanism of action by palmitoyl-CoA is provided by the finding that palmitoylpantetheine, which does not differ in its acyl group from palmitoyl-CoA, is an inhibitor instead of an activator of yeast pyruvate carboxylase. Modification of the CoA moiety of the acyl-CoA activator produced either inhibitory or inert compounds. Examples of inhibitory substances are acetyl- or palmitoylpantetheine and acetyl- or palmitoyl-4′-phosphopantetheine; an example of an inert derivative is acyl-3-dephospho-CoA (data not shown). The inhibitors increased the Hill coefficient for both palmitoyl-CoA and acetyl-CoA; it will be seen later that a different type of inhibitor, aspartate, also causes a considerable increase in the Hill coefficient for both of these activators.

When the CoA moiety of palmitoyl-CoA and acetyl-CoA is replaced by pantetheine, the two activators become inhibitors, suggesting that the CoA moiety plays an important role in the acyl activation of the enzyme. This observation suggests that the two acyl-CoAs act in basically the same fashion and that the function of the palmitoyl moiety of palmitoyl-CoA is not exerted through a nonspecific physical effect. The

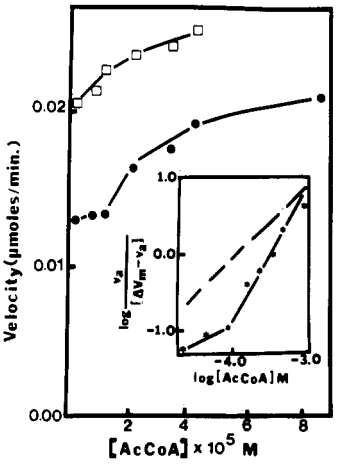

FIGURE 2: Effect of aspartate on the activation of yeast pyruvate carboxylase by acetyl-CoA. (□) No aspartate present; (●) 1 mM aspartate in the assay mix. The break in the line drawn for the aspartate curve was calculated from the point of inflection of the corresponding Hill plot [see insert: (○) no aspartate; (asterisks) 1 mM aspartate].

inhibition effected by palmitoylpantetheine occurred at concentrations very much higher than concentrations required for activation, suggesting again that the CoA moiety is important in both the binding and activating functions of palmitoyl-CoA.

Aspartate Inhibition. Aspartate has been reported to exert an antagonistic effect on the activation of yeast pyruvate carboxylase by acetyl-CoA (Palacián et al., 1966). In addition, the presence of aspartate considerably increased the Hill coefficient for acetyl-CoA, and conversely, acetyl-CoA increased the Hill coefficient for aspartate (Cazzulo & Stoppani, 1968). The effect of 1 mM aspartate on the activation of acetyl-CoA is complex (Figure 2). The lower curve, in which activation by acetyl-CoA in the presence of aspartate is shown, has a distinct inflection point at a concentration which approximates that required for half-activation in the absence of aspartate, i.e., the $K_{0.5}$ value. This inflection point is not seen in the upper curve in which aspartate is absent. The Hill plot (see insert to Figure 2) also shows the inflection point in the curve for activation by acetyl-CoA in the presence of aspartate. The slope of the curve at low concentrations of acetyl-CoA is actually less than that of the control curve (Hill coefficient less than 1 is indicative of negative cooperativity), while the slope at high acetyl-CoA concentrations is considerably higher than that of the control (Hill coefficient approaches 2 and the activation is positively cooperative).

The activation curve for palmitoyl-CoA (and pyruvate carboxylase from yeast), carried out in the presence of 5 mM aspartate (Figure 3), also has a distinct inflection point. As was also true with acetyl-CoA, the inflection point occurred at a concentration of palmitoyl-CoA similar to that required for half-activation of the enzyme in the absence of aspartate (the $K_{0.5}$). Hill plots of the same data also clearly show the presence of an inflection point in the presence of aspartate. At higher palmitoyl-CoA concentrations, the Hill coefficient was 1.84 compared to 1.1 for the control curve, showing a considerable increase in the cooperativity of the interaction between the enzyme and the acyl-CoA activator in the presence of aspartate. Table II summarizes the effect of 5 and 10 mM aspartate on the activating properties of acetyl- and palmitoyl-CoA. The presence of aspartate caused a considerable increase in the activation constants for both activators with an increase of about 80-fold for acetyl-CoA and about 60-fold for palmitoyl-CoA at 10 mM aspartate. The Hill coefficient for each activator was also increased about 1.7-fold in the

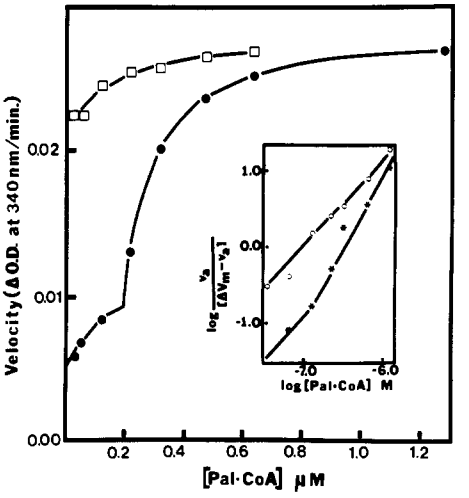

FIGURE 3: Effect of aspartate on the activation of yeast pyruvate carboxylase by palmitoyl-CoA. (□) No aspartate present; (●) 5 mM aspartate in the assay mix. The break in the line drawn for the aspartate curve was calculated as for Figure 2 [see insert of Hill plot: (○) no aspartate; (asterisks) 5 mM aspartate].

Table II: Effect of Aspartate on Activation by Acyl-CoA

aspartate concentration	activator: acetyl-CoA $K_{0.5}$ (μM)	n_H	V_{max}/V_0[a]	palmitoyl-CoA $K_{0.5}$ (μM)	n_H	V_{max}/V_0
no aspartate	10	0.98	1.25	0.012	0.84	1.31
5 mM aspartate	360	1.16	5.23	0.43	1.50	4.42
10 mM aspartate	800	1.70	6.29	0.70	1.43	5.47

[a] Maximal activity in the presence of activator (V_{max}, from extrapolation of a double-reciprocal plot of velocity vs. concentration of activator) divided by activity in the absence of activator (V_0). $K_{0.5}$ and n_H values were calculated from Hill plots run in the presence of 100 mM Tris-SO_4 (as the buffer), 6.7 mM $MgSO_4$, 10 mM potassium pyruvate, 3.3 mM K-ATP, and 40 mM $KHCO_3$.

presence of 10 mM aspartate. The close parallel in the effects of aspartate on the activating features of acetyl- and palmitoyl-CoA furnishes further proof that the general mechanism of action by these two effectors is very similar.

The influence of increasing amounts of aspartate on the activity of the enzyme is shown in the absence of acyl-CoA and in the presence of acetyl- and palmitoyl-CoA (Figure 4) each at a concentration twice its respective $K_{0.5}$. The results show clearly that the inhibition pattern produced by aspartate is similar, when either of the acyl-CoAs is present, to that observed when no acyl-CoA is present (i.e., the "basal activity"). In spite of the generally similar appearance of the three curves, Hill plots of the same data (not shown) gave Hill coefficients of 1.55, 1.32, and 1.22 and apparent K_i values of 2.88, 2.28, and 1.19 mM aspartate for no acyl-CoA, palmitoyl-CoA, and acetyl-CoA, respectively.

The foregoing results clearly demonstrated a high degree of interaction between acyl-CoA activation and aspartate inhibition but also suggest that the mechanism of interaction is a very complex one. The situation is further complicated by the effect of the inhibition on the basal activity of the enzyme. Although there are differences in detail between the interaction of aspartate with acetyl-CoA as compared with palmitoyl-CoA, it would appear that the latter acts by the same general mechanism as does acetyl-CoA.

Interaction of Acyl-CoA and the Substrates of the Reaction. One of the primary purposes of this study was to assess the

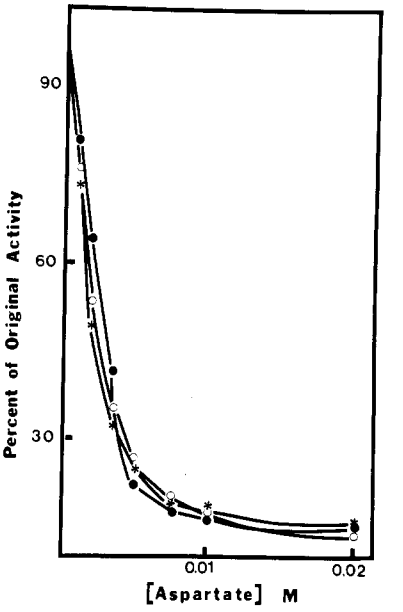

FIGURE 4: Effect of aspartate on the activity of yeast pyruvate carboxylase. (●) No acyl-CoA present; (asterisks) 8.4×10^{-5} M acetyl-CoA or (○) 2×10^{-6} M palmitoyl-CoA present in the assay mix.

mechanism by which acyl-CoA brings about activation of the yeast pyruvate carboxylase enzyme. The effect of acyl-CoA compounds on the apparent Michaelis constants of the substrates of the pyruvate carboxylase reaction indicated that the substrates were influenced differently by the presence of acyl-CoA. For some substrates the apparent maximum velocity was greatly affected while for others the K_m was more affected although both were affected in some manner. As reported for pyruvate carboxylase enzymes isolated from other species, Lineweaver–Burk plots for pyruvate appear nonlinear, showing an increase in slope as the concentration of pyruvate increased. The nonlinearity occurred whether acetyl-CoA was present or not. From the slopes and intercepts of the two linear regions of each curve, apparent K_m values were obtained at high and low pyruvate concentrations in the presence and absence of acetyl-CoA. In the absence of acetyl-CoA (and the presence of 3.3 mM ATP and 20 mM $KHCO_3$) at 0.04–1 mM pyruvate (as the K salt), an apparent K_m of 1.8 mM was obtained. In the presence of 0.05 mM acetyl-CoA at 0.04–0.4 mM pyruvate, an apparent K_m of 0.18 mM was obtained, and at 0.5–15 mM pyruvate, the apparent K_m was 0.55 mM. The K_m values decreased by 3.3-fold in the presence of acetyl-CoA at both low and high concentrations of pyruvate. These results suggest that pyruvate activates the enzyme and that acetyl-CoA increases the affinity of the enzyme for pyruvate. It should be noted that K^+ is also an activator of yeast pyruvate carboxylase (Ruiż-Amil et al., 1965) and that pyruvate was varied as the K salt in these studies. The activation observed with potassium pyruvate may be partly due to the K^+ although K^+ activation is linear, with the K_m for K^+ being 8.4 mM; therefore, small increases in the concentration of K^+ should have a relatively small effect.

There was a 2-fold increase in the maximum velocity in the presence of acetyl-CoA (0.08 mM along with 10 mM pyruvate and 20 mM $KHCO_3$) and essentially no change in the K_m for ATP (0.2 mM) under these assay conditions (Table III). Potassium bicarbonate showed an increase in the maximum velocity of only *1.1-fold* in the presence of 0.08 mM acetyl-CoA (10 mM pyruvate and 3.3 mM ATP) and a *4-fold* decrease in the K_m from 8.8 to 2.2 mM. When bicarbonate was

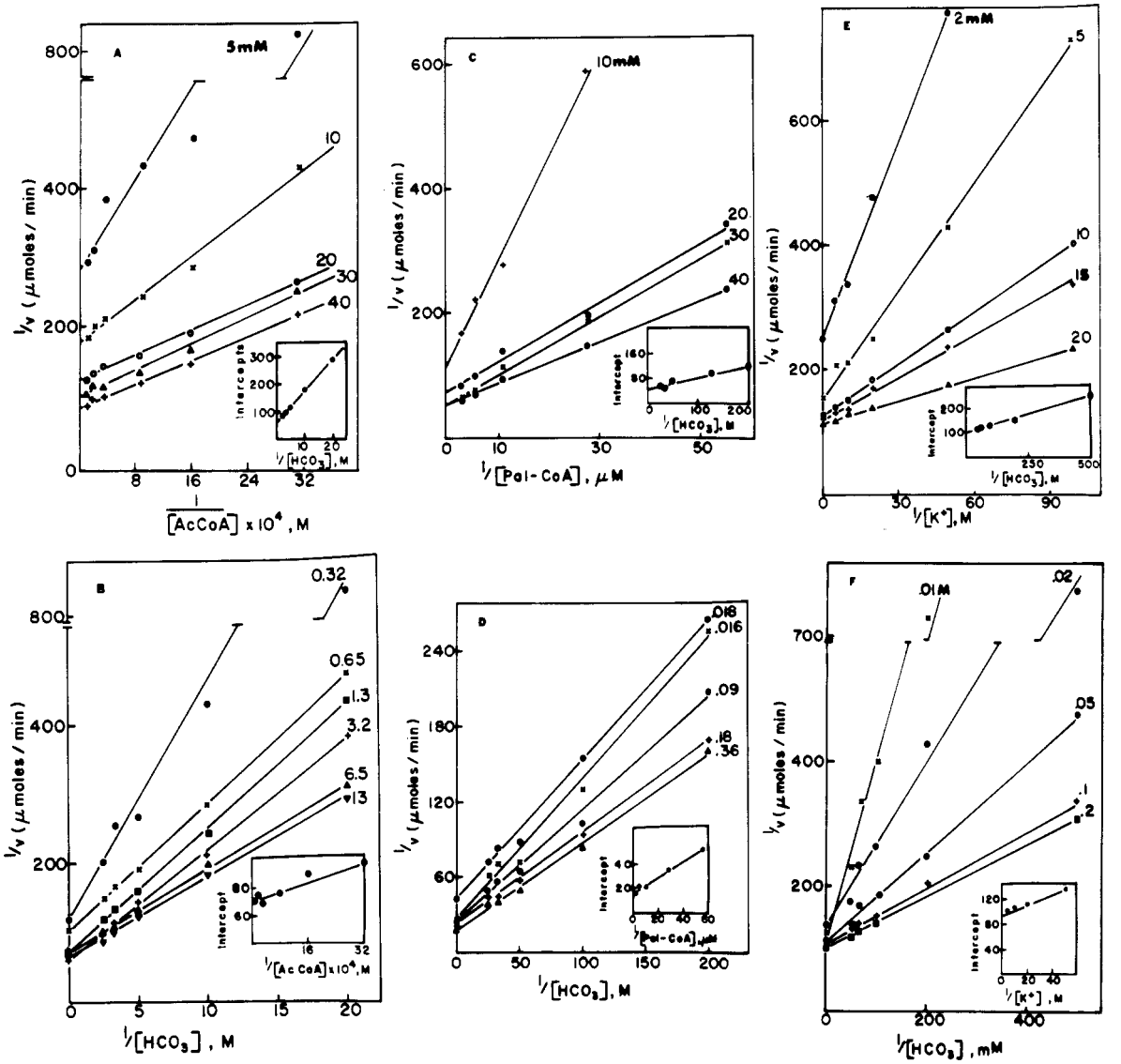

FIGURE 5: Lineweaver–Burk plots of acetyl-CoA vs. bicarbonate (and vice versa) (A and B), palmitoyl-CoA vs. bicarbonate (C and D), and K^+ vs. bicarbonate (E and F). Inserts are secondary plots of intercepts. Bicarbonate is varied as the tetramethylammonium salt. Velocities are given as activated velocities, i.e., activity after subtraction of very nominal basal activity.

varied as the tetramethylammonium salt, a *7.5-fold* increase in maximum velocity and a *1.9-fold* decrease in K_m were observed in the presence of 0.065 mM acetyl-CoA. These data suggest that acetyl-CoA has a very dramatic effect on the maximum velocity and, relatively speaking, a rather modest effect on the K_m of bicarbonate. The inference may be drawn that K^+ and acetyl-CoA are essentially competing or alternate activators in terms of their effects on the maximum velocity which may be obtained in the presence of varying bicarbonate.

In order to shed light on this problem, it was necessary to separate the activating effects of K^+ from those of the acyl-CoA. Attention was focused on the effect of the two activators, acetyl- and palmitoyl-CoA, on the affinity of the enzyme for bicarbonate, the substrate which seemed most affected by the presence of acyl-CoA. The following studies were carried out by doing primary and secondary plots of kinetic data obtained in initial velocity studies while varying an effector, acyl-CoA or K^+, against bicarbonate and vice versa while all other substrates were held at concentrations at least 10-fold greater than their apparent K_m values. The K_m values were determined from the secondary plots of intercepts.

The relationship of the Lineweaver–Burk plots of the activation by acetyl-CoA at changing fixed concentrations of bicarbonate changed from noncompetitive to uncompetitive as the level of bicarbonate was increased (Figure 5). These experiments were run without K^+ ion in order to obviate the complications arising from the presence of that ion. A secondary plot of the intercepts was reasonably linear and when

Table III: Effects of Acetyl-CoA on the Interactions of Yeast Pyruvate Carboxylase and Its Substrates[a]

substrate	apparent K_m (mM)		relative V_{max}	
	−acetyl-CoA	+acetyl-CoA	−acetyl-CoA	+acetyl-CoA
pyruvate (as K salt)				
high concentration	1.8	0.6	1	1.7[b]
low concentration	0.6	0.2		
ATP (K salt)	0.2	0.2	1	2[c]
HCO_3 (K salt)	8.8	2.2	1	1.1[c]
as the tetramethylammonium salt	35	19.4	1	7.5[d]

[a] Assay conditions were as follows: Tris-SO_4 (as the buffer), $MgSO_4$, K-ATP (3.3 mM), potassium pyruvate (10 mM), and $KHCO_3$ (20 mM). [b] Concentration of acetyl-CoA: 0.05 mM. [c] Concentration of acetyl-CoA: 0.08 mM. [d] Concentration of acetyl-CoA: 0.065 mM.

Table IV: Summary of the Effect of Various Activators of Pyruvate Carboxylase from Yeast on the Apparent K_m of Bicarbonate

activator (concn)[a]	K_m(bicarbonate) (mM)
K^+ (8.4×10^{-3} M)	3.1
palmitoyl-CoA (1.4×10^{-8} M)	11.0
acetyl-CoA (4×10^{-6} M)	19.2
no activator	37.0

[a] Assay conditions as described for Table III. Concentrations represent the limiting $K_{0.5}$ values calculated from the secondary plots of intercepts shown in Figure 5.

used to calculate the limiting K_m for bicarbonate gave a value of 19.1 mM compared to a value of 37 mM when acetyl-CoA as well as K^+ was absent.

Studies similar to those just described for acetyl-CoA were also carried out with palmitoyl-CoA. Double-reciprocal plots of palmitoyl-CoA concentration vs. velocity revealed a pattern that was complicated in a manner similar to that observed for acetyl-CoA. From a secondary plot of the intercepts, a K_m of 11 mM was obtained for bicarbonate. Primary plots of varying bicarbonate at changing fixed concentrations of palmitoyl-CoA were done as with acetyl-CoA; there was a suggestion of a change from noncompetitive to uncompetitive plots as the concentratin of bicarbonate increased. The $K_{0.5}$ for palmitoyl-CoA derived from the secondary intercept plot was 1.4×10^{-8} M compared to a value of 4.0×10^{-6} M for acetyl-CoA. The relationship between acyl-CoA and bicarbonate is more complex than that predicted from the rate equation of a simple two-substrate system. Palmitoyl-CoA is a more effective activator than acetyl-CoA in that it causes a 1.8-fold greater affinity of the enzyme for bicarbonate.

The effect of K^+ on the affinity of the enzyme for bicarbonate was also studied. The K_m for bicarbonate derived from secondary plots as mentioned above was 3.1 mM. These experiments were run in the absence of added acyl-CoA. The data suggest that each of the activators increased the affinity of the enzyme for bicarbonate with the order of effectiveness according to the K_m for bicarbonate being K^+, palmitoyl-CoA, and acetyl-CoA (see Table IV for a summary of these data). Both K^+ and acyl-CoA increase the affinity of the enzyme for bicarbonate; however, the concentration of K^+ required to give optimum affinity is quite high (16.8 mM). An important role for K^+ in the critical control of pyruvate carboxylase activity seems unlikely unless there is some very specific compartmentation involved since the K^+ concentration in the cell is about 17 mM (Conway & Gaffney, 1966) and other monovalent cations, such as ammonium ion, also activate. The intracellular concentrations of acyl-CoA compounds are hard to determine since these compounds are known to bind tightly to various proteins, e.g., BSA. A combination of K^+ and acyl-CoA is probably necessary to obtain optimum activity in the cell. These results suggest that K^+ alone can produce a maximum affinity for bicarbonate but that acyl-CoA can enhance this affinity at lower concentrations of K^+. If neither K^+ or acetyl-CoA were present, the observed activity of the enzyme was very low, usually 2% of the maximum activity in the presence of activators. Even this very low activity may be an artifact since ammonium ions can replace K^+ as an activator.

Discussion

All pyruvate carboxylase enzymes examined thus far are activated by a monovalent cation with K^+, Rb^+, Cs^+, and NH_4^+ serving with varying degrees of effectiveness (McClure et al., 1971a,b; Cazzulo & Stoppani, 1967; Barden & Scrutton, 1974). In addition, pyruvate and ADP, as well as aspartate and acyl-CoA, are likely candidates for regulators of yeast pyruvate carboxylase. The K_m values for pyruvate for most varieties of pyruvate carboxylases are in the range 0.14–0.2 mM (McClure et al., 1971a,b; Utter & Fung, 1971; Taylor et al., 1969). It seems likely that availability of pyruvate may be one of the regulatory factors of this enzyme (Easterbrook-Smith et al., 1979).

An earlier finding of Keech & Utter (1963) showed that ADP was an inhibitor of pyruvate carboxylase from chicken liver. Miller & Atkinson (1972) have shown that the enzyme from yeast is very sensitive to the energy charge (ratio of phosphorylated adenine nucleotides to total adenine nucleotides).

Chicken liver pyruvate carboxylase appears to be dependent on the presence of acetyl-CoA, even in the presence of very large concentrations of substrates. There is little evidence for any sort of interaction between acetyl-CoA and the substrates or monovalent activator, K^+ (Utter et al., 1975; Fung, 1972). Frey & Utter (1977) were able to carry out binding studies to show that chicken liver pyruvate carboxylase binds 3–4 mol of acetyl-CoA/mol of enzyme. This binding of acetyl-CoA appeared to be cooperative in nature with a Hill coefficient of about 2 (compared to a value of 3 for the catalytic n_H) although there are some problems inherent in these binding studies.

Scrutton (1974) and Ashman & Keech (1975) have shown that the enzymes from rat liver and sheep kidney differ in their response to acetyl-CoA compared to the avian enzyme. The degree of positive cooperativity is lower, about 2 instead of 3, and a significant fraction (as much as 25%) of the potential activity can be elicited in the absence of acetyl-CoA if substrates and K^+ are present at high concentrations. Ashman et al. (1973) and Scrutton & White (1973) have been able to desensitize pyruvate carboxylase from sheep kidney and rat liver, respectively, against activation by acetyl-CoA by treatment with trinitrobenzenesulfonate. This same inhibitor simply inactivates the enzyme from chicken liver.

Pyruvate carboxylase from yeast represents still another type of enzyme regarding its response to acyl-CoA. Although this enzyme can be stimulated up to 10-fold by acyl-CoA at low HCO_3^- and K^+ (Tolbert, 1970), when these substances are present at high concentrations, stimulation by acetyl-CoA is essentially absent. Treatment of yeast pyruvate carboxylase with trinitrobenzenesulfonic acid results in the loss of acetyl-CoA-independent activity at a slower rate than acetyl-CoA-dependent activity. Loss of response to acetyl-CoA activation by yeast pyruvate carboxylase does not lead to a parallel loss of response to inhibition by aspartate, indicating that separate sites may be involved for these two effectors.

The regulation of yeast pyruvate carboxylase by aspartate and acyl-CoA compounds may have a physiological role in maintaining a balance between catabolic and anaplerotic reactions during growth on compounds giving rise to three-carbon acids if L-aspartate is regarded as an end product of anaplerosis. Such control is exerted on phosphoenolpyruvate (PEP) carboxylase in the Enterobacteriaceae, where PEP rather than pyruvate is the substrate for CO_2 fixation (Cañovas & Kornberg, 1966).

Registry No. CoASH, 85-61-0; acetyl-CoA, 72-89-9; propionyl-CoA, 317-66-8; *n*-butyryl-CoA, 2140-48-9; *n*-hexanoyl-CoA, 5060-32-2; palmitoyl-CoA, 1763-10-6; stearoyl-CoA, 362-66-3; arachidoyl-CoA, 15895-27-9; oleoyl-CoA, 1716-06-9; palmitoleoyl-CoA, 18198-76-0; benzoyl-CoA, 6756-74-7; phenylacetyl-CoA, 7532-39-0;

K, 7440-09-7; ATP, 56-65-5; HCO_3, 71-52-3; pyruvate, 127-17-3; aspartate, 56-84-8; pyruvate carboxylase, 9014-19-1.

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