Regulation of Pyruvate Carboxylase by Coenzyme A and Acyl Coenzyme A Thio Esters*

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ABSTRACT: Pyruvate carboxylase has been purified 235-fold from dried baker's yeast. Like pyruvate carboxylase from a variety of other sources the purified enzyme from yeast is stimulated by acetyl coenzyme A. Unlike other documented systems, the yeast enzyme is stimulated by coenzyme A, acetoacetyl coenzyme A, palmityl coenzyme A, pantethine, and adenosine 3′,5′-cyclic phosphate. A partial explanation of the stimulation of yeast pyruvate carboxylase by coenzyme A and acyl thio esters is that these compounds specifically lower the K_m for bicarbonate.

In addition to the unique effector specificity, the yeast carboxylase also differs from vertebrate organ carboxylases in the kinetics of effector binding. Typical Michaelis–Menten kinetics are observed in a plot of reaction velocity *vs.* effector concentration. In vertebrate carboxylase preparations a similar plot has yielded higher order nonlinear kinetics. These data indicate major differences between the yeast and vertebrate organ pyruvate carboxylases.

The carboxylation of pyruvate (reaction 1) catalyzed by pyruvate carboxylase (EC 6.4.1.1) has been studied in a number of different organisms. These studies have emphasized that major differences exist in the effect of acetyl-CoA on the enzymatic activity of these various carboxylases. Utter and Keech (1963) have shown that avian liver and kidney pyruvate carboxylase require catalytic amounts of acetyl-CoA for enzymatic activity. In contrast, pyruvate carboxylase extracted from the mold *Aspergillus niger* (Bloom and Johnson, 1962) or from the bacterium *Pseudomonus citronellis* (Seubert and Remberger, 1961) is not affected by acetyl-CoA. Between these two extremes pyruvate carboxylase from a variety of sources (Henning *et al.*, 1963; Benedict, 1964; Fuller *et al.*, 1961) has been shown to be definitely stimulated by the addition of catalytic amounts of acetyl-CoA although these carboxylases do not require acetyl-CoA for enzymatic activity.

$$\text{pyruvate} + HCO_3^- + \text{ATP} \xrightarrow{Mg^{2+}} \text{OAA}^1 + \text{ADP} + P_i \quad (1)$$

The precise role of acetyl-CoA in the pyruvate carboxylase reaction is not known. Scrutton *et al.* (1965) and Benedict (1964) have shown reaction 1 to be composed of the following half-reactions

$$\text{ATP} + HCO_3^- + \text{biotin–enzyme} \rightleftharpoons^{Mg^{2+}} \text{ADP} + P_i + CO_2\text{–biotin–enzyme}$$

$$CO_2\text{–biotin–enzyme} + \text{pyruvate} \rightleftharpoons \text{OAA} + \text{biotin–enzyme}$$

In addition Gailiusis *et al.* (1964) have shown that acetyl-CoA is only necessary for the fixation of HCO_3^-. The transcarboxylation reaction does not require acetyl-CoA. Cooper and Benedict (1966) obtained kinetic evidence showing that acetyl-CoA causes a tertiary structural change in the carboxylase resulting in a sevenfold lowering of the K_m for bicarbonate. More recently Ling and Keech (1966) have interpreted their nonlinear Lineweaver–Burk plots as indicating a homotropic cooperative binding of more than one molecule of acetyl-CoA to each molecule of sheep kidney carboxylase.

A considerable body of physiological evidence has accumulated suggesting that pyruvate carboxylase may be a regulatory enzyme in gluconeogenesis. Keech and Ling (1966) have recently suggested that the liver and kidney carboxylases may have different physiological roles. The kidney enzyme may have a slightly different structure than the liver enzyme. We wish to present evidence which supports our earlier observations. Yeast and vertebrate organ pyruvate carboxylases catalyze a similar reaction. Yeast carboxylase has a quantitatively different effector response. In yeast, acetyl-CoA may be replaced by a variety of effectors. The action of these effectors is similar to acetyl-CoA.

Experimental Procedure

Materials. Medium-mesh diethylaminoethylcellulose, potassium pyruvate, *cis*-oxaloacetate, disodium adeno-

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: OAA, oxaloacetic acid.

TABLE I: Purification of Yeast Pyruvate Carboxylase.[a]

Fraction	Total Protein (mg)	Total Act. (cpm $\times 10^{-6}$)	Sp Act. (cpm $\times 10^{-3}$/mg of protein)	-Fold Purification
Crude	59,492	139	2.3	
Protamine sulfate	35,744	273	8.1	3.46
35–40% ammonium sulfate	3,672	186	50.9	21.72
Dialysate		271	82.4	35.17
DEAE-cellulose	58	31	552.5	235.61

[a] Purification of yeast pyruvate carboxylase. The reactions used were the same as indicated in Methods. Comparable quantities of protein from each fraction were used in the assay.

sine triphosphate, reduced glutathione, malic dehydrogenase, reduced pyridine nucleotide, *S*-acetoacetyl-CoA, *S*-palmitoyl-CoA, pantethine, and coenzyme A were purchased from Sigma Chemical Co. Acetyl-CoA was purchased from P-L Biochemical Corp. Sodium [^{14}C]-bicarbonate was purchased from New England Corp. Dried baker's yeast was obtained from Standard Brands Inc.

Preparation of DEAE-cellulose. DEAE-cellulose was exhaustively washed with 0.10 N KOH followed by a water rinse until the pH of the eluate was neutral. At this time the chromatographic material was exhaustively washed with 0.10 N HCl followed by a water rinse until the pH of the eluate was neutral. The washed DEAE-cellulose was suspended in 0.005 M phosphate buffer of the desired pH and stored at 4°.

Protein Determinations. Proteins were determined by the biuret method of Gornall *et al.* (1949) up to the DEAE-cellulose stage of enzyme purification. After this stage proteins were determined by the optical method of Warburg and Christian (1941).

Assay of Pyruvate Carboxylase. Enzymatic activity was assayed by the procedure of Gailiusis *et al.* (1964). The reaction mixture contained in micromoles per milliliter: 100, potassium phosphate buffer (pH 7.4); 5, GSH; 10, ATP; 10, potassium pyruvate; 65, potassium bicarbonate containing 3.25 μCi/μmole; 0.077, acetyl-CoA; 3, oxaloacetate; and 10, $MgCl_2$. Less than 300 μg of protein was added to this mixture. The reaction mixture was incubated at 37° for 15 min. The reaction was stopped by adding 1.0 ml of 6 N HCl containing 5 mg of 2,4-dinitrophenylhydrazine. After a 10-min postincubation, the mixture was extracted with 5.0 ml of ethyl acetate. A known aliquot (0.25 ml) of the ethyl acetate phase was transferred to an aluminum planchet. The amount of radioactivity on the planchet was determined with a Nuclear-Chicago gas flow counter (Geiger) operating at an efficiency of 19.7%.

An aliquot of the ethyl acetate layer was cochromatographed with authentic OAA and pyruvate DNP-hydrazones on Whatman No. 2 filter paper. The chromatogram was developed at 25° for 22 hr in a solvent mixture of butanol–ethanol and 0.5 N NH_4OH (7:1:2, v/v). The radioactivity was located in a band at R_F 0.17 which coincided with a band of authentic OAA–DNP-hydrazone. If any of the necessary cofactors for the carboxylation of pyruvate were deleted from the reaction mixture a negligible amount of radioactivity (less than 360 cpm) was located in the ethyl acetate phase.

The pyruvate carboxylase assay was also coupled to malic dehydrogenase. This allowed an assay of the OAA formed from the carboxylation of pyruvate. The net formation of OAA could be followed by the disappearance of NADH at 340 mμ or by the incorporation of [^{14}C]HCO_3^- into malate. For this radioassay the reaction was terminated by the addition of 1.0 ml of concentrated formic acid. The reaction mixture was evaporated to dryness and passed through a 1 × 12 cm Dowex 50 (H^+) resin column. The eluate was evaporated to dryness and the residue dissolved in H_2O. The H_2O extract was chromatogrammed on Whatman No. 3MM filter paper. The chromatogram was developed in a solvent of butanol–formic acid–H_2O (5:1:4, v/v). The radioactivity was located at a R_F of 0.43 which coincided exactly with known malic acid. The radioactive area was cut from the chromatogram and assayed for radioactivity in a Packard liquid scintillation spectrometer.

Results

Purification of Pyruvate Carboxylase. Yeast pyruvate carboxylase was solubilized from dried baker's yeast by the method of Gailiusis *et al.* (1964); 1300 g of dried yeast was ground to a medium fine powder, by means of a Sargent flour mill. The grinding was carried out at temperatures below 5°. The ground yeast was then added with stirring to 3 l. of 0.10 M pH 7.4 phosphate buffer. This mixture was allowed to autolyze at 37° with intermittent stirring until its volume had doubled due to gas production (a period of approximately 1–1.5 hr). Autolysis was followed by rapid cooling in ice and centrifugation of the cooled autolysate for 15 min at 7790*g* in a Sorvall RC-2 refrigerated centrifuge. The viscous supernatant was filtered through four layers of cheesecloth and recentrifuged 30 min at 27,000*g* to remove the remaining large particles and cell debris. The straw-colored supernatant was finally filtered through a thick mat of glass wool which removed a considerable amount of the fat which is abundant in this type of preparation. Pyruvate carboxylase was purified 235-fold by the procedures summarized in Table I.

Protamine Sulfate Treatment. Protamine sulfate (2%

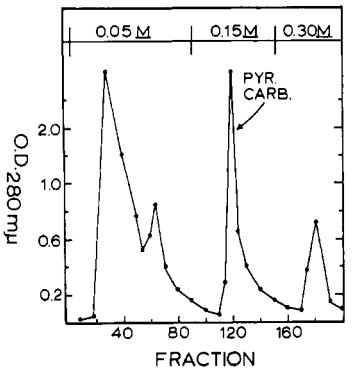

FIGURE 1: Elution pattern of yeast pyruvate carboxylase from DEAE-cellulose; 400 mg of the dialyzed preparation was placed on a 5.2 × 55 cm column which had been prepared as indicated in Methods. Elution was accomplished by applying pH 7.4 phosphate buffers to the column at the points indicated. All of these procedures were carried out at 4°.

solution, pH 6.7) was added with slow stirring to the soluble supernatant until a final concentration of 0.1 mg of protamine sulfate/mg of protein had been reached. Following 10-min slow stirring, the heavy beige precipitate was removed by centrifugation for 10 min at 27,000*g*. It was found that these contact times and concentrations of protamine sulfate were optimal. Significant deviations from them yielded either a supernatant containing large amounts of DNA or one lacking enzymatic activity.

Ammonium Sulfate Fractionation. Solid reagent grade ammonium sulfate was added slowly to the protamine sulfate supernatant. At the conclusion of a 30-min equilibration period the 0–35% pellet was removed by centrifugation. By the same procedure a 35–45% pellet was obtained. This pellet was suspended in a minimal amount (50–75 ml) of pH 7.4 phosphate buffer and dialyzed 1.5 hr against 7 l. of 0.005 M pH 7.4 phosphate buffer containing 3.9×10^{-4} M 2-mercaptoethanol. Following dialysis the preparation was stable for several days at −20°.

DEAE-cellulose Chromatography. The preparation

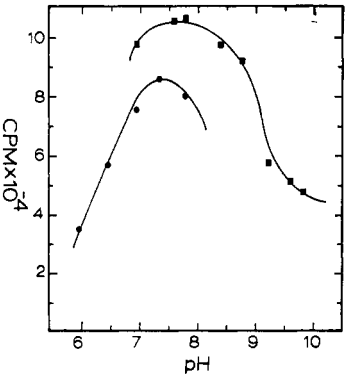

FIGURE 2: Effect of varying pH upon pyruvate carboxylase activity. The reaction mixture was identical with that used in Table II except that the buffer concentration was raised to 150 mM to ensure maintenance of the proper pH. (■) Tris and (●) phosphate.

TABLE II: The Stimulation of Yeast Pyruvate Carboxylase by CoA and Acyl-CoA Thio Esters.[a]

Compound	[^{14}C]2,4-DNP-hydrazone (cpm)	Rel Effect
None	52,760	1.00
Acetyl-CoA	194,680	3.69
Coenzyme A	174,680	3.31
Palmityl-CoA	203,140	3.85
Acetoacetyl-CoA	152,780	2.90
Pantethine	91,360	1.73
Adenosine 5′-diphosphate	71,460	1.35
Adenosine 3′,5′-cyclic phosphate	88,040	1.67

[a] Stimulation of yeast pyruvate carboxylase by acyl-CoA compounds. The reaction mixture was composed of the constituents, listed under Methods with the following exceptions: 10 mM HCO_3^- of specific activity 0.2 μCi/μmole and 50 μg of protein were used in place of those indicated. Each of the effectors (0.1 mg) was added to the respective assay mixtures with the assumption that with this great excess, differences in absolute concentration could be neglected.

(400–500 mg) was placed on a 5.2 × 55 cm DEAE-cellulose column which had been prepared as described earlier. Elution was accomplished by stepwise washing with pH 7.4 phosphate buffers containing 3.9×10^{-4} M mercaptoethanol. It is noteworthy that deletion of the 2-mercaptoethanol at any point resulted in rapid and complete loss of activity. The elution pattern of pyruvate carboxylase from the column is shown in Figure 1. As noted the enzyme was eluted as a single sharp peak in the presence of 0.15 M buffer. At this stage of purification the enzyme is stable for only 48 hr at −20°. All activity is lost within several hours at 25°.

Effect of pH on Pyruvate Carboxylase Activity. As shown in Figure 2, yeast pyruvate carboxylase exhibits a broad pH optimum centered around pH 7.4 or 7.6 depending upon the nature of the buffering system employed. This is in reasonable agreement with the pH 7.8 optimum observed by Utter and Keech (1963) for avian liver pyruvate carboxylase. It is considerably different from the pH 8.4 optimum observed for the sheep kidney carboxylase (Ling and Keech, 1966). Since it has been shown (Cooper *et al.*, 1967, 1968) that HCO_3^- is the active species of CO_2 in the pyruvate carboxylase reaction, a pH optimum differing from 7.4 would be unexpected.

Acetyl-CoA Requirement. The requirement for acetyl-CoA in the vertebrate systems is both absolute and specific. Keech and Utter (1963) found that CoA, butyryl-CoA, and acetylpantetheine were totally inactive in the avian liver pyruvate carboxylase reaction. Propionyl-CoA and crotonyl-CoA were 92 and 47% as effective as acetyl-CoA in this reaction. Scrutton and Ut-

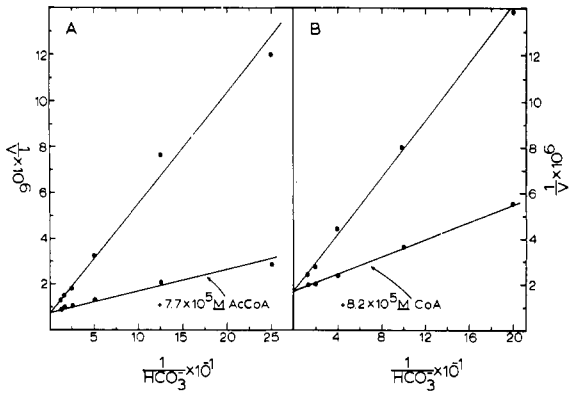

FIGURE 3: Effect of increasing concentrations of HCO_3^- upon carboxylase activity in the presence and absence of acetyl-CoA (B). Reaction conditions were identical with those of Table II.

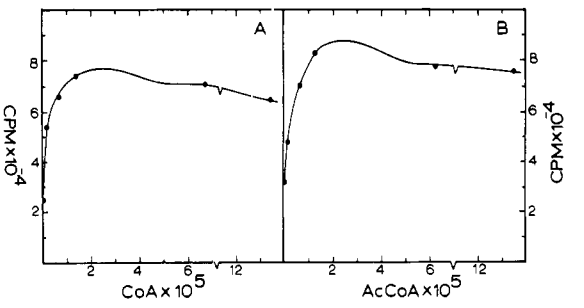

FIGURE 4: Effect of increasing concentrations of acetyl-CoA (A) and CoA (B) upon pyruvate carboxylase activity. The reaction conditions were identical with those used in Table II.

ter (1967) have shown that CoA is inactive in the avian liver pyruvate carboxylase reaction. Acetylpantetheine and malonyl-CoA are competitive inhibitors with respect to acetyl-CoA in this reaction. In contrast to these results CoA and a number of thio esters of CoA are active in stimulating the pyruvate carboxylase reaction in yeast (Table II). CoA is 90% as efficient in stimulating the enzymatic activity as acetyl-CoA. This partially confirms the results of Ruiz-Amil *et al.* (1965) who showed CoA to be 50% effective in replacing acetyl-CoA in the yeast pyruvate carboxylase reaction. Adding palmityl-CoA to the purified yeast pyruvate carboxylase reaction results in a greater stimulation than acetyl-CoA. The activity of these compounds in this reaction raises considerable question as to the possible mechanism of regulating pyruvate carboxylase by acetyl-CoA. These data suggest that yeast and avian enzymes are different with respect to their effector requirements.

In order to determine whether or not the modifiers were acting in a similar manner the effects of CoA and acetyl-CoA upon the K_m of the enzyme for HCO_3^- were compared. Such an approach seemed plausible since Cooper and Benedict (1966) have shown that acetyl-CoA will decrease the K_m of the enzyme for HCO_3^- by sevenfold. As shown in Figure 3A,B, CoA and acetyl-CoA both decrease the K_m of the enzyme for HCO_3^- by three- and fivefold, respectively. As documented earlier this decreased Michaelis constant is specific for bicarbonate. No change in the K_m for ATP and pyruvate is observed in the presence or absence of CoA or acetyl-CoA.

A second difference between the yeast enzyme and that of the sheep and avian liver is its response to increasing concentration of effectors. Ling and Keech (1966) observed a sigmoid curve when plotting velocity *vs.* effector concentration. As shown in Figure 4A,B, however, a similar plot using the yeast enzyme exhibits no sigmoid character.

Discussion

The results presented in this paper show that yeast pyruvate carboxylase is stimulated by CoA and different acyl-CoA thio esters. This response is different than the response of avian liver pyruvate carboxylase to different effectors. The activity of CoA in the yeast carboxylase reaction rules out the necessity of an activated CO_2–acyl-CoA effector intermediate in the reaction. Furthermore, the activities of CoA, palmityl-CoA, and acetyl-CoA in this reaction exempt acetyl-CoA from playing a unique role in stimulating yeast carboxylase.

The difference between the yeast and kidney enzyme is demonstrated by the way the two carboxylases bind acetyl-CoA. Keech and Ling (1966) have shown that sheep kidney pyruvate carboxylase exhibits homotropic interaction during the binding of acetyl-CoA. The velocity curve for acetyl-CoA in this reaction is sigmoidal. The yeast pyruvate carboxylase does not exhibit an homotropic interaction during the binding of CoA or acetyl-CoA. These velocity curves are hyperbolic, not sigmoidal. In the yeast system there is no interaction of effector sites. The effector interaction in the pyruvate carboxylase reaction which has been observed is between HCO_3^- binding and the effector. This is undoubtedly the reason that acyl-CoA thio esters affect the CO_2 fixation step and not the transcarboxylation step of yeast pyruvate carboxylase reaction (Gailiusis *et al.*, 1964).

In view of the significant differences that have been shown to exist between the yeast, avian, and sheep enzymes, it is difficult to suggest that pyruvate carboxylase from these different sources shares a common cellular regulatory role. The avian liver carboxylase has been shown (Utter *et al.*, 1964) to control the amount of pyruvate that is shuttled to PEP through the "abbreviated" dicarboxylic acid shuttle. According to this hypothesis, acetyl-CoA concentrations required for the operation of the trichloroacetic acid cycle are also sufficient to activate the avian pyruvate carboxylase. Such an activation of pyruvate carboxylase leads to a drain of pyruvate away from the trichloroacetic acid cycle toward the gluconeogenic precursor PEP through oxaloacetate. It is difficult to see how this unique control of avian pyruvate carboxylase by acetyl-CoA concentrations can be applied to yeast pyruvate carboxylase because (1) acetyl-CoA is not necessary for up to 50% of the total possible carboxylase activity and (2) CoA and palmityl-CoA are nearly as efficient effectors of yeast pyruvate carboxylase as acetyl-CoA. The responses of purified yeast pyruvate carboxylase to different effec-

tors may lead to a more general metabolic regulation of this enzyme reaction.

References

Benedict, C. R. (1964), *6th Intern. Congr. Biochem. 6*, 503.

Bloom, S. J., and Johnson, M. J. (1962), *J. Biol. Chem. 237*, 2718.

Cooper, T. G., and Benedict, C. R. (1966), *Biochem. Biophys. Res. Commun. 22*, 285.

Cooper, T. G., Tchen, T. T., and Benedict, C. R. (1967), *Plant Physiol. 42*, S-3.

Cooper, T. G., Tchen, T. T., Benedict, C. R., and Wood, H. G. (1968), *Federation Proc. 27*, 2088.

Fuller, R. C., Smillie, R. M., Sisler, E. C., and Kornberg, H. L. (1961), *J. Biol. Chem. 236*, 2140.

Gailiusis, J., Rinne, R. W., and Benedict, C. R. (1964), *Biochim. Biophys. Acta 92*, 595.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Henning, H. V., Seiffert, I., and Seubert, W. (1963), *Biochim. Biophys. Acta 77*, 345.

Keech, D. B., and Ling, A. (1966), *Biochem. Biophys. Res. Commun. 24*, 476.

Keech, D. B., and Utter, M. F. (1963), *J. Biol. Chem. 238*, 2609.

Ling, A., and Keech, D. B. (1966), *Biochem. Z. 344*, 367.

Ruiz-Amil, M., DeTorrontegui, G., Palacian, E., Catalina, L., and Losada, M. (1965), *J. Biol. Chem. 240*, 3485.

Scrutton, M. C., Keech, D. B., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 574.

Scrutton, M. C., and Utter, M. F. (1967), *J. Biol. Chem. 242*, 1723.

Seubert, W., and Remberger, U. (1961), *Biochem. Z 334*, 401.

Utter, M. F., and Keech, D. B. (1963), *J. Biol. Chem. 238*, 2603.

Utter, M. F., Keech, D. B., and Scrutton, M. C. (1964), *Advan. Enzyme Reg. 2*, 49.

Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.