Kinetic Characterization of Yeast Pyruvate Carboxylase Isozyme Pyc1†

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ABSTRACT: Yeast (*Saccharomyces cerevisiae*) is unusual in being the only organism thus far identified as having two genes for pyruvate carboxylase. The expression of the two isozymes Pyc1 and Pyc2 appears to be differentially regulated, and since both are expressed cytoplasmically, this suggests that they have different properties. To the present, little has been done to characterize these isozymes, and almost all of the published kinetic information on yeast pyruvate carboxylase comes from measurements of enzyme prepared from bakers' yeast which is likely to be a mixture of both isozymes. Here we have measured basic kinetic parameters for Pyc1 and found that the K_a of this isozyme for acetyl CoA is in the order of 8−10-fold higher than previously recorded, suggesting that Pyc1 and Pyc2 may be differentially regulated by this effector. Pyc1 is highly dependent on the presence of acetyl CoA for activity and in this respect is similar to chicken liver pyruvate carboxylase. However, unlike the chicken liver enzyme, the quaternary structure of the enzyme is quite stable in the absence of acetyl CoA, and the major locus of action of this effector appears to lie outside of the stimulation of the biotin carboxylation reaction.

Pyruvate carboxylase (EC 6.4.1.1) is a biotin-dependent enzyme that catalyzes the carboxylation of pyruvate to form oxaloacetate, and the overall reaction that it catalyzes proceeds in two steps as follows:

$$\text{MgATP} + \text{HCO}_3^- + \text{ENZ-biotin} \xrightleftharpoons{\text{Mg}^{2+},\ \text{acetyl CoA}} \text{MgADP} + \text{P}_\text{i} + \text{ENZ-biotin-CO}_2^- \quad (1)$$

$$\text{ENZ-biotin-CO}_2^- + \text{pyruvate} \rightleftharpoons \text{ENZ-biotin} + \text{oxaloacetate} \quad (2)$$

In the first partial reaction of pyruvate carboxylase, the biotin prosthetic group is carboxylated by HCO_3^- with concomitant cleavage of MgATP (reaction 1). The second partial reaction involves the carboxylation of pyruvate by carboxybiotin (reaction 2). Free Mg^{2+} and acetyl CoA are only required in reaction 1, but the degree of dependence on acetyl CoA for activity depends on the source of the enzyme (for a review, see ref *1*).

Current available evidence suggests that reaction 1 proceeds via a carboxyphosphate intermediate as shown in reaction 3 (see ref *2* for a review).

$$\begin{array}{ccccc} & & {}^{-2}\text{O}_3\text{POCO}_2^- & & \\ & & | & & \\ \text{ENZ-biotin + MgATP} & \rightleftharpoons & \text{ENZ-biotin} & \rightleftharpoons & \text{ENZ-biotin-CO}_2^- \\ \text{+ HCO}_3^- & & | & & \text{+ MgADP + Pi} \\ & & \text{MgADP} & & \end{array} \quad (3)$$

Attwood and Graneri (*3*) showed that chicken liver pyruvate carboxylase was capable of catalyzing the release of phosphate from MgATP in the presence of HCO_3^- and of Mg^{2+} in the absence of pyruvate and in the presence or absence of acetyl CoA. However, these steady-state reactions proceeded at less than 0.1% of the rate of the full pyruvate carboxylation reaction, and the authors suggested that the rate-limiting step in the catalytic cycle in the absence of pyruvate was the decarboxylation of the enzyme−carboxybiotin complex. Legge et al. (*4*) showed that acetyl CoA increased the rate constants for the approach to steady-state and enhanced the degree of coupling of ATP cleavage and carboxyenzyme formation. Legge et al. (*4*) presented evidence that the carboxyenzyme present in this type of experiment was in fact the enzyme−carboxybiotin complex, and thus when carboxyenzyme is referred to in the current work, it should be understood to mean enzyme−carboxybiotin complex. In the absence of acetyl CoA, the decarboxylation of carboxyphosphate is at least as rapid as carboxybiotin formation. Later, Branson and Attwood (*5*) showed that the cleavage of MgATP and carboxylation of the enzyme in the absence of pyruvate were more complicated in the chicken liver enzyme, in part owing to the reversible inactivation that the chicken enzyme undergoes on dilution.

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Saccharomyces cerevisiae has been shown to contain two genes for pyruvate carboxylase (*6, 7*), namely, PYC1 and PYC2. This yeast is the only organism known to have two genes for pyruvate carboxylase. The expression of the two genes of yeast pyruvate carboxylase is differentially regulated, and it is proposed that different metabolic functions are carried out by the different isoforms of the enzyme (*8, 9*). The two isozymes, Pyc1[1] and Pyc2, share 92% amino acid sequence homology (*7*), and both are expressed in the cytoplasm (*6*). Studies on yeast pyruvate carboxylase to date have been predominantly performed using enzyme isolated from bakers' yeast (*Saccharomyces cerevisiae*) and which has therefore potentially been a mixture of both of the isoenzymes.

Since Pyc1 and Pyc2 appear to be differentially regulated and have different metabolic roles, it may be expected that they have different properties. Only rudimentary characterization of the two isozymes has so far been performed on partially purified cell extracts. In this study, we have performed a basic kinetic characterization of Pyc1 and examined in greater detail the effects of acetyl CoA on the kinetics of the enzyme. These more detailed studies involved determination of the rate constants for the approach to steady-state of the formation of the carboxyenzyme complex and ATP cleavage.

MATERIALS AND METHODS

Yeast. Yeast strain DM18 (MATα, *ura3,yrp1, pyc1*Δ*::LEU2, pyc2*Δ*::HIS3)* was used with the PYC1 gene reinserted using pVT 100 vector to produce a strain of yeast that only expressed the Pyc1 isoenzyme (*8*). This yeast was grown with orbital shaking at 30 °C in a minimal medium [0.8% yeast nitrogen base (Difco), 1.1% casein amino acids (Becton Dickinson), 100 μg/L D-biotin (Sigma), 0.01% L-leucine, 0.01% L-tryptophan, 0.07% L-aspartate, 0.011% adenine, 0.011% L-tyrosine, with 4% glucose as the carbon source] for 24 h to an average of 18 g wet weight of yeast cells per 1 L of medium. The yeast were harvested by centrifugation and resuspended in a buffer of 100 mM Tris−acetate, pH 7.2, and 10 mM $MgCl_2$, and stored at −80 °C until required for enzyme preparation.

Preparation of Pyc1. Pyc1 was purified based on a method by Rohde et al. (*10*). The yeast was disrupted using bead-beater and glass beads, but the lipid extraction step was omitted. Chromatography on a DEAE-Sephacel column with a linear gradient from 20 to 320 mM ammonium sulfate was used, with further purification on an Avidin−Sepharose affinity column. The enzyme was precipitated with 55% (w/v) ammonium sulfate and stored at −80 °C in a storage solution composed of 0.1 M Tris-HCl, pH 7.2, 1.6 M sucrose, and 40 mM $(NH_4)_2SO_4$. Prior to the kinetic experiments, the enzyme was transferred from the storage solution to 0.1 M Tris-HCl, pH 7.8, by centrifuging through Sephadex G-25 as described by Helmerhorst and Stokes (*11*). The purity of the enzyme preparations used was estimated to be 88% by SDS−PAGE.

Other Materials. From Amersham Australia Pty. Ltd., $NaH^{14}CO_3$ was obtained as an aqueous solution at a specific radioactivity of 55 mCi/mmol, [^{14}C]biotin was obtained as a solid at a specific radioactivity of 50−62 mCi/mmol, and [γ-^{32}P]ATP was obtained as an aqueous solution of the triethylammonium salt at a specific radioactivity of approximately 3000 Ci/mmol. All other materials were high-purity preparations from commercial suppliers.

Spectrophotometric Pyruvate Carboxylase Assays. The conditions for this assay were described by Attwood and Cleland (*12*), except that the buffer used was 0.1 M Tris-HCl, pH 7.8. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of oxaloacetate/min at 30 °C. After transfer of the enzyme from storage buffer to 0.1 M Tris-HCl, pH 7.8, assays were preformed in triplicate at 30 °C. Steady-state turnover numbers for both the ATP cleavage in the absence of pyruvate and the overall pyruvate carboxylation reaction under conditions used in the pre-steady-state experiments were determined as above except the temperature was 20 °C and the concentration of HCO_3^- was 15 mM.

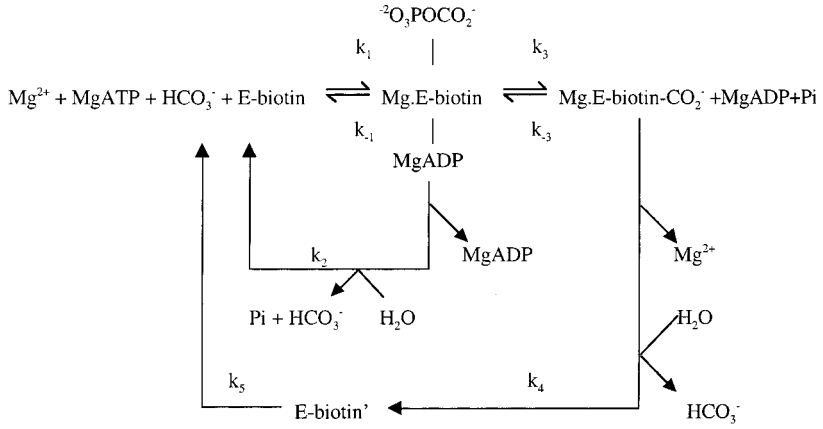
Biotin Determination. After removal from storage solution, aliquots of the enzyme solutions were set aside and stored at −80 °C for later determination of the biotin content. The enzyme solutions were incubated with 0.2% (w/v) chymotrypsin at 37 °C for 24 h. Pronase was then added to a final concentration of 0.45% (w/v), and the solutions were incubated for an additional 72 h at 37 °C. The solutions were then heated for 15 min at 100 °C before being used in the biotin assay described by Rylatt et al. (*13*).

Pre-Steady-State ATP Cleavage in the Absence or the Presence of Acetyl CoA. The effects of acetyl CoA on the kinetics of ATP cleavage were determined as described by Legge et al. (*4*). Briefly, two solutions were prepared in 0.1 M Tris-HCl, pH 7.8, with 7 mM $MgCl_2$, 15 mM $NaHCO_3$, in the absence or presence of 0.25 mM acetyl CoA. One solution contained the enzyme and the other [γ-^{32}P]ATP. The concentration of ATP in the reaction was 2.5 mM, with approximately 30 μCi/μmol of [γ-^{32}P]ATP. The final reaction concentration of the enzyme was 50 units/mL in the absence of acetyl CoA and 38 units/mL in the presence of acetyl CoA. These solutions were preincubated at 20 °C for at least 10 min before the reactions were initiated by mixing equal volumes of the enzyme and ATP solutions. Reactions were stopped at various times by addition of 2 M HCl (1.25 volume of the reaction solution). The enzyme was precipitated from the solution by the addition of 100% ice-cold trichloroacetic acid to a final concentration of 4.5%. The amount of P_i released from ATP was measured as described previously by Legge et al. (*4*). Reactions were performed on a Hi-Tech PQ/SF 53 preparative quench/stopped-flow spectrophotometer.

Approach to Steady-State of Carboxyenzyme Formation in the Absence or the Presence of Acetyl CoA. The effects of acetyl CoA on the kinetics of carboxyenzyme formation were determined as described by Legge et al. (*4*). The enzyme and ATP solutions were prepared, and the reactions were initiated by mixing equal volumes of these solutions as described above for the ATP cleavage experiments. There was no [γ-^{32}P]ATP in this case; however, the 15 mM $NaHCO_3$ was replaced with 12.5 mM $NaHCO_3$ and 2.5 mM $NaH^{14}CO_3$. The reactions were stopped at various times by the addition of an equal volume of a quench solution containing 200 mM pyruvate, 0.4 mM NADH, 22 units/mL

[1] Abbreviations: Pyc1, isoform 1 of pyruvate carboxylase coded for by the PYC1 gene; Pyc2, isoform 2 of pyruvate carboxylase coded for by the PYC2 gene; CLPC, chicken liver pyruvate carboxylase.

Scheme 1



malate dehydrogenase, 200 mM ethylenediaminetetraacetic acid (EDTA), and 400 mM $NaHCO_3$ adjusted to pH 7.8. Legge et al. (*4*) described previously that the EDTA forms complexes with Mg^{2+} in the reaction mixtures and stops the reaction; the large excess of $NaHCO_3$ dilutes out the $NaH^{14}CO_3$, reducing further incorporation of $^{14}CO_2^-$ into carboxyenzyme. The stable [^{14}C]malate formed from [^{14}C]oxaloacetate when the carboxyenzyme reacts with pyruvate was measured after the solutions were acidified to remove $NaH^{14}CO_3$. The final reaction concentration of the enzyme was 35 units/mL in the absence and in the presence of acetyl CoA. Reactions were performed on a Hi-Tech PQ/SF 53 preparative quench/stopped-flow spectrophotometer.

Carboxyenzyme Decay in the Absence or the Presence of Acetyl CoA. The rates of decay of the carboxyenzyme in the presence and absence of acetyl CoA were measured as described by Attwood and Wallace (*14*) except 0.1 M Tris-HCl buffer was used instead of 0.1 M *N*-ethylmorpholine hydrochloride buffer, in a final volume of 0.5 mL at 20 °C. The [^{14}C]carboxyenzyme complex was prepared as described for the carboxyenzyme formation experiments above and the reaction quenched with 200 mM EDTA/400 mM $NaHCO_3$. At specific time points, aliquots of the quenched reaction solution were removed and added to a transfer solution containing 0.1 M Tris-HCl, pH 7.8, 0.4 mM NADH, 28 units/mL malate dehydrogenase, and 200 mM sodium pyruvate. The radioactivity in the form of [^{14}C]malate was then determined as described above for the carboxyenzyme formation experiments. Control experiments were performed where the enzyme was added after the quench solution and pyruvate was omitted from the transfer solution.

Data Analysis. The kinetic data were analyzed using nonlinear least-squares regression analysis to fit the data to one of the following equations as described in the text:

$$y = A(1 - e^{-kt}) \quad \text{(i)}$$

$$y = A(1 - e^{-kt}) + rt \quad \text{(ii)}$$

$$y = A(1 - e^{-kt}) + A'(1 - e^{-k't}) \quad \text{(iii)}$$

$$CE = B(e^{-k_2 t}) \quad \text{(iv)}$$

$$v = V_{max}/(1 + K_a/[\text{acetyl CoA}]) + R \quad \text{(v)}$$

where y is either the number of moles of carboxyenzyme formed per mole of biotin or the number of moles of P_i released per mole of biotin; A and A' are the amplitude of the approach of the reaction to steady-state; k and k' are apparent first-order rate constants; r is the steady-state turnover number. In eq iv, CE is the picomoles of carboxyenzyme remaining at time t, B is the amplitude of the decay reaction, and k_2 is the apparent first-order rate constant for the decay of carboxyenzyme. In eq v, v is the velocity of the reaction at any [acetyl CoA], V_{max} is the velocity of the reaction in the presence of saturating [acetyl CoA], K_a is equal to the [acetyl CoA] required to give $v = V_{max}/2$, and R is the residual velocity of the reaction in the absence of acetyl CoA. Fitting of the data to Scheme 1 was performed by a nonlinear least-squares regression analysis/numerical integration method using the program Scientist. The numerical integration method is EPISODE (*15*), and the least-squares regression analysis is based on the Powell algorithm (*16*).

Table 1: K_ms for Reaction Substrates and K_a for Acetyl CoA[a]

substrate or effector	K_m or K_a (mM)
pyruvate	0.495 (±0.062)
bicarbonate	1.364 (±0.119)
MgATP	0.065 (±0.006)
acetyl CoA	0.079 (±0.009)

[a] The figures in parentheses are the standard errors of the estimates of the kinetic parameters derived from the nonlinear least-squares fits of the data to the Michaelis−Menten equation or, in the case of the acetyl CoA data, to eq v.

RESULTS

Table 1 shows the K_m values for the substrates pyruvate, bicarbonate, and MgATP and the K_a value for acetyl CoA. The K_a value for acetyl CoA was determined by fitting the measured reaction velocity vs [acetyl CoA] data to eq v. These values were obtained at 30 °C by spectrophotometric assay of pyruvate carboxylase activity at various concentrations of the effectors or substrates. k_{cat} for the reaction in the presence of saturating acetyl CoA was 89.79 ± 2.21 $s^{-1}$and in the absence of acetyl CoA was 3.9 ± 0.7 s^{-1}. Thus, in the absence of acetyl CoA, Pyc1 exhibits only about 4% of the activity observed with saturating acetyl CoA. Pyc1 exhibited no loss of enzymic activity over a period of 2 h when either kept at 400 units/mL at 0 °C or diluted to 4 units/mL at room temperature, although there was a 15% loss of activity when the enzyme was stored overnight at 4 °C at 400 units/mL.

To further investigate the effects of acetyl CoA, the kinetics of the approach to steady-state of ATP cleavage and

Table 2: Kinetic Parameters of Carboxyenzyme Formation and ATP Cleavage Reactions in the Presence and Absence of Acetyl CoA Derived from Nonlinear Least-Squares Analysis of the Data Shown in Figures 1 and 2 As Described under Materials and Methods[a]

	A (mol/mol of biotin)	k (s^{-1})	A' (mol/mol of biotin)	k' (s^{-1})	steady-state turnover number (s^{-1})
carboxyenzyme formation					
+AcCoA	0.08 (±0.01)	32.9 (±22.7)	0.21 (±0.02)	0.60 (±0.14)	
−AcCoA	0.18 (±0.02)	7.20 (±0.15)	0.08 (±0.04)	0.09 (±0.08)	
ATP cleavage					
+AcCoA	0.60 (±0.03)	7.44 (±0.59)			0.037 (±0.003)[b]
−AcCoA	0.36 (±0.02)	7.33 (±1.17)			0.014 (±0.002)[b]

[a] Figures in parentheses are the standard errors of the estimates of the kinetic parameters derived from the nonlinear least-squares regression analysis of the data to eq iii in the case of carboxyenzyme formation and eq ii in the case of ATP cleavage. [b] Mean and standard deviation of three determinations.

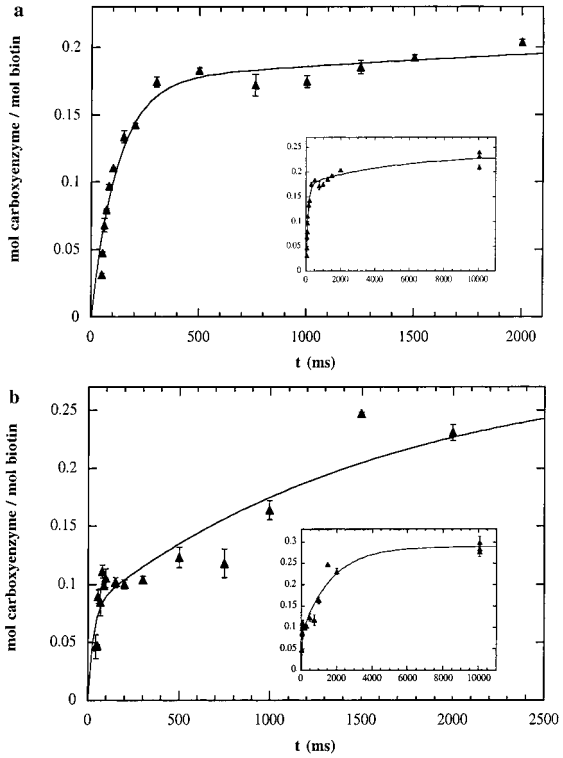


FIGURE 1: Kinetics of carboxyenzyme formation measured in the absence (a) and the presence (b) of 0.25 mM acetyl CoA at 20 °C in 0.1 M Tris-HCl, pH 7.8, with 7 mM $MgCl_2$, 2.5 mM ATP, 12.5 mM $NaHCO_3$, and 2.5 mM $NaH^{14}CO_3$. The solid lines represent nonlinear least-squares fits of the data to eq iii. The data points represent the mean, and the error bars represent the standard deviation of three determinations of carboxyenzyme at each time point. To determine better end points for the reactions, determinations of carboxyenzyme were also made after 10 s of reaction. The inset panels show the full time course, including these time points.

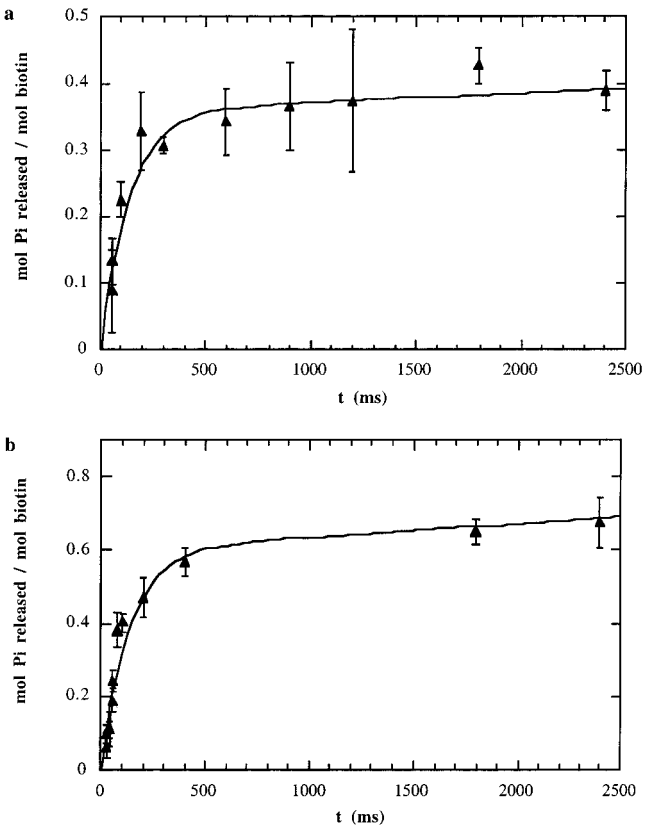


FIGURE 2: Kinetics of ATP cleavage were measured in the absence (a) and presence (b) of 0.25 mM acetyl CoA at 20 °C in 0.1 M Tris-HCl, pH 7.8, with 7 mM $MgCl_2$, 2.5 mM [γ-^{32}P]ATP, 15 mM $NaHCO_3$. P_i released/mol of biotin corresponds to the total moles of ATP cleaved per mole of biotin. The solid lines represent nonlinear least-squares fits of the data to eq ii with the value of r set at the value for the steady-state turnover number measured manually. The data points represent the mean, and the error bars represent the standard deviation of three determinations of P_i released from ATP at each time point.

formation of the carboxyenzyme were investigated. Figure 1 shows the approach to steady-state of the carboxyenzyme formation in the absence (a) and the presence (b) of acetyl CoA. Fits of the data to eq i were poor, and a biphasic process as represented by eq iii gave a better fit. The fitting of eq iii revealed an initial, rapid burst phase followed by a second, much slower phase of carboxyenzyme formation. The rapidity of the initial burst phase of carboxyenzyme formation in the presence of acetyl CoA and the difficulty of measuring time points faster than 50 ms have led to the large error in the estimate of the rate constant for the rapid phase of the formation of the carboxyenzyme complex. Values of the kinetic parameters derived from these fits are shown in Table 2. Overall, the amplitudes of carboxyenzyme formation are similar, but in the absence of acetyl CoA the amplitude of the fast phase of the reaction is twice that in the presence of acetyl CoA. The amplitude of the slow phase of the reaction in the absence of acetyl CoA is about a third of that in the presence of the effector. However, the rate constants of both phases of carboxyenzyme formation are higher in the presence of acetyl CoA compared to its absence, i.e., 4.6 times higher and 6.7 times higher for the fast and slow phases, respectively.

Figure 2 shows the approach to steady-state of ATP cleavage in the absence (a) and presence (b) of acetyl CoA. Both reactions are apparently first-order approaches to

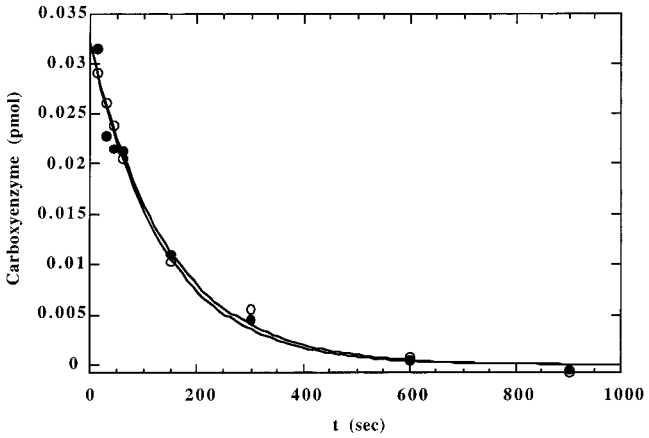


FIGURE 3: Decay of the carboxyenzyme complex was measured in the absence (○) and presence (●) of 0.25 mM acetyl CoA at 20 °C in 0.1 M Tris-HCl, pH 7.8, with 7 mM $MgCl_2$, 2.5 mM ATP, 12.5 mM $NaHCO_3$, and 2.5 mM $NaH^{14}CO_3$. The solid lines represent nonlinear least-squares fit of the data to eq iv.

steady-state. The rate constants for the approach to steady-state are the same in the presence and absence of acetyl CoA and are similar to that of the fast phase of carboxyenzyme formation in the absence of acetyl CoA. The amplitude of ATP cleavage in the approach to steady-state in the presence of acetyl CoA is about double that in the absence of acetyl CoA.

The steady-state rates of ATP cleavage in the absence of pyruvate (turnover number *r*) were determined in manual spectrophotometric assays and are shown in Table 2. In addition, the turnover numbers of the pyruvate carboxylation reaction under identical conditions of temperature and substrate concentrations used in the experiments to determine carboxyenzyme formation and ATP cleavage were determined. These rate constants were 25.5 ± 0.9 s^{-1} in the presence of acetyl CoA and 0.88 ± 0.03 s^{-1} in its absence (figures given are means ± SD of three measurements). In Table 2, the turnover number for ATP cleavage in the absence of pyruvate but presence of acetyl CoA is 2.6 times greater than that in its absence. The turnover numbers for ATP cleavage in the absence of pyruvate are 0.15% and 1.6% of those for the pyruvate carboxylation reaction in the presence and absence of acetyl CoA, respectively. Acetyl CoA has a greater effect on the complete pyruvate carboxylation reaction than on ATP cleavage in the absence of pyruvate, increasing the turnover number for the complete reaction by 29-fold. This effect of acetyl CoA on the pyruvate carboxylation reaction is similar to that observed in measurements at 30 °C (data not shown). The two sets of measurements at 20 and 30 °C suggest that the pyruvate carboxylation reaction has a Q_{10} of about 4 in both the presence and absence of acetyl CoA.

The kinetics of decay of carboxyenzyme at 20 °C in the presence and absence of acetyl CoA are shown in Figure 3. Fits of the data to eq iv gave apparent first-order rate constants of 0.0074 ± 0.0010 s^{-1} in the presence of 0.25 mM acetyl CoA and 0.0070 ± 0.0005 s^{-1} in its absence (values given are estimates obtained for the rate constants ± standard error by nonlinear least-squares regression fits of the data to eq iv). Thus, acetyl CoA has no apparent effect on the stability of carboxyenzyme under these conditions.

DISCUSSION

Most of the previous studies on yeast pyruvate carboxylase were performed using preparations of enzyme isolated from bakers' yeast and as such were likely to be a mixture of Pyc1 and Pyc2 isozymes. It is clear that both isozymes are expressed in *S. cerevisiae* although it would appear that the level of expression of each isoform is dependent on the growth conditions and point in the growth cycle (*8*, *9*). Stucka et al. (*7*) demonstrated that in mutant strains of yeast which had one or another PYC gene deleted, the remaining Pyc isozyme was expressed at sufficient levels to give enzyme activities comparable those present in the wild-type yeast; thus, each isozyme alone is capable of supporting growth of the yeast. Thus, we can say with some certainty that both isozymes of pyruvate carboxylase would have been present in preparations of enzyme from bakers' yeast used in previous studies, but the proportions of the two isozymes present is unclear.

The K_m for pyruvate shown in Table 1 is similar to that reported by Stucka et al. (*7*) for Pyc1 (0.45 mM) but is lower than the values reported by Ruiz-Amil et al. (*17*) (0.8 mM) and Myers et al. (*18*) (0.6 mM) for enzyme prepared from bakers' yeast. The K_m value for ATP shown in Table 1 is lower than previously reported values of 0.19 mM for Pyc1 (*7*) and about 0.2 mM for enzyme from bakers' yeast (*17*, *18*). Stucka et al. (*7*) found that Pyc2 had similar values of K_m for pyruvate and ATP as Pyc1, but did not measure other kinetic parameters. The K_m for bicarbonate shown in Table 1 is a little lower than that reported by Ruiz-Amil et al. (*17*) (2.4 mM) but much lower than that reported by Myers et al. (*18*) (19.1 mM). Our reaction conditions are similar to those used by Myers et al. (*18*) in that K^+, which is known to markedly reduce the K_m of the enzyme for bicarbonate, was not present in the reaction mixtures.

Apart from the difference in the K_m for bicarbonate described above, perhaps the most marked difference between the kinetic characteristics of Pyc1 and those reported for enzyme preparations from bakers' yeast is the effect of acetyl CoA on enzyme activity. Both Ruiz-Amil et al. (*17*) and Cazzulo and Stoppani (*19*) reported that in the absence of acetyl CoA, yeast pyruvate carboxylase showed 40−50% of the activity in the presence of a saturating concentration of this effector. However, in these studies the potassium salt of bicarbonate was used throughout. Tolbert (*20*) and Myers et al. (*18*) demonstrated that in the absence of K^+ and acetyl CoA, pyruvate carboxylase prepared from bakers' yeast exhibited only about 2% of the activity when acetyl CoA was saturating, and this is similar to our result for Pyc1 which showed about 4% activity in the absence of acetyl CoA. The K_as for acetyl CoA measured in a variety of studies on enzyme prepared from bakers' yeast are, however, fairly consistent: Ruiz-Amil et al. (*17*), ∼10 μM; Cazzulo and Stoppani (*19*), 9 μM; Cooper and Benedict (*21*), ∼5 μM; Myers et al. (*18*), 4 μM. All of these values are 8−20-fold lower than the K_a for Pyc1 shown in Table 1. This large difference in K_a between Pyc1 and enzyme preparations from bakers' yeast containing both Pyc1 and Pyc2 suggests that a major difference between these isozymes lies in their regulation by acetyl CoA, with Pyc2 having a much lower K_a for acetyl CoA than Pyc1. In the study of Myers et al. (*18*) where the K_a for acetyl CoA was determined under conditions most similar to those used in our experiments (no

K^+ present), the highest concentration of acetyl CoA used was 100 μM, and it thus is possible that the major contribution to the observed effect of acetyl CoA was from its action on Pyc2.

Pyc1 is highly dependent on acetyl CoA for activity and in this respect is similar to CLPC, which, in the absence of acetyl CoA, only exhibits about 1% of the activity observed in the presence of a saturating concentration of the effector (*22*). Part of the effect of acetyl CoA on CLPC is to prevent dilution inactivation and cold inactivation in which the native enzymic tetramer rapidly dissociates to form inactive enzyme species (*23, 24*). Branson and Attwood (*5*) recently reported that CLPC undergoes rapid loss of activity when diluted to concentrations as high as 35 units/mL. Pyc1, diluted to 4 units/mL at room temperature or kept on ice, failed to show any loss of activity over a period of 2 h and only 15% loss of activity over 24 h at 4 °C. Thus, Pyc1 does not have an inherently unstable quaternary structure in the way that CLPC has. Stucka et al. (*7*) reported that overnight storage of their Pyc1 preparation at 0 °C resulted in complete loss of activity but since this was only a partially purified preparation the presence of proteolytic enzyme activity may have contributed to this loss of activity. Another effect of acetyl CoA on CLPC is that it enhances the stability of the carboxyenzyme (*14*) whereas this was not the case for Pyc1 (see Figure 3).

Legge et al. (*4*) and Branson and Attwood (*5*) showed that a major locus of the effect of acetyl CoA on the activity of CLPC lay in the stimulation of ATP cleavage and carboxyenzyme formation. Acetyl CoA increased the apparent first-order rate constant of the approach to steady-state for ATP cleavage from 0.028 to 6.6 s^{-1} and for carboxyenzyme formation from 0.028 to 6.1 s^{-1}, increases of over 200-fold (*4*). With Pyc1, in both the presence and absence of acetyl CoA, the approach to steady-state of carboxyenzyme formation appears to comprise two first-order processes, while with ATP cleavage the process appears to be a single first-order reaction. Acetyl CoA had no effect on the rate constant for the approach to steady-state of ATP cleavage (see Table 2) which was about 7.4 s^{-1} in both the presence and absence of acetyl CoA. Thus, in the absence of acetyl CoA, the rate constant for approach to steady-state ATP cleavage is much higher in Pyc1 than in the chicken liver enzyme and is not increased in the presence of acetyl CoA. Comparisons of the rate constants for the approach to steady-state of carboxyenzyme formation in the presence and absence of acetyl CoA are more difficult owing to the large errors on two of the estimates (see Table 2). However, in general, acetyl CoA would appear to increase the rate constants of both phases of the reaction, although not as much as the 200-fold observed with CLPC.

A striking feature of the Pyc1 kinetics is the lower accumulation of carboxyenzyme in the steady-state compared to CLPC. In CLPC, the proportion of total enzyme present in the steady-state as carboxyenzyme was 57% in the presence of acetyl CoA and 68% in its absence (*4*). However, with Pyc1 the figures are 29% and 27%, respectively (see Table 2), suggesting that at steady-state enzyme is accumulating to a higher degree in an enzyme species other than carboxyenzyme.

Another feature of the approach to steady-state of carboxyenzyme formation is that it is biphasic in Pyc1 and monophasic in CLPC under similar conditions (*4*). A later study showed that in the presence of 10 mM Mg^{2+} the approach to steady-state was also biphasic in CLPC. However, this only occurred in the presence of acetyl CoA and occurred in both carboxyenzyme formation and ATP cleavage with the rate constants for the slow phases (2.1 and 5.5 s^{-1}, respectively) being much higher than those recorded for Pyc1 (*5*).

To analyze the observed kinetics of approach to steady-state for both ATP cleavage and carboxyenzyme formation in CLPC, Branson and Attwood (*5*) proposed a reaction model based on a simpler scheme of Legge et al. (*4*). Branson and Attwood (*5*) showed that the scheme could produce simulated data that closely matched the experimental data. Scheme 1 in the current work is based on the proposal of Branson and Attwood (*5*) with two simplifications. First, Branson and Attwood (*5*) had to account for the dilution inactivation of CLPC in the absence of acetyl CoA and subsequent reactivation on mixing with ATP and hence proposed that a proportion of enzyme is initially inactive, but becomes active during the reaction. Since Pyc1 does not undergo dilution inactivation, this part of the reaction model has been omitted from Scheme 1. Thus, at time zero, the enzyme exists in an equilibrium between two forms: E-biotin and E-biotin′, where the biotin is at the site of reaction 1 in E-biotin and reaction 2 in E-biotin′. Second, in CLPC it is known that carboxyenzyme can decarboxylate via a slow mechanism which does not involve dissociation of carboxybiotin from the site of the biotin carboxylation reaction. This has not been determined for Pyc1, but in CLPC, it accounts for at most 10% of the carboxyenzyme decarboxylation reaction (*5*) and has been omitted from Scheme 1.

In the studies by Legge et al. (*4*) and Branson and Attwood (*5*), simulated data were calculated for the reaction schemes using numerical integration methods and the values of the rate constants varied until the simulated data matched the experimental data. The Scientist program allows a combination of numerical integration and nonlinear least-squares regression analysis using differential equations to describe a reaction scheme (see Appendix). Using Scientist, the combined ATP cleavage and carboxyenzyme formation data in the absence of acetyl CoA were fitted to Scheme 1, and the process was repeated for the data in the presence of acetyl CoA. The following simplifications, as used by Branson and Attwood (*5*), were that k_{-1} and k_{-3} were fixed at zero, and k_4 was fixed at 0.007 s^{-1}, as determined from the measurements of decay of the carboxyenzyme shown in Figure 3. These fits and simulations of data for the other enzyme species in Scheme 1 are shown in Figure 4. As can be seen in Figure 4a, the fit of the data to Scheme 1 in the absence of acetyl CoA is good for both ATP cleavage and carboxyenzyme formation. In the presence of acetyl CoA, the fit is good for the ATP cleavage data, but less so for the carboxyenzyme data; however, a fit of the simulated carboxyenzyme formation data to eq iii gave values of A, A', and k that were similar to those shown in Table 2, with only the value of k' being markedly lower at 0.17 s^{-1}. A feature of both Figure 4a and Figure 4b is the high proportion of the enzyme present as E-biotin′ throughout the reaction. This differs from the situation in CLPC, especially in the presence of acetyl CoA and 10 mM Mg^{2+} where nearly all of the enzyme is present as carboxyenzyme after 2 s of reaction (*5*).

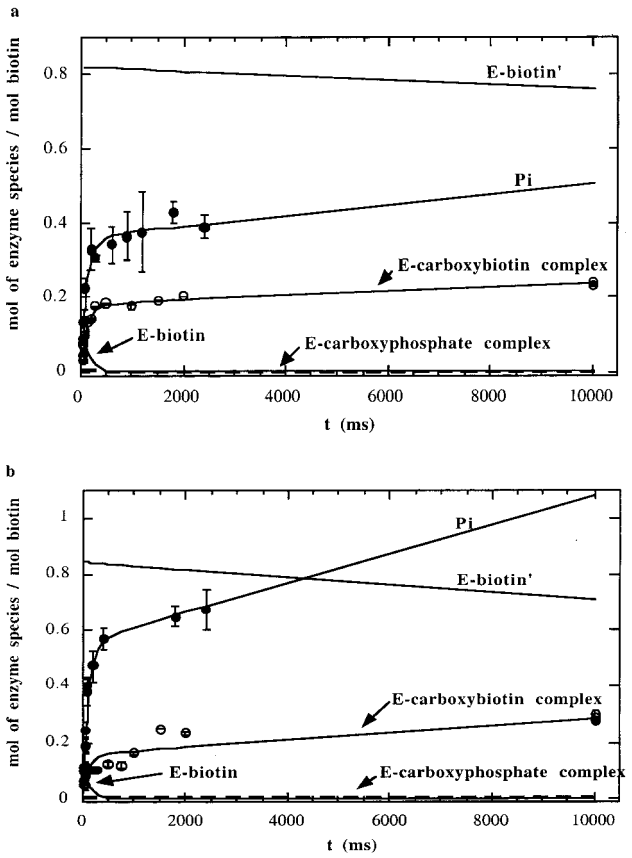


FIGURE 4: Data simulated from Scheme 1 in the absence of acetyl CoA (a) and the presence of acetyl CoA (b) using the parameters shown in Table 3.

Table 3: Parameter Values Used in the Simulations of Scheme 1 for the Reactions in the Presence or Absence of Acetyl CoA[a]

acetyl CoA	k_1	k_2	k_3	k_4	k_5	E-b_0[b]
absent	16.2	763	763	0.007	0.009	0.18
present	32.2	2111	819	0.007	0.019	0.16

[a] All values of rate constants have units of s^{-1}. [b] E-b_0 (=E-biotin$_0$) is the amount of this enzyme species present at zero time, expressed as a proportion of total enzyme, and has units of moles of enzyme species per mole of biotin.

The estimates of the rate constants and the value of E-biotin at zero time (E-biotin$_0$) are shown in Table 3. As Branson and Attwood (*5*) found, the values of k_2 and k_3 could be increased and fixed without affecting the fit of the data to Scheme 1, as long as they were maintained in the same ratio shown in Table 3. In the same way, these values could be reduced without effect as long as they were greater than those of k_1; in other words, k_1 is rate-limiting in ATP cleavage and carboxyenzyme formation from E-biotin. Branson and Attwood (*5*) found that acetyl CoA reduced the ratio of k_2/k_3 in CLPC, thus enhancing the coupling between ATP cleavage and biotin carboxylation to form carboxyenzyme. Acetyl CoA increased the minimum possible values of k_2 and k_3, but it was not possible to ascertain whether k_2 had decreased relative to k_3 or k_3 had increased relative to k_2 or a combination of both had occurred. In Pyc1, however, this ratio increases from 1 in the absence of acetyl CoA to 2.6 in its presence, one possibility being that acetyl CoA may make the carboxyphosphate intermediate more reactive, which also has the effect, under these conditions, of decreasing its stability and enhancing k_2.

From Table 3, it would appear that k_4 is rate-limiting in the catalytic cycle, followed by k_5 which is about 2-fold higher in the presence of acetyl CoA compared to that in its absence. Thus, acetyl CoA enhances to a degree the rate of movement of biotin from the site of reaction 2 to that of reaction 1. Acetyl CoA enhances the rate constant for ATP cleavage (k_1) by a factor of 2. The value of k_1 in both the presence and absence of acetyl CoA is greater than the turnover numbers for the complete pyruvate carboxylation reaction under corresponding conditions (25.5 and 0.88 s^{-1}, respectively). This suggests that ATP cleavage is not the rate-limiting step in the overall reaction in Pyc1. Unlike the reaction step governed by k_4, that governed by k_5 would be expected to be a normal part of the catalytic cycle in the full pyruvate carboxylation reaction. In Pyc1, the reaction governed by k_5 is too slow to be part of pyruvate carboxylation catalytic cycle in both the presence and the absence of acetyl CoA. Branson and Attwood (*5*) found that this step could be the rate-limiting step for the pyruvate carboxylation reaction in CLPC in the absence of acetyl CoA but was too slow to be part of the reaction in the presence of acetyl CoA. Branson and Attwood (*5*) suggested that the presence of pyruvate may enhance the rate of this reaction or enable it to proceed via a slightly different mechanism, and this would appear to be the case for Pyc1.

In summary, Pyc1 appears to have a much higher K_m for bicarbonate and K_a for acetyl CoA than values cited in the literature for enzyme isolated from baker's yeast, comprising a mixture of Pyc1 and Pyc2. This difference in K_a values for acetyl CoA suggests that Pyc1 and Pyc2 may be differentially regulated by acetyl CoA, with Pyc2 being fully activated at lower concentrations of this effector than Pyc1. In the absence of acetyl CoA, Pyc1 displays very low activity and is akin to CLPC in this respect. However, unlike in CLPC, where a major effect of acetyl CoA is to enhance the rate of the ATP cleavage step in the reaction mechanism, this is very much less marked in Pyc1, suggesting that another reaction step or steps, outside of those leading to the carboxylation of biotin, is (are) the major locus of action of acetyl CoA.

APPENDIX

The differential equations that were used in the nonlinear regression analysis and simulation of Scheme 1 were as follows:

$$[\text{E-biotin}]' = k_2[\text{Mg·E-biotin·}^{-}\text{O}_3\text{POCO}_2^{-}\text{·MgADP}] + k_5[\text{E-biotin}'] - k_1[\text{E-biotin}] \quad \text{(A1)}$$

$$[\text{Mg·E-biotin·}^{-}\text{O}_3\text{POCO}_2^{-}\text{·MgADP}]' = k_1[\text{E-biotin}] - (k_2 + k_3)[\text{Mg·E-biotin·}^{-}\text{O}_3\text{POCO}_2^{-}\text{·MgADP}] \quad \text{(A2)}$$

$$[\text{Mg·E-biotin-CO}_2^{-}]' = k_3[\text{Mg·E-biotin·}^{-}\text{O}_3\text{POCO}_2^{-}\text{·MgADP}] - k_4[\text{Mg·E-biotin-CO}_2^{-}] \quad \text{(A3)}$$

$$[\text{E-biotin}'] = k_4[\text{Mg·E-biotin-CO}_2^{-}] - k_5[\text{E-biotin}'] \quad \text{(A4)}$$

$$[\text{P}_i \text{ cleaved from ATP}]' = k_1[\text{E-biotin}] \quad \text{(A5)}$$

In eq A5, the rate of cleavage of P_i from ATP can be

expressed in this way since the reaction step governed by k_1 is the step in which the γ-phosphate is cleaved from ATP. The reaction is quenched in HCl and treated with trichloroacetic acid; the enzyme is thus denatured, and since carboxyphosphate is a very labile intermediate, this intermediate is almost certainly completely hydrolyzed, liberating P_i. Thus, the measured P_i includes that from carboxyphosphate, any enzyme-bound P_i, and free P_i, and so truly represents P_i cleaved from ATP. All concentrations are expressed as moles per mole of active enzyme present at the end of the reaction.

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