# Locus of Action of Acetyl CoA in the Biotin–Carboxylation Reaction of Pyruvate Carboxylase†

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ABSTRACT: The [$^{14}$C]carboxyphospho–enzyme complex formed by incubation of the enzyme with $H^{14}CO_3^-$, MgATP, and $Mg^{2+}$ was prepared and isolated by gel filtration as described by Phillips et al. [(1992) *Biochemistry 31*, 9445–9450]. When time courses of transfer of the [$^{14}$C]carboxyl group from the complex to pyruvate were studied, it was found that at the first time point (15 s) the formation of [$^{14}$C]oxalacetate was the same in the presence or absence of acetyl CoA. However, in the absence of acetyl CoA, the radioactivity fixed in [$^{14}$C]oxalacetate declined rapidly over the subsequent 15 min, whereas in the presence of acetyl CoA the formation of [$^{14}$C]oxalacetate continued up to about 10 min. The decline in [$^{14}$C]-oxalacetate in the absence of acetyl CoA was found to be due to enzyme-dependent decarboxylation of the oxalacetate by the enzyme. Incubation of the isolated [$^{14}$C]carboxyphospho–enzyme complex with MgADP and $Mg^{2+}$ resulted in no significant reduction in the formation of [$^{14}$C]oxalacetate on addition of acetyl CoA and pyruvate. Incubation of the isolated [$^{32}$P]carboxyphospho–enzyme complex with pyruvate resulted in no significant reduction in the formation of [$\gamma$-$^{32}$P]ATP on the addition of MgADP and $Mg^{2+}$. This new evidence casts doubt on the suggested locus of activation of the enzyme by acetyl CoA being the facilitation of the transfer of the carboxyl group from carboxyphosphate to biotin and indeed on the identity of the isolated enzyme intermediate [Phillips et al. (1992) *Biochemistry 31*, 9445–9450].

Pyruvate carboxylase (EC 6.4.1.1) is a biotin-dependent enzyme that catalyzes the following overall reaction:

$$\text{MgATP} + HCO_3^- + \text{pyruvate} \overset{Mg^{2+},\ \text{acetyl CoA}}{\rightleftharpoons} \text{MgADP} + P_i + \text{oxalacetate} \quad (1)$$

There is a considerable body of evidence [see Attwood and Keech (1984)] that reaction 1 proceeds in two stages: In the first stage (reaction 2) the biotin moiety that is covalently attached to the enzyme is carboxylated in a reaction that requires $Mg^{2+}$ and is stimulated by acetyl CoA. In the second stage, the carboxyl group is transferred from carboxybiotin to pyruvate to form oxalacetate (reaction 3) in a reaction that

$$\text{MgATP} + HCO_3^- + \text{ENZ–biotin} \overset{Mg^{2+},\ \text{acetyl CoA}}{\rightleftharpoons} \text{MgADP} + P_i + \text{ENZ–biotin–}CO_2^- \quad (2)$$

$$\text{ENZ–biotin–}CO_2^- + \text{pyruvate} \rightleftharpoons \text{ENZ–biotin} + \text{oxalacetate} \quad (3)$$

does not require $Mg^{2+}$. The role of acetyl CoA in reaction 3 is not clear since in studies on the pyruvate/oxalacetate isotope exchange reaction, acetyl CoA was found not to affect the exchange rate in the chicken liver enzyme (Scrutton et al., 1965) but did increase the rate by about 50% in the rat liver enzyme (McClure et al., 1971). What is apparent from direct measurements using the isolated enzyme–carboxybiotin complex (Goodall et al., 1981; Attwood et al., 1984) is that the transfer of the carboxyl group to pyruvate occurs readily in the absence of acetyl CoA. In chicken liver pyruvate carboxylase there is a very high dependence on the presence of acetyl CoA for activity, and under optimal conditions of high pyruvate concentrations, the highest maximum velocity of reaction in the absence of acetyl CoA has been reported as 2% of that in the presence of saturating acetyl CoA when the reaction was performed using higher than normal concentrations of pyruvate, $HCO_3^-$, and MgATP and in the presence of 100 mM $K^+$ (Phillips et al., 1992).

Reaction 2 is thought to proceed via an enzyme–carboxyphosphate complex as shown in reactions 4 and 5. The

$$\text{MgATP} + HCO_3^- + \text{ENZ–biotin} \overset{Mg^{2+}}{\rightleftharpoons} \left[\begin{array}{c} {}^{2-}O_3POCO_2^- \\ | \\ \text{ENZ–biotin} \end{array}\right] + \text{MgADP} \quad (4)$$

$$\left[\begin{array}{c} {}^{2-}O_3POCO_2^- \\ | \\ \text{ENZ–biotin} \end{array}\right] \overset{\text{acetyl CoA}}{\rightleftharpoons} \text{ENZ–biotin–}CO_2^- + P_i \quad (5)$$

evidence for this comes from work with propionyl CoA carboxylase using $HC^{18}O_3^-$ (Kaziro et al., 1962) in which $HCO_3^-$ was shown to directly participate in the cleavage of MgATP and from the fact that carbamyl phosphate, which is a structural analogue of carboxyphosphate, will phosphorylate MgADP in a reaction catalyzed by pyruvate carboxylase (Ashman & Keech, 1975; Attwood & Graneri, 1991). Recently, there have been reports of the isolation of a CPE[1] using chicken liver pyruvate carboxylase (Wallace et al., 1985; Phillips et al., 1992). The CPE was formed by incubation of

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[1] Abbreviations: CPE, carboxyphospho–enzyme complex, the prefix [$^{14}$C] indicates that the carboxyl group of the carboxyphospho–enzyme complex was derived from $H^{14}CO_3^-$, while the prefix [$^{32}$P] indicates that the phosphate group was derived from [$\gamma$-$^{32}$P]ATP; SD, standard deviation of the mean; *n*, number of determinations of experimental data used to calculate the mean; cpm, counts per minute; EDTA, ethylenediaminetetraacetic acid.

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the enzyme with $HCO_3^-$, ATP, and $Mg^{2+}$ in the absence of acetyl CoA and isolated by gel filtration (Phillips et al., 1992). When [$\gamma$-$^{32}$P]ATP was used, radioactivity could be transferred from the isolated [$^{32}$P]CPE to ADP to form [$\gamma$-$^{32}$P]ATP. When $H^{14}CO_3^-$ was used, radioactivity could be transferred from the isolated [$^{14}$C]CPE to pyruvate to form [$^{14}$C]oxalacetate, but at a significant rate only in the presence of acetyl CoA (Wallace et al., 1985). Wallace et al. (1985) and Phillips et al. (1992) thus proposed that the CPE was enzyme–carboxyphosphate and that the major locus of action of acetyl CoA in activating pyruvate carboxylase was in facilitating the transfer of the carboxyl group from carboxyphosphate to biotin.

The ability to form and isolate enzyme–carboxyphosphate complex (reaction 4) and then, by the addition of acetyl CoA, induce the transfer of the carboxyl group to biotin (reaction 5) appeared to offer a means of studying the individual steps in the reaction leading to the carboxylation of biotin. This work initially set out to do this; however, it was found that the apparent slowness of the isolated CPE to transfer its carboxyl group to pyruvate in the absence of acetyl CoA was due to an artifact of the assay. In this paper, the reactions that occur on the addition of pyruvate to the isolated [$^{14}$C]CPE are described. It is shown that transfer of the carboxyl group from the isolated CPE to pyruvate occurs equally well in the presence or absence of acetyl CoA. In addition, reaction of the CPE with MgADP in the presence of $Mg^{2+}$ consumes little of its carboxylating activity, while reaction with pyruvate consumes little of its phosphorylating activity. These data indicate that the CPE is not the enzyme–carboxyphosphate complex.

## MATERIALS AND METHODS

*Preparation and Assay of Chicken Liver Pyruvate Carboxylase.* Chicken liver pyruvate carboxylase was prepared to a specific activity of 35 units/mg as described by Goss et al. (1979) except that a DEAE-Sepharose column was used in place of the DEAE-Sephadex column. One unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 $\mu$mol of oxalacetate/min under saturating substrate conditions at 25 °C. Assays of pyruvate carboxylating activity were carried out spectrophotometrically as described by Attwood and Cleland (1986) at pH 7.8, where 0.1 M *N*-ethylmorpholine chloride replaced the Tricine buffer.

*Oxalacetate Decarboxylation Assays.* Assays of oxalacetate decarboxylating activity of pyruvate carboxylase in the presence or absence of 250 $\mu$M acetyl CoA at 25 °C in 0.1 M *N*-ethylmorpholine buffer and in the absence of any other substrates were also performed as described by Attwood and Cleland (1986) using 1 mM oxalacetate and 4.4 units of pyruvate carboxylase.

*Isolation of the CPE.* Initially, an isolation procedure using the rapid centrifuge desalting method of Helmerhorst and Stokes (1980) was assessed by processing the reaction mixture for the formation of [$^{14}$C]CPE similar to that described by Phillips et al. (1992), minus the enzyme. This procedure was found to be unsatisfactory since significant amounts of $H^{14}CO_3^-$, ATP, and $Mg^{2+}$ were present in the eluates from this procedure (data not shown). Thus, in all experiments described here the CPE was prepared and isolated exactly as described by Phillips et al. (1992) except that an 83 × 0.7 cm column of Sephadex G-25 was used to isolate the complex in place of a 45 × 1.4 cm column. Fractions of 1.2–1.4 mL were collected, and total radioactivity present in each fraction was measured by placing 50 $\mu$L of each fraction into 5 mL of scintillation fluid containing Triton X-100 and counting. Fractions were kept on ice until required for the time course experiments and for spectrophotometric assay of enzyme activity. Where the [$^{14}$C]CPE was prepared, the amount of transferrable [$^{14}$C]carboxyl group present in fractions thought to contain the complex was measured essentially as described by Phillips et al. (1992), i.e., 25 $\mu$L of each fraction was incubated with 25 $\mu$L of 0.1 M *N*-ethylmorpholine containing 500 $\mu$M acetyl CoA and either 20 or 200 mM pyruvate for 5 min at 25 °C. The reactions were then stopped by the addition of 25 $\mu$L of 1 M HCl saturated with semicarbazide–HCl, which also served to stabilize the [$^{14}$C]oxalacetate. The acid-stable radioactivity corresponding to the [$^{14}$C]oxalacetate was quantitated as described by Attwood et al. (1984). Where the [$^{32}$P]CPE was isolated, total radioactivity and the amount of transferrable $^{32}P_i$ present in fractions thought to contain the complex were measured in a similar way to that described by Phillips et al. (1992). For the transfers to ADP, 200 $\mu$L of each fraction was incubated for 15 min at 30 °C with 25 $\mu$L of water and 25 $\mu$L of 1 M *N*-ethylmorpholine hydrochloride, pH 7.8, containing 64 mM $MgCl_2$ with or without 20 mM ADP. The reactions were stopped by the addition of 50 $\mu$L of ice-cold 5 M $HClO_4$ and the samples placed on ice for 10 min; the denatured protein was then pelleted by centrifugation in a microfuge. The [$\gamma$-$^{32}$P]ATP was then separated from $^{32}P_i$ as described by Rubio et al. (1981), and the radioactivity in the resultant solution of [$\gamma$-$^{32}$P]ATP was determined using Cherenkov counting. The radioactivity of the samples without ADP was subtracted from that of those with ADP present to give an accurate measure of radioactivity due to [$\gamma$-$^{32}$P]ATP formation by transfer of $^{32}P_i$ from the [$^{32}$P]CPE.

*Time Courses for the Transfer of [$^{14}$C]Carboxyl Groups to Pyruvate from the [$^{14}$C]CPE.* When most of the transferrable $^{14}$C was found in a single fraction from the Sephadex G-25 column, only this fraction was used in subsequent experiments. Frequently, however, the bulk of the transferrable $^{14}$C was split between two fractions; in this case the fractions were pooled and used in subsequent experiments. Aliquots of the enzyme complex were mixed with an equal volumn of transfer mix comprising 0.1 M *N*-ethylmorpholine, pH 7.8, containing either 20 or 200 mM pyruvate and either 0 or 500 $\mu$M acetyl CoA. The reaction mixtures thus contained 10 or 100 mM pyruvate and 0 or 250 $\mu$M acetyl CoA. At various times after mixing, 50-$\mu$L aliquots were removed from the reaction mixtures and added to 25 $\mu$L of 1 M HCl saturated with semicarbazide–HCl to stop the reactions and stabilize the [$^{14}$C]oxalacetate. The radioactivity in the samples was then determined as described above. Thus, each experiment comprised a time course in the presence of acetyl CoA run simultaneously with one in its absence. Where the newly formed [$^{14}$C]oxalacetate was converted to [$^{14}$C]malate, the transfer mixes also contained 22 units/mL of malate dehydrogenase and 0.4 mM NADH.

*Inhibition of [$^{14}$C]Oxalacetate Decarboxylation by Avidin.* In each experiment, two time courses were initiated as above using transfer mixes containing 20 mM pyruvate and no acetyl CoA. The initial 50-$\mu$L aliquots were removed as usual at 15 s, but at 1 min, 1 mg of avidin was added to one reaction mixture while to the second reaction mixture was added 1 mg of avidin that had been incubated with biotin that was present in 10-fold excess over the biotin-binding capacity of the avidin. These additions were made in volumes of solution that comprised 14% of the volumes of the reaction mixtures. The experiments then proceeded as described above.

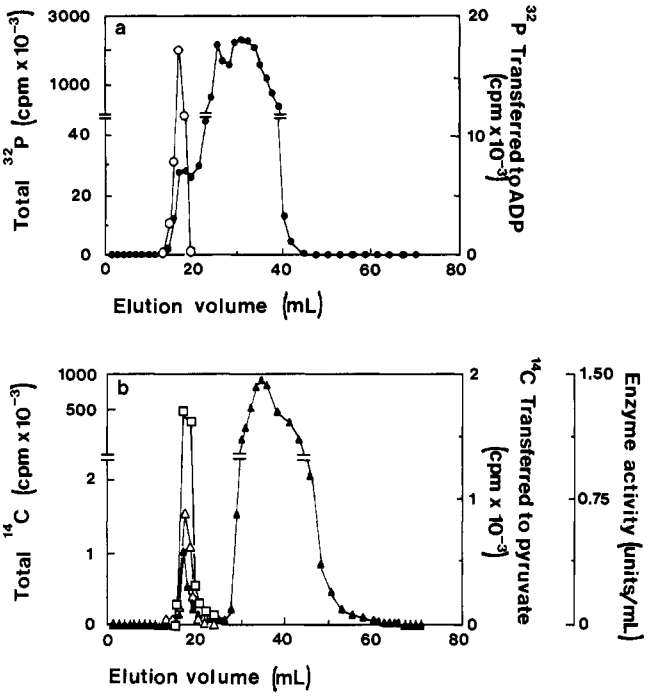


FIGURE 1: Isolation of the carboxyphospho–enzyme complex. To prepare the CPE from chicken liver pyruvate carboxylase, the enzyme was incubated with $Mg^{2+}$, ATP, and $HCO_3^-$ as described under Materials and Methods, where either (a) [$\gamma$-$^{32}$P]ATP or (b) $H^{14}CO_3^-$ was used. The isolation of the complexes was achieved by gel filtration on Sephadex G-25 at 4 °C with elution by 0.1 M *N*-ethylmorpholine acetate, pH 7.2, at 0.6 mL/min, and subsequent assays of the fractions are described under Materials and Methods. Symbols: enzyme activity (□); $^{14}$C transferred to pyruvate (△); $^{32}$P transferred to ADP (○); total radioactivity $^{14}$C (▲); $^{32}$P (●). The data are expressed as counts per minute in the aliquot of each fraction that was assayed.

*Quenching of [$^{14}$C]Carboxyl Group Transfer Reactions with $H^{12}CO_3^-$ and EDTA.* In the experiments where further fixation of $H^{14}CO_3^-$ was quenched on addition of the transfer mixes, these mixes also contained 400 mM $NaH^{12}CO_3$ and 200 mM EDTA (pH adjusted to 7.8) so that the final concentrations of EDTA and $H^{12}CO_3^-$ in the reaction mixtures were 200 and 100 mM, respectively. Controls were performed in which the transfer mixes contained no pyruvate and the acid-stable counts per minute in these controls were subtracted from the corresponding sample acid-stable counts per minute. At the concentrations described above, the combination of EDTA and $H^{12}CO_3^-$ was shown to completely inhibit the incorporation of $^{14}$C from $H^{14}CO_3^-$ into [$^{14}$C]oxalacetate in the reaction catalyzed by pyruvate carboxylase in the presence of acetyl CoA, ATP, and $Mg^{2+}$ (data not shown).

*Incubation of the [$^{14}$C]CPE with $Mg^{2+}$ and MgADP prior to Pyruvate.* To 90-$\mu$L aliquots of the enzyme complex on ice was added 10 $\mu$L of ice-cold water (solution A) or solution A with 1 mM glucose and 25 units of hexokinase (solution B), solution B with 80 mM $MgCl_2$ (solution C), or solution C with 20 mM ADP (solution D). The purpose of the glucose and hexokinase was to remove any ATP formed in the reaction between the ADP and the [$^{14}$C]CPE and thus prevent this ATP being used by pyruvate carboxylase in any further reactions. After 15 min on ice, 25-$\mu$L aliquots from each sample were added to 25 $\mu$L of transfer mix containing 500 $\mu$M acetyl CoA and 20 mM pyruvate. After 15 s at 25 °C, 25 $\mu$L of 1 M HCl saturated with semicarbazide–HCl was added to each sample and the acid-stable counts per minute determined as described above.

*Incubation of the [$^{32}$P]CPE with Pyruvate prior to Transfer to MgATP.* Isolated [$^{32}$P]CPE was incubated on ice for 15 min with 0.24 mM NADH and 11 units/mL malate dehydrogenase in the presence or absence of 10 mM pyruvate. Aliquots of these solutions were added to transfer solutions containing either $MgCl_2$ or $MgCl_2$ plus ADP, and the radioactivity incorporated into [$\gamma$-$^{32}$P]ATP was measured as described above. The purpose of the NADH and malate dehydrogenase was to ensure the removal of the oxalacetate formed by the reaction between the CPE and pyruvate and hence prevent its utilization by pyruvate carboxylase to synthesize [$\gamma$-$^{32}$P]ATP from any $^{32}P_i$ present and the added ADP.

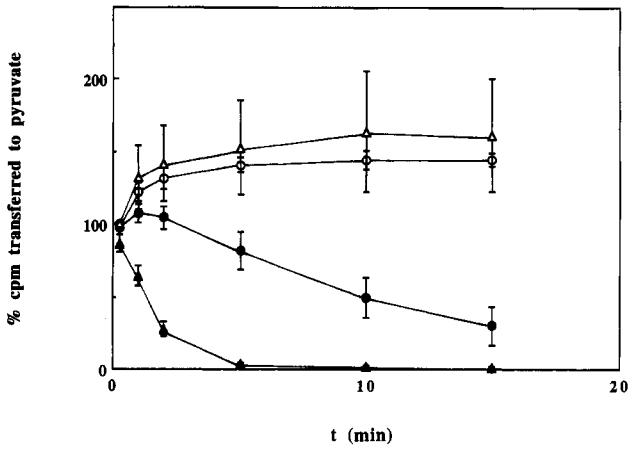


FIGURE 2: Time courses for the transfer of [$^{14}$C]carboxyl groups to pyruvate from the isolated [$^{14}$C]carboxyphospho–enzyme complex in the presence or absence of acetyl CoA. The transfers were performed at 25 °C by adding an equal volume of the complex to 0.1 M *N*-ethylmorpholine chloride, pH 7.8, with (open symbols) or without (solid symbols) 500 $\mu$M acetyl CoA with either 20 (△, ▲; $n$ = 3) or 200 mM pyruvate (○, ●; $n$ = 7). The error bars indicate the SD of the mean of $n$ determinations. At various times thereafter, aliquots from the reaction mixture were added to semicarbazide–HCl in HCl to stop the reaction and stabilize the [$^{14}$C]oxalacetate. Data are expressed as a percent of the acid-stable counts per minute transferred to pyruvate in the presence of acetyl CoA at 15 s in each experiment, and there were $n$ experiments.

## RESULTS

*Isolation of the CPE.* The elution profiles of reaction mixtures containing either $H^{14}CO_3^-$ or [$\gamma$-$^{32}$P]ATP from the Sephadex G-25 column are shown in Figure 1. As can be seen in Figure 1a, the peak of total radioactivity that eluted at about 17 mL, ahead of the major peaks of radioactivity ([$\gamma$-$^{32}$P]ATP and $^{32}P_i$), also coincided with a peak of radioactivity transferred to ADP. Similarly, in Figure 1b there is a peak of total radioactivity that eluted at about 17 mL, ahead of the major peak of radioactivity ($H^{14}CO_3^-$), that also coincided with a peak of acid-stable radioactivity transferred to pyruvate and a peak of enzymic activity. These peaks also eluted in approximately the same volume as the corresponding peak seen in Figure 1a. Both elution profiles are similar to those observed by Phillips et al. (1992).

*Time Courses for the Transfer of [$^{14}$C]Carboxyl Groups to Pyruvate from the Isolated [$^{14}$C]CPE in the Presence or Absence of Acetyl CoA.* Figure 2 shows time courses for the transfers to 10 or 100 mM pyruvate in the presence or absence of acetyl CoA at 25 °C. The absolute counts per minute transferred from the CPE to pyruvate depended on the exact amount of pyruvate carboxylase used to prepare the complex and the distribution between fractions of the complex eluted from the Sephadex G-25. In addition, since the isolated complex spontaneously decarboxylates with time (Phillips et al., 1992), the absolute counts per minute transferred to

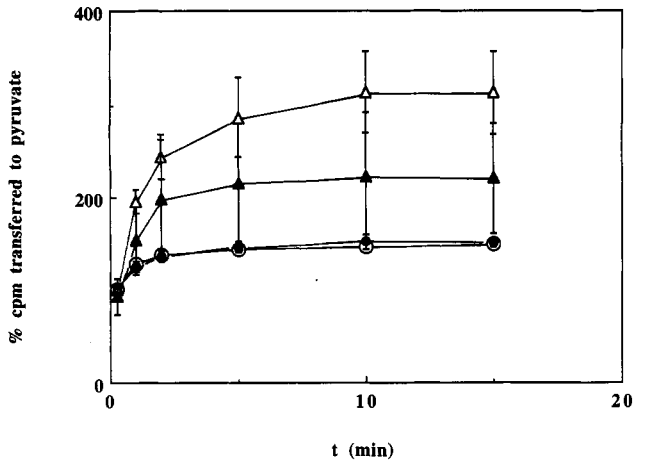


FIGURE 3: Effect of conversion of newly formed [$^{14}$C]oxalacetate to [$^{14}$C]malate on the time courses of transfer of [$^{14}$C]carboxyl groups to pyruvate from the isolated [$^{14}$C]carboxyphospho–enzyme complex. The transfers were performed at 25 °C by adding an equal volume of the complex to 0.1 M *N*-ethylmorpholine chloride, pH 7.8, containing 22 units/mL malate dehydrogenase and 0.4 mM NADH with (open symbols) or without (solid symbols) 500 $\mu$M acetyl CoA with either 20 (△, ▲; $n$ = 4) or 200 mM pyruvate (○, ●; $n$ = 3). At various times thereafter, aliquots from the reaction mixture were added to semicarbazide–HCl in HCl to stop the reaction. The error bars indicate the SD of the mean of $n$ determinations. Data are expressed as a percent of the acid-stable counts per minute transferred to pyruvate in the presence of acetyl CoA at 15 s in each experiment, and there were $n$ experiments.

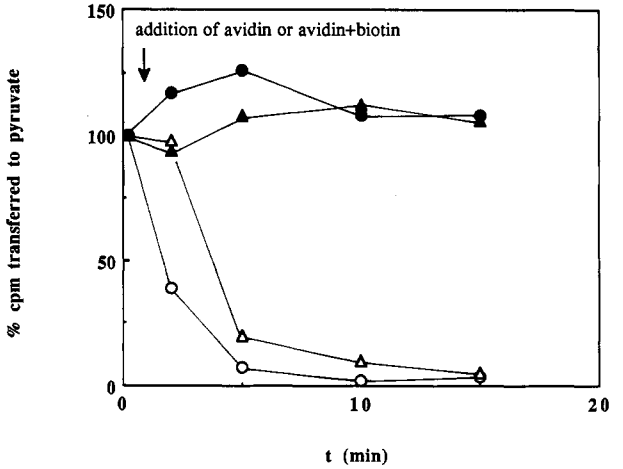


FIGURE 4: Effect of avidin on transfer of [$^{14}$C]carboxyl groups to pyruvate from the isolated [$^{14}$C]carboxyphospho–enzyme complex. This shows two experiments where transfers were performed at 25 °C by adding an equal volume of the complex to 0.1 M *N*-ethylmorpholine chloride, pH 7.8, containing 20 mM pyruvate and no acetyl CoA and at various times thereafter adding aliquots from the reaction mixture to semicarbazide–HCl in HCl to stop the reaction and stabilize the [$^{14}$C]oxalacetate. At 1 min after the start of the transfer, as indicated in the figure, 1 mg of avidin (solid symbols) or 1 mg of avidin that had been preincubated with a 10-fold excess of biotin (open symbols) was added to each reaction mixture. Data are expressed as a percent of the acid-stable counts per minute transferred to pyruvate at 15 s.

pyruvate also depended on the time after isolation of the complex that the time course was measured. The average maximum (15-min time point) acid-stable counts per minute measured in the presence of acetyl CoA were 854 ± 177 (±SD; $n$ = 12) with 100 mM pyruvate and 380 ± 117 (±SD; $n$ = 8) with 10 mM pyruvate. To compare between experiments, the radioactivity transferred to pyruvate is expressed as a percentage of that transferred at 15 s in the presence of acetyl CoA in the other time course of the experiment. In the presence of acetyl CoA there was a rise in the radioactivity fixed in [$^{14}$C]oxalacetate over the first 10 min with both 10 and 100 mM pyruvate. In the absence of acetyl CoA, however, over the 15 min time course there was a net decline in radioactivity fixed in [$^{14}$C]oxalacetate with both 10 and 100 mM pyruvate compared to the 15-s time point. The decline in acid-stable radioactivity was much more rapid with 10 mM pyruvate ($t_{1/2} \approx 1$ min) than with 100 mM pyruvate ($t_{1/2} \approx 7$ min), and in the latter case there was a small increase in radioactivity up to 1 min, whereas with 10 mM pyruvate there was an immediate decline in radioactivity fixed. At the initial time points of 15 s, there appear to be only small differences in radioactivity fixed when the data obtained in the presence or absence of acetyl CoA are compared. These data show that there is a very rapid initial formation of [$^{14}$C]oxalacetate which is not dependent on the presence of acetyl CoA. In the absence of acetyl CoA, however, the newly formed [$^{14}$C]oxalacetate is rapidly decarboxylated, and this process is inhibited in the presence of high pyruvate concentrations. To confirm this interpretation of the results, transfers of radioactivity from the [$^{14}$C]CPE to pyruvate were performed in the presence of malate dehydrogenase and NADH, so that the newly formed [$^{14}$C]oxalacetate was immediately converted to the more stable [$^{14}$C]malate. The results of this experiment are shown in Figure 3. With both 10 and 100 mM pyruvate in the absence of acetyl CoA, there was no time-dependent loss of acid-stable counts per minute. With 100 mM pyruvate, the time courses in the presence or absence of acetyl CoA were almost identical. With 10 mM pyruvate, with the exception of the 15-s time point, the transfer of radioactivity to pyruvate in the absence of acetyl CoA was, in general, lower than that in its presence. These results indicate that the cause of the time-dependent loss of acid-stable counts per minute in the absence of acetyl CoA observed in Figure 2 was decarboxylation of the newly formed [$^{14}$C]oxalacetate and that conversion of the [$^{14}$C]oxalacetate to [$^{14}$C]malate prevents this loss of acid-stable counts per minute.

*Inhibition of [$^{14}$C]Oxalacetate Decarboxylation by Avidin.* Figure 4 shows the effect of the addition of avidin on the stability of [$^{14}$C]oxalacetate formed by the transfer of radioactivity from the [$^{14}$C]CPE to 10 mM pyruvate in the absence of acetyl CoA. Addition of the avidin prevented any loss of acid-stable counts per minute, while addition of avidin that had been preincubated with excess biotin so that all of the biotin-binding sites on the avidin were occupied by free biotin did not prevent this loss. Avidin binds biotin with high affinity and is a potent inhibitor of pyruvate carboxylase (Duggleby et al., 1982); thus, the stability of the acid-stable counts per minute in the presence of avidin indicates that the time-dependent loss of acid-stable counts per minute shown in Figure 2 is due to the decarboxylation of the newly formed [$^{14}$C]oxalacetate by pyruvate carboxylase.

*Oxalacetate Decarboxylation by Pyruvate Carboxylase in the Absence of Other Substrates.* In the absence of any other substrates, the rate of pyruvate carboxylase-catalyzed oxalacetate decarboxylation in the presence of acetyl CoA was $1.10 \times 10^{-2} \pm 0.04 \times 10^{-2}$ $\mu$mol min$^{-1}$ mg$^{-1}$ (±SD; $n$ = 4), which is about 0.031% of the $V_{max}$ of pyruvate carboxylating activity. In the absence of acetyl CoA, however, the rate of oxalacetate decarboxylation was $1.83 \times 10^{-2} \pm 0.06 \times 10^{-2}$ $\mu$mol min$^{-1}$ mg$^{-1}$ (±SD; $n$ = 4). Thus, there is a 40% inhibition of pyruvate carboxylase-catalyzed oxalacetate decarboxylation in the presence of acetyl CoA.

*Slow, Time-Dependent Increase in Acid-Stable Counts per Minute on Transfer of [$^{14}$C]Carboxyl Groups from the [$^{14}$C]CPE to Pyruvate in the Presence of Acetyl CoA.* Figure

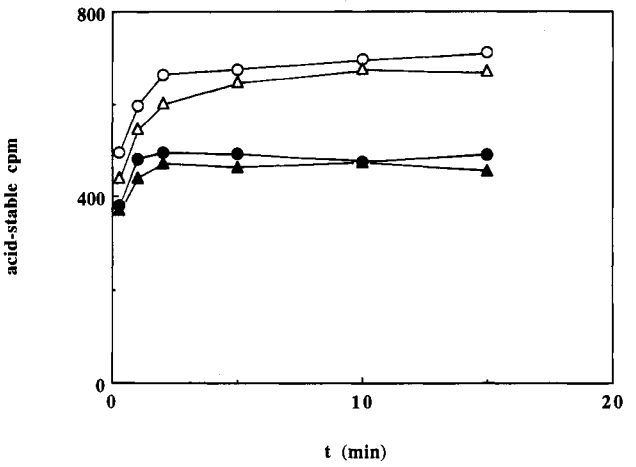


FIGURE 5: Effects of the quenching agent EDTA + $H^{12}CO_3^-$ on transfer of [$^{14}$C]carboxyl groups to pyruvate from the isolated [$^{14}$C]carboxyphospho–enzyme complex. This shows two experiments in which transfers were performed at 25 °C by adding an equal volume of the complex to 0.1 M *N*-ethylmorpholine chloride, pH 7.8, containing 200 mM pyruvate and 500 μM acetyl CoA and in the presence (solid symbols) or absence (open symbols) of 400 mM $HCO_3^-$ and 200 mM EDTA. At various times thereafter, aliquots from the reaction mixture were added to semicarbazide–HCl in HCl to stop the reaction and stabilize the [$^{14}$C]oxalacetate.

Table I: Effects of Preincubation of the Putative [$^{14}$C]Carboxyphospho–Enzyme Complex with $Mg^{2+}$ and MgADP prior to and during [$^{14}$C]Carboxyl Group Transfer to Pyruvate

| treatment prior to transfer to pyruvate[a] | acid-stable cpm ± SD ($n$ = 6)[b] |
|---|---|
| solution A ($H_2O$) | 269 ± 10 |
| solution B (glucose, hexokinase) | 208 ± 16 |
| solution C (glucose, hexokinase, $MgCl_2$) | 190 ± 17 |
| solution D (glucose, hexokinase, $MgCl_2$, ADP) | 189 ● 43 |

[a] For details of solution composition see Materials and Methods. [b] Combined data from two separate experiments.

5 shows the effect of EDTA and $H^{12}CO_3^-$ on the time course of transfer of [$^{14}$C]carboxyl groups to pyruvate in the presence of acetyl CoA. As can be seen, the presence of these quenching agents that inhibit the formation of [$^{14}$C]oxalacetate from any $H^{14}CO_3^-$ that coelutes with the [$^{14}$C]CPE from the Sephadex G-25 column to some degree inhibited the slow increase in acid-stable counts per minute seen over the 15 min of the time course. In the presence of the quenching agents, the acid-stable counts per minute at all time points were lower than in their absence and no further increase in acid-stable counts per minute occurred after 1 min, whereas in the absence of quenching agents, the counts per minute increased by about 19% after the 1-min time point. These results indicate that there are two components to the increase in acid-stable counts per minute observed in the presence of acetyl CoA over the 15-min time course. There is a slow component that is caused by fixation of $H^{14}CO_3^-$ into [$^{14}$C]oxalacetate, probably due to coelution from the Sephadex G-25 column of small amounts of $H^{14}CO_3^-$, MgATP, and $Mg^{2+}$ with the enzyme. There is also a faster component that may correspond to the last stages of the [$^{14}$C]carboxyl group transfer from the [$^{14}$C]CPE to pyruvate.

*Effect of Incubation of the [$^{14}$C]CPE with $Mg^{2+}$ and MgADP prior to [$^{14}$C]Carboxyl Group Transfer to Pyruvate.* The results of these experiments are shown in Table I. Incubation of the [$^{14}$C]CPE with glucose and hexokinase (solution B) reduced the subsequent formation of [$^{14}$C]oxalacetate on addition of pyruvate by 23% relative to incubation with water (solution A), whereas there was little difference in the formation of [$^{14}$C]oxalacetate among preincubations of the [$^{14}$C]CPE with solution B, C, or D. The reduction in the formation of [$^{14}$C]oxcalacetate caused by preincubation with glucose and hexokinase may be due to the removal of MgATP that coeluted with the CPE. This would result in the abolition of [$^{14}$C]oxalacetate formed from $H^{14}CO_3^-$ on subsequent addition of pyruvate. This reduction in [$^{14}$C]oxalacetate formed corresponds well with the approximately 20% reduction in [$^{14}$C]oxalacetate formation at 15 s observed in Figure 5 when EDTA and $HCO_3^-$ were present to prevent $^{14}$C incorporation into oxalacetate from $H^{14}CO_3^-$. The lack of effect of solutions B, C, and D clearly shows that preincubation of the isolated [$^{14}$C]CPE with $Mg^{2+}$ and ADP under these conditions and the presence of $Mg^{2+}$ and ADP during the transfer reaction with pyruvate had no significant effect on the ability of the [$^{14}$C]CPE to transfer its [$^{14}$C]carboxyl group to pyruvate.

*Effect of Incubation of [$^{32}$P]CPE with Pyruvate prior to Transfer to MgADP.* In reactions in which the [$^{32}$P]CPE had been preincubated with pyruvate prior to MgADP, the radioactivity transferred to form [$\gamma$-$^{32}$P]ATP was 4087 ± 340 cpm (±SD; $n$ = 4), whereas when pyruvate was absent from the preincubation mixture the radioactivity transferred to form [$\gamma$-$^{32}$P]ATP was 4391 ± 709 cpm (±SD; $n$ = 4). This clearly shows that reaction with pyruvate has little effect on the phosphorylating ability of the CPE.

## DISCUSSION

An enzymic complex was prepared by incubating the pyruvate carboxylase with $HCO_3^-$, $Mg^{2+}$, and MgATP in the absence of acetyl CoA and isolated by gel filtration chromatography on a Sephadex G-25 column according to the method of Phillips et al. (1992). This complex had a similar elution profile and similar properties to those detailed by Phillips et al. (1992), who described the complex as the carboxyphospho–enzyme complex (CPE). Wallace et al. (1985) reported that incubation of the [$^{14}$C]CPE with 10 mM pyruvate in the absence of acetyl CoA at 25 °C for 5 min resulted in the apparent transfer of [$^{14}$C]carboxyl groups to pyruvate that was only 6.5% of that in the presence of acetyl CoA. The 5-min time points in Figure 2 for the transfer reaction with 10 mM pyruvate show that the acid-stable counts per minute formed in the absence of pyruvate is only about 2% of that in the presence of acetyl CoA. Taking these time points in isolation from the rest of the time course, it would appear that very little transfer of [$^{14}$C]carboxyl groups from the [$^{14}$C]CPE to pyruvate occurred in the absence of acetyl CoA. However, the time courses of [$^{14}$C]carboxyl group transfer from the [$^{14}$C]CPE to pyruvate show that, initially, there was similar formation of [$^{14}$C]oxalacetate in the presence or absence of acetyl CoA. Subsequently, in the absence of acetyl CoA, the radioactivity fixed in [$^{14}$C]oxalacetate was lost. When the [$^{14}$C]oxalacetate was converted to [$^{14}$C]malate, which is not a substrate for pyruvate carboxylase and which is much less susceptible to spontaneous decarboxylation than oxalacetate since it cannot form an enol intermediate (O'Leary, 1977), the time-dependent loss of acid-stable counts per minute in the absence of acetyl CoA did not occur. This clearly indicates that the cause of the time-dependent loss of acid-stable counts per minute was the decarboxylation of [$^{14}$C]oxalacetate.

The inhibition of this decarboxylation of [$^{14}$C]oxalacetate by avidin shows that pyruvate carboxylase itself catalyzed the decarboxylation reaction. Attwood and Cleland (1986) have

shown that in the presence of acetyl CoA, pyruvate carboxylase is capable of catalyzing the decarboxylation of 1 mM oxalacetate in the absence of $P_i$ and MgADP at a rate that is 0.039% of the rate of the pyruvate carboxylation reaction. This is in reasonable agreement with the value measured here of 0.031% in the experiments to directly measure pyruvate carboxylase-catalyzed decarboxylation of oxalacetate. However, in these decarboxylation experiments it was found that the rate of decarboxylation of oxalacetate in the absence of acetyl CoA was about 1.7 times that in its presence. This indicates that acetyl CoA has a direct inhibitory effect on the decarboxylation reaction that may in part explain why, in the carboxyl group transfer time courses, no loss of [$^{14}$C]oxalacetate was evident in the presence of acetyl CoA. Another effect of acetyl CoA, at least in sheep kidney enzyme (Ashman et al., 1972), is to lower the $K_m$ for pyruvate. Thus, in the presence of acetyl CoA, the pyruvate present in the carboxyl group transfer reactions was able to more effectively compete with the [$^{14}$C]oxalacetate for the binding site on pyruvate carboxylase and hence inhibit the decarboxylation reaction. The fact that the rate of decarboxylation of [$^{14}$C]oxalacetate in the presence of 100 mM pyruvate was much lower than with 10 mM pyruvate supports the idea that competition for the active site of the enzyme between pyruvate and [$^{14}$C]oxalacetate reduces the rate of decarboxylation of the latter.

A feature of the carboxyl group transfer time courses was that at the first time point (15 s) the acid-stable counts per minute formed in the presence or absence of acetyl CoA were very similar (see Figure 2). This suggests that carboxyl group transfer from the [$^{14}$C]CPE to pyruvate proceeds equally well in the absence of acetyl CoA as in its presence. Figure 3 shows transfer time courses under conditions where the transfer of [$^{14}$C]carboxyl groups from [$^{14}$C]CPE to pyruvate was optimized and decarboxylation of the newly formed [$^{14}$C]oxalacetate was minimized; i.e., the transfer was performed in the presence of 100 mM pyruvate and the [$^{14}$C]oxalacetate was converted to [$^{14}$C]malate. Under these conditions, the time courses of [$^{14}$C]oxalacetate formation in the presence and absence of acetyl CoA were almost identical, suggesting that acetyl CoA has little effect on the ability of the [$^{14}$C]CPE to carboxylate pyruvate.

In the experiments where [$^{14}$C]carboxyl group transfer from [$^{14}$C]CPE to pyruvate was performed in the presence of EDTA and $H^{12}CO_3^-$, the slow part of the formation of [$^{14}$C]oxalacetate that occurred in the presence of acetyl CoA on the 0.25–15-min time scale was abolished. Since EDTA and $H^{12}CO_3^-$ in the concentrations used were found to completely inhibit the pyruvate carboxylation that utilizes $HCO_3^-$, this suggests that the slow formation of [$^{14}$C]oxalacetate that was abolished by these agents was due to fixation of $H^{14}CO_3^-$ into [$^{14}$C]oxalacetate. From Figure 5, this reaction represents about 20% of the [$^{14}$C]oxalacetate formed up to 15 s and about 32% of the total [$^{14}$C]oxalacetate formed over the overall 15-min time course. These results strongly suggest that, despite the apparent good separation of the [$^{14}$C]CPE from $H^{14}CO_3^-$ on the Sephadex G-25 column, some $H^{14}CO_3^-$ coeluted with the enzyme and also some ATP and $Mg^{2+}$. Phillips et al. (1992) found that all of the $^{14}$C that coeluted with the [$^{14}$C]CPE was fixed into [$^{14}$C]oxalacetate and concluded from this that all of the $^{14}$C in the [$^{14}$C]CPE was present as [$^{14}$C]carboxyphosphate. However, Phillips et al. (1992) also found that a small amount of ATP coeluted with the CPE, and thus it is possible that some of the $^{14}$C in the [$^{14}$C]CPE was present as $H^{14}CO_3^-$ that was fixed into [$^{14}$C]oxalacetate in a reaction involving the ATP.

Higher formation of [$^{14}$C]malate was observed with 10 mM pyruvate in the presence of acetyl CoA compared with that in its absence; 10 mM pyruvate is unlikely to be saturating in the absence of acetyl CoA (Ashman et al., 1972), and this would allow what Easterbrook-Smith et al. (1976) termed "a hydrolytic leak" to occur. When pyruvate is not saturating, there is inefficient transfer of the carboxyl group from carboxybiotin to pyruvate, and thus the catalytic cycle of the enzyme is completed by decarboxylation of the carboxybiotin to form $CO_2$ or $HCO_3^-$ (Easterbrook-Smith et al., 1976) instead of oxalacetate formation. The fact that with 100 mM pyruvate there is almost identical formation of [$^{14}$C]malate in the presence or absence of acetyl CoA suggests that saturation of the enzyme with pyruvate and hence the hydrolytic leak are important. This may also explain the generally lower efficiency of carboxyl group transfer to 10 mM pyruvate compared to 100 mM pyruvate, although lower competition between pyruvate and [$^{14}$C]oxalacetate for the enzyme active site at 10 mM pyruvate even in the presence of acetyl CoA is also likely to be an important factor.

Phillips et al. (1992) showed that incubation of the [$^{32}$P]CPE with $Mg^{2+}$ and ADP resulted in the formation of [$\gamma$-$^{32}$P]ATP and on the basis of this evidence, together with the ability of the [$^{14}$C]CPE to carboxylate pyruvate, suggested that the enzyme complex that they had isolated did indeed contain the carboxyphosphate intermediate. In addition, Phillips et al. (1992) also showed that if acetyl CoA was present during the preparation or isolation of the CPE prepared with [$\gamma$-$^{32}$P]ATP, [$^{32}$P]CPE could not be detected. From this result and the apparent requirement for acetyl CoA for the transfer of [$^{14}$C]carboxyl groups from [$^{14}$C]CPE to pyruvate these authors suggested that acetyl CoA induces the transfer of the carboxyl group from carboxyphosphate to biotin and thus destroys the carboxyphosphate. If the enzyme species formed by incubation of pyruvate carboxylase with $Mg^{2+}$, ATP, and $HCO_3^-$ is truly the enzyme–carboxyphosphate complex, one would expect incubation of the [$^{14}$C]CPE with $Mg^{2+}$ and ADP to result in the formation of ATP and the destruction of carboxyphosphate and hence make impossible the formation of [$^{14}$C]oxalacetate on the addition of pyruvate. Table I shows the results of such experiments, and it is clear that $Mg^{2+}$ and ADP did not impair the ability of the [$^{14}$C]CPE to carboxylate pyruvate. The formation of [$^{14}$C]oxalacetate was not due to pyruvate carboxylation from $H^{14}CO_3^-$ since any ATP should have been removed by the hexokinase, and since the transfer reaction was only allowed to proceed for 15 s, this mainly eliminated the slower $H^{14}CO_3^-$-dependent carboxylation reaction described above. In addition, if the CPE were the enzyme–carboxyphosphate complex, one would expect that incubation of the [$^{32}$P]CPE with pyruvate should result in the formation of oxalacetate and the destruction of carboxyphosphate, making impossible the formation of [$\gamma$-$^{32}$P]ATP on addition of $Mg^{2+}$ and ADP. Clearly this did not occur, and removal of any oxalacetate by malate dehydrogenase ensured that [$\gamma$-$^{32}$P]ATP formation was not due to the reverse reaction of pyruvate carboxylase in which oxalacetate, MgADP, and $^{32}P_i$ are substrates.

The above two sets of experiments indicate that the CPE is not the enzyme–carboxyphosphate complex. The CPE behaves in a similar way to the enzyme–carboxybiotin complex with respect to its ability to carboxylate pyruvate in the absence of any added cofactor, including acetyl CoA (Attwood et al., 1984). In the experiments presented here, acetyl CoA does not appear to stimulate pyruvate carboxylation from the CPE, and the work of Goodall et al. (1981) and Attwood and Wallace

(1986) suggests that acetyl CoA has only small effects on the rate of carboxyl group transfer from the enzyme–carboxybiotin complex. It will be necessary, however, to study the reaction during the first 15 s using quenched-flow techniques to determine if acetyl CoA does induce any acceleration of the carboxylation reaction from the CPE. The work presented here suggests that the transferrable carboxyl group in the CPE is incorporated into carboxybiotin and preincubation of the CPE with $Mg^{2+}$ and MgADP in the absence of $P_i$ would not be expected to affect the carboxylating ability of carboxybiotin. If the CPE does contain carboxybiotin, this implies that this intermediate is able to form in the absence of acetyl CoA. Thus, at present, there is only evidence to show that a major role of acetyl CoA in the overall pyruvate carboxylase reaction is to stimulate the cleavage of MgATP (Attwood & Graneri, 1991, 1992).

If the carboxyl group of the CPE is incorporated in carboxybiotin, in what form is the phosphate that can phosphorylate ADP? A phosphorylated intermediate that has been proposed to be involved in the reaction mechanism of pyruvate carboxylase is *O*-phosphobiotin (Lynen, 1967; Kluger & Adawadkar, 1976; Kluger et al., 1979), formed as a result of the direct phosphorylation of the carbonyl oxygen of biotin by MgATP. *O*-Phosphobiotin is then proposed to react with $HCO_3^-$, either to directly form carboxybiotin in a concerted reaction or to form carboxyphosphate and then carboxybiotin. However, attempts to stabilize and isolate *O*-phosphobiotin have failed (Phillips et al., 1992), and the other available experimental evidence favors a biotin carboxylation reaction in which carboxyphosphate formed directly by reaction between ATP and $HCO_3^-$ is the biotin-carboxylating agent [see Knowles (1989)]. If there is no phosphorylated enzyme intermediate in the reaction pathway of biotin carboxylation, perhaps the CPE is phosphorylated as a result of a reaction that does not normally occur in the pyruvate carboxylase reaction. The variability of the ratios $^{32}P$/biotin in the [$^{32}P$]CPE and $^{14}C$/biotin in the [$^{14}C$]CPE reported by Phillips et al. (1992) may also indicate that phosphorylation does not occur as a result of the formation of a reaction pathway intermediate as such a variation in stoichiometry would not be expected. The overall reaction of chicken liver pyruvate carboxylase is highly dependent on the presence of acetyl CoA, and as Phillips et al. (1992) demonstrated, acetyl CoA prevented the formation of [$^{32}P$]CPE. Thus, it is possible that in the presence of acetyl CoA, in the normal reaction pathway, the phosphorylation reaction does not occur to any great extent.

As a result of the findings presented here, there is now only evidence that a major locus of action of acetyl CoA in activating pyruvate carboxylase lies in enhancing the rate of $HCO_3^-$-dependent ATP cleavage (Attwood & Graneri, 1992). Clearly there needs to be further characterization of the CPE, especially the nature of the phosphorylation of the enzyme, and this laboratory is currently engaged in further investigation of the biotin–carboxylation reaction.

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