Isolation of a Carboxyphosphate Intermediate and the Locus of Acetyl-CoA Action in the Pyruvate Carboxylase Reaction†

Nelson F. B. Phillips,‡ Mark A. Snoswell, Anne Chapman-Smith, D. Bruce Keech, and John C. Wallace*

Department of Biochemistry, University of Adelaide, Adelaide, South Australia, Australia

Received March 31, 1992; Revised Manuscript Received June 26, 1992

ABSTRACT: When chicken liver pyruvate carboxylase was incubated with either $H^{14}CO_3^-$ or γ-[^{32}P]ATP, a labeled carboxyphospho–enzyme intermediate could be isolated. The complex was catalytically competent, as determined by its subsequent ability to transfer either $^{14}CO_2$ to pyruvate or ^{32}P to ADP. While the carboxyphospho–enzyme complex was inherently unstable and the stoichiometry of the transfer was variable depending on experimental conditions, both the [^{14}C]carboxyphospho–enzyme and the carboxy[^{32}P]phospho–enzyme had similar half-lives. Acetyl-CoA was shown to be involved in the conversion of the carboxyphospho–enzyme complex to the more stable carboxybiotin–enzyme species, which was consistent with the effects of acetyl-CoA on isotope exchange reactions involving ATP. We were unable to detect the formation of a phosphorylated biotin derivative during the ATP cleavage reaction. In the presence of K^+ and at pH 9.5, the acetyl-CoA-independent activity of chicken liver pyruvate carboxylase approached 2% of the acetyl-CoA-stimulated rate, which represents a 30-fold increase on previously reported activity for this enzyme.

As part of an ongoing investigation into the reaction pathway of the biotin-dependent enzyme, pyruvate carboxylase (PC;[1] EC 6.4.1.1), and the locus of action of the allosteric activator, acetyl-CoA, we have considered the following established information:

(a) Under equilibrium conditions, PC catalyzes four clearly defined isotope exchange reactions (Scrutton et al., 1965; McClure et al., 1971a; Ashman & Keech, 1975)

$$\text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{ADP} \quad (1)$$

$$\text{ATP} \overset{Mg^{2+},\text{acetyl-CoA}}{\rightleftharpoons} P_i \quad (2)$$

$$\text{pyruvate} \rightleftharpoons \text{oxalacetate} \quad (3)$$

$$HCO_3^- \overset{\text{acetyl-CoA}}{\underset{ADP,\,P_i}{\rightleftharpoons}} \text{oxalacetate} \quad (4)$$

While reactions 1 and 3 can proceed in the absence of acetyl-CoA, the presence of the activator increases the rate of all four isotope exchange reactions (Ashman et al., 1972; Attwood & Keech, 1985).

(b) When assayed under identical conditions, reactions 1 and 2 proceed at the same rate (McClure et al., 1971b), indicating that, although the observed ATP/ADP exchange is not inhibited by avidin (Ashman & Keech, 1975), it is a part of the main reaction sequence. However, the two processes can be uncoupled by a partial denaturation of the enzyme (Wallace et al., 1985).

On the basis of these data, the overall reaction catalyzed by PC

$$\text{ATP} + HCO_3^- + \text{pyruvate} \overset{Mg^{2+},\text{acetyl-CoA}}{\rightleftharpoons} \text{ADP} + P_i + \text{oxalacetate} \quad (5)$$

can be broken down into the following minimal steps:

$$\text{ENZ–biotin} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{ATP–ENZ–biotin} \quad (6)$$

$$\text{ATP–ENZ–biotin} + HCO_3^- \rightleftharpoons \left[\begin{array}{l} P_i \\ \vdots \\ COO^- \end{array}\!\!>\text{ENZ–biotin}\right] + \text{ADP} \quad (7)$$

$$\left[\begin{array}{l} P_i \\ \vdots \\ COO^- \end{array}\!\!>\text{ENZ–biotin}\right] \overset{\text{acetyl-CoA}}{\rightleftharpoons} \text{ENZ–biotin–}CO_2^- + P_i \quad (8)$$

$$\text{ENZ–biotin–}CO_2^- + \text{pyruvate} \rightleftharpoons \text{ENZ–biotin} + \text{oxalacetate} \quad (9)$$

In support of this sequence of events for the carboxylation of the biotin moiety there is the ample evidence that biotin is not directly involved in the cleavage of ATP [see Knowles (1989)]. Climent and Rubio (1986) have shown that biotin does not participate in HCO_3^--dependent ATP cleavage for biotin carboxylase from *Escherichia coli*. Additionally, the experiments of Kaziro et al. (1962), using $HC^{18}O_3^-$ to investigate the mechanism of propionyl-CoA carboxylase (EC 6.4.1.3), showed that bicarbonate participates directly in the cleavage of the β–γ phosphoryl bond of ATP (eq 7).

In this paper, we demonstrate the formation of a carboxyphosphate–enzyme complex of PC prior to the carboxylation of biotin (see eq 8) and show that one of the effects of acetyl-CoA occurs during the subsequent transfer of the "activated" carboxyl group to the biotin moiety. A preliminary account of this work has been presented elsewhere (Wallace et al., 1985).

MATERIALS AND METHODS

Preparation and Assay of Pyruvate Carboxylase. Chicken liver pyruvate carboxylase was prepared as described by Goss

† This work was supported by a grant (D278/15094) from the Australian Research Grants Commission (to D.B.K. and J.C.W.). N.F.B.P. was the recipient of a University of Adelaide Postgraduate Research Award.

* Correspondence: Department of Biochemistry, University of Adelaide, GPO Box 498, Adelaide SA 5001, Australia.

‡ Present address: Departments of Biochemistry and Medicine, Case Western Reserve University, Cleveland, OH 44106.

[1] Abbreviations: PC, pyruvate carboxylase (EC 6.4.1.1); PEI, polyethylenimine.

0006-2960/92/0431-9445$03.00/0 © 1992 American Chemical Society

et al. (1979) to a specific activity of 25–32 unit/mg. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of oxalacetate/min at 25 °C. The catalytic activity of the enzyme was assayed either by the spectrophotometric or the radiochemical method described by Keech and Utter (1963), with the modifications detailed in the figure legends. The ATP:ADP and ATP:P_i isotope exchange rates were measured as described by Ashman and Keech (1975). All enzyme assays were carried out at pH 7.8, with redistilled *N*-ethylmorpholine replacing Tris buffer.

Isolation of Carboxyphospho–Enzyme Complex. In order to isolate the carboxyphospho–enzyme complex, chicken liver PC (60–100 unit/mL) was incubated for 15 min at 4 °C with either $H^{14}CO_3^-$ or [^{32}P]ATP, in the absence of acetyl-CoA. The reaction mixture contained 2 mM ATP, 8 mM $MgCl_2$, 1 mM $NaHCO_3^-$, and 100 mM *N*-ethylmorpholine chloride, pH 7.8. The carboxyphospho–enzyme complex was separated from the reactants by gel filtration at 4 °C on Sephadex-G25 (45 × 1.4 cm), equilibrated, and eluted with 100 mM *N*-ethylmorpholine acetate, pH 7.2, at a flow rate of 0.63 mL/min. Modifications are detailed in the figure legends.

Determination of Stability and Catalytic Competence of Carboxyphospho–Enzyme Complex. Stability and catalytic competence were determined by measuring the transfer of the [^{14}C]carboxyl or [^{32}P]phosphoryl group from the isolated carboxyphospho–enzyme complex to pyruvate or ADP, respectively. Transfer of the ^{14}C-carboxyl group to pyruvate was determined by incubating the isolated complex with 10 mM pyruvate in 100 mM *N*-ethylmorpholine chloride, pH 7.8, and, where indicated, 0.25 mM acetyl-CoA, for 5 min at 25 °C. The oxalacetate formed was stabilized and quantitated as described by Easterbrook-Smith et al. (1976). Transfer of $^{32}P_i$ from the isolated complex to ADP was detected by incubation in the same buffer in the presence of 8 mM $MgCl_2$ and 2 mM ADP for 15 min at 30 °C. The reaction was stopped by the addition of formic acid and the products analyzed by PEI thin-layer chromatography as described for the ATP isotope exchange reactions (Ashman & Keech, 1975).

Diazomethylation and Tryptic Digestion of Carboxyphospho–Enzyme Complex. Diazomethane was prepared by the procedure described by Vogel (1967) and reacted with the enzyme by a modification of the method of Powers and Meister (1978). After addition of the diazomethane solution, esterification was allowed to proceed for 1 h at room temperature in the dark. The ethereal solution was removed, and the precipitate was washed with 0.1 M potassium phosphate buffer, pH 7.2, and then resuspended in preparation for the tryptic digestion. Tryptic digests, separation of the biotin-containing peptide, and quantitation of the biotin content of the enzyme samples were as described by Rylatt et al. (1977).

Other materials were as previously described (Ashman et al., 1972; Ashman & Keech, 1975), or of analytical reagent grade.

RESULTS

Evidence for Carboxyphospho–Enzyme Complex. When chicken liver PC is incubated with MgATP and HCO_3^-, the formation of a carboxyphospho–enzyme complex can be inferred from the scheme depicted in eqs 6 and 7. In order to test this hypothesis, these components were incubated together in the presence of either $H^{14}CO_3^-$ or γ-[^{32}P]ATP, and then the enzyme was separated from the small, water-soluble components by passage of the reaction mixture through a Sephadex-G25 column at 4 °C. The results of experiments where either isotope was used are shown in Figure 1. When PC was incubated with $H^{14}CO_3^-$ and ATP in the absence of acetyl-CoA, ^{14}C-labeled radioactivity was found to coelute with PC activity (Figure 1a). The fact that the [^{14}C]carboxyl group could be transferred to pyruvate when the complex was incubated with acetyl-CoA and pyruvate (Figure 1a) indicated that the isolated [^{14}C]carboxyphospho–enzyme complex was catalytically competent. Since essentially all the enzyme-bound radioactivity was trapped as oxalacetate, the $H^{14}CO_3^-$ was not binding nonspecifically to the enzyme.

Likewise, when PC was incubated with γ-[^{32}P]ATP and HCO_3^-, ^{32}P-labeled radioactivity eluted with PC activity (Figure 1c), and the isolated carboxy[^{32}P]phospho–enzyme complex was shown to be catalytically competent by demonstrating that the [^{32}P]phosphoryl group could be transferred to ADP (Figure 1c). However, unlike the [^{14}C]carboxy group transfer to pyruvate, the isolated carboxy[^{32}P]phospho–enzyme complex was able to transfer the [^{32}P]phosphoryl group to ADP without the addition of acetyl-CoA (Figure 1c). When the incubation temperature for the formation of the carboxy-[^{32}P]phospho–enzyme complex was raised to 25 °C, and the complex fractionated on Sephadex G-25 at 4 °C, no ^{32}P was associated with the enzyme, suggesting that the radioactivity was due to a labile enzyme-bound phosphoryl group. A control experiment where U-[^{14}C]ATP replaced γ-[^{32}P]ATP demonstrated that the ^{32}P-labeled radioactivity eluting with the enzyme did not represent tightly bound ATP. In this case, the amount of ^{14}C-labeled radioactivity in the PC peak was less than 10% of the theoretical maximum, based on the biotin content of the PC sample, whereas the ^{32}P-labeled enzyme contained 60% of the maximum.

Effect of Acetyl-CoA on Carboxyphospho–Enzyme Complex. The minimal requirement for the formation of the carboxyphospho–enzyme complex described in the previous section was enzyme, MgATP, and HCO_3^- (see eq 7). In addition, previous investigations (Scrutton et al., 1965) have shown that the presence of acetyl-CoA in the reaction mixture stabilizes the enzyme-bound $^{14}COO^-$ group (eq 8) in a form which can be transferred to pyruvate (eq 9). This was also the case in this study (cf. Figure 1, panels a and b). However, the carboxy[^{32}P]phospho–enzyme complex could not be detected if the acetyl-CoA was present during either the incubation or the isolation procedures. The shoulder of radioactivity apparently associated with the PC activity peak when incubation included acetyl-CoA (Figure 1d) probably represents incomplete separation of released $^{32}P_i$. The material was shown by PEI thin-layer chromatography to be 94% $^{32}P_i$, which was less than 10% of the PC content when quantitated on the basis of nanomoles of $^{32}P_i$/nanomole of biotin. To demonstrate the existence of the enzyme-bound phospho moiety, ADP had to be added to the isolated complex to act as a phosphate acceptor before the activator was added (see Figure 1, panels c and d). This suggests that acetyl-CoA labilizes the carboxyphospho moiety in such a way as to allow the carboxyl group to be transferred to the biotin molecule and release the phosphoryl group into the medium.

Stability of Isolated Carboxyphosphate–Enzyme Complex. Analysis of the catalytic competence and stoichiometry of the labeled PC complexes isolated by Sephadex chromatography was carried out by determining their ability to transfer ^{14}C or ^{32}P to pyruvate or ADP, respectively, as described under Materials and Methods. The radioactivity present, and transferred, was expressed as a proportion of the biotin content for each enzyme-containing fraction, which was assayed as described under Materials and Methods. The [^{14}C]carboxyphospho–enzyme complex formed in the absence of acetyl-CoA (Figure 1a) was catalytically competent, as indicated by

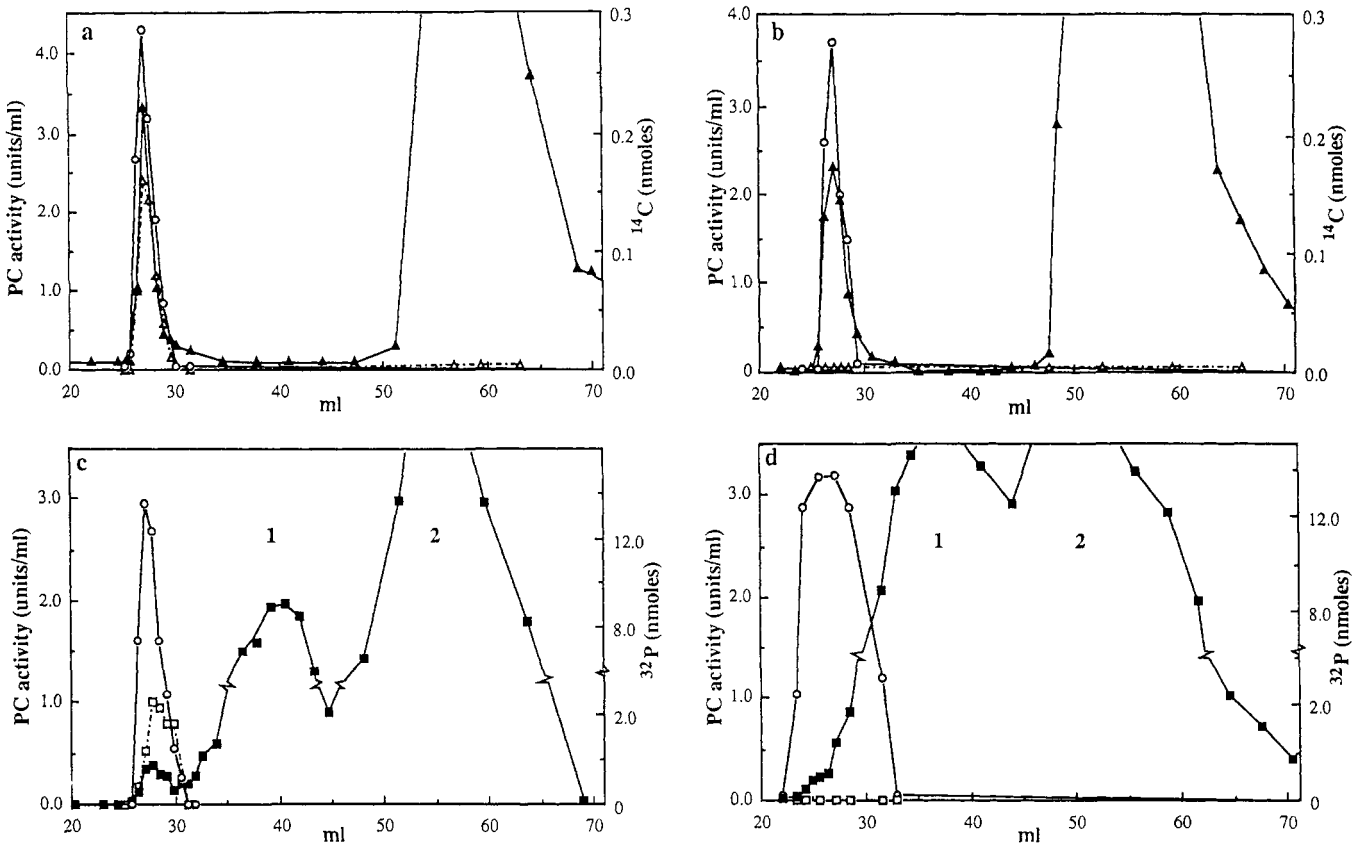

FIGURE 1: Preparation and isolation of the carboxyphospho–enzyme complex. Chicken liver PC was incubated with HCO_3^- and MgATP in the presence of $H^{14}CO_3^-$ (panels a and b) or γ-[^{32}P]ATP (panels c and d), and the enzyme was isolated from the substrates by gel filtration at 4 °C, as described under Materials and Methods. The material eluted from the Sephadex-G25 column was tested, as described under Materials and Methods, for enzyme activity, total radioactivity, and, where appropriate, radioactivity which could be transferred to pyruvate or ADP. (Panel a) The reaction was carried out in the absence of acetyl-CoA, and eluted material was collected into tubes containing acetyl-CoA at a final concentration of 0.25 mM. (Panels b and c) Both the reaction and collection of fractions were carried out in the absence of acetyl-CoA. (Panel d) The reaction mix included 0.25 mM acetyl-CoA, and fractions were collected in the absence of acetyl-CoA. Symbols: enzyme activity (○); ^{14}C transferred to pyruvate (△); ^{32}P transferred to ADP (□); total radioactivity: ^{14}C (▲), ^{32}P (■). Peaks 1 and 2 were determined by PEI thin-layer chromatography to be P_i and ATP, respectively.

its subsequent ability to transfer ^{14}C to pyruvate in the presence of acetyl-CoA, and all the enzyme-bound radioactivity could be fixed in this manner. However, in spite of many attempts to optimize the experimental conditions, we were never able to observe more than about 25% of the amount theoretically possible, based on the biotin content of the PC sample. That result implies that 75% of the biotin molecules in the enzyme did not become carboxylated on subsequent addition of acetyl-CoA, under the conditions used in these experiments. In contrast, the isolated carboxy[^{32}P]phospho–enzyme complex (Figure 1c) approached 60% of the theoretical maximum. However, although the complex was catalytically competent, only about 50% of the enzyme-bound radioactivity could be transferred back to ADP, which is consistent with the catalytically competent amount of [^{14}C]carboxyl in the complex.

Examination of the spontaneous decay of the isolated intermediate demonstrated that the half-life of the [^{14}C]carboxyphospho–enzyme complex at 0 °C (48.5 min) and that of the carboxy[^{32}P]phospho–enzyme complex, (46.1 min) were similar when catalytic competence was used as a measure of stability (Figure 2). The corresponding half-lives at 4 °C were 28.0 and 25.7 min, respectively.

Varying pH values had diverse effects on the formation and stability of enzyme-bound [^{14}C]carboxyl and [^{32}P]phosphoryl groups. Whereas at low pH values the amount of enzyme-bound [^{14}C]carboxyl groups which could be subsequently transferred to pyruvate was relatively small, there was a significant increase as alkalinity was increased (Figure 3a). In contrast, the amount of [^{32}P]phosphate associated with the enzyme which could be transferred to ADP appeared to be unaffected in the range from pH 6.2 to 8.5 (Figure 3a). However, once bound to the enzyme, the stability of the activated carboxyl group appeared to be unaffected over the pH range from 6.2 to 8.5, while the phosphate adduct was

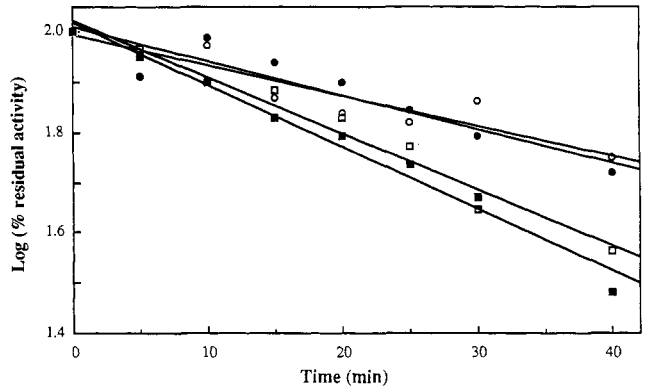

FIGURE 2: Stability of the carboxyphospho–enzyme complex at 0 and 4 °C. Carboxyphospho–enzyme complex labeled with either ^{32}P or ^{14}C was prepared by incubating PC with MgATP and HCO_3^- as described under Materials and Methods and was isolated at 4 °C by the rapid centrifuge desalting technique of Helmerhorst and Stokes (1980). The subsequent stability of the complex at either 0 or 4 °C was determined by the ability of samples removed at various times to transfer [^{14}C]carboxyl to pyruvate (in the presence of 0.25 mM acetyl-CoA) and $^{32}P_i$ to ADP as described under Materials and Methods. [^{14}C]Carboxyl transferred to pyruvate at 0 °C (○) and 4 °C (□); $^{32}P_i$ transferred to ADP at 0 °C (●) and 4 °C (■).

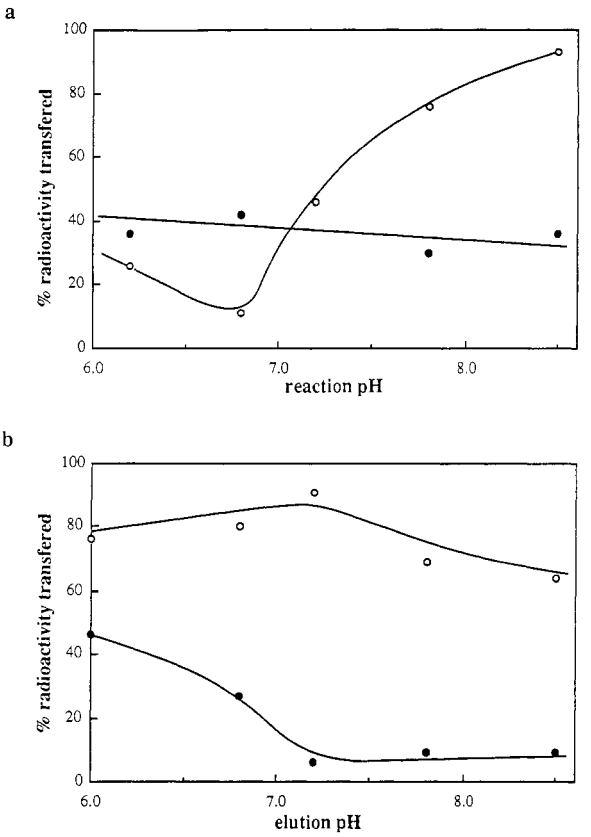

FIGURE 3: Effect of reaction and elution pH on the catalytic competence of the carboxyphospho–enzyme complex. Carboxyphospho–enzyme complex labeled with either ^{32}P or ^{14}C was prepared and isolated as described under Materials and Methods except that either the pH of (panel a) the reaction mixture or (panel b) the elution buffer was varied as indicated. The buffering system was *N*-ethylmorpholine acetate, with an elution pH of 7.2 when the reaction pH was varied and a reaction pH of 7.8 when the elution pH was varied. Catalytic competence was determined by the ability to transfer [^{14}C]carboxyl to pyruvate, in the presence of 0.25 mM acetyl-CoA, (○) and $^{32}P_i$ to ADP (●), as described under Materials and Methods.

most stable under mildly acidic conditions (Figure 3b); its stability and capacity to transfer back to ADP was markedly reduced on the alkaline side of neutrality. The presence of 10 mM Mg^{2+} in the elution buffer had no effect on the newly formed carboxyl group but reduced the stability of the enzyme-bound phosphate adduct 5-fold. The influence of various buffer anions on the stability of enzyme-bound ^{14}C and ^{32}P was complex. Taking the amount of label associated with the eluting enzyme in the presence of chloride ions as 100%, when acetate was the anion during both the reaction and elution, enzyme-bound [^{14}C]carboxyl increased to 114% and enzyme-bound [^{32}P]phosphoryl increased to 173%. On the other hand, sulfate ions reduced the amount of both enzyme-bound [^{14}C]-carboxyl and [^{32}P]phosphoryl groups by 55% and 95%, respectively.

Lack of Evidence for o-Phosphobiotin Intermediate. Since we have been unable to detect "free" carboxyphosphate in the reaction solution after treatment with diazomethane (Wallace et al., 1985), the possibility that the reactive enzyme intermediate is an *o*-phosphobiotin derivative (Kluger & Adawadkar, 1976; Kluger et al., 1979) was investigated. Following incubation of PC with γ-[^{32}P]ATP under the conditions described under Materials and Methods for formation of the carboxyphospho–enzyme complex, the putative intermediate was stabilized as the methyl phospho ester form with diazomethane (see Materials and Methods). The tryptic digest was incubated with avidin, and the biotin-containing peptide was separated by gel filtration as described by Rylatt et al. (1977). Analysis of the biotin content of both the material complexed with avidin and the radioactive material following gel filtration clearly demonstrated that no ^{32}P was associated with the biotin peptide. Treatment of the PC sample with avidin prior to incubation with labeled ATP did not affect the outcome. Control experiments indicated that the negative result was not due to the inability of avidin to bind a methylated phospho ester form of biotin. A sample of the diazomethylated ^{32}P-labeled enzyme was acid hydrolyzed in 6 M HCl after digestion with pronase (1:10 w/w, 4 h at 37 °C). At various time intervals, the biotin content of the hydrolyzed material was determined as described under Materials and Methods. A phospho ester bond with the carbonyl group of biotin would be hydrolyzed by treatment with acid, thus allowing the biotin to complex with avidin in the biotin assay procedure. In fact, 96% of the biotin in the diazomethylated ^{32}P-labeled PC sample was complexed with avidin in the biotin assay, even before acid hydrolysis, and this did not increase with time, suggesting that the phosphorylated enzyme species was not a phosphobiotin derivative.

Effect of Acetyl-CoA on Isotopic Exchange Reactions Involving ATP. The reaction sequence shown in eqs 6–8 is consistent with previous observations that chicken liver PC catalyzes an isotope exchange between ATP and ADP which proceeds in the absence of acetyl-CoA (eq 1), whereas the ATP:P_i isotope-exchange reaction (eq 2) has an absolute requirement for the activator (Scrutton & Utter, 1965). However, a combination of acetyl-CoA and P_i was shown to stimulate the ATP:ADP exchange rate (Wallace et al., 1985). In terms of the proposed reaction sequence in eqs 6–8, when acetyl-CoA is added to the reaction mixture, (i) a new enzyme species, enzyme–biotin–CO_2, is formed and (ii) P_i is released into the medium. In order for eq 8 to proceed in the back reaction when acetyl-CoA is present, the concentration of P_i must be increased. Investigation of the effect of varying concentrations of acetyl-CoA on the isotope-exchange reactions involving ATP (Figure 4) showed that while acetyl-CoA was not required for the ATP:ADP exchange, a slight stimulation of the ATP:ADP exchange occurred at concentrations up to 0.1 mM, after which the rate reverted to the basal level as the concentration was further increased (Figure 4a). the ATP:P_i exchange reaction, which was dependent on acetyl-CoA, was also maximally stimulated by 0.1 mM acetyl-CoA, and at higher concentrations a reduction in the exchange rate was observed (Figure 4b). The overall forward reaction catalyzed by chicken liver PC (eq 5) is not inhibited by concentrations of acetyl-CoA above 0.1 mM (Ashman et al., 1972). Thus, it appears that the formation of the enzyme–biotin–CO_2 species on addition of acetyl-CoA (eq 8) reduces the concentration of the other enzyme species involved in the reactions and, hence, reduces the rate of exchange.

Acetyl-CoA-Independent Reaction. Although early reports indicated that chicken liver PC had an absolute requirement for acetyl-CoA (Scrutton et al., 1965), a small but detectable rate of carboxylation of pyruvate by chicken liver PC in the absence of acetyl-CoA was reported by Ashman et al. (1972). We have examined the assay conditions for this acetyl-CoA-independent carboxylation of pyruvate and have found that the rate of enzymic activity was enhanced when enzyme and substrate concentrations were increased. Increasing the K^+ concentration stimulated enzyme activity both in the presence and in the absence of acetyl-CoA, with the most pronounced effect being that on the independent activity at high PC and substrate concentrations (Figure 5). In addition, in contrast to the acetyl-CoA-stimulated activity, there was a 7-fold increase in the acetyl-CoA-independent reaction velocity when

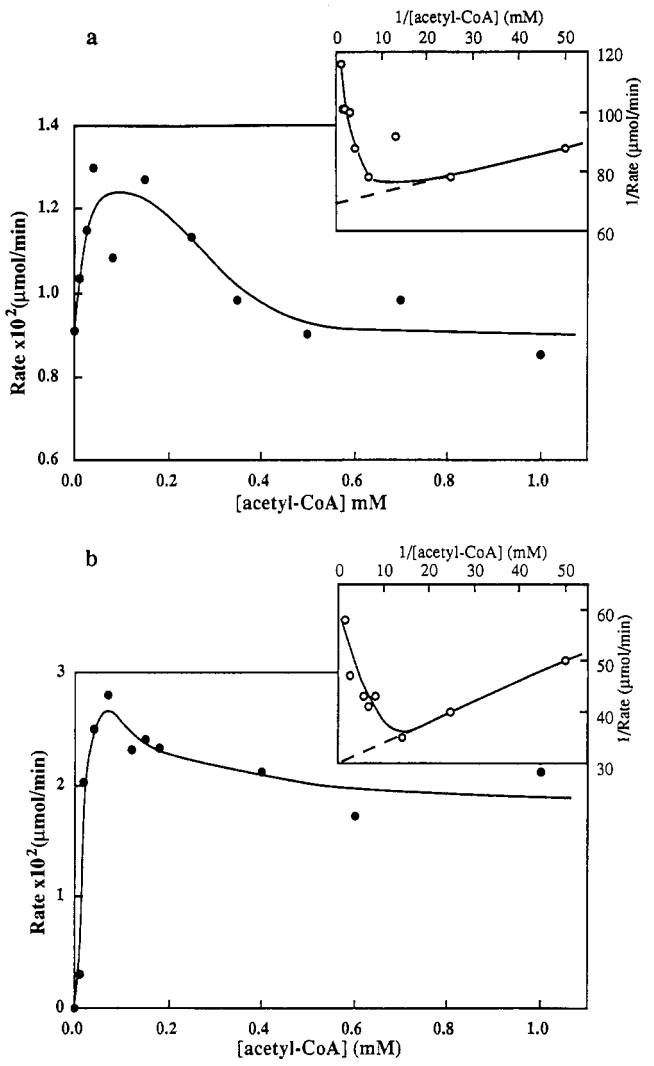

FIGURE 4: Effect of acetyl-CoA on (panel a) ATP:ADP and (panel b) ATP:P_i exchange activity of chicken liver PC. Isotopic-exchange reactions were carried out as described under Materials and Methods, except that the concentration of acetyl-CoA was varied. The data points are the mean of the rate obtained at three different times during the exchange reaction. Insets show the data plotted in double reciprocal form.

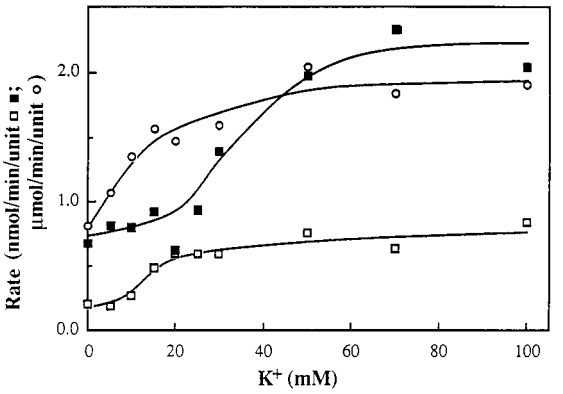

FIGURE 5: Effect of [K^+] on the acetyl-CoA-dependent and -independent activities of chicken liver PC. Activity [μmol min^{-1} (unit of PC added)$^{-1}$] in the presence of acetyl-CoA (○) was assayed by the procedure described under Materials and Methods except that [K^+] was varied as indicated. The Cl^- ion was maintained at 100 mM with *N*-ethylmorpholine chloride, pH 7.8. Acetyl-CoA-independent activity [nmol min^{-1} (unit of PC added)$^{-1}$] was assayed in the same manner with normal substrate concentrations (2.5 mM MgATP, 10 mM pyruvate, 10 mM HCO_3^-; □) or high substrate concentrations (5 mM MgATP, 40 mM pyruvate, 40 mM HCO_3^-; ■) and varying [K^+]. Acetyl-CoA was omitted from the assay system, and the assays were initiated with 1 unit of PC.

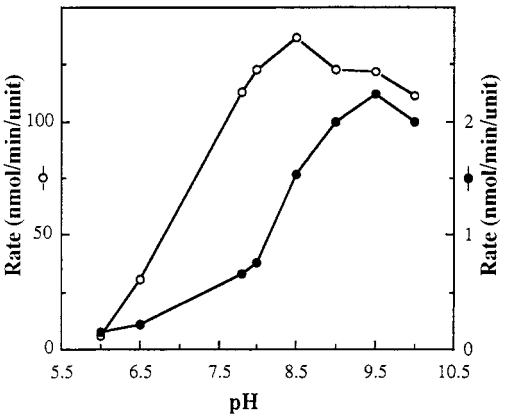

FIGURE 6: Effect of pH on the acetyl-CoA-dependent and -independent activities of chicken liver PC. Acetyl-CoA-dependent activity (○) was assayed by the procedure described under Materials and Methods. The acetyl-CoA-independent activity (●) was assayed similarly but with high substrate concentrations (5 mM MgATP, 40 mM pyruvate, 40 mM HCO_3^-), 100 mM [K^+], 1 unit of PC, and acetyl-CoA omitted from the assay system. The pH of the *N*-ethylmorpholine chloride buffer was varied as indicated.

the pH of the assay system was increased from pH 7.8 to pH 9.5 (Figure 6). Thus, under these conditions, the acetyl-CoA-independent activity approached 2% of the acetyl-CoA-stimulated rate.

DISCUSSION

The data presented in this paper provide evidence for the formation, isolation, and partial characterization of a carboxyphospho–enzyme–biotin complex from PC. Although the existence of the complex was inferred from isotope-exchange studies carried out under equilibrium conditions, there is no reason to suspect that the enzymic species is not a normal participant in the reaction pathway under steady-state conditions. In terms of the reaction sequence, the formation of the carboxyphospho–enzyme complex precedes the carboxylation of biotin, which results in the formation of an enzyme–biotin–CO_2 complex. Using the [γ-^{17}O,γ-^{18}O]-γ-phosphorothioate of ATP, Hansen and Knowles (1985) demonstrated that biotin carboxylation in chicken liver PC proceeds with stereochemical inversion at phosphorus, which is consistent with the formation of a carboxyphospho–enzyme complex prior to the carboxylation of biotin. In addition, this stereochemical outcome is further evidence against the phosphorylation of biotin during the reaction. The observed sequence similarity between carbamoyl-phosphate synthetase and the region in the N-terminal half of yeast PC identified as the probable ATP-binding domain (Lim et al., 1988) tends to support a mechanistic similarity in terms of the formation of a carboxyphosphate intermediate. The results of the study presented here are in agreement with the first part of the general mechanistic pathway proposed by Knowles (1989) for the biotin carboxylases. However, the fact that the ratio between the two radioactive labels in the isolated carboxyphospho–enzyme complex is variable, depending upon the experimental conditions, suggests that during the reaction (eqs 6–8), other intermediates may be formed in the interval between the formation of the carboxyphosphate derivative and the relatively stable carboxybiotin.

During the isolation of the carboxyphospho–enzyme complex, essentially two species of intermediate appear to be formed: (i) the carboxyphospho–enzyme intermediate containing both phosphoryl and carboxyl groups which are enzymically competent, present at 25–30% of the theoretical maximum, and (ii) a nonproductive phosphoryl–enzyme

complex (probably formed from the hydrolysis of the carboxyl group) with about 30% of the possible radioactivity. Hence, when the whole enzyme sample was analyzed for ^{32}P content, 60% of the enzyme was considered to be "loaded" with ^{32}P, and of this only half was enzymically competent. The formation of this nonproductive phosphoryl–enzyme complex during the isolation procedure may account for our inability to trap more than 25% of the theoretically possible carboxyl groups as oxaloacetate.

The relative stoichiometries of the two radioactive species bound in the isolated carboxyphospho–enzyme complex (Figure 1) indicate that first the catalytic competence of the phosphoryl group, in terms of the ability to transfer $^{32}P_i$ to ADP, is dependent on the presence of the carboxyl group on the complex. This is consistent with the sequence of events proposed in eqs 6 and 7. Secondly, binding of ADP in the reverse reaction of eq 7 requires the presence of the carboxyl group on the enzyme. This interpretation is supported by the observation that ADP is required for the release of HCO_3^- in the HCO_3^-:oxalacetate isotope-exchange reaction (Scrutton & Utter, 1965). The similarity of the half-lives of the differently labeled enzyme species determined from the decay profiles (Figure 2) is consistent with the carboxyl and phosphoryl groups being part of the same complex, thus suggesting that the carboxyphospho moiety of the isolated intermediate is enzyme-bound. It is apparent that the catalytically competent form of the carboxyphospho–enzyme complex is labile and is degraded in the absence of acetyl-CoA in a nonsynchronous manner, i.e., the rate of dissociation of the carboxyl group is greater than that of the phosphoryl group.

The isotope-exchange reactions involving ATP indicate that, following ATP cleavage, the release of ADP occurs without requiring acetyl-CoA. Similarly, the formation of ATP from ADP and carbamoyl phosphate catalyzed by PC was shown to be stimulated by, but not dependent on, acetyl-CoA (Attwood & Graneri, 1991). The effect of acetyl-CoA both on the formation of the carboxyphospho–enzyme complex and on the ATP:P_i isotope-exchange reaction suggests that the activator mediates the release of P_i from the carboxyphospho–enzyme complex. The presence of acetyl-CoA facilitates the formation of, and shifts the equilibrium towards, the carboxybiotin intermediate (eq 8). This is consistent with our observation that the [^{14}C]carboxy–enzyme complex which was formed in the absence of acetyl-CoA was incapable of transfering any significant amount of radioactivity to pyruvate unless acetyl-CoA was present in the pyruvate solution (Figure 1b). Hence, it appears that it is the reaction shown in eq 8 which is sensitive to the presence of acetyl-CoA. It is apparent that acetyl-CoA participates in the interconversion between the two carboxylated enzyme forms. Whether this occurs by facilitating the transfer of the carboxyl group to the biotin or by labilizing the carboxyphosphate complex to release CO_2 in the vicinity of the biotin moiety is unclear [see also Knowles (1989)].

The observation that the acetyl-CoA-independent activity of chicken liver PC can approach 2% of the activator-stimulated rate, with high substrate and enzyme levels and in the presence of K^+ ions, represents a 30-fold increase over the value reported previously for acetyl-CoA-independent activity in chicken liver PC (Ashman et al., 1972). Barden and Scrutton (1974) have reported that for chicken liver PC, while the apparent K_a for K^+ is independent of acetyl-CoA, K^+ binds after the bicarbonate ion and has an effect on events both before and after the carboxylation of biotin. It appears that one of the functions of acetyl-CoA in the reaction mechanism is to facilitate the cleavage of the carboxyphospho moiety, with the cleavage occurring between the carbon and the oxygen of the C–O–P linkage. The question arises as to how PC carries out the transition between the carboxyphospho–enzyme form and the carboxy–biotin–enzyme form in the absence of acetyl-CoA. In conjunction with the observation that an ionizing group with a pK value of 8.6 is involved in the acetyl-CoA activation process (Scrutton & Utter, 1967), the altered pH profile of the acetyl-CoA-independent activity as compared with the dependent activity suggests that the ionization of an amino acid residue involved in the interconversion of the two carboxylated enzyme forms may be facilitated by acetyl-CoA (or K^+). From the fact that K^+ will replace acetyl-CoA, *albeit* poorly, it would appear that acetyl-CoA is the normal biological stimulator of pyruvate carboxylase.

REFERENCES

Ashman, L. K., & Keech, D. B. (1975) *J. Biol. Chem. 250*, 14–21.

Ashman, L. K., Keech, D. B., Wallace, J. C., & Nielsen, J. (1972) *J. Biol. Chem. 247*, 5818–5824.

Attwood, P. V., & Keech, D. B. (1985) *Curr. Top. Cell. Regul. 23*, 1–55.

Attwood, P. V., & Graneri, B. D. L. A. (1991) *Biochem. J. 273*, 443–448.

Barden, R. E., & Scrutton, M. C. (1974) *J. Biol. Chem. 249*, 4829–4838.

Climent, I., & Rubio, V. (1986) *Arch. Biochem. Biophys. 251*, 465–470.

Easterbrook-Smith, S. B., Hudson, P. J., Goss, N. H., Keech, D. B., & Wallace, J. C. (1976) *Arch. Biochem. Biophys. 176*, 709–720.

Goss, N. H., Dyer, P. Y., Keech, D. B., & Wallace, J. C. (1979) *J. Biol. Chem. 254*, 1734–1739.

Hansen, D. E., & Knowles, J. R. (1985) *J. Am. Chem. Soc. 107*, 8304–8305.

Helmerhorst, E., & Stokes, G. B. (1980) *Anal. Biochem. 104*, 130–135.

Kaziro, Y., Hass, L. F., Boyer, P. D., & Ochoa, S. (1962) *J. Biol. Chem. 237*, 1460–1468.

Keech, D. B., & Utter, M. F. (1963) *J. Biol. Chem. 238*, 2609–2614.

Kluger, R., & Adawadkar, P. D. (1976) *J. Am. Chem. Soc. 98*, 3741–3742.

Kluger, R., Davis, P. P., & Adawadkar, P. D. (1979) *J. Am. Chem. Soc. 101*, 5995–6000.

Knowles, J. R. (1989) *Annu. Rev. Biochem. 58*, 195–221.

Lim, F., Morris, C. P., Occhiodoro, F., & Wallace, J. C. (1988) *J. Biol. Chem. 263*, 11493–11497.

McClure, W. R., Lardy, H. A., & Cleland, W. W. (1971a) *J. Biol. Chem. 246*, 3584–3590.

McClure, W. R., Lardy, H. A., Wagner, M., & Cleland, W. W. (1971b) *J. Biol. Chem. 246*, 3579–3583.

Powers, S. G., & Meister, A. (1978) *J. Biol. Chem. 253*, 1258–1265.

Rylatt, D. B., Keech, D. B., & Wallace, J. C. (1977) *Arch. Biochem. Biophys. 183*, 113–122.

Scrutton, M. C., & Utter, M. F. (1965) *J. Biol. Chem. 240*, 3714–3723.

Scrutton, M. C., & Utter, M. F. (1967) *J. Biol. Chem. 242*, 1723–1735.

Scrutton, M. C., Keech, D. B., & Utter, M. F. (1965) *J. Biol. Chem. 240*, 574–581.

Vogel, I. A. (1967) in *Text Book of Practical Organic Chemistry*, 3rd ed., p 967, Longmans, Green & Co. Ltd., London.

Wallace, J. C., Phillips, N. B., Snoswell, M. A., Goodall, G. J., Attwood, P. V., & Keech, D. B. (1985). *Ann. N.Y. Acad. Sci. 447*, 169–188.

Registry No. PC, 9014-19-1; K^+, 7440-09-7; acetyl-CoA, 72-89-9.