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On the Intermediacy of Carboxyphosphate in Biotin-Dependent Carboxylations†

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ABSTRACT: In the ATP-dependent carboxylation of biotin that is catalyzed by most biotin-dependent carboxylases, a fundamental mechanistic question is whether the ATP activates bicarbonate (via the formation of carboxyphosphate as an intermediate) or whether the ATP activates biotin (via the formation of *O*-phosphobiotin). We have resorted to three mechanistic tests using the biotin carboxylase subunit of acetyl-CoA carboxylase from *Escherichia coli*: positional isotope exchange, intermediate trapping, and ^{18}O tracer experiments on the ATPase activity. First, no catalysis of positional isotope exchange in adenosine 5′-([α,β-$^{18}O,\beta,\beta$-$^{18}O_2$]triphosphate) was observed when either biotin or bicarbonate was absent, nor was any exchange seen in the presence of both *N*-1-methylbiotin and bicarbonate. Second, the putative carboxyphosphate intermediate could not be trapped as its trimethyl ester, under conditions of incubation and analysis where the authentic triester was shown to be adequately stable. In the third test, however, we showed that the ATPase activity of biotin carboxylase that is seen in the absence of biotin, an activity that is known to parallel the normal carboxylase reaction when biotin is present, occurs with the transfer of an ^{18}O label directly from [^{18}O]bicarbonate into the product P_i. This result suggests that the bicarbonate-dependent biotin-independent ATPase reaction catalyzed by biotin carboxylase goes via carboxyphosphate and that the carboxylation of biotin itself may proceed analogously.

The carboxylation reactions that are catalyzed by biotin-dependent enzymes all involve 1-carboxybiotin (*N*-1-carboxybiotin), the intermediacy of which has been demonstrated both by direct trapping (Lynen et al., 1959, 1961; Knappe et al., 1961) and by experiments that have shown the chemical and kinetic competence of the synthetic material (Guchhait et al., 1974b). The *N*-1-carboxybiotin intermediate is normally produced from biotin and bicarbonate in an ATP-dependent reaction that is common to all biotin-dependent carboxylases (except transcarboxylase, where the carboxyl group donor is a β-keto or a β-thioester acid derivative). The mechanism by which the carboxylation of the N-1 ureido nitrogen of biotin is driven by ATP has remained a vexatious issue, despite considerable experimental scrutiny (Kaziro et al., 1962; Moss & Lane, 1971; Wood & Barden, 1977; Kluger et al., 1979; Attwood & Keech, 1984; Hansen & Knowles, 1985). There have been three favored pathways proposed that seek in different ways to overcome the two mechanistic problems: that biotin is a poor nucleophile and that bicarbonate is a poor electrophile (see Figure 1). Each of these three pathways accommodates the important finding that one of the three bicarbonate oxygens ends up in product phosphate in every turnover of a biotin-dependent carboxylase (Kaziro et al., 1962). In mechanism 1 (stepwise), bicarbonate is phosphorylated by ATP to form the highly reactive mixed-anhydride carboxyphosphate, which is then attacked by the N-1 nitrogen of enzyme-bound biotin (either directly or after collapse to enzyme-bound CO_2; Sauers et al., 1975) to yield carboxybiotin. This pathway has the attraction of involving a precedented species, carboxyphosphate (Powers & Meister, 1976, 1978; Wimmer et al., 1979), though this is balanced by the evident lack of nucleophilicity of the ureido nitrogen of biotin (Caplow, 1965; Caplow & Yager, 1967; Bruice & Hegarty, 1970). In mechanism 2 (concerted), ATP is used to activate biotin rather than bicarbonate by phosphorylation on the ureido *oxygen* to give the reactive species *O*-phosphobiotin. In a subsequent (chemically unprecedented)

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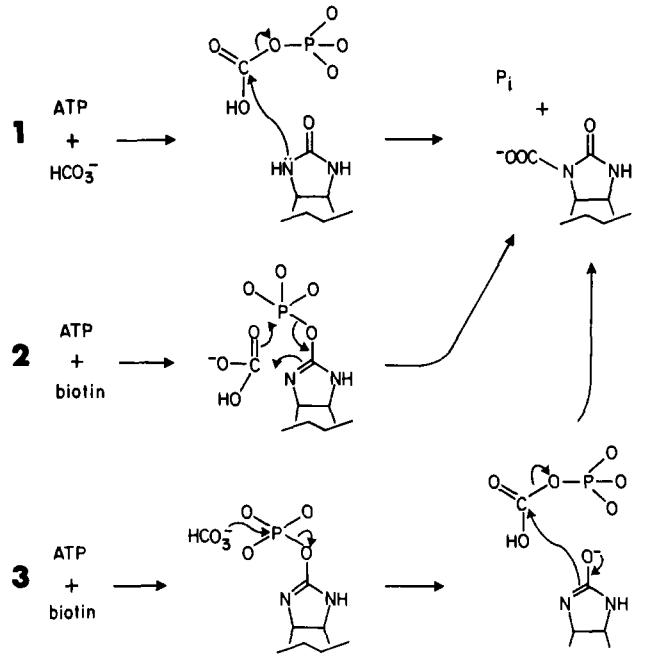

FIGURE 1: Mechanistic possibilities for the ATP-dependent carboxylation of biotin by bicarbonate, catalyzed by biotin carboxylase.

six-electron pericyclic process, biotin becomes carboxylated on N-1 and phosphate is lost. Finally, mechanism 3, in analogy to the preferred pathway for the enzyme phosphoenolpyruvate carboxylase (Hansen & Knowles, 1982), involves the formation of *O*-phosphobiotin that is attacked by bicarbonate to yield carboxyphosphate and the isourea form of biotin. These two reactive species then collapse to give the products (Figure 1).

Although both chemical and enzymic analogy made mechanism 3 the most attractive, our recent stereochemical studies (Hansen & Knowles, 1985) have, sadly, cast this route into disfavor. Thus, when the stereochemical fate of the γ phospho group of adenosine [γ-S_P]-5′-*O*-([$\beta\gamma$-^{17}O, γ-^{17}O, ^{18}O]-3-thiotriphosphate) was followed in the reaction catalyzed by pyruvate carboxylase, *inversion* of the configuration at phosphorus was seen. On the reasonable basis that each act of enzyme-catalyzed phospho group transfer involves inversion of the configuration at phosphorus (Knowles, 1980), this result eliminated mechanism 3. While it has been suggested that the generation of carboxyphosphate from *O*-phosphobiotin might proceed by a pseudorotatory route with retention of configuration for this step (Kluger et al., 1979), the lack of enzymic precedent for pseudorotatory pathways and the corpus of knowledge on enzymic phospho transfer persuade us to remove mechanism 3 from further consideration. Pathways 1 and 2 thus remain. The experiments described in this paper focus on the distinction between these two pathways, and the results reported provide evidence in favor of mechanism 1.

Experimental Procedures

Materials

Adenosine 5′-([α,β-^{18}O, β,β-$^{18}O_2$]Triphosphate) was prepared as described by Hassett et al. (1982). The distribution of ^{18}O was evaluated by ^{31}P NMR and found to be as follows: α,β bridge $^{18}O_1$, 79%; α peripheral positions $^{18}O_1$, 11%; α,β bridge $^{18}O_1$ with β peripheral positions $^{18}O_2$, 68%; α,β bridge $^{18}O_1$, with a β peripheral position $^{18}O_1$, 10%; β peripheral positions $^{18}O_2$, 16%; β peripheral position $^{18}O_1$, 3%; γ peripheral positions $^{18}O_3$, 2%; γ peripheral positions $^{18}O_2$, 2%; and γ peripheral position $^{18}O_1$, 3%. Since the critical resonance for the positional isotope exchange experiments is that of the γ phosphorus, the fact that 93% of the oxygens attached to the γ phoshorus are ^{16}O allowed the positional exchange from the peripheral β positions (which are 95.5% ^{18}O) to be readily measured.

Dimethyl phosphate was prepared from dimethyl phosphorochloridate (Sosnovsky & Zaret, 1969) by hydrolysis in aqueous pyridine. The diester was purified by chromatography on a column of Dowex AG-1 [equilibrated with 25 mM triethylammonium bicarbonate buffer, pH 7.5, and eluted with a linear gradient (25–200 mM) of the same buffer]. The material was converted to the sodium salt by passage down a column of Dowex 50 (Na^+ form).

Trimethyl Carboxyphosphate. Sodium dimethyl phosphate (210 mg) suspended in dry dioxane (10 mL) was refluxed with methyl chloroformate (10 equiv) for 2 h. After the NaCl was removed by filtration and the solvent was removed by evaporation under reduced pressure, the resulting oil (160 mg) was purified by HPLC on a column of Zorbax C-8 (Du Pont, Wilmington, DE) eluted with acetonitrile–water (34:66 v/v). The product was extracted from the appropriate fractions into ethyl acetate, and this solution was dried over anhydrous sodium sulfate. After removal of the solvent, the product had ^{1}H NMR ($CDCl_3$) δ 3.90 (d, 6 H, J = 11.7 Hz) and 3.95 (s, 3 H). Fast atom bombardment mass spectrometry (in 3-nitrobenzene) gave $M^+ + 1$ at m/z 185.

N-1-Methylbiotin was prepared as follows. Sodium hydride (2 mmol) was washed with *n*-pentane and then suspended in dimethylformamide (5 mL). After the suspension was cooled to −20 °C, biotin methyl ester (prepared from biotin and diazomethane, 2 mmol) and methyl iodide (2 mmol) were added at −20 °C. The mixture was stirred and allowed to warm to room temperature over 2 h. Water was then added, and the product was extracted into ethyl acetate. After removal of the solvent, the products were chromatographed on a column of silica gel in CH_2Cl_2. *N*-1,*N*-3-Dimethylbiotin methyl ester was eluted by CH_2Cl_2–methanol (99:1 v/v) and *N*-1- and N-3-monomethylbiotin methyl esters eluted together in CH_2Cl_2–methanol (98:2 v/v). The esters (160 mg) were treated with aqueous ammonia (5 mL) in methanol (5 mL) at room temperature for 20 h. The four products (the *N*-1-methyl and *N*-3-methyl acids and amides) were separated by chromatography first on DEAE-Sephadex in triethylammonium bicarbonate buffer, pH 7.5, followed by HPLC on ODS-H-2151 (Senshukagaku, Tokyo) eluting with acetonitrile–triethylammonium phosphate buffer, pH 6.9 (14:86 v/v). *N*-1-Methylbiotin was recrystallized from hot water and had ^{1}H NMR (pyridine-d_5) δ 7.6 (1 H, br s, N*H*), 4.24 (1 H, m, collapses to dd with D_2O, J = 7.9 and 4.25 Hz, C-4 ring-fused *H*), 4.18 (1 H, dd, J = 7.9 and 4.5 Hz, C-5 ring-fused *H*), 3.23 (1 H, m), 2.94 (1 H, d, J = 12.9 Hz), 2.80 (3 H, s, NCH_3), 2.79 (1 H, dd, J = 12.9 and 4.5 Hz), 2.46 (2 H, t, J = 7.3 Hz, CH_2CO_2H), and 1.9–1.5 (6 H, m). *N*-3-Methylbiotin had ^{1}H NMR (pyridine-d_5) δ 7.7 (1 H, br s, N*H*), 4.43 (1 H, m), 3.94 (1 H, dd, J = 8.4 and 4.8 Hz), 3.23 (1 H, m), 2.92 (3 H, s, NCH_3), 2.87 (2 H, m), 2.51 (2 H, t, J = 7.1 Hz), and 1.9–1.5 (6 H, m).

Biotin carboxylase was isolated from *Escherichia coli* K12 (555 g, from the Grain Processing Corp., Muscatine, IA) by modification of the method of Guchhait et al. (1974a). Chromatography on phosphocellulose was omitted, and the enzyme fraction after the DEAE-cellulose column was further chromatographed on CM-Sephadex C-50 (eluting with a linear gradient of 10–200 mM sodium phosphate buffer) and then on hydroxyapatite (eluting with a linear gradient of 10–200 mM sodium phosphate buffer). The purified enzyme was

>90% homogeneous on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and had a specific catalytic activity of 3.3 units/mg. The value cited by Guchhait et al. (1974a) is 3.6 units/mg.

Methods

Ultraviolet absorbance measurements were made with a Perkin-Elmer 554 spectrophotometer. HPLC analyses were done on a Waters 510 instrument equipped with a Waters 490 multiwavelength detector and automated gradient controller. Scintillation counting was performed on a Beckman LS1081 instrument. NMR measurements were made on a Bruker AM300 spectrometer. Mass spectrometric data were collected in either EI or FAB mode by use of a Kratos MS 50L double-focusing instrument.

Positional isotope exchange experiments were done as follows. The reaction mixtures (2 mL) all contained 50 mM HEPES buffer, pH 7.5, labeled ATP (5 mM), $MgCl_2$ (8 mM), reduced glutathione (3 mM), bovine serum albumin (0.6 mg/mL), ethanol (10% v/v), and purified biotin carboxylase (0.15–1.2 units). Five experiments were performed, with additions (a) potassium bicarbonate (8 mM), (b) biotin (50 mM), (c) *N*-1-methylbiotin (50 mM) and potassium bicarbonate (8 mM), (d) *N*-1-methylbiotin amide (50 mM) and potassium bicarbonate (8 mM), or (e) biotin (50 mM), potassium bicarbonate (8 mM), and potassium phosphate (250 mM). The incubation mixture lacking bicarbonate [that is, (b)] was first sparged with N_2 overnight, the enzyme being contained in a small dialysis bag during the sparging process. The incubations were at 30 °C. (a–c) were incubated for 200 min, (d) was incubated for 15 h, and (e) was incubated for 14 h. As a check on the labeled ATP and the analytical procedures, a sample of ATP (3 mM) was subjected to complete positional isotope exchange by incubation with creatine (10 mM), EDTA (2 mM), $Mg(OAc)_2$ (5 mM), 2-mercaptoethanol (10 mM), and creatine kinase (10 units) in 100 mM imidazole–HCl buffer, pH 6.8. After 6 h at 30 °C, complete scrambling of the peripheral β-$^{18}O_2$ labels into the β,γ bridge position was found.

Diazomethane trapping experiments for carboxyphosphate were done as follows. The incubation mixture contained: [γ-^{32}P]ATP (0.4 mM, 10–50 mCi), potassium bicarbonate (8 mM), biotinol (20 mM; control experiments showed that use of biotin itself leads to substantial decomposition of any trimethyl carboxyphosphate present, presumably because of the higher pH at the end of the quench with excess diazomethane that results from the methylation of biotin's carboxylate group), reduced glutathione (3 mM), $MgCl_2$ (8 mM), bovine serum albumin (0.6 mg/mL), ethanol (10% v/v), and purified biotin carboxylase (0.2–2.4 units, 1.2–14.4 nmol) in 50 mM triethanolamine–HCl buffer, pH 7.4 (total volume, 100 μL). Analogous experiments were also performed in the absence of biotinol. After 2–20 min at room temperature, excess diazomethane in solution in ether–acetonitrile (1 mL) containing unlabeled trimethyl carboxyphosphate as a marker was added, followed rapidly by ethyl acetate (1 mL) and water (1 mL). The mixture was vortexed, and then the organic layer was left over anhydrous sodium sulfate for 20 min. After careful evaporation to dryness, the residue was redissolved in acetonitrile (50 μL) and subjected to reverse-phase HPLC. The column was monitored for ^{32}P.

Tracer ^{18}O experiments to detect the transfer of ^{18}O from [^{18}O]bicarbonate to inorganic phosphate were done as follows. The reaction mixture contained ATP (1 mM), reduced glutathione (8 mM), $MgCl_2$ (8 mM), KCl (50 mM), bovine serum albumin (0.6 mg/mL), ethanol (10% v/v), and biotin carboxylase (10 units) in 100 mM triethanolamine–HCl buffer, pH 7.9 (2 mL). This solution was added to potassium [^{18}O]bicarbonate [16 μmol, prepared by freeze-drying an appropriate volume of unlabeled potassium bicarbonate that had been allowed to equilibrate with $H_2{}^{18}O$ (>95% atom excess) under Ar for 24 h at room temperature] and the mixture left at 30 °C for 14 min. The reaction was then stopped by the addition of ethanol (4 mL). Inorganic phosphate (420 nmol) was isolated by ion-exchange chromatography first on DEAE-Sephadex and then on Dowex AG-1, and the free acid was obtained by passage through Dowex 50 (H^+ form). The phosphoric acid sample was then converted to trimethyl phosphate by treatment with diazomethane and analyzed by mass spectrometry.

Results and Discussion

The two routes for the ATP-dependent carboxylation of biotin that we must consider (mechanisms 1 and 2 of Figure 1) are very different. One involves phosphorylation of bicarbonate to carboxyphosphate, and the other proceeds via the formation of *O*-phosphobiotin. The evident chemical lability of each of these species clearly precludes any attempt to investigate the competence of exogenously added synthetic material. Thus, Sauers et al. (1975) have estimated the half-life of carboxyphosphate in neutral aqueous solution to be on the order of 70 ms, and efforts by Blonski et al. (1984) to isolate *O*-phosphobiotin analogues (as distinct from their S-phospho counterparts) have proved unsuccessful. To distinguish between the two mechanisms, therefore, we must use other approaches, and we discuss here the results of four different methods of attack: (a) the use of substrate analogues; (b) the investigation of isotope-exchange processes; (c) efforts to trap the putative intermediate directly; and (d) ^{18}O tracer experiments.

Substrate Analogues. When carbamyl phosphate is incubated with ADP and either *E. coli* acetyl-CoA carboxylase (Polakis et al., 1972, 1974) or pyruvate carboxylase from sheep liver (Ashman & Keech, 1975), ATP is formed. The presumption that carbamyl phosphate acts as a surrogate for the putative carboxyphosphate intermediate led to mechanism 1 being favored. Yet Kluger et al. (1979) have argued forcefully that carbamyl phosphate could equally well be an analogue of *O*-phosphobiotin in mechanism 2 and that this unnatural catalytic reaction (of ATP production from carbamyl phosphate and ADP) is not mechanistically conclusive. In other experiments, Ashman and Keech (1975) found that phosphonoacetate (another stable analogue of the putative carboxyphosphate) is a noncompetitive inhibitor of pyruvate carboxylase, and, although the binding is not very tight, this finding is also consistent with reaction via mechanism 1. Overall, while these results are suggestive, they do not provide an unambiguous distinction between mechanisms.

Isotope Exchanges. If the carboxylation of biotin followed a simple ping-pong pathway, isotope-exchange experiments would distinguish between mechanisms 1 and 2. Unfortunately, neither a rapid carboxylase-catalyzed ATP/ADP exchange in the absence of biotin (which would support 1) nor an ATP/ADP exchange in the complete absence of bicarbonate (which would suggest 2) has been observed (Scrutton & Utter, 1965; Barden et al., 1972; Polakis et al., 1974; Ashman & Keech, 1975; Wallace et al., 1985). The failure to observe partial exchange reactions is not uncommon, of course, and may derive either from the need to assemble all the reaction components before any chemistry, even of partial reactions, can occur (that is, "substrate synergism") or from the fact that product release does not occur until all

chemical processes at the active site are complete. In the present case, this second possibility would mean that ADP could only leave if biotin (mechanism 1) or bicarbonate (mechanism 2) were bound. Such behavior can often be uncovered by studying the catalysis of positional isotope exchange in appropriately labeled [^{18}O]ATP by the methods developed by Midelfort and Rose (1976). We chose first, therefore, to investigate the existence of a carboxylase-catalyzed exchange of ^{18}O from the peripheral β positions of substrate ATP into the β,γ bridge position of reisolated material. Although in unpublished experiments M. Wimmer had failed to observe any significant positional isotope exchange catalyzed by pyruvate carboxylase or propionyl-CoA carboxylase (Mary Wimmer, private communication), we have reinvestigated this question using the purified biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase (Guchhait et al., 1974a). Use of this enzyme system has the advantage of allowing us to investigate the exchange reaction in the absence of biotin, as well as to examine the possible synergistic effects of free biotin analogues such as *N*-1-methylbiotin, as has been earlier explored for the carbamyl phosphate reaction by Lane and his collaborators (Polakis et al., 1974).

Adenosine 5′-([α,β-^{18}O, β,β-$^{18}O_2$]triphosphate) was used as the substrate in positional isotope exchange experiments, the reaction progress being followed by ^{31}P NMR. Five incubations were performed: (a) in the absence of biotin; (b) in the absence of bicarbonate; (c) where biotin was replaced by *N*-1-methylbiotin; (d) where biotin was replaced by *N*-1-methylbiotin amide; and (e) with the complete reaction mixture in the presence of P_i. At the end of each incubation, the labeled ATP was reisolated by ion-exchange chromatography on DEAE-Sephadex, and the positions of the ^{18}O labels were established by ^{31}P NMR. In none of the five incubations was any significant positional isotope exchange observed. Control experiments established that the biotin carboxylase always retained full catalytic activity. Less than 3% of the ATP had been hydrolyzed to ADP by the end of incubations a and c, 11% at the end of incubation d, and 28% in incubation e.

From these experiments, we find that no positional isotope exchange processes are catalyzed by biotin carboxylase. When either biotin or bicarbonate is absent, no positional isotope exchange is observed. The presence of *N*-1-methylbiotin [which Polakis et al. (1974) had shown to be an effective synergistic agent in accelerating the rate of ATP synthesis from ADP and carbamyl phosphate] failed to produce a detectable exchange rate. Even in the complete system (that is, including ATP, bicarbonate, biotin, P_i, and enzyme), no positional exchange was observed. Omission of ethanol [which is normally present to activate the enzyme (Guchhait et al., 1974a)] had no effect on the outcome of these experiments. These findings are in agreement with the results obtained both by M. Wimmer (private communication) and, very recently, by Tipton and Cleland (1988b). In general, there are two reasons why the positional isotope exchange test can fail. First, the relevant intermediate state (in this case, enzyme-bound ADP and carboxyphosphate for mechanism 1 or enzyme-bound ADP and *O*-phosphobiotin for mechanism 2) may partition forward so strongly that the resynthesis of isotopically scrambled ATP is too rare an event to be detected. This explanation certainly cannot be ruled out, granting the expected high chemical reactivity of each of the putative intermediates, carboxyphosphate or *O*-phosphobiotin. The second reason why a positional isotope exchange reaction can fail is if the β phospho group of enzyme-bound ADP is not torsionally free. Such suppression of phospho group rotation has been suggested before [e.g. by Hilscher et al. (1985) in the case of argininosuccinate synthetase] and may be the reason why, for biotin carboxylase, no positional isotope scrambling can be observed.

Intermediate Trapping. In an effort to identify the intermediate, if any, involved in the enzyme-catalyzed carboxylation of biotin, we next followed the direct trapping approach. In 1976, Powers and Meister reported the trapping of carboxyphosphate as its trimethyl ester from reaction mixtures containing glutamine-dependent carbamyl phosphate synthetase at the steady state (Powers & Meister, 1976, 1978). Accordingly, we established a trapping protocol using diazomethane and a subsequent workup and chromatographic separation that would allow the hoped-for product (trimethyl carboxyphosphate, which was independently synthesized and characterized) to be identified. From all trapping experiments, a small peak of radioactivity was seen at the retention volume of authentic trimethyl carboxyphosphate, following a large peak of trimethyl phosphate. The size of the radioactive peak coeluting with authentic trimethyl carboxyphosphate did not, however, exceed that found from control incubations in the absence of enzyme, and the expected correlation between the amount of enzyme in the incubation and the size of the notional trimethyl carboxyphosphate peak was not satisfied. These results, along with those from experiments using different quenching methods and incubation conditions as well as the alterntive use of [^{14}C]bicarbonate, suggested that the small amount of trimethyl carboxyphosphate we observed comes from the *nonenzymic* formation of carboxyphosphate by attack of bicarbonate on ATP (or, more probably, of bicarbonate on ATP methyl esters formed during the quench). Evidently, if carboxyphosphate is formed in the active site of the carboxylase, it is either at such low levels in the steady state or is so inaccessible to external diazomethane (such sequestration would hardly be surprising for a labile intermediate) that none can be detected in direct trapping experiments. Other failures to trap presumed carboxyphosphate intermediates have been reported for pyruvate carboxylase (Wallace et al., 1985) and for phosphoenolpyruvate carboxylase (Fujita et al., 1984).

^{18}O Tracer Experiments. It has recently been shown that biotin carboxylase possesses a low ATPase activity in the absence of biotin, an activity that appears to be an intrinsic property of the carboxylase (Climent & Rubio, 1986; Tipton & Cleland, 1988a). Thus, the ATPase activity is dependent upon the presence of bicarbonate, is increased by the presence of 10% ethanol to the same degree as the normal biotin carboxylation reaction, shows the same magnesium ion and potassium ion dependence, and displays a thermal stability equivalent to that of the normal reaction. This hydrolysis of ATP seems, therefore, to be catalyzed by the carboxylase in what could well be a diversion from the normal fate of carboxyphosphate when biotin is present as an acceptor. Yet it can also be argued that the observed ATPase activity is adventitious and that the dependence of this reaction on the presence of bicarbonate derives from some conformational activation of the carboxylase. To prove that the activity indeed comes from the hydrolytic diversion of a carboxyphosphate intermediate, we must show that bicarbonate is involved covalently. This can be done by establishing that the P_i produced from ATP in the absence of biotin derives its fourth oxygen from bicarbonate rather than from water.

First, the bicarbonate-dependent ATPase activity of purified biotin carboxylase in the absence of biotin was confirmed and found to be approximately 0.5% of the rate from the normal carboxylation reaction (Figure 2), in good agreement with the

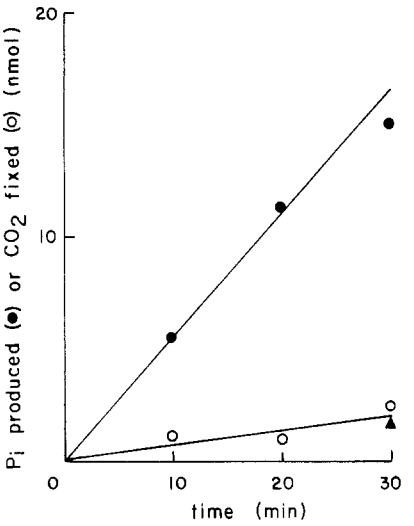

FIGURE 2: ATPase activity of biotin carboxylase in the absence of biotin. (●) P_i produced from an incubation (1 mL) containing ATP (1 mM), $KHCO_3$ (8 mM), $MgCl_2$ (8 mM), reduced glutathione (3 mM), KCl (5 mM), bovine serum albumin (0.6 mg/mL), and biotin carboxylase (0.1 unit) in 100 mM triethanolamine–HCl buffer, pH 8.0, containing ethanol (10% v/v) at 30 °C. (▲) As above, except in the absence of enzyme. (○) As above in the presence of enzyme, except that [^{14}C]bicarbonate was used, and the amount of CO_2 fixed was measured according to the method of Guchhait et al. (1974a).

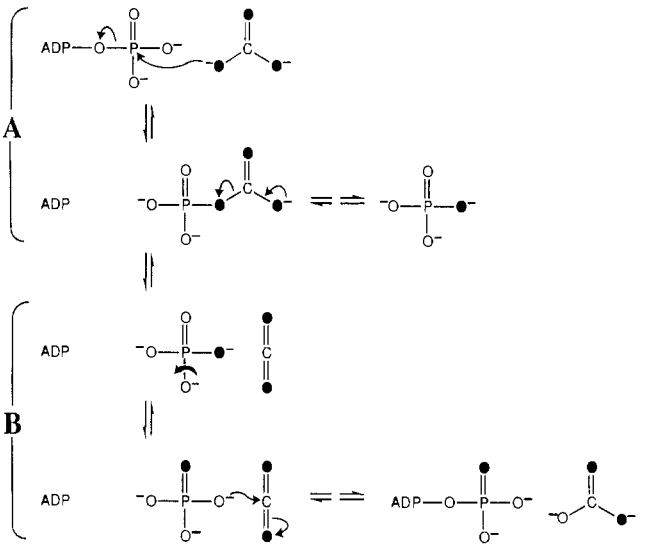

FIGURE 3: Pathways for (A) bicarbonate-dependent ATPase reaction via the intermediacy of carboxyphosphate and for (B) the transient production of enzyme-bound CO_2 that could in principle lead to the exchange of an ^{18}O label from bicarbonate into reisolated ATP.

original finding of Climent and Rubio (1986). That this activity is not due to a very low biotin contamination was proved by the fact that the level of [^{14}C]bicarbonate converted to an involatile form (fixed) is unaffected by the presence or absence of enzyme in control incubations. The experiment to determine the source of the fourth oxygen of P_i deriving from the bicarbonate-dependent ATPase reaction was then performed by incubation of ATP with [^{18}O]bicarbonate in buffered enzyme solution in $H_2{}^{16}O$. After 14 min at 30 °C, the reaction was stopped and the P_i isolated. Conversion of this P_i to trimethyl phosphate with diazomethane then allowed mass spectrometric analysis. The relative intensity of M^+ (the molecular ion at 140) and $M^+ + 2$ (at 142) was then compared with that observed in the inorganic phosphate that derives from a control reaction of [^{18}O]bicarbonate with 0.5% the quantity of enzyme in the *presence* of biotin for the same incubation time. The different amount of enzyme used in the "plus biotin" control roughly compensated for the slower rate of ATP consumption in the "minus biotin" experiment. The ratio of $(M^+ + 2)/M^+$ in the minus biotin experiment was about 70% of that found in the plus biotin control, showing that *in the ATPase reaction of biotin carboxylase in the absence of biotin, the fourth oxygen of P_i derives not from water but from bicarbonate.* The level of ^{18}O observed in the P_i is reasonable, on the basis that the ^{18}O content of the original bicarbonate is about 95% and that the half-life of ^{18}O in bicarbonate in $H_2{}^{16}O$ at pH 7.9 and 37 °C has been reported to be 7.8 min (Wimmer et al., 1979). These results indicated that the biotin-independent ATPase reaction catalyzed by biotin carboxylase in the presence of bicarbonate occurs via the intermediacy of carboxyphosphate (Figure 3A) and strengthen the view that the carboxylation of biotin itself proceeds analogously.

Finally, while carboxyphosphate is an attractive first intermediate in the carboxylation of biotin, Jencks and his group have argued persuasively (Sauers et al., 1975) that carboxyphosphate might collapse to enzyme-bound CO_2, which should be a *more* electrophilic species for the carboxylation either of biotin or (in subsequent steps of the action of carboxylases) of carbon enolates. According to this view, the role of ATP would be to dehydrate bicarbonate and to deliver a reactive molecule of CO_2 to appropriate nucleophiles at the active site. If this theory is correct, there is a chance that a positional isotope exchange, one involving the tumbling of P_i at the active site (Figure 3B), could lead to the incorporation of ^{18}O from [^{18}O]bicarbonate into remaining ATP. That such a phenomenon is possible is suggested by the results of Fujita et al. (1984) with bicarbonate-dependent phosphoenolpyruvate carboxylase. In these experiments, when the substrate analogue phosphoenol-α-ketobutyrate was incubated with the enzyme in the presence of [^{18}O]bicarbonate, multiple incorporations of ^{18}O into the liberated P_i were observed, which could (at least in principle) arise from the tumbling of the enzyme-bound P_i. In the present case, such positional isotope exchange and reaction reversal would lead to the appearance of ^{18}O in the remaining ATP. When the reisolated ATP was subjected to fast atom bombardment mass spectrometry, however, *no* ^{18}O incorporation was observed. This finding is mirrored by that made by Tipton and Cleland (1988b) using γ-labeled ATP in $H_2{}^{16}O$.

The results reported in the present paper can be compared with those obtained for the enzyme carbamyl-phosphate synthetase, which has several features in common with the biotin-dependent carboxylases and which one might expect would share a similar mechanism. For the synthetase, positional isotope exchange experiments and direct trapping studies have indicated the intermediacy of carboxyphosphate, and this enzyme also shows a bicarbonate-dependent ATPase activity in which ^{18}O label is transferred from bicarbonate to P_i (Wimmer et al., 1979). For biotin carboxylase, in contrast, the carboxyphosphate intermediate is so tightly sequestered and/or so short-lived that it eludes detection by positional isotope exchange or by direct trapping. Yet the fact that the bicarbonate-dependent ATPase reaction catalyzed by biotin carboxylase in the absence of biotin produces P_i containing a single ^{18}O label that derives from bicarbonate indicates that the enzyme catalyzes the formation of a carboxyphosphate intermediate. It is then most economical to suppose that the same species is formed and is a necessary intermediate in the complete reaction that occurs in the *presence* of the cosubstrate biotin.

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