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Carbon-13 and Deuterium Isotope Effects on the Catalytic Reactions of Biotin Carboxylase†

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ABSTRACT: ^{13}C and ^{2}H kinetic isotope effects have been used to investigate the mechanism of enzymic biotin carboxylation. $^{D}(V/K)$ is 0.50 in 80% D_2O at pD 8.0 for the forward reaction and 0.57 at pD 8.5 for the phosphorylation of ADP by carbamoyl phosphate. These values approach the theoretical maximum limit for a reaction in which a proton is transferred from a sulfhydryl to a nitrogen or oxygen base. Therefore, it appears that this portion of the reaction is at or near equilibrium. $^{13}(V/K)$ at pH 8 is 1.007; the small magnitude of this number suggests that the reaction is almost fully committed by the time the carbon-sensitive steps are reached. There does not appear to be a reverse commitment to the reaction under the conditions in which $^{13}(V/K)$ was determined. A large forward commitment is consistent with the failure to observe positional isotope exchange from the $\beta\gamma$-bridge position to the β-nonbridge position in [$^{18}O_4$]ATP or washout of ^{18}O from the γ-nonbridge positions. Transfer of ^{18}O from bicarbonate to inorganic phosphate in the forward reaction was clearly observed, however. These observations suggest that biotin carboxylase exists in two distinct forms which differ in the protonation states of the two active-site bases, one of which is a sulfhydryl. Only when the sulfhydryl is ionized and the second base protonated can catalysis take place. Carboxylation of biotin is postulated to occur via a pathway in which carboxyphosphate is formed by nucleophilic attack of bicarbonate on ATP. Decarboxylation of carboxyphosphate in the active site generates CO_2, which serves to carboxylate the isourea tautomer of biotin that is generated by the removal of the proton on N1′ by the ionized sulfhydryl.

The biotin carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase catalyzes the MgATP-dependent carboxylation of biotin, which delivers the carboxyl group to acetyl CoA at the active site of the carboxyltransferase subunit. Polakis et al. (1974) demonstrated that biotin carboxylase, in common with other ATP- and biotin-dependent carboxylases, uses bicarbonate as the source of the carboxyl group and that during the course of the reaction ATP is hydrolyzed to ADP and inorganic phosphate.

A bewildering variety of mechanisms has been advanced for the ATP-dependent carboxylation of biotin. For years, the prime mechanistic constraint has been the observation of Kaziro et al. (1962) that ^{18}O from substrate bicarbonate is incorporated into inorganic phosphate and the carboxylated product in the ratio of 1:2. Various investigators have also found that carbamoyl phosphate will serve as a phosphoryl group donor in the biotin carboxylase (Polakis et al., 1972) or pyruvate carboxylase (Ashman & Keech, 1975) catalyzed phosphorylation of ADP, suggesting that the normal biotin carboxylation reaction proceeds via a carbonic-phosphoric anhydride intermediate ("carboxyphosphate"), although this interpretation has been disputed (Kluger et al., 1979). Recently, Hansen and Knowles (1985) have further limited the possible mechanisms of biotin carboxylation by determining that the γ phosphate of ATP undergoes inversion of configuration during turnover by chicken liver pyruvate carboxylase.

We have determined the ^{2}H and ^{13}C kinetic isotope effects on the carboxylation of biotin catalyzed by biotin carboxylase with the aim of further paring down the mechanistic possi-

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bilities. These data, in conjunction with investigations of isotope-exchange reactions at the γ phosphate of ATP, lead us to argue in favor of a mechanism of biotin carboxylation in which carboxyphosphate is formed by nucleophilic attack by bicarbonate on ATP and then decomposes in the enzyme active site to yield CO_2 and phosphate. Carboxybiotin is then formed by nucleophilic attack on the CO_2 by the isourea tautomer of biotin.

Materials and Methods

Biotin carboxylase was isolated from wild-type *E. coli* B as described in the preceding paper (Tipton & Cleland, 1988b). D_2O (99.8 atom %) was from Aldrich. $H_2{}^{18}O$ was from the Mound Facility of the Monsanto Research Corp.

ATP stock solutions were calibrated by end-point assay with hexokinase and glucose-6-phosphate dehydrogenase. ADP was calibrated by end-point assay with pyruvate kinase and lactate dehydrogenase. Carbamoyl phosphate solutions were calibrated by end-point assay with carbamate kinase, hexokinase, and glucose-6-phosphate dehydrogenase. Bicarbonate stock solutions were calibrated by manometric measurement of the CO_2 released upon acidification.

^{13}C Isotope Effect Methodology. Determination of $^{13}(V/K)$ for biotin carboxylation was carreid out by using the procedure described by O'Leary (1980) with some modifications required by the transient nature of carboxybiotin. Low-conversion samples were prepared by placing solutions containing all the components of the reaction mixture except bicarbonate, EtOH, and enzyme in 25-mL screw-top centrifuge tubes and degassing overnight by sparging with N_2. EtOH was degassed separately by sparging with N_2. The appropriate volume of degassed EtOH was added to each tube and sparged for an additional 15 min. The tubes were sealed with airtight septa, and $KHCO_3$ was added via syringe from a 0.79 M stock solution. The complete reaction mixture contained the following components: 9 mM ATP, 14 mM $MgCl_2$, 66 mM biotin, 15% (v/v) EtOH, and 70 mM HCO_3^- in HEPES[1] at pH 7.9 in a total volume of 7 mL. $^{13}(V/K)_{D_2O}$ was measured under these conditions at pD 7.8. In another set of experiments the ATP level was reduced to 0.44 mM, and the ATP was recycled by using phosphoenolpyruvate and pyruvate kinase. These experiments were carried out at pH 8.1 and 7.1. To determine the effect of the divalent metal ion on $^{13}(V/K)$, 6.8 mM $MnCl_2$ was used in place of $MgCl_2$; the reaction was carried out in 200 mM HEPES at pH 7.6.

Reactions were initiated by addition of biotin carboxylase as a suspension in 60% ammonium sulfate. To minimize contamination of the samples by decarboxylation of the carboxybiotin, sufficient enzyme was added so that 20–30 μmol of carboxybiotin was formed within 12–20 min; this required approximately 3 units of biotin carboxylase. To determine the extent of the reaction, an aliquot was removed just prior to quenching and placed in ice; the amount of ATP that was turned over was then determined by enzymic end-point assay. Reactions were quenched and carboxybiotin was stabilized by addition of 50 μL of saturated CO_2-free KOH, followed by addition of 2.5 mL of a solution of 2.0 M $BaCl_2$/0.1 M $Ba(OH)_2$ (this represents a 10-fold excess of Ba^{2+} over HCO_3^-). The $BaCO_3$ formed from the unreacted HCO_3^- was removed from the solution by centrifugation for 15 min at 20000*g* at 4 °C. The supernatant, which contained the carboxybiotin and no $BaCO_3$, was removed to a nitrogen-flushed CO_2-isolated vessel of the type described by O'Leary (1980) by using a syringe equipped with a stopcock. The carboxybiotin was then decarboxylated by adding concentrated H_2SO_4; the CO_2 was collected and purified with a high-vacuum line and its isotopic content determined with a Finnegan Delta-E isotope ratio mass spectrometer. High-conversion samples were generated by acidifying aliquots of the stock $KHCO_3$ solution and isolating and analyzing the CO_2 as above.

^{18}O Exchange Studies. (a) Exchange within and from ATP. γ,γ,γ-Nonbridge, $\beta\gamma$-bridge [$^{18}O_4$]ATP was synthesized by the method of Werhli et al. (1965), using the modifications described by Midelfort and Rose (1976), and purified by chromatography on DEAE-Sephadex A-25. The [$^{18}O_4$]P_i used in the synthesis of the [$^{18}O_4$]ATP was synthesized as described by Hackney et al. (1980). Positional isotope exchange from the $\beta\gamma$-bridge position to the β-nonbridge position and washout of ^{18}O from the γ-nonbridge positions were assayed for by examination of the residual [^{18}O]ATP with ^{31}P NMR. ^{31}P NMR spectra were obtained on a Bruker AM-400 spectrometer operating at 161.9 MHz with a 10-mm probe. Proton-coupled 16K spectra were acquired by using a 50-Hz sweep width and a 10-μs pulse. The free induction decays were subjected to Gaussian multiplication and exponential multiplication prior to Fourier transformation in order to enhance resolution.

The exchange experiments were carried out with 13.8 mM [$^{18}O_4$]ATP, 50 mM biotin, 4.7 mM $KHCO_3$, 19.9 mM $MgCl_2$, and 15% (v/v) EtOH in 200 mM HEPES, pH 8.0, in a total volume of 10 mL. Reaction was initiated by addition of 0.2 unit of biotin carboxylase. To ensure that no reverse reaction occurred, the inorganic phosphate that was formed during turnover by biotin carboxylase was removed from the reaction with a coupling system consisting of 500 units of phosphorylase *a*, 115 units of phosphoglucomutase, 125 units of glucose-6-phosphate dehydrogenase, 20 mM NADP, and 2 mg/mL glycogen. To monitor the progress of reaction, aliquots were removed and assayed for ATP content by using a hexokinase, glucose-6-phosphate dehydrogenase end-point assay. In one experiment 20 mM ADP was present in addition to the other components listed above; in this case, 0.8 unit of biotin carboxylase was used to initiate the reaction.

Periodically, aliquots containing from 30 to 50 μmol of ATP were removed from the reaction and quenched by the addition of 100 μmol of EDTA; the enzyme was denatured by vigorous vortexing in the presence of 5 drops of CCl_4. The aliquots were then frozen until the ATP could be isolated. Because of the presence of glycogen in the samples, the ATP was purified before the NMR analysis. Samples were loaded into 2.5 × 25 cm columns of DEAE-Sephadex A-25 equilibrated in 100 mM triethylammonium bicarbonate. The ATP was eluted with a gradient from 100 mM to 1 M TEAB over 1 L. Fractions containing ATP were pooled, and the buffer was removed by rotary evaporation. Samples were prepared for NMR analysis by dissolving the ATP in 2 mL of 200 mM CHES, pH 9.0, 0.5 mL of D_2O, and 0.25 mL of 250 mM EDTA. Samples prepared in this way had a final pH of approximately 9.3.

Exchange reactions were also looked for in the bicarbonate-dependent biotin-independent ATPase reaction catalyzed by biotin carboxylase. Biotin carboxylase (3.5 units) was incubated with 9.3 mM [$^{18}O_4$]ATP, 39.5 mM $KHCO_3$, 12 mM $MgCl_2$, and 15% (v/v) EtOH in 200 mM HEPES, pH 8.0, in a total volume of 10 mL. In this experiment there

[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)-tetraacetic acid; TEAB, triethylammonium bicarbonate; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; PEP, Phosphoenolpyruvate.

was no need to take precautions to ensure that the reaction was irreversible; also, the ATP was not purified before analysis. Aliquots were removed from the reaction periodically, and the enzyme was removed by ultrafiltration using a Centricon 10. NMR samples were prepared as above.

(*b*) *Exchange from Bicarbonate.* $KHC^{18}O_3$ was prepared by dissolving 0.6 mmol of K_2CO_3 and 0.2 mmol of $KHCO_3$ in 200 μL of $H_2{}^{18}O$. The solution was allowed to stand for 24 h at room temperature before use. Biotin carboxylase, which is routinely stored as a suspension in a 50 mM potassium phosphate buffer that is 60% saturated with ammonium sulfate, was dialyzed before use against 50 mM HEPES, pH 7.8, containing 1 mM DTT and 20% (v/v) glycerol. Solutions that were to be used in the exchange reaction were degassed before use by sparging with N_2. The exchange reaction was carried out in a 2-mL solution containing 3.3 units of biotin carboxylase, 1 mM ATP, 4 mM $MgCl_2$, 50 mM biotin, and 15% (v/v) EtOH. The reaction was initiated by adding a volume of the $KHC^{18}O_3$ stock solution sufficient to bring the reaction to 50 mM in bicarbonate. The reaction was terminated after 5 min by removal of the enzyme via ultrafiltration with a Centricon 30. D_2O (500 μL) was added to the filtrate, and the sample was analyzed by ^{31}P NMR as described above.

Solvent Deuterium Isotope Effects. Steady-state kinetic assays were carried out in 1-mL total volume, 1-cm path length cuvettes by using a Beckman DU monochrometer equipped with a Gilford OD converter and a 10-mV chart recorder. The pH's of assay solutions were measured with a Beckman Model Phi 21 pH meter. pD's were calculated according to the equation pD = pH + 0.4 (Schowen, 1977). Buffers were adjusted to the desired pH with KOH, and potassium salts of all substrates were used whenever possible. Biotin carboxylase catalyzed carboxylation of biotin was monitored by coupling ATP turnover to NADH oxidation via the action of pyruvate kinase and lactate dehydrogenase. Typical assays contained 0.1 mM NADH, 0.5 mM phosphoenolpyruvate, 3 units of pyruvate kinase, and 5 units of lactate dehydrogenase. NADH oxidation was monitored by following the change in absorbance at 340 nm.

Data Analysis. The steady-state kinetic data were fitted to eq 1 using a BASIC translation of the FORTRAN program of Cleland (1979).

$$v = VA/(K + A) \quad (1)$$

Carbon isotope effects were calculated from the mass ratios of the isolated CO_2 by using eq 2, where R_p is the mass ratio

$$^{13}(V/K) = \log (1 - f)/\log [(1 - fR_p/R_0)] \quad (2)$$

of ^{13}C to ^{12}C in the CO_2 at fraction of reaction f and R_0 is the mass ratio at $f = 0$. Because carboxybiotin decarboxylated to a small extent during the course of the experiment, the data were corrected to account for the change in the isotopic composition of the carboxybiotin due to decarboxylation. Equation 3 describes the correction that was applied. R is the correction

$$\log R = (1 - {}^{13}k)[\log (1 - f^*)]/{}^{13}k \quad (3)$$

factor by which the mass ratio of the product CO_2 was divided in order to arrive at a corrected value of R_p. In eq 3, ^{13}k is the isotope effect on nonenzymatic decarboxylation of carboxybiotin, which was determined to be 1.024 at pH 8 (Tipton & Cleland, 1988c), and f^* is the extent to which the carboxybiotin decarboxylated; this was calculated from the duration of the experiment and the previously determined rate constant for decarboxylation of carboxybiotin (Tipton & Cleland, 1988c). This correction actually overcorrects the data slighly because it assumes a model in which the carboxybiotin is formed instantaneously and then decarboxylates over the course of the reaction; in the real situation, of course, carboxybiotin formation and decomposition occur simultaneously. The correction turned out to be quite small because generation of carboxybiotin was accomplished in 12 min or less, and f^* was on the order of 0.05. The correction increased $^{13}(V/K)$ by approximately 0.1%. Because $^{13}k_{D_2O}$ is not known, $^{13}(V/K)_{D_2O}$ was determined with samples generated at 2 °C, so that decarboxylation of biotin did not occur to an appreciable extent, and therefore no correction of the data was required.

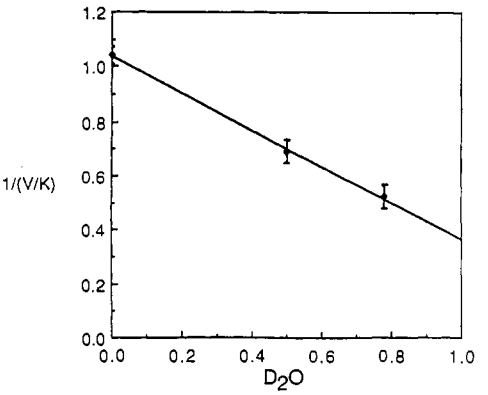


FIGURE 1: Proton inventory of the forward reaction of biotin carboxylase at pL 8.0. Biotin was 40 mM, ATP was 0.5 mM, $MgCl_2$ was 8 mM, and HCO_3^- was varied between 1.6 mM and 20 mM. Initial velocity data were fitted to eq 1.

RESULTS

$^{13}(V/K)$ for Biotin Carboxylation. At pH 7.9, $^{13}(V/K)$ was 1.0073 ± 0.0005, in a system in which ATP was not recycled. At pH 8.1, with ATP recycled with pyruvate kinase and PEP, $^{13}(V/K)$ was 1.0069 ± 0.0009. $^{13}(V/K)_{D_2O}$ at pD 7.8 was 1.0086 ± 0.0002. When Mg was replaced by Mn at pH 7.6, $^{13}(V/K)$ was 1.004 ± 0.001. Each value represents the average of three determinations.

Solvent Deuterium Isotope Effect. The results of a proton inventory carried out for the forward reaction catalyzed by biotin carboxylase at pH(D) 8.0 are shown in Figure 1. Each point represents two separate determinations of V/K. In 80% D_2O, $^D(V/K)$ was 0.50 ± 0.09. ^DV was 0.80 ± 0.02. The solvent deuterium isotope effect was also determined for the biotin carboxylase catalyzed phosphorylation of ADP by carbamoyl phosphate at pH(D) 8.5. In 80% D_2O, $^D(V/K)$ was 0.57 ± 0.05, and ^DV was 0.67 ± 0.03. The $V/K_{(biotin)}$ pD profile is displaced toward higher pH by 0.4 unit relative to the corresponding pH profile (data not shown).

Exchange from Bicarbonate. Figure 2 shows the ^{31}P NMR spectrum of the inorganic phosphate produced by the catalytic reaction of biotin carboxylase in the presence of $HC^{18}O_3^-$. Two peaks are observed, one due to $[^{16}O_4]P_i$, which represents reaction with bicarbonate from which the ^{18}O has already washed out, and the second peak, 0.02 ppm upfield, due to $[^{18}O^{16}O_3]P_i$, which arises from reaction of the ATP with bicarbonate that is still labeled with ^{18}O.

Exchange from ATP. Representative ^{31}P NMR spectra of the ATP isolated from the exchange experiments are shown in Figure 3. Schemes I and II outline the potential exchange reactions in the biotin carboxylase reaction. Positional isotope exchange from the $\beta\gamma$-bridge position to the β-nonbridge position would be apparent in the β-phosphate signal by the appearance of a peak approximately 0.01 ppm upfield of the signal shifted by the bridging oxygen. Washout of label from the nonbridging γ oxygens would appear in the signal from the γ phosphate; the upfield peaks representing the most highly

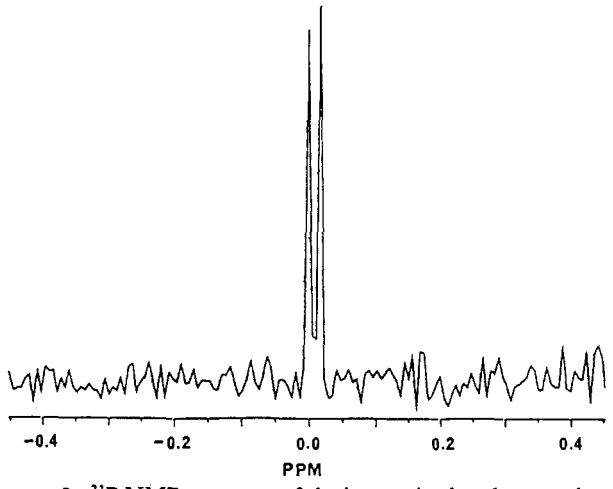


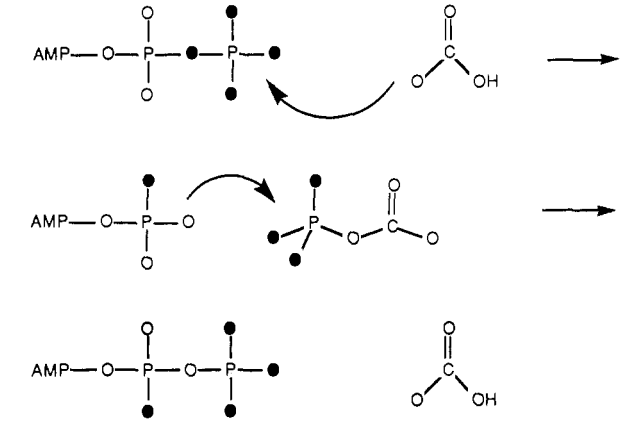
FIGURE 2: ^{31}P NMR spectrum of the inorganic phosphate produced in the forward reaction of biotin carboxylase from $HC^{18}O_3^-$ and ATP. Conditions of the experiment are presented in the text.

labeled phosphates would decrease in intensity, and the downfield peaks caused by the less highly labeled phosphates would increase. As shown in Figure 3, there is no change in the apperance of the spectra over time. This is true for the normal biotin carboxylation linked reaction in the absence and presence of inhibitory levels of ADP and for the ATPase reaction.

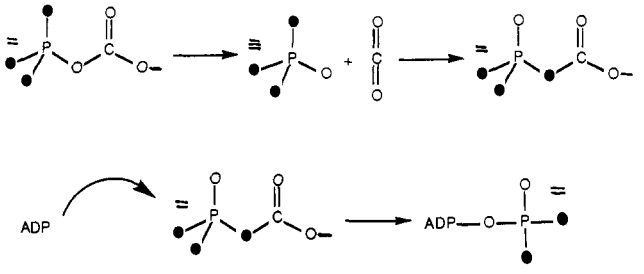
Discussion

Enzymic carboxylation of biotin entails a number of proton transfers and several potentially carbon isotope sensitive steps. When carboxyphosphate is initially formed by nucleophilic attack of bicarbonate on the γ phosphate of ATP, the carboxyl group bears a proton that must be removed before carboxylation of biotin can occur. Although the phosphoryl group of carboxyphosphate is dianionic, the leaving-group phosphate must gain a proton in order to leave as a dianion. Lastly, the proton on N1′ of biotin must be removed in order to enhance

Scheme I



Scheme II



the nucleophilicity of the biotin. The carbon atom derived from bicarbonate must undergo motion along the reaction coordinate and changes in bonding at several steps along the pathway leading to biotin carboxylation, which may or may not be reflected in the measured ^{13}C isotope effects. The formation of carboxyphosphate entails a secondary carbon isotope effect; a primary carbon isotope effect or effects will occur in the transfer of the carboxyl group from carboxyphosphate to biotin, whether this occurs in one step or via free CO_2. We have therefore measured $^{13}(V/K)$ in H_2O and D_2O and $^{D}(V/K)$ for the biotin carboxylase catalyzed carboxylation

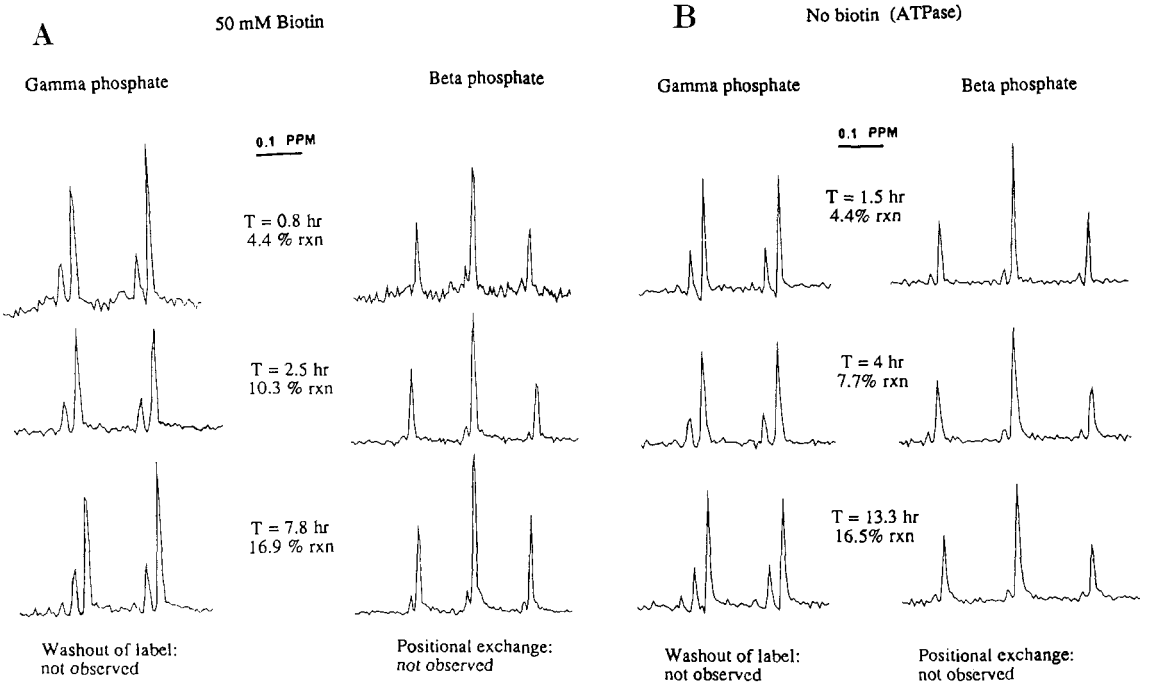


FIGURE 3: Representative ^{31}P NMR spectra of $[^{18}O_4]$ATP recovered from incubations with biotin carboxylase. Conditions of the experiment are presented in the text. The spectra shown are for the normal forward reaction in the absence of inhibitory levels of ADP (A) and for the ATPase reaction (B).

of free biotin; these numbers allow us to make a number of deductions about the chemical mechanism of the reaction.

The carbon isotope effect, $^{13}(V/K)$, was determined to be 1.007 at pH 8. The small magnitude of this number precludes making detailed mechanistic deductions but does suggest that a large forward commitment is attenuating the expression of ^{13}k in $^{13}(V/K)$. This conclusion is consistent with the results of the isotope-exchange experiments, in which we failed to see any evidence of partitioning from enzyme-bound intermediates. Failure to see exchange of ^{18}O from the $\beta\gamma$ bridge to the β-nonbridge position, which appears to be a common property of biotin enzymes, may have one of three causes. First, one must consider the possibility that the intermediate which allows positional exchange to occur, in this case, carboxyphosphate, does not exist. The weight of the evidence to date suggests that this possibility be ruled out. The exchange of ^{18}O from bicarbonate to inorganic phosphate, clearly shown in Figure 2, corroborates the landmark experiments of Kaziro et al. (1962) and demonstrates that the bicarbonate and the γ phosphate of ATP interact during the course of the reaction. Furthermore, Wimmer et al. (1979) have demonstrated via positional isotope-exchange experiments that carboxyphosphate can exist long enough in an enzyme active site to be a viable intermediate. The analysis of Hansen and Knowles (1985) of the stereochemistry at the phosphorus center in the pyruvate carboxylase catalyzed carboxylation of biotin strongly suggests the intermediacy of carboxyphosphate. The second possible reason for failure to observe positional isotope exchange is that the terminal phosphate of ADP is not torsionally symmetrical. In the preceding paper we discussed evidence which led us to suggest that the nucleotide is coordinated to two divalent metal ions in the active site of biotin carboxylase. Such close coordination may well preclude rotation of the β phosphate. The final possible explanation for the failure to observe exchange is that the reaction is committed to proceed to completion by the time the intermediate is formed. This explanation, which is by no means exclusive of the second one, also provides a rationale for the small magnitude of $^{13}(V/K)$.

The same three reasons can be invoked to explain the failure to observe washout of ^{18}O from the γ-nonbridge positions, which would occur by the mechanism shown in Scheme II. If biotin carboxylation proceeded by nucleophilic attack directly on carboxyphosphate, then no label could be exchanged from the phosphate. We will argue below, however, that a mechanism which proceeds through free CO_2 and phosphate is much more attractive. If the phosphate formed in the active site had no torsional symmetry, then carboxyphosphate would reform with the oxygens in their original arrangement, and no washout would result. We have no data that allow us to evaluate this possibility. The third possibility is that the reaction is committed by the time the CO_2 and phosphate are formed and that partitioning back to ATP and HCO_3^- is simply too unfavorable.

The diminution of $^{13}(V/K)$ when Mn(II) replaces Mg(II) is also consistent with the idea that the forward reaction has a large commitment. Mn tightens the binding of all of the substrates of biotin carboxylase by at least an order of magnitude, which would also tend to increase the forward commitment. In the general case $^{13}(V/K)$ is also a function of the reverse commitment of the reaction (Cleland, 1982). In reactions dominated by a large reverse commitment, $^{13}(V/K)$ approaches $^{13}K_{eq}$. The reverse commitment of the carboxylation of biotin appears to be very small, because $^{13}(V/K)$ does not change regardless of whether or not ADP is recycled, which precludes reversal of the reaction.

Interpretation of $^{13}(V/K)$ is difficult because it reflects several carbon-sensitive steps. However, the ^{13}C isotope effects on a number of model reactions that allow the calculation of $^{13}K_{eq}$ for carboxyphosphate formation from ATP and bicarbonate have been measured (Tipton & Cleland, 1988a). The ^{13}C equilibrium isotope effect for formation of carbamoyl phosphate from bicarbonate, ammonia, and ATP was found to be 0.9983, which indicates that ^{13}C enriches in carbamoyl phosphate by about 0.2% relative to bicarbonate. The fractionation factor of carbamate relative to aqueous CO_2 was also determined and found to be 1.011, which is larger than the fractionation factor of bicarbonate by 0.2%. One can calculate that the equilibrium isotope effect on formation of carboxyphosphate should differ from that of carbamoyl phospate by the difference made by the presence of the NH_2 group. The NH_2 group stiffens the bonds to carbon by 0.2%, and the equilibrium isotope effect on carbamoyl phosphate formation is 0.2% inverse, so one can calculate that the equilibrium isotope effect on formation of carboxyphosphate is 1.00. Attempts to characterize the contributions of the various carbon-sensitive steps to the overall measured value of $^{13}(V/K)$ by determining $^{13}(V/K)$ for the carbamoyl phosphate reaction failed because the decomposition of carbamoyl phosphate occurred with a very large normal isotope effect at a greater rate than that of the enzyme-catalyzed reaction.

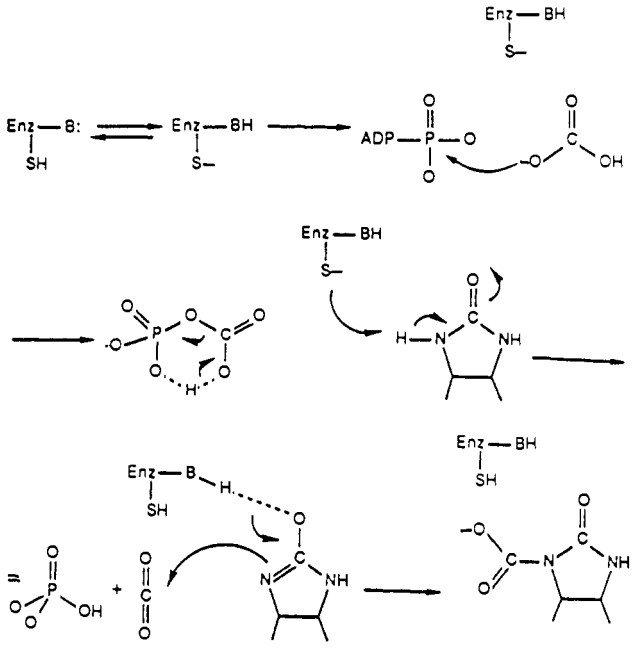
The solvent deuterium isotope effect on biotin carboxylation was decidedly inverse, 0.50 in 80% D_2O at pD 8. Current knowledge of fractionation factors provides only one explanation of these data: that a protonated sulfhydryl group loses its proton during the course of the reaction. The fractionation factor of sulfhydryl groups is 0.4–0.5 (Cleland, 1987), which is dramatically different from the values of approximately unity that are seen with oxygen and nitrogen bases. The full expression of this fractionation factor suggests that, in contrast to the carbon isotope sensitive steps, the proton-sensitive step reflected in this measurement is coming to equilibrium, and the measurement therefore reflects almost all of the full equilibrium isotope effect. The observation that the pD profile is displaced from the pH profile by 0.4 unit is also consistent with this observation.

In the preceding paper, we presented evidence for the existence of a sulfhydryl group at the enzyme active site that is necessary for catalysis. This evidence consisted of observations of the inactivation of biotin carboxylase by a number of sulfhydryl-modifying reagents and the protection by substrates against inactivation. The observation of an inverse solvent isotope effect is fully consistent with these observations and is difficult to explain without invoking participation of a sulfhydryl group in the catalytic reaction. To evaluate the possibility that D_2O causes a rate enhancement by some nonspecific effect on the conformation of the protein, we carried out a proton inventory of the carboxylation reaction. The inverse of the pseudo-first-order rate constant varied linearly with the fraction of D_2O present in the reaction, indicating that only one proton is in motion in the transition state.

Somewhat surprisingly, the carbamoyl phosphate reaction also exhibited a large inverse deuterium isotope effect of 0.57 on V/K. In the preceding section, we discussed the pH dependence of the kinetic parameters of the biotin carboxylase catalyzed reactions and noted that the pH dependence of the carbamoyl phosphate reaction is the same as that of the normal forward reaction, even though no acid–base catalysis is required.

Thus, any mechanism that purports to explain the mechanisms of the biotin carboxylase catalyzed reactions must be

Scheme III



consistent with the following experimental observations: (1) Both forward and reverse reactions proceed with large inverse deuterium isotope effects. (2) The forward and reverse reactions occur from enzyme in the same protonation state. (3) The active site contains a catalytic base with a pK of 6.6 that must be unprotonated for initiation of the catalytic cycle. (4) The active site also contains a sulfhydryl that is initially protonated but which loses its proton at the start of the catalytic cycle and is reprotonated before the cycle repeats. Scheme III shows a catalytic mechanism for the carboxylation of biotin that we believe is consistent with all the experimental observations and is attractive on chemical grounds.

The inverse deuterium isotope effect is explained by the presence of two enzyme forms, only one of which is active. The inactive form, which represents the majority of the protein at any given time, contains the protonated sulfhydryl and the unprotonated second catalytic base. The active form contains the ionized sulfhydryl and the protonated second base. This form of the enzyme is required for catalysis of the transphosphorylation reaction in either direction. The active and inactive forms are equilibrium, and the proton transfer from the sulfhydryl to the second base is responsible for the rate enhancement attained in D_2O. Whether binding of the substrates causes a protein conformation change that induces the proton shift or whether the substrates bind only to the correctly ionized form of the enzyme cannot be determined from our data, although the observation of a pK in the V profiles that is not seen in V/K pH profiles certainly suggests that a protein conformation occurs when the substrates are bound to the enzyme. Once all the substrates are bound in the active site and the bases are correctly protonated, the carboxyphosphate transfers its proton from one end to the other, which allows it to decarboxylate. The ionized sulfhydryl removes the proton from N1′ of biotin, and the isourea tautomer of biotin attacks the CO_2.

The carbamoyl phosphate reaction, in which carbamoyl phosphate is presumably acting as an analogue of carboxyphosphate, is also catalyzed only by the second form of the enzyme, which explains the observed pH dependence and deuterium isotope effect.

Besides explaining the deuterium isotope effect data, this mechanism has a number of attractive features about it. First, it provides a mechanism for the tautomerization of biotin, which the model studies of Hegarty et al. (1969) have demonstrated to be necessary for the enhancement of the nucleophilicity of biotin. Second, the mechanism postulates that a sulfhydryl group catalyzes the tautomerization; such a suggestion is not without precedent. Attwood et al. (1986) measured the ^2H and ^{13}C isotope effects on the reverse of the second half-reaction catalyzed by the biotin-dependent enzyme pyruvate carboxylase and found that these interdependent isotope effects were consistent with each other only if one supposed that a sulfhydyl group acted to tautomerize biotin. In addition, Hudsen et al. (1975) have been able to demonstrate specific labeling of a cysteine residue at the active site of the second half-reaction of pyruvate carboxylase.

The carboxylation reaction is proposed to proceed via free CO_2, a mechanism that is supported by the model studies of Sauers et al. (1975). The alternative to this pathway is for biotin to attack carboxyphosphate directly. However, it is very unlikely that biotin could attack carboxyphosphate when the carboxyl group is ionized, because a dianionic tetrahedral intermediate would result. One could postulate that the carboxyl group remains protonated, which might enchance its electrophilicity. There are several problems with this mechanism, however. Since the dianion of phosphate was found to inhibit the carbamoyl phosphate dependent reaction, we concluded in the previous paper that the dianion of phosphate is the product of the forward reaction. Therefore, a proton transfer to the phosphate must occur at some point in the mechanism in order to generate the dianion. Also, if one considers the reverse reaction, this proposal would require that carboxybiotin be protonated before carboxyphosphate could be formed. The protonated carboxybiotin would then have to suffer nucleophilic attack from the trianion of inorganic phosphate to generate carboxyphosphate in the correct protonation state to produce bicarbonate upon phosphorylation of ATP. Formation of the trianion of inorganic phosphate in the enzyme active site would require the participation of an as yet undetected acid–base catalyst.

The relative timing of proton-sensitive steps and carbon-sensitive steps can be determined by using the multiple isotope technique of Hermes et al. (1982), in which concerted reactions are identified by virtue of the fact that deuteriation of the substrate causes an increase in $^{13}(V/K)$ relative to $^{13}(V/K)$ measured with protio substrate. Thus, it is tempting to conclude from the measured value of $^{13}(V/K)_{D_2O}$ of 1.0086 that a proton transfer is occurring in concert with carbon atom motion. A number of schemes can be envisioned in which proton motion coincides with carbon motion: for example, concerted deprotonation of the carboxyl group of carboxyphosphate and decarboxylation of carboxyphosphate or concerted decarboxylation of carboxyphosphate and tautomerization of biotin. Either one of these possibilities seems reasonable, but the difference between $^{13}(V/K)_{H_2O}$ and $^{13}(V/K)_{D_2O}$ is small enough that it may be prudent not to draw too many conclusions from these data.

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Atypical Abasic Sites Generated by Neocarzinostatin at Sequence-Specific Cytidylate Residues in Oligodeoxynucleotides†

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ABSTRACT: Neocarzinostatin chromophore produces alkali-labile, abasic sites at cytidylate residues in AGC sequences in oligonucleotides in their duplex form. Glutathione is the preferred thiol activator of the drug in the formation of these lesions. The phosphodiester linkages on each side of the abasic site are intact, but when treated with alkali, breaks are formed with phosphate moieties at each end. Similar properties are exhibited by the abasic lesions produced at the purine residue to which the C in AGC is base-paired on the complementary strand. The abasic sites at C residues differ from those produced by acid-induced depurination in the much greater lability of the phosphodiester linkages on both sides of the deoxyribose, in the inability of $NaBH_4$ to prevent alkali-induced cleavage, and in the relative resistance to apurinic/apyrimidinic endonucleases. The importance of DNA microstructure in determining attack site specificity in abasic site formation at C residues is shown not only by the requirement for the sequence AGC but also by the findings that substitution of G by I 5′ to the C decreases the attack at C, whereas placement of an I opposite the C markedly enhances the reaction. Quantitation of the abstraction of ^3H into the drug from C residues in AGC specifically labeled in the deoxyribose at C-5′ or C-1′,2′ suggests that, in contrast to the attack at C-5′ in the induction of direct strand breaks at T residues, abasic site formation at C residues may involve attack at C-1′. Each type of lesion may exist on the complementary strands of the same DNA molecule, forming a double-stranded lesion.

The DNA-damaging antibiotic neocarzinostatin (NCS)[1] consists of a labile nonprotein chromophore complexed with its apoprotein [reviewed in Goldberg (1986)]. The biologically active nonprotein chromophore has a structure comprised of three subunits: a 5-methyl-7-methoxynaphthoate and a 2,6-dideoxy-2-(methylamino)galactose moiety interlinked by a C_{12} subunit containing a bicyclo[7.3.0]dodecadiyne system bearing acyclic carbonate moiety and an epoxide. NCS chromophore binds to DNA by an intercalative mechanism and causes a variety of lesions of which spontaneous base release (T > A ≫ C > G) and single-strand breaks are the most predominant. The mechanism of base release is not clearly understood. The strand breaks occur in a thiol- and O_2-dependent reaction primarily at thymidylic and deoxyadenylic acid residues (T > A ≫ C > G) and have mainly (>80%) a nucleoside aldehyde at their 5′-ends and a PO_4 at their 3′-termini. A reaction mechanism proposed to account for these products

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[1] Abbreviations: NCS, neocarzinostatin; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

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