Catalytic Mechanism of Biotin Carboxylase: Steady-State Kinetic Investigations†

Peter A. Tipton and W. W. Cleland*

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Received August 24, 1987; Revised Manuscript Received January 28, 1988

ABSTRACT: Biotin carboxylase was purified from *Escherichia coli* by a new procedure, and its steady-state kinetic parameters were examined. MgATP and bicarbonate add to the enzyme randomly, followed by addition of biotin. Both bicarbonate and MgATP add in rapid equilibrium. A catalytic base with a pK of 6.6 is observed in V/K profiles. Inactivation studies also revealed a sulfhydryl group in the active site that is essential for catalysis. It is proposed that the acid–base catalysts are necessary for the tautomerization of biotin, which presumably enhances its nucleophilicity toward the carboxyl group donor. A second enzymic group with a pK of 6.6, whose role is unknown, is seen in V_{max} profiles. The pH profiles for the biotin carboxylase catalyzed phosphorylation of ADP by carbamoyl phosphate have the same shape as the profiles for the forward reaction, which demonstrates that the enzymic bases assume the same protonation states for catalysis of transphosphorylation in either direction. The lack of reactivity of thionucleotide analogues of ATP when Mg is used as the divalent metal ion suggests that both metal ions required for reaction coordinate to the nucleotide. The second metal ion appears to be absolutely required for reaction and not merely an activator of the reaction. Characterization of a bicarbonate-dependent biotin-independent ATPase activity strongly suggests that carboxylation proceeds via a carboxyphosphate intermediate.

Acetyl-CoA carboxylase exists in *Escherichia coli* as a complex of three functionally defined subunits that are isolated separately (Guchhait et al., 1974a). The biotin carboxylase subunit catalyzes the first half-reaction, the MgATP-dependent carboxylation of enzyme-bound biotin:

$$MgATP + HCO_3^- + \text{biotin-Enz} \xrightleftharpoons{M^{2+}} MgADP + P_i + CO_2\text{-biotin-Enz}$$

The biotin cofactor is covalently linked to the second subunit, the biotin carboxyl carrier protein, via an amide linkage to the ϵ-amino group of a specific lysine (Sutton et al., 1977; Kondo et al., 1983). The third subunit, the carboxyltransferase subunit, catalyzes the transfer of the carboxyl group from carboxybiotin to acetyl CoA to form malonyl CoA. The overall reaction represents the committed step in fatty acid biosynthesis.

Biotin carboxylase is one of only two biotin-dependent carboxylases that will accept free biotin as an alternate substrate in lieu of the biotin carboxyl carrier protein [the other is β-methylcrotonyl-CoA carboxylase (Moss & Lane, 1971)] and so presents itself as an ideal system with which to study the mechanism of biotin carboxylation in detail. Indeed, the seminal studies of Lane's group (Guchhait et al., 1974b; Polakis et al., 1974) on biotin carboxylase effectively resolved the controversy regarding the structure of carboxybiotin in favor of the N1′-carboxylated structure. We have investigated the steady-state kinetic behavior of biotin carboxylase in order to define more closely possible chemical mechanisms. The normal reaction, the MgATP-dependent carboxylation of biotin, and a biotin-independent ATPase activity have been investigated, as well as the biotin carboxylase catalyzed phosphorylation of MgADP by carbamoyl phosphate, which is considered to be a model of the reverse reaction in which carbamoyl phosphate acts as an analogue of the putative carboxyphosphate intermediate. In the following paper (Tipton & Cleland, 1988) we address the nature of intermediates along the pathway to biotin carboxylation in more detail on the basis of evidence from isotope-exchange studies and kinetic isotope effect experiments.

Materials and Methods

E. coli B cells grown to $^3/_4$ log phase on enriched media were obtained from Grain Processing Corp., Muscatine, IA, and stored at −80 °C. $NaH^{14}CO_3$ was from Amersham. Hydroxylapatite and DEAE-Bio-Gel A were from Bio-Rad; Reactive Green A-19 agarose, ADP–agarose (ADP linked to the agarose via the ribose hydroxyls), and 50% aqueous poly(ethylene imine) were purchased from Sigma. Ammonium sulfate was obtained from Schwarz-Mann, and CC31 cellulose powder was obtained from Whatman. Trisacryl[1] GF05 was from LKB.

Initial velocity studies were carried out on a Cary 118 spectrophotometer or a Beckman DU monochrometer equipped with a Gilford OD converter and a 10-mV recorder. Buffers were adjusted to the desired pH with KOH, and potassium salts of all reagents were used whenever possible. MES, HEPES, and CHES (all 200 mM) were used in the appropriate ranges.

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, which were made fresh before each experiment, were calibrated by end-point assay with carbamate kinase, hexokinase, and glucose-

†This work was sponsored by a grant from the National Institutes of Health (GM 18938). P.A.T. was a trainee on Grant 5T32-GM07215 from the National Institutes of Health.

[1] Abbreviations: Trisacryl, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-2-propenamide; MES, 4-morpholineethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DEAE, diethylaminoethyl; NEM, *N*-ethylmaleimide; 2-PDS, 2-pyridyl disulfide; EDTA, (ethylenedinitrilo)-tetraacetic acid; DTT, dithiothreitol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; PEI, poly(ethylene imine); SDS, sodium dodecyl sulfate.

0006-2960/88/0427-4317$01.50/0

6-phosphate dehydrogenase. The dissociation constant for MgATP under the experimental conditions used was determined by using the equation of Adolfsen and Mondrianakis (1978). Bicarbonate stock solutions were calibrated by manometric measurement of the CO_2 released upon acidification. $MgCl_2$ stock solutions were calibrated with calmagite (Chauhan & Ray Sarkar, 1969), and $MnCl_2$ stock solutions were calibrated with Eriochrome Black. Inorganic phosphate stock solutions were calibrated by the method of Carter and Karl (1982).

(S_P)-ATP-α-S, ATP-γ-S, and ADP-β-S were purchased from Boehringer Mannheim and were purified before use by chromatography on DEAE-Sephadex A-25. R_P and S_P isomers of ATP-β-S were synthesized enzymatically by the procedures of Eckstein and Goody (1976).

Assays. Spectrophotometric assays were routinely performed in 1.0 mL total volume in 1-cm path length self-masking quartz cuvettes. When the exact concentration of bicarbonate was crucial, assays were performed in stoppered cuvettes using degassed buffer and NADH. Temperature was maintained at 25 °C with a circulating water bath. Unless otherwise noted, all assays contained 15 % (v/v) ethanol. Reaction in the forward direction was followed by monitoring the oxidation of NADH at 340 nm via the coupled reactions of pyruvate kinase and lactate dehydrogenase; assays contained 0.1 mM NADH, 0.5 mM phosphoenolpyruvate, 3 units of pyruvate kinase, and 5 units of lactate dehydrogenase. Assays in the direction of ATP synthesis were followed by monitoring production of NADPH at 340 nm via the coupled reactions of hexokinase and glucose-6-phosphate dehydrogenase. These assays contained 0.5 mM glucose, 0.5 mM NADP, 5 units of hexokinase, and 5 units of glucose-6-phosphate dehydrogenase. For direct detection of carboxybiotin formation, formation of [^{14}C]carboxybiotin from $H^{14}CO_3^-$ was monitored by the procedure of Guchhait et al. (1974a).

Inactivation Studies. 2-Pyridyl disulfide was from Sigma, methyl methanethiosulfonate was from Aldrich, and *N*-ethylmaleimide was from Calbiochem. Experiments were carried out by incubating biotin carboxylase with the inactivating reagent in the presence or absence of substrates and removing aliquots periodically to check for remaining activity. The aliquots were of sufficiently small volume compared to the volume in which the remaining activity was determined that the inactivating reagent was diluted 20-fold. Enzyme activity was taken to be proportional to the velocity of the reaction catalyzed by the aliquot in 40 mM biotin, 1 mM ATP, 2 mM $MgCl_2$, and 20 mM $KHCO_3$ in 15% (v/v) ethanol at pH 8.0 in 200 mM HEPES. NEM and 2-PDS were typically used at 1 mM; methyl methanethiolsulfonate was used at 10 μM.

Data Analysis. Data were fitted to the appropriate rate equations by using the FORTRAN programs of Cleland (1979). If it was not obvious from graphical analysis of the data to which equation the data should be fitted, they were fitted to each potentially appropriate equation, and the equation with the lowest sum of the squares of the residuals was deemed the appropriate equation. In no case did the data fit more than one equation equally well.

When one substrate was varied at fixed levels of the other substrates, the data were fitted to eq 1.

$$v = \frac{VA}{K + A} \tag{1}$$

When two substrates were varied at fixed levels of the others, the data were fitted to eq 2, 3, or 4, which describe patterns that intersect on the ordinate (equilibrium ordered), intersect off of the ordinate (sequential), or consist of parallel lines (ping-pong), respectively.

$$v = \frac{VAB}{K_bA + AB + K_{ia}K_b} \tag{2}$$

$$v = \frac{VAB}{K_aB + K_bA + AB + K_{ia}K_b} \tag{3}$$

$$v = \frac{VAB}{K_aB + K_bA + AB} \tag{4}$$

The initial velocity data used to determine the kinetic mechanism under conditions of saturating Mg were fitted to eq 5, where A, B, and C are the concentrations of biotin, ATP, and bicarbonate, respectively.

$$v = \frac{VABC}{\text{const} + (\text{coeff } B)B + (\text{coeff } C)C + K_aBC + K_bAC + ABC} \tag{5}$$

The pH profiles for biotin carboxylase were fitted to eq 6, for cases in which one group ionizes, and to eq 7 when two groups ionize. In these equations, y is V, V/K, or $1/K_i$, C is the value of this parameter at high pH, and K_1 and K_2 are acid dissociation constants.

$$\log y = \log [C/(1 + H/K_1)] \tag{6}$$

$$\log y = \log [C/(1 + H/K_1 + H^2/K_1/K_2)] \tag{7}$$

The data for the pH dependence of the rate constant of inactivation of biotin carboxylase were fitted to eq 8, where C is the value of k_{inact} at low pH.

$$\log (k_{inact}) = \log [C(1 + K_1/H)] \tag{8}$$

Competitive inhibition data were fitted to eq 9.

$$v = \frac{VA}{K(1 + I/K_i) + A} \tag{9}$$

Rate constants for inactivation of biotin carboxylase by the sulfhydryl-modifying reagents were calculated from least-squares fits of the data plotted as ln (v_t/v_0) versus time, where v_0 is the initial activity, v_t is the activity at time t, and the slope of the line is the negative of the rate constant for inactivation.

Purification of Biotin Carboxylase. A new procedure for purification of biotin carboxylase, based on the method presented by Guchhait et al. (1974a), was used. Biotin carboxylase activity was measured as described by Guchhait et al. (1974a), except assays were carried out at 25 °C instead of 37 °C.

All operations except the cell cracking were performed at 4 °C. The cracking buffer consisted of 100 mM potassium phosphate, pH 7.5, 1 mM dithiothreitol, and 1 mM EDTA. Buffer A was 10 mM potassium phosphate, pH 7.0, and 1 mM DTT, and buffer B was 20 mM Tris, pH 7.8, containing 1 mM DTT, and 10% (v/v) glycerol.

Frozen cell paste (250 g) was resuspended in 1 L of cracking buffer and stirred overnight. Cells were broken by passage 2 times through a precooled Manton-Gaulin cell press operating at 8000 psi. The cell extract was centrifuged at 25000g for 45 min and the pelleted cell debris discarded.

Nucleic acids were removed from the supernatant by precipitation with poly(ethylene imine) (PEI). Before each use, the PEI was diluted 10-fold into cracking buffer and the pH was readjusted to 7.5 with HCl. A volume of PEI equal to 4% of the supernatant volume was added dropwise to the protein solution with stirring. The suspension was stirred for 15 min after addition of the PEI was complete, and the pre-

cipitated material was removed by centrifugation at 15000*g* for 15 min.

The supernatant was slowly brought to 60% saturation by addition of crystalline ammonium sulfate and stirred for an additional 20 min. Precipitated protein was recovered by centrifugation at 15000*g* for 15 min and the supernatant discarded. The pellet was resuspended in a minimum volume of cracking buffer and dialyzed against 6 L of buffer A.

The dialyzed protein was then chromatographed on hydroxylapatite. To ensure adequate flow rates, Bio-Rad HTP was mixed 1:1 (by dry weights) with Whatman CC31 cellulose powder. Protein was applied to a 4.0 × 25 cm column equilibrated in buffer A and washed for 1 h with buffer A. Biotin carboxylase was eluted from the column with a gradient from buffer A to 500 mM potassium phosphate, pH 7.0, containing 1 mM DTT and 1 mM EDTA over 1 L. Biotin carboxylase eluted at approximately 200 mM phosphate. Active fractions were pooled and precipitated by addition of ammonium sulfate to 60% saturation. Protein was recovered by centrifugation, resuspended in the cracking buffer, and dialyzed against 4 L of buffer A.

The dialyzed protein was applied to a 4.0 × 17 cm column of DEAE-Bio-Gel A equilibrated in buffer A. Biotin carboxylase did not bind to this column and was eluted by washing with buffer A. Active fractions were pooled and precipitated with ammonium sulfate. The concentrated protein was desalted by passage over a column of Trisacryl GF05 equilibrated in 20 mM Tris, pH 7.8, and 1 mM DTT.

The desalted protein was applied to a 2.0 × 25 cm column of Reactive Green A-19 agarose equilibrated in buffer B. The column was washed for 30 min after addition of the protein, and then biotin carboxylase was eluted by washing with a gradient from 0 to 500 mM KCl in buffer B over 500 mL. Biotin carboxylase eluted with approximately 350 mM KCl. Active fractions were pooled and concentrated to 5–10 mL by ultrafiltration with Amicon YM30 membranes. It was found that biotin carboxylase binds tightly to Amicon PM-type membranes; use of the YM membranes was therefore imperative. The protein was desalted by repeated dilution with buffer B and concentration by ultrafiltration.

The concentrated protein was loaded onto a 2.0 × 6.0 cm column of ADP–agarose equilibrated in buffer B. The resin contained ADP covalently linked to the agarose via the ribose hydroxyls; resin in which the ADP was bound to the agarose via the N6 amino group had no affinity for biotin carboxylase. After loading was completed, the flow was shut off for 30 min to facilitate binding of the protein to the resin. The column was then washed with buffer B until the absorbance at 280 nm of the effluent stabilized, and biotin carboxylase was eluted with 100 mL of buffer B containing 10 mM $MgCl_2$ and 5 mM ATP.

Active fractions were pooled and concentrated via ultrafiltration to approximately 3 mL. The concentrated protein was placed in a dialysis bag that was placed in 1 L of 60% saturated ammonium sulfate in 50 mM potassium phosphate, pH 7.0, containing 1 mM DTT and 20% (v/v) glycerol. After 2 days the flocculated protein was recovered by centrifugation and stored as a suspension in the above buffer.

Results

Purification of Biotin Carboxylase. Results of the purification protocol outlined above are shown in Table I. SDS–polyacrylamide gel electrophoresis demonstrated that the enzyme thus prepared is not homogeneous, but no competing activities could be detected spectrophotometrically. The relative simplicity of the protocol offered advantages over that described by Guchhait et al. (1974a), with which we were unable to obtain reproducible results.

Table I: Purification of Biotin Carboxylase from 250 g of *E. coli* B Cell Paste

step	total activity[a]	protein[b] (mg)	specific activity (units/mg)	yield[c] (%)
cell-free extract	34.9	18 373	0.0019	100
poly(ethylene imine)	34.9	18 480	0.0019	100
0–60% ammonium sulfate	18.6	10 780	0.0017	89
hydroxylapatite	30.2	1 130	0.0267	87
DEAE-Bio-Gel A	42.3	950	0.045	121
Reactive Green A-19 agarose	38.7	167.4	0.231	111
ADP agarose	21.8	18.9	1.15	62

[a] Activity determined by fixation of $H^{14}CO_3^-$ at 25 °C. [b] Protein determined by the method of Bradford. [c] The apparent total number of units of biotin carboxylase routinely increased at different stages of the purification. It is not known if this is due to removal of inhibitors or if there is another cause.

Table II: Kinetic Constants for the Biotin Carboxylase Catalyzed Carboxylation of Biotin under Conditions of Saturating Mg[a]

V_{max}	1.29 ± 0.15	σ	0.011
K_a	71.7 ± 12.8	const/coeff *B*	0.090 ± 0.026
K_b	0.056 ± 0.015	const/coeff *C*	7.35 ± 3.00
coeff *B*	114.5 ± 23.8	coeff C/K_b	25.1 ± 12.3
coeff *C*	1.40 ± 0.39	coeff C/K_a	0.020 ± 0.007
const	10.3 ± 1.9	coeff B/K_a	1.60 ± 0.33

[a] The second and third values in the second column are dissociation constants from binary EB and EC complexes, while the other three are dissociation constants of C, B, or A from ternary EBC or EAC complexes. Michaelis constants and dissociation constants in mM; velocities in μmol min^{-1} (unit of enzyme)$^{-1}$. Kinetic constants determined by nonlinear least-squares fit to eq 5. All assays were carried out at 25 °C by using 0.002–0.005 unit of biotin carboxylase per assay. Biotin was varied between 20 and 50 mM. Bicarbonate was varied between 3 and 13.5 mM. ATP was varied between 17 and 430 μM. $MgCl_2$ was 8.0 mM. Reactions were run in 200 mM HEPES, pH 8.0. A, B, and C, are biotin, MgATP, and HCO_3^-, respectively.

Kinetic Mechanism. Initial velocity data at fixed saturating Mg^{2+} yielded intersecting patterns in all cases, which demonstrates that addition of all substrates is sequential. Reciprocal plots in which bicarbonate was varied at different levels of MgATP were curved downward, suggesting that there is significant negative cooperativity in the binding of bicarbonate.

The entire set of initial velocity data obtained by the sequential variation of ATP, bicarbonate, and biotin concentrations at fixed levels of the others was fitted to different equations describing various terreactant mechanisms until a reasonable fit was obtained. Criteria for concluding that the data were well fitted were minimization of the residuals of the fit and well-defined values for all the terms in the equation. The data were best fitted by eq 5; Table II lists values of each term in the equation defined by the computer fit, along with σ, the square root of the sum of the squares of the residuals.

For the initial velocity studies done with varying levels of Mg, a value of 40 μM was calculated from the equation of Adolfsen and Mondrianakis (1978) as the dissociation constant of MgATP under the conditions of ionic strength and pH that were used. Assays were carried out in the presence of 40 mM biotin and 12 mM HCO_3^- in 200 mM HEPES at pH 8.0. Free Mg^{2+} was varied from 0.3 to 1.5 mM; MgATP was varied from 50 to 250 μM. Plots of initial velocity data with free Mg^{2+} as the variable substrate at different levels of MgATP show lines intersecting on the ordinate, indicating that MgATP adds in rapid equilibrium prior to Mg^{2+} addition. The Michaelis constant for Mg^{2+} was determined to be 0.57 ± 0.18 mM, the dissociation constant of MgATP from enzyme was

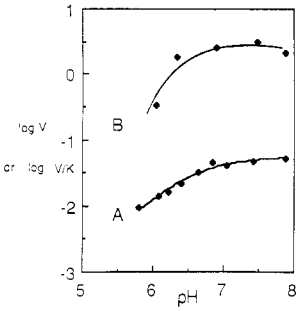


FIGURE 1: pH profiles for the carboxylation of biotin. Biotin is the variable substrate in both cases. (A) V/K profile with Mg. Assays were carried out in the presence of 8.2 mM biotin, 0.5 mM ATP, 2 mM $MgCl_2$, 4 mM HCO_3^-, and 5% (v/v) EtOH. The line is drawn according to eq 6 (B) V profile with Co. Assays were carried out in the presence of 0.1 mM ATP, 0.2 mM $CoCl_2$, 20 mM [HCO_3^- + CO_2], between 2 and 33 mM biotin, and 15% (v/v) EtOH. The line is drawn by hand.

0.24 ± 0.07 mM, and σ for the fit was 0.020.

When Mn was substituted for Mg, a value of 10 μM was used as the dissociation constant for MnATP. Initial velocity data were obtained in the presence of 40 mM biotin and 20 mM HCO_3^- in 200 mM HEPES at pH 7.5. Free Mn^{2+} was varied from 2 to 15 μM; MnATP was varied between 5 and 40 μM. These data also yielded a pattern of lines intersecting on the ordinate when plotted with free Mn^{2+} as the variable substrate. The K_m for Mn^{2+} was 11 ± 1 μM, and the dissociation constant of MnATP from enzyme was 17 ± 2 μM, with a value of 0.013 for σ for the overall fit.

When bicarbonate was varied at different levels of Mg^{2+}, the lines appeared parallel at high levels of Mg^{2+} and became more obviously intersecting at lower Mg^{2+} levels. These data were obtained in the presence of 40 mM biotin and 0.5 mM MgATP in 200 mM HEPES at pH 8.0. Bicarbonate was varied between 1.6 and 40 mM, and free Mg^{2+} was varied between 0.2 and 6.9 mM.

To achieve very low levels of free Mg^{2+}, the total level of Mg was held at 0.5 mM and ATP was added at 0.5, 1.0, and 2.0 mM. This yielded free Mg^{2+} concentrations of 5, 15, and 97 μM, respectively; other experimental conditions were as above. Under these conditions the apparent $V/K_{bicarbonate}$ [μmol/(min·mM)] was 0.14 ± 0.03 at 5 μM Mg^{2+}, 0.18 ± 0.03 at 15 μM Mg^{2+}, and 0.50 ± 0.11 at 97 μM Mg^{2+}. The apparent V_{max} (μmol/min) was 0.097 ± 0.004 at 5 μM Mg^{2+}, 0.26 ± 0.01 at 15 μM Mg^{2+}, and 0.47 ± 0.03 at 97 μM Mg^{2+}.

pH Kinetics. Figure 1A shows the variation of log (V/K_{biotin}) with pH in which Mg was used as the divalent metal. Under these conditions the K_m for biotin is around 80 mM, and initial velocities determined at 8 mM biotin were taken to be directly proportional to V/K, so no data allowing the calculation of V_{max} under these conditions were obtained. The volume of stock $KHCO_3$ solution added to each cuvette was increased as the pH was decreased in order to keep the concentration of HCO_3^- constant. Thus, the pK seen at 6.55 ± 0.05 is due to a group on the enzyme and is not the pK of bicarbonate. In experiments in which the HCO_3^- concentration is not maintained at a fixed level across the pH range, the V/K profile reaches a slope of 2 at low pH, and the two pK's are not distinguishable. It should also be noted that the data in Figure 1A were obtained in only 5% (v/v) ethanol to minimize perturbation of the pK by the presence of the organic solvent.

By substituting Co for Mg, which significantly lowers the K_m of biotin, the variation of V with pH can be determined and is shown in Figure 1. The V/K profile looks similar to

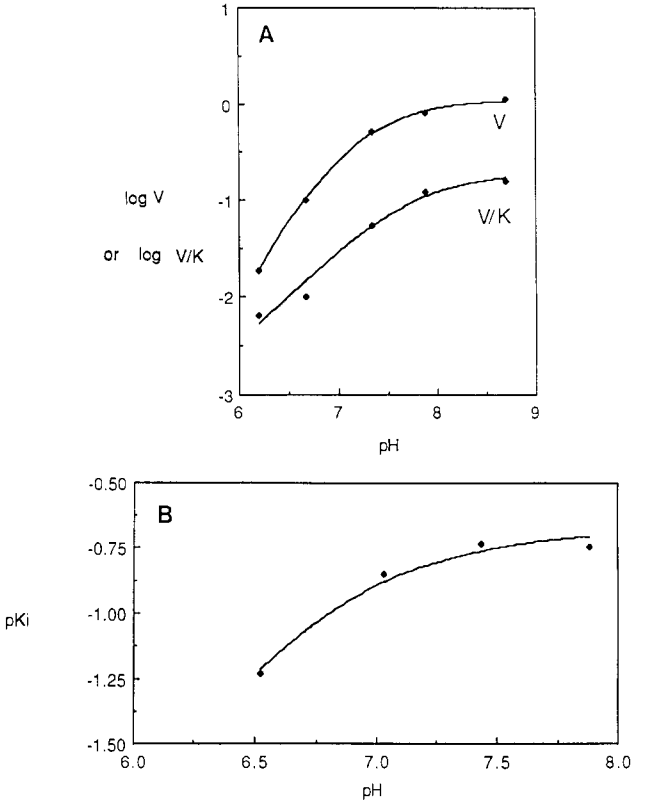


FIGURE 2: pH profiles for the biotin carboxylase catalyzed phosphorylation of ADP by carbamoyl phosphate. Carbamoyl phosphate is the variable substrate. (A) V/K and V profiles. Assays were carried out in the presence of 50 mM biotin, 8 mM $MgCl_2$, 1.4 mM ADP, 10% (v/v) EtOH, and between 2 and 20 mM carbamoyl phosphate. (B) pK_i profile. Inorganic phosphate, varied between 0 and 9.6 mM, was used as a competitive inhibitor versus carbamoyl phosphate. The lines were drawn according to eq 6 for the V/K profile and according to eq 7 for the V and pK_i profiles.

the profile obtained with Mg, except that the pK of HCO_3^- is also seen, because its concentration was not maintained at a constant level across the pH range. However, the V profile breaks more sharply than the V/K profiles; it is apparent that an ionizable group which is not seen in the V/K profiles appears in the V profile. The two pK's in the V/K profile (one of which is due to bicarbonate) are not distinguishable and are determined by the fit to eq 7 to be 5.90 ± 0.19.

The pH profiles for the biotin carboxylase catalyzed phosphorylation of MgADP by carbamoyl phosphate were also determined and are shown in Figure 2. V/K decreases with decreasing pH, defining a pK of 7.74 ± 0.16. Two pK's are apparent in the V profile; these were determined by fitting of the data to eq 7 to be 7.14 ± 0.07 and 6.91 ± 0.08. Inorganic phosphate was found to be a competitive inhibitor versus carbamoyl phosphate, and the pH dependence of the inhibition is also shown in Figure 2. This pK_i profile shows two indistinguishable pK's that were determined to be 6.46 ± 0.06 by fitting of the data to eq 7.

Inactivation Studies. Biotin carboxylase was found to be susceptible to inactivation by a number of sulfhydryl-modifying reagents. 2-Pyridyl disulfide (1 mM) and *N*-ethylmaleimide (0.75 mM) inactivated biotin carboxylase with rate constants of 0.22 and 0.12 min^{-1}, respectively, at pH 8.0. Methyl methanethiolsulfonate (10 μM) inactivated biotin carboxylase at pH 8.0 with a rate constant of 0.07 min^{-1}. In the presence of 40 mM biotin and saturating concentrations of Mg, ATP, and bicarbonate, complete protection against inactivation by NEM was observed, and the rate of inactivation by 2-PDS was reduced 7-fold.

Table III: Protection of Biotin Carboxylase from Inactivation by 1 mM 2-PDS

substrates[a]	k_{inact} (min^{-1})	substrates[a]	k_{inact} (min^{-1})
none	0.17	EtOH, CoATP, Co	0.07
EtOH	0.28	EtOH, bicarbonate, CoATP, Co	0.03
EtOH, biotin	0.35		
EtOH, bicarbonate	0.10	EtOH, biotin, bicarbonate, CoATP, Co	0.03
EtOH, bicarbonate, Co	0.12		
EtOH, CoATP	0.07		

[a] The concentrations of the substrates, when present, were as follows: EtOH, 15% (v/v); biotin, 50 mM; bicarbonate, 25.3 mM; CoATP, 100 μM; free Co, 100 μM. Reactions were carried out in 200 mM HEPES, pH 8.0 at 25 °C.

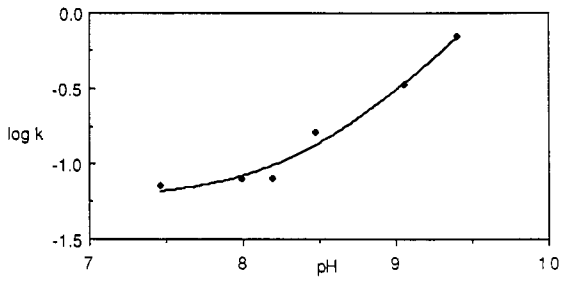


FIGURE 3: Inactivation of biotin carboxylase by *N*-ethylmaleimide as a function of pH. Biotin carboxylase was incubated with 750 μM NEM, 4 mM HCO_3^-, 0.24 mM ATP, and 0.8 mM $MgCl_2$. The line was drawn according to eq 8.

Inactivation by 2-PDS was partially reversible; biotin carboxylase inactivated to less than 10% of its initial activity by incubation with 1 mM 2-PDS for 20 min regained 50% of its activity after 10 min further incubation with 4 mM dithiothreitol. Biotin carboxylase could be cyanoylated by adding 50 mM KCN to enzyme that had been inactivated by 2-PDS. Enzyme thus treated did not regain activity upon addition of DTT.

The effectiveness of various combinations of substrates in providing protection against inactivation by 2-PDS was investigated, and the results are listed in Table III. Biotin offered no protection at all, while Co, CoATP, and bicarbonate proved to be as effective as the full complement of substrates. CoATP and bicarbonate singly also offered some protection.

The pH dependence of the rate constant for inactivation by NEM was determined in the presence of levels of Co, ATP, and bicarbonate that were 20% of the concentration which gave complete protection. As shown in Figure 3, the rate of inactivation is constant at about 0.08 min^{-1} below pH 8 and increases a factor of 10 per pH unit above pH 8.

Iodoacetamide, 5,5′-dithiobis(2-nitrobenzoic acid), and diethyl pyrocarbonate, which specifically modifies histidine residues, were also investigated as potential inactivators of biotin carboxylase and were found to be ineffective.

Thionucleotides. (S_P)-ATP-α-S, S_p- and (R_P)-ATP-β-S, and ATP-γ-S were investigated as substrates for biotin carboxylase in the forward direction. ADP-β-S was tested as a substrate for the reverse reaction from carbamoyl phosphate. Table IV lists the kinetic parameters of the thionucleotide substrates for which there is a facile spectrophotometric assay, using Mg, Mn, or Co. It should be noted that the hexokinase, glucose-6-phosphate dehydrogenase coupled assay used to monitor production of ATP-β-S from ADP-β-S detects formation of only the Λ isomer; therefore, the stereospecificity of the phosphorylation reaction remains undefined. Table V compares the ability of the ATP analogues to support biotin carboxylation on the basis of incorporation of $H^{14}CO_3^-$ into [^{14}C]carboxybiotin.

Table IV: Kinetic Parameters of Thionucleotides as Substrates for Biotin Carboxylase, pH 7.5

substrate	V/K [μmol/(min·μM)]	V_{max} (μmol/min)	K_m (μM)
	Biotin Carboxylation Reaction[a]		
MgATP	0.028 ± 0.001	1.54 ± 0.03	54.6 ± 2.6
CoATP	0.212 ± 0.026	1.60 ± 0.05	7.6 ● 1.1
MnATP	0.343 ± 0.036	0.84 ± 0.01	2.5 ± 0.2
Mg[(S_P)-ATP-β-S]	*b*	*b*	*b*
Co[(S_P)-ATP-β-S]	0.099 ± 0.003	2.84 ± 0.07	28.6 ± 1.4
Mn[(S_P)-ATP-β-S]	0.094 ± 0.009	2.07 ± 0.16	21.9 ± 3.8
Mg[(S_P)-ATP-α-S]	*b*	*b*	*b*
Co[(S_P)-ATP-α-S]	0.015 ± 0.003	0.171 ± 0.007	11.4 ± 2.5
Mn[(S_P)-ATP-α-S]	0.004 ± 0.001	0.059 ± 0.005	15.4 ± 5.9
	ADP Phosphorylation Reaction[c]		
MgADP	0.0021 ± 0.0001	0.084 ± 0.002	39.8 ± 2.2
CoADP	0.0024 ± 0.0001	0.186 ± 0.015	76.9 ± 11.8
MnADP	0.044 ± 0.004	0.055 ± 0.001	1.26 ± 0.15
MgADP-β-S[d]	*b*	*b*	*b*
CoADP-β-S[d]	0.00036 ± 0.00003	0.020 ± 0.001	53.8 ± 6.3
MnADP-β-S[d]	0.00091 ± 0.00004	0.0168 ± 0.0002	18.4 ± 1.1

[a] Biotin, 40 mM; bicarbonate, 20 mM; nucleotide or thionucleotide varied from 10 to 200 μM; Mg, 2.0 mM, or Co, 0.6 mM, or Mn, 0.25 mM; EtOH, 15% (v/v). [b] Too slow to determine. [c] Biotin, 40 mM; carbamoyl phosphate, 6 mM; ADP or ADP-β-S varied from 20 to 200 μM; Mg, 4.4 mM, or Co, 0.2 mM, or Mn, 0.2 mM; EtOH, 15% (v/v). [d] Kinetic parameters are for the formation of the Λ isomer of ATP-β-S only.

Table V: Biotin Carboxylase Catalyzed Formation of Carboxybiotin with Thionucleotides[a]

substrate	Mg	Co	Mn
ATP	(100)	95	78
ATP-α-S	8	76	43
(S_P)-ATP-β-S	13	21	14
(R_P)-ATP-β-S	3	27	14
ATP-γ-S	2	8	2

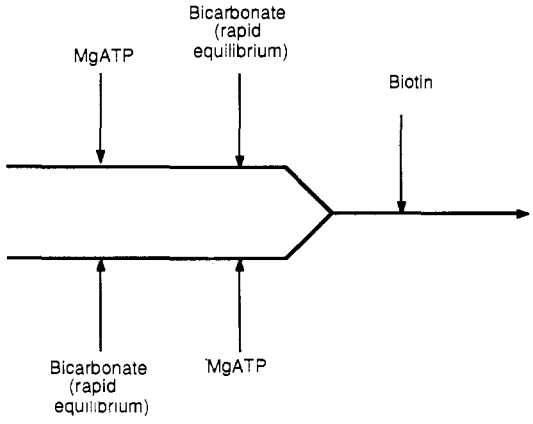
[a] Relative amounts of carboxybiotin formed during 10-min incubation. Nucleotides and thionucleotides, 100 μM; Co and Mn, 200 μM; Mg, 1.5 mM; biotin, 50 mM; bicarbonate, 8 mM; EtOH, 15% (v/v). Reactions performed at pH 7.5 with Co and Mn and at pH 8.0 with Mg.

ATPase Activity. Biotin carboxylase was found to catalyze ATP hydrolysis in the absence of biotin. Like the normal biotin carboxylation reaction, the ATPase reaction also required bicarbonate, MgATP, and free Mg^{2+}. Kinetic patterns were obtained by varying each of these substrates in turn. Strikingly, MgATP still adds in rapid equilibrium order fashion; the Michaelis constant for free Mg^{2+} is 0.43 ± 0.16 mM, and the dissociation constant of MgATP from the enzyme is 0.62 ± 0.24 mM. When bicarbonate is varied at different levels of Mg, the reciprocal plots appear very nearly parallel. Fitting these data to eq 4 for ping-pong patterns yields values of 2.2 ± 0.6 mM for the Michaelis constant of Mg and 2.7 ± 0.8 mM for the Michaelis constant of bicarbonate. When MgATP is varied at different levels of bicarbonate, an intersecting pattern results.

Discussion

Biotin carboxylase is a pseudoquadreactant enzyme. Biotin, MgATP, and bicarbonate are true substrates, and free Mg^{2+}, whose concentration affects the velocity of the reaction but does not change during the course of the reaction, can be

Scheme I



considered to be a pseudosubstrate. To simplify the determination of the kinetic mechanism, initial velocity patterns were obtained under conditions of saturating Mg. This effectively reduces the reaction to a terreactant system but, of course, precludes reaching any conclusion about how the free Mg^{2+} fits into the kinetic scheme.

The conclusion that the data best fit eq 5 was reached by first fitting the data to the equation describing a fully random terreactant mechanism:

$$v = VABC/[\text{const} + (\text{coeff } A)A + (\text{coeff } B)B + (\text{coeff } C)C + K_aBC + K_bAC + K_cAB + ABC]$$

Any term that was undefined by this fit was removed from the equation, and the data were refitted to the new equation. The process was repeated until an equation was arrived at in which all the terms were well-defined.

Equation 5 is degenerate in that three different kinetic mechanisms are described by it (Viola & Cleland, 1982). All have in common the rapid equilibrium addition of bicarbonate, which is manifested in the rate equation by the absence of a Michaelis constant for bicarbonate, and the lack of an enzyme–biotin complex, manifested by the absence of an A term; all contain a constant term which indicates that addition of substrates is sequential. The mechanisms differ in the binary and ternary complexes that are present. The first possible mechanism is a completely ordered mechanism in which bicarbonate adds first, followed by MgATP and then biotin. In the second mechanism, addition of bicarbonate and MgATP is random, and biotin can add to the enzyme–bicarbonate complex or to the enzyme–bicarbonate–MgATP complex. In the third mechanism, addition of bicarbonate and MgATP is random, but the ternary enzyme–bicarbonate–MgATP complex must always form before biotin can add. The first mechanism appears unlikely on the basis of the data shown in Table III. Protection by MgATP of biotin carboxylase from inactivation by 2-PDS demonstrates that an enzyme–MgATP complex does form; however, these data do not prove that the complex formed is productive. We have no data which serve to eliminate the possibility that an enzyme–bicarbonate–biotin complex forms, as postulated in the second mechanism; however, such a kinetic mechanism makes little sense from the viewpoint of catalytic efficiency of the enzyme. Considering that acetyl-CoA carboxylase is a ping-pong enzyme with two active sites, the third kinetic mechanism, shown in Scheme I, is appealing, because it would allow for maximum catalytic efficiency. While the biotin is occupied at the active site of the second half-reaction, the substrates of the first half-reaction could be adding at their active site so that all the components of the reaction are in place as soon as the cofactor returns. Such a sequence of events is allowed by the second mechanism as well but is not necessary. Inhibition studies would be extremely helpful to further define the kinetic mechanism. However, no suitable inhibitors were found; nitrate, azide, formate, borate, and cyanate were all tested as possible inhibitors versus bicarbonate, but none appeared to be anything more than mildly inhibitory at levels up to 100 mM. Thiocyanate was also tested and did inhibit biotin carboxylase; however, the inhibition was noncompetitive versus all the substrates, and the inhibition constants were 20 mM or greater. Analogues of biotin were also tested as potential inhibitors. Neither imidazolidone, imidazolidenethione, diaminobiotin, nor a racemic mixture of biotin sulfoxides was inhibitory.

Inspection of the terms in the second column of Table II reveals the synergism in the binding of MgATP and bicarbonate to biotin carboxylase. The dissociation constant of MgATP from the MgATP–enzyme complex is 4.5-fold higher than the dissociation constant of MgATP from the MgATP–HCO_3^-–enzyme complex. Similarly, the dissociation constant of HCO_3^- from the HCO_3^-–enzyme complex is 4.5-fold higher than the dissociation constant of HCO_3^- from the ternary complex.

When metal-ATP concentrations are varied at different levels of free metal, a pattern in which the lines intersect on the ordinate is obtained, indicating that metal-ATP also adds in rapid equilibrium, and addition of the second divalent metal ion serves to lock this substrate onto the enzyme. The pattern obtained when bicarbonate is varied at different levels of free Mg is more difficult to interpret. At high Mg concentrations, the lines are almost parallel, but as the metal level is decreased the lines become more obviously intersecting. When these data are plotted with free metal as the variable substrate, the lines are curved downward, which makes clear that there is significant negative cooperativity in the binding of the metal. These data do not, however, offer information about when the second metal ion binds to the enzyme. All the available information is consistent with the kinetic mechanism shown in Scheme I, which neglects the second metal ion.

The pH dependence of the biotin carboxylase catalyzed carboxylation of biotin is shown in Figure 1. In the experiment from which the data in Figure 1A are drawn, the level of HCO_3^- was maintained at a constant level across the pH range, so the pK that is defined is not the pK of bicarbonate. None of the other substrates have pK's in the experimental range, so the pK must be that of a group on the enzyme. Furthermore, because V/K reflects events occurring on the enzyme only between the time of the binding of the variable substrate, in this case biotin, and the release of the first product and because biotin adds last, one can conclude that the observed pH dependence of the reaction does not encompass the formation of the MgATP–bicarbonate–enzyme complex to which biotin adds. (This statement must be qualified by the caveat that it is true only if MgATP is saturating across the pH range, as it was in the above experiment.) Therefore, it seems very probable that the observed base acts in a catalytic role. Model studies have suggested that while the urea tautomer of biotin is extremely nonnucleophilic, the isourea tautomer is 10 orders of magnitude more nucleophilic (Hegarty et al., 1969; Kondo et al., 1983). Thus, a likely role for the base observed in the V/K profile is to remove the proton from N1′ of biotin to generate the much more reactive isourea tautomer. However, solvent deuterium isotope effect data presented and discussed in detail in the following paper suggest that this base does not remove the amide proton from biotin. Instead, its role is to ionize an active-site sulfhydryl that tautomerizes biotin. Fry

et al. (1985) have determined that the uncatalyzed rate of exchange of the biotin N1′ proton is sufficient to keep up with enzyme turnover. However, Attwood and Cleland (1986) have also detected a catalytic base in the biotin-dependent enzyme pyruvate carboxylase, which they proposed served to tautomerize biotin. Therefore, it appears that enzymic catalysis of the removal of the proton may be a common feature of biotin-dependent enzymes.

Because biotin carboxylase is susceptible to inactivation by sulfhydryl-modifying reagents, the rate constant for inactivation was measured as a function of pH to determine whether or not a correlation exists between the pK's seen in the V and V/K profiles and the pK of the sulfhydryl that is being alkylated. As shown in Figure 3, the pK of the susceptible sulfhydryl is greater than 9.5, beyond the experimentally accessible range. The conditions under which these data were collected were designed to mimic the conditions of the V/K_{biotin} profile, in which the concentration of biotin is extrapolated to zero and the other substrates are saturating. In the inactivation experiments, biotin is absent, but the other substrates are not truly saturating because saturation precludes inactivation. Thus, the inactivation experiment only approaches the V/K_{biotin} conditions, but it still seems extremely unlikely that this difference could explain the difference in pK of 3 or more pH units seen in the two experiments if these pK's arose from the same base. It seems most reasonable to conclude that the base which is alkylated by the sulfhydryl-modifying reagents cannot be identified with either of the bases whose pK's are seen in the pH profiles. Because its pK is beyond the range explored in the pH profiles, its ionization state remained constant (protonated) throughout those experiments and was thus undetected. Although the data presented here do not speak to the role of the sulfhydryl in the catalytic reaction, the fact that even modifications which do not add much steric bulk to the active site, such as S-methylation and cyanoylation, completely destroy activity suggests that this sulfhydryl plays an active role in catalysis. In the following paper, data are presented that do suggest a role for the sulfhydryl group.

When Co is substituted for Mg in the forward reaction, the K_m's of the substrates are lowered by approximately an order of magnitude. Therefore, one can vary biotin levels and determine the pH dependence of V as well as V/K. The V profile has the same shape as the V/K profiles with Mg and Co, but reaches a slope greater than that seen in the V/K profiles. This suggests that there is another pH-sensitive step in the catalytic cycle which occurs either before or after the steps between biotin binding and release of the first product. This additional ionization could reflect a pH dependence on product release or may be indicative of a pH-dependent protein conformation change. It is interesting to note that pyruvate carboxylase also shows one more ionizable group in the V profile than is seen in the V/K profile for the reaction in which oxalacetate decarboxylation is linked to ATP formation (Attwood & Cleland, 1986).

Figure 2 shows the pH profiles for the phosphorylation of MgADP by carbamoyl phosphate, a reaction in which biotin does not participate chemically but is necessary for reaction (Polakis et al., 1974). Like the profiles for the carboxylation reaction, the V/K profile breaks to a slope of 1, but the V profile reaches a slope of 2. Phosphorylation of ADP by carbamoyl phosphate should not require acid–base catalysis, so the reason for the pH dependence of the reaction is not obvious. To clarify the situation, inorganic phosphate, which is a competitive inhibitor versus carbamoyl phosphate, was used to obtain a pK_i profile. Such profiles show the pK's of the substrates and inhibitor, when in the experimental range, and also the true pK's of groups necessary for binding of substrates. The pK_i profile, also shown in Figure 2, reaches a slope of 2 and defines two nondistinguishable pK's at 6.5. One of these is presumably the pK of inorganic phosphate and the other the second pK seen in the V profiles.

The most reasonable explanation of these data is that the pK's seen in the pH profiles of the carbamoyl phosphate reaction arise from the same groups whose pK's are seen in the pH profiles of the forward reaction. Because biotin derivatives in which N1′ is blocked will support the carbamoyl phosphate reaction (Polakis et al., 1974), it appears that acid–base catalysts do not play an active role in the carbamoyl phosphate reaction. The data presented here, however, argue that the enzyme will catalyze phosphoryl transfer only when it is in one specific protonation state.

Several different experiments addressed the question of the role of the second metal ion in the catalytic reaction. It is clear that a divalent metal ion in addition to the one which coordinates to the nucleotide increases the velocity of the reaction. Other experiments (data not shown) demonstrate that the second metal ion plays a role in the carbamoyl phosphate reaction as well. These experiments do not, however, determine whether the second metal ion serves to stimulate the reaction above some basal velocity that is maintained in its absence or whether it is truly required in order for reaction to occur. Another way to state the question is, do the reciprocal plots obtained when Mg is varied at different levels of bicarbonate, and which are concave downward, approach an asymptote, or do the lines continue upward indefinitely? To distinguish between these possibilities, the reaction kinetics were measured at extremely low levels of free Mg. To accomplish this, the total Mg level was fixed at 0.5 mM, and reaction velocity was measured as a function of bicarbonate concentration in the presence of 0.5 mM total ATP, 1.0 mM total ATP, and 2.0 mM total ATP. Under these conditions MgATP is saturating, and the level of free Mg is very low. The reaction velocity was observed to decrease as the level of free Mg decreased. This decrease is not due to competitive inhibition by free ATP, because MgATP is saturating. Because the reaction velocity is dependent on the free Mg^{2+} concentration even at $^1/_{100}$ its K_m (the K_m for free Mg^{2+} is about 500 μM), it does not appear that the reaction would proceed in the absence of free divalent metal ion. To ensure that carboxylation of biotin was not becoming uncoupled from ATP hydrolysis at low Mg ATP hydrolysis, biotin carboxylation was observed to continue to depend on the concentration of free Mg^{2+}, even at very low levels of Mg^{2+} (data not shown). This observation is in agreement with the results of Allen et al. (1984), who demonstrated that free divalent metal ion is absolutely required by the biotin-dependent enzyme propionyl-CoA carboxylase when the exchange inert complex cobalt(III) tetraammine ATP is used as the substrate in place of MgATP.

Use of thionucleotide analogues of ATP as substrates for biotin carboxylase shed some light on the binding site of the second metal ion. Neither isomer of ATP-β-S was a very good substrate for biotin carboxylase when Mg was used. (S_P)-ATP-β-S, which can be used in a coupled assay, was a very slow substrate; the kinetic parameters could not be determined because measurable velocities were obtained only with high levels of thionucleotide (100–500 μM), where the reaction is subject to substrate inhibition. However, ATP-β-S is not chemically incompetent because it becomes a good substrate when Mn or Co is substituted for Mg. When these alternate metal ions were used, the reaction proceeded with a significant

velocity at substrate concentrations an order of magnitude lower than those required to see reaction with Mg. The data in Table V indicate that biotin carboxylase can utilize (S_P)-ATP-β-S with Mg, but a 15-fold higher level of Mg was required to see reaction, compared to the levels of Mn and Co that were used. (R_P)-ATP-β-S cannot be assayed easily with a spectrophotometric assay, but the data in Table V reveal that biotin carboxylase does not utilize it when the divalent metal ion is Mg. (S_P)-ATP-α-S showed behavior similar to that seen with the ATP-β-S isomers: The thionucleotide was not a substrate with Mg but was with Mn or Co. One of the most acute differences between Mg and Mn or Co is that Mg is a much harder Lewis acid, and coordination with sulfur is therefore unfavorable. The lack of reactivity of the thionucleotides with Mg therefore suggests that both nonbridging oxygens of the β-phosphate and at least one nonbridging oxygen of the α-phosphate normally coordinate to divalent metal ions in the active site of biotin carboxylase. Because it is difficult to imagine a conformation of the nucleotide in which all three of these phosphate oxygens are ligands of one metal ion, the most likely explanation of the data is that the nucleotide is coordinated to both of the divalent metal ions. Thus, these data serve to place the binding site of the second divalent metal ion in the active site, adjacent to the nucleotide binding site.

ATP-γ-S was a very poor substrate regardless of the divalent metal ion present. Hansen and Knowles (1985) also found ATP-γ-S to be a very slow substrate for pyruvate carboxylase. Therefore, one cannot determine if the lack of reactivity is due to unfavorable interactions between the thiophosphoryl group and the metal ions in the active site or if ATP-γ-S is not chemically competent to support the reaction. Certainly it is not clear, a priori, why carboxythiophosphate could not be formed or why it would be unreactive once it was formed.

Whether biotin carboxylase forms one isomer of ATP-β-S preferentially when ADP-β-S is used in the carbamoyl phosphate reaction was not determined. Because of the stereospecificity of hexokinase, which was used in the coupled assay, only formation of the Λ isomer of ATP-β-S was detected.

Climent and Rubio (1986) were the first to demonstrate that biotin carboxylase has a bicarbonate-dependent ATPase activity in the absence of biotin that proceeds at about 0.5% the rate of reaction coupled to biotin carboxylation. This observation has important ramifications for the mechanism of enzyme-catalyzed biotin carboxylation, and we have further characterized the kinetic properties of this reaction. Although the protein used in our studies was not completely homogeneous, the dependence of the reaction on the same substrates as the normal biotin carboxylase reaction and the observation that the ATPase reaction is stimulated by ethanol to the same extent as the overall reaction lead us to conclude that we are describing an intrinsic activity of biotin carboxylase and not that of a contaminant of the enzyme preparation. Climent and Rubio (1986) have demonstrated that the ATPase reaction is also stimulated to the same extent as the overall reaction by potassium ion and that the heat stabilities of the reactions are identical. The dependence of the ATPase activity on the presence of bicarbonate immediately suggests that ATP hydrolysis occurs via the intermediacy of carboxyphosphate, which then presumably decomposes to yield CO_2 and inorganic phosphate in the absence of an acceptor for the carboxyl group. The kinetic mechanism of the overall reaction shown in Scheme I suggests that all the components necessary for the ATPase reaction can bind to the enzyme in the absence of biotin and, indeed, the kinetic patterns obtained for the ATPase reaction are qualitatively similar to the ones seen in the overall reaction. When free Mg is varied at different levels of bicarbonate, the reciprocal lines appear nearly parallel and exhibit the same negative cooperativity seen in the overall reaction at low levels of Mg. In both reactions MgATP adds in rapid equilibrium followed by free Mg. The kinetic behavior of bicarbonate is different in the ATPase reaction, however. In reactions coupled to biotin carboxylation bicarbonate adds in rapid equilibrium, but when bicarbonate is varied at different levels of MgATP in the ATPase reaction, an intersecting pattern in which the lines do not intersect on the ordinate results.

These observations are difficult to rationalize in terms of the phosphobiotin mechanism advanced by Kluger (Kluger & Adawadker, 1976; Kluger et al., 1979), in which the ureido oxygen of biotin in phosphorylated by ATP and the phosphobiotin then attacks bicarbonate directly. Hansen and Knowles (1985) have also pointed out that the stereochemistry of phospho transfer in the pyruvate carboxylase reaction is difficult to reconcile with a phosphobiotin mechanism. A wholly concerted mechanism, in which nucleophilic attack by biotin on bicarbonate occurs concomitantly with attack by bicarbonate on ATP, is also rendered suspect by the demonstration that ATP hydrolysis can occur in the absence of biotin. The bicarbonate-dependent biotin-independent ATPase activity is consistent with a mechanism in which the bicarbonate attacks the γ phosphoryl of ATP to form carboxyphosphate. In the absence of an acceptor for the carboxyl group, carboxyphosphate decarboxylates to effect net hydrolysis of ATP. Hansen and Knowles (1985) also concluded that their results could be most simply interpreted in terms of a carboxyphosphate mechanism.

In summary, the data presented here demonstrate that there are at least two bases at the active site of biotin carboxylase; one of these is a sulfhydryl. In addition, the protonation state of another base is important for the catalytic reaction. Whether it is involved in substrate binding or conformation changes of the protein cannot be distinguished from these data. The acid–base catalysts at the active site are believed to catalyze the tautomerization of biotin. The nucleotide appears to be coordinated to two divalent metal ions at the active site. Bicarbonate-dependent enzyme-catalyzed hydrolysis of ATP in the absence of biotin suggests that the normal catalytic reaction proceeds via a carboxyphosphate intermediate. In the following paper, data are presented that allow us to draw more detailed conclusions regarding the nature of the other intermediates along the pathway to biotin carboxylation.

REFERENCES

Adolfsen, R., & Mondrianakis, E. N. (1978) *J. Biol. Chem. 253*, 4378.

Allen, C. M., Jr., & Jones, M. E. (1964) *Biochemistry 3*, 1238.

Allen, S. H. G., Treble, D. H., Peterson, D. D., & Botti, C. F., Jr. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1781.

Attwood, P. V., & Cleland, W. W. (1986) *Biochemistry 25*, 8191.

Attwood, P. V., Tipton, P. A., & Cleland, W. W. (1986) *Biochemistry 25*, 8197.

Carter, S. G., & Karl, D. W. (1982) *J. Biochem. Biophys. Methods 7*, 7.

Chauhan, U. P. S., & Ray Sarkar, B. C. (1969) *Anal. Biochem. 32*, 70.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

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

Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685.

Fry, D. C., Fox, T. L., Lane, M. D., & Mildvan, A. S. (1985) *J. Am. Chem. Soc. 107*, 7659.
Guchhait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J., & Lane, M. D. (1974a) *J. Biol. Chem. 249*, 6633.
Guchhait, R. B., Polakis, S. E., Hollis, D., Fenselau, C., & Lane, M. D. (1974b) *J. Biol. Chem. 249*, 6646.
Hansen, D. E., & Knowles, J. R. (1985) *J. Am. Chem. Soc. 107*, 8304.
Hegarty, A. F., Bruice, T. C., & Benkovic, S. J. (1969) *J. Chem. Soc., Chem. Commun.*, 1173.
Kluger, R., & Adawadker, P. D. (1976) *J. Am. Chem. Soc. 98*, 3741.
Kluger, R., Davis, P. P., & Adawadkar, P. D. (1979) *J. Am. Chem. Soc. 101*, 5995.
Kondo, H., Miura, K., & Sunamoto, J. (1982) *Tetrahedron Lett. 23*, 659.
Kondo, H., Uno, S., Moriuchi, F., Sunamoto, J., Ogushi, S., & Tsuru, D. (1983) *Bull. Chem. Soc. Jpn. 56*, 1176.
Moss, J., & Lane, M. D. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 35*, 321.
Polakis, S., Guchhait, R. B., Zwergel, E. E., Lane, M. D., & Cooper, T. G. (1974) *J. Biol. Chem. 249*, 6657.
Sauers, C. K., Jencks, W. P., & Groh, S. (1975) *J. Am. Chem. Soc. 97*, 5546.
Sutton, M. R., Fall, R. R., Nervi, A. M., Alberts, A. W., Vagelos, P. R., & Bradshaw, R. A. (1977) *J. Biol. Chem. 252*, 3934.
Tipton, P. A., & Cleland, W. W. (1988) *Biochemistry* (following paper in this issue).
Viola, R. E., & Cleland, W. W. (1982) *Methods Enzymol. 87*, 353.

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

Peter A. Tipton and W. W. Cleland*
Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706
Received August 24, 1987; Revised Manuscript Received January 28, 1988

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-

† This work was sponsored by a grant from the National Institute of Health (GM 18938). P.A.T. was a trainee on Grant 5T32-GM07215 from the National Institutes of Health. A major portion of the funding for the isotope ratio mass spectrometer was provided by a grant from NSF (PCM-8218027). ^{31}P spectra were obtained at the National Magnetic Resonance Facility at Madison, which is supported by a grant from the NIH (RR02301).

0006-2960/88/0427-4325$01.50/0