Do Cysteine 230 and Lysine 238 of Biotin Carboxylase Play a Role in the Activation of Biotin?†

Keith L. Levert, Robert B. Lloyd, and Grover L. Waldrop*

Division of Biochemistry and Molecular Biology, Louisiana State University, Baton Rouge, Louisiana 70803-1715

Received November 18, 1999; Revised Manuscript Received January 21, 2000

ABSTRACT: Biotin carboxylase from *Escherichia coli* catalyzes the ATP-dependent carboxylation of biotin and is one component of the multienzyme complex acetyl-CoA carboxylase, which catalyzes the committed step in long-chain fatty acid synthesis. For the carboxylation of biotin to occur, biotin must be deprotonated at its N1′ position. Kinetic investigations, including solvent isotope effects and enzyme inactivation by *N*-ethylmaleimide, suggested a catalytic role for a cysteine residue and led to the proposal of a mechanism for the deprotonation of biotin. The proposed pathway suggests a catalytic base removes a proton from a nearby cysteine residue, forming a thiolate anion, which then abstracts the proton from biotin. Inactivation studies of pyruvate carboxylase, which has an analogous mode of action to biotin carboxylase, suggests the catalytic base in this reaction is a lysine residue. Using the crystal structure of biotin carboxylase, cysteine 230 and lysine 238 were identified as the likely active-site residues that act as this acid−base pair. To test the hypothesis that cysteine 230 and lysine 238 act as an acid−base pair to deprotonate biotin, site-directed mutagenesis was used to mutate cysteine 230 to alanine (C230A) and lysine 238 to glutamine (K238Q). Mutations at either residue resulted in a 50-fold increase in the K_m for ATP. The C230A mutation had no effect on the formation of carboxybiotin, indicating that cysteine 230 does not play a role in the deprotonation of biotin. However, the K238Q mutation resulted in no formation of carboxybiotin, which showed that lysine 238 has a role in the carboxylation reaction. *N*-Ethylmaleimide was found to inactivate the C230A mutant but not the K238Q mutant, suggesting that *N*-ethylmaleimide is reacting with lysine 238 and not cysteine 230. The pH dependence of *N*-ethylmaleimide inactivation revealed that the p*K* value for lysine 238 was 9.4 or higher, suggesting lysine 238 is not a catalytic base. Thus, the results suggest that cysteine 230 and lysine 238 do not act as an acid−base pair in the deprotonation of biotin.

Biotin, or vitamin H, serves as a cofactor for a diverse group of enzymes that catalyze carboxylation reactions. These biotin-dependent carboxylases are involved in many vital metabolic pathways such as gluconeogenesis, fatty acid synthesis, and amino acid catabolism. The reactions catalyzed by this family of enzymes follow the same two-step pathway:

$$\text{enzyme}-\text{biotin} + \text{MgATP} + \text{HCO}_3^- \overset{\text{Mg}^{2+}}{\rightleftharpoons} \text{enzyme}-\text{biotin}-\text{CO}_2^- + \text{MgADP} + \text{Pi} \quad (1)$$

$$\text{enzyme}-\text{biotin}-\text{CO}_2^- + \text{acceptor} \rightleftharpoons \text{acceptor}-\text{CO}_2^- + \text{enzyme}-\text{biotin} \quad (2)$$

The first partial reaction involves the carboxylation of biotin at the N1′ position. This is accomplished by the ATP-dependent phosphorylation of bicarbonate, the source of CO_2, forming a reactive carboxyphosphate intermediate. The carboxyl group is then transferred to biotin, which is covalently linked to the enzyme through an amide linkage to the side chain of a specific lysine residue. In the second half of the reaction the carboxylate group is transferred from carboxybiotin to an acceptor molecule. The acceptor will vary depending on the enzyme. Pyruvate carboxylase, for example, utilizes pyruvate as an acceptor while acetyl-CoA carboxylase utilizes acetyl-CoA as its substrate.

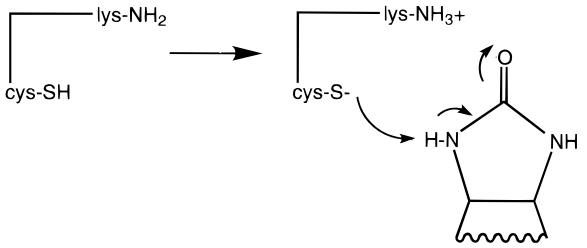
The first half-reaction, which is catalyzed by all biotin-dependent carboxylases, involves the deprotonation of the 1′ nitrogen of biotin. Mechanistic studies utilizing the biotin carboxylase component of *Escherichia coli* acetyl-CoA carboxylase and pyruvate carboxylase have suggested a possible mechanism for the deprotonation of biotin. Tipton and Cleland found a large inverse solvent isotope effect in the reaction catalyzed by biotin carboxylase, which suggests a sulfhydryl group is involved in a proton transfer (*1*). Participation of an active-site cysteine residue in catalysis by biotin carboxylase was further supported by the observation that *N*-ethylmaleimide inactivated the enzyme while the substrates protected against the inactivation (*2*). Werneburg and Ash found that *o*-phthalaldehyde (*o*-PA) inactivated pyruvate carboxylase by cross-linking cysteine and lysine residues and that substrates protected against inactivation, suggesting that the modified cysteine and lysine residues

† This research was supported by grants from the NIH (GM51261) and the Petroleum Research Fund of the American Chemical Society (32234-AC4) to G.L.W.

* To whom correspondence should be addressed at the Department of Biological Sciences, Louisiana State University, Life Sciences Building, Room 508, Baton Rouge, LA 70803-1715. Phone: (225) 388-5209. Fax: (225) 388-4638. E-mail: gwaldro@lsu.edu.

10.1021/bi992662a CCC: $19.00
Published on Web 03/21/2000

Scheme 1



were in the active site (*3*). All this evidence taken together led several groups (*1*−*5*) to propose an active-site lysine residue removes the proton from the thiol group of a nearby cysteine residue. The thiolate anion then abstracts the N1′ proton of biotin while the protonated lysine residue stabilizes the negative charge on the ureido oxygen (Scheme 1).

Using the crystal structure of *E. coli* biotin carboxylase (*6*), the only three-dimensional structure of a biotin-dependent carboxylase, Jitrapakdee et al. proposed that the cysteine and lysine residues acting as acid−base catalysts are Cys230 and Lys238 (*7*). These residues are located in the active site of biotin carboxylase, and the sulfur atom of Cys230 and ϵ-amino group of Lys238 are sufficiently close (4.2 Å) to allow for cross-linking of these two residues by *o*-PA. Cys230 and Lys238 are also strictly conserved in all biotin-dependent carboxylases, suggesting they play a role in the catalytic mechanism (*8*).

The biotin carboxylase component of *E. coli* acetyl-CoA carboxylase has been used as a model system for mechanistic studies of biotin carboxylation because this enzyme retains activity when isolated from the multienzyme complex. Moreover, biotin carboxylase is able to utilize free biotin as a substrate, thereby simplifying kinetic analysis (*9*). A system for site-directed mutagenesis studies of *E. coli* biotin carboxylase has been described recently which allows for overexpression and purification of mutant forms of biotin carboxylase that are free of the contaminating wild-type enzyme derived from the chromosomal copy of the gene (*10*). This system was used to test directly the hypothesis that Cys230 and Lys238 act as an acid−base pair to remove the N1′ proton from biotin.

MATERIALS AND METHODS

Chemicals and Enzymes. Primers were synthesized by Life Technologies GibcoBRL. Sodium bicarbonate labeled with ^{14}C was from Amersham and had a specific activity of 0.1 mCi/mmol. His-binding resin and restriction-grade thrombin were from Novagen. Pyruvate kinase was from Boehringer Mannheim. All other reagents were from Sigma or Aldrich.

Site-Directed Mutagenesis and Enzyme Purification. Site-directed mutagenesis and purification of wild-type and mutant enzymes were as previously described by Blanchard et al. (*10*). Cys230 was mutated to alanine, and Lys238 was mutated to glutamine. The lysine to glutamine substitution is more isosteric than an alanine replacement, and the amide side chain cannot act as a base. The pairs of internal primers used to make each site-directed mutant were as follows (the bases that were changed are underlined): for C230A, 5′-GCGGAACGTGACGCCTCCATGCAACGC-3′ and 3′-CGCCTTGCACTGCGGAGGTACGTTGCG-5′ and, for K238Q, 5′-GCCGCCACCAGCAAGTGGTCGAAGA-3′ and 3′-CGGCGGTGGTCGTTCACCAGCTTCT-5′. The entire gene of each mutant form of biotin carboxylase was sequenced to verify the mutation and confirm there were no other changes in the sequence.

Enzyme Assays. The rate of ATP hydrolysis by biotin carboxylase was determined spectrophotometrically by coupling the production of ADP to pyruvate kinase and lactate dehydrogenase, and following the oxidation of NADH at 340 nm (*10*). When the concentration of ATP was varied above 2 mM (2−25 mM), the concentration of $MgCl_2$ was increased to 50 mM, ensuring the formation of MgATP complex. Solvent deuterium isotope effects on ATP hydrolysis by biotin carboxylase were determined in 80% D_2O and at a pD of 8.0, where pD values were calculated according to the equation pD = pH + 0.4 (*11*). When the concentration of bicarbonate was varied, all solutions were degassed to lower the endogenous levels of bicarbonate (*10*).

The rate of ATP synthesis from ADP and carbamoyl phosphate was determined spectrophotometrically with the coupled enzyme system of hexokinase and glucose-6-phosphate dehydrogenase, where the production of NADPH was followed at 340 nm (*10*). Data were collected using a Uvikon 810 (Kontron Instruments) spectrophotometer interfaced to a PC equipped with a data acquisition program. Reactions were initiated by the addition of enzyme, and were held at 25 °C by a circulating water bath. Kinetic parameters were calculated per active site using a MW of 50 000 for the biotin carboxylase monomer (biotin carboxylase exists as a homodimer).

To determine whether there was a stoichiometric production of ADP and carboxybiotin, the amount of carboxybiotin produced by biotin carboxylase was determined using a [^{14}C]-bicarbonate fixation assay and compared to the production of ADP as described by Blanchard et al. (*10*).

Inactivation by NEM. Enzyme modification by *N*-ethylmaleimide (NEM) was performed in 10 mM Hepes and 500 mM KCl, pH 7.0. The reaction was initiated by the addition of NEM to a final concentration of 6 mM. Aliquots were removed at regular time intervals and assayed for residual activity. The reaction velocity of each aliquot, for the wild-type enzyme, was determined in 100 mM biotin, 3 mM ATP, 15 mM bicarbonate, and 8 mM $MgCl_2$ at pH 8.0 in 100 mM Hepes. When reaction velocities were determined, for both mutants, the concentrations of ATP and $MgCl_2$ were increased to 20 and 50 mM, respectively. The rate constants for inactivation by NEM were determined by least-squares analysis of a plot of $\ln(v_t/v_o)$ versus time, where v_t is the velocity at time t, v_o is the initial velocity, and the slope of the line is equal to the negative of the rate constant. The pH dependence of the rate of inactivation was determined by incubating biotin carboxylase with 70 μM NEM in a 100 mM solution of the appropriate buffer. The buffers used and their pH ranges were as follows: Hepes, pH 7.0−8.0; TAPS, pH 8.0−9.0; CHES, pH 9.0−9.5.

Data Analysis. The K_m and k_{cat} were determined by fitting the velocity versus substrate concentration data to the Michaelis−Menten equation using the nonlinear regression program Enzfitter.

The data for the pH dependence on the rate of inactivation were fitted to the log form of eq 1, where y represents the

$$y = C/(1 + H/K_a) \quad (1)$$

Table 1: Kinetic Parameters for the Bicarbonate-Dependent ATPase Reaction[a]

	k_{cat} (min^{-1})	$K_{m_{ATP}}$ (mM)	$K_{m_{HCO_3^-}}$ *(mM)*
WT	0.073 ± 0.001[b]	0.081 ± 0.003[b]	0.37 ± 0.04[b]
C230A	0.102 ± 0.001	4.23 ± 0.13	0.70 ± 0.10
K238Q	0.117 ± 0.002	6.87 ± 0.27	*c*

[a] The k_{cat} and K_m values for ATP were determined by varying ATP at a constant saturating level of bicarbonate. The K_m value for bicarbonate for the C230A mutant was determined by varying bicarbonate at a constant nonsaturating level of ATP (20 mM) and biotin (75 mM). The standard errors in k_{cat} and K_m were determined from the nonlinear regression analysis. [b] These values were taken from ref 10. [c] Refer to the text for results.

rate of inactivation at a particular pH value, *C* represents the pH-independent value of the rate constant, K_a is an acid dissociation constant, and *H* is the hydrogen ion concentration.

RESULTS

Bicarbonate-Dependent ATP Hydrolysis. Biotin carboxylase from *E. coli* catalyzes a slow bicarbonate-dependent ATP hydrolysis reaction in the absence of biotin (*12*) (reaction 3). Current evidence suggests this reaction proceeds through

$$\mathrm{MgATP} + \mathrm{H_2O} \xrightarrow{\mathrm{HCO_3^-}} \mathrm{MgADP} + \mathrm{Pi} \qquad (3)$$

the formation of carboxyphoshate, which rapidly decomposes in the absence of biotin (*13*). The Michaelis constants for ATP and bicarbonate and the k_{cat} of this partial reaction were determined for the wild-type enzyme and both mutants of biotin carboxylase (Table 1). The k_{cat} values for the C230A and K238Q mutants were equivalent to the k_{cat} for wild-type biotin carboxylase. In contrast, a 50-fold increase in the Michaelis constant for ATP was observed for both mutant enzymes.

The Michaelis constant for bicarbonate was determined at fixed, nonsaturating levels of ATP and biotin.[1] Therefore, the K_m values for bicarbonate reported here are apparent K_m values. The apparent K_m values for bicarbonate for wild-type biotin carboxylase and the C230A mutant were similar. However, for K238Q there was no change in the reaction rate with increasing concentrations of bicarbonate (0.5−7.1 mM). This may be due to a decrease in the Michaelis constant for bicarbonate to a value lower than the concentration of bicarbonate that remained in the degassed buffer. Thus, the endogenous level of bicarbonate (0.5 mM at pH 8.0, ref *14*) was enough to saturate the enzyme.

Biotin-Dependent ATP Hydrolysis. The rate of ATP hydrolysis by wild-type biotin carboxylase is significantly increased in the presence of biotin. The initial velocity of ATP hydrolysis as a function of increasing amounts of biotin for wild-type biotin carboxylase and the two mutants is shown in Figure 1. The k_{cat} of the biotin-dependent ATPase for the wild-type enzyme was 73.8 ± 3.8 min^{-1}, which is 1000-fold faster than the k_{cat} of the rate of ATP hydrolysis in the absence of biotin (Table 1). The k_{cat} for the C230A mutant was 30.8 ± 1.6 min^{-1}, slightly less than 50% of the velocity for the wild-type enzyme. The Michaelis constant for biotin also was relatively unaffected by the C230A mutation. Wild-type biotin carboxylase and the C230A mutant had K_m values for biotin of 122 ± 17 and 167 ± 18 mM, respectively. In contrast to wild-type biotin carboxylase and the C230A mutant, the K238Q mutation had a drastic effect on the biotin-dependent ATP hydrolysis reaction. As can be seen in Figure 1, increasing concentrations of biotin had very little effect on the rate of ATP hydrolysis for the K238Q mutant. The reaction rate was equivalent to the k_{cat} for the bicarbonate-dependent ATPase reaction (Table 1).

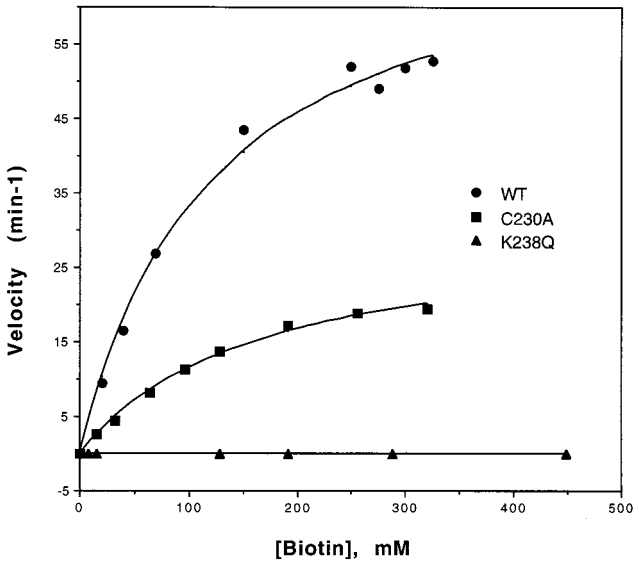


FIGURE 1: Effect of biotin on the ATP hydrolysis reaction for wild-type biotin carboxylase (WT) and both mutants of biotin carboxylase, C230A and K238Q. Initial velocities were measured at a saturating level of ATP and bicarbonate with increasing levels of biotin. The initial velocities represent the velocity divided by the concentration of active sites. The points are the experimental velocities, and the lines for the wild-type and C230A mutant are derived from the best fit of the data to the Michaelis−Menten equation.

It should be noted that a previous study of the K238Q mutant enzyme found the maximal velocity was decreased only 4-fold while the K_m for biotin was equivalent to that observed for the wild-type enzyme (*15*). A possible explanation for the difference between the findings of Kazuta et al. and the results reported here is that Kazuta et al. did not demonstrate a method for separating the mutant enzyme from the wild-type enzyme derived from the chromosomal copy of the bacterial genome. Thus, the maximal velocity and Michaelis constant for biotin that were reported for the K238Q mutant were probably the result of contaminating wild-type biotin carboxylase.

The assay for the biotin-dependent hydrolysis of ATP measured the rate of production of ADP using the coupling enzymes pyruvate kinase and lactate dehydrogenase. The C230A and K238Q mutations clearly did not affect the ability of biotin carboxylase to hydrolyze ATP; however, this method did not measure the ability of biotin carboxylase to transfer the carboxyl group from carboxyphosphate to biotin.

[1] Biotin was added to increase the initial velocity, which allowed less enzyme to be added to the assay mixture. It was necessary to add a small volume of enzyme to minimize the endogenous level of bicarbonate. The endogenous level of bicarbonate at pH 8.0 is 0.5 mM (*14*), which is close to the Michaelis constant for bicarbonate in biotin carboxylase. This makes it difficult to vary the level of bicarbonate. The endogenous level of bicarbonate could be removed from all the components of the assay except the enzyme. Hence, it was necessary to minimize the amount of enzyme added.

Table 2: Kinetic Parameters for the ATP Synthesis Reaction[a]

	k_{cat} (min^{-1})	$K_{m_{ADP}}$ (mM)	$K_{m_{CbmP}}$ (mM)
WT	0.31 ± 0.01	0.19 ± 0.01	4.84 ± 0.24
C230A	0.42 ± 0.01	0.62 ± 0.05	7.94 ± 0.31
K238Q	0.43 ± 0.01	1.2 ± 0.1	3.13 ± 0.27

[a] The kinetic parameters were determined by varying carbamoyl phosphate (CbmP) at a constant saturating level of ADP or varying ADP at a constant saturating level of CbmP. The standard errors in k_{cat} and K_m were determined from the nonlinear regression analysis.

An assay using radiolabeled bicarbonate was used to measure the amount of carboxybiotin produced by wild-type biotin carboxylase and the two mutant enzymes. The amount of ADP was also measured to determine whether there was a stoichiometric formation of ADP and carboxybiotin. The ratio of the micromoles of carboxybiotin formed to the micromoles of ADP formed for the wild type, C230A, and K238Q was 1.00, 1.07, and 0, respectively. The wild-type enzyme and the C230A mutant both demonstrated a 1:1 stoichiometry for the production of ADP and carboxybiotin. Thus, the C230A mutation had no effect on the ability of biotin carboxylase to produce carboxybiotin. Although the K238Q mutant was able to hydrolyze ATP, it did not produce a measurable quantity of carboxybiotin. This suggested the production of carboxybiotin had been uncoupled from ATP hydrolysis by this mutation. Alternatively, biotin may not have been able to bind to the K238Q mutant enzyme. To distinguish between these two possibilities, another model reaction catalyzed by biotin carboxylase was examined.

ATP Synthesis Reaction. Biotin carboxylase from *E. coli* will catalyze an ATP synthesis reaction (reaction 4). In this

$$\text{MgADP} + \text{carbamoyl-P} \rightarrow \text{MgATP} + CO_2 + NH_3 \quad (4)$$

reaction, a phosphate group is transferred from carbamoyl phosphate to ADP, forming ATP and carbamate, which rapidly decomposes into carbon dioxide and ammonia. This reaction is believed to represent the reverse reaction, with carbamoyl phosphate acting as a substrate analogue of the putative carboxyphosphate intermediate (*16*). The rate of this reaction is increased in the presence of biotin, which does not participate in the chemistry of the reaction but is thought to activate the enzyme via a conformational change (*16*). Thus, the degree of activation of the reaction by biotin can be used to assess whether biotin binds to the K238Q mutant enzyme. First, however, the effect of the two mutations on the Michaelis constants for carbamoyl phosphate and ADP and the k_{cat} of the reaction in the absence of biotin were determined (Table 2). The mutations did not have a significant effect on either the k_{cat} of the reaction or the binding of carbamoyl phosphate. In addition, only a moderate effect on the K_m of ADP was observed. It is important to note that biotin carboxylase will catalyze the ATP synthesis reaction in the absence of biotin. Though it has been previously reported that biotin is absolutely required for ATP synthesis activity (*16*), our results have shown this is not correct. Biotin simply stimulates the ATP synthesis activity.

To evaluate the level of stimulation of this reaction by biotin for wild-type biotin carboxylase and the two mutants, initial velocities were measured at a saturating concentration of ADP and carbamoyl phosphate, both in the absence of biotin and in the presence of 64 mM biotin. The degree of stimulation by biotin in the C230A mutant was similar to that observed in the wild-type enzyme, a 22.8 ± 0.9-fold and 19.8 ± 2.0-fold increase, respectively. This is consistent with the fact that the C230A mutation did not significantly affect the Michaelis constant for biotin. The rate of ATP synthesis catalyzed by K238Q was stimulated 1.50 ± 0.03-fold. Although this degree of stimulation by biotin was small compared to that of the wild type, it suggested biotin was able to bind to the K238Q mutant enzyme.

Table 3: Solvent Deuterium Isotope Effects[a]

	biotin-dependent ATPase		HCO_3^--dependent ATPase	
	$^D(k_{cat})$	$^D(k_{cat}/K_m)$	$^D(k_{cat})$	$^D(k_{cat}/K_m)$
WT	0.63 ± 0.08	0.45 ± 0.10	0.86 ± 0.10	0.21 ± 0.09
C230A	1.07 ± 0.12	1.15 ± 0.40	1.19 ± 0.04	0.32 ± 0.04
K238Q	*b*	*b*	1.05 ± 0.03	0.22 ± 0.07

[a] Solvent isotope effects were determined in 80% D_2O. For the biotin-dependent ATPase reaction, biotin was varied for the wild-type enzyme at saturating levels of ATP (3 mM) and bicarbonate (15 mM), while for the C230A mutant ATP was held constant at 20 mM and bicarbonate was saturating at 15 mM. For the bicarbonate-dependent ATPase reaction, ATP was varied at a saturating concentration of bicarbonate (15 mM). The error was calculated by standard propagation of the errors from the k_{cat} and k_{cat}/K_m. [b] The concentration of biotin did not have an effect on the rate of ATP hydrolysis by the K238Q mutant.

Solvent Deuterium Isotope Effects. While the site-directed mutagenesis studies were a direct test of whether Cys230 and Lys238 were involved in removing the proton from the 1′ nitrogen of biotin, it was of interest to see what effect these two mutations had on the solvent isotope effect and chemical inactivation by *N*-ethylmaleimide observed in the wild-type enzyme. The inverse solvent isotope effect and chemical inactivation by *N*-ethylmaleimide for wild-type biotin carboxylase were two of the principal pieces of evidence suggesting a cysteine was involved in the catalytic mechanism (*1*, *2*).

Biotin carboxylase from *E. coli* exhibits an inverse solvent isotope effect on both the k_{cat} and k_{cat}/K_m parameters (*1*). Since thiol groups have inverse fractionation factors, Tipton and Cleland proposed a catalytic mechanism involving a cysteine residue undergoing proton exchange (*1*). We have confirmed there is an inverse kinetic solvent isotope effect on the k_{cat} and k_{cat}/K_m parameters of the biotin-dependent ATPase reaction for the wild-type enzyme (Table 3) and have extended the analysis by measuring the solvent isotope effect on the bicarbonate-dependent ATPase reaction. If the origin of the inverse solvent isotope effect was due solely to a cysteine residue removing the proton from the 1′ nitrogen of biotin, then an inverse solvent isotope effect would not be observed in the bicarbonate-dependent ATPase reaction because biotin is not involved in this reaction. However, as shown in Table 3, an inverse solvent isotope effect on both k_{cat} and k_{cat}/K_m was observed for the bicarbonate-dependent ATPase reaction for wild-type biotin carboxylase. Thus, the solvent isotope effect remained inverse in a reaction that did not involve biotin. Since the bicarbonate-dependent ATPase reaction is the first part of the entire reaction involving biotin, the data suggest the source of the inverse solvent isotope effect in the biotin-dependent ATPase reaction may solely or in part be due to the inverse kinetic solvent isotope effect

of the bicarbonate-dependent ATPase reaction.[2] Thus, an inverse kinetic solvent isotope effect should not be used to infer a role for cysteine in the deprotonation of biotin.

Since studies of enzyme-catalyzed reactions performed in D_2O result in protic exchanges at many sites on both the enzyme and substrate, solvent isotope effects are often difficult to interpret. Thus, the cause of the inverse solvent isotope effect observed for biotin carboxylase is not readily apparent. Proton exchange involving a thiol group is not the only explanation for inverse solvent isotope effects. For some enzymes, including thermolysin (*17*) and carbonic anhydrases (*18*), inverse solvent isotope effects have been attributed to metal ions. In addition, it has been suggested low-barrier hydrogen bonds might also result in isotope effects of less than 1 (*19*). At this time it is difficult to suggest a cause for the solvent isotope effects observed in biotin carboxylase, but it is unlikely they arise from a proton exchange involving the 1′ nitrogen of biotin.

Table 3 also shows the kinetic solvent isotope effects for both mutants of biotin carboxylase. In the biotin-dependent ATPase reaction, the C230A mutant had, within error, a kinetic solvent isotope effect of unity on both kinetic parameters. This change in solvent isotope effect brought about by the C230A mutation suggests that Cys230 may be responsible for the inverse kinetic solvent isotope effect. However, an alternative interpretation consistent with all of the data is the mutation caused a change in the rate-determining step of the reaction, so that the isotope-sensitive step is no longer rate-limiting. For the bicarbonate-dependent ATPase reaction, neither mutation had an effect on the kinetic solvent isotope effect on the k_{cat}/K_m parameter. In contrast, the kinetic solvent isotope effect on the k_{cat} for the bicarbonate-dependent ATPase reaction was normal for both C230A and K238Q. A change in the isotope effect on the k_{cat} but not on the k_{cat}/K_m suggests both mutations have resulted in a change in the ratio to catalysis.[3] This means that the rate of a non-isotope-sensitive step that occurs after the first irreversible step (e.g., product release) has become rate-limiting.

Inactivation by NEM. NEM is often used to inactivate enzymes presumably by reacting with the thiol group of cysteine residues. Tipton and Cleland found that *E. coli* biotin carboxylase is inactivated by NEM, and the inactivation is blocked by the presence of all three substrates (*2*). The inactivation of biotin carboxylase by NEM was one of the principal pieces of evidence implicating a cysteine in the active site of biotin carboxylase. The crystal structure of biotin carboxylase revealed Cys230 as the only cysteine in the active site of the enzyme (*6*), which suggested this residue as the site of NEM modification. This hypothesis was tested by determining the rate of inactivation by NEM for both the C230A and K238Q mutants. If NEM is reacting with Cys230, then the C230A mutant should show little to no inactivation, whereas the K238Q mutant should be inactivated by NEM. The rate of inactivation by NEM of wild-type biotin carboxylase and the two mutants, C230A and K238Q, is shown in Figure 2. The C230A mutant was inactivated by NEM at a rate comparable to that of the wild-type enzyme, 0.045 ± 0.002 and 0.060 ± 0.004 min^{-1}, respectively. Surprisingly, the K238Q mutant was not rapidly inactivated by NEM. There was a slow loss of activity observed for the K238Q mutant, which could be attributed to the precipitation of the enzyme that occurred in the presence of NEM and not by active-site modification. These results suggest that NEM may be reacting with Lys238 and not Cys230. Even though NEM reacts preferentially with cysteine residues, it has been shown to react with lysine as well (*22*). Moreover, modification of Lys238 by NEM and not Cys230 is consistent with the observation that the K238Q mutation results in a dramatic decrease in enzyme activity, whereas mutation of Cys230 affects biotin carboxylase activity only slightly.

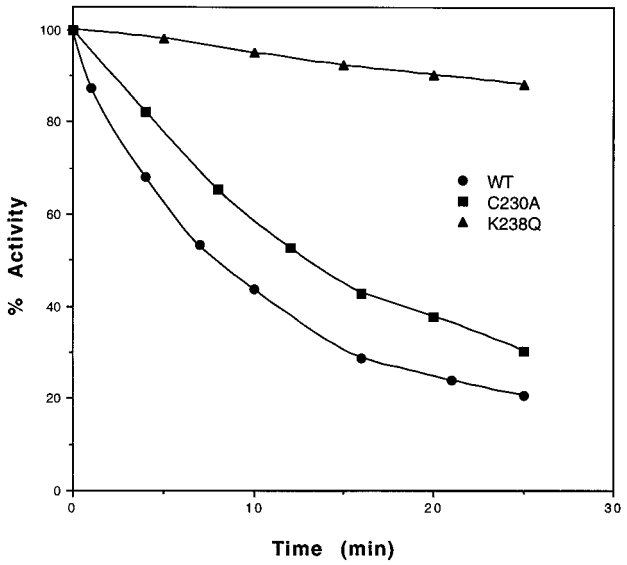


FIGURE 2: Inactivation by NEM for wild-type biotin carboxylase (WT) and both mutants of biotin carboxylase, C230A and K238Q. Each enzyme was incubated with 6 mM NEM at pH 7.0. Aliquots were removed at regular time intervals and assayed for remaining activity. The residual activity is expressed as the percent activity compared to the activity at time zero.

The modification of Lys238 by NEM provides a means to measure the p*K* of Lys238 by determining the pH dependence of the rate of inactivation. The pH dependence of the rate of inactivation of biotin carboxylase is shown in Figure 3. It was not possible to measure the rate of inactivation higher than pH 9.5 because of the loss of enzyme activity. Because a complete pH profile could not be obtained, the p*K* value can only be estimated to be 9.4 or higher. This high p*K* value suggests Lys238 is protonated at physiological pH, and carries a positive charge at the onset of the enzymatic reaction.

DISCUSSION

The objective of this study is to determine whether Cys230 and Lys238 act in concert to remove the proton from the 1′ nitrogen of biotin. The results of the bicarbonate-dependent

[2] This conclusion is valid if the reaction of bicarbonate and ATP is rate-limiting for the overall catalysis. To the extent that the isotope effects for the bicarbonate-dependent ATPase reaction and the overall reaction with biotin were very similar, the simplest explanation is that the reaction of bicarbonate and ATP to form carboxyphosphate is rate-limiting for the overall catalysis. If this were not the case, then one would have to postulate a more complicated mechanism involving two different proton transfers that both happen to give the same isotope effect.

[3] The ratio to catalysis is the sum of the ratios of the rate constant for the isotope-sensitive step to the net rate constants for each of the other forward steps (*20*, *21*).

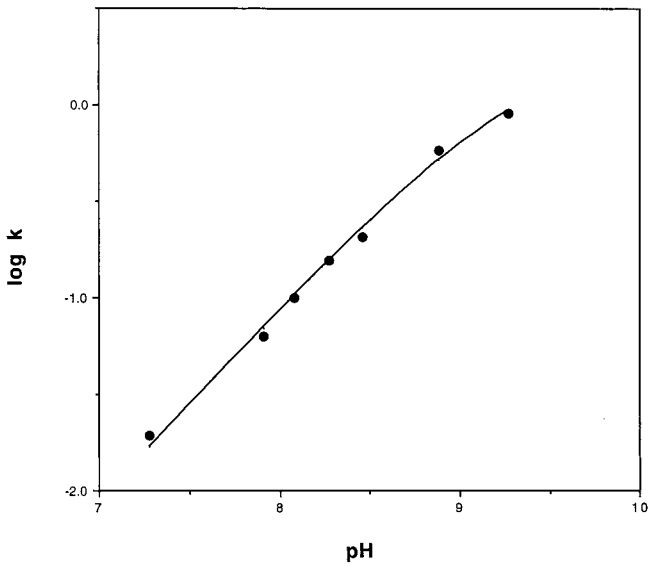


FIGURE 3: Inactivation of wild-type biotin carboxylase by NEM as a function of pH. The rate of inactivation was determined by incubating biotin carboxylase with 70 μM NEM at increasing pH values. The data points represent the experimentally determined inactivation rates, and the line is the best fit of the data to the log form of eq 1.

ATPase assay are consistent with the proposed roles of Cys230 and Lys238 in the catalytic mechanism of biotin carboxylase. The fact that neither mutation affected the k_{cat} of the bicarbonate-dependent ATP hydrolysis assay suggests that neither Cys230 nor Lys238 is essential for formation of the carboxyphosphate intermediate. Biotin does not participate in the bicarbonate-dependent ATP reaction. Therefore, residues that are supposed to interact with biotin would not be essential to the bicarbonate-dependent ATPase activity. However, the increase in K_m values for ATP for both C230A and K238Q compared to that of the wild type suggests these residues are involved in binding ATP, either directly or indirectly. In fact, Artymiuk et al. postulated that Lys238 interacted with ATP on the basis of a comparison of the three-dimensional structures of biotin carboxylase and D-Ala:D-Ala ligase (*23*). Biotin carboxylase and D-Ala:D-Ala ligase were found to have very similar three-dimensional structures despite having very little sequence homology. The common feature between biotin carboxylase and D-Ala:D-Ala ligase is they catalyze an ATP-dependent ligation of a carboxyl group to a nitrogen atom via an acyl phosphate intermediate. The crystal structure of D-Ala:D-Ala ligase contained a substrate analog bound at the active site that allowed the interactions between the analog and protein to be determined. The D-Ala:D-Ala ligase structure suggested the side chain amine of the residue equivalent to Lys238 in D-Ala:D-Ala ligase, Lys215, was bound to the γ-phosphate of ATP (*24*). This is consistent with affinity labeling of biotin carboxylase with adenosine diphosphopyridoxal, which also suggested Lys238 interacts with the γ-phosphate of ATP (*15*).

While the results of the bicarbonate-dependent ATPase assay are consistent with the proposed roles of Cys230 and Lys238, the results of the biotin-dependent ATPase assay argue strongly against Cys230 and Lys238 acting in concert to remove the proton from the 1′ nitrogen of biotin. The data from the biotin-dependent ATPase assay suggest that Cys230 in not an essential residue in the catalytic mechanism of biotin carboxylase because the k_{cat} of this reaction is not drastically affected by the C230A mutation. Also, the lack of a significant change in the K_m for biotin for the C230A mutant suggests Cys230 does not have any interaction with biotin. Most importantly, if Cys230 acted as a catalytic base to remove the 1′ nitrogen of biotin to allow for carboxylation, then very little carboxybiotin should be formed compared to ATP hydrolyzed. A 1:1 ratio for the production of ADP and carboxybiotin clearly showed that the C230A mutation does not diminish the ability of biotin carboxylase to carboxylate biotin. Thus, it is very unlikely Cys230 acts as a catalytic base to remove the proton from the 1′ nitrogen of biotin.

In contrast to the C230A mutation, the K238Q mutation had a significant effect on the ability of biotin carboxylase to produce carboxybiotin. While the K238Q mutant retained the ability to hydrolyze ATP in the presence of bicarbonate at a rate equivalent to that of the wild type (Table 1), there was no increase in the rate of ATP hydrolysis in the presence of biotin, as well as no detectable formation of carboxybiotin. Stimulation of the ATP synthesis activity by biotin suggested that biotin could bind to the K238Q mutant. However, the small degree of activation of the ATP synthesis reaction in the K238Q mutant may indicate the affinity of biotin for biotin carboxylase has been significantly decreased by the K238Q mutation. All of these results strongly suggest that Lys238 plays a critical role in carboxyl transfer from carboxyphosphate to biotin. This leads to the question of whether Lys238 might directly abstract the N1′ proton of biotin, but the pH dependence of the inactivation by NEM suggests this residue has a pK of 9.4 or greater, which makes it an unlikely candidate for a catalytic base. Consequently, all of the data taken as a whole indicate that Cys230 and Lys238 do not act as a pair of acid−base catalysts to remove the proton from the 1′ nitrogen of biotin.

Although Lys238 may not be a catalytic base, the data show that it does play an important role in the transfer of the carboxyl group from carboxyphosphate to biotin. Any postulated role for Lys238 in the catalytic mechanism must be consistent with the following observations: (1) Lys238 interacts with the γ-phosphate of ATP, (2) Lys238 has an interaction with biotin, and (3) Lys238 carries a positive charge. A possible role for Lys238 in the catalytic mechanism of biotin carboxylase stems from the work of Shi and Walsh (*25*). They mutated the lysine residue equivalent to Lys238 in D-Ala:D-Ala ligase, Lys215, and concluded that the protonated ϵ-amino group interacted with the γ-phosphate of ATP and helped to orient the γ-phosphate for transfer to D-alanine. In an analogous manner for biotin carboxylase, Lys238 interacts with the γ-phosphate of ATP and in turn the phosphate group of carboxyphosphate, either through an electrostatic interaction or via hydrogen-bonding, and helps to orient the carboxyphosphate intermediate for carboxyl transfer to biotin. Concurrently, the positively charged lysine residue could interact with the carbonyl oxygen of biotin, again through either a hydrogen bond or an electrostatic interaction. This would promote the tautomerization of the biotin molecule to the imidate form, which is more nucleophilic than the ureido form (*26*).

Finally, since neither Cys230 nor Lys238 acts as a catalytic base to remove the proton from the 1′ nitrogen of biotin, the question remains as to how the proton from the 1′

nitrogen of biotin is removed. Perhaps an active-site amino acid is not involved at all. An alternative hypothesis begins with the observation that transfer of the carboxyl group from carboxyphosphate to biotin requires one of the phosphate oxygens of carboxyphosphate to be protonated. Thus, one of the phosphate oxygens of carboxyphosphate may act as a catalytic base to abstract the proton from the 1′ nitrogen of biotin. This is a very economical mechanism, and similar substrate-assisted catalytic mechanisms have been proposed for aspartate transcarbamylase (*27*) and the GTPase activity of the protein ras (*28*).

REFERENCES

1. Tipton, P. A., and Cleland, W. W. (1988) *Biochemistry 27*, 4325−4331.
2. Tipton, P. A., and Cleland, W. W. (1988) *Biochemistry 27*, 4317−4325.
3. Werneburg, B. G., and Ash, D. E. (1993) *Arch. Biochem. Biophys. 303*, 214−221.
4. Attwood, P. V., Tipton, P. A., and Cleland, W. W. (1986) *Biochemistry 25*, 8197−8205.
5. Attwood, P. V. (1995) *Int. J. Biochem. Cell Biol. 27*, 231−249.
6. Waldrop, G. L., Rayment, I., and Holden, H. M. (1994) *Biochemistry 33*, 10249−10256.
7. Jitrapakdee, S., Booker, G. W., Cassady, A. I., and Wallace, J. C. (1996) *Biochem. J. 316*, 631−637.
8. Kondo, H., Shiratsuchi, K., Yoshimoto, T., Masuda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M., and Tanabe, T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9730−9733.
9. Moss, J., and Lane, M. D. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 35*, 321−442.
10. Blanchard, C. Z., Lee, Y. M., Frantom, P. A., and Waldrop, G. L. (1999) *Biochemistry 38*, 3393−3400.
11. Glasoe, P. K., and Long, F. A. (1960) *J. Phys. Chem. 64*, 188.
12. Climent, I., and Rubio, V. (1986) *Arch. Biochem. Biophys. 251*, 465−470.
13. Ogita, T., and Knowles, J. R. (1988) *Biochemistry 27*, 8028−8033.
14. Asada, K. (1982) in *Biological Carboxylations in Organic and Bio-organic Chemistry of Carbon Dioxide* (Inoue, S., and Yamazaki, N., Eds.) John Wiley & Sons, New York.
15. Kazuta, Y., Tokunaga, E., Aramaki, E., and Kondo, H. (1998) *FEBS Lett. 427*, 377−380.
16. Polakis, S. E., Guchhait, R. B., Zwergel, E. E., Lane, M. D., and Cooper, T. G. (1974) *J. Biol. Chem. 249*, 6657−6667.
17. Stein, R. L. (1988) *J. Am. Chem. Soc. 110*, 7907−7908.
18. Kassebaum, J. W., and Silverman, D. N. (1989) *J. Am. Chem. Soc. 111*, 2691−2696.
19. Cleland, W. W. (1992) *Biochemistry 31*, 317−319.
20. Northrop, D. B. (1982) *Methods Enzymol. 87*, 607−625.
21. Cook, P. F., and Cleland, W. W. (1981) *Biochemistry 20*, 1790−1796.
22. Brewer, C. F., and Riehm, J. P. (1967) *Anal. Biochem. 18*, 248−255.
23. Artymiuk, P. J., Poirrette, A. R., Rice, D. W., and Willett, P. (1996) *Nat. Struct. Biol. 3*, 128−132.
24. Fan, C., Moews, P. C., Walsh, C. T., and Knox, J. R. (1994) *Science 266*, 439−443.
25. Shi, Y., and Walsh, C. T. (1995) *Biochemistry 34*, 2768−2776.
26. Hegarty, A. F., Bruice, T. C., and Benkovic, S. J. (1969) *J. Chem. Soc., Chem. Commun*. 1173−1174.
27. Gouaux, J. E., Krause, K. L., and Lipscomb, W. N. (1987) *Biochem. Biophys. Res. Commun. 142*, 893−897.
28. Schweins, T., Geyer, H., Scheffzek, K., Warshel, A., Kalbitzer, H. R., and Wittinghofer, A. (1995) *Nat. Struct. Biol. 2*, 36−44.

BI992662A