ATP-Dependent Enolization of Acetone by Acetone Carboxylase from *Rhodobacter capsulatus*†

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ABSTRACT: Acetone carboxylase catalyzes the carboxylation of acetone to acetoacetate with concomitant hydrolysis of ATP to AMP and two inorganic phosphates. The biochemical, molecular, and genetic properties of acetone carboxylase suggest it represents a fundamentally new class of carboxylase. As the initial step in catalysis, an α-proton from an inherently basic ($pK_a = 20$) methyl group is abstracted to generate the requisite carbanion for attack on CO_2. In the present study α-proton abstraction from acetone has been investigated by using gas chromatography/mass spectrometry to follow proton−deuteron exchange between D_6-acetone and water. Acetone carboxylase-catalyzed proton−deuteron exchange was dependent upon the presence of ATP, Mg^{2+}, and a monovalent cation (K^+, Rb^+, NH_4^+), and produced mixtures of isotopomers, ranging from singly exchanged H_1D_5- to fully exchanged H_6-acetone. The initial rate of isotopic exchange was higher than k_{cat} for acetone carboxylation. The time course of isotopic exchange showed that multiple exchange events occur for each acetone-binding event, and there was a 1:1 stoichiometric relationship between molecules of ATP hydrolyzed and the sum of new acetone isotopomers formed. ADP rather than AMP was formed as the predominant product of ATP hydrolysis during isotopic exchange. The stimulation of $H^{+-}D^+$ exchange and ATP hydrolysis by K^+ followed saturation kinetics, with apparent K_m values of 13.6 and 14.2 mM for the two activities, respectively. The rate of H^+ exchange into D_6-acetone was greater than the rate of D^+ exchange into H_6-acetone. There was an observable solvent (H_2O vs D_2O) isotope effect (1.7) for acetone carboxylation but no discernible substrate (H_6- vs D_6-acetone) isotope effect. It is proposed that α-proton abstraction from acetone occurs in concert with transfer of the γ-phosphoryl group of ATP to the carbonyl oxygen, generating phosphoenol acetone as the activated nucleophile for attack on CO_2.

Acetone is a toxic molecule that is produced biologically by the spontaneous and enzyme-catalyzed decarboxylation of acetoacetate. Acetone undergoes further metabolic transformations in microbes and higher organisms (*1*−*3*), and a variety of diverse bacteria have been found to grow using acetone as a source of carbon and energy (*4*−*9*). Studies of acetone-utilizing bacteria have shown that the pathway of acetone metabolism involves carboxylation of acetone to acetoacetate, which is subsequently converted into acetyl-CoA[1] for entry into central metabolism (*2*).

Acetone carboxylases (EC 6.4.1.6) have been purified and characterized from *Xanthobacter autotrophicus* and *Rhodobacter capsulatus*, two bacteria capable of growth with acetone (*10*, *11*). The biochemical, molecular, and genetic characterizations of these enzymes reveal that they are essentially identical, unique from all other organic carboxylases that have been characterized to date (*10*−*12*). Both enzymes are expressed at very high levels (∼25% of soluble cell protein) in acetone-grown cells, have $\alpha_2\beta_2\gamma_2$ quaternary structures, are encoded by operons consisting of the genes *acxABC*, share 70−84% identity for the individual subunits, and catalyze the ATP-dependent carboxylation of acetone with the unprecedented production of AMP and inorganic phosphate according to eq 1.

$$CH_3COCH_3 + CO_2 + ATP + 2H_2O \rightarrow CH_3COCH_2COO^- + H^+ + AMP + 2P_i \quad (1)$$

Biotin is not associated with purified acetone carboxylase and is not a required cofactor for the enzyme (*10*, *11*). Interestingly, recent studies have shown that manganese is an essential, stoichiometric ($2Mn^{2+}/\alpha_2\beta_2\gamma_2$ oligomer), and tightly bound cofactor for acetone carboxylase (*12*). EPR studies of acetone carboxylase indicated that two mononuclear manganese(II) sites are present in each $\alpha_2\beta_2\gamma_2$ oligomer, and that manganese is involved in nucleotide binding and activation during catalysis (*12*).

As the initial step in catalysis, all organic substrate carboxylations require the formation of an anionic species (carbanion) for attack on electrophilic CO_2 or an activated CO_2 species (e.g., carboxyphosphate), and various strategies have been developed for carbanion stabilization (*13*). Classic

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[1] Abbreviations: CoA, coenzyme A; EC, enzyme classification; EPR, electron paramagnetic resonance; β-HBDH, β-hydroxybutyrate dehydrogenase; GC/MS, gas chromatography/mass spectrometry; IPTG, isopropyl thiogalactoside; LB, Lucia−Bertini; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PEP, phosphoenol pyruvate; PLP, pyridoxal phosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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carboxylase substrates such as acetyl-CoA, pyruvate, and propionyl-CoA contain an electrophilic carbonyl α to the C−H bond to be cleaved, allowing stabilization of the carbanion via keto−enolate tautomerization (*13*). Acetone shares this structural feature with one noticeable difference: there is no additional electron-withdrawing group on symmetric acetone to increase the acidity of protons α to the carbonyl group. Thus, a central question concerning acetone carboxylation is how the requisite carbanion (and associated enolate) is formed for this simplest of ketones, for which the methyl protons have a very high pK_a value of 20 (*14*). It is possible that acetone carboxylase employs a unique strategy for carbanion stabilization, reflected in the unprecedented reaction stoichiometry (eq 1) and unique biochemical properties of the enzyme.

To gain insights into the strategy of acetone carboxylation by acetone carboxylase, we have, in the present work, examined acetone carbanion formation in the absence of CO_2. To accomplish this, we relied on the volatility of acetone to develop a gas chromatography/mass spectrometry (GC/MS) assay that allows for the analysis of total isotopic exchange catalyzed by acetone carboxylase, as well as the composition of individual isotopomers formed from isotopic exchange. The results of these studies suggest a role for γ-phosphoryl group transfer from ATP in generation and stabilization of the acetone carbanion (enolate). Additional kinetic isotopic studies of acetone carboxylation are presented and, together with the isotopic exchange studies, are used to formulate a proposed mechanism for acetone carboxylation involving phosphoenol acetone as an intermediate.

EXPERIMENTAL PROCEDURES

Materials. D_6-acetone (99.5 atom % D), D_2O (99.9 atom % D), and nucleoside phosphates were purchased from Sigma-Aldrich Chemicals. H_6-acetone (HPLC grade), potassium acetate (enzyme grade), and $MgCl_2$ (ACS grade) were purchased from Fisher Scientific. MOPS buffer (ULTROL grade, free acid) was purchased from Calbiochem. All other chemicals were of the highest purity available.

Growth of Bacteria and Enzyme Purification. Growth of *R. capsulatus* strain B10 and purification of acetone carboxylase were performed as described previously (*12*). The growth medium for expression of acetone carboxylase contained 50 μM $MnCl_2$, which is the optimal concentration for stoichiometric incorporation of manganese(II) into the enzyme (*12*).

GC/MS Analysis of Isotopic Exchange. Assays for measuring isotopic exchange were conducted in 9 mL serum vials crimp sealed with butyl rubber stoppers. The reaction mixtures contained, unless otherwise noted, a 1 mL total volume of 50 mM MOPS buffer, pH 7.6, 11 mM $MgCl_2$, 80 mM potassium acetate, 10 mM ATP, 4 mM H_6- or D_6-acetone, and acetone carboxylase (0.2−1 mg of protein). The assay components were first prepared as fresh concentrated stock solutions (1 M each except ATP, which was 0.5 M) in H_2O, which were then added to either H_2O or D_2O to the desired final concentrations. ATP stocks were prepared in 100 mM MOPS, pH 7.6. The final concentrations of D_2O in the solutions were calculated on the basis of the volumes of H_2O stocks added to the solutions. Assay vials containing all components except acetone were sealed and repeatedly evacuated and flushed with N_2 gas that had been scrubbed of CO_2 and O_2 by passage over columns containing a copper-based catalyst (for removal of O_2) and Ascarite II (for removal of CO_2). Assays were then initiated by the addition of D_6- or H_6-acetone. Assay vials were shaken at 200 cycles min^{-1} in a 30 °C water bath. At desired time points the vials were transferred to a 75 °C water bath for 7 min to terminate the enzyme reaction and increase the volatility of acetone. As controls, assay vials containing all components except acetone carboxylase were analyzed identically. Samples of 1 mL from the headspace of the vials were then injected into a Shimadzu GC-17A gas chromatograph which contained a Restech XTT-5 capillary column (30 × 0.25 cm). The GC injection port temperature was 120 °C, and the GC oven was programmed to ramp from the initial temperature of 100 °C to 200 °C in 15 min with a helium flow rate of 0.7 mL/min with splitless injection. The gas chromatograph was interfaced with a GCMS-QP500 quadruple mass spectrometer, and the interface temperature between the gas chromatograph and mass spectrometer was 280 °C. The MS scan interval was set at 0.35 s, and the detector was set at 1.44 kV. The mass spectrometer *m*/*z* ratio was set to target the molecular ion of the compound of interest to increase sensitivity. For acetone analysis, the *m*/*z* ratio was set from 55 to 67 or ±3.0 mass units of the possible isotopomers ranging from H_6 to D_6. For analysis of other potential substrates (acetaldehyde, 2-butanone, 2-pentanone, 3-pentanone, DMSO, 2-propanol) the *m*/*z* ratio was set accordingly. Initial data analysis was conducted with the software provided by Shimadzu, MS workstation CLASS-5000, and later with SigmaPlot 9.0. The data provided by the Shimadzu software reported peak intensities relative to that of the most abundant peak, which was labeled 100%. These data were imported into SigmaPlot, and the peak heights were normalized and converted to acetone concentration for each isotopomer. Control samples were prepared identically but without enzyme present. The acetone concentrations for any isotopomers that were present in the control vials were taken as background and subtracted from the experimental data for reporting concentrations.

Gas Chromatographic Assays. Acetone carboxylation assays were performed in 9 mL serum vials containing ATP (10 mM), $MgCl_2$ (11 mM), potassium acetate (80 mM), MOPS buffer (50 mM), pH 7.6, potassium bicarbonate (40 mM), carbon dioxide (10 mM), and acetone carboxylase (0.15−0.7 mg), in a total volume of 1 mL. The assays were initiated by the addition of 4 mM H_6- or D_6-acetone. Assay vials were shaken at 250 cycles min^{-1} in a 30 °C water bath. Acetone carboxylation was monitored by GC analysis as previously described (*15*).

Quantification of Nucleotides by HPLC. Samples (5 μL) were removed from the assay vials used for GC/MS analysis of isotopic exchange just prior to quenching of the assays. The samples were diluted with 45 μL of H_2O, followed by the injection of 25 μL of the samples onto a Phenomenex Synergi 4u Hydro-RP 80A column (250 × 4.6 mm) plumbed to a Shimadzu LC-10AT HPLC system. Nucleotides were resolved isocratically with a mobile phase of 25 mM potassium phosphate buffer, pH 5.8, at a linear flow rate of 2.5 mL/min. Nucleotides were detected by absorbance at 259 nm using a Shimadzu SPD-10A UV−vis detector interfaced to a Shimadzu CR501 Chromatopac integrator. Standard

solutions of ATP, ADP, and AMP were prepared and the concentrations of the solutions determined using the extinction coefficient 15.4 mM^{-1} cm^{-1}. Standard curves generated for nucleotide standards were used to quantify ATP, ADP, and AMP in enzyme assays.

Expression and Purification of Recombinant β-Hydroxybutyrate Dehydrogenase (β-HBDH). The plasmid pKKT5×3-HBDH containing the cloned β-HBDH gene from *Rhodobacter* sp. DSMZ 12077 (*16*) was a gift from Dr. Alfred M. Engel of Roche Diagnostics. *Escherichia coli* JM109 was transformed with the plasmid using standard protocols (*17*). Cells were grown in a 12 L capacity Microferm fermenter at 37 °C on LB medium containing 100 μg/mL ampicillin. Once an optical density (A_{600}) of 1 was obtained, IPTG (1 mM) was added to the fermenter. After a 4 h induction period the cells were concentrated using a tangential flow filtration system (Millipore Corp.) and pelleted by centrifugation. The cell paste was washed once with 50 mM potassium phosphate buffer, pH 7.2, then frozen in liquid nitrogen, and stored at −80 °C.

Purification of Recombinant β-HBDH. The procedure outlined by Krüger et al. (*16*) was followed for purification of recombinant β-HBDH with slight modifications noted below. The frozen cell paste was thawed in 3 volumes of 50 mM potassium phosphate buffer, pH 7.6, and the cells were disrupted by a French press. The disrupted cells were centrifuged at 34000*g* for 1 h at 4 °C. Ammonium sulfate was added to the clarified cell extract to a concentration of 1.5 M. This suspension was then centrifuged at 184000*g* for 30 min, and the supernatant was applied to a 5 × 10 cm column of Phenyl Sepharose (Amersham Biosciences). The column was washed with 3 column volumes of 50 mM phosphate, pH 7.6, containing 1.5 M ammonium sulfate. The column was then developed with a 10 column volume linear gradient from 1.5 to 0 M ammonium sulfate. Fractions containing β-HBDH activity and with purity >95% as analyzed by SDS−PAGE were combined, concentrated by ultrafiltration, and pelleted into liquid nitrogen.

Coupled Spectrophotometric Assay for Acetone Carboxylase Activity. A continuous spectrophotometric assay was developed that couples acetoacetate production by acetone carboxylase to acetoacetate reduction and NADH oxidation by β-HBDH. Assays were conducted in 2 mL anaerobic quartz cuvettes that contained 1 mL of assay components. Buffers, H_2O, D_2O, and stock solutions were made anoxic by sparging or by repeated evacuation and flushing with N_2 on a vacuum manifold, except for potassium bicarbonate solutions, which were prepared by adding anoxic water to a degassed vial containing $KHCO_3$ solid. The stock solutions were the same as those noted for GC/MS assays of isotopic exchange, except that the MOPS buffer stock solution was 2 M rather than 1 M. The concentrations of assay components in the cuvettes were 50 mM MOPS, pH 7.6, 10 mM ATP, 80 mM potassium acetate, 11 mM $MgCl_2$, 0.2 mM NADH, 40 mM $KHCO_3$, and 10 mM CO_2. The bulk solvent was H_2O and/or D_2O, and the relative concentrations of each were determined on the basis of the amounts of H_2O, D_2O, and H_2O-based stock solutions added to the assays. After all of the assay components had been mixed and equilibrated at 30 °C for several minutes, acetone carboxylase (0.04−0.15 mg) and β-HBDH (0.345 mg, yielding 90 units of activity) were added to the cuvettes. The amount of β-HBDH added to the assays was severalfold higher than that required for saturation of rates. To initiate assays, H_6-acetone or D_6-acetone was added to the desired concentration from appropriate stock solutions prepared in H_2O or D_2O. Assays were performed at 30 °C in a Shimadzu UV-2401 spectrophotometer containing a water-jacketed cell holder for temperature control. The change in absorbance at 340 nm over time was correlated with micromoles of β-hydroxybutyrate produced using the extinction coefficient for NADH (6.22 mM^{-1} cm^{-1}).

Data Analysis. Nonlinear and linear regression analyses and curve fitting to first-order rate laws were performed using the software SigmaPlot (v 9.0).

RESULTS

Proton−Deuteron Exchange Catalyzed by Acetone Carboxylase As Visualized by GC/MS. A central question concerning the mechanism of acetone carboxylation is how acetone carboxylase generates and stabilizes the necessary carbanion (enolate) for catalysis, and whether this enolate forms in the absence of CO_2. The classic technique to study carbanion formation is to examine the enzyme-catalyzed exchange of protons between the substrate and solvent in the absence of a second substrate, as illustrated, for example, by the early studies of Rose and co-workers with pyruvate carboxylase (*18*, *19*). Tritium is typically employed for such studies, and scintillation counting is used for quantification of exchange.

In considering studying possible isotopic exchange catalyzed by acetone carboxylase, it occurred to us that GC/MS analysis of proton−deuteron exchange might be a useful tool for this analysis. Acetone is volatile and uncharged, allowing its analysis from the headspace of vials with no need for derivatization or extraction. Since D_6-acetone and D_2O are readily available, the analysis can be conducted with fully labeled substrate or solvent rather than relying on the tracer levels used in radioactive analyses. An additional potential advantage of GC/MS over radioactive analysis is that the composition of individual isotopomers can be determined, allowing the quantification of individual isotopomers formed from D_6-acetone (in H_2O) or H_6-acetone (in D_2O).

For initial analyses, different concentrations of acetone carboxylase were incubated in H_2O with all assay components required for acetone carboxylation (Mg^{2+}, ATP, K^+) and with 4 mM D_6-acetone as the substrate (no CO_2 present). After a 300 min incubation period, acetone was removed from the headspace and analyzed by GC/MS. As shown in Figure 1A, significant exchange of H into D_6-acetone occurred under these conditions when acetone carboxylase was included in the assay mixture. Interestingly, for all concentrations of enzyme examined, a mixture of all of the possible isotopomers was formed, ranging from fully exchanged H_6-acetone (molecular ion peak 58) to singly exchanged H_1D_5-acetone (molecular ion peak 63). The amount of total exchange observed was proportional to the amount of enzyme added to the assay; this is most clearly seen by focusing on the decreasing sizes of the fully deuterated (mass 64) peak and the increasing sizes of the fully protonated (mass 58) peak as the concentration of protein in the assays was increased. Acetone carboxylase-catalyzed deuteron−proton exchange occurred only in the

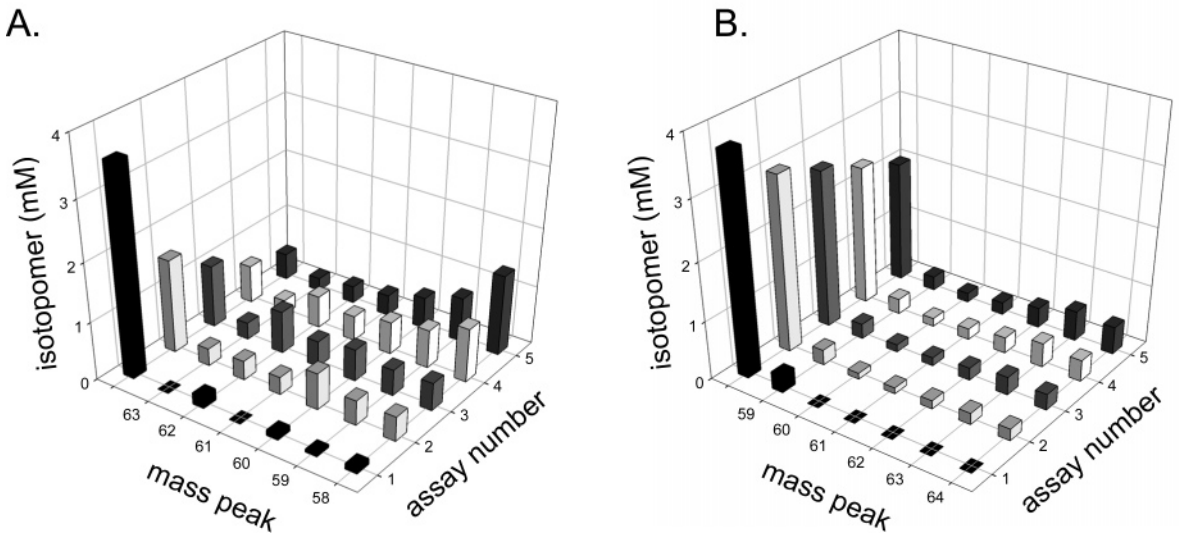


FIGURE 1: GC/MS analysis of proton−deuteron exchange between acetone and solvent catalyzed by acetone carboxylase. Assays were performed as described in the Experimental Procedures using 4 mM D_6- or H_6-acetone as substrate. The chromatograms shown were obtained after a 300 min incubation period. Assay numbers 1−5 were conducted with the following amounts of enzyme present: 1, no enzyme present; 2, 0.25 mg of enzyme; 3, 0.50 mg of enzyme; 4, 1.0 mg of enzyme; 5, 1.5 mg of enzyme. Panel A: D_6-acetone as substrate in H_2O solvent. Panel B: H_6-acetone as substrate in solvent which was ~80% D_2O (v/v).

presence of the cofactors shown previously to be required for acetone carboxylation, i.e., Mg^{2+}, ATP, and K^+. The exclusion of any one of these cofactors from the reaction mixture abolished the stimulation of deuteron−proton exchange (data not shown).

The isotopic exchange assay was repeated under conditions identical to those of Figure 1A, but this time using H_6-acetone as the substrate and conducting the assay in solvent that contained ~80% D_2O, such that deuteron incorporation into H_6-acetone was measured. As shown in Figure 1B, the same general trend for isotopic exchange was observed, with H_6-acetone being converted into a mixture of all possible isotopomers. However, the extent of exchange in these fixed time assays was significantly less when D incorporation into H_6-acetone was followed.

Correlation among Isotopic Exchange, ATP Hydrolysis, and Formation of ATP Hydrolysis Products. As shown in eq 1, the carboxylation of acetone to acetoacetate occurs with the formation of AMP and two inorganic phosphates, an unprecedented reaction stoichiometry for an ATP-dependent carboxylase. Of potential relevance to the present work, acetone carboxylase has been shown to exhibit acetone-dependent ATPase activity in the absence of the second substrate CO_2, but exhibits no ATPase activity with CO_2 alone (*10*, *11*). This acetone-dependent ATPase activity occurs with no discernible change in acetone concentration and produces phosphate at a rate comparable to the rate of phosphate production observed for the complete carboxylation reaction (*10*, *11*). There is, however, a significant difference in the pattern of ATP hydrolysis products seen for the complete assay and with acetone alone: the acetone-dependent ATPase activity results in the production of *both* ADP and AMP as ATP hydrolysis products, with ADP being formed as the major hydrolysis product (*10*, *11*).

It is likely that the acetone-dependent ATPase activity of acetone carboxylase arises from the need to hydrolyze ATP to overcome the thermodynamic barrier for γ-proton abstraction from acetone. If this is the case, there should be a correlation between the rates of ATP hydrolysis and proton−deuteron exchange catalyzed by acetone carboxylase. To investigate this, the time courses of proton−deuteron exchange and ATP hydrolysis were followed simultaneously over a 400 min period (Figure 2A). For quantification of exchange activity, two formulas were used to focus on either the total concentration of new isotopomers formed (eq 2) or the total number of protons exchanged (eq 3). These formulas are presented for the situation where D_6-acetone is used as the substrate and H_2O is the solvent. Comparable equations can be used to quantify exchange activity with H_6-acetone in D_2O solvent.

$$[\text{isotopomers}] = \sum[H_nD_{6-n}\text{-acetone}] \quad (2)$$

$$[\text{deuterons}] = \sum n[H_nD_{6-n}\text{-acetone}] \quad (3)$$

As shown in Figure 2A, ATP hydrolysis occurred concomitant with proton exchange into D_6-acetone. Interestingly, for each time point, the total number of deuterons exchanged was much greater than the amount of ATP hydrolyzed, showing that a single ATP hydrolysis event is not required for each proton−deuteron exchange. However, a 1:1 stoichiometric relationship was seen between the sum of the new isotopomers formed and the amount of ATP hydrolyzed at each time point. The relationship between the number of isotopomers formed and ATP hydrolysis products is presented in Figure 2B. Consistent with the results of the previous studies of acetone-dependent ATPase activity (*10*, *11*), ADP was formed as the predominant product (~80−85%) of ATP hydrolysis during this time course, while AMP was formed as a minor product (15−20%). Importantly, there is no evidence that ATP is directly hydrolyzed to AMP and inorganic pyrophosphate by acetone carboxylase, and the $\alpha-\beta$ and $\beta-\gamma$ phosphodiester bonds are believed to be cleaved sequentially (*10*). Thus, the formation of AMP is

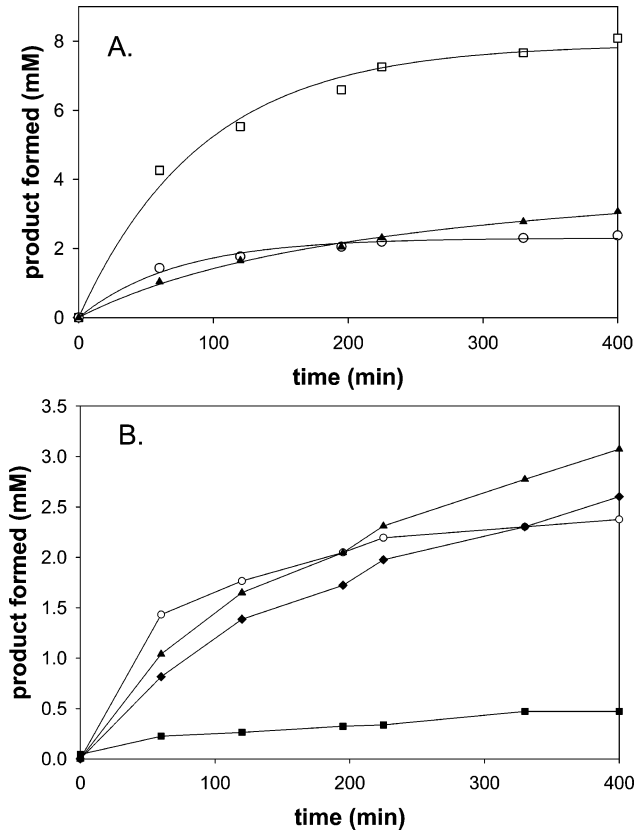


FIGURE 2: Relation between proton−deuteron exchange and ATP hydrolysis. Assays were performed with 0.35 mg of protein and with 4 mM D_6-acetone as substrate in H_2O solvent. Panel A: Relation among total proton exchange, sum of new isotopomers, and ATP hydrolyzed. The curves were fit using the equation for a first-order reaction as described by Rose (*19*). The symbols are as follows: (□) total proton−deuteron exchange, (○) sum of new isotopomers observed, (▲) ATP hydrolyzed. Panel B: Relation between the sum of new isotopomers and ATP hydrolysis products. The symbols are as follows: (○) sum of new isotopomers observed, (▲) ATP hydrolyzed, (◆) ADP formed, (■) AMP formed.

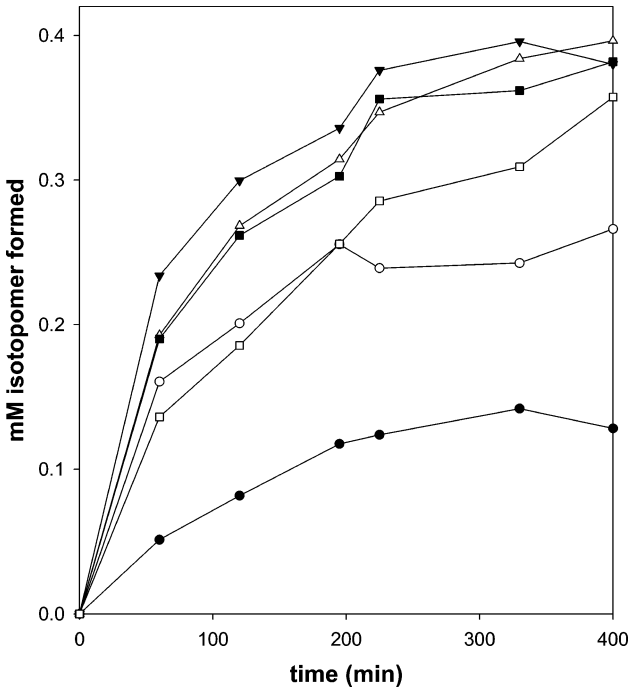


FIGURE 3: Time course of isotopomer formation catalyzed by acetone carboxylase. The data were obtained from additional analysis of the experiment described for Figure 2. The symbols are as follows: (●) mass peak 63, (○) mass peak 62, (▼) mass peak 61, (▽) mass peak 60, (■) mass peak 59, (□) mass peak 58.

indicative of prior formation of ADP. As shown in Figure 2B, the sum of ADP and AMP formed, and thus the number of $\beta-\gamma$ phosphodiester bonds cleaved, correlates reasonably well with the number of isotopomers formed for each time point.

Time Courses of Isotopomer Production from Proton−Deuteron Exchange. The results of Figures 1 and 2 raise the question of how individual isotopomers are formed from proton−deuteron exchange into D_6-acetone. If each proton−deuteron exchange event requires dissociation followed by rebinding of a new molecule of acetone, then mass peak 63 (i.e., H_1D_5-acetone) would be expected to increase first, followed by sequential increases in the intensities of mass peak 62 (H_2D_4-acetone), mass peak 61 (H_3D_3-acetone), and so on, with associated decreases in the intensities of the larger mass peaks. As shown in Figure 3, which is an extension of the experiment described in Figure 2, this result was not seen: mass peak 61, wherein three protons have been exchanged, formed at the highest rate, followed closely by mass peaks 60 and 59. Mass peak 58, the fully exchanged product, was formed at the fourth fastest rate in this experiment. Mass peak 63, the singly exchanged product, actually formed at the slowest rate for this experiment, while mass peak 62 formed at the second slowest rate. Thus, it appears that multiple exchanges can occur for each acetone binding event, with a tendency toward three exchanges per binding event for the experimental conditions of Figure 3. It should be noted that some variability in the rates of formation of individual peaks is seen when this experiment is repeated: in some cases mass peak 63 has been observed to accumulate slightly faster than that of the representative experiment of Figures 2 and 3, while in some cases mass peak 61 forms at a rate slightly lower than, for example, those of mass peaks 59 and 60. These slight variabilities from individual experiments do not change the overall conclusion that multiple exchange events occur preferentially over single exchange events per acetone binding.

Nucleotide Requirement for Proton−Deuteron Exchange. Previous studies have shown that nucleoside triphosphates other than ATP (i.e., GTP, CTP, ITP, XTP, UTP, and TTP) are incapable of supporting acetone carboxylation by acetone carboxylase (*10*, *11*). The nucleotide requirement for proton−deuteron exchange was investigated by examining the same range of nucleoside triphosphates, in addition to nonhydrolyzable ATP analogues. As shown in Table 1, a small amount of exchange activity was observed in the presence of XTP and CTP, while no activity was seen with GTP, TTP, UTP, ITP, or dATP. The nonhydrolyzable ATP analogues AMP−CPP and AMP−PNP did not support proton−deuteron exchange, providing further evidence that ATP hydrolysis is required for exchange activity. Likewise, ADP, pyrophosphate, phosphate, and the combinations of ADP + phosphate and AMP + pyrophosphate did not stimulate exchange. The latter observations are noteworthy in the context of prior studies of pyruvate carboxylase-catalyzed isotopic exchange between solvent and pyruvate (*18*). For pyruvate carboxylase, phosphate and various phosphate analogues were capable of stimulating exchange activity as well as or better than ATP,

Table 1: Nucleotide Specificity of Proton−Deuteron Exchange Catalyzed by Acetone Carboxylase[a]

nucleotide (10 mM)	concn of deuterons exchanged (mM)	sum of concns of new isotopomers (mM)
ATP	8.05 ± 0.38	2.01 ± 0.06
AMP−PNP	ND[b]	ND
AMP−CPP	ND	ND
dATP	ND	ND
ADP + P_i	ND	ND
ADP + PP_i	ND	ND
AMP + P_i	ND	ND
AMP + PP_i	ND	ND
XTP	1.25 ± 0.34	0.31 ± 0.12
CTP	0.97 ± 0.05	0.24 ± 0.01
GTP	ND	ND
TTP	ND	ND
UTP	ND	ND
ITP	ND	ND

[a] Assays contained 0.54 mg of protein and 4 mM D_6-acetone as substrate. Reactions were terminated after 180 min. Results represent the average of three assays with associated standard deviations. [b] ND = no detectable activity above background.

showing that phosphodiester bond hydrolysis is not required for the exchange (*18*). In contrast, the results of Figure 2 and Table 1 show that acetone carboxylase-catalyzed isotopic exchange is obligately dependent on ATP hydrolysis. This is perhaps not surprising, given the 1000-fold higher basicity of acetone protons relative to those on the methyl group of pyruvate (*13*, *14*).

Monovalent Metal Ion Requirement for Acetone Carboxylase-Catalyzed Proton−Deuteron Exchange and ATP Hydrolysis. Monovalent ions are commonly required for or stimulate the activity of ATP-dependent enzymes (*20*, *21*). Potassium ion was shown in previous studies to stimulate acetone carboxylase activity (*10*, *11*). However, the absolute requirement for this ion was not rigorously established, since the source of bicarbonate used in the assays was always the potassium salt. The requirement of K^+ was therefore investigated using the proton−deuteron exchange assay developed in this study. As shown in Figure 4, the extent of proton−deuteron exchange catalyzed by acetone carboxylase was dramatically affected by the concentration of K^+ present in the assay. Each of the three activities measured (total deuterons exchanged, sum of new isotopomers formed, ATP hydrolyzed) increased in a saturable fashion in response to increasing [K^+]. In agreement with the results of Figure 2, there was a direct correlation between the sum of isotopomers formed and the amount of ATP hydrolyzed for each [K^+], while the total number of deuterons exchanged was much higher. The apparent K_m values for K^+ stimulation of the three activities were calculated to be 13.6 ± 1.6, 10.6, and 14.2 ± 2.6 mM for total deuterons exchanged, sum of isotopomers formed, and amount of ATP hydrolyzed, respectively.

In a separate experiment, the monovalent ion specificity of the exchange reaction was investigated. As shown in Table 2, both Rb^+ and NH_4^+ were effective substitutes for K^+ in stimulating proton−deuteron exchange. In contrast, the smaller Li^+ and Na^+ ions were not effective at stimulating exchange activity. These results are in general agreement with those observed for other enzymes (*20*), including the prototype enzyme pyruvate carboxylase (*18*). As seen in Table 1, for each stimulatory monovalent ion, there is good

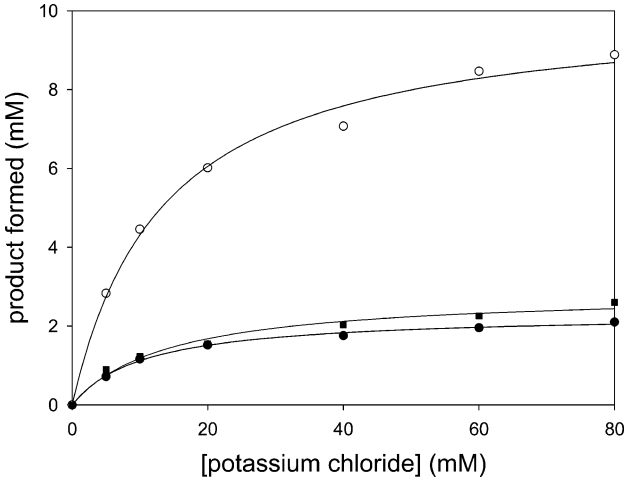


FIGURE 4: Effect of potassium ion concentration on proton−deuteron exchange and ATP hydrolysis rates. Assays were performed with 0.35 mg of protein and with 4 mM D_6-acetone as substrate in H_2O solvent. Assays were terminated after 320 min. The symbols are as follows: (○) total deuterons exchanged, (●) sum of new acetone isotopomers formed, (■) amount of ATP hydrolyzed.

Table 2: Monovalent Metal Ion Stimulation of Proton−Deuteron Exchange and ATP Hydrolysis[a]

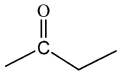
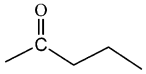
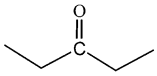
monovalent ion	sum of concns of new acetone isotopomers formed (mM)	concn of deuterons exchanged (mM)	concn of ATP cleaved (mM)
none	0.08 ± 0.14	0.16 ± 0.54	0.00 ± 0.09
Li^+	0.08 ± 0.15	0.29 ± 0.45	0.05 ± 0.19
Na^+	0.00 ± 0.11	0.00 ± 0.38	0.00 ± 0.01
K^+	0.47 ± 0.09	1.54 ± 0.32	0.67 ± 0.12
Rb^+	0.77 ± 0.15	2.72 ± 0.52	0.75 ± 0.14
NH_4^+	0.40 ± 0.09	2.30 ± 0.51	0.74 ± 0.21

[a] Assays were conducted as described in the Experimental Procedures using 0.16 mg of acetone carboxylase. All monovalent ions were chloride salts and were added at a concentration of 80 mM. Assays were initiated with the addition of 4 mM D_6-acetone. Reactions were terminated after 300 min and analyzed by GC/MS. Data represent the average of four reactions with associated standard deviations. Background exchange was not subtracted for this experiment.

agreement between the sum of isotopomers formed and the amount of ATP hydrolyzed in these fixed time assays.

Alternate Substrates in the Proton−Deuteron Exchange Assay. Acetone carboxylase from *X. autotrophicus* has been shown previously to carboxylate 2-butanone, forming 2-ketovalerate as product, at a rate 46% of the rate of acetone carboxylation (*10*). Other ketones, including 2-pentanone, 3-pentanone, and 2-hexanone, were not substrates for *X. autotrophicus* acetone carboxylase, nor was acetaldehyde, pyruvate, or phosphoenol pyruvate (*10*). Since carboxylation of an alternative substrate presumably requires enolization, we asked whether proton−deuteron exchange could be observed with 2-butanone, as well as a range of substrate analogues. The previous study of alternative substrates was conducted with the *X. autotrophicus* acetone carboxylase, while the enzyme used for the present studies is the *R. capsulatus* enzyme. Thus, each of the substrates and analogues tested was assayed for carboxylation activity as well as proton−deuteron exchange activity, in case the enzymes from the two sources exhibit any differences in substrate specificity.

Table 3: Substrate Specificity of Acetone Carboxylase-Catalyzed H^+/D^+ Exchange[a]

Compound	Structure	ATP cleaved (mM)	New isotopomers formed	new isotopomers (mM)	H^+ exchanged (mM)	% carboxylation activity[b]
Acetaldehyde		ND[b]	0	ND[c]	ND[c]	ND[d]
Acetone		2.51	6	2.29	8.78	100
Butanone		1.08	3	1.05	1.56	47
2-pentanone		0.19	2	0.22	0.33	ND[d]
3-pentanone		0.03	1	0.03	0.03	ND[d]
dimethylsulfoxide		ND[b]	0	ND[c]	ND[c]	ND[d]
isopropanol		ND[b]	0	ND[c]	ND[c]	ND[d]

[a] Proton exchange assays contained 0.4 mg of acetone carboxylase, 10 mM ATP, 11 mM $MgCl_2$, 80 mM potassium acetate, and 50 mM MOPS/D_2O, pH 7.6. All assays were initiated by the addition of 4 mM substrate from a freshly prepared stock. Assays were terminated after 320 min. [b] Substrate carboxylation was analyzed by gas chromatography. [c] ND = no activity detected above background.

As shown in Table 3, 2-butanone was a substrate for *R. capsulatus* acetone carboxylase, exhibiting a rate comparable to that reported earlier for the *X. autotrophicus* enzyme, while no detectable activity was observed with 2-pentanone and 3-pentanone. These results agree exactly with the results reported for *X. autotrophicus* acetone carboxylase (*10*). The additional compounds tested were also not transformed at any detectable rate by acetone carboxylase.

To observe possible isotopic exchange with these alternate compounds, assays were conducted with the unlabeled compounds in buffer containing ~80% D_2O; thus, the exchange of deuterons into substrate was measured rather than the exchange of protons into deuterated substrate. As shown in Table 3, proton−deuteron exchange occurred for 2-butanone at a relative rate consistent with the 46% activity observed in the carboxylation assay (Table 1). Low but reproducible exchange activity was also observed for both 2-pentanone (~10% of the acetone exchange rate) and 3-pentanone (~1% of the acetone exchange rate) even though no carboxylation of these compounds could be detected. As for acetone, the number of new isotopomers of 2-butanone and 2-pentone that were formed during these assays agreed well with the amount of ATP hydrolyzed. Interestingly, only three new isotopomers of 2-butanone were observed as products of proton−deuteron exchange, as opposed to six isotopomers of acetone being observed. The masses of the three new isotopomers of 2-butanone were 73, 74, and 75, or those for which one, two, or three protons had been replaced by deuterons. The likely explanation for this result is that only the α-methyl protons of asymmetric 2-butanone are exchanged by acetone carboxylase. This idea is supported by the observation that 2-ketovalerate is the sole product of 2-butanone carboxylation (*10*).

Acetone Enolization Assay Considerations and Determination of Initial Rates. The GC/MS assay for isotopic exchange described above provides a powerful tool for examining total proton−deuteron exchange, determining the identity of multiple isotopomers formed, and correlating exchange with ATP hydrolysis. One limitation of the assay is sensitivity, requiring prolonged incubation times to observe enough exchange for accurate quantification of products. As can be seen from Figures 2 and 3, the assays do not remain linear for the entire assay time periods examined. This can be partially attributed to the nature of the reaction itself, wherein the equilibrium where deuterons in acetone molecules have been fully exchanged by protons is approached in a first-order fashion (*18*). Acetone molecules for which deuterons have been replaced by protons will continue to exchange new protons for protons from solution, resulting in lower observed rates of new isotopomer formation, even if the actual exchange rate remains constant. The apparent uncoupling between ATP hydrolysis and new isotopomer formation seen for time points beyond 200 min in Figure 2 probably arises from this phenomenon. An additional consideration is the extent of ATP hydrolysis which occurs during the course of the assays, which will of necessity shift the equilibrium of the reaction and slow the rate of exchange.

These considerations do not affect the design or interpretation of experiments presented thus far, as they are not intended to provide initial rate data. Still, an estimation of initial rates of isotopic exchange would be useful, as the rate of exchange can then be compared to the overall rate of acetone carboxylation to gain possible information about rate-limiting steps. An examination of Figures 2 and 3, and additional analyses of shorter time courses not shown here, show that isotopic exchange rates are fairly linear for the first 60 min of reaction. Thus, specific activities for isotopic

exchange and ATP hydrolysis were calculated from 60 min fixed time point assays with D_6-acetone as the substrate. The observed specific activities, reported as turnover numbers per $\alpha\beta\gamma$ protomeric unit, were as follows: total proton exchange rate, 51 ± 6 min^{-1}; rate of formation of new isotopomers, 18 ± 2 min^{-1}; rate of ATP hydrolysis, 18 ± 3 min^{-1}.

Continuous Spectrophotometric Assay for Measurement of Acetone Carboxylation Rates and Kinetic Parameters. Previous studies of acetone carboxylation relied on two assays to measure activity: conversion of acetone to acetoacetate by gas chromatography and measurement of phosphate production using a coupled enzyme assay. Both of these assays have limitations for kinetic analyses: the gas chromatographic assay is not very sensitive and is discontinuous, making it ineffective in acetone K_m determination, while phosphate production can be uncoupled from acetone carboxylation, as noted above, complicating analyses where CO_2 concentrations are varied. An ideal assay would rely on measurement of the carboxylation product acetoacetate in real time. This can potentially be achieved by adding β-HBDH and NADH, such that the coupled reduction of acetoacetate to β-hydroxybutyrate can be measured spectrophotometrically by measuring conversion of NADH to NAD^+ as shown in eq 4.

$$\text{acetoacetate} + \text{NADH} + H^+ \rightarrow \beta\text{-hydroxybutyrate} + NAD^+ \quad (4)$$

For this assay to be practical, a large amount of coupling enzyme must be added to ensure that conversion of acetoacetate to β-hydroxybutyrate is not rate-limiting, and the cost of commercially available β-HBDH is prohibitively high. Recently, an overexpression system was described for producing large quantities of highly active and thermally stable β-HBDH (*16*). This expression system was used as the source of β-HBDH for development of the desired coupled assay. Using saturating concentrations of β-HBDH, the observed rates of NADH oxidation using the coupled assay were approximately 85% of the rates observed for direct measurement of acetoacetate by gas chromatography over a range of acetone concentrations.

Using the newly developed coupled enzyme assay, the following kinetic parameters were determined: $k_{cat} = 29 \pm 0.4$ min^{-1} (per $\alpha\beta\gamma$ protomer) and $K_{m,app,acetone} = 29 \pm 3$ μM. As noted above, the total proton exchange rate, indicative of carbanion formation, occurred with an estimated rate of 51 min^{-1}, while the ATP hydrolysis rate observed in the absence of CO_2 occurred at a slower rate of 18 min^{-1}. Thus, the rate of acetone enolization as measured by proton−deuteron exchange is faster than, and thus compatible with, k_{cat} for the overall acetone carboxylation reaction when CO_2 is present.

Investigation of Possible Solvent and Substrate Isotope Effects. An intriguing result of Figure 1 is the difference in extent of proton−deuteron exchange for the combinations D_6-acetone/H_2O (Figure 1A and all subsequent exchange experiments to now) and H_6-acetone/D_2O (Figure 1B). For the experiment in Figure 1, the total number of protons (from H_2O) exchanged into D_6-acetone was, on average, higher than the total number of deuterons (from D_2O) exchanged into H_6-acetone. This difference is greater than can be accounted for by the amount of H_2O present in the D_2O solvent of Figure 1B (20%). The difference in exchange rates suggests the possibility that a solvent isotope effect is present. To investigate this, the effect of [D_2O] on acetone carboxylation rates at nominally saturating concentrations of substrates was determined. As shown in Figure 5, there was a direct relationship between the percentage of H_2O in the assay and the observed rate of acetone carboxylation. The linearity of the data indicates that a single proton-transfer event is responsible for the observed isotope effect (*22*). The ratio $k_H/k_D = 1.7 \pm 0.1$ was determined from linear regression analysis of the data in Figure 5. This observed isotope effect for the carboxylation reaction is in general agreement with the results of Figure 1, which showed that the extent of isotopic exchange in these side by side fixed time point assays was higher for the combination H_2O/D_6-acetone than for D_2O/H_6-acetone.

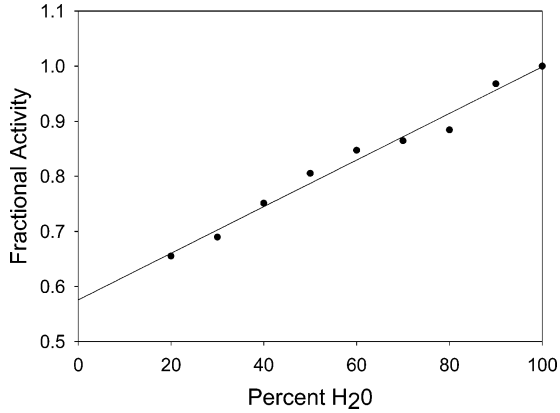


FIGURE 5: Effect of solvent isotope composition on the rate of acetone carboxylation. Buffers with varying concentrations of H_2O and D_2O were prepared as described in the Experimental Procedures. Acetone carboxylation was measured with 0.11 mg of acetone carboxylase and 4 mM H_6-acetone as substrate using the β-hydroxybutyrate dehydrogenase coupled enzyme assay also described in the Experimental Procedures. Rates were derived from the linear portion of the progress curves of A_{340} vs time (typically 30−180 s of reaction). Rates are reported as fractions of the rates observed in solvent, which was 100% H_2O. Data points represent the average of two assays.

The possibility of a substrate isotope exchange was investigated by conducting a kinetic analysis of D_6-acetone vs H_6-acetone carboxylation in H_2O solvent. As shown in Figure 6, there was no discernible substrate isotope effect ($k_H/k_D = 1.0$), and the derived K_m and V_{max} values for H_6- and D_6-acetone were identical within experimental error.

DISCUSSION

As noted in the introduction, acetone carboxylase is somewhat enigmatic among the ATP-dependent carboxylases: it does not require biotin, does not share sequence identity with other carboxylases, and forms AMP and inorganic phosphate as ATP hydrolysis products. Acetone carboxylation is thermodynamically unfavorable ($\Delta G^{\circ\prime} =$ 17.1 kJ/mol), and the reverse reaction, acetoacetate decarboxylation, occurs spontaneously in aqueous solution. Interestingly, one of the first enzymes to be studied extensively mechanistically is acetoacetate decarboxylase from *Clostridium acetobutylicum*, which catalyzes the decarboxylation of acetoacetate to acetone and CO_2 (*23*, *24*). Acetoacetate decarboxylase does not require exogenous cofactors, is a

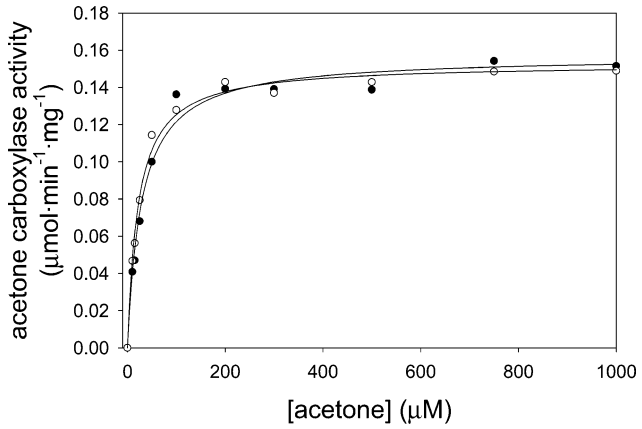


FIGURE 6: Comparison of H_6- and D_6-acetone as substrates for acetone carboxylase. Acetone carboxylation was measured with 0.047 mg of acetone carboxylase and various concentrations of H_6- or D_6-acetone as substrate using the β-hydroxybutyrate dehydrogenase coupled enzyme assay described in the Experimental Procedures. Data points shown are the average of three assays. The curve fits were obtained by nonlinear regression analysis of the data using SigmaPlot. The symbols are as follows: (○) D_6-acetone, (●) H_6-acetone.

multimer of identical 29000 M_r subunits, and forms a Schiff base adduct between acetoacetate and the ϵ-amino group of an active site lysine residue to facilitate C−C bond cleavage (*25*, *26*). Acetone carboxylase does not share sequence homology with acetoacetate decarboxylase, does not form a Schiff base during catalysis, and obligately requires ATP for catalysis (*10*, *11*). Despite the thermodynamic favorability of acetoacetate decarboxylation, we have not been able to observe this activity with acetone carboxylase under any conditions (data not shown). Likewise, acetoacetate decarboxylase does not catalyze acetone carboxylation under any circumstances. Thus, the pathways and strategies for acetone carboxylation and acetoacetate decarboxylation are fundamentally different.

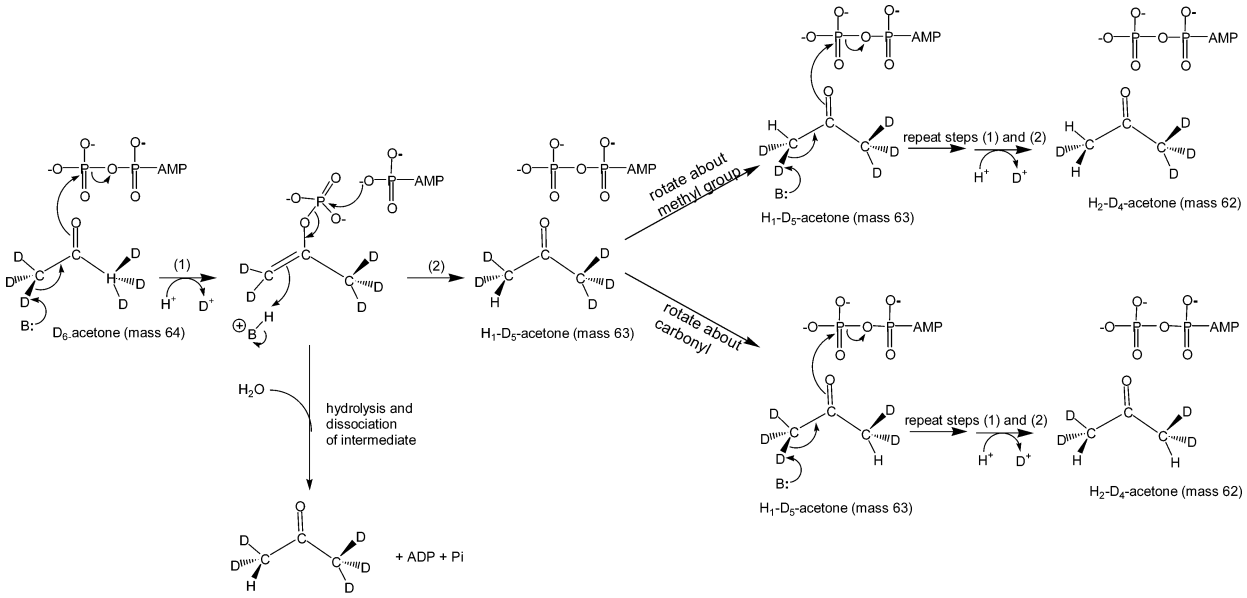
A central question regarding acetone carboxylase is how the enzyme forms and stabilizes the necessary carbanion required for nucleophilic attack on CO_2 in the C−C bond forming step, and whether this carbanion forms in the absence of the second substrate CO_2. In the absence of a Schiff base adduct, the only strategy available for acetone carbanion stabilization is keto−enol tautomerization. The results presented in this paper clearly show that acetone carboxylase is capable of forming the acetone carbanion. The fact that isotopic exchange occurs at a rate faster than k_{cat} for acetone carboxylation is important. For a concerted reaction, α-H^+ abstraction would occur at a high rate only in the presence of CO_2, and isotopic exchange between α-CH and solvent would not occur, or would occur at a very low rate. The fact that acetone carboxylase catalyzes α-H^+ abstraction in the absence of CO_2 at a rate consistent with the overall rate of reaction provides strong evidence that the carbanion, and hence enol acetone, is an intermediate in the reaction pathway. Indeed, Irwin Rose has stated that exchange between α-CH and water in the absence of the second substrate can be construed as direct evidence for an enol intermediate in catalysis (*19*).

In the absence of a cationic imine, metal ion catalysis can provide a second strategy to stabilize an enolate formed from α-H^+ abstraction (*27*). It is thus noteworthy to point out that acetone carboxylase contains tightly bound manganese(II) ion, in addition to requiring Mg^{2+} and K^+ for catalysis (*12*). It is likely that one or more of these metals are involved in enol acetone stabilization. The low acidity of the α-CH of acetone suggests, however, that additional factors are required for α-H^+ abstraction and carbanion stabilization. The results presented in this paper indicate that ATP hydrolysis provides the additional driving force for this process, and provide insights into how this is accomplished. Three observations are important in this context. First, the amount of ATP hydrolysis that occurs is correlated with the sum of new acetone isotopomers formed rather than with the total number of protons exchanged (Figure 2A). Second, isotopic exchange occurs with the production of ADP rather than AMP as the primary hydrolysis product (Figure 2B), in stark contrast to the carboxylation stoichiometry, where AMP is the only nucleotide hydrolysis product (eq 1). Third, isotopomers that have been subject to multiple exchanges form more readily than singly exchanged acetone (Figure 3). These results indicate that the ATP-dependent binding and activation of a single acetone molecule is sufficient to allow multiple proton exchanges into the bound acetone, and that hydrolysis of the $\beta-\gamma$ but not the $\alpha-\beta$ phosphodiester bond is required for exchange.

Scheme 1 provides a model that accounts for these observations. According to this model, α-H^+ abstraction from acetone occurs in concert with γ-phosphoryl group transfer from ATP to acetone, thereby stabilizing enol acetone as phosphoenol acetone and forming enzyme-bound ADP. In the absence of the electrophile CO_2, phosphoenol acetone can either hydrolyze and dissociate from the active site, in which case acetone with a singly exchanged proton will be formed, or reabstract a proton from solution in concert with re-formation of ATP (Scheme 1). Precedents for re-formation of ATP from an unstable phosphorylated intermediate exist as in, for example, carbamoyl-phosphate synthetase (proposed carboxyphosphate intermediate) and glutamine synthetase (proposed γ-glutamyl phosphate intermediate) (*28*). Repeating this cycle several times successively, without phosphoenol acetone hydrolysis, could result in three proton−deuteron exchange events on the methyl group oriented for general-base abstraction of a proton (assuming free rotation about this methyl group). If bound acetone is also able to rotate about the carbonyl carbon, complete exchange of deuterons for protons could conceivably occur for each acetone binding event (Scheme 1).

The proposed phosphoenol acetone intermediate is structurally similar to PEP, which is the substrate for PEP carboxylase. While PEP is a relatively stable molecule in aqueous solution, the proposed phosphoenol acetone is unstable, and therefore cannot be directly trapped or visualized. In PEP carboxylase, the phosphoryl group of PEP is transferred to bicarbonate from carboxyphosphate and enol pyruvate (*13*, *29*). Enol pyruvate then serves as the electrophile to attack CO_2 formed from carboxyphosphate decomposition (*13*, *29*). With this precedent in mind, the question is raised as to how phosphoenol acetone, if indeed it is formed, reacts with CO_2 (or bicarbonate). At present, it is unclear whether CO_2 or bicarbonate is the substrate for acetone carboxylase. An analysis of the active species is complicated by the very slow turnover of the enzyme (29 min^{-1}), the rapid equilibration of CO_2 and bicarbonate in

Scheme 1



aqueous solution, and the relative insensitivity of the GC assay used previously for measuring activity. The coupled assay described herein provides a more sensitive measurement of activity, and preliminary studies comparing CO_2 vs HCO_3^- as substrate over short time courses indicate that HCO_3^- is the enzyme substrate, although this needs to be pursued in more detail (data not shown). Importantly, acetone carboxylation requires the additional hydrolysis of the $\alpha-\beta$ phosphoryl bond of ADP (eq 1). Perhaps carboxyphosphate is formed from $\alpha-\beta$ phosphoryl group transfer from the bound ADP shown in Scheme 1. Once formed, carboxyphosphate would provide the necessary activated CO_2 species for C−C bond formation, and the reaction would be completed by concerted hydrolysis of phosphoenol acetone to generate enol acetone for attack on CO_2. While speculative, this scenario is consistent with the observation that ADP rather than AMP is the predominant product of CO_2-independent proton−deuteron exchange in acetone, while no ADP is seen for the complete carboxylation reaction. It should be noted that acetone carboxylase exhibits no ATPase or ADPase activity in the presence of CO_2 alone. Thus, if phosphoanhydride bond hydrolysis is required for activation of bicarbonate, this event occurs only when acetone is also present.

Interestingly, XTP and CTP supported proton−deuteron exchange rates between 10% and 15% of those observed with ATP (Table 1), but neither XTP nor CTP was capable of supporting acetone carboxylation. A possible explanation for this observation is that these nucleotides can provide the phosphoryl group for phosphoenol acetone production, but not for the subsequent phosphoryl group transfer required for carboxylation.

Returning to acetoacetate decarboxylase, it should be noted that this enzyme is also capable of catalyzing the exchange of protons into high concentrations (500 mM) of D_6-acetone, via the intermediacy of the Schiff base adduct discussed above (*30*). This result demonstrates that the Schiff base adduct of acetone is sufficiently stable to allow proton−deuteron exchange, but not sufficiently reactive for C−C bond formation with CO_2. This is, perhaps, not surprising, since Schiff base adducts, including those that form with PLP, are observed for decarboxylation reactions but not for carboxylations (*27*).

The abstraction of an α-H^+ from acetone might be expected to be a rate-limiting step in acetone carboxylation, but the lack of a substrate isotope effect (Figure 6) shows that this is not the case. The presence of a significant solvent isotope effect (Figure 5) suggests instead that H^+ abstraction from water is partially rate limiting (*31*). A likely origin for this effect is the need to generate hydroxide ion for attack on one or more of the high-energy phosphate compounds that are used (ATP, ADP) or possibly formed (phosphoenolacetone, carboxyphosphate?) during catalysis.

In summary, the mechanistic studies presented in this paper reinforce the previous biochemical, spectroscopic, and genetic studies suggesting that acetone carboxylase employs a novel strategy for substrate carboxylation. The isotopic exchange studies presented herein have allowed the formulation of a working model for the initial step in acetone carboxylation, wherein phosphoanhydride bond cleavage is required for α-H^+ abstraction and stabilization of an activated enol acetone intermediate (Scheme 1). While these studies do not prove the intermediacy of phosphoenol acetone in the reaction, they do provide a framework for the design of additional experiments to test the proposed mechanism.

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