Mycobacterium tuberculosis Ketopantoate Hydroxymethyltransferase: Tetrahydrofolate-Independent Hydroxymethyltransferase and Enolization Reactions with α-Keto Acids†

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ABSTRACT: The *panB* gene that encodes ketopantoate hydroxymethyltransferase has been cloned from *Mycobacterium tuberculosis*, expressed, and purified to homogeneity. ^{1}H NMR spectroscopy was used to determine the rate of (i) tetrahydrofolate-independent hydroxymethyltransferase chemistry between formaldehyde and α-ketoisovalerate and (ii) deuterium exchange in the methylenetetrahydrofolate-independent enolization of α-ketoisovalerate and other α-keto acids, catalyzed by PanB. These studies have demonstrated that substrate enolization by PanB is divalent metal-dependent with a preference of $Mg^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+} > Ca^{2+}$. The rate of enolization is pH-dependent with optimal activity in the range of 7.0−7.5. The pH profile was bell-shaped, depending on the ionization state of two ionizable groups with apparent p*K* values of 6.2 and 8.3. Enolization and isotope exchange occurs with some α-keto acids (e.g., pyruvate and α-ketobutyrate), resulting in the complete exchange of all *β*-hydrogens. Enzyme-catalyzed enolization and isotope exchange occur with other long-chain and branched α-keto acids, resulting in the stereospecific exchange of only one of the *β*-hydrogen atoms. These results are discussed in the context of steric restrictions present in the enzyme active site and the stereochemistry of base-catalyzed isotope exchange.

Pantothenate is a key precursor for the biosynthesis of two essential cofactors, coenzyme A (CoA) and acyl carrier protein (ACP). Although pantothenate is obtained in the diet of mammals, microorganisms and plants must synthesize it by the pathway (*1*−*3*) shown in Figure 1. The first unique step in pantothenate biosynthesis is the formation of ketopantoate from N_5,N_{10}-methylenetetrahydrofolate and α-ketoisovalerate, the direct precursor of L-valine. This reaction is catalyzed by the product of the *panB* gene encoding ketopantoate hydroxymethyltransferase (PanB),[1] which has been functionally identified in *Escherichia coli* (*4*−*6*), *Aspergillus nidulans* (*7*), and *Salmonella typhimurium* (*8*). The product of the *panE* gene, ketopantoate reductase, reduces ketopantoate to form hydroxypantoate, and the final step in pantothenate biosynthesis is catalyzed by the *panC*-encoded pantothenate synthetase, which catalyzes the ATP-dependent condensation of hydroxypantoate and *β*-alanine (generated by the *panD*-encoded aspartate 1-decarboxylase) to form pantothenate. Previous reports have suggested that either PanC (*9*) or PanD (*10*) catalyzes the rate-limiting step in pantothenate synthesis. Recently, however, Rubio et al. have shown that a mutation in the promotor of the *panB* gene, which results in the overexpression of PanB, caused an increase in pantothenate and coenzyme A synthesis in *Salmonella enterica* (*11*). These studies suggest that PanB is, in fact, the rate-limiting reaction in pantothenate biosynthesis and that the hydroxymethyltransferase might be an attractive target for inhibitor design.

There are few well-characterized examples of hydroxymethyltransferases, with serine hydroxymethyltransferase being the most thoroughly studied (*12*−*16*). This enzyme contains PLP and, after transimination with L-serine, catalyzes the cleavage of the Cα−C*β* bond to generate formaldehyde and glycine. In the presence of THF, the formaldehyde condenses with the THF to generate N_5,N_{10}-methylenetetrahydrofolate. This C1 fragment is then used by thymidylate synthase in the synthesis of DNA precursors, as well as in other metabolic pathways, including pantothenate biosynthesis. There are few published reports on ketopantoate hydroxymethyltransferase, and early studies of the *E. coli* enzyme used only partially purified preparations (*4*) and discontinuous assays (*5*).

Although no chemical mechanism has been proposed for this reaction, inspection of the substrate of PanB, α-ketoisovalerate, suggests that the ketopantoate hydroxymethyltransferase may catalyze the reaction via an initial *β*-proton abstraction of the activated C−H bond and formation of a resonance-stabilized carbanion of α-ketoisovalerate, followed by direct attack by the enolate on the methylene group of N_5,N_{10}-methylenetetrahydrofolate, resulting in the formation of ketopantoate.

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[1] Abbreviations: PanB, ketopantoate hydroxymethyltransferase; DHF, dihydrofolate; THF, tetrahydrofolate; IPTG, isopropyl 1-thio-*β*-galactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEA, triethanolamine; NMR, nuclear magnetic resonance.

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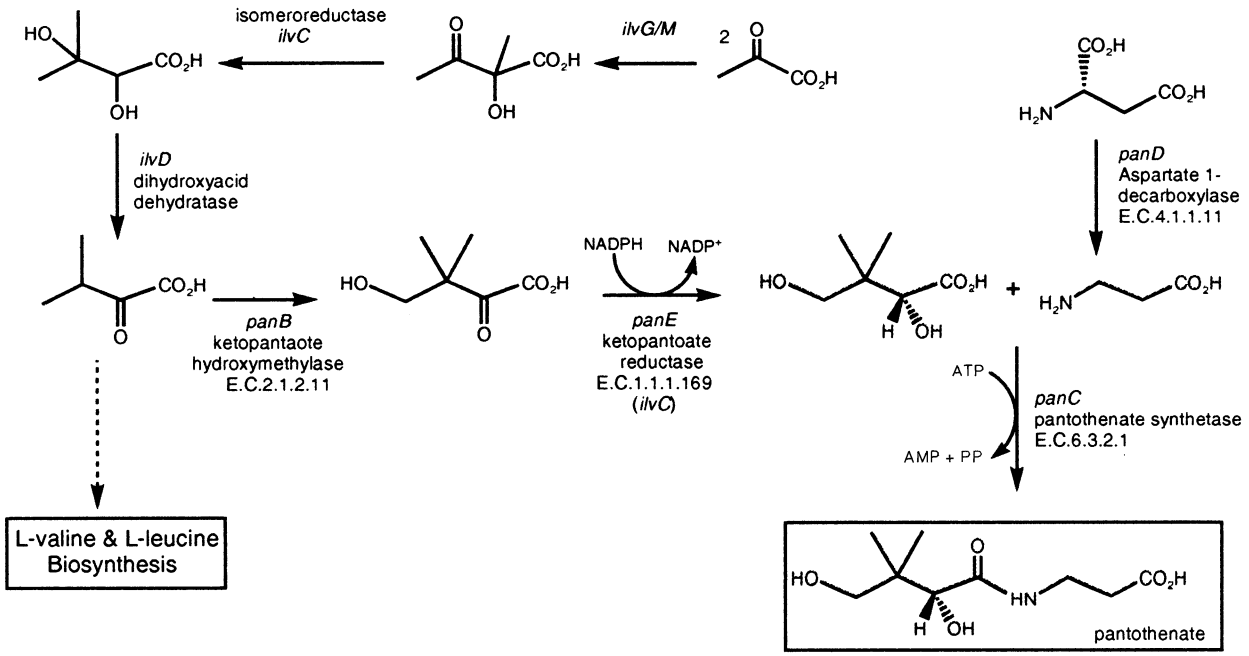

FIGURE 1: Pantothenate biosynthesis in bacteria, plants, and yeast.

In this report, we describe the cloning, expression, and purification of the *Mycobacterium tuberculosis* ketopantoate hydroxymethyltransferase. ^{1}H NMR spectroscopy has been used to examine α-keto acid enolization by following the rate of deuterium exchange of the β-protons of various α-keto acids in the methylenetetrahydrofolate-independent reactions of α-keto acids with PanB. On the basis of these observations, we propose a mechanism for this metalloenzyme and define the stereochemistry of the enzyme-catalyzed enolization.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotide primers used for PCR amplification were synthesized by Life Technologies (Gaithersberg, MD); *Nde*I and *Eco*RI restriction enzymes were purchased from New England Biolabs (Beverly, MA); pET23a(+) vector DNA and *E. coli* BL21(DE3) cells were from Novagen (Madison, WI). *M. tuberculosis* H37Rv genomic DNA was a generous gift from W. R. Jacobs (AECOM). α-Keto acids, dihydrofolate, tetrahydrofolate, formaldehyde, L-glutamic acid, pyridoxal 5′-phosphate monohydrate (PLP), ninhydrin, and *E. coli* branched-chain amino acid transaminase (BCAT) were purchased from Sigma (St. Louis, MO). Deuterium oxide, acetic acid-d_4, and Tris-d_{11} were purchased from Cambridge Isotope Laboratories (Andover, MA).

Cloning and Expression of the M. tuberculosis Ketopantoate Hydroxymethyltransferase Gene (panB). An N-terminal primer contained a *Nde*I restriction site overlapping the initiation codon and 22 downstream base pairs of the panB coding sequence: 5′-ATTCCATATGTCTGAGCAGA-CTATCTATG-3′. The C-terminal primer contained an *Eco*RI restriction site, the termination codon, and 19 upstream base pairs of the panB anti-codon sequence: 5′-CGGATTTCT-CAGAAACTGTGTTCGTCAGC-3′. These primers were used to amplify the *panB* gene from genomic *M. tuberculosis* DNA using standard PCR protocols. The PCR products were separated on a 1% low-melting agarose gel, digested with *Nde*I and *Eco*RI, and ligated into a previously digested pET23a(+) expression vector. The recombinant plasmid was used to directly transform competent *E. coli* BL21(DE3) cells. The transformed cells were grown at 37 °C in Luria−Bertani medium containing 50 μg/mL carbenicillin and grown to an OD_{600} of approximately 0.7. IPTG was added to a final concentration of 1 mM to induce transcription of the T7 polymerase, and the cells were allowed to grow an additional 4 h before harvesting by centrifugation at 5000 rpm. Analysis of crude extracts by SDS−PAGE stained with Coomassie blue demonstrated large amounts of a soluble 29 kDa protein.

Overexpression and Purification of M. tuberculosis PanB. Six liters of Luria−Bertani medium containing 50 μg/mL carbenicillin was inoculated with 1 mL of stationary phase *E. coli* BL21(DE3)/pET-23a(+)/*panB* culture. Fermentation at 37 °C was continued to an OD_{600} of approximately 0.5 and was followed by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation after 4 h, and the cell paste was resuspended in 40 mM triethanolamine (TEA), pH 7.8, prior to storage at −70 °C. All subsequent steps were performed at 4 °C. Frozen cells were thawed and lysed by sonication after the addition of 10 mg of lysozyme and one protease inhibitor tablet (Complete protease inhibitor cocktail tablet, Boehringer Mannheim). The crude extract was obtained by centrifugation at 19000 rpm for 1 h. Nucleic acids were precipitated by the addition of streptomycin sulfate (1% w/v final) to the supernatant. After being stirred for 30 min the supernatant was pelleted at 15000 rpm for 45 min. The resulting supernatant was dialyzed against 20 mM TEA (pH 7.8) for 2 h. The precipitate was removed by centrifugation for 30 min at 19 000 rpm. The clear supernatant was loaded onto a 400 mL Q-Sepharose Fast Flow anion-exchange column equilibrated in 25 mM TEA (pH 7.8) and eluted at 2 mL/min with a 1500 mL 0−1 M linear NaCl gradient. Fractions were analyzed by SDS−PAGE, and those containing PanB were pooled and concentrated (YM-10, Amicon) to 10 mL.

An equal volume of 2 M ammonium sulfate was added to this sample, and the resultant solution was stirred for 30 min before being pelleted at 15 000 rpm for 30 min. The 1 M $(NH_4)_2SO_4$ supernatant was applied to a 2.4 cm × 16 cm phenyl-Sepharose column equilibrated in 1 M ammonium sulfate/20 mM TEA (pH 7.8). The protein was eluted at 2 mL/min with a 1−0 M linear $(NH_4)_2SO_4$ gradient. Fractions that exhibited a single band on SDS−PAGE with Coomassie blue staining were pooled.

Measurement of Ketopantoate Hydroxymethyltransferase (PanB) Activity. Ketopantoate hydroxymethyltransferase activity was assayed spectrophotometrically by coupling the PanB-catalyzed formation of ketopantoate, from α-ketoisovalerate and N_5,N_{10}-methylenetetrahydrofolate, to ketopantoate reductase (PanE, Figure 1). The decrease in absorbance of NADPH at 340 nm ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) was measured at 37 °C using a UVIKON XL spectrophotometer with a circulating water bath and thermospacers. Assays were performed in 50 mM Hepes (pH 7.5) containing 4 mM $MgCl_2$, 100 μM NADPH, 4 μg of PanE, 24 μg of PanB, and varying concentrations of α-ketoisovalerate and N_5,N_{10}-methylenetetrahydrofolate in a total volume of 1 mL. N_5,N_{10}-Methylenetetrahydrofolate was prepared by mixing 25 mM formaldehyde and 25 mM tetrahydrofolate in the assay buffer and incubating for 5 min at 37 °C. Initial velocity kinetic data were analyzed by Lineweaver−Burk analysis and fitted to the equation

$$v = VAB/(K_{iA}K_B + K_AB + K_BA + AB) \quad (1)$$

where V is the maximal velocity, A and B are the concentrations of the substrates, K_A and K_B are the Michaelis constants for substrates A and B, and K_{iA} is the inhibition constant for substrate A.

Ketopantoate Formation from Formaldehyde and α-Ketoisovalerate. Solutions for the NMR studies contained 100 mM Tris-d_{11} (pH 7.0), 50 mM α-ketoisovalerate, 50 mM formaldehyde, and 4 mM $MgCl_2$ in a total volume of 1 mL. Assays were performed with and without 200 μM dihydrofolate and were initiated by the addition of 100 μg of PanB. Proton NMR spectra were recorded and rates calculated as described below.

Enolization of α-Ketoisovalerate by ^{1}H NMR Spectroscopy of Hydrogen Exchange Rates. Enolization rates were determined by monitoring the exchange of the β-hydrogen of α-ketoisovalerate in D_2O. Solutions for the NMR enolization studies contained 100 mM Tris-d_{11} (pH 7.0), 50 mM α-ketoisovalerate, and 4 mM $MgCl_2$ in a total volume of 1 mL. Assays were initiated by the addition of 30 μg of PanB. Proton NMR spectra were recorded at 26 °C at 300 MHz with a Bruker WM-300 spectrometer. Spectra were acquired every 5 min, and kinetic data were obtained by calculating the integrated intensity of the remaining β-hydrogen compared to the integrated intensity of the original β-methyl hydrogen peak. The exchange rates were determined by analyzing the integrated intensity versus time and fitting the data to eq 2, using a nonlinear least squares routine in Sigma Plot:

$$y = A_i \exp(-k_{ex}t) \quad (2)$$

where A_i is the initial fraction of hydrogens ($i = 1$), t is time, and k_{ex} is the rate of exchange. The $k_{enolization}$ of α-ketoisovalerate was determined using the equation:

$$k_{enol} = (k_{ex}\ min^{-1})([S_i])/[E] \quad (3)$$

where k_{ex} is the rate of exchange, $[S_i]$ is the initial substrate concentration, and [E] is the enzyme concentration. This method was used for other α-keto acid substrates examined.

Stereochemistry of the C3 Hydrogen Abstraction and Deuterium Exchange of α-Ketoisocaproate. The enolization reaction contained 10 mM Tris-d_{11} (pH 7.0), 10 mM α-ketoisocaproate, and 4 mM $MgCl_2$ in a total volume of 1 mL. Assays were initiated by the addition of 500 μg of PanB. The transamination reaction contained the 1 mL enolization reaction, 10 mM Tris-d_{11} (pH 7.0), 10 mM glutamic acid, 10 μM pyridoxal 5′-phosphate, and 2 nmol of *E. coli* branched-chain amino acid transaminase (BCAT) in a total volume of 5 mL. The reaction was incubated at 37 °C, and the production of L-leucine was followed by thin-layer chromatography [solvent: 96% ethanol−34% ammonium hydroxide (70:30)] and visualized with ninhydrin. The resulting solution was lyophilized to dryness and taken up in a 5% NaOD/D_2O solution for NMR analysis.

pH Profiles. The enolization rates of α-ketoisovalerate were measured over the pH range of 5.5−9.5 using a Tris-d_{11}/acetate-d_4 buffer system. Assays were performed at 26 °C in 100 mM Tris-d_{11}/acetate-d_4 buffer containing 4 mM $MgCl_2$ and 50 mM α-ketoisovalerate. The reactions were initiated by the addition of enzyme. The pH of each sample was determined after data acquisition using an Accumet model 20 pH meter equipped with a microelectrode. The rates of enolization at each pH were calculated as described above and fitted to the equation:

$$\log k_{enolization} = \log[C/(1 + H/K_a + K_b/H)] \quad (4)$$

where C is the pH-independent plateau value, K_a is the ionization constant for the acidic group, K_b is the ionization constant for the basic group, and H is the hydrogen ion concentration.

Effects of Metal Ions on the Rate of Enolization. The effects of divalent cations (Mg^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, and Ni^{2+}), as their Cl^- salts, on the enolization rate of α-ketoisovalerate were determined by ^{1}H NMR spectroscopy as described above. Reaction mixtures contained 50 mM α-ketoisovalerate and 0.2−10 mM metal ion in 100 mM Tris-d_{11}/acetate-d_4 (pH 7.0). The reaction was initiated by the addition of ketopantoate hydroxymethyltransferase. The rate of hydrogen exchange was calculated as described above, and the data were fitted to the equation

$$k_{enol} = k_{ex}[S]/K_m + [S] \quad (5)$$

where k_{ex} is the rate of hydrogen exchange, [S] is the substrate concentration, and K_m is the Michaelis constant for the metal.

RESULTS

Purification and Properties of the Recombinant Ketopantoate Hydroxymethyltransferase. The *M. tuberculosis* ketopantoate hydroxymethyltransferase was heterologously overexpressed by cloning the corresponding gene into the

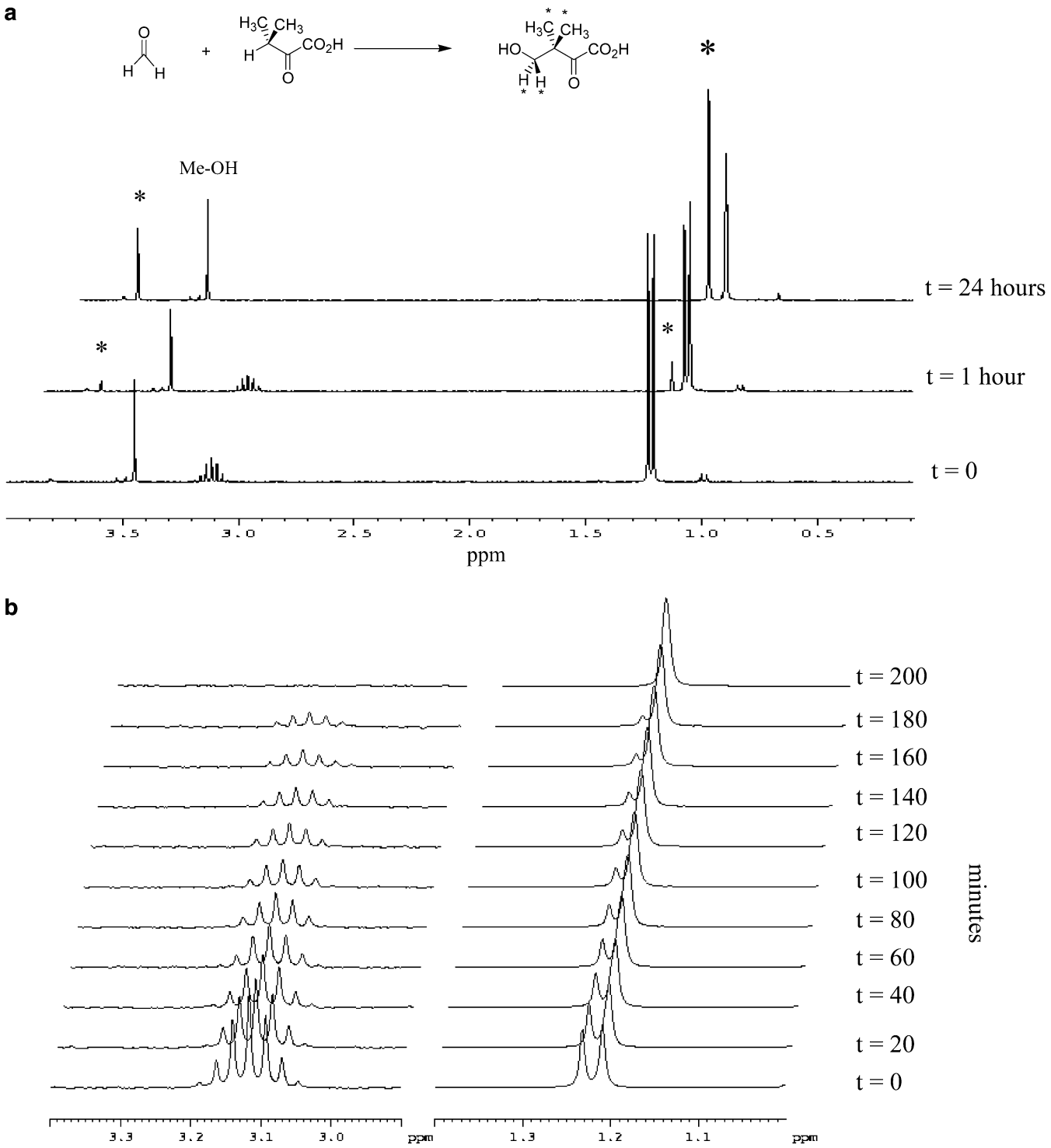

FIGURE 2: (a) 300 MHz ^{1}H NMR spectrum of the PanB-catalyzed methylenetetrahydrofolate-independent formation of ketopantoate. Ketopantoate equivalent methyl groups and C4 hydrogens are labeled with an asterisk at 1.24 and 3.75 ppm, respectively. (b) 300 MHz ^{1}H NMR spectra of the PanB-catalyzed enolization of α-ketoisovalerate. Reactions were run in Tris-d_{11}/acetate-d_4 (pH 7.0) at 26 °C.

pET23a(+) expression vector. After induction with IPTG, *E. coli* BL21(DE3) cells containing this vector expressed a soluble protein with the expected monomer mass as determined by SDS−PAGE. The protein was purified to homogeneity with a final yield of 150 mg from 12 g of *E. coli* cells, using consecutive Fast Flow Q-Sepherose anion-exchange column chromatography and hydrophobic interaction chromatography on a phenyl-Sepharose column. The subunit molecular mass of ketopantoate hydroxymethyltransferase as determined by SDS−PAGE is ca. 29 kDa, consistent with the molecular mass of 29366 Da calculated from the amino acid sequence of the *M. tuberculosis* enzyme. The native molecular mass was estimated at 120 kDa using a Sephacryl S-200 HR gel filtration column, calibrated with Bio-Rad molecular weight markers, suggesting that the native enzyme exits as a tetramer. The N-terminal amino acid sequence determined, SEQTIYGANT, both confirms the expression and purification of the correct gene product and is consistent with the predicted amino acid sequence lacking the N-terminal methionine residue. Analysis of purified PanB

by electrospray ionization/mass spectrometry demonstrated that the enzyme has a subunit molecular mass of 29202 Da, compared to the expected molecular mass calculated from the amino acid sequence minus the initiating methionine residue (29205 Da).

Determination of Ketopantoate Hydroxymethyltransferase (PanB) Activity. Ketopantoate hydroxymethyltransferase activity was determined spectrophotometrically by following the decrease in absorbance of NADPH at 340 nm ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) by coupling the PanB-catalyzed formation of ketopantoate to ketopantoate reduction, catalyzed by ketopantoate reductase. Initial velocities, determined with varying concentrations of α-ketoisovalerate and N_5,N_{10}-methylenetetrahydrofolate, were linear and proportional to the amount of added enzyme (data not shown). The intersecting lines revealed in double reciprocal plots of the initial velocity using either α-ketoisovalerate or N_5,N_{10}-methylenetetrahydrofolate suggest a sequential kinetic mechanism for ketopantoate hydroxymethyltransferase (data not shown). The data were fitted to eq 1, yielding K_m values of 240 ± 40 μM for α-ketoisovalerate and 820 ± 240 μM for N_5,N_{10}-methylenetetrahydrofolate, with k_{cat} equal to 47 ± 6 min^{-1}.

Methylenetetrahydrofolate-Independent Formation of Ketopantoate. Ketopantoate hydroxymethyltransferase (PanB) is able to utilize free formaldehyde and α-ketoisovalerate to form ketopantoate in a methylenetetrahydrofolate-independent reaction (Figure 2a) as revealed by ^{1}H NMR. The α-ketoisovalerate resonances corresponding to the two equivalent methyl groups can be seen as a doublet at 1.24 ppm, and the β-proton was observed as a septet centered at 3.12 ppm. Ketopantoate formation can be seen as the disappearance of these signals and the corresponding appearance of a singlet at 1.40 ppm, corresponding to the methyl groups of ketopantoate and a singlet centered at 3.75 ppm corresponding to the C4 hydrogens of ketopantoate. Enzyme catalyzed enolization, and deuterium exchange of α-ketoisovalerate causes the disappearance of the β-proton septet and the collapse of the methyl group doublet to a singlet. These experiments were also performed in the presence of 200 μM dihydrofolate. The k_{obs} for the reaction was calculated to be 135 min^{-1} for the cofactor-independent reaction and 239 min^{-1} in the presence of dihydrofolate. Of the other α-keto acids tested, only α-keto-β-methylvalerate was a substrate. We estimate the conversion to product to be less than 14%.

PanB-Catalyzed Enolization of α-Ketoisovalerate. A stack plot of the 300 MHz ^{1}H NMR spectra of the PanB-catalyzed enolization of α-ketoisovalerate, at 26 °C, is shown in Figure 2b. The resonances corresponding to the two equivalent methyl groups were observed as a doublet at 1.24 ppm, and the β-proton was observed as a septet centered at 3.12 ppm. As observed in the THF-independent reaction of formaldehyde and α-ketoisovalerate, enzyme catalyzed enolization, and deuterium exchange causes the disappearance of the β-proton septet and the collapse of the methyl group doublet to a singlet.

The determination of the extent of exchange at any time after enzyme addition was calculated from the integrated intensity of the β-hydrogen septet to the integrated intensity of the methyl hydrogen resonances. The exchange rates (k_{ex}) were determined by analyzing the β-hydrogen resonance every 5 min and fitting to eq 2 using a nonlinear, least squares method. The exchange rates were used to calculate the rate of enolization (eq 3) of α-ketoisovalerate, which was found to be 728 min^{-1}. The addition of 200 μM dihydrofolate did not significantly change the rate of α-ketoisovalerate enolization.

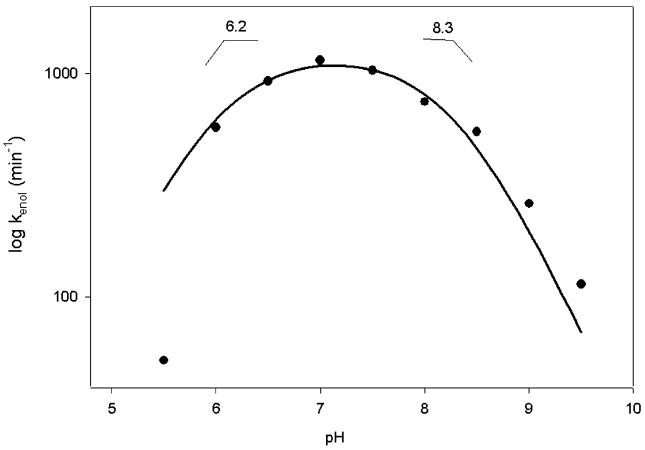

FIGURE 3: pH dependence of the $k_{enolization}$ of α-ketoisovalerate using ^{1}H NMR spectroscopy. The symbols represent experimental values, while the curve is fitted to the data using eq 4.

Table 1: Kinetic Parameters of the Effects of Divalent Metals on *M. tuberculosis* Ketopantoate Hydroxymethyltransferase Enolization of α-Ketoisovalerate

metal	K_m (mM)	k_{enol} (min^{-1})
Mg^{2+}	0.61 ± 0.10	852 ± 41
Co^{2+}	0.08 ± 0.02	298 ± 11
Zn^{2+}	0.33 ± 0.17	299 ± 44
Ni^{2+}	0.45 ± 0.29	56 ± 9
Ca^{2+}	0.27 ± 0.15	27 ± 3

Determination of the Absolute Stereochemistry of Enolization. Since the keto acid substrates could not be used directly to determine the absolute stereochemistry of enolization, the monodeuterated form of α-ketoisocaproate was synthesized as described above. This compound was reacted with *E. coli* branched-chain amino acid transaminase (BCAT), using L-glutamic acid as the amino donor, to form the corresponding monodeuterated L-leucine product. The proton chemical shifts of L-leucine have been previously assigned (*17*), and the two nonequivalent β-protons are centered at 1.42 and 1.33 ppm for the H^{3S} and H^{3R} protons, respectively. The enzyme catalyzed enolization, and deuterium exchange of α-ketoisocaproate and subsequent transimination to L-leucine result in the appearance of a single β-proton resonance at 1.33 ppm, arguing that it is the *pro-S* position of the α-keto acid that is exchanged (data not shown).

pH Dependence on the Rate of Enolization. The pH dependence of the enolization reaction was measured over the pH range of 5.5−9.5, using magnesium as the metal activator, in a Tris-d_{11}/acetate-d_4 buffer system. As seen in Figure 3, the plot of the pH dependence of $k_{enolization}$ is a bell-shaped curve with k_{enol} decreasing with an apparent slope of 1 at lower pH values and a slope of −1 at higher pH values. Fits of the data in the pH range of 6.0−9.5 to eq 4 yielded a pK for the acidic group of 6.2 ± 0.3 and a pK for the basic group of 8.3 ± 0.3.

Effects of Metals Ions on Enzyme Activity. The ability of various divalent metal ions to promote the enolization of

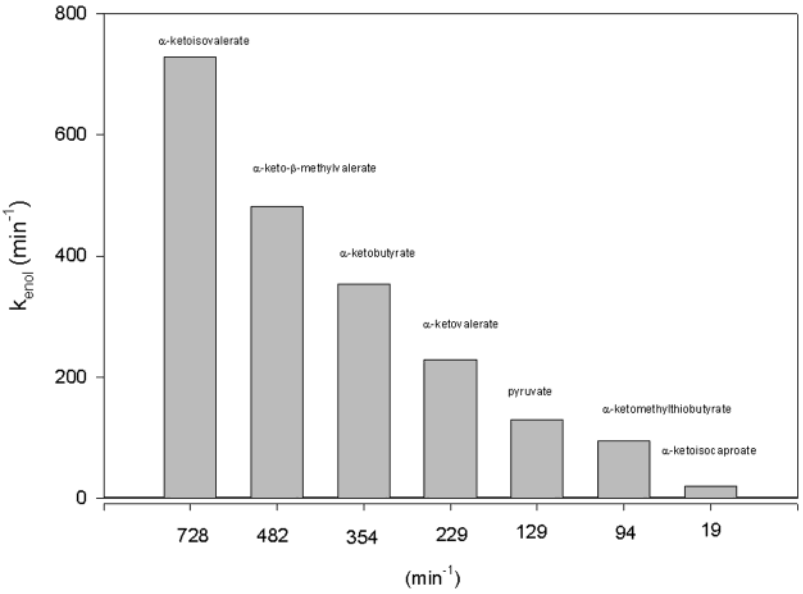

FIGURE 4: Substrate specificity of the PanB-catalyzed enolization of α-keto acids.

α-ketoisovalerate is shown in Table 1. Ketopantoate hydroxymethyltransferase is inactive in the absence of divalent metals. Maximal enolization occurs in the presence of Mg^{2+}, intermediate activity with Co^{2+} and Zn^{2+}, and minimal activity with Ni^{2+} and Ca^{2+}.

Substrate Specificity of Ketopantoate Hydroxymethyltransferase. Ketopantoate hydroxymethyltransferase can catalyze the enolization of a number of α-keto acids (Figure 4). Of the α-keto acids tested, the order of preference exhibited by PanB was α-ketoisovalerate > α-keto-*β*-methylvalerate > α-ketobutyrate > α-ketovalerate > pyruvate > α-keto-methylthiobutyrate > α-ketoisocaproate.

DISCUSSION

Pantothenate is a precursor of coenzyme A (CoA) and acyl carrier protein (ACP) in a pathway that is essential in bacteria, yeast, and plants (*18*). Biochemical investigations have shown that the first unique step in pantothenate biosynthesis is the hydroxymethylation of α-ketoisovalerate using N_5,N_{10}-methylenetetrahydrofolate as a formaldehyde source to generate ketopantoate. This reaction is catalyzed by the *panB*-encoded ketopantoate hydroxymethyltransferase.

Ketopantoate hydroxymethyltransferase from *M. tuberculosis* was cloned on the basis of the published genome sequence (*19*). The overexpressed and purified protein yielded 150 mg of homogeneous enzyme from 12 g of cells. Concentrated solutions (20 mg/mL) of the enzyme are water white, and there is no evidence for the presence of enzyme-bound PLP nor is the activity dependent on added PLP. The molecular mass of the monomer is predicted to be 29366 Da, while the observed monomer molecular mass by ESI-MS was 29202 Da, consistent with the predicted amino acid sequence lacking the N-terminal methionine residue. This was confirmed by the N-terminal amino acid sequence results. Gel filtration analysis suggests that the native enzyme exits as a tetramer. Previously, the *E. coli* and *A. nidulans* PanB were reported to exist in vivo as a hexamer and an octamer, respectively (*6*, *7*).

In this study, we have assayed ketopantoate hydroxymethyltransferase activity spectrophotometrically by coupling the PanB-catalyzed formation of ketopantoate to the reaction of the NADPH-dependent ketopantoate reductase (PanE, Figure 1), which uses ketopantoate as a substrate. The decrease in absorbance of NADPH at 340 nm was followed spectrophotometrically. The intersecting initial velocity pattern observed with α-ketoisovalerate and N_5,N_{10}-methylenetetrahydrofolate suggests a sequential kinetic mechanism. The kinetic parameters determined from fits of the initial velocity data provided K_m values of 240 μM for α-ketoisovalerate and 820 μM for N_5,N_{10}-methylenetetrahydrofolate, with V_{max} equal to 1.6 μmol min^{-1} mg^{-1} (k_{cat} = 47 min^{-1}). These results are comparable to the apparent K_m value of 180 μM for α-ketoisovalerate and V_{max} of 2.8 μmol min^{-1} mg^{-1} reported for the *E. coli* enzyme (*5*). In the previous studies of the *E. coli* enzyme, K_m values for methylenetetrahydrofolate were not reported due to enzyme inhibition by tetrahydrofolate and formaldehyde. Our studies revealed no inhibition at concentrations of methylenetetrahydrofolate (i.e., eqimolar formaldehyde and tetrahydrofolate) up to 2 mM in the coupled assay used. However, this assay is limited by the absorbance of methylenetetrahydrofolate at 340 nm (λ_{max} 412 nm) so higher concentrations could not be used in the spectrophotometric assay.

Using ^{1}H NMR spectroscopy, we have shown that PanB catalyzes the methylenetetrahydrofolate-independent synthesis of ketopantoate from free formaldehyde and α-keto-isovalerate at an initial rate of 135 min^{-1} (Figure 2a). The addition of 200 μM dihydrofolate increases the rate to 239 min^{-1}. However, these initial rates decrease in a time-dependent manner, presumably because of inhibition by formaldehyde that can react with the ϵ-amino group of lysine residues. Since free formaldehyde is unlikely to be the natural substrate, the remainder of this paper examines the methylene-tetrahydrofolate-independent PanB-catalyzed enolization of α-keto acids.

The ketopantoate hydroxymethyltransferase-catalyzed enolization of α-ketoisovalerate in D_2O results in the exchange

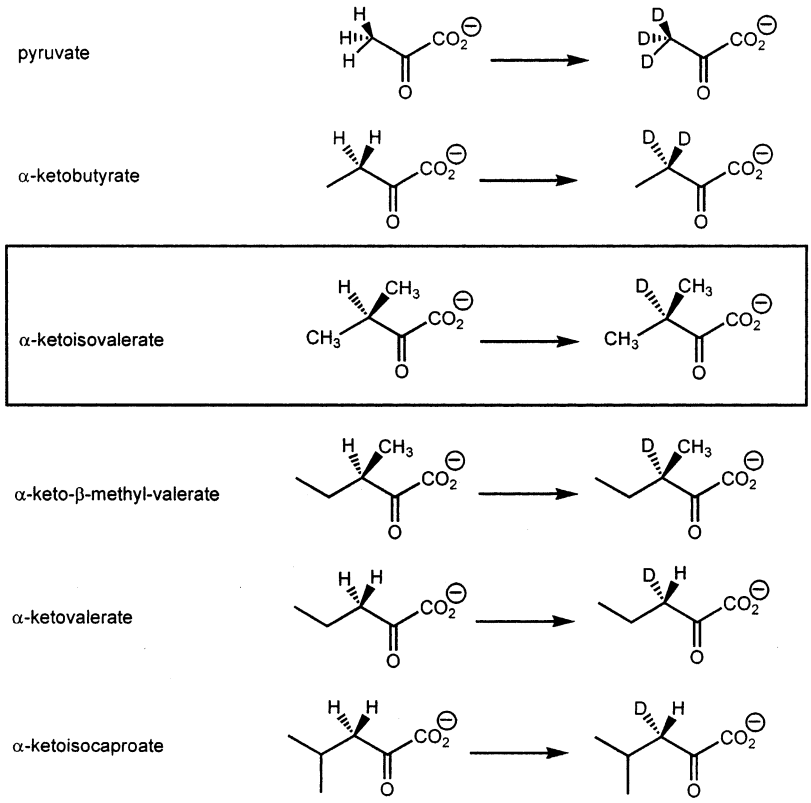

FIGURE 5: PanB-catalyzed hydrogen exchange of α-keto acids containing 1, 2, or 3 β-hydrogens.

of the β-hydrogen of α-ketoisovalerate for deuterium in a methylenetetrahydrofolate-independent reaction. This confirms that the first step in the reaction leading to ketopantoate formation is the enolization of α-ketoisovalerate to form the stabilized carbanion. The requirement for added divalent metal for this reaction suggests that the *M. tuberculosis* ketopantoate hydroxymethyltransferase binds metal ions that assist in the polarization of the carbonyl group and stabilize the enolate anion.

The pH dependence of the enolization reaction suggests that the deprotonation of a group exhibiting a p*K* value of 8.3 ± 0.3 and the protonation of a group exhibiting a p*K* value of 6.2 ± 0.3 cause a loss of enolization activity. The group that exhibits a p*K* value of 6.2 is most likely an enzyme group that acts as a general base to initiate catalysis via the abstraction of the β-hydrogen of α-ketoisovalerate. Possible roles for the group whose p*K* value is 8.3 could be to maintain the general base in the proper ionization state during the enolization reaction, to donate a hydrogen bond to the carboxyl group of the substrate, or to aid in coordination of the metal. The rapid decrease in the rate of enolization of α-ketoisovalerate at pH values below 5.5 does not represent the p*K* value of a single ionizable group of the substrate, as these keto acids have very low p*K* values (e.g., pyruvate p*K* = 2.39), but may represent the loss of proper protein folding at pH values lower than 5.5.

We have examined the substrate specificity of *M. tuberculosis* ketopantoate hydroxymethyltransferase and have found that this enzyme is able to catalyze the enolization of a variety of α-keto acids as substrates. Figure 5 shows the number of β-hydrogens exchanged on the various α-keto acids examined. All of the β-hydrogens of pyruvate and α-ketobutyrate exchanged while only one of the two β-hydrogens of α-ketovalerate, α-ketocaproate, and α-keto-β-methylvalerate exchanged. Our studies have shown that the PanB is stereospecific and abstracts the H^S β-proton of its α-keto acid substrates. We propose the following for this differentiation by the PanB-catalyzed enolization of α-keto acids (Figure 6). The active site of the *M. tuberculosis* PanB may contain a "pocket" in which the C3 methyl or hydrogen substituents of the α-keto acids are accommodated. With α-ketobutyrate and pyruvate, after abstraction of the first hydrogen and exchange, the free rotation around the C2−C3 bond will allow for the presumptive enzymatic base to abstract the remaining hydrogens. If the C3 methyl or hydrogen of α-ketovalerate and α-ketocaproate is oriented to allow the first hydrogen to exchange, rotation around the C2−C3 of these α-keto acids would place an ethyl or isopropyl group into the pocket, and this apparently cannot occur. On the other hand, the active site appears to be able to accommodate the alkyl chains of the longer α-keto acids, such as α-keto-β-methylvalerate and α-ketomethylthiobutyrate, although these are slower substrates.

Combined with our steady-state kinetic data, we propose a chemical mechanism for *M. tuberculosis* ketopantoate hydroxymethyltransferase (Figure 7). After binding of both α-ketoisovalerate and N_5,N_{10}-methylenetetrahydrofolate to the Mg^{2+}−enzyme complex, the active site base, presumably the unprotonated group exhibiting a p*K* of 6.2, removes the β-hydrogen of α-ketoisovalerate to form a metal ion-stabilized enolate. The enolate collapses and nucleophilically attacks either free formaldehyde (not shown) or the methylene group of N_5,N_{10}-methylenetetrahydrofolate, resulting in cleavage of the N5 or N10 bond of the cofactor (as shown) to the methylene group. The subsequent hydrolysis of the

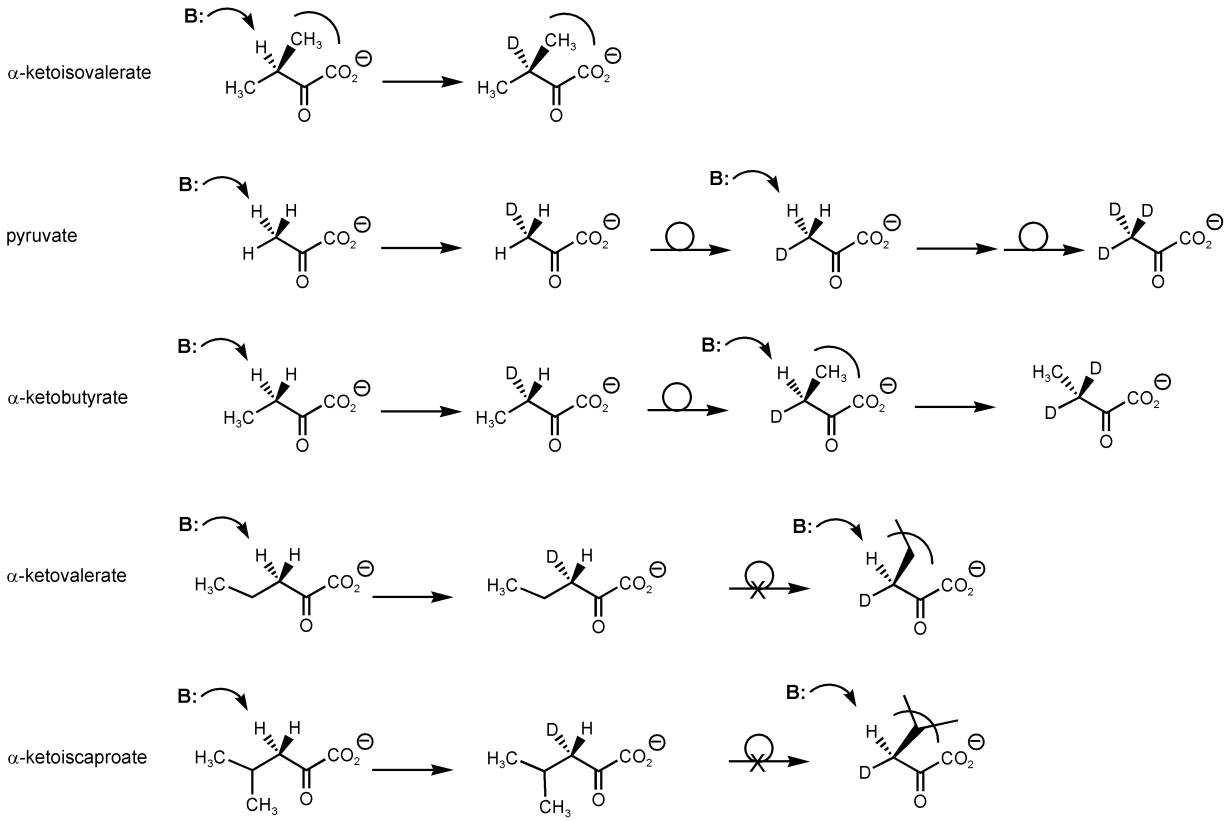

FIGURE 6: Mechanistic rationale for the extent and stereochemistry of the PanB-catalyzed enolization of α-keto acids.

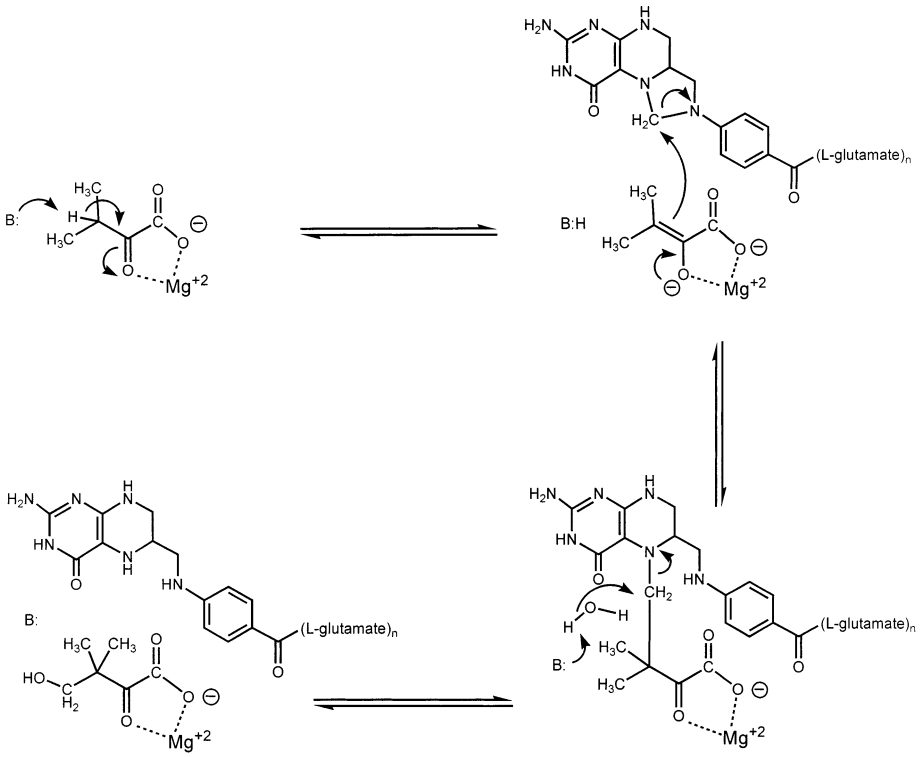

FIGURE 7: Proposed chemical mechanism for hydroxymethyl transfer from N_5,N_{10}-methylenetetrahydrofolate to α-ketoisovalerate catalyzed by *M. tuberculosis* ketopantoate hydroxymethyltransferase.

C4−N5 bond yields the products, ketopantoate and tetrahydrofolate.

The data presented in this paper represent the first mechanistic and stereochemical information about *M. tuberculosis* ketopantoate hydroxymethyltransferase, the first unique step in pantothenate biosynthesis. Structural information will be of key importance in determining the mechanism of PanB, as well as aid in the development of inhibitors against this essential enzyme involved in the rate-limiting step in pantothenate biosynthesis in *M. tuberculosis*.

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