Kinetic and Mechanistic Analysis of the *E. coli panE*-Encoded Ketopantoate Reductase†

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Received November 19, 1999; Revised Manuscript Received January 20, 2000

ABSTRACT: Ketopantoate reductase (EC 1.1.1.169) catalyzes the NADPH-dependent reduction of α-ketopantoate to form D-(−)-pantoate in the pantothenate/coenzyme A biosynthetic pathway. The enzyme encoded by the *panE* gene from *E. coli* K12 was overexpressed and purified to homogeneity. The native enzyme exists in solution as a monomer with a molecular mass of 34 000 Da. The steady-state initial velocity and product inhibition patterns are consistent with an ordered sequential kinetic mechanism in which NADPH binding is followed by ketopantoate binding, and pantoate release precedes $NADP^+$ release. The pH dependence of the kinetic parameters *V* and *V/K* for substrates in both the forward and reverse reactions suggests the involvement of a single general acid/base in the catalytic mechanism. An enzyme group exhibiting a p*K* value of 8.4 ± 0.2 functions as a general acid in the direction of the ketopantoate reduction, while an enzyme group exhibiting a p*K* value of 7.8 ± 0.2 serves as a general base in the direction of pantoate oxidation. The stereospecific transfer of the *pro-S* hydrogen atom of NADPH to the C-2 position of ketopantoate was demonstrated by ^1H NMR spectroscopy. Primary deuterium kinetic isotope effects of 1.3 and 1.5 on V_{for} and V/K_{NADPH}, respectively, and 2.1 and 1.3 on V_{rev} and V/K_{HP}, respectively, suggest that hydride transfer is not rate-limiting in catalysis. Solvent kinetic isotope effects of 1.3 on both V_{for} and V/K_{KP}, and 1.4 and 1.5 on V_{rev} and V/K_{HP}, respectively, support this conclusion. The apparent equilibrium constant, K_{eq}', of 676 at pH 7.5 and the standard free energy change, ΔG, of −14 kcal/mol suggest that ketopantoate reductase reaction is very favorable in the physiologically important direction of pantoate formation.

Ketopantoate reductase (EC 1.1.1.169), encoded by the *panE* gene, is an NADPH[1]-dependent enzyme that catalyzes the synthesis of D-pantoate from α-ketopantoate in the early steps of pantothenate biosynthesis uniquely found in bacteria, yeast, and plants (Scheme 1). D-Pantoate is subsequently condensed with β-alanine to form pantothenate, which is a vitamin of the B group and is a nutritional requirement in mammals. Pantothenate is a key precursor for the biosynthesis of coenzyme A (*1*). The reduction of α-ketopantoate can also be catalyzed by acetohydroxy acid isomeroreductase, (EC 1.1.1.86), the product of *ilvC* gene, which physiologically catalyzes the formation of α,β-dihydroxy-β-methylvalerate and α,β-dihydroxyisovalerate from α-aceto-α-hydroxybutyrate and α-acetolactate, respectively, in isoleucine and valine biosynthesis (*2*). It has been suggested that both *panE* and *ilvC* are responsible for the biosynthesis of pantoate from ketopantoate in vivo (*2, 3*). Acetohydroxy acid isomeroreductase requires Mg^{2+} for the reduction of ketopantoate, whereas ketopantoate reductase does not (*4, 5*). Frodyma and Downs have reported that the *apbA* gene product, required for the function of the alternative pyrimidine biosynthetic (APB) pathway (*6*), catalyzes the NADPH-dependent reduction of α-ketopantoate to form pantoate, and demonstrated that the *apbA* and *panE* genes in *Salmonella typhimurium* are identical (*7, 8*).

The sequence of the *panE* gene encoding ketopantoate reductase has been reported from *Salmonella typhimurium*, and *Escherichia coli* (*6, 9*). Comparison of the predicted amino acid sequences revealed 82% identity between the *E. coli* and *S. typhimurium* proteins. Ketopantoate reductase activity has been reported in cell-free extracts of *Salmonella typhimurium, Saccharomyces cerevisiae, Pseudomonas maltophilia 845, and Escherichia coli* (*5, 7, 8*), and the enzyme has been purified to homogeneity and partially characterized from *Pseudomonas maltophilia 845* and *Salmonella typhimurium* (*5, 7*). The kinetic mechanism of the sequence-unrelated, high molecular weight *P. maltophilia* enzyme has been reported to be an ordered sequential mechanism; however, the kinetic mechanism of the highly homologous *S. typhimurium* reductase was reported to be ping-pong (*7*). The *E. coli* ketopantoate reductase has been partially purified from the wild-type strain (*10*), but no detailed kinetic or mechanistic experiments have been performed. In this paper, we describe the cloning, expression, and purification of the *E. coli* ketopantoate reductase, and present the kinetic and

† This work was supported by NIH Grant AI33696.

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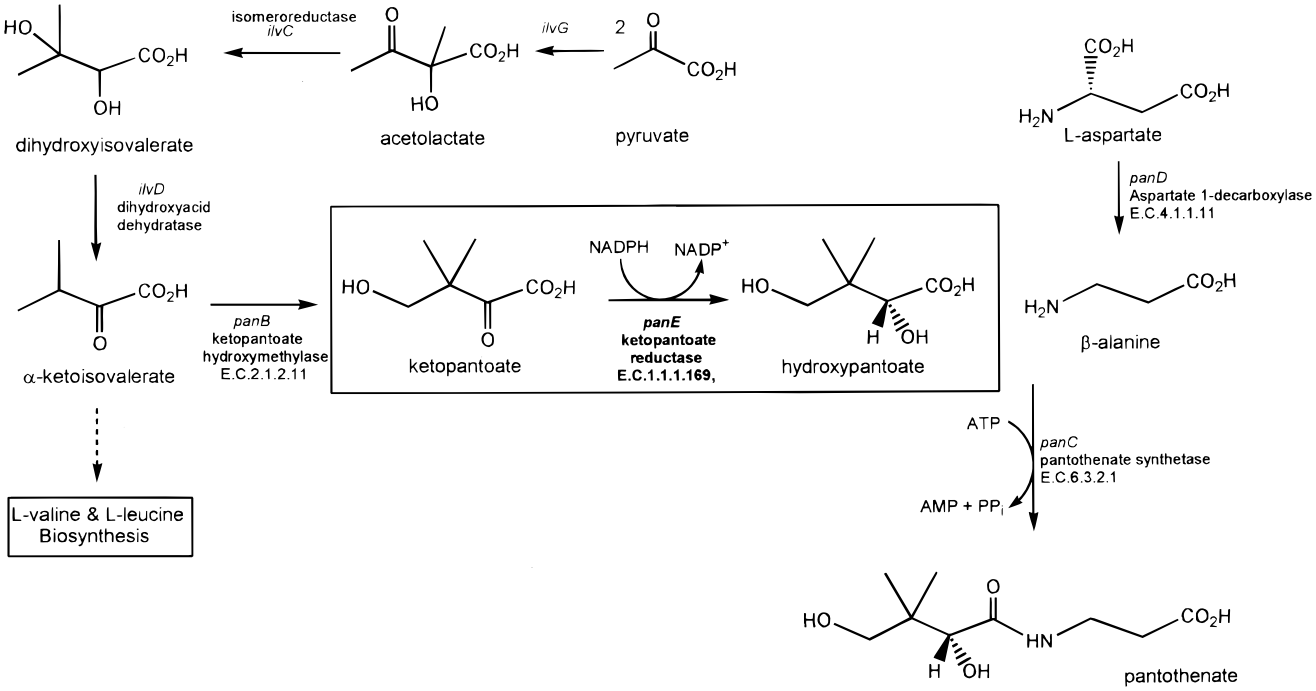
[1] Abbreviations: KPR, ketopantoate reductase; KP, α-ketopantoate; HP, D-(−)-pantoate; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized β-nicotinamide adenine dinucleotide phosphate; APB, alternative pyrimidine biosynthetic; IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Mes, 4-morpholinoethanesulfonic acid; Epps, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; Ches, cyclohexylaminoethanesulfonic acid; TEA, triethanolamine.

10.1021/bi992676g CCC: $19.00
Published on Web 03/07/2000

Scheme 1



chemical mechanism of this enzyme. ^{1}H NMR spectroscopy was used to determine the stereospecificity of hydride transfer in ketopantoate reductase reaction, and this information allowed us to determine the primary deuterium kinetic isotope effect on this highly unusual secondary alcohol dehydrogenase.

MATERIALS AND METHODS

Materials. Ketopantoyl lactone, pantoyl lactone, D-glucose-*1-d*, and ethyl-d_5 alcohol-*d* were obtained from Aldrich. NADPH, NADP$^+$, IPTG, ATP, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIV), hexokinase from yeast, alcohol dehydrogenase from *Thermoanaerobium brockii*, yeast aldehyde dehydrogenase, and all buffer components were purchased from Sigma. D_2O (>99.8 atom % excess) was from Cambridge Isotope Laboratories. All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. *Pfu* DNA polymerase was from Stratagene. pET23a(+) plasmid and *E. coli* strain BL21(DE3) were obtained from Novagen. All chromatographic supports were from Pharmacia.

Preparation of Ketopantoic Acid and Pantoic Acid. Ketopantoyl lactone was hydrolyzed in 1 mM NaOH. The resulting solution was then lyophilized, and the desired product (ketopantoic acid) was recrystallized from EtOH−H_2O (*11*, *12*): ^{1}H NMR (300 MHz, D_2O) δ 3.77 (s, 2 H), δ 1.30 (s, 6 H). Pantoic acid was prepared from pantoyl lactone using NaOH as previously described (*12*): ^{1}H NMR (300 MHz, D_2O) δ 3.92 (s, 1 H), δ 3.57 (dd, 2 H), and δ 1.02 (d, 6 H). [2-^{2}H]Pantoate was enzymatically prepared from ketopantoate with *E. coli* ketopantoate reductase using [4*S*-^{2}H]NADPH. [2-^{2}H]Pantoate was purified on a DEAE 52 column and lyophilized: ^{1}H NMR (300 MHz, D_2O) δ 3.57 (dd, 2 H) and δ 1.02 (d, 6 H).

Preparation of 4S- and 4R-Deuterated Reduced Nucleotides. NADP$^+$ was purified on a FPLC Mono Q anion-exchange column as previously described (*13*). 4*S*-Deuterated NADPH was prepared by enzymatic reduction of the oxidized nucleotide with *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase using glucose-*1-d* as the ^{2}H source (*14*). [4*S*-^{2}H] NADPH was purified on a Mono Q column, and the fractions with absorbance ratios $A_{260}/A_{340} \leq 2.3$ were pooled. 4*R*-Deuterated NADPH was prepared from ethyl alcohol-d_5 and NADP$^+$ using *Thermoanaerobium brokii* alcohol dehydrogenase (*14*). [4*R*-^{2}H]NADPH was purified on a Mono Q column, and fractions with absorbance ratios $A_{260}/A_{340} \leq 2.3$ were pooled. The concentrations of 4*S*- and 4*R*-deuterated NADPH were determined by enzymatic end-point assays with yeast glutathione reductase in the presence of excess oxidized glutathione.

Cloning and Expression. The *panE* gene, encoding ketopantoate reductase, was obtained by PCR amplification of the gene from genomic DNA of *E. coli* K12 using *Pfu* DNA polymerase and two primers (5′-ATTCCATATGAAAATTACCGTATTGGGATGC-3′; 5′-GCGGATCCTCACTACCAGGGGCGAGGCAAAC-3′) containing *Nde*I and *Bam*HI restriction sites, respectively. The *panE* gene was inserted into an *Nde*I- and *Bam*HI-digested pET23a(+) plasmid. The recombinant plasmid was transformed into competent cells of *E. coli* strain BL21(DE3). The BL21(DE3) cells containing pET23a(+):*panE* were grown at 37 °C to an A_{600} of 0.5 in LB medium containing 50 μg/mL carbenicillin. IPTG was added to the culture (final concentration of IPTG = 0.5 mM), and growth was continued for an additional 4 h at 37 °C.

Purification of Ketopantoate Reductase. Eight grams of cells was suspended in 40 mL of 40 mM triethanolamine hydrochloride, pH 7.8, containing protease inhibitors (Boehringer Mannheim) and 8 mg of lysozyme, and stirred for 30 min at 4 °C. The cells were disrupted by sonication, and cell debris was removed by centrifugation for 45 min at 11 000 rpm. Nucleic acids were precipitated by the addition of streptomycin sulfate (1% w/v final) to the supernatant, and the nucleic acids were pelleted by centrifugation for 45 min at 11 000 rpm. The supernatant was dialyzed against

20 mM triethanolamine hydrochloride, pH 7.8, and the precipitate which formed during dialysis was removed by ultracentrifugation. The supernatant was applied to a 140 mL Fast Flow Q-sepharose anion-exchange column, and proteins were eluted with a linear 0−0.8 M NaCl gradient. The enzyme activity was eluted between 0.5 and 0.6 M NaCl. Active fractions were pooled, and the solution was concentrated to 10 mL, and ammonium sulfate was added to a final concentration of 1 M. After centrifugation to remove insoluble materials, the supernatant was applied to a 2.5 × 18 cm Phenyl Sepharose column equilibrated in 20 mM triethanolamine hydrochloride, pH 7.8, containing 1 M $(NH_4)_2SO_4$. The enzyme was eluted with a linear 1−0 M $(NH_4)_2SO_4$ gradient at 1 mL/min. The active fractions were pooled, concentrated (PM10, Amicon) to 4 mL, and applied to a 1.6 × 70 cm Superdex 200 (Pharmacia) gel filtration column equilibrated with 50 mM Hepes, pH 7.5, 50 mM NaCl. The enzyme was eluted at 0.5 mL/min, and the active fractions, which exhibited a single band on SDS−PAGE with Coomassie blue staining, were pooled.

Protein Analysis. Protein concentrations were determined using a Bio-Rad protein assay kit using bovine serum albumin as a standard. The subunit molecular mass of ketopantoate reductase was determined by SDS−polyacrylamide gel electrophoresis according to the method of Laemmli (*15*). The native molecular weight was estimated by using a Superdex 200 gel filtration column calibrated using Bio-Rad molecular weight markers. Electrospray ionization/mass spectrometry was performed on the purified enzyme to determine the subunit molecular mass of ketopantoate reductase.

Steady-State Kinetic Studies. Initial velocities of ketopantoate reductase activity were determined by monitoring the decrease in the absorbance of NADPH at 340 nm (ϵ_{340} = 6.22 mM^{-1} cm^{-1}) at 25 °C using a UVIKON 943 spectrophotometer with a circulating water bath and thermospacers. A typical reaction contained 100 mM Hepes, pH 7.5, 100 μM NADPH, and 1 mM sodium ketopantoate in a total volume of 1.0 mL at 25 °C. The reaction was initiated by the addition of ketopantoate reductase (≤10 μL). The reverse reaction was measured by monitoring the increase at 340 nm accompanying $NADP^+$ reduction. A typical assay contained 100 mM Hepes, pH 7.5, 1 mM $NADP^+$, 3 mM pantoate, and the reaction was initiated by addition of ketopantoate reductase (≤10 μL) in a final volumn of 1.0 mL at 25 °C.

Primary Deuterium and Solvent Kinetic Isotope Effects. Primary deuterium kinetic isotope effects were determined at a saturating concentration of ketopantoate (1 mM) at varied concentrations of [4*S*-^{1}H]- and [4*S*-^{2}H]NADPH or [4*R*-^{2}H]-NADPH (5−25 μM) by monitoring the decrease in absorbance of NADPH at 340 nm at 25 °C. Primary kinetic isotope effects in the reverse direction were determined at a saturating concentration of $NADP^+$ (1 mM) at varied concentrations of [2-^{1}H]pantoate and [2-^{2}H]pantoate (70−540 μM) by monitoring the increase in absorbance of NADPH at 340 nm at pH 7.5, 25 °C. Solvent kinetic isotope effects on *V* and *V/K* for both forward and reverse directions were measured at a saturating concentration of NADPH (50 μM) or $NADP^+$ (1 mM) at varied concentrations of KP (40−200 μM) or HP (400−2000 μM) in 100 mM Hepes prepared in H_2O or 99.8% D_2O at pH(D) 7.5, 25 °C. Proton inventories were measured at various mole fractions of D_2O between 0 and 0.95 in buffer containing 100 mM Hepes, 50 μM NADPH or 1 mM $NADP^+$, 1 mM KP or 3 mM HP at pH 7.5, 25 °C.

pH Profiles. Buffers were prepared by titrating their acid forms to the desired pH value with NaOH and filtered through a 0.22 μm Millipore filter. pH studies were carried out as described by Wong and Blanchard (*16*). The following buffers at 100 mM concentration were used to maintain the pH of reaction: Mes (pH 5.9−6.7), Bis-Tris (pH 5.9−7.2), Hepes (pH 6.8−8.0), Epps (pH 7.3−8.7), Ches (pH 8.6−9.6). The kinetic parameters, *V* and *V/K* for NADPH, were determined by varying the concentration of NADPH between 5 and 25 μM at a saturating concentration of ketopantoate (1 mM). The *V/K* value for ketopantoate was determined by varying the concentration of ketopantoate between 40 and 640 μM at a saturating concentration of NADPH (200 μM). The *V* and *V/K* values for $NADP^+$ were determined by varying the concentration of $NADP^+$ between 5 and 250 μM at a saturating concentration of pantoate (3 mM), while the *V/K* value for pantoate was determined by varying the concentration of pantoate between 320 and 2560 μM at a saturating $NADP^+$ concentration (1 mM). The pH values were measured after each enzyme reaction. The log values of *V* and *V/K* at each pH were plotted against pH.

Stereochemistry of Hydride Transfer. ^{1}H NMR was used to determine the stereospecificity of hydride transfer in the ketopantoate reductase-catalyzed reaction using stereospecifically labeled β-NADPH. ^{1}H NMR spectra were recorded on a Bruker DRX 300 MHz spectrometer in D_2O at 25 °C. Reaction mixtures contained 2.5 mM [4*S*-^{2}H]- or [4*R*-^{2}H]-NADPH, 1 mM ketopantoate, and 7 μg of ketopantoate reductase (KPR) in 2 mL of 25 mM potassium phosphate buffer, pH 7.5. After incubation for 30 min at 25 °C, the solution was filtered through a YM 10 Amicon membrane, and then applied to a DEAE 52 column. The alcohol product was eluted with 2 mL of D_2O, followed by lyophilization. The product was then dissolved in 0.6 mL of D_2O, and analyzed by ^{1}H NMR.

Determination of Equilibrium Constant. Solutions contained 0.1 mM NADPH, 1 mM ketopantoate, 1 mM $NADP^+$, 10 mM pantoate, and 7 μg of KPR in 1 mL of 100 mM Hepes, pH 7.5. The absorbance at 340 nm was measured before the addition of KPR. The reaction was incubated at 25 °C for 40 min, and the absorbance at 340 nm was determined. The apparent K_{eq}' and free energy were calculated by eqs 1 and 2, respectively

$$K_{eq}' = [NADP^+][\text{pantoate}]/[\text{NADPH}][\text{ketopantoate}] \quad (1)$$

$$\Delta G = -2.3RT \log K_{eq} \quad (2)$$

where K_{eq} equals $K_{eq}'/[H^+]$, *R* is the gas constant, and *T* is the absolute temperature.

Data Analysis. Reciprocal initial velocities were plotted against reciprocal substrate concentrations. Data were fitted to the appropriate rate equations by using the programs developed by Cleland (*17*). The individual substrate saturation kinetic data were fitted to eq 3. Kinetic data conforming to a sequential initial velocity pattern were fitted to eq 4. Data for competitive, uncompetitive, and noncompetitive inhibition were fitted to eqs 5, 6, and 7, respectively. Data

Table 1: Purification of *E. coli* Ketopantoate Reductase

fraction	total protein (mg)	total units (μmol/min)	sp act. (units/mg)	% yield	fold purification
crude extract	1520	3040	2.0	100	1
Fast Q Sepharose	240	2379	9.9	78	5
Phenyl Sepharose	107	1964	18.4	65	9.2
Superdex 200	88	1672	19	55	9.5

for pH profiles that showed the decrease in log V or log (V/K) with a slope of -1.0 as the pH was increased were fitted to eq 8. Data for pH profiles that showed a decrease in log V or log (V/K) with a slope of $+1.0$ as the pH was decreased were fitted to eq 9. Data for bell-shaped pH profiles that showed limiting slopes of $+1.0$ and -1.0 were fitted to eq 10.

$$v = VA/(K + A) \quad (3)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (4)$$

$$v = VA/[K(1 + I/K_{is}) + A] \quad (5)$$

$$v = VA/[K + (1 + I/K_{ii})A] \quad (6)$$

$$v = VA/[K(1 + I/K_{is}) + (1 + I/K_{ii})A] \quad (7)$$

$$\log Y = \log[C/(1.0 + K/H^+)] \quad (8)$$

$$\log Y = \log[C/(1 + H^+/K_1)] \quad (9)$$

$$\log Y = \log[C/(1 + H^+/K_1 + K_2/H^+)] \quad (10)$$

Data for fitting the primary deuterium and solvent kinetic isotope effect were fitted to eq 11:

$$v = VA/[K(1.0 + F_iE_{V/K}) + A(1.0 + F_iE_v)] \quad (11)$$

where F_i is the fraction of deuterium label ($F_i = 0.0$ and 1.0 for hydrogen- and deuterium-containing NADPH), $E_{V/K}$ is the isotope effect minus 1 on V/K, and E_v is the isotope effect minus 1 on V.

RESULTS

Expression, Purification, and Properties of Ketopantoate Reductase. Overexpression of the *panE* gene in *E. coli* BL21-(DE3) strain yielded highly active, soluble ketopantoate reductase with a molecular mass of 33 kDa on SDS−PAGE. Ketopantoate reductase (KPR) was purified to homogeneity in a yield of 88 mg from 8 g of cells in the four-step purification summarized in Table 1. The enzyme was purified 9.5-fold in 55% overall recovery. Gel filtration chromatography on a Superdex 200 column was used to estimate the molecular weight of the native enzyme as 33 000, suggesting the native enzyme exists as a monomer. Electrospray mass spectrometry analysis showed that the subunit of the enzyme has a molecular mass of 33 866, consistent with the molecular weight of 33 870 predicted from the amino acid sequence of *E. coli* ketopantoate reductase. The KPR activity observed in the NADPH-dependent reduction of ketopantoate was unaffected by the addition of magnesium.

Steady-State Kinetic Studies. Initial velocity patterns were obtained when NADPH was varied at different fixed levels of ketopantoate. Double reciprocal plots gave converging lines intersecting to the left of the ordinate, suggesting a sequential kinetic mechanism. In the reverse reaction, the same pattern was observed when $NADP^+$ was the variable substrate using several fixed levels of D-pantoate. The determined kinetic parameters are given in Table 2. Product inhibition studies were performed by using $NADP^+$, which exhibited competitive inhibition versus NADPH ($K_{is} = 52 \pm 2$ μM) and noncompetitive inhibition versus ketopantoate ($K_{is} = 230 \pm 40$ μM; $K_{ii} = 160 \pm 30$ μM) in the direction of ketopantoate reduction. Pantoate exhibited noncompetitive inhibition versus both ketopantoate ($K_{is} = 420 \pm 80$ μM; $K_{ii} = 220 \pm 20$ μM) and NADPH ($K_{is} = 500 \pm 90$ μM; $K_{ii} = 270 \pm 30$ μM) (Table 3).

Table 2: Kinetic Parameters of *E. coli* Ketopantoate Reductase

substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	V/K
NADPH	4.0 ± 0.4	13.8 ± 0.4	3.5
KP	120 ± 8		0.11
$NADP^+$	7 ± 2	0.90 ± 0.03	0.13
HP	260 ± 40		0.0034

Table 3: Product Inhibition of *E. coli* Ketopantoate Reductase

varied substrate	product inhibitor	K_{is} (μM)	K_{ii} (μM)	fixed substrate (μM)	inhibition
NADPH	$NADP^+$	52 ± 2		KP (600)	C
KP	$NADP^+$	230 ± 40	160 ± 30	NADPH (50)	NC
NADPH	HP	420 ± 80	220 ± 20	KP (600)	NC
KP	HP	500 ± 90	270 ± 30	NADPH (50)	NC

Stereospecificity of Hydride Transfer. The stereospecifically labeled [4*S*-^{2}H]- and [4*R*-^{2}H]NADPH were prepared from either D-glucose-*1-d* or ethyl alcohol-d_5 using *L. mesenteroides* glucose-6-phosphate dehydrogenase or *T. brokii* alcohol dehydrogenase, respectively. The hydride transfer specificity of ketopantoate reductase was determined by ^{1}H NMR analysis, based on the resonance of the C_2-H of pantoate. In ketopantoate reductase catalysis, the deuterium of [4*S*-^{2}H]NADPH was transferred to ketopantoate, resulting in the formation of [2-^{2}H]pantoate. This result was confirmed using [4*R*-^{2}H]NADPH, with the product [2-^{1}H]pantoate exhibiting a signal at δ 3.92 (Figure 1).

Equilibrium Constant and Free Energy. The equilibrium constant was obtained by measuring the concentrations of substrate and product at equilibrium. The experimentally determined K_{eq}' at pH 7.5 is 676, in agreement with the value for K_{eq}' of 980 calculated from the kinetic constants determined at pH 7.5 using the Haldane equation for a sequential Bi-Bi kinetic mechanism, eq 12 (18):

$$K_{eq} = (V^f/V^r)^2(K_pK_q/K_AK_B) \quad (12)$$

The free energy change, $\Delta G = -14$ kcal/mol, was calculated using eq 2.

pH Profiles. The pH dependence of the kinetic parameters in the forward and reverse directions was determined over the pH range of 5.9−9.6 using a variable concentration of one substrate at a saturating concentration of the other substrate. The log value of the maximum velocity of KP reduction decreases at high pH as a single group with a pK value of 8.4 ± 0.2 is deprotonated (Figure 2). The V/K value for ketopantoate decreases above a pK value of 8.1 ± 0.2 as

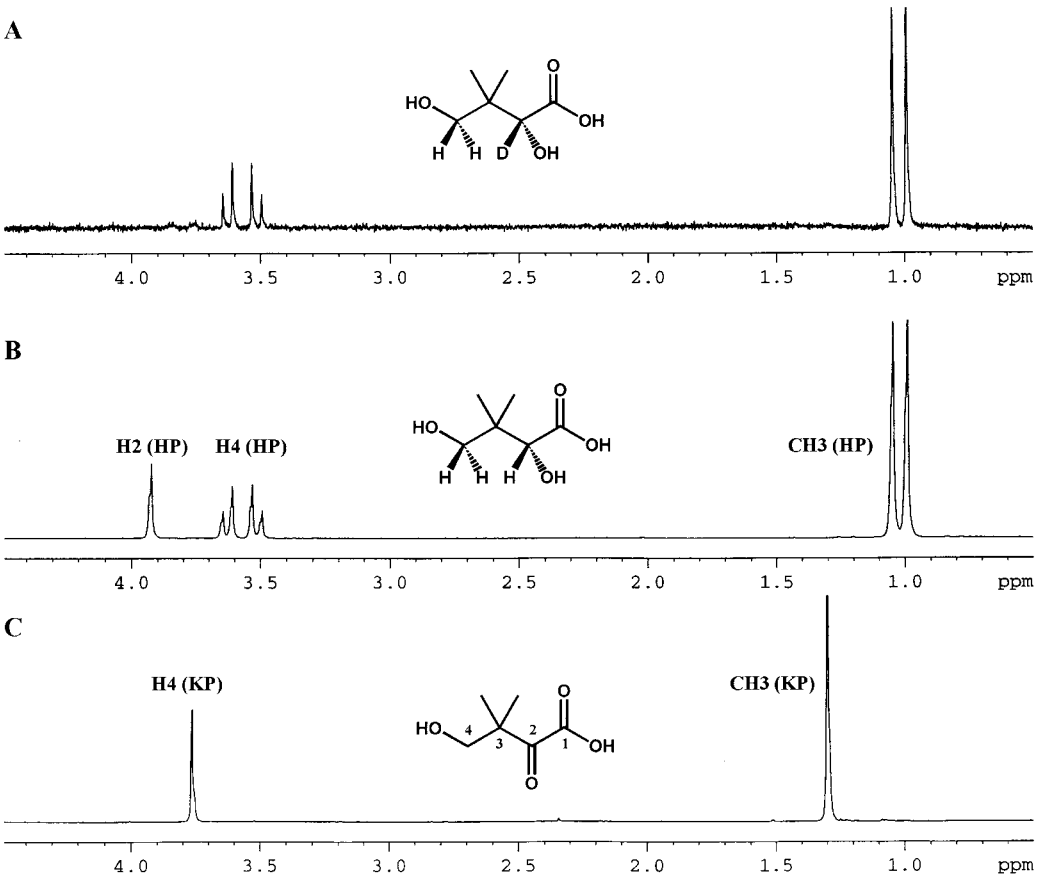


FIGURE 1: ^{1}H NMR spectra of (A) reaction product isolated from the reduction of α-ketopantoate with [4*S*-^{2}H]NADPH, (B) D-pantoate, and (C) α-ketopantoate.

a single group is deprotonated. The pH dependence of *V/K* for NADPH decreases as a result of the protonation of a group with a p*K* of 6.2 ± 0.2 and as a result of deprotonation of another group with a p*K* value of 8.7 ± 0.2. The pH dependence of the kinetic parameters for the reverse reaction is shown in Figure 3. The log *V* decreases as a single group exhibiting a p*K* value of 7.8 ± 0.2 is protonated. The *V/K* values for $NADP^+$ and pantoate decrease as single groups exhibiting p*K* values of 7.6 ± 0.2 and 8.1 ± 0.2, respectively, are protonated.

Deuterium and Solvent Kinetic Isotope Effects. Deuterium kinetic isotope effects were obtained by direct comparison of the initial velocities of oxidation of NADPH and [4*S*-4-^{2}H]NADPH concomitant with ketopantoate reduction. Primary or secondary deuterium kinetic isotope effects were determined using varying concentrations of either [4*S*-^{1}H]- and [4*S*-^{2}H]NADPH or [4*R*-^{1}H]- and [4*R*-^{2}H]NADPH, respectively, at a saturating concentration of ketopantoate at pH 7.5, 25 °C. Primary deuterium kinetic isotope effects on *V* and *V/K* using [4*S*-^{2}H]NADPH exhibit values of 1.33 ± 0.02 and 1.54 ± 0.04, respectively (Figure 4). Secondary deuterium kinetic isotope effects on *V* and *V/K* for [4*R*-^{2}H]-NADPH exhibit values of 1.19 ± 0.05 and 1.20 ± 0.07, respectively (data not shown). Primary deuterium kinetic isotope effects on *V* and *V/K* for pantoate oxidation were determined using [2-^{1}H]- and [2-^{2}H]pantoate at a saturating concentration of $NADP^+$ (1 mM) as shown in Figure 5. The values of the primary deuterium isotope effects on *V* and *V/K* for [2-^{2}H]pantoate are 2.1 ± 0.1 and 1.3 ± 0.1, respectively. Solvent kinetic isotope effects on V_{for} and V/K_{KP} exhibit values of 1.32 ± 0.07 and 1.27 ± 0.08, respectively (data not shown), and solvent kinetic isotope effects on V_{rev} and V/K_{HP} exhibit values of 1.37 ± 0.06 and 1.52 ± 0.11, respectively (Figure 6). A linear relationship between the maximal velocities and mole fraction of D_2O were observed in proton inventory measurements for both forward and reverse directions (Figure 6).

DISCUSSION

Pantothenate is a vitamin required by mammals for the synthesis of CoA, and is produced in bacteria and plants in three steps from α-ketoisovalerate by the action of the *panB*-, *panE*-, *panD*-, and *panC*-encoded enzymes (*19*−*22*) (Scheme 1). The *E. coli* ketopantoate reductase has been partially purified from cell extracts and shown to catalyze the NADPH-dependent reduction of ketopantoate. This same activity was identified in a screen for thiamin auxotrophs in *Salmonella typhimurium* that revealed a gene, termed *apbA*, that catalyzed NADPH-dependent ketopantoate reduction and was proposed to be involved in this *a*lternative *p*yrimidine *b*iosynthetic pathway (*7*, *8*). This gene sequence predicted an enzyme of 271 amino acids whose sequence was 82% identical to the corresponding *E. coli panE*-encoded reductase. Sequence-based searches for other orthologues of ketopantoate reductase revealed five additional putative reductases, exhibiting between 25 and 29% sequence identity, but no other similar sequences (Figure 7). Given the common chemistry shared between ketopantoate reductase and a large number of secondary alcohol dehydrogenases, it is extremely unusual to have identified no other analogous proteins. For

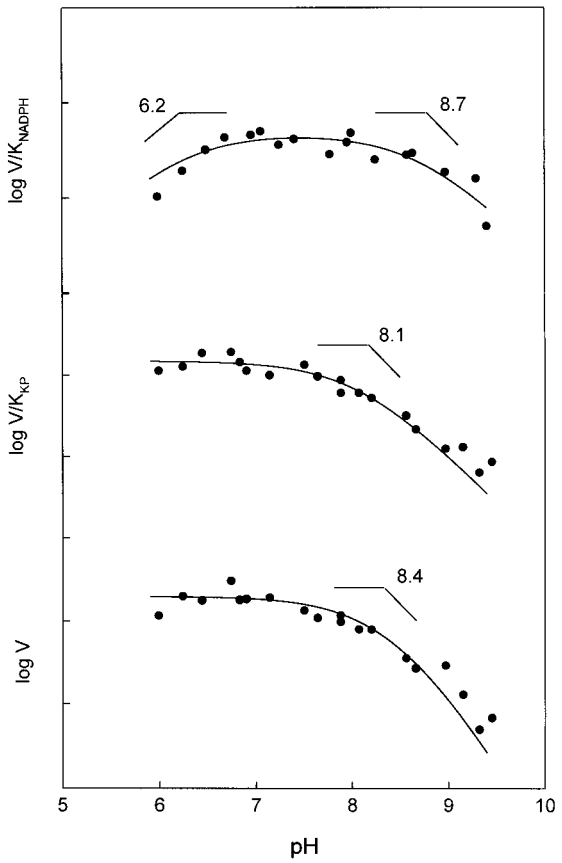


FIGURE 2: pH dependence of kinetic parameters for ketopantoate reduction. The experimental points are fits of the data to eq 3, and the curves for V (bottom) and V/K_{KP} (middle) are fits of the data to eq 8. The pH dependence of V/K_{NADPH} (top) is fit to eq 10.

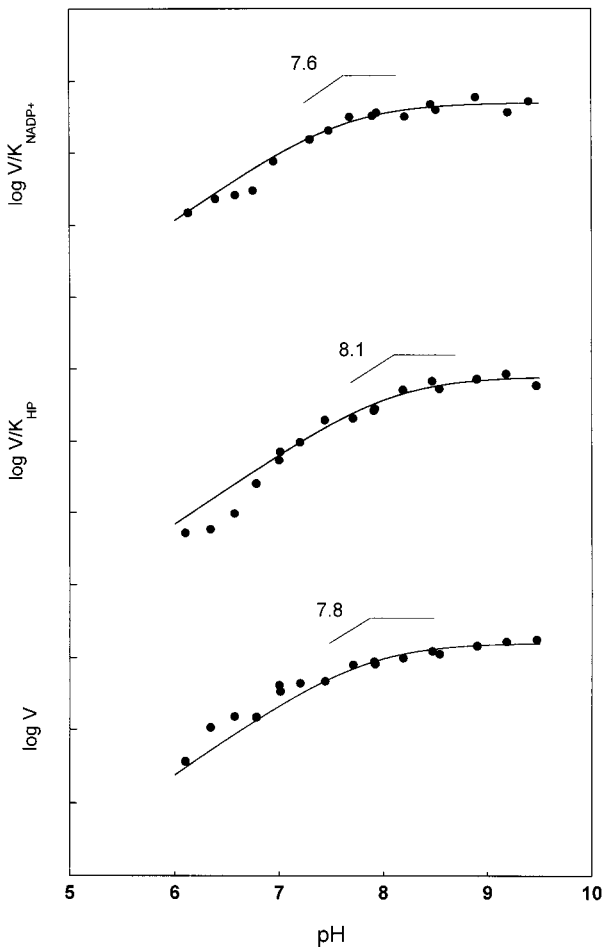


FIGURE 3: pH dependence of kinetic parameters for pantoate oxidation. The experimental points are fits of the data to eq 3, and the curves for V (bottom), V/K_{HP} (middle), and V/K_{NADP^+} (top) are fits of the data to eq 9.

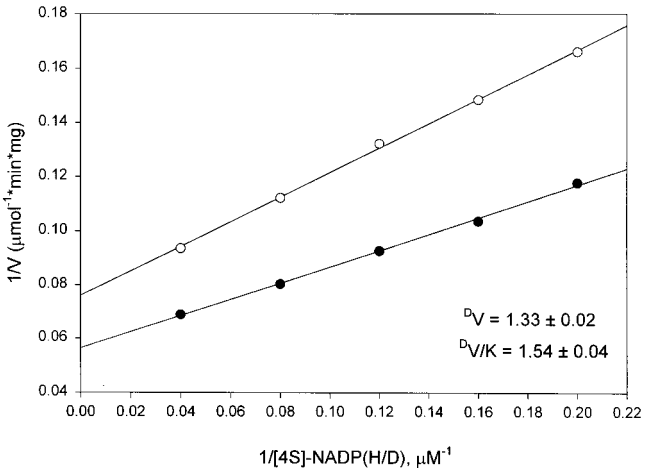


FIGURE 4: Primary deuterium isotope effects for the ketopantoate reductase reaction. The experimental values were determined using [4*S*-^{1}H]NADPH (●) and [4*S*-^{2}H]NADPH (○) at saturating concentration of ketopantoate.

this reason, we have performed a detailed series of kinetic, stereochemical, and mechanistic studies on the recombinant *E. coli* reductase.

The synthesis of D-(−)-pantoate from ketopantoate is catalyzed by both the *panE*-encoded ketopantoate reductase and the *ilvC*-encoded acetohydroxy acid isomeroreductase (*2*, *3*). The *E. coli panE* ketopantoate reductase activity is not dependent on added divalent metal ions, while the presence of Mg^{2+} is essential for the reduction of ketopantoate by acetohydroxy acid isomeroreductase (*2*). Metal ions play an important role in the electrostatic stabilization of the transition state of other secondary alcohol dehydrogenases, such as Zn^{2+} in alcohol dehydrogenase (*23*) and Mg^{2+} in isocitrate dehydrogenase (*24*). The divalent metal ion-independent activity of ketopantoate reductase suggests that active site residues may serve the same function as Mg^{2+} or Zn^{2+} in the stabilization of the transition state in isocitrate dehydrogenase or alcohol dehydrogenase.

Expression and Purification. The *E. coli* ketopantoate reductase has never been purified, and no detailed characterization of the enzyme has been reported (*5*). In this study, we constructed an overexpression plasmid containing the *E. coli panE* gene, expressed it in BL21(DE3) cells, and obtained large amounts of soluble, highly active enzyme. The reductase was purified to homogeneity in a yield of 88 mg of reductase from 8 g of cells by using consecutive anion-exchange, Phenyl Sepharose, and gel filtration column chromatography. The subunit molecular weight of the enzyme was determined by electrospray mass spectrometry to be 33 866, in agreement with the molecular weight of 33 870 predicted from the amino acid sequence of the *E. coli* enzyme. The native molecular weight of the enzyme was estimated by gel filtration on a Superdex 200 column to be 33 000, suggesting that the *E. coli* enzyme exists a monomer. This monomeric form is similar to that demonstrated for the *S. typhimurium* enzyme (*7*), while KPR of

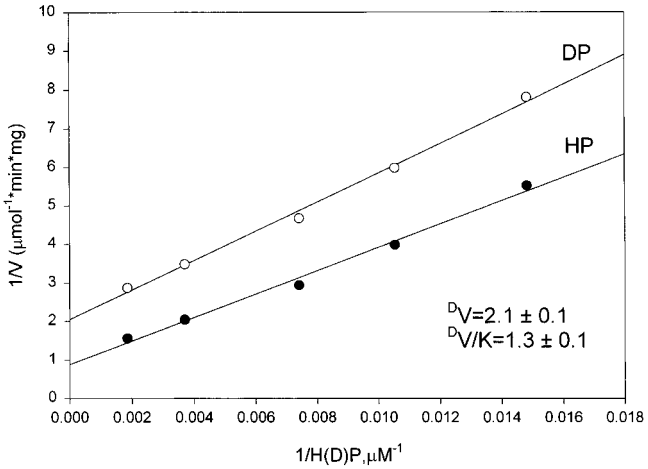


FIGURE 5: Primary deuterium isotope effects for the ketopantoate reductase reaction. The experimental values were determined using [2-^{1}H]pantoate (●) and [2-^{2}H]pantoate (○).

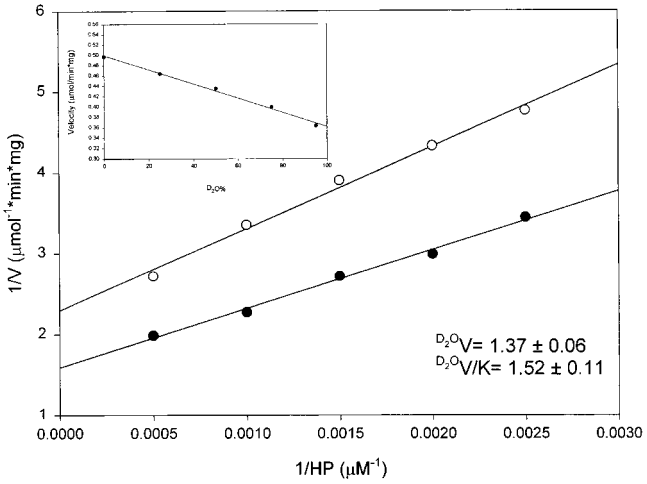


FIGURE 6: Solvent kinetic isotope effects on the ketopantoate reductase reaction. Data in the reverse direction determined in H_2O (●) and in D_2O (○). Insets: Proton inventory experiments.

Pseudomonas maltophilia 845 has been reported to be oligomer composed of 3−5 subunits with a molecular weight of 30 500 (*5*).

Kinetic Mechanism. The intersecting pattern observed in double-reciprocal plots in both the forward and reverse reactions catalyzed by *E. coli* ketopantoate reductase suggests a sequential kinetic mechanism. In product inhibition studies, $NADP^+$ exhibited linear, competitive inhibition versus NADPH and noncompetitive inhibition versus ketopantoate, while hydroxypantoate exhibited linear, noncompetitive inhibition versus both NADPH and ketopantoate. These kinetic data are consistent with an ordered kinetic mechanism in which NADPH binding is followed by ketopantoate binding and with $NADP^+$ release following hydroxypantoate release (Scheme 2). The purified *Pseudomonas maltophilia* ketopantoate reductase has been reported to utilize this same ordered mechanism (*5*), as have many secondary alcohol dehydrogenases, including yeast and liver alcohol dehydrogenase (*25, 26*), glycerol dehydrogenase (*27*), and progesterone 5α-reductase (*28*).

The equilibrium constant, K_{eq}', value determined experimentally agrees with that calculated for an ordered sequential Bi-Bi kinetic mechanism using eq 12 and the kinetic parameters in Table 2. The ΔG value of −14 kcal/mol suggests that the reaction is very favorable in the physiologically relevant direction of ketopantoate reduction.

Stereochemistry. The stereospecificity of hydride transfer in numerous NAD(P)H-dependent oxidoreductases has been reported (*29*) and separated into two classes, A and B. For ketopantoate reductase, the stereospecificity of hydride transfer was determined by ^{1}H NMR spectroscopy of enzymatically generated D-(−)-pantoate formed by using stereospecifically labeled β-NADPH. The formation of C-2-deuterated pantoate using [4*S*-^{2}H]NADPH indicates that the *pro-S* hydrogen at the C-4 position of the nicotinamide ring of NADPH was transferred to ketopantoate, demonstrating that *E. coli* ketopantoate reductase is a B-specific enzyme, similar to results obtained with partially purified *E. coli* and yeast ketopantoate reductases (*30*). In contrast, most secondary alcohol dehydrogenases exhibiting ordered kinetic mechanisms are A-specific, including malate dehydrogenase (*31*) and alcohol dehydrogenase (*32*). This difference in the stereospecificity of hydride transfer between dehydrogenases may reflect the convergent evolution of the two classes of dehydrogenases.

Isotope Effects. Small primary deuterium kinetic isotope effects in the forward direction on V and V/K of 1.33 ± 0.02 and 1.54 ± 0.04, respectively, were obtained using [4*S*-^{1}H]- and [4*S*-^{2}H]NADPH. Secondary deuterium kinetic isotope effects on V and V/K of 1.19 ± 0.05 and 1.20 ± 0.07, respectively, were obtained using [4*R*-^{1}H]- and [4*R*-^{2}H]-NADPH. The primary deuterium kinetic isotope effects in the reverse direction on V and V/K of 2.1 ± 0.1 and 1.3 ± 0.1 were obtained using [2-^{1}H]- or [2-^{2}H]pantoate. The small primary deuterium kinetic isotope effects suggest that hydride transfer is unlikely to be a rate-limiting step at pH 7.5, as has been reported previously for alcohol dehydrogenase in the direction of acetaldehyde reduction (*33*). The observed secondary deuterium kinetic isotope effects on ketopantoate reduction are close to the predicted equilibrium isotope effect (1/0.87) for reduced nucleotide oxidation (*34*), suggesting that the chemical interconversion of substrates and products may be fast relative to substrate/product dissociation. The small solvent kinetic isotope effects on V_{for} and V/K_{KP} of 1.32 ± 0.07 and 1.27 ± 0.08, respectively, and V_{rev} and V/K_{HP} of 1.37 ± 0.06 and 1.52 ± 0.11, respectively, further indicate that the chemical reaction may not be a rate-limiting step in ketopantoate reductase catalysis.

pH Dependence of the Kinetic Parameters. The pH dependence of V in the forward direction suggests that the deprotonation of a single group exhibiting a pK value of 8.4 ± 0.2 causes the catalytic rate to decrease. A similar pH dependence is observed for V/K_{KP}, with this kinetic parameter decreasing as a single group exhibiting a pK value of 8.1 ± 0.2 is deprotonated. V/K_{NADPH} decreases as a result of the protonation of one group exhibiting a pK value of 6.2 ± 0.2 and the deprotonation of a second group exhibiting a pK value of 8.7 ± 0.2. The group exhibiting a pK value of 6.2 ± 0.2 was assigned to the 2′-phosphate of NADPH, since the solution pK of this group has been reported as 6.5 (*35*), representing preferential binding of the dianionic form of the phosphate monoester. The group whose deprotonation decreases V/K_{NADPH} is possibly an enzyme group that interacts with the 2′-phosphate, since NADPH does not contain any ionizable groups in this region. The group whose

```
                1                                                          60
E. coli         ~~~MKITVLG CGALGQLWLT ALCKQGHEVQ GWLRVPQPYC SVN....LVE TDGSIFNESL
S. typhimurium  ~~~MKITVLG CGALGQLWLS ALCKHGHDVQ GWLRVPQPYC SVN....LID TDGSFFNESL
P. horikoshii   ~~~MKIYILG AGAIGSLVGG LLANVGEDVT LIGRGR.HIE AINKRGLMIE .GLTNLKINT
P. abyssi       ~~~MKIYILG AGAIGSLFGG LLANAGEDVL LIGRDP.HVS AINEKGLKIV .GIKDLNVKV
A. fulgidus     MRVQRVQIMG AGALGSLVGA LIQLAGYDVI FVARGK.QLE AL.KKGLRVS .GLKNAELKV
A. aeolicus     ~MVMKFLIVG VGAIGSAYLA FLTRAGHEAA GLVRRN.PVN RIKVEG..IW .GEFEIPVKT
B. subtilus     ~~~MKIGIIG GGSVGLLCAY YLSLY.HDVT VVTRRQEQAA AIQSEGIRLY KGGEEFRADC
Consensus       ---------G -G--G----- ---------- ---R------ ---------- ----------

               61                                                          120
E. coli         TANDPDFLAT SDLLLVTLKA WQVSDAVKSL ASTLPVTTPI LLIHNGMGTI EELQNI..QQ
S. typhimurium  TANDPDFLAK SELLLVTLKA GFRRSTNPGV ..NLPVTSPI LLIHNGMGTI EELQNI..QQ
P. horikoshii   KATTSIPGAK PDLIILTTKS YSTDDALNSA KDIV.RDTWV LSLQNGIGNE EKIME.LG.G
P. abyssi       EATTRVPEEK PDLIVLATKS YSTIEALKSA RHIV.KGSWV LSIQNGIGNE DKIIE.FG.G
A. fulgidus     YCTSQ.PED. ADITFVTVKA YDTETVAKKL AEVD.AGV.V CSLQNGVGNE EILAK.YC.R
A. aeolicus     FTKVEEVPFI PDIVIISVKS YDTEEALKKV KPVVGENTFI MIAQNGYGNY EKAVEIYGEG
B. subtilus     SADTSINSDF .DLLVVTVKQ HQLQSVFSSL ERIGKTN..I LFLQNGMGHI HDLKDWHVGH
Consensus       ---------- --------K- ---------- ---------- ----NG-G-- ----------

               121                                                         180
E. coli         PLLMGTTTHA A.RRDGNVII HVANGITHIG ...PARQQDG DYSYLADILQ TVLPDVAWHN
S. typhimurium  PMLMGTITHA A..RDGNIII HVANGTTHIG ...PAREQDG DYSYLADILQ GVLPDVAWHN
P. horikoshii   RPIGGITTNG AVLKEPGVVE WRGRGITLIG LYPKGR..NE FVEEVKETFN RAGLETEVTE
P. abyssi       KAIGGITTNG AMVEAPGVIK WTGKGVTIIG LYPQGK..EK FIEKVADVFN SADIETHVSE
A. fulgidus     KVLGGVTTYG ANLKDYGHVV YAGEGYTYVG EMDGRV..SG EAEMVAEVLR DAGMRAEAVN
A. aeolicus     KVILSRIIFG SKVIKPGHIR ITVSADEVVI GDPSGKIDEE FLKNLARTFT EAGIPTRYER
B. subtilus     SIYVGIVEHG AVRKSDTAVD HTGLGAIKWS AFDDAEPDR. ..LNILFQHN HSDFPIYYET
Consensus       ---------- ---------- ---------- ---------- ---------- ----------

               181                                                         240
E. coli         NIRAELWRKL AVNCVINPLT AIWNCPNGEL RHHP...QEI MQICEEVAAV IEREGHHTSA
S. typhimurium  NIRAEMWRKL AVNCVINPLT ALWNCPNGEL RHHT...DEI NAICEEVAAV IEREGYHTSA
P. horikoshii   NIIGWKWAKT IVNSAINPIG AILEVKNGAI KDNDYLLSIA VEVVKEGCKI ALQNGI....
P. abyssi       NIISWIWAKA IVNSAINPIG TLLEVKNKVI RENDFLLSMA MEVVKEGCRV ALQNGI....
A. fulgidus     DIEFRIWAKA VVNAAINPIT AICRVKNGEV VRNPHLWEVA RAVADEGRQV MARMGY....
A. aeolicus     DVYKYLVDKI IYNSALNPLG ALFEVNYGSL AENPHTKELM NRVIDEIFQV IEKAKLPCFW
B. subtilus     DWYRLLTGKL IVNACINPLT ALLQVKNGEL LTTPAYLAFM KLVFQEACRI L.........K
Consensus       --------K- --N---NP-- ---------- ---------- -----E---- ----------

               241                                                         300
E. coli         EDL....RDY VMQVIDATAE NISSMLQDIR ALRHTEIDYI NGFLLRRARA HGIAVPENTR
S. typhimurium  DDL....RYY VEQVIDSTAE NISSMFEDVR AMRHTEIDYI TGYLLKRARV HGLAFRKIAA
P. horikoshii   .KFDISPMEL LIQTLEQTRE NYNSMLQDIW RGKRTEIDFI NGKIIEYAKL VNLEAPLNFL
P. abyssi       .EFDVPPMDL FFQTLEQTRE NYNSMLQDIW RGKKTEVDYI NGKIVEYAKA VNLEAPMNLL
A. fulgidus     .EFDAA..SE VRKVAEMTAE NRSSMLQDLE RGKRTEVEFI NGAIVKKGEE FGIDCAVNRT
A. aeolicus     KSADEYKKVF YEKLIPPTAE HYPSMLEDVK KGK.TEIEAL NGAIVELGKK YGVSTPTNEF
B. subtilus     LENEEKAWER VQAVCGQTKE NRSSMLVDVI GGRQTEADAI IGYLLKEASL QGLDAVHLEF
Consensus       ---------- -------T-E ---SM--D-- ----TE---- -G-------- ----------

               301                  323
E. coli         LFEMVKRKES EYERIGTGLP RPW
S. typhimurium  CLKW~~~~~~ ~~~~~~~~~~ ~~~
P. horikoshii   LWALVKAKES ..LGGGSK~~ ~~~
P. abyssi       LWGLIKGKEA ..LEGKK~~~ ~~~
A. fulgidus     LLNLVRGVES .GL~~~~~~~ ~~~
A. aeolicus     ITKMVKAKEL FNLKDT~~~~ ~~~
B. subtilus     LYGSIKALER NTNKVF~~~~ ~~~
Consensus       ---------- ---------- ---
```

FIGURE 7: Alignment of the amino acid sequences of seven bacterial ketopantoate reductases. Consensus residues are those conserved between all seven sequences. This alignment was generated by using the PILEUP program of the GCG package. The GenBank accession numbers are as follows: *E. coli* (AE000148); *S. typhimurium* (P37402); *P. horikoshii* (O50098); *P. abyssi* (AJ248285); *A. fulgidus* (O28578); *A. aeolicus* (O67619); *B. subtilus* (O34661).

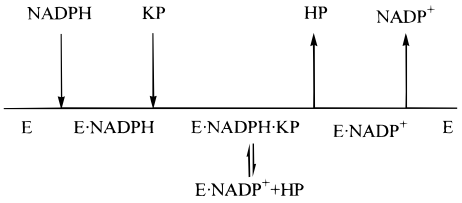
deprotonation decreases V/K_{KP} and V in the forward direction is likely a group which must be protonated to donate a hydrogen bond to the carbonyl group of ketopantoate and to function as a general acid. The pK value exhibited by this group in the V/K_{KP} profile is displaced outward in the V_{for} profile, as seen for other dehydrogenases (*36*).

The pH dependence of V/K_{NADP^+} reveals that the protonation of a single group exhibiting a pK value of 7.6 decreases this kinetic parameter. This value is much too high to assign to the 2′-phosphate of the nucleotide, and we cannot assign it to anything other than an enzyme side chain, possibly the aspartate or glutamate residue observed in the consensus nucleotide sequence that interacts with the adenosyl ribose hydroxyl groups. Both V/K_{HP} and V_{rev} are influenced by the ionization state of a single group, exhibiting a pK value of 8.06 and 7.82, respectively. The protonation of this group decreases both V/K_{HP} and V_{rev}, and is likely to be a general base, required both to accept a hydrogen bond from the 2-hydroxyl group of HP and also to initiate catalysis in this direction by proton removal from the hydroxyl group.

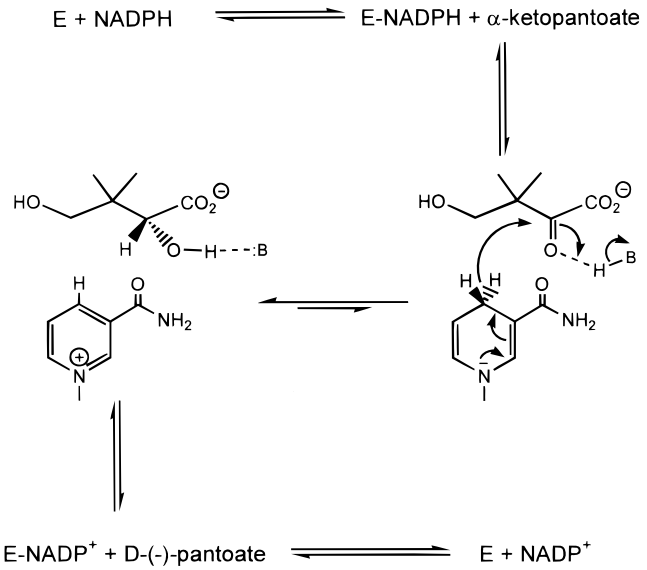
Such opposite pH dependence of the maximum velocities in the forward and reverse directions of ketopantoate

Scheme 2

Kinetic Mechanism



Scheme 3



reductase suggests a general acid−base chemical mechanism (Scheme 3) in which a single group is required to be in opposite ionization states for catalysis in the two directions. In the direction of KP reduction, after the sequential binding of NADPH and KP, the C-4S hydride is transferred from NADPH to the C-2 carbonyl of ketopantoate which is hydrogen bonded to the protonated form of an enzyme catalytic residue. Whether a discrete alkoxide intermediate is present or protonation is concerted with hydride transfer is unknown at this time. Deprotonation of this enzyme group, exhibiting a pK value of 8.06 in the E−NADPH complex and 8.38 in the E−NADPH−KP complex, leads to a decrease in both V/K_{KP} and the maximum velocity. In the reverse reaction, this same catalytic group functions as a general base to accept a hydrogen bond from the C-2 hydroxyl of pantoate. Catalysis in this direction is initiated by proton removal to generate an alkoxide, which collapses with transfer of a hydride ion from C-2 of pantoate to $NADP^+$, generating ketopantoate and NADPH. Protonation of this group, exhibiting a pK value of 8.06 in the E−$NADP^+$ complex and 7.82 in the E−$NADP^+$−HP complex, leads to a decrease in both V/K_{HP} and the maximum velocity in the reverse direction. This general acid−base mechanism has been documented for both the yeast and liver alcohol dehydrogenase catalyzed reactions (*33*), and the studies reported here suggest a similar chemical mechanism for ketopantoate reductase.

This conservation of chemical mechanism occurs despite no significant primary structural similarity between bacterial ketopantoate reductases and other secondary alcohol dehydrogenases. Among this large family of enzymes, there are two representative classes: the large (300−380 amino acids), multimeric alcohol dehydrogenases that contain divalent metals (*37*), as exemplified by the Zn-containing yeast alcohol dehydrogenase; and the smaller (240−280 amino acids) mono- or multimeric alcohol dehydrogenases that do not require metal ions for activity (*38*), as exemplified by the hydroxysteroid dehydrogenases. In the former class, the nucleotide binding sequence motif (GXGXXG) is found in the middle of the primary sequence, while in the latter class a different motif (GXXXGXG) is found at the amino terminus of the coding sequence. In the former class, the hydride ion transferred to the carbonyl-containing substrate is the *pro-R* hydrogen, while in the latter class, the hydride ion transferred to the carbonyl-containing substrate is the *pro-S* hydrogen. Finally, residues that function in catalysis in the former class include the metal ion and a conserved histidine residue, while a conserved YXASK sequence is responsible for carbonyl polarization, and alkoxide protonation in the latter case. Ketopantoate reductase shares features with each of these classes. The highly conserved GXGXXG nucleotide binding motif among the ketopantoate reductases is most similar to the large, metal-dependent, multimeric alcohol dehydrogenases, but is located at the amino terminus. Catalysis occurs via hydride transfer of the *pro-S* hydrogen of NADPH, similar to the stereochemistry observed for members of the short-chain alcohol dehydrogenase class (*38*). Finally, the ketopantoate reductases do not require metal ions for activity, like the short-chain alcohol dehydrogenase class, and can exist both in monomeric native states, as demonstrated here for the *E. coli* reductase, and in multimeric states, as reported for the reductase from *Pseudomonas maltophilia* 845 (*5*). Although these properties suggest that ketopantoate reductases are more similar to the short-chain class, the lack of overall sequence homology or the conserved YXASK sequence involved in catalysis suggests that the reductase must use other enzyme side chains to promote carbonyl polarization and alkoxide protonation. Alignment of the seven bacterial ketopantoate reductase sequences (Figure 7) reveals no conserved histidine residues, but two conserved lysine residues (K72 and K176 in the *E. coli* reductase sequence) and four conserved acidic residues (E210, E240, D248, and E256 in the *E. coli* reductase sequence) that may function in catalysis. Mutagenesis of these residues and evaluation of the effect of their replacement on catalysis are underway.

ACKNOWLEDGMENT

We thank Dr. Xiangyang Chen for providing ketopantoic acid and Mr. Mehul Patel for technical assistance.

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BI992676G