Substrate Specificity and Kinetic Isotope Effect Analysis of the *Eschericia coli* Ketopantoate Reductase†

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ABSTRACT: Ketopantoate reductase (EC 1.1.1.169), an enzyme in the pantothenate biosynthetic pathway, catalyzes the NADPH-dependent reduction of α-ketopantoate to form D-(−)-pantoate. The enzyme exhibits high specificity for ketopantoate, with *V* and *V/K* for ketopantoate being 5- and 365-fold higher than those values for α-ketoisovalerate and 20- and 648-fold higher than those values for α-keto-*β*-methyl-*n*-valerate, respectively. For pyridine nucleotides, *V/K* for *β*-NADPH is 3−500-fold higher than that for other nucleotide substrates. The magnitude of the primary deuterium kinetic isotope effects on *V* and *V/K* varied substantially when different ketoacid and pyridine nucleotide substrates were used. The small primary deuterium kinetic isotope effects observed using NADPH and NHDPH suggest that the chemical step is not rate-limiting, while larger primary deuterium isotope effects were observed for poor ketoacid and pyridine nucleotide substrates, indicating that the chemical reaction has become partially or completely rate-limiting. The pH dependence of ^{D}V using ketopantoate was observed to vary from a value of 1.1 at low pH to a value of 2.5 at high pH, while the magnitude of $^{D}V/K_{NADPH}$ and $^{D}V/K_{KP}$ were pH-independent. The value of ^{D}V is large and pH-independent when α-keto-*β*-methyl-*n*-valerate was used as the ketoacid substrate. Solvent kinetic isotope effects of 2.2 and 1.2 on *V* and *V/K*, respectively, were observed with α-keto-*β*-methyl-*n*-valerate. Rapid reaction analysis of NADPH oxidation using ketopantoate showed no "burst" phase, suggesting that product-release steps are not rate-limiting and the cause of the small observed kinetic isotope effects with this substrate pair. Large primary deuterium isotope effects on *V* and *V/K* using 3-APADPH in steady-state experiments, equivalent to the isotope effect observed in single turnover studies, suggests that chemistry is rate-limiting for this poorer reductant. These results are discussed in terms of a kinetic and chemical mechanism for the enzyme.

Ketopantoate reductase (EC 1.1.1.169) catalyzes the formation of D-pantoate from α-ketopantoate in the biosynthesis of pantothenate in bacteria, yeast, and plants (Scheme 1). Pantothenate (vitamin B_5) is synthesized by the subsequent condensation of D-pantoate with *β*-alanine and is used for the biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP) (*1*). CoA and ACP are important acyl group carriers and are involved in numerous metabolic processes including fatty acid synthesis and oxidization, hormone synthesis and regulation, transcription, protein transport, enzyme activation, and cell signaling (*2*−*7*). CoASH is the major intracellular thiol in *Staphylococcus aureus* and is thought to function like glutathione in this human pathogen (*8*, *9*). CoA and ACP are also important in the biosynthesis of polyketides and nonribosomal peptide biosynthesis (*10*, *11*). Both ketopantoate reductase and acetohydroxy acid isomeroreductase are able to catalyze the reduction of ketopantoate; the latter also catalyzes the formation of α,*β*-dihydroxy-*β*-methylvalerate and α,*β*-dihydroxyisovalerate from α-aceto-α-hydroxybutyrate and α-acetolactate, respectively, in the biosynthesis of isoleucine and valine (*12*). Mg^{2+} is required for ketopantoate reduction catalyzed by acetohydroxy acid isomeroreductase but not by ketopantoate reductase (*13*, *14*). The *panE* gene encoding ketopantoate reductase has been demonstrated to be identical to the *apbA* gene, which was identified in the *a*lternative *p*yrimidine *b*iosynthetic (apb) pathway in *Salmonella typhimurium* (*15*, *16*).

Ketopantoate reductase has been purified and partially characterized from *Pseudomonas maltophilia* 845 and *S. typhimurium* (*14*, *15*). We have previously cloned, overexpressed, purified, and preliminarily characterized the *Escherichia coli panE*-encoded ketopantoate reductase (*17*, *18*). It is monomeric with a molecular mass of 34000 Da and catalyzes the stereospecific transfer of the 4*S*-hydrogen of NADPH[1] to ketopantoate. On the basis of steady-state product and dead-end inhibition studies, the kinetic mechanism was suggested to be ordered, with NADPH binding followed by ketopantoate binding and $NADP^+$ release

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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; IPTG, isopropyl 1-thio-*β*-D-galactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEA, triethanolamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; tNADPH, reduced *β*-thionicotinamide adenine dinucleotide phosphate; 3-APADPH, reduced *β*-3-acetylpyridine adenine dinucleotide phosphate; α-NADPH, reduced α-nicotinamide adenine dinucleotide phosphate; NHDPH, reduced *β*-nicotinamide hypoxanthine dinucleotide phosphate; 3′-NADPH, reduced *β*-3′-nicotinamide adenine dinucleotide phosphate.

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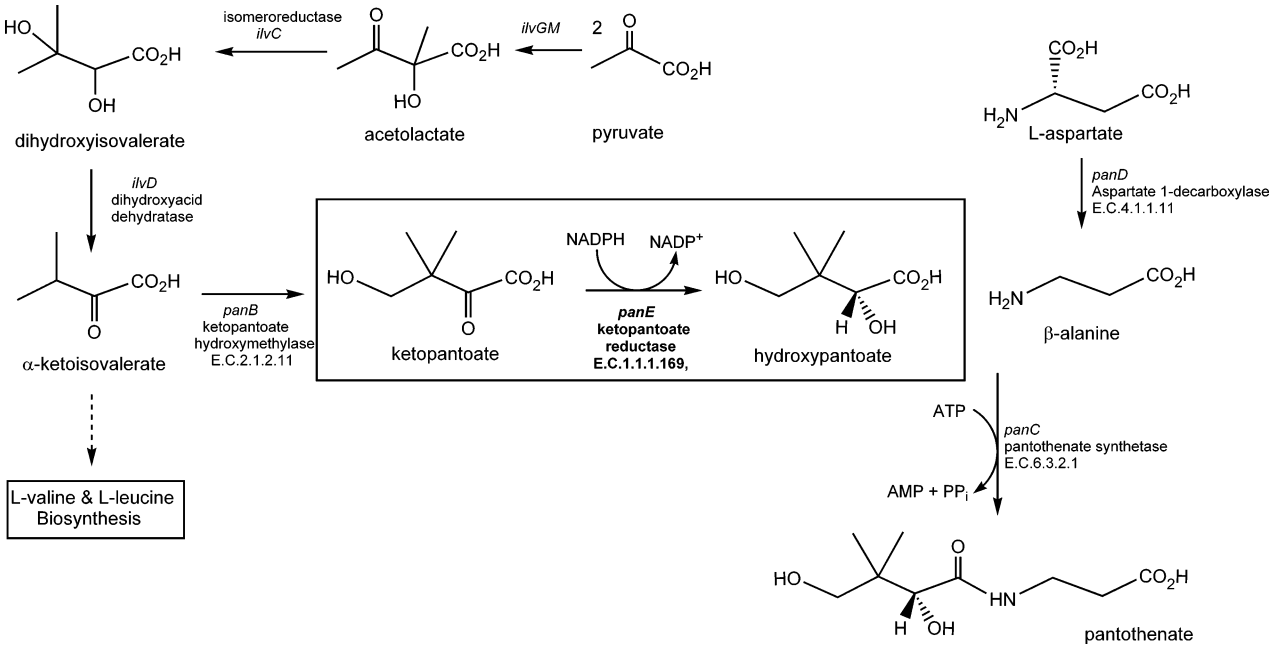
Scheme 1: Pantothenate Biosynthetic Pathway in Bacteria, Yeast, and Plants



following hydroxypantoate release. The pH dependence of *V* and *V/K* for the *E. coli* enzyme suggests the involvement of a general acid/base in the catalytic mechanism. Ketopantoate reductase uses Lys176 and Glu256 residues as key residues to function in catalysis and substrate binding (*18*). The importance of Lys176 and Glu256 residues for substrate binding and catalysis has been confirmed in the reported three-dimensional structure of the *E. coli* ketopantoate reductase, showing these residues in the presumed active site of the apoenzyme (*19*). The small primary and solvent deuterium kinetic isotope effects in the forward and reverse directions suggest that the chemical reaction is not rate-limiting when ketopantoate and NADPH were used as substrates (*17*) and that one or more physical steps are rate-limiting. In the present study, we investigate the enzyme's specificity for various ketoacid and pyridine nucleotide substrates and determine the magnitudes of various isotope effects using these nonphysiological substrates.

MATERIALS AND METHODS

Materials. Ketopantoyl lactone, α-ketoisovalerate, α-keto-β-methyl-*n*-valerate, D-glucose, and D-glucose-1-*d* were obtained from Aldrich. All oxidized pyridine nucleotides, IPTG, ATP, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (type XXIV), hexokinase from yeast, and all buffer components were purchased from Sigma. D_2O (99.9 atom %) was purchased from Cambridge Isotope Laboratory. All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. *Pfu* DNA polymerase was from Stratagene. The pET23a(+) plasmid and *E. coli* strain BL21(DE3) were obtained from Novagen. All chromatographic supports were from Pharmacia.

Preparation of Ketopantoic 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 (*20*, *21*). ^{1}H NMR (300 MHz, D_2O): δ 3.77 (s, 2H), δ 1.30 (s, 6H).

Preparation of 4S-Labeled Reduced Nucleotides. All 4*S*-protonated and -deuterated nucleotides were prepared by enzymatic reduction of the oxidized nucleotides with *L. mesenteroides* glucose-6-phosphate dehydrogenase and hexokinase using glucose-1-*h* or glucose-1-*d* as the ^{1}H or ^{2}H source (*22*). 4*S*-Protonated or -deuterated nucleotides were purified on a Mono Q column, and the fractions with appropriate absorbance ratios (i.e., NADPH, $A_{260}/A_{340} \leq 2.3$; tNADPH, $A_{260}/A_{395} \leq 1.3$; 3-APADPH, $A_{260}/A_{363} \leq 1.5$; NHDPH, $A_{249}/A_{340} \leq 2.0$) were pooled. The concentrations of 4*S*-labeled reduced nucleotides were determined by enzymatic end-point assays with yeast glutathione reductase in the presence of excess oxidized glutathione.

Expression and Purification of Ketopantoate Reductase. The *E. coli panE* gene was overexpressed in the *E. coli* strain BL21(DE3), and the enzyme was purified to homogeneity as previously described (*17*). Protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as a standard. The purity of ketopantoate reductase was determined by SDS−polyacrylamide gel electrophoresis according to the method of Laemmli (*23*). Electrospray ionization/mass spectrometry was performed on the purified enzyme to confirm the molecular mass of the expressed proteins.

Steady-State Kinetic Studies. Initial velocities of ketopantoate reductase activity were determined by monitoring the decrease in absorbance of NADPH at 340 nm ($\epsilon_{340} = 6.22$ 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, 200 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 ($\leq$10 μL).

Primary Deuterium and Solvent Kinetic Isotope Effects. Primary deuterium kinetic isotope effects were determined either at a saturating concentration of ketoacid using variable concentrations of (4*S*)-4-^{1}H- and (4*S*)-4-^{2}H-labeled reduced pyridine nucleotide or at a saturating concentration of (4*S*)-4-^{1}H- and (4*S*)-4-^{2}H-labeled reduced pyridine nucleotide using variable concentrations of ketoacid. In addition, isotope effects on the kinetic parameters *V*, V/K_a, V/K_b, and K_{ia} were

Table 1: Kinetic Parameters and Primary and Solvent Kinetic Isotope Effects of Ketoacids for *E. coli* Ketopantoate Reductase

ketoacid	K_m (mM)	V_{max} (μmol min^{-1}mg^{-1})	V/K	^{D}V	$^{D}V/K_{NADPH}$	^{D_2O}V	$^{D_2O}V/K$
α-ketopantoate	0.12 ± 0.01	14 ± 0.4	117	1.33 ± 0.02 (H_2O)[a] 1.16 ± 0.11 (D_2O)	1.54 ± 0.04 (H_2O)[a] 1.04 ± 0.07 (D_2O)	1.32 ± 0.07	1.27 ± 0.08
α-ketoisovalerate	8.7 ± 1.2	2.8 ± 0.1	0.32	2.7 ± 0.1	2.7 ± 0.2	nd[b]	nd
α-keto-β-methyl-*n*-valerate	3.8 ± 0.4	0.69 ± 0.02	0.18	3.9 ± 0.1 (H_2O) 3.9 ± 0.1 (D_2O)	1.9 ± 0.2 (H_2O) 2.9 ± 0.2 (D_2O)	2.20 ± 0.05	1.2 ± 0.03

[a] Determined at a fixed, saturating concentration of ketopantoate. For comparison, the isotope effects determined from eq 3 are $^{D}V = 1.11 \pm 0.07$, $^{D}V/K_{NADPH} = 1.2 \pm 0.3$, $^{D}V/K_{KP} = 1.3 \pm 0.2$, and $^{D}K_{iNADPH} = 0.9 \pm 0.4$. [b] nd: not determined.

determined by varying the concentrations of [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH at five fixed concentrations of ketopantoate. Solvent kinetic isotope effects on *V* and *V/K* were measured at a saturating concentration of NADPH (200 μM) at variable concentrations of α-keto-β-methyl-*n*-valerate (1−10 mM) in 100 mM Hepes prepared in H_2O or 99.9% D_2O at pH(D) 7.5, at 25 °C. Multiple isotope effects on *V* and *V/K* were determined at a saturating concentration of ketopantoate (1 mM) and at variable concentrations of [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH in 100 mM HEPES prepared in H_2O or 99.9% D_2O at pH(D) 7.5, at 25 °C.

pH Dependence of Primary Deuterium Kinetic Isotope Effects. The following buffers at 100 mM concentration were used to maintain the pH of reaction: MES (5.9−6.7), HEPES (6.8−8.2), and CHES (8.6−9.6). The pH dependence of the primary deuterium kinetic isotope effects on *V* and *V/K* were measured by comparing the initial velocities for the oxidation of [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH at a saturating concentration of either ketopantoate or α-keto-β-methyl-*n*-valerate at various pH values at 25 °C. The pH values were measured after each enzyme reaction.

Rapid Kinetics. Stopped-flow experiments were performed with an Applied Photophysics π*-180 spectrophotometer (SX 18 MV) (mixing dead time = 1.5 ms). The oxidation of reduced pyridine nucleotide was monitored at 340 nm. Reactions were carried out at 25 °C in 100 mM HEPES, pH 7.5. Reactions were initiated by rapidly mixing equal volumes of substrates (1 mM ketopantoate and 200 μM NADPH) and enzyme (0, 15, 30, and 60 μM) into the flow cell. Data acquisition and analysis were carried out with Applied Photophysics software. Primary deuterium kinetic isotope effects on *V* and *V/K* were obtained using [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH or 3-APADPH at a saturating concentration of ketopantoate (1 mM) in 100 mM HEPES, pH 7.5 at 25 °C.

Isothermal Titration Calorimetry. Calorimetric titrations were conducted using a MCS microcalorimeter (Microcal, Inc., Northampton, MA). Enzyme was dialyzed against 50 mM HEPES buffer, pH 7.5 at 4 °C, and the concentration of the enzyme used in the experiment was 110 μM. NADPH and ketopantoate were prepared in the same buffer at concentrations of 2 and 20 mM, respectively. In individual titrations, injections of 7 μL of the ligand solution were made via the computer-controlled microsyringe into the 1.5 mL enzyme solution at intervals of 4 min, while being stirred at 300 rpm, at 27 °C. The data were fitted to a theoretical titration curve using software supplied by Microcal, Inc., with ΔH (the enthalpy change in kcal mol^{-1}), K_a (the binding constant in M^{-1}), and *n* (the number of binding sites per monomer) as adjustable parameters. The thermodynamic parameters ΔG and ΔS were calculated from eqs 4 and 5.

Data Analysis. The steady-state kinetic data were fitted to the appropriate rate equations by using the programs developed by Cleland (*24*). The individual substrate saturation kinetic data were fitted to eq 1. Primary deuterium kinetic isotope effect data were fitted to the eq 2:

$$v = VA/(K + A) \quad (1)$$

$$v = VA/[K(1.0 + F_iE_{V/K}) + A(1.0 + F_iE_V)] \quad (2)$$

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 one on *V/K*, and E_v is the isotope effect minus one on *V*. Isotope effects on the kinetic parameters, *V*, V/K_a, V/K_b, and K_{ia} were determined from fits of the initial velocity patterns using either [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH using eq 3:

$$v = VAB/\{[K_{ia}K_b(1 + F_iE_{K_{ia}}) + K_bA](1 + F_iE_{V/K_b}) + K_aB(1 + F_iE_{V/K_a}) + AB(1 + F_iE_v)\} \quad (3)$$

where the determined values of *E* for each of the kinetic constants represent the isotope effect on that kinetic parameter minus one.

For the calculation of thermodynamic binding parameters

$$\Delta G = -RT \ln K_a \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

where *T* is the absolute temperature and $R = 1.98$ cal mol^{-1} K^{-1}.

RESULTS

Ketoacid and Pyridine Nucleotide Specificity. The specificity of the *E. coli* ketopantoate reductase for ketoacid substrates was determined at saturating concentrations of NADPH (200 μM) at pH 7.5. Compared to ketopantoate, α-ketoisovalerate and α-keto-β-methyl-*n*-valerate exhibit lower V_{max} and higher K_m values (Table 1). The *V/K* value for ketopantoate is 365- and 648-fold higher than those determined for α-ketoisovalerate and α-keto-β-methyl-*n*-valerate, respectively. No detectable activity was observed with other ketoacids including pyruvate, α-ketovalerate, α-ketobutyrate, 2-oxohexanoic acid, α-ketoisocaproic acid, benzoylformic acid, and phenylpyruvate.

The kinetic parameters of six pyridine nucleotide substrates were measured at saturating concentrations of ketopantoate (1 mM) and are summarized in Table 2. Relative *V* and *V/K* values were determined by comparing the *V* and *V/K* value for each pyridine nucleotide analogue to that of β-NADPH

Table 2: Kinetic Parameters and Primary Deuterium Isotope Effects Exhibited by Nucleotide Substrates

nucleotide	rel V (%)	K_m (μM)	V/K_{rel}	^DV	$^DV/K$
β-NADPH	100	4 ± 0.4	100	1.33 ± 0.02	1.54 ± 0.04
NHDPH	498	120 ± 10	16.6	1.21 ± 0.09	1.15 ± 0.06
tNADPH	31.3	18.0 ± 2.6	7.0	2.8 ± 0.4	1.2 ± 0.2
3-APADPH	72	7.8 ± 0.5	36.9	3.0 ± 0.2	1.9 ± 0.3
α-NADPH	1.54	36 ± 3	0.17	nd[a]	nd
3′-NADPH	384	75 ± 20	20.5	nd	nd

[a] nd: not determined.

at the same time under identical experimental conditions. NHDPH and 3′-NADPH exhibited 5- and 4-fold higher maximal velocities than β-NADPH but also 30- and 19-fold higher K_m values, respectively. α-NADPH, tNADPH, and 3-APADPH exhibited lower V_{max} and higher K_m values compared to β-NADPH. The magnitude of the value of V/K obtained with all alternate pyridine nucleotide substrates is lower than that with β-NADPH (Table 2).

Primary Deuterium Kinetic Isotope Effects. Primary deuterium kinetic isotope effects were determined in two ways: by determining the isotope effects on V, V/K_a, V/K_b, and K_{ia} by comparing initial velocity patterns obtained with either [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH or by varying the concentrations of (4*S*)-4-^{1}H- and (4*S*)-4-^{2}H-labeled reduced pyridine nucleotide, at a fixed, saturating concentration of ketoacid substrates or by varying the concentrations of ketoacid substrate at a fixed, saturating concentration of either [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH at pH 7.5, at 25 °C. Primary deuterium kinetic isotope effects on V and V/K_{NADPH} exhibited values of 2.67 ± 0.04 and 2.73 ± 0.17 using α-ketoisovalerate and 3.9 ± 0.1 and 1.9 ± 0.2 using α-keto-β-methyl-*n*-valerate as ketoacid substrates, respectively (Table 1). At a fixed, saturating concentration of ketopantoate (1 mM), the primary deuterium kinetic isotope effects on both V and V/K were determined for various reduced pyridine nucleotide substrates (Table 2). Small primary deuterium isotope effects on V and V/K were observed with NADPH and NHDPH, while larger isotope effects using tNADPH and 3-APADPH were observed on V, with more modest increases in the isotope effects on V/K.

pH Dependence of Primary Deuterium Kinetic Isotope Effects. The effect of pH on the primary deuterium kinetic isotope effects on both V and V/K_{NADPH} were determined by comparing the rate of [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH oxidation at saturating concentrations of ketopantoate (1 mM) at pH values between 6.5 and 9.6 (Figure 1). The primary deuterium kinetic isotope effect on V exhibited its maximal value of 2.5 ± 0.2 at high pH values and decreased to a value of 1.1 ± 0.1 at low pH values, but the magnitudes of $^DV/K_{NADPH}$ were small ($\sim$1.5) and pH-independent. The magnitude of $^DV/K_{KP}$ is also pH-independent with an average value of $\sim$1.8. The pH profile we previously determined shows that the log of the maximum velocity decreases at high pH as a single group with a pK value of 8.4 ± 0.2 is deprotonated (Figure 1). At a saturating concentration of α-keto-β-methyl-*n*-valerate, the primary deuterium kinetic isotope effects on V are pH-independent (Figure 2).

Solvent and Multiple Kinetic Isotope Effects. Solvent kinetic isotope effects on V and V/K exhibit values of 2.20 $\pm$ 0.05 and 1.20 $\pm$ 0.03 at a saturating concentration of

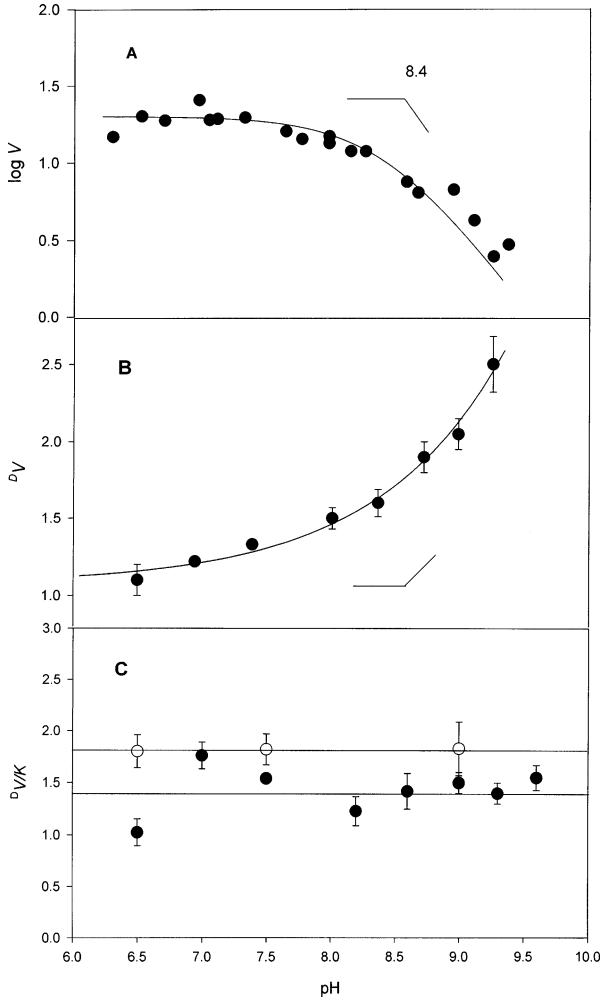


FIGURE 1: pH dependence of the primary deuterium kinetic isotope effects on the ketopantoate reductase reaction: (A) pH profile of V_{max}; (B) pH dependence of ^DV; (C) pH dependence of the $^DV/K$ values for NADPH (filled circles) and ketopantoate (open circles). Individual values of ^DV and $^DV/K$ are fits of the data to eq 2.

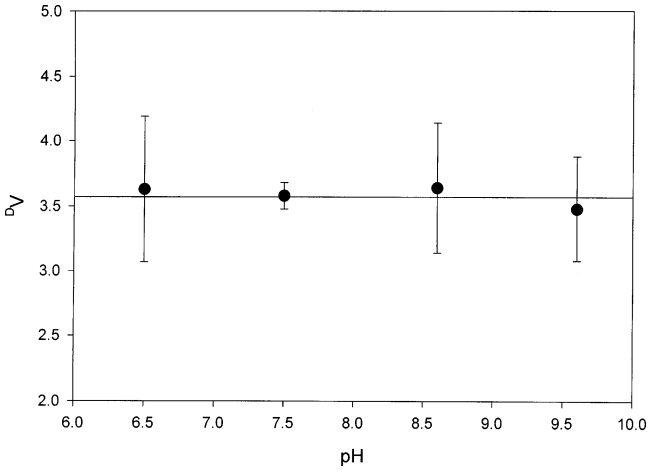


FIGURE 2: Effect of pH on the primary deuterium kinetic isotope effect on V values determined using [(4*S*)-4-^{1}H]NADPH and [(4*S*)-4-^{2}H]NADPH as the variable substrates at a saturating concentration of α-keto-β-methyl-*n*-valerate.

NADPH and at variable concentrations of α-keto-β-methyl-*n*-valerate, respectively (Figure 3). The primary deuterium kinetic isotope effects on V and V/K, determined at a saturating concentration of α-ketopantoate and variable

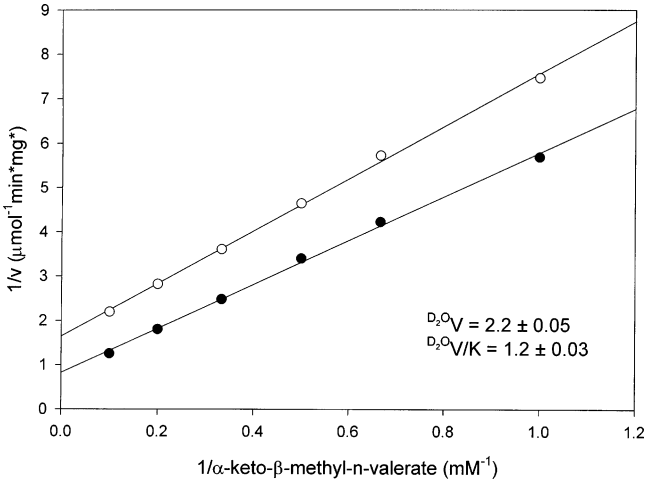


FIGURE 3: Solvent kinetic isotope effects using α-keto-*β*-methyl-*n*-valerate as the variable substrate at a fixed, saturating [NADPH]. Data were measured in H_2O (●) and in D_2O (○).

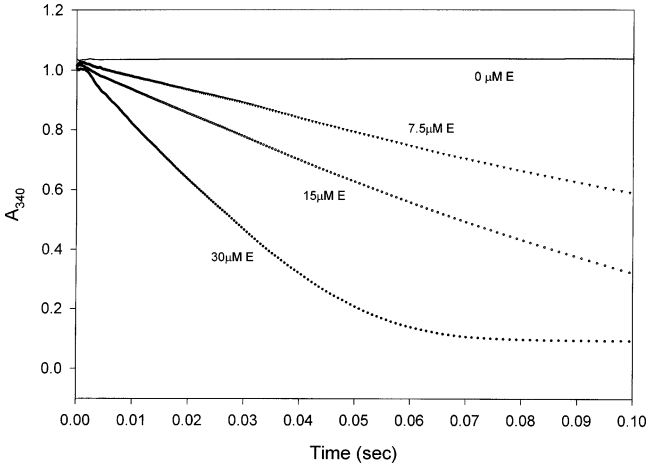


FIGURE 4: Time course for the oxidation of NADPH. Reactions were carried out at 25 °C in 50 mM Hepes, pH 7.5, using 170 μM NADPH, 1 mM ketopantoate, and 0, 7.5, 15, and 30 μM enzyme.

concentrations of [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]NADPH, were 1.33 ± 0.02 and 1.54 ± 0.04 in H_2O and 1.16 ± 0.11 and 1.04 ± 0.07 when determined in D_2O (Table 1).

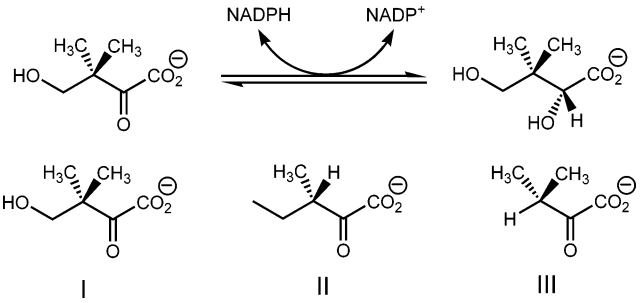
Isothermal Titration Calorimetry. The binding of NADPH and ketopantoate to the free enzyme was determined by ITC. Ketopantoate reductase exhibited high affinity for NADPH with a calculated K_d value of 2.1 ± 0.3 μM, while no interaction between ketopantoate (at concentrations up to 20 mM) and the free enzyme was detected by ITC (data not shown).

Rapid Kinetics. Rapid reaction studies of ketopantoate reductase were determined by measuring the decrease in absorbance of 340 nm upon the oxidation of NADPH or 3-APADPH using a rapid mixing, stopped-flow instrument. Stopped-flow traces for the NADPH oxidation using 0−30 μM ketopantoate reductase are presented in Figure 4. No "burst" phase was observed. Under rapid kinetic conditions, the primary deuterium kinetic isotope effects on nucleotide oxidation using [(4*S*)-4-^{1}H]- and [(4*S*)-4-^{2}H]pyridine nucleotides were $^Dk_{ox}$ = 1.32 ± 0.02 for NADPH and $^Dk_{ox}$ = 3.5 ± 0.1 for 3-APADPH, respectively.

DISCUSSION

Ketopantoate reductase is the second of four enzymes that convert α-ketoisovalerate, a precursor of L-valine, into pantothenate. The *E. coli* enzyme has been kinetically characterized (*17*), highly conserved residues were mutated, and the altered forms of the enzyme were studied (*18*). The reductase uses a general acid/base chemical mechanism in catalysis (*17*, *18*), as reported for both long- and short-chain alcohol dehydrogenases (*25*, *26*). Ketopantoate reductase exhibits properties that are more similar to those of the short-chain alcohol dehydrogenase family, including size, metal ion dependence, and domain organization. However, unlike other short-chain alcohol dehydrogenase family members which possess a conserved YXASK motif used in catalysis (*27*), ketopantoate reductase lacks this sequence motif and instead uses K176 and E256 residues for catalysis and ketopantoate/pantoate binding (*18*). The three-dimensional structure of the *E. coli* enzyme was recently reported and revealed an overall structure consisting on an N-terminal nucleotide binding domain and an α-helical C-terminal domain, proposed to be the site of interaction with ketopantoate (*19*). Both K176 and E256 were shown to be at the domain interface where substrates were proposed to bind. This wealth of kinetic and structural information encouraged us to further characterize the reaction mechanism using alternate substrates and to determine the magnitude of isotope effects and rate-limiting steps in the reaction.

Scheme 2: (Top) Reaction Catalyzed by Ketopantoate Reductase and (Bottom) Structures of Ketopantoate (**I**), α-Keto-*β*-methyl-*n*-valerate (**II**), and α-Ketoisovalerate (**III**)



Substrate Specificity. We examined a series of α-ketoacids as potential substrates for ketopantoate reductase. Ketopantoate reductase is relatively specific for its physiological substrate ketopantoate, and α-ketoisovalerate and α-keto-*β*-methyl-*n*-valerate, structurally similar to ketopantoate (Scheme 2), exhibited relatively poor substrate activity with higher K_m and lower V_{max} values. No detectable activity was observed with other ketoacids. The *V/K* values for α-ketoisovalerate and for α-keto-*β*-methyl-*n*-valerate are 365- and 648-fold lower than that for ketopantoate, respectively (Table 1), suggesting that the *γ*-hydroxyl group of ketopantoate, previously proposed to contribute to enzyme binding (*18*, *19*), is important for activity with ketopantoate reductase.

Six pyridine nucleotides were examined for their activity with ketopantoate reductase. Of these, tNADPH, 3-APADPH, and α-NADPH exhibited lower maximum velocities and higher K_m values when compared to *β*-NADPH. tNADPH and 3-APADPH differ in the nature of their nicotinamide substituents and possess more positive reduction potentials. However, NHDPH and 3′-NADPH, which have the same reduction potential as *β*-NADPH, exhibited 5 and 4 times higher V_{max} values than that of *β*-NADPH, possibly reflecting the more rapid dissociation of the oxidized forms of the two

nucleotides compared to β-NADP$^+$. A higher maximum velocity with NHDPH than with β-NADPH was observed previously in studies of trypanothione reductase (*28*). However, compared to β-NADPH, all pyridine nucleotide substrates exhibit lower *V/K* values (Table 2). While activity could be demonstrated using NADH, saturation kinetics could not be demonstrated using NADH as a substrate at concentrations of up to 400 μM.

Isotope Effects. The previously reported steady-state kinetic analysis of the KPR reaction suggested that the reductase utilizes an ordered, sequential kinetic mechanism (*17*), with NADPH binding preceding ketopantoate binding and pantoate release preceding NADP$^+$ release. A more sensitive method to determine the degree of randomness in a sequential, bireactant mechanism is the determination and analysis of isotope effects on the kinetic parameters and the pH dependence of these effects.

We have previously reported small primary kinetic isotope effects on both *V* and V/K_{NADPH} using ketopantoate and either [(4*S*)-4-^{1}H]- or [(4*S*)-4-^{2}H]NADPH and the reductase (*17*). The nonunitary value of $^DV/K_{NADPH}$ is inconsistent with our other steady-state kinetic studies, suggesting an ordered kinetic mechanism. However, the small value of $^DV/K_{NADPH}$ relative to its standard error suggested that the kinetic mechanism was predominantly ordered.

To obtain information concerning the ability of NADPH and ketopantoate to bind to the free enzyme, we determined substrate binding directly using isothermal titration calorimetry. NADPH binds to the free enzyme and exhibits a K_d value of 2.1 ± 0.3 μM, compared to the steady-state K_m value of 4 μM (Table 2). We could not demonstrate KP binding to the free enzyme, suggesting either that KP binds only to the E−NADPH complex (ordered kinetic mechanism) or that the binding of KP to the free enzyme is not enthalpically driven, a possibility we view as unlikely.

Small primary deuterium kinetic isotope effects on both *V* and *V/K* were observed when NADPH and NHDPH were used as reductants of ketopantoate (Table 2), suggesting that these nucleotides have a high commitment to catalysis and that the hydride transfer step is not entirely rate-limiting in the overall reaction (*17*). Significantly larger primary deuterium kinetic isotope effects on *V* were observed when (4*S*)-4-^{1}H- and (4*S*)-4-^{2}H-labeled tNADPH and 3-APADPH were used, suggesting that the hydride transfer to C_2 of ketopantoate for these two weaker nucleotide reductants becomes slower and more rate-limiting. The magnitude of $^DV/K$ for these two nucleotide substrates, particularly 3-APADPH, where the magnitude of $^DV/K$ is statistically much greater than one, argues that, at least for this nucleotide substrate, the kinetic mechanism is random. It is unclear from these studies alone whether the primary deuterium kinetic isotope effect on hydride transfer by these reduced nucleotides (i.e., the intrinsic isotope effect) is similar to what we estimate for NADPH (see below). The magnitude of ^DV can be strongly influenced by the rates of steps that are not related to hydride transfer. For example, in the reactions catalyzed by *Drosophila melanogaster* alcohol dehydrogenase (*29*, *30*), D-glyceraldehyde-3-phosphate dehydrogenase (*31*), and lactate dehydrogenase (*32*), the dissociation of the product nucleotide from the active site, or the isomerization of enzyme−product complexes, is the rate-limiting step in the reaction. Thus small, or unitary, kinetic isotope effects on *V* are observed in these reactions. This does not appear to be relevant to the KPR-catalyzed reaction, as discussed below.

In the present studies, we have also determined the primary deuterium isotope effects on *V* and V/K_{NADPH} using α-ketoisovalerate ($^DV = 2.7$ and $^DV/K_{NADPH} = 2.7$) and α-keto-β-methyl-*n*-valerate ($^DV = 3.9$ and $^DV/K_{NADPH} = 1.9$), respectively. Both α-ketoisovalerate and α-keto-β-methyl-*n*-valerate are poor substrates for KPR, and these data indicate that the hydride transfer step has become significantly rate-limiting when these poor ketoacid substrates were used. The statistically significant, nonunitary values of $^DV/K_{NADPH}$ when these substrates are used suggest that the kinetic mechanism is not ordered with NADPH binding required before alternative ketoacid binding.

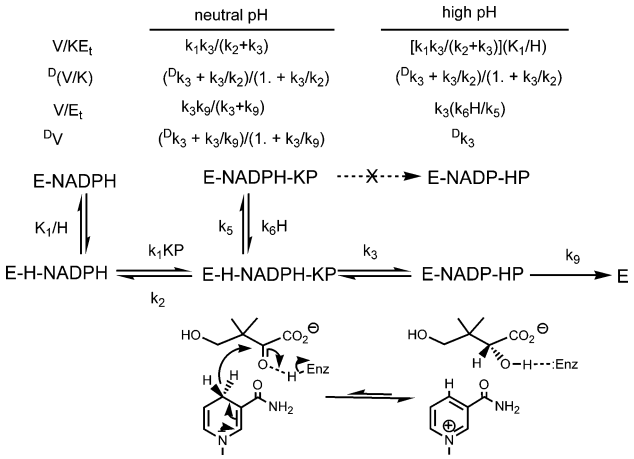
Rapid Kinetics. To assess the possible slow dissociation of NADP$^+$ from the enzyme as the cause for the low value of ^DV, we performed rapid kinetic studies. If the dissociation of product is completely rate-limiting, then the absorbance change would be predicted to exhibit a burst phase, followed by a linear phase that corresponds to the rate observed in steady-state experiments in the reduction of ketopantoate. However, no burst phase was observed in rapid kinetic studies performed at pH 7.5 (Figure 4), arguing that product release is not completely rate-limiting at this pH value.[2]

pH Dependence of the Primary Deuterium Isotope Effects. We have previously shown that the deprotonation of an enzyme group (p*K* value = 8.4) causes the catalytic rate to decrease in the reduction of ketopantoate (*17*, *18*). We suggested that Lys176 must be protonated to polarize the carbonyl and ultimately donate a proton to the alkoxide generated after hydride transfer to the carbonyl (*17*, *18*). We thus posited that the primary deuterium kinetic isotope effect would be affected by the protonation state of this group. In fact, as shown in Figure 1B, the primary deuterium kinetic isotope effect on *V* is pH-dependent. The magnitude of ^DV, determined with NADPH and ketopantoate, varies with pH, increasing from a value of 1.1 at low pH values to a value of 2.5 at high pH values. For comparison, we show the pH dependence of *V* and suggest that as the catalytic rate decreases as a result of the deprotonation of K176, the rate-limiting nature of the hydride transfer step increases. This is reflected in the increasing magnitude of ^DV as the pH increases. The magnitude of both $^DV/K_{NADPH}$ and $^DV/K_{KP}$ are pH-independent and less than ^DV at high pH values (Figure 1C).

In their theoretical analysis of the pH dependence of isotope effects, Cook and Cleland (*33*) described all possible situations where an isotope-dependent step is part of a pH-dependent portion of the mechanism. A model which we have used to analyze our data is shown in Scheme 3. In this model, at the optimal pH where the enzyme is protonated,

[2] The amplitude of a "burst" will depend on (a) the fraction of enzyme that is catalytically active, (b) the ratio of the rate constants for product formation and product dissociation, (c) the ratio of the rate constants for reverse catalytic reaction and product dissociation, and (d) the internal catalytic equilibrium constant (*37*). While all of these may contribute to the inability to experimentally observe a burst, our analysis of the kinetic isotope effects suggests that at pH 7.5 catalysis is only 7−8 times faster than the net rate constant for product dissociation. In our model, only at significantly lower pH values (pH <6), where NADPH is not stable, will chemistry become fast enough to observe completely rate-limiting product release and a significant burst.

Scheme 3: Model of Ketopantoate Reductase Used To Interpret the pH Dependence of the Isotope Effects[a]



[a] From ref *33*.

binding of NADPH occurs to generate EH−NADPH. This complex can be deprotonated to form E−NADPH, which is incapable of binding ketopantoate. Binding of KP to EH−NADPH generates the precatalytic ternary complex, which is converted into the product complex in the catalytic,and isotope-sensitive step, k_3. Deprotonation of EH−NADPH−KP at high pH values leads to an E−NADPH−KP complex that is incapable of reaction and from which KP cannot dissociate to generate the E−NADPH complex. The derived expressions for *V* and *V/K* and the isotope effects on those parameters (*33*) at neutral and high pH values are shown in Scheme 3. Both V/K_{KP} and *V* are pH-dependent, decreasing as the enzyme is deprotonated at high pH values, as observed experimentally (*17*). $^DV/K_{NADPH}$ and $^DV/K_{KP}$ are predicted to be pH-independent, but ^DV is predicted to be pH-dependent, increasing and approaching $^Dk_3$ as the pH increases, as observed experimentally (Figure 1B,C). We believe the magnitude of $^Dk_3$ to be between 3.3 and 3.7, based on the magnitude of $^DV/K_{NADPH}$ measured for the severely catalytically impaired K176A mutant form of the reductase (*18*). Significantly, only in this mechanism can the magnitude of ^DV exceed that of $^DV/K$. The molecular basis of the inability of KP to dissociate from the unprotonated E−NADPH−KP complex is unclear at this time and must await an ongoing structural analysis of the reductase with substrates/products bound at the active site (*19*). Such behavior appears not to have been previously observed with other enzymes.

The analysis described above suggests that the use of a slow substrate, for which catalysis and hydride transfer are largely or completely rate-limiting, should show significantly less dependence of the kinetic isotope effect on the pH value where it is determined. When α-keto-*β*-methyl-*n*-valerate was used as a substrate, the primary deuterium isotope effect on *V* is independent of pH, with values of ^DV ranging from 3.5 to 3.6 between pH 6.5 and pH 9.6 (Figure 2). This suggests that the hydride transfer step is rate-limiting under all pH conditions. The magnitude of the pH-independent primary deuterium kinetic isotope effect with this poor ketoacid substrate and NADPH is also similar to that observed for ketopantoate and NADPH using the K176A mutant and is likely close to the intrinsic primary deuterium kinetic isotope effect for hydride transfer from NADPH to ketoacid substrates, including ketopantoate.

Solvent Kinetic Isotope Effect. The small solvent kinetic isotope effect on *V* (1.3) using ketopantoate and NADPH confirms the non-rate-limiting nature of proton transfer steps in catalysis. Using α-keto-*β*-methyl-*n*-valerate, the ^{D_2O}V is larger and equal to 2.2 (Figure 3), suggesting that the proton transfer step has also slowed in this reaction. The nonequivalence of the solvent kinetic isotope effect on *V* and *V/K* using α-keto-*β*-methyl-*n*-valerate suggests that hydride transfer and alkoxide protonation may kinetically occur in two distinct steps, as proposed recently for the reaction catalyzed by xylose reductase (*34*). If a discrete alkoxide intermediate forms in the reaction, an isotope effect on the slow protonation of the alkoxide will be diminished by the presence of a second isotope effect on a step involved in alkoxide generation (*35*, *36*). The primary deuterium kinetic isotope effects on *V* and *V/K* are 1.33 ± 0.02 and 1.54 ± 0.04 in H_2O and are reduced to 1.16 ± 0.11 and 1.04 ± 0.07 in D_2O, respectively (Table 2). The magnitude of these differences is realistically not large enough to argue that hydride transfer and protonation occur in separate steps. When these studies are repeated using the alternative ketoacid substrate α-keto-*β*-methyl-*n*-valerate, the primary deuterium kinetic isotope effects on *V* and V/K_{NADPH} of 3.9 and 1.9 in H_2O are unaffected and slightly elevated, respectively, in D_2O (^DV = 3.9 and $^DV/K_{NADPH}$ = 2.9). The lack of a significant difference of ^DV in H_2O and D_2O at neutral pH, considering the large solvent kinetic isotope effect on *V*, is most compatible with a synchronous hydride transfer and proton transfer. This interpretation is strongly supported by the observation that, at high pH values, all deuterium kinetic isotope effects are positive. At these high pH values, one would predict that alkoxide protonation would become very slow, causing the reverse commitment to increase until, in the limit, all isotope effects would approach the equilibrium isotope effect, $^DK_{eq}$, which would be inverse in this direction. This is obviously not what we observe experimentally, ruling out a discrete alkoxide intermediate in the reaction.

Conclusions. Ketopantoate reductase catalyzes a typical pyridine nucleotide-dependent reduction of an α-ketoacid. However, it differs significantly from members of both the short-chain and long-chain dehydrogenase families in the enzymatic groups that it uses to assist in catalysis. The data presented here support a kinetic mechanism that is ordered with the natural substrates. The unusual pH dependence of the isotope effects suggests an unusual substrate trapping accompanying deprotonation of a catalytic group. The nature of this interaction will await a more detailed structural examination of the enzyme in complexes with bound substrates.

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