# Identification of Active Site Residues in *E. coli* Ketopantoate Reductase by Mutagenesis and Chemical Rescue†

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ABSTRACT: Ketopantoate reductase (EC 1.1.1.169) catalyzes the NADPH-dependent reduction of α-ketopantoate to D-(−)-pantoate in the biosynthesis of pantothenate. 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. To identify residues involved in catalysis and substrate binding, we mutated the following six strictly conserved residues to Ala: Lys72, Lys176, Glu210, Glu240, Asp248, and Glu256. Of these, the K176A and E256A mutant enzymes showed 233- and 42-fold decreases in $V_{max}$, and 336- and 63-fold increases in the $K_m$ value of ketopantoate, respectively, while the other mutants exhibited WT kinetic properties. The $V_{max}$ for the K176A and E256A mutant enzymes was markedly increased, up to 25% and 75% of the wild-type level, by exogenously added primary amines and formate, respectively. The rescue efficiencies for the K176A and E256A mutant enzymes were dependent on the molecular volume of rescue agents, as anticipated for a finite active site volume. The protonated form of the amine is responsible for recovery of activity, suggesting that Lys176 functions as a general acid in catalysis of ketopantoate reduction. The rescue efficiencies for the K176A mutant by primary amines were independent of the p$K_a$ value of the rescue agents (Brønsted coefficient, $\alpha = -0.004 \pm 0.008$). Insensitivity to acid strength suggests that the chemical reaction is not rate-limiting, consistent with (a) the catalytic efficiency of the wild-type enzyme ($k_{cat}/K_m = 2 \times 10^6\ M^{-1}\ s^{-1}$) and (b) the small primary deuterium kinetic isotope effects, $^DV = 1.3$ and $^DV/K = 1.5$, observed for the wild-type enzyme. Larger primary deuterium isotope effects on *V* and *V*/*K* were observed for the K176A mutant ($^DV = 3.0$, $^DV/K = 3.7$) but decreased nearly to WT values as the concentration of ethylamine was increased. The nearly WT activity of the E256A mutant in the presence of formate argues for an important role for this residue in substrate binding. The double mutant (K176A/E256A) has no detectable ketopantoate reductase activity. These results indicate that Lys176 and Glu256 of the *E. coli* ketopantoate reductase are active site residues, and we propose specific roles for each in binding ketopantoate and catalysis.

Ketopantoate reductase (EC 1.1.1.169) catalyzes the conversion of α-ketopantoate to D-pantoate, which is subsequently condensed with β-alanine to form pantothenate in bacteria, yeast, and plants (Scheme 1). Pantothenate is a B-group vitamin, and is the key precursor for biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP) (*1*). Both CoA and ACP are essential cofactors for cell growth and involved in many metabolic reactions and biosynthetic pathways (*2*). CoA and ACP thioesters are important intermediates in fatty acid biosynthesis (*2*, *3*). CoASH is the major intracellular thiol in *Staphylococcus aureus* and is thought to function like glutathione in this human pathogen (*4*, *5*). CoA and ACP also play important roles in the biosynthesis of polyketides and nonribosomal peptide biosynthesis (*6*, *7*). The reduction of ketopantoate can be catalyzed by both ketopantoate reductase and acetohydroxy acid isomeroreductase; 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 isoleucine and valine biosynthesis (*8*). Ketopantoate reduction catalyzed by acetohydroxy acid isomeroreductase is $Mg^{2+}$-dependent while reduction by ketopantoate reductase is not (*9*, *10*). The *panE* gene encoding ketopantoate reductase has been demonstrated to be identical to the *apbA* gene, which is involved in the alternative pyrimidine biosynthetic (apb) pathway in *Salmonella typhimurium* (*11*, *12*). Ketopantoate reductase has been purified and partially characterized from *Pseudomonas maltophilia* 845 and *Salmonella typhimurium* (*10*, *11*). We have previously cloned, overexpressed, purified, and preliminarily characterized the *E. coli panE*-encoded ketopantoate reductase (*13*). It is monomeric with a molecular mass of 34 000 Da, and catalyzes the stereospecific transfer of the 4*S*-hydrogen of NADPH[1] to ketopantoate. The steady-state kinetic mechanism is ordered with NADPH binding first followed by ketopantoate binding and $NADP^+$ release following hydroxypantoate release. The pH dependence of

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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. Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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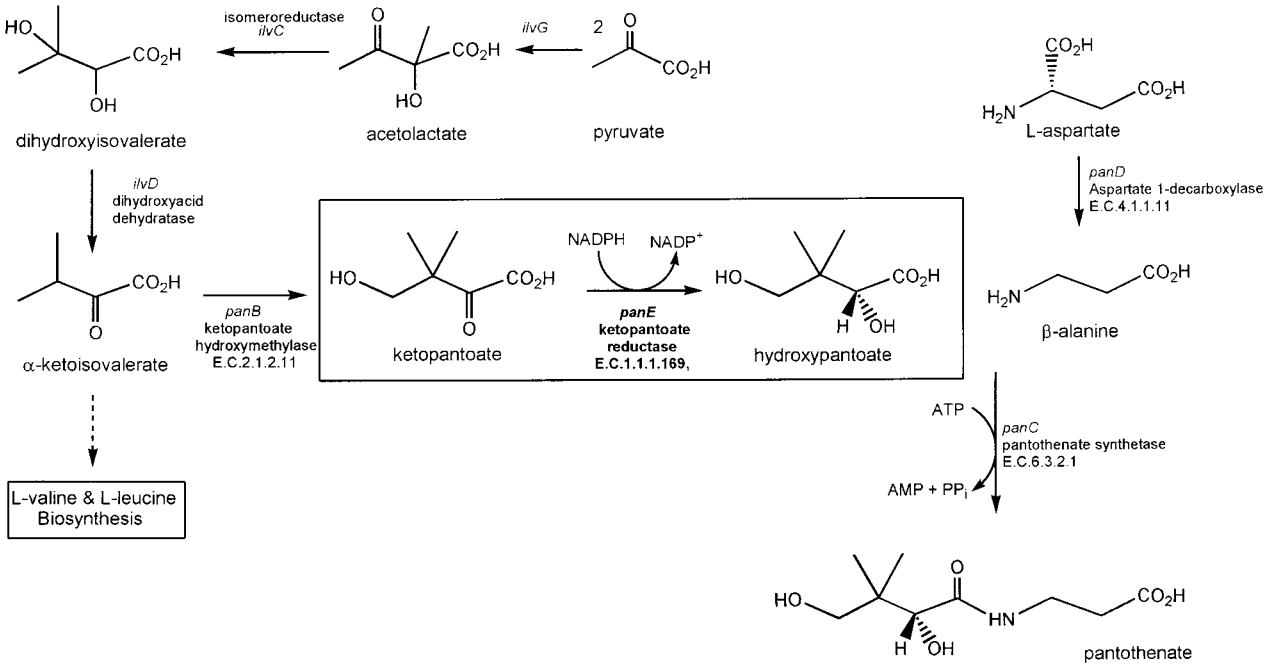
Scheme 1: Pantothenate Biosynthetic Pathway in Bacteria, Yeast, and Plants




*V* and *V/K* for the *E. coli* enzyme suggests the involvement of a general acid/base in the catalytic mechanism.

Multiple sequence alignment of seven known bacterial ketopantoate reductases reveals no conserved histidine or tyrosine residues, the acid/base groups observed in the active site of liver and *Drosophila melanogaster* alcohol dehydrogenases (*14*, *15*), but does reveal two completely conserved lysine residues (*E. coli* K72 and K176) and four conserved acidic residues (*E. coli* E210, E240, D248, and E256) that may function as general acid/base groups. To identify active site and catalytic residues, we mutated these six strictly conserved residues in *E. coli* ketopantoate reductase. Substitution of alanine residues for either Lys176 or Glu256 caused significant reduction in the catalytic efficiency of the enzyme, but the other mutants exhibited WT activity. The roles of these residues in the active site are proposed and discussed.

## MATERIALS AND METHODS

*Materials.* Ketopantoyl lactone, D-glucose-1-*d*, and all amines were obtained from Aldrich. Sodium formate, sodium acetate, NADPH, $NADP^+$, IPTG, ATP, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Type XXIV), hexokinase from yeast, and all buffer components were purchased from Sigma. All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. *Pfu* DNA polymerase and the QuickChange Site-Directed Mutagenesis Kit were from Stratagene. The pET 23a(+) 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−$H_2O$ (*16*, *17*). $^1H$ NMR (300 MHz, $D_2O$): $\delta$ 3.77 (s, 2H), 1.30 (s, 6H).

*Preparation of [4S-4-$^2$H]NADPH.* $NADP^+$ was purified on a FPLC Mono Q anion-exchange column as previously described (*18*). [4S-4-$^2$H]NADPH was prepared by enzymatic reduction of the oxidized nucleotide with *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase using glucose-1-*d* as the $^2H$ source (*19*). [4*S*-4-$^2$H]NADPH was purified on a Mono Q column, and the fractions with absorbance ratios $A_{260}/A_{340} \leq 2.3$ were pooled. The concentration of [4*S*-4-$^2$H]NADPH was determined by enzymatic end-point assays with yeast glutathione reductase in the presence of excess oxidized glutathione.

*Mutagenesis.* The singly mutated genes corresponding to K72A, K176A, K176C, E210A, E240A, D248A, E256A, and E256D were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and pET23a(+):*panE* as the template. The double mutant K176A/E256A was constructed in the same manner using the mutagenic primers for E256A and the template vector of the single mutant K176A. The mutant genes were sequenced in their entirety to ensure that no unexpected mutations occurred.

*Expression and Purification of the Wild-Type and Mutant Enzymes.* Both wild-type and mutant genes were expressed in the *E. coli* strain BL21(DE3). The BL21(DE3) cells containing pET23a(+):*panE* or mutant gene were grown at 37 °C to an $A_{600}$ of 0.5 in LB medium containing 50 $\mu$g/mL carbenicillin. IPTG (0.5 mM) was added to the culture, and growth was continued for an additional 4 h at 37 °C. The wild-type and mutant enzymes were purified to homogeneity as previously described (*13*). Protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as a standard. The purity of WT and mutant ketopantoate reductases was determined by SDS−polyacrylamide gel electrophoresis according to the method of Laemmli (*20*). Electrospray ionization/mass spectrometry was performed on both the purified wild-type and mutant enzymes 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 $\mu$M NADPH, and 1 mM (for WT) or 10−160 mM (for mutant enzymes) sodium ketopantoate in a total volume of 1.0 mL at 25 °C. The reaction was initiated by the addition of ketopantoate reductase (≤10 $\mu$L).

*Primary Deuterium Kinetic Isotope Effects.* Primary deuterium kinetic isotope effects were determined at a saturating concentration of ketopantoate (1 mM for WT and 160 mM for mutant enzymes) at varied concentrations of [4*S*-4-$^1$H]- and [4*S*-4-$^2$H]NADPH by monitoring the decrease in absorbance of NADPH at 340 nm at 25 °C.

*Chemical Rescue of the K176A and E256A Mutants.* All amines, formate, and acetate were prepared in 100 mM Hepes at the desired pH and concentration. The chemical rescue effects of primary amines for K176A, or of formate and acetate for E256A, were determined by measuring the initial velocity of ketopantoate reduction at 25 °C as a function of added amine or carboxylic acid. Assay mixtures contained 100 mM Hepes, pH 7.5, 200 $\mu$M NADPH, 10 mM ketopantoate and various concentrations of rescue agents (12.5−600 mM for 10 different primary amines, 4−192 mM for formate and acetate) in a total volume of 1.0 mL at 25 °C. The reaction was initiated by addition of mutant enzyme (≤10 $\mu$L). Rescue parameters ($V_{max}$ and $K_m^{amine}$) for ketopantoate reduction by added primary amines were obtained from fits of initial velocities versus concentration of amines to eq 1. With primary amines (p$K_a$ ≤ 8.2), the kinetic parameters were corrected for the fraction of the protonated amine as previously described (*21*, *22*). Such correction was not required for amines with a p$K_a$ ≥ 9.0 (≥96% acid at pH 7.5). Similarly, no such correction was required for formate and acetate (>99% base at pH 7.5). The molecular volume for rescue agents was calculated by using the program of SPARTAN SGI Version 5.1.3 OpenGL (Wave function Inc., Irvine, CA).

*Alkylation of K176C Mutant.* Alkylation of K176C was performed as previously described (*23*). The reaction solution contained 20 mM TEA-HCl, pH 7.8, 50 mM bromoethylamine, and 3 mg of K176C in a total volume of 1.0 mL. After incubation for 1 h at 25 °C, the reaction mixture was dialyzed against 5 mM ammonium acetate at pH 6.5, and 1 mM DTT was added to remove residual bromoethylamine. Electrospray ionization/mass spectrometry was performed on both the unalkylated and alkylated K176C mutant enzymes to evaluate the efficiencies of alkylation.

*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 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, respectively), $E_{V/K}$ is the isotope effect minus 1 on *V*/*K*, and $E_v$ is the isotope effect minus 1 on *V*.

## RESULTS

*Mutagenesis.* Sequence alignments of seven known bacterial ketopantoate reductases revealed conserved residues of interest for mutagenesis studies in the *E. coli* enzyme (Figure 1). Alanine mutations were introduced at residues K72, K176, E210, E240, D248, and E256, which were completely conserved in all seven protein sequences. Furthermore, K176C, E256D, and the double mutant K176A/E256A were constructed to evaluate the importance of these two residues in catalysis. All mutant genes were verified by nucleotide sequencing.

*Expression and Purification.* Expression of all mutants genes of ketopantoate reductase in *E. coli* strain BL21(DE3) cells produced soluble protein at levels comparable to the wild-type enzyme. The K176A, K176C, E256A, and E256D mutant enzymes were purified to homogeneity in a yield of 20−60 mg from 3−6 g of cells using a procedure similar to that used for purifying the wild-type enzyme (*13*).

*Kinetic Characterization.* The activity of ketopantoate reductase was examined in crude soluble extracts expressing all mutant enzymes. Of these, the K176A and E256A mutant enzymes exhibited extremely low KPR activity while the other mutants showed essentially wild-type enzyme activity. The kinetic parameters determined for purified, homogeneous WT and selected mutant enzymes are listed in Table 1. The K176A and E256A mutant enzymes exhibited 233- and 42-fold decreases in $V_{max}$, and 336- and 63-fold increases in the $K_m$ value of ketopantoate, respectively. The E256D mutant enzyme exhibited a 3-fold lower $V_{max}$, and an 8-fold increase in $K_m$ for ketopantoate, respectively (Table 1). Replacement of K176 and E256 residues with alanine does not significantly affect the $K_m$ for NADPH (Table 1). No KPR activity could be detected in the K176A/E256A double mutant.

*Chemical Rescue of K176A Mutant by Amines.* Compared with the wild-type enzyme, the *V*/*K* value for ketopantoate determined for the K176A mutant is decreased 4.8 × $10^4$-fold, indicating that Lys176 is important for ketopantoate binding and catalysis. The $V_{max}$ of the K176A mutant enzyme increased 59-fold (to a level equal to 25% of that for the wild-type enzyme), and the $K_m$ value for ketopantoate decreased 25-fold by exogenously added 300 mM ethylamine. Ten other primary amines were also tested and shown to be effective in rescuing the K176A mutant (Table 2). Saturation kinetics were observed in these experiments for all primary amines. Addition of up to 600 mM amine did not significantly affect the activity of WT enzyme, arguing against a nonspecific activation by amines. The chemical rescue of the K176A mutant with amines further supports an essential role for Lys176 in catalysis. The rescue efficiency of 2,2,2-trifluoroethylamine (p$K_a$ = 5.7) was more efficient at pH 6.0 ($V_{max}$ = 3.6 ± 0.4 $\mu$mol $min^{-1}$ $mg^{-1}$) than at pH 7.5 ($V_{max}$ = 0.43 ± 0.06 $\mu$mol $min^{-1}$ $mg^{-1}$), suggesting that the protonated form of the amine functions as a general acid in catalysis. While the activity of the K176A mutant was markedly increased by addition of methyl-, ethyl-, and propylamine, the branched isopropylamine and bulkier butylamine were less effective (Table 2). A plot of log ($V/K_{amine}$) vs molecular volume for amines suggests that the efficiency of chemical rescue is determined by the molecular volumes of rescue agents (Figure 2A). A Brønsted

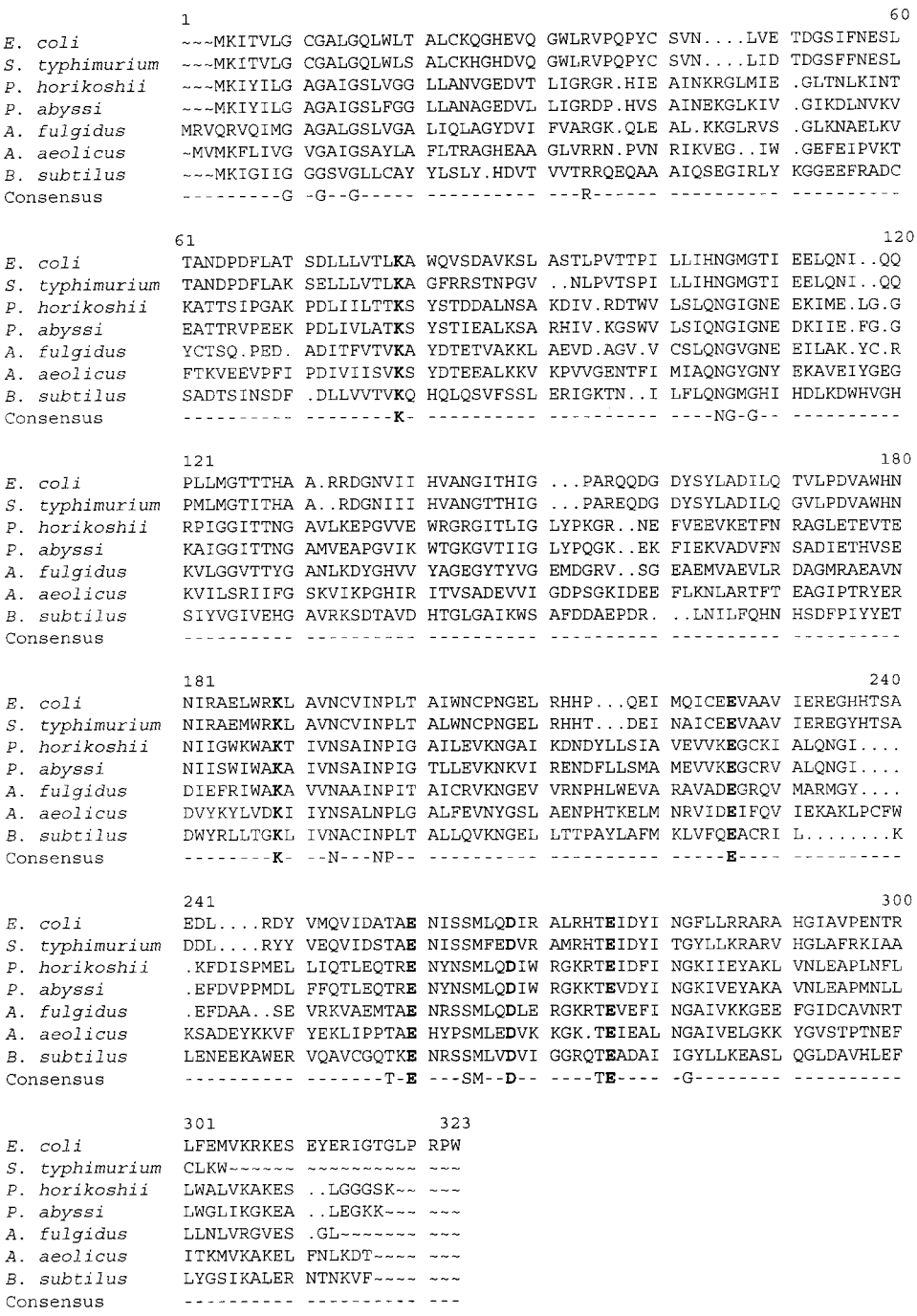



FIGURE 1: Alignment of the amino acid sequences of seven bacterial ketopantoate reductases. Consensus residues are those conserved between all seven sequences. Boldface residues are those subjected to mutagenesis. This alignment was generated by using the PILEUP program of the GCG package. The GenBank accession numbers are as follows: *Escherichia coli* (AE000148); *Salmonella typhimurium* (P37402); *Pyrococcus horikoshii* (O50098); *Pyrococcus abyssi* (AJ248285); *Archaeoglobus fulgidus* (O28578); *Aquifex aeolicus* (O67619); *Bacillus subtilus* (O34661).

plot of log ($V/K_{amine}$) versus the p$K_a$ value of nine amines showed that the rescue efficiencies do not depend on the p$K_a$ of the rescue agents (Brønsted coefficient, $\alpha = -0.004 \pm 0.008$) (Figure 2B).

*Chemical Rescue of K176C Mutant by Alkylation of Cysteine.* The K176C mutant enzyme showed low (9% of WT enzyme), but significant, KPR activity, suggesting that the cysteine residue may be partially able to serve as a general acid in catalysis. Alkylation of cysteine in the K176C mutant with bromoethylamine led to a 5-fold increase in $V_{max}$, equivalent to 51% of the WT enzyme activity, and a 2-fold decrease in $K_m$.

*Chemical Rescue of E256A Mutant by Formate and Acetate.* The E256A mutant had a 42-fold lower $V_{max}$ and a 63-fold higher $K_m$ for ketopantoate, giving an overall $1.8 \times 10^3$-fold decrease in $V/K_{KP}$ compared to wild-type enzyme (Table 1). The activity of the E256A mutant could be increased to 75% of that for the WT enzyme in the presence of 200 mM formate (molecular volume = 53.9 Å$^3$), while the larger acetate (molecular volume = 73.4 Å$^3$) was less effective (maximally increased to 10% of that for the WT enzyme, Figure 3). The formate-dependent increase in activity of the E256A mutant exhibited saturation kinetics. The efficiency of chemical rescue with formate of the E256A

Table 1: Kinetic Parameters of WT and Mutants of *E. coli* Ketopantoate Reductase

| ketopantoate reductase | $V_{rel}$[b] | NADPH | | KP | |
|---|---|---|---|---|---|
| | | $K_m$ ($\mu$M) | $V/K_{rel}$ | $K_m$ ($\mu$M) | $V/K_{rel}$ |
| WT | 100 | 4.0 ± 0.4 | 100 | 120 ± 8 | 100 |
| K176A | 0.43 | 16 ± 3 | 0.11 | 40000 ± 6000 | 0.002 |
| K176C | 9.3 | 6.6 ± 1.6 | 7.1 | 70 ± 10 | 17 |
| K176C[a] | 51.1 | 3.8 ± 0.3 | 54 | 38 ± 9 | 145 |
| E256A | 2.4 | 2.0 ± 0.3 | 4.7 | 7500 ± 2900 | 0.05 |
| E256D | 34.4 | 2.9 ± 0.6 | 42.3 | 950 ± 290 | 4.6 |
| K176A/E256A | nd[c] | nd | nd | nd | nd |

[a] Alkylation with bromoethylamine. [b] WT $V_{max}$ = 14.0 ± 0.4 $\mu$mol min$^{-1}$ mg$^{-1}$. [c] nd: not detectable.

mutant was more efficient at pH 7.2 ($V_{max}$ = 3.3 ± 0.2 $\mu$mol min$^{-1}$ mg$^{-1}$) than at pH 5.9 ($V_{max}$ = 1.3 ± 0.1 $\mu$mol min$^{-1}$ mg$^{-1}$), suggesting that the anionic form of formic acid (p$K_a$ = 3.7) is responsible for the increased activity.

*Deuterium Kinetic Isotope Effects.* Primary deuterium kinetic isotope effects were determined using varying concentrations of [4*S*-4-$^1$H]- and [4*S*-4-$^2$H]NADPH at a fixed, saturating concentration of ketopantoate at pH 7.5, 25 °C. Primary deuterium kinetic isotope effects on $V$ and $V/K_{NADPH}$ exhibited values of 3.0 ± 0.3 and 3.7 ± 0.3 using the K176A mutant (Figure 4) and 1.1 ± 0.1 and 3.3 ± 0.4 using the E256A mutant (Table 3; data not shown), respectively. In the presence of increasing concentrations of ethylamine, the primary deuterium kinetic isotope effects on both $V$ and $V/K$ for the K176A mutant enzyme decreased (Figure 5), and approached the values observed for the WT enzyme ($^DV$ = 1.3 and $^DV/K$ = 1.5) at high levels of added ethylamine (Table 3).

## DISCUSSION

The pH dependence of the kinetic parameters $V$ and $V/K$ for substrates in both the forward and reverse reactions catalyzed by *E. coli* ketopantoate reductase is consistent with a chemical mechanism involving a general acid−base catalyst (*13*). The reactions catalyzed by both yeast and liver alcohol dehydrogenase have also been reported to use a general acid/base chemical mechanism in catalysis (*25*). Despite this conservation of chemical mechanism, there is no significant sequence homology between bacterial ketopantoate reductase and other alcohol dehydrogenases. Sequence alignment of the seven reported bacterial ketopantoate reductases (Figure 1) reveals no conserved histidine or tyrosine residues, the acid/base groups observed in the active sites of liver and *Drosophila melanogaster* alcohol dehydrogenases. This alignment does, however, reveal two completely conserved lysine residues (*E. coli* K72 and K176) and four conserved acidic residues (*E. coli* E210, E240, D248, and E256). Lysine, glutamate, and aspartate residues have been reported to function catalytically as general acid and bases in many enzymatic reactions (*26*−*28*). These six conserved residues were individually replaced by alanine using site-directed mutagenesis. The K176A and E256A mutant enzymes showed extremely low KPR activity in crude extracts, while expression of the other four mutants exhibited activity in extracts similar to the expressed WT enzyme. We thus prepared homogeneous mutant enzymes to probe whether Lys176 and Glu256 are essential for catalysis and what their roles might be.

*Chemical Rescue of K176 Mutants.* Mutation of K176 to alanine resulted in a dramatic impairment of catalytic activity, and in apparent substrate affinity, compared to the wild-type enzyme (Table 1). This suggests that Lys176 may be involved in both catalysis and ketopantoate binding. Both catalysis and substrate binding by K176A can be enhanced by exogenously added primary amines (Table 2). Toney and Kirsch first demonstrated that catalytic activity can be restored to the inactive K258A mutant of *E. coli* aspartate aminotransferase by exogenous amines (*29*, *30*). Chemical rescue with amines has subsequently been reported for the K80 mutant of leucine dehydrogenase (*26*), the K329 mutant of ribulose-1,5-bisphosphate carboxylase/oxygenase (*31*), the K358 mutant of lactose permease (*32*), and the K296 mutant of rhodopsin (*33*). In the present study, the maximum velocity of the K176A mutant was increased from 0.4% to 25% that of the WT enzyme by the addition of 300 mM ethylamine. Similarly, the $K_m$ for ketopantoate decreased 25-fold in the presence of 300 mM ethylamine. In addition to the restorative effects of ethylamine on the reductase activity of the K176A mutant, significant effects were also observed for 10 other amines (Table 2). These data strongly suggest that the amino group of a series of primary amines can compensate for the loss of function in the K176A mutant, and support K176 as an active site residue involved in both catalysis and ketopantoate binding.

The K176C mutant exhibits low, but significant, KPR activity (9% of WT, Table 1), suggesting that the cysteine residue, perhaps via an intervening water molecule, can also promote general acid catalysis in ketopantoate reduction. The ability of cysteine to serve as a general acid/base catalyst has been documented in PLP-independent racemases, including diaminopimelate epimerase (*34*), glutamate racemase (*35*), and proline racemase (*36*). The low KPR activity of the K176C mutant can be restored to 51% of $V$ and 145% of $V/K$ of WT enzyme by alkylation with bromoethylamine (Table 1). These data further support our hypothesis that Lys176 is a residue essential for catalysis.

The general acid/base catalytic function of lysine residues at enzyme active sites has been reported for many amino acid dehydrogenases, including glutamate dehydrogenase (*37*), leucine dehydrogenase (*26*), and more recently phenylalanine dehydrogenase (*38*). The protonated form of K78 in phenylalanine dehydrogenase interacts with the α-carbonyl oxygen of keto acid (*38*). To probe the protonation state of the amine that could restore activity, we probed the pH dependence of rescue for amines with low p$K_a$ values. Rescue by 2,2,2-trifluoroethylamine (p$K_a$ = 5.7) was considerably more efficient at pH 6.0 than at pH 7.5, indicating that the protonated form of the amine is responsible for rescue. This result strongly supports the suggestion that ϵ-amino group of Lys176 functions as a general acid in ketopantoate reduction. A 336-fold increase in $K_{KP}$ by mutation of K176 to alanine and a 25-fold decrease in $K_{KP}$ by rescue of K176A with protonated ethylamine suggest that ϵ-amino group of Lys176 is also involved in binding of substrate in protonated form. In previously reported studies of the pH dependence of the ketopantoate reductase reaction, it was shown that both $V$ and $V/K$ in the direction of ketopantoate reduction decrease as the pH increases, suggesting a group exhibiting a p$K_a$ value of 8.4 must be protonated to donate a hydrogen bond to the carbonyl group of ketopantoate and to function as a

Table 2: Amine Rescue of Ketopantoate Reduction Activity of K176A Mutant

| amine | p$K_a$ | molecular[a] volume (Å$^3$) | $K_m^{amine}$ (M) | $V_{max}$ ($\mu$mol min$^{-1}$ mg$^{-1}$) | $V/K_{amine}$ |
|---|---|---|---|---|---|
| methyl- | 10.6 | 53.2 | 0.15 ± 0.02 | 2.9 ± 0.2 | 19.9 |
| ethyl- | 10.6 | 73.8 | 0.14 ± 0.01 | 3.5 ± 0.1 | 25.0 |
| propyl- | 10.5 | 94.2 | 0.11 ± 0.01 | 3.2 ± 0.1 | 30.0 |
| isopropyl- | 10.6 | 93.6 | 0.65 ± 0.06 | 1.3 ± 0.1 | 2.0 |
| butyl- | 10.6 | 114.6 | 0.13 ± 0.02 | 0.61 ± 0.04 | 4.9 |
| ethanol- | 9.5 | 84.7 | 0.17 ± 0.03 | 2.4 ± 0.2 | 13.9 |
| ammonia | 9.2 | 31.9 | 0.22 ± 0.02 | 1.26 ± 0.05 | 5.7 |
| 2-fluoroethyl- | 9.0 | 80.5 | 0.15 ± 0.02 | 2.8 ± 0.1 | 19.3 |
| propargyl-[b] | 8.2 | 82.3 | 0.065 ± 0.008 | 2.6 ± 0.1 | 39.8 |
| 2-cyanoethyl-[b] | 7.7 | 97.7 | 0.07 ± 0.04 | 0.27 ± 0.02 | 3.8 |
| 2,2,2-trifluoroethyl-[b] | 5.7 | 94.2 | 0.27 ± 0.09 | 10 ± 1 | 37.3 |

[a] The molecular volume for amines was calculated by using the program SPARTAN SGI Version 5.1.3 OpenGL (Wavefunction Inc., Irvine, CA). [b] Corrected for the fraction of protonated amine.

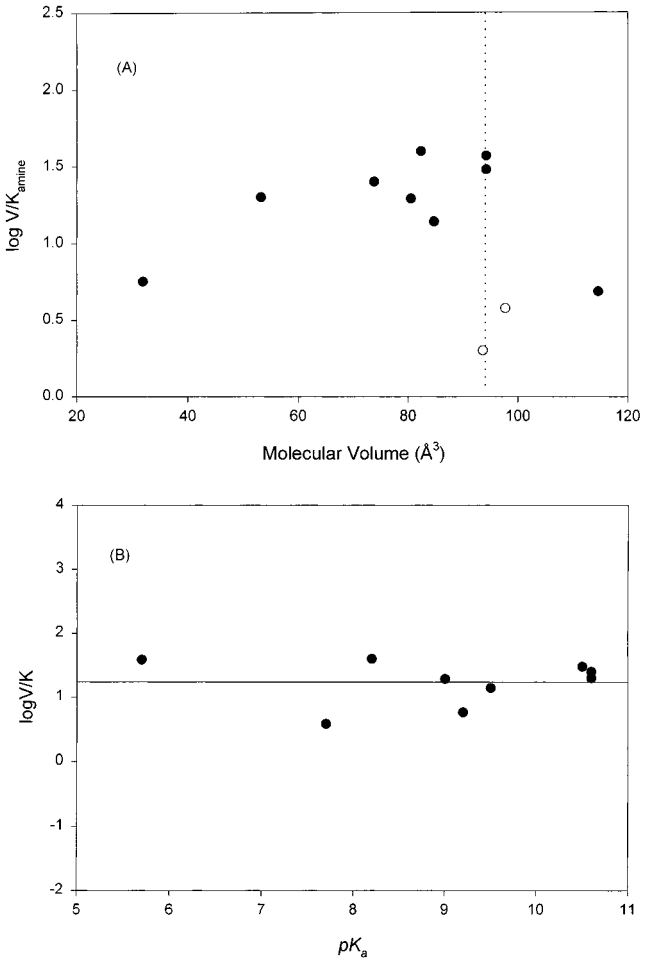



FIGURE 2: (A) Relationship between log (*V/K*) and molecular volume of amines (Table 2). The dotted vertical line is drawn at 94.2 Å$^3$. Filled circles represent saturated unbranched alkylamines, while the unfilled circles represent isopropylamine and cyanoethylamine. (B) A Brønsted plot showing the relationship between log (*V/K*) and the p$K_a$ value of amines (Table 2).

general acid (*13*). All of the present data argue that Lys176 is the residue whose p$K_a$ value of 8.4 is observed in the pH profiles and which functions to bind ketopantoate and act as the general acid.

*Correlation between Rescue Efficiency and Molecular Volume.* Using differently sized amines (Figure 2A), rescue of KPR activity of the K176A mutant shows a strong correlation with molecular volume. The branched isopropylamine and larger *n*-butylamine are less effective in restoring the reductase activity of the K176A mutant, suggesting a steric restraint for isopropylamine or butylamine. Optimal rescue was obtained with amines whose volume matches the volume vacated by conversion of lysine to alanine (94.2 Å$^3$, Table 2).

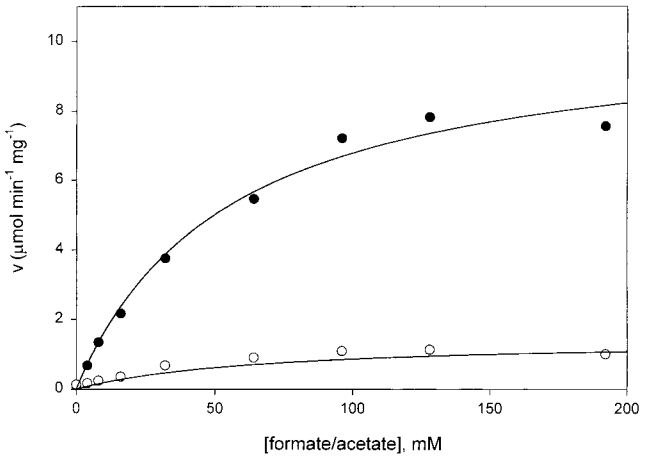



FIGURE 3: Effect of formate (●) and acetate (○) on ketopantoate reduction by E256A mutant. The $V_{max}$ values were determined at pH 7.5 with varying concentrations of formate and acetate. The smooth lines are fits of the data to eq 1, and yield $V_{max} = 10.5 \pm 0.7$ $\mu$mol min$^{-1}$ mg$^{-1}$ and $K_m = 55 \pm 10$ mM for formate and $V_{max} = 1.5 \pm 0.2$ $\mu$mol min$^{-1}$ mg$^{-1}$ and $K_m = 69 \pm 12$ mM for acetate.

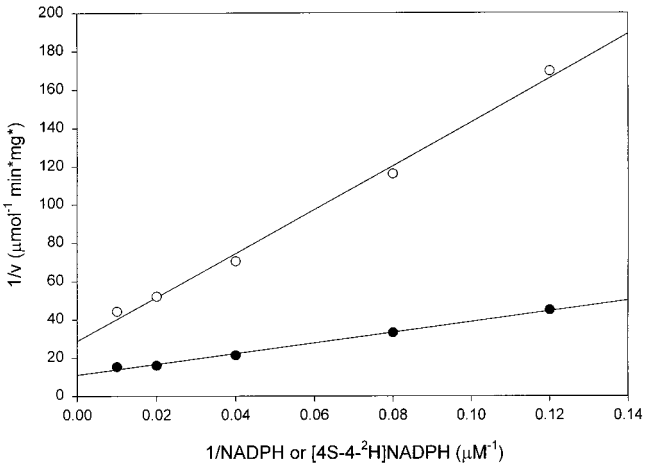



FIGURE 4: Primary deuterium isotope effects for ketopantoate reduction by K176A mutant. The data were determined using [4*S*-4-$^1$H]NADPH (●) and [4*S*-4-$^2$H]NADPH (○) at saturaing concentration of ketopantoate. The lines through the data are the fits of the data to eq 2 and yield values for $^DV = 3.0 \pm 0.3$ and $^DV/K = 3.7 \pm 0.3$.

Table 3: Primary Deuterium Isotope Effects Exhibited by WT and Mutant Ketopantoate Reductases

| KPR | rescue agents | $^{D}V$ | $^{D}V/K$ |
|---|---|---|---|
| WT | − | 1.33 ± 0.02 | 1.54 ± 0.04 |
| K176A | − | 3.0 ± 0.3 | 3.7 ± 0.3 |
| | ethylamine (450 mM) | 1.5 ± 0.3 | 2.0 ± 0.1 |
| E256A | − | 1.1 ± 0.1 | 3.3 ± 0.5 |

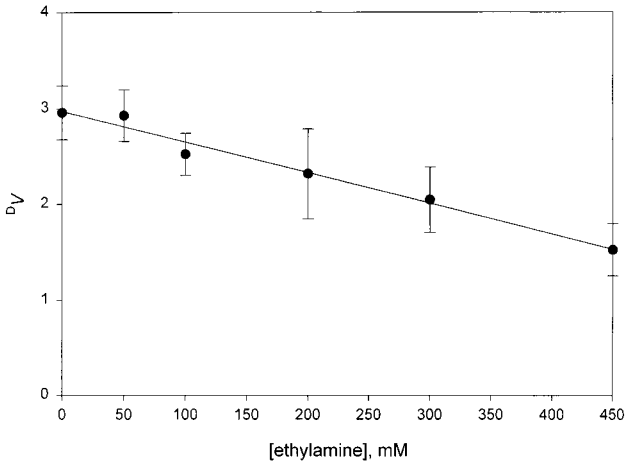



FIGURE 5: Effect of ethylamine concentration on primary deuterium isotope effects for ketopantoate reduction by the K176A mutant.

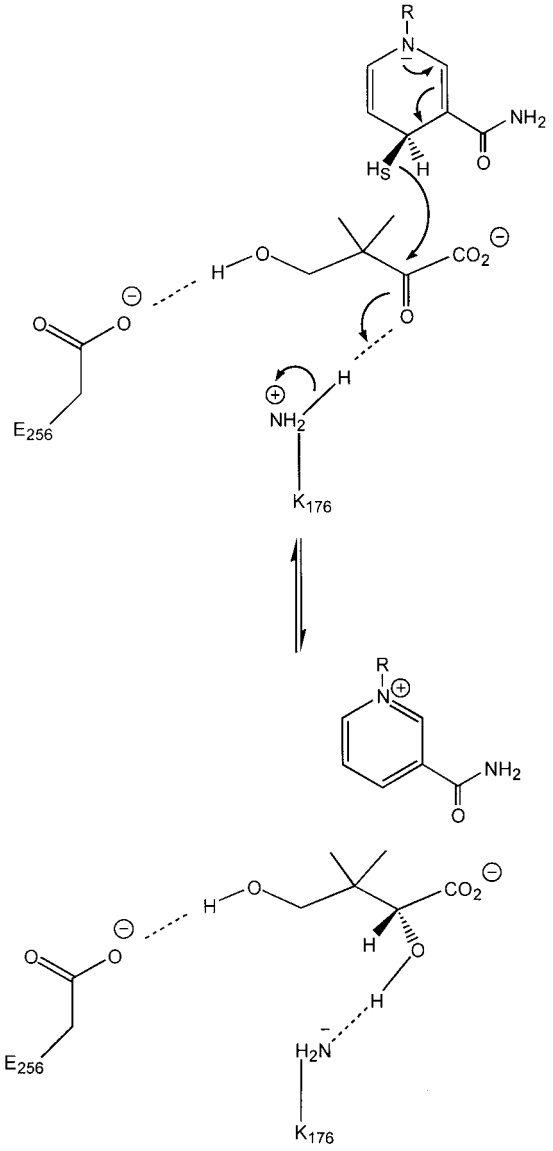
*Brønsted Coefficient (α).* The slope of a plot of log ($V/K_{amine}$) versus $pK_a^{amine}$ (Brønsted plot) can give information concerning the nature of the rate-limiting transition state. Moderate sensitivity of rates to acid strength ($\alpha \approx -0.5$) suggests predominantly rate-limiting proton transfer. Insensitivity to acid strength ($\alpha \approx 0$) suggests that proton transfer is not rate-limiting but some step prior to proton transfer is rate-limiting (*39*). Total insensitivity to acid strength ($\alpha = -0.004 \pm 0.008$, Figure 2B) for rescue of KPR activity of the K176A mutant strongly suggests that chemistry is not rate-limiting. This conclusion is consistent with (a) the small primary deuterium kinetic isotope effects on *V* and *V/K* using [4*S*-4-$^2$H]NADPH as the variable substrate in wild-type KPR and (b) the larger isotope effects for the K176A mutant which decrease as the concentration of added ethylamine increases (Figure 5).

*Chemical Rescue of E256 Mutants.* The E256A mutant exhibited a 42-fold decrease in $V_{max}$ and a 63-fold increase in $K_m$ for ketopantoate compared to wild-type enzyme (Table 1). Ketopantoate reductase activity of the E256A mutant can be greatly increased by addition of formate, which compensates for the loss of the carboxymethyl group by mutation of E256 to alanine. A similar restoration of catalytic activity for mutation of glutamate to alanine by formate was also reported for *E. coli* cytidine deaminase (*40*). Formate restores the KPR activity of the E256A mutant to 75% of that of WT enzyme, while the bulkier acetate anion only enhances the activity to 10% of that for WT enzyme, suggesting that the efficiency of rescue is also sensitive to the size of the rescue agent. The catalytic rate of E256A in the presence of formate was twice as high at pH 7.2 as at pH 5.9, indicating that the anionic species of formic acid is responsible for the KPR activity rather than its conjugate acid. Only a modest reduction in the KPR activity (34% of WT enzyme) was observed with the E256D mutant (Table 1).

*Deuterium Kinetic Isotope Effects.* The primary deuterium kinetic isotope effects for K176A and E256A were obtained using [4*S*-4-$^1$H]- and [4*S*-4-$^2$H]NADPH. Large primary deuterium kinetic isotope effects for K176A on *V* and *V/K* of 3.0 ± 0.3 and 3.7 ± 0.3, respectively, were observed at pH 7.5. The large primary deuterium isotope effects exhibited by K176A suggest that hydride transfer has become significantly rate-limiting. We have previously reported small primary deuterium kinetic isotope effects for wild-type enzyme on *V* and *V/K* of 1.33 ± 0.02 and 1.54 ± 0.04, respectively, suggesting that the chemical reaction is unlikely to be a slow step in the overall reaction catalyzed by the WT enzyme. However, the larger primary deuterium isotope effects exhibited by K176A can be reduced to $^{D}V = 1.5 \pm 0.3$ and $^{D}V/K = 2.0 \pm 0.1$ by the addition of 450 mM ethylamine (Figure 5), suggesting that the rate-limiting chemistry in the mutant can be enhanced to the point where it is no longer rate-limiting by addition of ethylamine. The E256A mutant exhibited only a small primary kinetic isotope effect on $^{D}V$ (1.1 ± 0.1) but a large primary deuterium kinetic isotope effect on $^{D}V/K$ (3.3 ± 0.4). For an ordered kinetic mechanism, demonstrated previously for ketopantoate reductase (*13*), the magnitude of $^{D}V$ reflects the relative rate of the isotope-sensitive hydride transfer step to all subsequent steps, including the release of products, enzyme isomerizations, etc. For the K176A mutant, the reduction in the rate of the chemical reaction causes $^{D}V$ to increase to 3.0. For the E256A mutant, the observed magnitude of $^{D}V$ suggests that the catalytic step has not been similarly affected by this replacement and that one or more of these other steps is slower that catalysis. On the other hand, the magnitude of $^{D}V/K$ reflects the relative rate of the isotope-sensitive chemical reaction to steps between the binding of the isotopically labeled substrate, NADPH, and the first irreversible step, in the present case D-pantoate release. The magnitude of $^{D}V/K$ observed for the E256A mutant argues that D-pantoate release has been accelerated by the replacement of E256 by alanine. If catalysis was significantly decreased by this mutation, one would predict both $^{D}V/K$ and $^{D}V$ to increase relative to their magnitude observed for the WT enzyme, as was observed for the K176A mutant. We therefore conclude that E256A functions in D-pantoate (and ketopantoate) binding, and the E256A mutant exhibits an enhanced rate of release of D-pantoate, as evidenced by the magnitude of $^{D}V/K$ (Table 3) and a reduced apparent affinity (63-fold) for ketopantoate (Table 1).

*Proposed Functions of K176 and E256.* Based on our previous studies (*13*) and those reported here, we propose that K176 functions as the general acid in the direction of ketopantoate reduction. By hydrogen bonding to the C2 carbonyl of ketopantoate, K176 effectively polarizes the carbonyl and facilitates hydride transfer to C2 from NADPH. Whether hydride transfer generates an intermediate alkoxide, which is subsequently protonated by K176, or whether hydride transfer and protonation are concerted remains to be experimentally addressed. The data presented here also allow us to propose a role for E256, as shown in Scheme 2. As discussed above, we do not feel that E256 can function as an auxiliary catalytic component, but rather contributes to ketopantoate and D-pantoate binding. Of the possible interactions, an interaction with the C1 carboxylate would be electrostatically unfavorable, leaving the interaction be-

Scheme 2: Proposed Roles for Lys176 and Glu256 in the Mechanism of *E. coli* Ketopantoate Reductase




tween the C4 alcohol and E256 as the most likely cause of the kinetotype exhibited by the E256A mutant. The determination of the three-dimensional structure of the E−$NADP^+$−ketopantoate complex is underway, and will provide evidence for, or against, the proposed functions of these residues.

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