JOURNAL OF BACTERIOLOGY, Jan. 1983, p. 259–269
0021-9193/83/010259-11$02.00/0

Vol. 153, No. 1

Role of Acetohydroxy Acid Isomeroreductase in Biosynthesis of Pantothenic Acid in *Salmonella typhimurium*

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Received 2 August 1982/Accepted 11 October 1982

Structural genes have been identified for all of the enzymes involved in the biosynthesis of pantothenic acid in *Salmonella typhimurium* and *Escherichia coli* K-12, with the exception of ketopantoic acid reductase, which catalyzes the conversion of α-ketopantoate to pantoate. The acetohydroxy acid isomeroreductase from *S. typhimurium* efficiently bound α-ketopantoate (K_m = 0.25 mM) and catalyzed its reduction at 1/20 the rate at which α-acetolactate was reduced. Since two enzymes could apparently participate in the synthesis of pantoate, a *S. typhimurium ilvC8* strain was mutagenized to derive strains completely blocked in the conversion of α-ketopantoate to pantoate. Several isolates were obtained that grew in isoleucine-valine medium supplemented with either pantoate or pantothenate, but not in the same medium supplemented with α-ketopantoate or β-alanine. The mutations that conferred pantoate auxotrophy (designated *panE*) to these isolates appeared to be clustered, but were not linked to *panB* or *panC*. All *panE* strains tested had greatly reduced levels of ketopantoic acid reductase (3 to 12% of the activity present in DU201). The capacity of the isomeroreductase to synthesize pantoate in vivo was assessed by determining the growth requirements of *ilvC*$^+$ derivatives of *panE ilvC8* strains. These strains required either α-ketopantoate, pantoate, or pantothenate when the isomeroreductase was present at low levels; when the synthesis of isomeroreductase was induced, *panE ilvC*$^+$ strains grew in unsupplemented medium. These phenotypes indicate that a high level of isomeroreductase is sufficient for the synthesis of pantoate. *panE ilvC*$^+$ strains also grew in medium supplemented with lysine and methionine. This phenotype resembles that of some *S. typhimurium ilvG* mutants (e.g., DU501) which are partially blocked in the biosynthesis of coenzyme A and are limited for succinyl coenzyme A. *panE ilvC*$^+$ strains which lack the acetohydroxy acid synthases required only methionine for growth (in the presence of leucine, isoleucine, and valine). This and other evidence suggested that the synthesis of pantoic acid by isomeroreductase was blocked by the α-acetohydroxy acids and that pantoic acid synthesis was enhanced in the absence of these intermediates, even when the isomeroreductase was at low levels. *panE ilvC*$^+$ strains reverted to pantothenate independence. Several of these revertants were shown to have elevated isomeroreductase levels under noninduced and induced conditions; the suppressing mutation in each revertant was shown to be closely linked to *ilvC* by P22 transduction. This procedure presents a means for obtaining mutants with altered regulation of isomeroreductase.

Pantoic acid, an intermediate in the pantothenic acid biosynthetic pathway, is formed by the reduction of α-ketopantoic acid (5, 13) (Fig. 1). This reaction is apparently catalyzed by ketopantoic acid reductase in *Escherichia coli* K-12 (12, 25, 26). Although structural genes for all of the other pantothenic acid biosynthetic enzymes have been identified and mapped in *E. coli* K-12 and in *Salmonella typhimurium*, the gene encoding ketopantoic acid reductase has not been identified in either species (2, 7, 8, 10). The absence of mutants deficient in this reaction suggests that more than one enzyme is able to catalyze the conversion of α-ketopantoate to pantoate.

A candidate for such an enzyme is acetohydroxy acid isomeroreductase, the product of the *ilvC* gene. This enzyme catalyzes the second common step in isoleucine and valine biosynthesis, i.e., the formation of α,β-dihydroxy-β-methylvalerate and α,β-dihydroxyisovalerate from α-aceto-α-hydroxybutyrate and α-aceto-

lactate, respectively (Fig. 1 and 2). The conversion of the α-acetohydroxy acids to these intermediates is initiated with the migration of the α-alkyl group of the acetohydroxy acid to the β-carbon to form the α-keto-β-hydroxy acid which is subsequently reduced to the α,β-dihydroxy acid by using the cosubstrate NADPH (Fig. 2). The isomerized intermediates, α-keto-β-hydroxy-β-methylvalerate and α-keto-β-hydroxyisovalerate, bear striking resemblance to α-ketopantoate (α-keto-β,β-dimethyl-γ-hydroxybutyrate, Fig. 2). In the work presented here we show that purified isomeroreductase catalyzes the reduction of α-ketopantoate and that mutation in a gene which we designate *panE* leads to a requirement for pantoate when present in strains bearing an *ilvC* mutation. Conditions are described under which *panE ilvC*$^+$ strains grow in medium lacking pantothenate or its specific precursors.

MATERIALS AND METHODS

Chemicals and enzyme assays. Sodium α-ketoisovalerate, DL-pantoyl lactone, D-pantoyl lactone, and calcium D-pantothenate were obtained from the Sigma Chemical Co., St. Louis, Mo. α-Ketopantoyl lactone was prepared from DL-pantoyl lactone by the method of Lipton and Strong (15). Potassium α-ketopantoate to be used in either enzyme or nutritional studies was prepared by titrating solutions of α-ketopantoyl lactone to pH 7.5 with KOH. Potassium D-pantoate was prepared by mixing 100 mM D-pantoyl lactone with an equimolar amount of KOH and allowing the solution to stand for 5 h to complete the hydrolysis. Sodium α-aceto-α-hydroxybutyrate and sodium α-acetolactate were prepared by saponification of the methyl esters, which were the generous gifts of Frank Armstrong.

Acetohydroxy acid isomeroreductase was purified from *S. typhimurium* by the method of Hofler et al. (11) and was assayed by the method of Arfin and Umbarger (1). Isomeroreductase assays used for the determination of kinetic constants were performed with purified enzyme at 30°C; all other isomeroreductase assays were performed at room temperature. α-Aceto-α-hydroxybutyrate was used exclusively as the substrate in the determination of isomeroreductase levels in crude extracts. When the isomeroreductase in crude extract was tested for the ability to catalyze D-pantoate synthesis, 5 mM potassium α-ketopantoate (pH 7.5) was added to the standard reaction mixture in place of α-aceto-α-hydroxybutyrate. One unit of isomeroreductase activity is defined as the amount of enzyme required to oxidize 1 nmol of NADPH per min in the standard assay.

Threonine deaminase (6), ketopantoate hydroxymethyltransferase (20, 24), and acetohydroxy acid synthases I and II (23) were assayed as previously described; acetohydroxy acid synthase II was measured in reaction mixtures containing 1 mM valine. Ketopantoic acid reductase was assayed by the method of King and Wilken (13), except that the pH of the sodium acetate buffer was raised from 5.0 to 5.5. The activity of ketopantoyl lactone reductase in crude extracts was estimated by the method of Wilken et al. (26).

Cell-free extracts were prepared from batch cultures as previously reported (3, 20). Protein concentrations were determined by the method of Lowry et al. (16).

Characterization of product of isomeroreductase reaction with α-ketopantoate as substrate. Acetohydroxy acid isomeroreductase was observed to catalyze the oxidation of NADPH in the presence of α-ketopantoic acid. The (reduced) product of this reaction was characterized (i) by feeding the product to a series of mutants blocked in consecutive steps of the pantothenic acid pathway and (ii) by gas-liquid chromatography of the lactonized substrate and product along with authentic ketopantoyl lactone and D-pantoyl lactone.

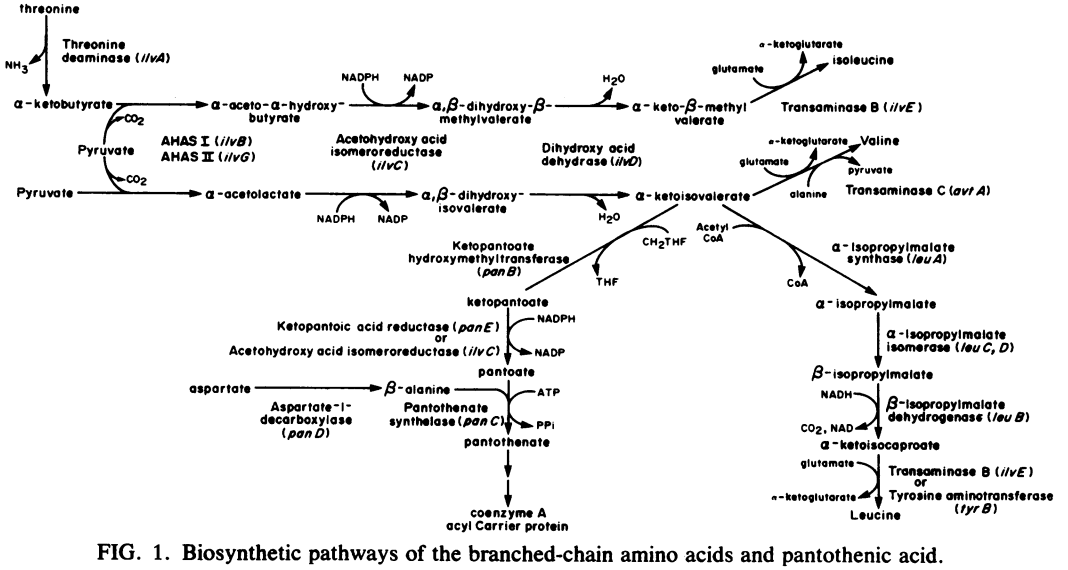

FIG. 1. Biosynthetic pathways of the branched-chain amino acids and pantothenic acid.

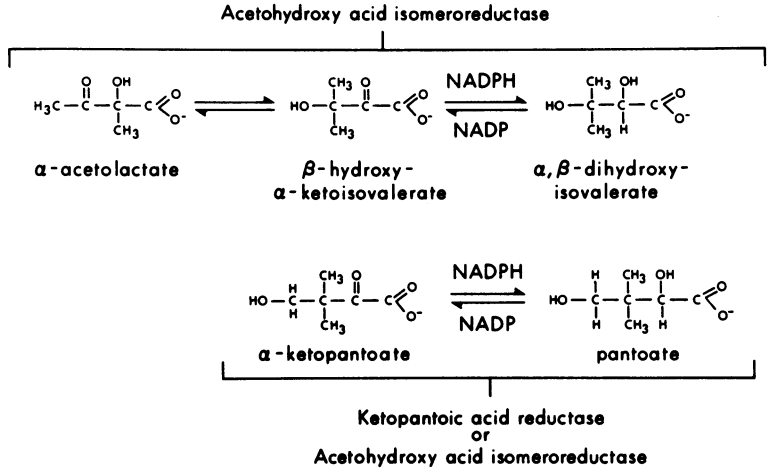

FIG. 2. Comparison of acetohydroxy acid isomeroreductase and ketopantoic acid reductase reactions.

The material for auxanographic studies was obtained by incubating 9 U of acetohydroxy acid isomeroreductase for 24 h at 37°C in reaction mixture (20 ml) that contained the following: potassium phosphate (pH 7.5), 4,000 μmol; $MgCl_2$, 80 μmol; NADP 10 μmol; glucose 6-phosphate dehydrogenase (Sigma) 16 U; potassium α-ketopantoate (pH 7.0), 200 μmol; and glucose 6-phosphate, 200 μmol. An additional 200 μmol of potassium α-ketopantoate (pH 7.0) and 200 μmol of glucose 6-phosphate were added 12 h after the reaction was initiated. At the end of the reaction period, the reaction mixture was centrifuged for 10 min to remove debris. The supernatant fluid was decanted, titrated to pH 5.0 with hydrochloric acid, and allowed to stand at room temperature for 1 h. The supernatant fluid was then adjusted to pH 7.0 with sodium hydroxide and extracted with two 10-ml portions of ethyl acetate. The ethyl acetate layers were carefully removed and rotary evaporated to dryness. The resulting material was dissolved in 2 ml of deionized water and filter sterilized. Samples (0.2 ml) of this material were spread onto isoleucine-valine agar medium. This medium was then streaked with cultures of *panC2* (pantothenate auxotroph), DU6543 (pantoate requirer), DU6544 (ketopantoate auxotroph), or an *ilvC*$^+$ derivative of DU7410 (ketopantoate auxotroph) to determine whether pantoate was present.

The material for chromatographic analysis was prepared by incubating 7.5 U of acetohydroxy acid isomeroreductase for 30 h at room temperature with a solution (2 ml) containing the following: potassium phosphate (pH 7.5), 400 μmol; potassium α-ketopantoate (pH 7.0), 8 μmol; NADPH, 16 μmol; $MgCl_2$, 8 μmol. Portions (0.5 ml) of the reaction mixture were removed at the beginning and the end of the reaction period, titrated to pH 2.0 with hydrochloric acid, and allowed to stand for 30 min to form the lactones of ketopantoate and pantoate. These solutions were then extracted with chloroform (0.6 ml); the chloroform layer was then dispensed into 1-dram (ca. 3.7-ml) vials and rotary evaporated to dryness. The material in the vials was then dissolved in a small volume (1 to 2 μl) of chloroform and analyzed on a Becker gas chromatograph (Packard Instrument Co.; model 417) equipped with a flame ionization detector (set at 250°C) and with a 6-ft (ca. 1.83-m) by 2-mm glass column packed with SP2330 (100-200 mesh) Supelcoport (Supelco, Inc., Bellafonte, Pa.). The column temperature was maintained at 225°C, and nitrogen was used as the carrier. Air and hydrogen gas rates were adjusted for optimal flame ionization detection. Solutions of authentic α-ketopantoate and D-pantoate were also titrated to pH 2.0, extracted into chloroform, evaporated to dryness, and used as standards. Complete resolution of ketopantoyl lactone and D-pantoyl lactone standards was obtained; ketopantoyl lactone eluted at 2.71 min, and D-pantoyl lactone eluted at 2.86 min after sample injection under the chromatographic conditions described above. (Ketopantoyl lactone and pantoyl lactone from reaction mixtures were identified by comparison of their retention times to those of the standards.)

Bacterial strains and culture media. All strains described in this paper are derivatives of *S. typhimurium* LT2 (Table 1). The minimal medium used in all experiments was that of Davis and Mingioli (9) which was modified by raising the concentration of glucose to 0.5% and omitting the citrate; the concentration of potassium phosphate and ammonium sulfate was doubled in medium to be used for growth experiments or in the 250-ml batch cultures used for the preparation of cell-free extracts.

Mutagenesis and analysis of nutritional requirements. *S. typhimurium* strain DU201 (*ilvC8*) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and enriched for pantothenic acid auxotrophs by the general methods of Miller (18). Ampicillin (20 μg/ml) was used to counterselect prototrophs in the first two cycles, and D-cycloserine (2 μmol/ml) was used in the third cycle. Isolates were initially characterized by streaking a saline suspension of a single colony onto isoleucine (50 μg/ml)–valine (100 μg/ml) agar minimal medium supplemented with either 0.2 mM potassium α-ketopantoate (25.6 μg/ml), 0.2 mM potassium D-pantoate (26 μg/ml), or calcium D-pantothenate (20 μg/ml) and onto unsupplemented isoleucine-valine

TABLE 1. Strain list

Strain	Genotype	Growth requirements	Source
LT2	Wild type	None	Laboratory collection
DU201	*ilvC8*	Isoleucine and valine	F. Armstrong
DU6543	*ilvC8 panE54*	Isoleucine, valine, and pantothenate	MNNG[a] mutagenesis of DU201
DU6563	*ilvC8 panE56*	Isoleucine, valine, and pantothenate	MNNG mutagenesis of DU201
DU6583	*ilvC8 panE58*	Isoleucine, valine, and pantothenate	MNNG mutagenesis of DU201
DU6611	*ilvC8 panE61*	Isoleucine, valine, and pantothenate	MNNG mutagenesis of DU201
DU6544	*ilvC*$^+$ *panE54*	α-Ketopantoate, pantoate, or pantothenate	P22 transduction of DU6543
DU6564	*ilvC*$^+$ *panE56*	α-Ketopantoate, pantoate, or pantothenate	P22 transduction of DU6563
DU6584	*ilvC*$^+$ *panE58*	α-Ketopantoate, pantoate, or pantothenate	P22 transduction of DU6583
DU6545	*ilvC*$^+$ *panE54 sup6*	None (stimulated by valine)	Pan$^+$ revertant of DU6544
DU6546	*ilvC*$^+$ *panE54 sup45*	Valine or α-ketoisovalerate	Pan$^+$ revertant of DU6544
DU6540	*ilvC*$^+$ *panE54 ΔilvGE605 ilvB1 (zia-1*::TN*10)*	Leucine, isoleucine, valine, and pantothenate	See text
DU6601	*ilvC*$^+$ *panE54 ΔilvGE605 ilvB1 (zia-1*::TN*10) sup601*	Leucine, isoleucine, and valine	Pan$^+$ revertant of DU6540
DU6603	*ilvC*$^+$ *panE54 ΔilvGE605 ilvB1 (zia-1*::Tn*10) sup603*	Leucine, isoleucine, and valine	Pan$^+$ revertant of DU6540
DU6604	*ilvC*$^+$ *panE54 ΔilvGE605 ilvB1 (zia-1*::Tn*10) sup604*	Leucine, isoleucine, and valine	Pan$^+$ revertant of DU6540
DU6801	*ilvC*$^+$ *panE54 ilvG*$^+$ *ilvB sup601*	Valine	See text
DU6803	*ilvC*$^+$ *panE54 ilvG*$^+$ *ilvB*$^+$ *sup603*	None	See text
DU6804	*ilvC*$^+$ *panE54 ilvG*$^+$ *ilvB*$^+$ *sup604*	None (stimulated by valine)	See text
PanC	*panC2*	Pantothenate	K. Sanderson
DU7410	*panB41 ilvC8*	Isoleucine, valine, and α-ketopantoate	MNNG mutagenesis of DU201
DU8020	*panD2 ilvC8*	Isoleucine, valine, and β-alanine	UV mutagenesis of DU201
DU8123	*panD123 ilvC8*	Isoleucine, valine, and β-alanine	MNNG mutagenesis of DU201

[a] MNNG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine.

agar medium. Pantothenate auxotrophs that grew on D-pantoate- but not α-ketopantoate-supplemented medium were selected for further analysis. Isolates with phenotypes corresponding to *panB*, *panC*, and *panD* mutant strains were also obtained by this procedure.

Growth rates were determined by measuring the increase in the optical density of log-phase cultures with a Klett-Summerson colorimeter equipped with a no. 42 (blue) filter; growth rates are expressed as exponential growth rate constants (generations per hour). Starter cultures for these experiments were grown overnight in nutrient broth at 37°C. To minimize nutrient carryover, cells were washed once or twice in saline and diluted 10-fold into minimal Davis-Mingioli medium. A 0.1-ml sample of the washed, diluted cells was used to inoculate 5 ml of medium. Cultures were incubated in sidearm flasks at 37°C with shaking. Metabolic intermediates and end products were added to liquid media at the following concentrations: sodium α-ketoisovalerate, 50 μg/ml; potassium α-ketopantoate and potassium D-pantoate, 40 μg/ml; calcium D-pantothenate, 20 μg/ml, L-methionine, 50 μg/ml; L-leucine, 100 μg/ml; L-lysine, 20 μg/ml; diaminopimelic acid (mixture of LL, DD, and *meso* isomers), 20 μg/ml; and δ-aminolevulinic acid, 20 μg/ml.

Strain construction and selection of Pan$^+$ revertants. P22-mediated transductions were performed as previously described (17). Strains DU6543 (*panE543 ilvC8*) and DU6563 (*panE563 ilvC8*) were transduced to *ilvC*$^+$ with HT105/4 phage (22) grown on *S. typhimurium* LT2 by plating transductants onto pantothenate-supplemented medium. Pan$^+$ revertants of *panE* mutant strains (e.g., DU6544) were obtained by diluting overnight nutrient broth cultures into saline, plating 10^6 to 10^8 cells onto minimal or leucine-isoleucine-valine agar medium, and incubating at 37°C for 48 h. The linkage of *panE* suppressor mutations to *ilvC* was determined by transducing strain DU6543 with phage grown on a suppressor strain (e.g., DU6545) and selecting *ilvC*$^+$ transductants on pantothenate-supplemented medium. Cotransduction of the suppressor mutation into the recipient strain was determined by scoring the recipient on minimal or valine agar medium.

RESULTS

Reduction of α-ketopantoate by acetohydroxy acid isomeroreductase. A preparation of isomeroreductase that was judged to be greater than 95% pure by electrophoretic analysis under denaturing conditions was used to determine kinetic parameters with α-ketopantoate as substrate; the K_m was 0.25 mM and the V_{max} was 0.136

μmol/min per mg. The apparent affinity of the enzyme for α-ketopantoate was similar to those for the substrates α-acetolactate (K_m, 0.30 mM) and α-acetohydroxybutyrate (K_m, 0.79 mM). The rate of reduction of α-ketopantoate, however, was approximately 20-fold lower than that of α-acetolactate (V_{max} = 2.45 μmol/min per mg). The reduction of α-ketopantoate to form pantoate specifically required NADPH; NADH was inactive. Arfin and Umbarger (1) have shown that Mg^{2+} is required for the reduction of α-keto-β-hydroxyisovalerate by purified isomeroreductase. We found that Mg^{2+} was required for the reduction of α-ketopantoate by isomeroreductase. Arfin and Umbarger (1) have also shown that the isomerized intermediate of α-acetolactate, α-keto-β-hydroxyisovalerate, is reduced by isomeroreductase, but, as with α-ketopantoate, the rate of reduction is less (10-fold) than with α-acetolactate. We showed by standard kinetic analysis that α-ketopantoate appears to be a competitive inhibitor of the conversion of α-aceto-α-hydroxybutyrate to α,β-dihydroxy-β-methylvalerate. The K_i computed from conventional Lineweaver-Burke plots was approximately 0.4 mM, which was close to the K_m value obtained with α-ketopantoate.

Formation of pantoic acid from α-ketopantoic acid in the isomeroreductase-catalyzed reaction. The reduced product of the isomeroreductase reaction with α-ketopantoate as substrate was shown to be pantoic acid (pantoyl lactone) by two criteria. (i) Material extracted from isomeroreductase reaction mixtures could support the growth of α-ketopantoate–pantoate auxotrophs, DU6544 (*panE54*) and DU7410 (*panB41*), as well as a strict pantoate auxotroph, DU6543 (*panE54 ilvC8*); this material could not support the growth of a *panC* mutant. (ii) Material extracted from reaction mixtures was analyzed by gas-liquid chromatography. This material contained two substances that had the same retention times as ketopantoyl lactone and pantoyl lactone standards. The peak corresponding to ketopantoyl lactone (retention time, 2.71 min) decreased, and the pantoyl lactone peak (retention time, 3.15 min) increased during the course of the reaction.

Ketopantoic acid reductase activity in crude extracts of *S. typhimurium*. Crude extracts of *S. typhimurium* containing isomeroreductase catalyzed the reduction of α-ketopantoate under conditions that are optimal for the activity of the purified enzyme (pH 7.5, 4 mM Mg^{2+}). Extracts of *ilvC* mutant strains would not catalyze the reduction of α-ketopantoate under these conditions. Crude extracts of *ilvC* and *ilvC*$^+$ strains contained an additional ketopantoic acid reductase activity that is optimal at pH 5.0, is Mg^{2+} independent, and requires NADPH. This activity probably is homologous with an activity in extracts of *E. coli* which was previously described (H. L. King, Jr., and D. R. Wilken, Fed. Proc. abstr. 476, 1972). Evidence is presented below to suggest that this activity functions as a ketopantoic acid reductase.

Selection and identification of *S. typhimurium* strains defective in ketopantoic acid reductase. *S. typhimurium* strain DU201 (*ilvC8*) was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (18) and enriched for pantothenic acid auxotrophs by the ampicillin selection technique (18). Four different phenotypic classes of pantothenate-requiring auxotrophs were obtained. Three of these corresponded to mutants already described: *panB* mutants, which require either α-ketopantoate, pantoate, or pantothenate; *panC* mutants, which require pantothenate; and *panD* mutants, which require β-alanine or pantothenate. Mutant strains in the remaining class, which we designate *panE*, were distinguished by their ability to grow in minimal medium supplemented with pantoate or pantothenate, but not in minimal medium supplemented with α-ketopantoate or β-alanine. On the basis of this phenotype, strains DU6543, DU6563, DU6583, and DU6611 were characterized as mutants that were unable to convert α-ketopantoate to pantoate. The growth rates of DU6543 and DU6563 in the presence of isoleucine and valine and other compounds are shown in Table 2. All four strains had substantially reduced levels of the Mg^{2+}-independent ketopantoic acid reductase described above (3.2 to 12% of the activity present in DU201; Table 2, data not shown). Both DU6543 and DU6563 had wild-type levels of ketopantoate hydroxymethyltransferase (data not shown). A ketopantoyl lactone reductase activity was detected in extracts of *S. typhimurium* LT2 (*ilvC8*); strains DU6543 and DU6563 also had the lactone reductase activity at approximately the same level (2.3 to 3.5 nmol/min per mg of protein). This observation suggests that ketopantoyl lactone does not play an important role in pantothenate biosynthesis.

Role of the α-acetohydroxy acid isomeroreductase in pantothenic acid biosynthesis. To determine the capacity of the isomeroreductase to catalyze the reduction of α-ketopantoate in vivo, the *ilvC*$^+$ gene was introduced into three *panE ilvC8* mutants, DU6543, DU6563, and DU6583, by P22-mediated transduction. The resulting strains, DU6544, DU6564, and DU6584, all retained the requirement for pantoate or pantothenate and gained the ability to grow in the presence of α-ketopantoate in the presence or absence of isoleucine and valine. Growth rates of DU6544 and DU6564 in the presence of pantothenic acid intermediates are shown in

TABLE 2. Exponential growth rates and ketopantoic acid reductase activities of DU201, DU6543, and DU6563

Strain	Growth rate (generations/h) in the following media:					Ketopantoic acid reductase[b] activity (nmol/min per mg)
	IV[a]	IV + α-keto-pantoate	IV + pantoate	IV + panto-thenate	IV + lysine + methionine + δ-ALA[c]	
DU201 (*ilvC8 panE*$^+$)	1.01	1.06	1.11	1.05	1.13	23.5 (100)[d]
DU6543 (*ilvC8 panE54*)	0[e]	0.21	0.88	1.05	0[e]	2.82 (12)
DU6563 (*ilvC8 panE56*)	0[e]	0.19	0.76	0.68	0[e]	0.74 (3.2)

[a] IV, Isoleucine-valine medium.
[b] Ketopantoic acid reductase assays were performed at pH 5.5 as described in the text on cells grown in minimal medium supplemented with pantothenate, isoleucine, and valine.
[c] δ-ALA, δ-Aminolevulinate.
[d] Values within parentheses refer to the percentage of ketopantoic reductase activity in a given strain divided by the specific activity in DU201.
[e] Growth stopped abruptly after three to four doublings.

Table 3; apparently, the isomeroreductase at uninduced levels is sufficient for pantothenic acid biosynthesis when abundant quantities of α-ketopantoate are present. The phenotype of *panE* mutant strains (DU6544, DU6564, and DU6584) may explain why none has been identified. In the presence of a functional isomeroreductase, these mutants are phenotypically identical to *panB* mutants (i.e., both require α-ketopantoate) and could only be distinguished by the appropriate assays.

The requirement of strain DU6544 for pantothenate or its precursors could be abrogated by inducing the synthesis of the isomeroreductase. Induction was accomplished by inoculating DU6544 onto minimal agar medium supplemented with ~0.5 μmol of α-aceto-α-hydroxybutyrate. Luxurious growth was observed within 24 h; no growth was observed in the absence of α-aceto-α-hydroxybutyrate on minimal medium. This observation indicates that an elevated level of isomeroreductase allows pantoate to by synthesized either (i) by raising the level of α-ketoisovalerate and concomitantly α-ketopantoate or (ii) by increasing the rate of conversion of α-ketopantoate to pantoate. The former explanation is unlikely in view of the observation that neither valine (100 μg/ml) nor α-ketoisovalerate (50 μg/ml) permitted the growth of DU6544, DU6564, or DU6584 (Table 3, data not shown for DU6584). Also, it should be noted that the inducer used in the experiment described above was the isoleucine intermediate, not the α-ketoisovalerate (valine) intermediate, therefore precluding the possibility that the inducer was merely serving as a source of α-ketoisovalerate which might serve to alleviate the ketopantoate requirement.

Lysine-methionine requirement of *panE* mutants. We have recently demonstrated that the biosynthesis of pantothenic acid in a *S. typhimurium ilvG* mutant strain (DU501) is partially blocked and that the concomitant decrease in coenzyme A and succinyl coenzyme A in this strain is manifested as a requirement for methionine (20). In addition, inhibition of the growth of

TABLE 3. Exponential growth rate of DU6544 and DU6564 in various media

Strain	Growth rate (generations/h) in the following media:															
	IV[a]	IV + α-ke-to-pan-toate	IV + pan-toate	IV + pan-tothe-nate	IV + methio-nine	IV + lysine + me-thio-nine	IV + ly-sine	IV + α-ke-toiso-valer-ate	Min-imal	α-Ke-topan-toate	Pan-toate	Panto-thenate	Methio-nine	Lysine + methio-nine	Lysine	α-Keto-isoval-erate
DU6544 (*ilvC*$^+$ *panE54*)	0[b]	0.968	1.18	1.30	0	1.09	0	0[b]	0	0.87	0.90	0.923	0	0.67	0	0[b]
DU6564 (*ilvC*$^+$ *panE56*)	0[b]	0.882	1.30	1.03	0	0.674	0	0[b]	0	1.15	1.18	1.39	0	0.86	0[b]	0[b]

[a] IV, Isoleucine-valine medium.
[b] No growth was observed within 24 h, growth observed after this period was due to the appearance of Pan$^+$ revertants.

wild-type *S. typhimurium* by inhibitors of pantothenic acid biosynthesis (e.g., salicylic acid) can be reversed by the amino acids that require succinyl coenzyme A for their biosynthesis, i.e., lysine and methionine (20). Methionine, but not lysine, reverses growth inhibition at the minimum inhibitory concentration of salicylic acid; at higher concentrations, both lysine and methionine are required to reverse inhibition. At the highest concentrations tested, only pantothenate reverses growth inhibition (20). Since the requirement for pantothenate, methionine and lysine, or methionine alone reflects the degree to which the pantothenic acid pathway is blocked, these requirements can be used to assess the synthesis of pantothenic acid in the *panE* mutant strains. Strains DU6543 (*ilvC8 panE54*) and DU6563 (*ilvC8 panE54*) did not grow in isoleucine-valine medium supplemented with either methionine, lysine, or a combination of lysine, methionine, and δ-aminolevulinate (Table 2). The growth of the *ilvC*$^+$ derivatives, DU6544 and DU6563, was not stimulated by either lysine or methionine alone; however, these strains would grow in medium supplemented with both lysine and methionine in the presence or absence of isoleucine and valine (Table 3). These phenotypes indicate that the isomeroreductase at uninduced levels synthesizes a sufficient amount of pantoate-pantothenate for all cellular functions with the exception of two of the succinyl coenzyme A-requiring biosynthetic pathways.

Suppression of the pantoate requirement of *panE* strains by *ilvYC* regulatory mutations. *panE ilvC8* strains (e.g., DU6543, DU6563, and DU6583) reverted to Pan$^+$ at a low frequency (less than 5 revertants per 10^8 cells) when spread on isoleucine-valine medium. *panE ilvC*$^+$ strains (e.g., DU6544 and DU6564) reverted to Pan$^+$ at a much greater frequency (~1,000 revertants per 10^8 cells) on the same medium or on minimal medium. These observations strongly suggest that most mechanisms of suppression of the pantoate requirement of *panE* strains require a functional isomeroreductase. Fourteen Pan$^+$ revertants of DU6544 were selected from either minimal or leucine-isoleucine-valine medium and assayed for ketopantoic acid reductase and isomeroreductase. None of these isolates had ketopantoic acid reductase activity above that in DU6544; all exhibited elevated levels of isomeroreductase when grown under the conditions in which they were originally selected (data not shown). Two of the revertants that were selected on leucine-isoleucine-valine medium, DU6545 and DU6546, were chosen for further analysis. The growth of DU6545 was stimulated by valine, whereas DU6546 required valine for growth; the significance of these phenotypes will be explained below. Both revertants maintained elevated isomeroreductase under repressing conditions (Table 4). The levels of threonine deaminase and acetohydroxy acid synthases I and II in the revertants approximated those found in the parent and responded normally to repression by the branched-chain amino acids (Table 4), therefore suggesting that the apparent constitutive expression of *ilvC* was for reasons other than enhanced internal induction by the acetohydroxy acids. P22-mediated transductional analysis of DU6545 and DU6546 showed that the suppressing mutation in each of these strains is tightly linked to *ilvC*: 96% (48 of 50) in the case

TABLE 4. Specific activities of acetohydroxy acid isomeroreductase, acetohydroxy acid synthases I and II, and threonine deaminase in DU6544 and Pan$^+$ revertants

Strain	Growth conditions[a]	Sp act (nmol/min per mg)			
		Acetohydroxy acid isomeroreductase	Acetohydroxy acid synthase		Threonine deaminase
			I	II	
DU6544 (*panE54 sup*$^+$)	Pan	56.8	9.50	3.22	132
	LIVP	19.0	5.42	1.44	50.4
DU6545 (*panE54 sup6*)	Pan	440	4.88	9.37	149
	LIVP	332	3.08	3.72	62.6
DU6546 (*panE54 sup45*)	Pan	128	11.8	5.80	107
	LIVP	95.5	3.80	4.75	61.0
DU6801 (*panE54 sup601*)	Pan	231	6.65	6.15	252
	LIVP	256	5.60	2.49	98
DU6803 (*panE54 sup603*)	Pan	497	4.50	6.28	150
	LIVP	562	0.975	1.32	27.0
DU6804 (*panE54 sup604*)	Pan	344	10.2	6.45	224
	LIVP	367	7.60	4.45	66.5

[a] Pan, Growth medium for preparation of cell extracts was supplemented with pantothenate (20 μg/ml); LIVP, growth medium was supplemented with leucine (100 μg/ml), isoleucine (50 μg/ml), valine (100 μg/ml), and pantothenate (20 μg/ml).

of the DU6545 suppressor and 73% (29 of 40) in the case of the DU6546 suppressor. These data suggest that the suppressing mutations might lie in the regulatory region of *ilvC* or in *ilvY*, the positive regulatory effector for *ilvC* (4).

To eliminate the classes of Pan$^+$ revertants whose suppressing mechanism depends on elevation of the α-acetohydroxy acids, a *panE* strain which lacked acetohydroxy acid synthases I and II was constructed. This strain, DU6540 (*panE54 ΔilvGE605 ilvB1* (*zia-1*::Tn*10*) *ilvC*$^+$), which required leucine, isoleucine, valine, and pantothenate for growth, yielded 10-fold fewer revertants than did strain DU6543 (*panE54 ilvG*$^+$ *ilvE*$^+$ *ilvB*$^+$ *ilvC*$^+$) when plated onto leucine-isoleucine-valine medium. Three Pan$^+$ revertants of DU6540, DU6601, DU6603, and DU6604, were selected for genetic and enzymic analysis. The isomeroreductase level in each revertant was elevated at least 40-fold over the level in the parental strain (Table 5) when revertants were grown in leucine-isoleucine-valine medium. The suppressing mutation in each revertant was at least 73% linked to *ilvC8* by P22 transduction (Table 5). Linkage between *ilvC* and the suppressor mutation was determined by crossing P22 phage grown on a given suppressor strain into DU6543 (*panE54 ilvC8*) and selecting for *ilvC*$^+$ transductants on pantothenate medium. Cotransduction of the suppressor mutation was determined by scoring onto either minimal or valine medium, since several *ilvB*$^+$ *ilvG*$^+$ suppressor strains selected on leucine-isoleucine-valine medium were observed to be either valine bradytrophs (e.g., DU6545) or valine auxotrophs (e.g., DU6546). This scoring procedure revealed that the *ilvC*$^+$ *sup* derivative of DU6543, DU6803 (*panE54 sup603 ilvB*$^+$ *ilvG*$^+$), was a prototroph and was not stimulated by valine. DU6801 (*panE54 sup601*) was a valine auxotroph, and DU6804 (*panE54 sup604*) was a valine bradytroph. A correlation exits between the phenotype of these derivatives and their isomeroreductase levels; this correlation will be discussed below.

The parental suppressor strains, DU6601, DU6603, and DU6604, lack both acetohydroxy acid synthases I and II, and no inducers can be made in vivo; therefore the regulation of the isomeroreductase was studied in the *ilvB*$^+$ *ilvG*$^+$ derivatives DU6801, DU6803, and DU6804, where the synthesis of the inducers could be regulated by the branched-chain amino acids. The isomeroreductase levels remained high in these strains and did not respond to repressing conditions. Threonine deaminase and acetohydroxy acid synthases I and II were present at the same levels as in DU6544 (*panE54 sup*$^+$ *ilvB*$^+$ *ilvG*$^+$) and were repressed by the branched-chain amino acids (Table 4).

Inhibition of pantoate synthesis in vivo by α-acetohydoxy acids. Nutritional characterization of DU6540 (*panE54 ΔilvGE605 ilvB1 zia-1*::Tn*10*) revealed that this strain could grow on leucine-isoleucine-valine medium supplemented with lysine and methionine or supplemented with methionine alone. This surprising phenotype indicated that pantothenic acid biosynthesis was facilitated in this strain, in spite of the fact that the isomeroreductase is maximally repressed (Table 5), pantoate cannot be synthesized in any significant quantity in the complete absence of isomeroreductase, however, since DU6543 (*panE54 ilvC8*) and DU6563 (*panE56 ilvC8*) will not grow on either leucine-isoleucine-valine medium or leucine-isoleucine-valine medium supplemented with lysine and methionine. These observations suggested that pantoate synthesis by the isomeroreductase was enhanced by raising the ratio of α-ketopantoate to its competitive substrates (α-acetolactate and α-aceto-α-hydroxybutyrate). Four lines of evidence support this view: (i) α-ketopantoate competed with α-aceto-α-hydroxybutyrate for binding to the isomeroreductase (see above); (ii) although 1 mM α-aceto-α-hydroxybutyrate stimulated the growth of DU6544, 10 mM α-aceto-α-hydroxybutyrate had no effect; (iii) valine enhanced the physiological suppression of the pantothenate requirement of DU6544 by α-aceto-α-hydroxy-

TABLE 5. Specific activities of isomeroreductase and cotransduction of suppressor mutations in DU6540 and Pan$^+$ revertants

Strain	Growth medium[a]	Isomeroreductase sp act (nmol/min per mg)	Elevation (fold)	% Cotransduction of *sup* to *ilvC8*
DU6540 (*sup*$^+$)	LIVP	4.60	1	ND[b]
DU6601 (*sup601*)	LIV	188	41	73 (66/90)
DU6603 (*sup603*)	LIV	522	113	76 (25/33)
DU6604 (*sup604*)	LIV	423	92	85 (39/46)

[a] LIV, Growth medium was supplemented with leucine (100 μg/ml), isoleucine (50 μg/ml), valine (100 μg/ml), and pantothenate (20 μg/ml); LIV, growth medium was supplemented with leucine (100 μg/ml), isoleucine (50 μg/ml), and valine (100 μg/ml).

[b] ND, Not determined.

butyrate (data not shown), presumably by inhibition of the formation of the α-acetohydroxy acids by acetohydroxy acid synthase I; and (iv) several of the *panE* suppressor strains (e.g., DU6545, DU6546, DU6801, and DU6804) were either valine bradytrophs or valine auxotrophs. A positive correlation exists between the magnitude of isomeroreductase production and the degree of valine independence of the suppressor strains; this suggests that the higher the level of isomeroreductase, the less the requirement for blocking the synthesis of the α-acetohydroxy acids.

The effect of valine in the facilitation of suppression by α-aceto-α-hydroxybutyrate and in the stimulation of growth of the suppressor strains could also be explained by proposing that added valine raises the level of α-ketoisovalerate and, hence, enhances the synthesis of α-ketopantoate. This explanation is unlikely since valine or α-ketoisovalerate does not stimulate the growth of *panE* strains (e.g., DU6544 and DU6564) in which the synthesis of the α-acetohydroxy acids cannot be curtailed by endproduct inhibition.

Genetic mapping of the *panE* locus. An effort was made to determine whether the mutations which confer pantoate auxotrophy are linked to the *panBC* gene cluster, located at min 4.5 on the *S. typhimurium* LT2 linkage map (21). The linkage of *panE* to *panC* was tested by growing P22 phage on strains DU6543 and DU6563 and transducing a *panC2* strain to *pan*$^+$ in the presence of either α-ketopantoate or pantoate; no α-ketopantoate- or pantoate-requiring transductants were obtained with either donor *panE* mutant (200 of each scored). The linkage of *panE* to *panB* was tested by transducing DU6563 (*panE56 ilvC8*) to pantoate independence with phage grown on strain DU7410 (*panB41*) in the presence of isoleucine, valine, and α-ketopantoate; no α-ketopantoate-requiring transductants were obtained (200 transductants scored).

To determine whether the mutations in *panE* strains DU6543 DU6563, and DU6611 were located in the same region, a P22 lysate of DU6564 (*panE56 ilvC*$^+$) was used to transduce strains DU6543 (*panE54 ilvC8*) and DU6611 (*panE54 ilvC8*) to *panE*$^+$ or to *ilvC*$^+$. There were approximately 30-fold fewer *panE*$^+$ transductants than *ilvC*$^+$ transducants in the case of DU6543 and 300-fold fewer in the case of DU6611; the frequency of *panE*$^+$ transductants was approximately the same as the frequency of *ilvC*$^+$ tranductants in analogous crosses where the donor phage was grown on the wild type (*panE*$^+$ *ilvC*$^+$). These data indicate that the *panE* mutations in strains DU6543, DU6564, and DU6611 are clustered in the same region.

In the course of our mutagenesis, we obtained several β-alanine or pantothenate auxotrophs. The mutation conferring the β-alanine requirement to two genetically distinct mutant strains, DU8020 and DU8123, was found to be linked to *panC* by P22 transduction: 99.1% in the case of DU8020 (112 β-alanine auxotrophs out of 113 total *panC*$^+$ transductants), and 97.4% in the case of DU8123 (112 β-alanine auxotrophs out of 115 total *panC*$^+$ transductants). These auxotrophs may represent *panD* mutants which lack aspartate-1-decarboxylase (7). If so, this finding conflicts with the report by Ortega et al. (19) that *panD* is lcoated at min 89 on the *S. typhimurium* linkage map (21); alternatively, the mutants could represent a gene that encodes a second, heterologous subunit of asparate-1-decarboxylase. Cronan and co-workers have shown that the *panD* gene in *E. coli* K-12 lies between *panB* and *panC* at min 3 (8).

The β-alanine requirement of DU8020 and DU8123 provided an additional marker for determining the location of *panE*; however, no linkage was observed between the two genes in P22-mediated transductions. Time of entry studies using *S. typhimurium* Hfr donors and *panE* recipients are being conducted to determine the general location of *panE*.

DISCUSSION

We have demonstrated that the acetohydroxy acid isomeroreductase can catalyze the reduction of α-ketopantoic acid both in vivo and in vitro. *S. typhimurium* strains that lack both ketopantoic acid rductase and the isomeroreductase (e.g., DU6543) strictly require either pantoate or pantothenate in addition to isoleucine and valine; these strains are strongly blocked in the conversion of α-ketopantoate to pantoate. The extent to which the isomeroreductase synthesizes pantoic acid is reflected in the growth requirements of *ilvC*$^+$ derivatives of DU6543 and DU6563. DU6544 and DU6564 require either ketopantoate, pantoate, or pantothenate; the requirement can be removed by inducing the synthesis of the isomeroreductase. This phenotype indicates that the isomeroreductase alone is sufficient for the biosynthesis of pantoate from α-ketopantoate if (i) the level of α-ketopantoate is raised or (ii) elevated levels of this enzyme are present.

Induced levels of isomeroreductase increase the synthesis of pantoate by increasing the rate at which α-ketopantoate is reduced. Elevated levels of α-ketoisovalerate do not overcome the block in pantoate synthesis and do not contribute to suppression of the requirement when the isomeroreductase is induced: (i) the growth of *panE* mutant strains is not stimulated by valine or α-ketoisovalerate; (ii) induction of isomerore-

ductase (with α-aceto-α-hydroxybutyrate) suppresses the pantoate requirement in the presence of excess leucine, isoleucine, and valine, i.e., when the synthesis of α-ketoisovalerate is limited; and (iii) genetic suppression by elevation of isomeroreductase can occur in strains in which the anabolic formation of α-ketoisovalerate is completely blocked.

A combination of lysine and methionine supported the growth of *panE* mutants. This dual requirement has been observed in other *S. typhimurium* strains in which the biosynthesis of coenzyme A or succinyl coenzyme A is curtailed. Nonpolarigenic *ilv*G mutants strains accumulate α-ketobutyrate, which inhibits synthesis of α-ketopantoate by ketopantoate hydroxymethyltransferase (20). *ilv*G mutant strains that synthesize an isoleucine-insensitive form of threonine deaminase are more severely intoxicated owing to the unbridled production of α-ketobutyrate; these strains require methionine and lysine for optimal growth (20). Strains deficient in α-ketoglutarate dehydrogenase (*sucA*) have a growth requirement for succinate or a combination of methionine and lysine (14). The ability of methionine-lysine medium to support the growth of *panE* (*ilvC*$^+$) strains suggests that the biosynthesis of pantothenate is partially blocked and that the isomeroreductase synthesizes an adequate amount of pantoate and coenzyme A for all cellular functions, with the exception of methionine and lysine biosynthesis.

An additional aspect of the in vivo ability of isomeroreductase to serve as a ketopantoic acid reductase is related to the flux of the α-acetohydroxy acids through the branched-chain amino acid biosynthetic pathway. It clearly appears that the presence of the α-acetohydroxy acids prevents the conversion of α-ketopantoate to pantoate by the isomeroreductase. In the presence of functional α-acetohydroxy acid synthases, high levels of isomeroreductase were required to alleviate the reductase deficiency in *panE* mutant strains (i.e., the lysine-methionine requirement), but in a strain (DU6540) where the synthesis of the acetohydroxy acid intermediates was completely blocked, the residual uninduced level was able to synthesize pantoate (and coenzyme A) in quantities sufficient for all the cellular functions, with the exception of methionine biosynthesis.

Conditions that limit the supply of leucine or valine (or both) might also limit the biosynthesis of pantothenic acid. Abrogation of the pantoate requirement of *panE* mutants by induction of the isomeroreductase suggests a mechanism by which pantothenic acid biosynthesis could be regulated by the level of leucine, isoleucine, or valine. *S. typhimurium* responds to branched-chain amino acid limitation by derepression of *ilvGEDA* and *ilvB* gene products and by induction of the isomeroreductase when the level of the α-acetohydroxy acids increases. This response would enhance the synthesis of pantoic acid as well as the synthesis of the branched-chain amino acids.

Five spontaneously arising Pan$^+$ revertants of DU6544 (*panE54 ilvC*$^+$) exhibited elevated isomeroreductase. The suppressing mutation in each strain was linked to *ilvC* by P22 transduction and may represent alterations in the *ilvY* gene product or in the *ilvC* promoter. Selection for Pan$^+$ revertants of *panE* strains that lack both acetohydroxy acid synthases I and II provides a method for obtaining mutants with altered expression of the *ilvC* gene or *ilvY* gene.

ACKNOWLEDGMENTS

We thank Barbara Murphy for assistance in selection of some of the Pan$^+$ revertants. We thank Robert W. Wheat for assistance with the gas-liquid chromatographic analysis. We also thank Dorothy Thompson for excellent technical assistance and Dayle Wilkins for typing the manuscript.

This investigation was supported by Public Health Service grant GM12551 from the National Institute of General Medical Sciences. D.A.P. is a trainee in the University Program in Genetics supported by Public Health Service grant GM02254 from the National Institute of General Medical Sciences.

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