

## Acetohydroxy Acid Synthase Is a Target for Leucine-Containing Peptide Toxicity in *Escherichia coli*

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Received 13 July 1981/Accepted 18 August 1981

Acetohydroxy acid synthase from a mutant resistant to leucine-containing peptides was insensitive to leucine inhibition. It is concluded that acetohydroxy acid synthase is a target for the toxicity of the high concentrations of leucine brought into *Escherichia coli* K-12 by leucine-containing peptides.

Leucine-containing peptides (LCP) enter *Escherichia coli* cells via three distinct peptide transport systems: Opp, an oligopeptide permease; Opp-I, a specialized tripeptide permease; and Dpp, a dipeptide permease (for a review see references 2 and 13). These transport systems are considered to be constitutive and not affected by either the peptide or its degradation products. Because LCP are probably taken in and cleaved faster than leucine is metabolized, leucine is accumulated intracellularly and reaches abnormally high concentrations (20). These high concentrations of leucine inhibit the growth of *E. coli* K-12 cells (12, 18). Leucine in its free form is nontoxic to *E. coli* K-12 strains, since its uptake is regulated by repression through its own intracellular concentration (14-16). Inhibition of growth by LCP occurs immediately after addition of the peptides (20, 23) and is relieved when 0.4 mM isoleucine is added (20). We have concluded, therefore, that leucine at high intracellular concentration interferes with one of the enzymes in the isoleucine pathway (20).

A common way to identify a target for a toxic agent is by comparing resistant mutants that are altered in the target site with their parental strain. The toxicity of LCP provides us with a good tool for the isolation of such mutants. As dipeptides are transported into *E. coli* via the Dpp and the Opp systems (2, 13), the probability of obtaining resistance to a leucine-containing dipeptide by a Dpp<sup>-</sup> alteration should have been very low (2, 7). Thus, we selected for the required mutants with either Gly-Leu or Phe-Leu, which were shown to possess high toxicity towards the strain used in this study, strain I (20).

Spontaneous mutants resistant to Phe-Leu (PLR) were isolated from cell cultures that had been grown after a long lag (about 1,600 min) in the minimal liquid medium VBf (VB medium [22] containing 10 µg of tryptophan per ml and 20 µg of cysteine per ml) in the presence of 0.2

mM Phe-Leu. Spontaneous Gly-Leu resistant mutants (GLR) were isolated by direct selection on solid VBf plates containing 0.5 mM toxic dipeptide. Mutants (PLR and GLR) were tested and found to maintain their parental characteristics, i.e., Trp<sup>-</sup>, Cys<sup>-</sup>, microscopic morphology, morphology of colonies, and growth rates. However, whereas all GLR mutants were completely resistant to Gly-Leu, only half of the PLR mutants possessed complete resistance to Phe-Leu. The frequencies of spontaneous GLR mutants in three unselected populations of strain I (suspensions of fresh isolated colonies that had been grown on the rich medium LB [11]) were  $1.1 \times 10^{-6}$ ,  $1.2 \times 10^{-6}$ , and  $3.8 \times 10^{-7}$ .

Resistance to leucine-containing dipeptides could arise for any of the following reasons: (i) a loss of the ability to transport the dipeptide (Dpp<sup>-</sup>), (ii) a loss of intracellular peptidase activity (Pep<sup>-</sup>), or (iii) quantitative or qualitative changes of the high-leucine target site (Leu<sup>r</sup>). A way to distinguish among these possibilities is by studying the utilization and toxicity of several di- and tripeptides with the independent mutants. As dipeptides are transported into *E. coli* cells via one nonstringent system, Dpp<sup>-</sup> mutants should lose their ability to utilize dipeptides as nutritional sources and should gain resistance to toxic dipeptides, whereas toxic tripeptides should still inhibit the growth of these mutants. The Pep<sup>-</sup> mutants should not utilize peptides as amino acid sources and should be resistant to peptides containing a toxic agent. The Leu<sup>r</sup> mutants should be resistant to all di- and tripeptides containing leucine, but should still grow normally when dipeptides are supplemented as nutritional sources. Such mutants would also be sensitive to peptides which contain a toxic agent other than leucine. However, since the mode or toxicity of valine in *E. coli* K-12 might be similar to that of leucine (brought by LCP), Leu<sup>r</sup> mutants may gain resistance to valine and valine-containing peptides.

Based on the results of the growth experi-

ment, the isolated GLR and PLR mutants could be separated into two main groups, Dpp<sup>-</sup> and Leu<sup>r</sup>. The growth response of representatives of these groups, GLR-3 for Leu<sup>r</sup> and GLR-5 for Dpp<sup>-</sup>, is shown in Table 1. No Pep<sup>-</sup> mutants were detected among the 12 independent mutants tested. The lack of Pep<sup>-</sup> mutants accords with the existence of a number of peptidases in *E. coli* K-12, most of which lack amino acid specificity (17, 19). It is surprising, however, that several Dpp<sup>-</sup> mutants were obtained within our GLR and PLR mutants. Our findings do correlate with those of Vonder Haar and Umbarger (23), who were successful in isolating spontaneous Dpp<sup>-</sup> mutants while selecting for GLR mutants. A possible explanation for these results may be that the specific dipeptides used for selection of the mutants (Gly-Leu and Phe-Leu) have very low or no affinity for the Opp system. A Dpp<sup>-</sup> mutant would, in this case, result from a single gene mutation at the *dpp* gene. The Opp system of such mutants should operate normally, as shown in this study by the mutants' sensitivity to the toxic tripeptides Orn<sub>3</sub> and Val-Gly-Gly. Of the Leu<sup>r</sup> mutants, GLR-3 was chosen for further investigations.

The toxicity of LCP in *E. coli* was assumed to be caused by the accumulation of their degradation product, leucine, to abnormally high concentrations, which interfere with the biosynthe-

sis of isoleucine by blocking one of the enzymes in its pathway (20). One candidate for the inhibition of LCP is the first enzyme in the biosynthetic pathway of isoleucine, threonine deaminase, which has already been shown to be inhibited by leucine (6, 23). This suggestion is supported by the observation that the inhibition of growth caused by LCP is cured with the addition of isoleucine alone (20). However, threonine deaminase activity was inhibited by leucine to equal degrees in crude extracts from both the Leu<sup>r</sup> mutant, GLR-3, and its parent, I, indicating the lack of involvement of the enzyme in LCP toxicity. Threonine deaminase was assayed as described by Burns (5). Absorption was measured at 530 mμ with a Micro-Sample spectrophotometer (Gilford 300N), and leucine effect was tested at various concentrations (up to 30 mM). Four independent experiments were carried out in duplicate.

The finding that *E. coli* B is resistant to LCP (20) may indicate another target for leucine sensitivity—acetohydroxy acid synthase (AHAS), which is the first enzyme in the biosynthetic pathway of valine, and also the second in the isoleucine pathway. Unlike *E. coli* K-12, *E. coli* B possesses an isoenzyme of AHAS, the product of *ilvG*, which is resistant to valine (21). This isoenzyme might be responsible for the lack of sensitivity of the B strain to LCP. This

TABLE 1. Growth studies with *E. coli* K-12 strain I and two of its GLR mutants

Agent <sup>a</sup>	Parental strain I			GLR-3			GLR-5		
	Δ-lag <sup>b</sup> (min)	GT <sup>c</sup> (min)	Yield <sup>d</sup> (KU)	Δ-lag (min)	GT (min)	Yield (KU)	Δ-lag (min)	GT (min)	Yield (KU)
VB + C	>1,500	∞	0	>1,500	∞	0	>1,500	∞	0
VB + T	>1,500	∞	0	>1,500	∞	0	>1,500	∞	0
VBf	0	85	100	0	85	112	0	88	110
VB + C + TG	0	85	105	0	86	107	>1,500	∞	0
VB + C + TP	-15	87	100	0	85	102	>1,500	∞	0
VBf + Val	>1,500	∞	0	>1,500	∞	0	>1,500	∞	0
VBf + VG	>1,500	∞	0	>1,500	∞	0	-20	85	103
VBf + VGG	>1,500	∞	0	>1,500	∞	0	>1,500	∞	0
VBf + GL	1,450	∞	2	60	80	105	0	80	105
VBf + PL	>1,500	∞	0	50	87	105	15	85	109
VBf + AL	1,380	∞	5	60	90	100	0	85	98
VBf + Leu <sub>3</sub>	1,400	∞	5	45	85	110	1,420	∞	3
VBf + LGG	>1,500	∞	0	30	85	106	1,350	∞	6
VBf + LPA	1,350	∞	8	60	83	108	1,300	∞	9
VBf + Orn <sub>3</sub>	1,400	∞	4	1,450	∞	2	>1,500	∞	0

<sup>a</sup> Abbreviations are: C, L-cysteine; T, L-tryptophan; TG, Trp-Gly; TP, Trp-Phe; VG, Val-Gly; VGG, Val-Gly-Gly; GL, Gly-Leu; PL, Phe-Leu; AL, Ala-Leu; Leu<sub>3</sub>, Leu-Leu-Leu; LGG, Leu-Gly-Gly; LPA, Leu-Phe-Ala (all purchased from Sigma Chemical Co., St. Louis, Mo.); and Orn<sub>3</sub>, Orn-Orn-Orn × 4HCl (purchased from Miles-Yeda). TG and TP were tested at 0.05 mM. Val, VG, and VGG were all at 0.1 mM. GL, PL, AL, Leu<sub>3</sub>, LGG, and LPA were supplemented at 0.25 mM.

<sup>b</sup> Δ-lag is the difference in minutes in attaining growth between the control culture (VBf) and the respective culture.

<sup>c</sup> GT, Generation time.

<sup>d</sup> The final yields in Klett units (KU) of the cultures were determined after 1,500 min of growth under the conditions described previously (20).

enzyme has already been reported to be inhibited by all of the three branched-chain amino acids in some enteric bacteria (1, 21), whereas in *E. coli* K-12 the inhibition of AHAS by leucine was not detected (10) or was small (4, 8). Under our experimental conditions leucine significantly inhibited AHAS activity in the crude extract of strain I, whereas the activity in LCP-resistant mutant GLR-3 was hardly affected, even in the presence of 30 mM leucine (Fig. 1). As GLR-3 is a spontaneous mutant isolated with a mutation

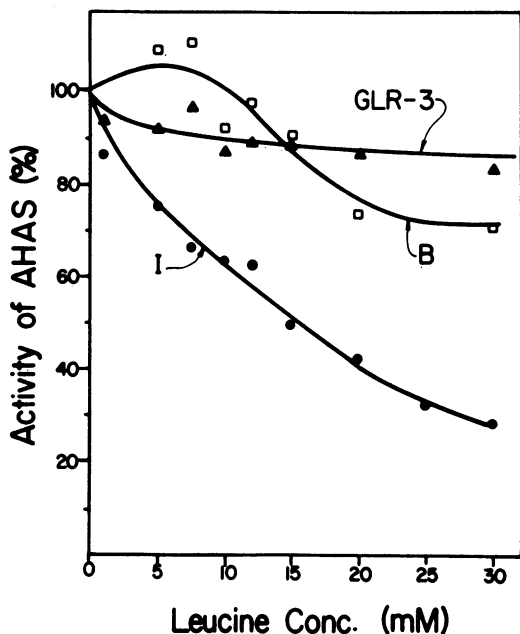


FIG. 1. Leucine effect on AHAS activity. Bacteria were grown in Vbf to late exponential phase (40 klett units). Cells were washed twice with an equal volume of 0.1 M sodium phosphate buffer (pH 7.3) and concentrated to approximately  $6 \times 10^9$  bacteria per ml. Extracts were prepared by sonication with an MSE sonicator for five successive 1-min treatments with 1-min intervals between them, in the cold. The buffered solutions used for extraction and the procedure for determining AHAS activity were as described by Bauler et al. and Blatt et al. (3, 4), except that the solution contained 0.1 M sodium phosphate buffer at pH 7.3, and aggregates produced either during the acetoin reaction or during the color reaction were excluded by centrifugation (10 min,  $3,000 \times g$ , room temperature). The colored reaction product was measured at 540 nm with a Micro-Sample spectrophotometer (Gilford 300N). Leucine effects were tested in crude extracts of the LCP-sensitive strain I (●), as well as of the LCP-resistant strains *E. coli* B (□) and mutant GLR-3 (▲). Each point is the average of four independent experiments which were carried out in duplicate. One-hundred percent activities for the parent strain I, mutant GLR-3, and *E. coli* B were 18.2, 19.4, and 23.2 nmol of acetoin, respectively, per mg of protein per min.

rate of approximately  $10^{-6}$ , it is most probably a single gene mutant which is modified only in its high leucine target site (Leu<sup>r</sup>). The correlation found between the Leu<sup>r</sup> characteristics of this mutant and the in vitro resistance of its AHAS activity to high concentrations of leucine indicates that AHAS is indeed a target for LCP toxicity in *E. coli* K-12. This notion is supported by our findings that AHAS activity in extracts of the LCP-resistant strain *E. coli* B was also relatively resistant to leucine (Fig. 1). Finally, it is noteworthy that isoenzymes AHAS-I (the product of *ilvB*) and AHAS-III (the product of *ilvH* and *ilvI*), which are normally the only ones present in *E. coli* K-12, are not subjected to repression or derepression by isoleucine (8, 9, 21). Inhibition of AHAS-I and AHAS-III caused by excess leucine generated from LCP may, therefore lead to an inhibition of growth without the complicating effects of derepression due to isoleucine starvation.

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