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Isoleucine and Valine Metabolism in *Escherichia coli*

XIX. Inhibition of Isoleucine Biosynthesis by Glycyl-Leucine

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The inhibition of growth of the K-12 strain of *Escherichia coli* by glycyl-L-leucine observed originally by Simmonds and co-workers was investigated. The inhibition was reversed by isoleucine and those precursors of isoleucine beyond threonine in the biosynthetic pathway. Threonine reversed the inhibition poorly. With heavy cell suspensions, the inhibition was transient: the onset of growth followed the disappearance of the dipeptide from the medium and the appearance of glycine and leucine. Glycyl-leucine was shown to be an inhibitor of threonine deaminase (EC 4.2.1.16 L-threonine hydro-lyase [deaminating]). One kind of glycyl-leucine-resistant mutant had a threonine deaminase that was resistant to isoleucine and glycyl-leucine inhibition. The pattern of glycyl-leucine inhibition is compared with those of inhibition by isoleucine and by the weaker inhibitors leucine and valine.

Simmonds and co-workers (4, 7) first noted the inhibitory effect of glycyl-L-leucine on *Escherichia coli* strain K-12. This inhibition was discovered during their studies on peptidase activity and peptide utilization for growth by various auxotrophs. The inhibition was also exerted by leucyl-glycine and tri- and tetra-peptides of glycine and leucine. Glycyl- and leucyl-peptides of other amino acids or other aminoacyl-peptides of glycine or leucine did not show the inhibitory effect.

The inhibitory effect of glycyl-leucine was encountered recently in our laboratory while we were studying growth inhibition caused by 4-azaleucine. Since azaleucine inhibition is reversed by leucine, various leucine analogues and derivatives were tested for their capacity to reverse the inhibition. Glycyl-leucine and leucyl-glycine were among those tested, but these compounds were found to cause more severe inhibition than azaleucine itself. This finding proved to be of further interest when it was noted that the inhibition was reversed by isoleucine. Thus, the possibility was raised that glycyl-leucine and leucyl-glycine might have interefered with isoleucine biosynthesis (via the pathway shown in Fig. 1) or with isoleucine utilization. This paper describes experiments undertaken to explain the inhibitory effect of glycyl-leucine on *E. coli* K-12.

MATERIALS AND METHODS

Organisms. The bacterial strains used in this study are listed in Table 1.

Media, preparation of cell extracts, and enzymatic assays. The cells were grown at 37 C in the minimal medium of Davis and Mingioli (1) supplemented with 0.5% glucose. Growth was determined by measuring turbidity with a Klett-Summerson photocolorimeter with a no. 42 (blue) filter (1 Klett unit = 4.3×10^6 cells per ml). Cells were harvested by centrifugation at 4 C and washed twice with 0.05 M phosphate buffer, *p*H 7.5. The cells were resuspended in eight times their wet weight of the same buffer and disrupted with a Branson 125-w Sonifier. The disrupted suspension was centrifuged at 15,000 rev/min for 20 min in the SS-1 rotor of a Sorvall model RC-2 refrigerated centrifuge. Enzymatic assays were performed immediately after centrifugation by methods previously described (2, 9). Protein concentration was determined by the biuret method (3). Enzyme activities are expressed as micromoles of product formed per minute per milligram of protein.

Inhibition and reversal studies on seeded plates. The sensitivity of various strains to glycyl-leucine was examined by preparing minimal agar plates seeded with cells of the strain to be tested. Glycyl-leucine (0.05 ml of a 0.1 M solution) was placed on the plate and the plate was incubated for 20 hr. Sensitive strains showed clear zones of inhibition about 2.5 cm in diameter. Resistant strains showed no inhibition zones. Reversal was tested by placing a drop of the compound to be tested close to the drop of glycyl-leucine. Reversal of inhibition re-

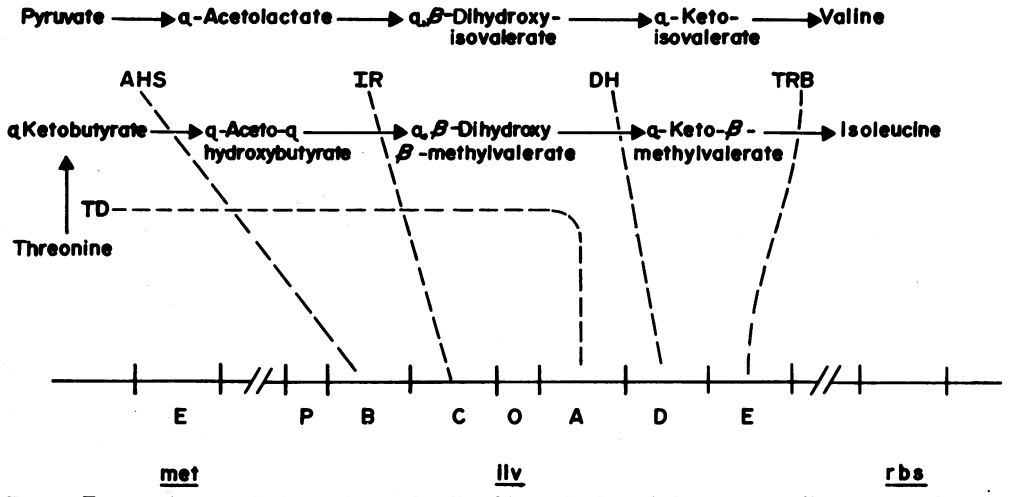

FIG. 1. *Enzymatic steps in isoleucine and valine biosynthesis and the corresponding structural genes. In addition to the structural genes, two controlling elements, ilvO and ilvP, are represented. Modified from Ramakrishnan and Adelberg (6).*

TABLE 1. *Bacterial strains*

Strain	Relevant genotype	Gene products derepressed[a]	Sensitive to glycyl-leucine	Source
K-12		None	Yes	
CU2501	*ilvO468*	*ilvADE*	No	Val^r prototroph isolated from K-12 by J. H. Jackson
CU1018	*ilvS505, ilvT504, ilvU506*	*ilvADE*	Yes	Formerly Tir-8 (8); thiaisoleucine resistant strain
CU5002	*azl-1*	*ilvADE, ilvB, leuABCD*	Yes	Isolated from an Hfr Met$^-$ as an Azl^r by S. Dwyer
CU5117	*azl-1, ilvF465*	*ilvADE, ilvB, leuABCD* (AHS not valine-sensitive)	No	Isolated from CU5002 by W. J. Pledger
CU37	*ilvB497*	None (AHS not valine-sensitive)	Yes	Val^r prototroph isolated from K-12 by W. J. Pledger
CU47	*ilvF465*	None (AHS not valine-sensitive)	Yes	Val^r transductant of K-12 using CU5117 as donor; prepared by W. J. Pledger

[a] AHS = acetohydroxy acid synthetase.

sulted in a flattening of one side of the nearly circular inhibition zone or in a crescent-shaped inhibition zone.

Isolation of glycyl-leucine-resistant strains. Cells resistant to glycyl-leucine were isolated by spreading 0.1 ml of a log-phase minimal medium-grown culture of *E. coli* K-12 (wild type) on minimal agar plates supplemented with 5 mM glycyl-leucine. The plates were incubated for 72 hr. Resistant colonies were picked and streaked on the same kind of plates to isolate single colonies.

Chemicals. Glycyl-L-leucine was purchased from Calbiochem and from Sigma Chemical Co. L-Isoleucine, L-valine, L-α-aminobutyrate, and rifampin were purchased from Calbiochem. L-Leucine, α-ketobutyrate, and chloramphenicol were purchased from Sigma Chemical Co. The isoleucine precursors, α-acetohydroxybutyrate and dihydroxymethylvalerate, were purchased from Reef Laboratory (Santa Paula, Calif.).

RESULTS

Growth inhibition of wild-type strain K-12 by glycyl-leucine. As shown in Fig. 2, the addition of 1 mM glycyl-leucine to a log-phase culture of *E. coli* K-12 in minimal medium resulted in a strong inhibition of growth lasting from 5 to 6 hr. Thereafter, the cells

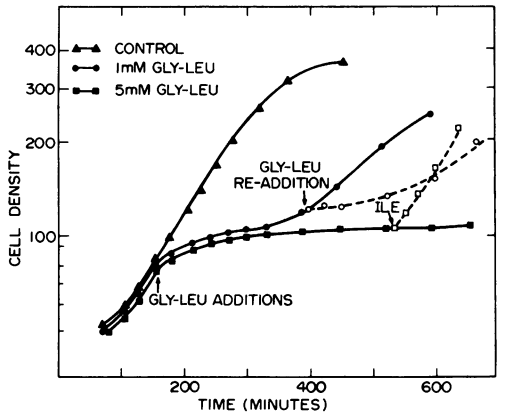

FIG. 2. *Effect of glycyl-leucine on the growth of E. coli K-12. Cell density is expressed in Klett units.*

resumed the normal growth rate. Readdition of 1 mM glycyl-leucine at this time resulted in another inhibitory period of similar duration. The inhibitory period, when 5 mM glycyl-leucine was employed, lasted over 12 hr. Adding 0.5 mM isoleucine to the cells at any time during the inhibition led to a complete and immediate reversal of the inhibition.

Experiments were performed (data not shown) in which samples of the growth medium of cells inhibited by glycyl-leucine were removed and assayed qualitatively for the presence of leucine, glycine, and glycyl-leucine. It was found that glycyl-leucine progressively disappeared from the medium while glycine and leucine increased. The time at which the cells recovered from the inhibition corresponded to the complete disappearance of glycyl-leucine from the medium.

Effect of glycyl-leucine on mutants with altered regulation of the isoleucine- and valine-forming enzymes. Since the glycyl-leucine inhibition was reversed by isoleucine, it was of interest to examine a series of mutants that had genetically altered regulation of isoleucine and valine biosynthesis. The first of these to be examined was a valine-resistant derivative of strain K-12, strain CU2501. This strain has derepressed levels of the enzymes specified by the *ilvADE* operon because of an *ilvO* mutation. It was resistant to glycyl-leucine. That high levels of the *ilvADE* gene products per se do not yield glycyl-leucine resistance was indicated by the sensitivity of strain CU1018 (formerly Tir-8). This strain is presumably derepressed because the three lesions it bears result in a low isoleucyl-transfer ribonucleic acid synthetase activity (8). Strain CU5002, a strain with derepression of all of the repressible enzymes involved in branched-chain amino acid biosynthesis, is also sensitive to glycyl-leucine.

Of these three strains, the only one resistant to glycyl-leucine was that which was resistant to valine as well. Therefore, it was of interest to examine strain CU5117, a derivative of strain CU5002, that in addition to being derepressed was also resistant to valine. Like the *ilvO* mutant, strain CU2501, strain CU5117 was also resistant to glycyl-leucine. The correlation between valine and glycyl-leucine resistance was lost when the lesion in this strain, which caused the formation of an acetohydroxy acid synthetase insensitive to valine, was transferred to strain K-12. The transductant, strain CU47, which lacked the derepression shown by strain CU5117 but had received the valine resistance marker from the resistant strain, was, nevertheless, not resistant to glycyl-leucine. The feedback-insensitive lesion in this strain, however, is not in *ilvB*, which was shown by Ramakrishnan and Adelberg (5) to be the structural gene for acetohydroxy acid synthetase, but is in *ilvF*, which is unlinked to the *ilv* region (W. J. Pledger, *personal communication*). Another Val^r strain, CU43, in which valine resistance of the acetohydroxy acid synthetase is due to an *ilvB* lesion, is also sensitive to glycyl-leucine. These relationships are also summarized in Table 1.

These comparisons with different strains showed clearly that glycyl-leucine and valine inhibition of the K-12 strain were different. Just as valine resistance in *ilvO* mutations (such as strain CU2501 described above) cannot be explained at present, the glycyl-leucine resistance of these strains is also paradoxical. A possible trivial basis for the odd patterns of resistance, of course, might be the extent to which these strains overproduce isoleucine. This possibility has not been investigated.

Effect of glycyl-leucine on levels of the isoleucine- and valine-forming enzymes. Since inhibition by glycyl-leucine appeared to result in an isoleucine limitation, the levels of the isoleucine and valine biosynthetic enzymes during the inhibitory period were examined. As shown in Fig. 3, threonine deaminase, the product of the *ilvA* gene of the *ilvADE* operon (see Fig. 1), underwent a 5- to 10-fold derepression during glycyl-leucine inhibition. The *ilvD* gene product, dihydroxy acid dehydrase, was also slightly derepressed but to a lesser extent. The presence of chloramphenicol or rifampin prevented the derepression of both enzymes, indicating that the increases in en-

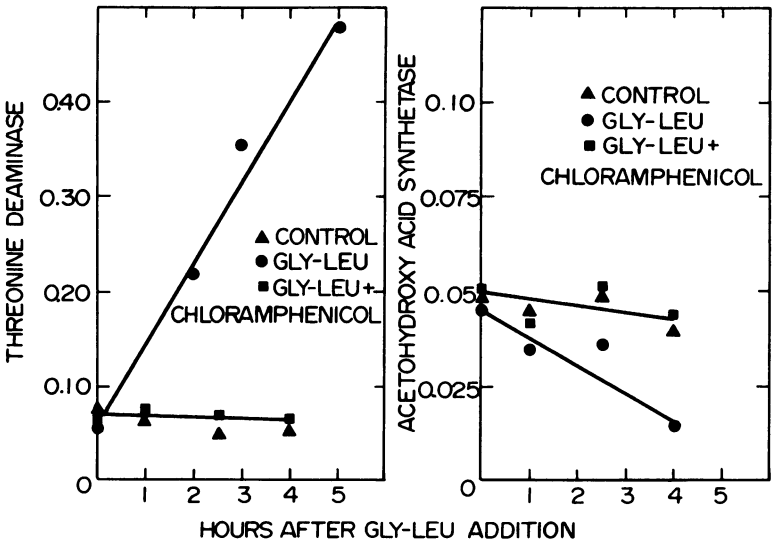

FIG. 3. *Effect of glycyl-leucine on threonine deaminase and acetohydroxy acid synthetase levels. Glycyl-leucine (1 mM) was added to log-phase cultures of E. coli K-12. Samples were removed at 1-hr intervals, and threonine deaminase and acetohydroxy acid synthetase levels were measured. Enzyme activities are expressed in micromoles per minute per milligram of protein. The level of chloramphenicol was 200 μg/ml.*

zyme activity were not due to a maturation or activation of inactive enzyme molecules already present. However, the *ilvB* gene product, acetohydroxy acid synthetase, was *repressed* threefold during the same period (Fig. 3). These results are consistent with the idea that glycyl-leucine causes an isoleucine limitation, since either a valine or leucine limitation would have caused derepression of the *ilvB* gene product as well as the *ilvADE* gene products. As shown earlier, only the *ilvADE* operon is under the multivalent control of all three branched-chain amino acids (2, 10).

In vitro effects of glycyl-leucine on the isoleucine and valine biosynthetic enzymes. The derepression pattern observed during glycyl-leucine inhibition is similar to that observed during valine inhibition in strain K-12 (2). Valine inhibits acetohydroxy acid synthetase, which is the first enzyme of its biosynthetic pathway and the second enzyme of the isoleucine pathway (Fig. 1). Thus, feedback inhibition by valine of acetohydroxy acid synthetase results in an isoleucine limitation and in a derepression of the *ilvADE* operon (2). The apparent similarities between the two inhibitory effects prompted an examination of the isoleucine and valine biosynthetic enzymes for one sensitive to glycyl-leucine. All five of the isoleucine and valine biosynthetic enzymes were therefore tested for in vitro inhibition by glycyl-leucine although, according to our present ideas, threonine deaminase and transaminase B would seem to be the only enzymes that might be subjected to inhibition without causing a concurrent valine limitation. None of the enzymes (nor the isoleucine-activating enzyme) was sensitive to 10 mM glycyl-leucine under the standard assay conditions. Closer attention was drawn to threonine deaminase as a possible site of interaction with glycyl-leucine when it was noted that isoleucine precursors beyond the threonine deamination step were capable of complete reversal of glycyl-leucine inhibition, whereas threonine itself reversed very poorly.

Since threonine deaminase becomes less sensitive to inhibition by isoleucine as the *p*H of the assay mixture is raised (R. O. Burns, *personal communication*), the possibility arose that perhaps the *p*H of the standard threonine deaminase assay (*p*H 8) was above the range of sensitivity of the enzyme to glycyl-leucine. Threonine deaminase was therefore tested for sensitivity to glycyl-leucine at *p*H 7. As is shown in Fig. 4, it was found to be sensitive at this *p*H and exhibited negative cooperativity with respect to substrate saturation similar to that observed when isoleucine was the inhibitor. (The cooperativity shown in Fig. 4 for isoleucine would be similar at *p*H 7 except that the enzyme is more sensitive to isoleucine at *p*H 7 than at *p*H 8.) It was different from the patterns of inhibition shown when inhibitory

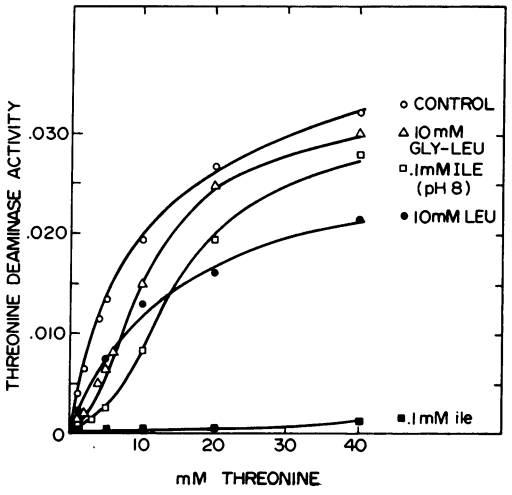

FIG. 4. *Effects of glycyl-leucine, leucine, and isoleucine on the activity of threonine deaminase in extracts of strain K-12. Control assays and assays in the presence of glycyl-leucine and leucine were performed at pH 7. At pH 8, the inhibition by leucine was the same as at pH 7. The assay in the presence of isoleucine was performed at pH 8. Assays performed at pH 7 in the presence of 0.1 mM isoleucine resulted in complete inhibition of threonine deaminase at all but the highest threonine concentrations. Enzyme activity is expressed in micromoles per minute per milligram of protein.*

concentrations of leucine were employed. The effect of an inhibitory concentration of valine is like that shown with leucine; i.e. it does not induce cooperativity.

Glycyl-leucine resistant mutants. Glycyl-leucine-resistant strains were isolated as described in Materials and Methods and were found to be of two major types. The first type seemed to have a defect in peptide uptake or utilization. The reason for this conclusion was that the mutants that were resistant to glycyl-leucine were also found to be resistant to glycyl-valine, but were still sensitive to valine. Wild-type cells are as sensitive to glycyl-valine as to valine, presumably because they are able to take up the peptide rapidly and cleave it. The glycyl-leucine resistance in these strains was found to be fairly unstable and was sometimes lost after a few transfers on nutrient medium or on storage in the absence of glycyl-leucine.

The second type of glycyl-leucine-resistant mutant was found to exhibit a threonine deaminase activity that was insensitive to inhibition by isoleucine. These mutants did not lose their resistance during storage or during growth in the absence of glycyl-leucine. When threonine deaminase from strain CU45 (*ilvA518*), one of the second type of glycyl-leucine-resistant mutants, was examined, it was found that it had also lost its sensitivity to glycyl-leucine at *p*H 7 (Fig. 5). To contrast further glycyl-leucine and valine inhibition, it should be emphasized that strain CU45 appears to be as sensitive to valine as its parent, strain K-12.

DISCUSSION

The growth rate of *E. coli* K-12 is unaffected by the addition of leucine or glycine, or both, to the medium in the concentration at which the peptide is inhibitory. The addition of leucine to cells in minimal medium resulted in a derepression of the *ilvADE* operon similar to that found with glycyl-leucine. This effect of leucine alone, although superficially similar to the glycyl-leucine effect on *ilvADE* derepression, is thought to be a different phenomenon. The studies on growth inhibition reported in this paper showed that glycyl-leucine itself was responsible for the inhibition, since recovery from the inhibition corresponded with complete disappearance of glycyl-leucine from the medium. The return of inhibition upon readdition of glycyl-leucine after recovery indicated that the disappearance of glycyl-leucine and not the formation of some product was necessary for recovery from inhibition. Leucine itself did not inhibit growth, and, during recovery from glycyl-leucine inhibition, the leucine levels were at their highest. The derepres-

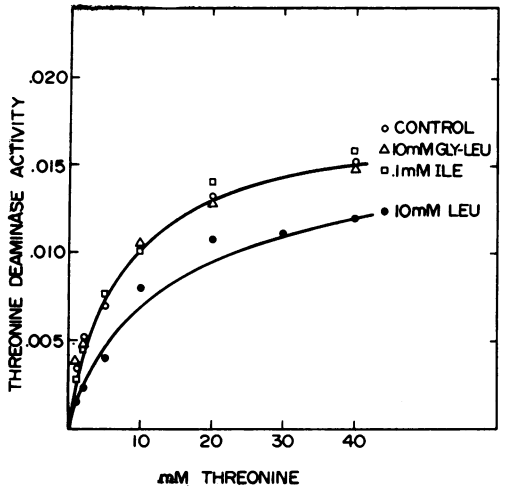

FIG. 5. *Effects of glycyl-leucine, leucine, and isoleucine on the activity of threonine deaminase in extracts of strain CU45. All assays were performed at pH 7. Enzyme activity is expressed in micromoles per minute per milligram of protein.*

sion of the *ilvADE* operon may be caused by the inhibition of threonine deaminase by leucine (Fig. 4). This inhibition could conceivably cause an isoleucine limitation signal resulting in derepression of the *ilvADE* gene cluster, but not sufficiently "limiting" to reduce the growth rate. However, this effect of leucine itself on threonine deaminase is thought not to be related to the glycyl-leucine effect for two reasons. First, the kinetics of inhibition of threonine deaminase by leucine and glycyl-leucine are different: leucine did not increase the substrate cooperativity as glycyl-leucine and isoleucine did. Second, the threonine deaminase activities in glycyl-leucine-resistant mutants are insensitive to isoleucine and glycyl-leucine but are still sensitive to leucine (Fig. 5).

Thus, it seems likely that glycyl-leucine inhibits threonine deaminase from *E. coli* K-12 by binding at the isoleucine site on the enzyme, resulting in an isoleucine limitation strong enough to derepress the *ilvADE* gene cluster and to reduce the growth rate of the cells.

It should be pointed out that coincidence of the isoleucine and glycyl-leucine patterns of inhibition and resistance are in accord with the idea of their binding at the same site, but they do not prove it. It will be extremely difficult with threonine deaminase, or any regulatory protein for that matter, to decide between ligands competing for the same site and ligands inducing conformational changes that obliterate the binding site for the competing ligand. Thus, the question whether glycyl-leucine and isoleucine bind at the same site is almost unanswerable until direct observation of that binding site can be made. However, if they are the same, glycyl-leucine might prove to be a useful ligand in studying threonine deaminase. It would thus be possible to obtain binding at the isoleucine site on threonine deaminase under conditions that are actually isoleucine-limiting. It thus might be possible to resolve the effects of isoleucine as a feedback inhibitor from its effect as a part of the multivalent repression control of gene expression in the *ilvADE* operon.

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