Vol. 89, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 27, 1979 Pages 650-658

THE ilvEDA OPERON OF ESCHERICHIA COLI K12 ENCODES ONLY ONE VALINE-α-KETOGLUTARATE TRANSAMINASE ACTIVITY

Craig W. Adams, Robert P. Lawther, and G. Wesley Hatfield

Department of Microbiology
College of Medicine
University of California, Irvine
Irvine, California 92717

Received June 18,1979

SUMMARY: Transaminase B of E. coli K12 was purified to apparent homogeneity as measured by SDS acrylamide gel electrophoresis, immunoelectrophoresis, and amino terminal sequence analysis. The valine- and isoleucine-α-ketoglutarate dependent transaminase activities of pure enzyme as well as crude extracts were characterized by immunologic and kinetic methods. The data disprove the existence of a separate valine-α-ketoglutarate transaminase within the ilvEDA operon.

Three separate transaminase activities have been described in Escherichia coli K12, which can be used in the biosynthesis of the branched chain amino acids, isoleucine, valine, and leucine (1). These enzymes are: the α-ketoglutarate dependent transaminase B, which has been shown to utilize as substrates isoleucine, valine, and leucine as well as phenylalanine, methionine, and glutamate (2,3,4); the pyruvate dependent transaminase C, which utilizes valine as well as alanine and α-aminobutyrate (5,6); and the α-ketoglutarate dependent aromatic amino acid transaminase, which can utilize leucine as well as aromatic amino acids, glutamate, aspartate, and methionine (7,8,9,10). The primary branched chain amino acid transaminase, transaminase B, is the product of the ilvE gene which is located in the ilvEDA operon. The expression of this operon is controlled by multivalent repression exerted by the branched chain amino acids (11).

Recently, yet another valine-α-ketoglutarate activity has been proposed. The product of a postulated "ilvJ" locus, which has been mapped between ilvE12 and ilvD (12). However, genetic (5) and biochemical (2) analyses by other workers have not been in agreement with the existence of this additional transaminase activity.

In order to resolve the question concerning the existence of an ilvJ encoded valine-α-ketoglutarate transaminase, we have purified transaminase B

This work was supported in part by NSF grant PCM78-08564 A03 and NIH grant GM-24330-02 to G.W.H.

0006-291X/79/140650-09$01.00/0

from E. coli K12 and confirmed that this single enzyme catalyzes the α-ketoglutarate dependent transamination of both isoleucine and valine. Furthermore, we have shown that both activities are simultaneously precipitated as a single polypeptide chain from crude extracts of E. coli K12 with monospecific antisera directed against purified transaminase B. These results together with additional kinetic analyses disprove the existence of an ilvJ encoded α-ketoglutarate dependent valine transaminase activity in E. coli K12.

METHODS

Media. The minimal medium employed was M63 (13) supplemented with 0.5% glucose, 1 mM $MgCl_2$, 50 μg/ml tryptophan, 5 μg/ml thiamine HCl. Except where noted, isoleucine (5 μg/ml), leucine (5 μg/ml), and valine (11.6 μg/ml) were also added.

Bacterial Strains. T3l-4-4 (thi-1, $trpE_{am}9829$, $trpA_{am}9761$) was constructed in this laboratory (13). T3l-4-505 (thi-1, $trpE_{am}9829$, trpA9761 Δ ilvCADE2049) was constructed in this laboratory (R. P. Lawther) with T3l-4-4, using CU505 arb^+ as a donor. CU2 was obtained from H. E. Umbarger.

Crude Extracts. Bacterial cultures for enzyme assays were inoculated from fresh overnight cultures and grown to late log phase (100 Klett units measured in a Klett-Summerson colorimeter equipped with a No. 54 green filter). The cultures were chilled on ice, harvested by centrifugation at 3000 x g for 10 minutes, washed once with TB buffer (15 mM sodium α-ketoglutarate, 1 mM pyridoxal-5'-monophosphate, 200 mM Tris HCl, pH 7.8). The bacterial pellets were resuspended in 1 ml TB buffer and disrupted by sonication with four 15 second bursts at 200 watts. Extracts were clarified by centrifugation at 6000 x g for 15 minutes. All manipulations were performed at 4C.

Enzyme Assays. Transaminase B assays were performed by the method of Duggan and Wechslar (14) except as noted. Assay mixtures contained 15 mM sodium α-ketoglutarate, 1 mM pyridoxal-5'-monophosphate and 200 mM Tris HCl (pH 7.8) in addition to [^{14}C] L-isoleucine (46 μCi/mmol or [^{14}C] L-valine (33 μCi/mmol). 300 μl of assay mixture were pre-incubated at 37C prior to addition of enzyme extract. To stop the reaction, 300 μl of 2 N HCl containing 0.3% dinitrophenylhydrazine were added to each tube, followed immediately by the addition of 300 μl of toluene. This biphasic solution was vortexed vigorously at 30 second intervals for 10 minutes and at 5 minute intervals for another 20 minutes. 2 ml of distilled water were added to each tube and the mixture vortexed. After centrifugation at 3000 x g for 10 minutes, 150 μl of the toluene layer were removed and the radioactivity measured by scintillation counting (the counts were corrected for 40% quenching).

Purification of Transaminase B. Transaminase B was purified from an E. coli K12 strain, T3l-4-505, containing the ColEl plasmid pLC30-17. This plasmid contains the entire ilv gene cluster (Lawther and Hatfield, unpublished results). The plasmid encoded activity was induced by chloramphenicol amplification according to the method of Hersfeld et al. (16). The enzyme was purified by the method of Monnier et al. (2) except that this method was followed by an additional DEAE Sephadex A-50 column chromatography. A 1.6 cm x 3.6 cm column of DEAE Sephadex A-50 was equilibrated with 20 mM potassium phosphate, pH 6.8, containing 0.2 mM pyridoxal-5'-monophosphate; 2 mM sodium α-ketoglutarate; and 0.3 M potassium chloride. The enzyme was eluted in a pure form with a linear gradient of 0.3 M to 0.5 M potassium chloride.

Amino Terminal Sequence Analysis of Transaminase B. The amino terminal sequence of transaminase B was determined using a modified Beckman automated sequenator, Model 890B, according to the methods described by Hunkapiller and Hood (17).

Preparation of Antisera. Complete Freunds adjuvant containing .33 mgs of transaminase B was injected intermuscularly at four separate sites into New Zealand rabbits at day 0. Injections of .33 mgs transaminase B in incomplete Freunds adjuvant were given on the 14th and 21st days. At 4 and 5 weeks, 40 ml bleeds were made followed by the injection of an equal volume of phosphate buffered salts. Terminal heart punctures were made at 6 weeks. After clotting at 4C, erythrocytes and fibrin were removed by centrifugation. All experiments were conducted using the same lot of immune sera. Immunoelectrophoresis (18) and Ouchterlony immunodiffusion (19) were performed using purified transaminase B and whole serum.

Immune Precipitation. Antisera was incubated with crude extract for 1 hour at 4C. Protein A-Sepharose was added and vortexed several times for 5 minutes. After centrifugation of the beads (15,000 x g, 2 min) the supernatants were assayed for remaining activity. For immunprecipitation, crude extracts were prepared from 50 ml of overnight cultures containing minimal media, isoleucine (5 µg/ml), leucine (5 µg/ml), valine (11.6 µg/ml) and 30 µCi of [^{35}S]-methionine. After centrifugation (100,000 x g, 1 hour) 20 µl of antisera was added to Eppendorf microfuge tubes containing equal counts of each extract (12.3 x 10^6 cpms in approximately 1.5 ml). The extracts were incubated with the antisera for 1 hour followed by the addition of 5 µl of packed protein A-Sepharose. The Sepharose beads were washed 3 times with 1.5 mls of potassium phosphate buffer (0.1 M, pH 7.2) and three times with a solution containing 0.1% SDS and 0.05% NP40. The samples were prepared for SDS polyacrylamide gel electrophoresis and equal volumes (50 µl) of each sample were electrophoresed (15). After Commassie Blue staining, gels were treated with ENHANCE (New England Nuclear), dried, and exposed to Kodak X-Omat film for 2 days at -70C.

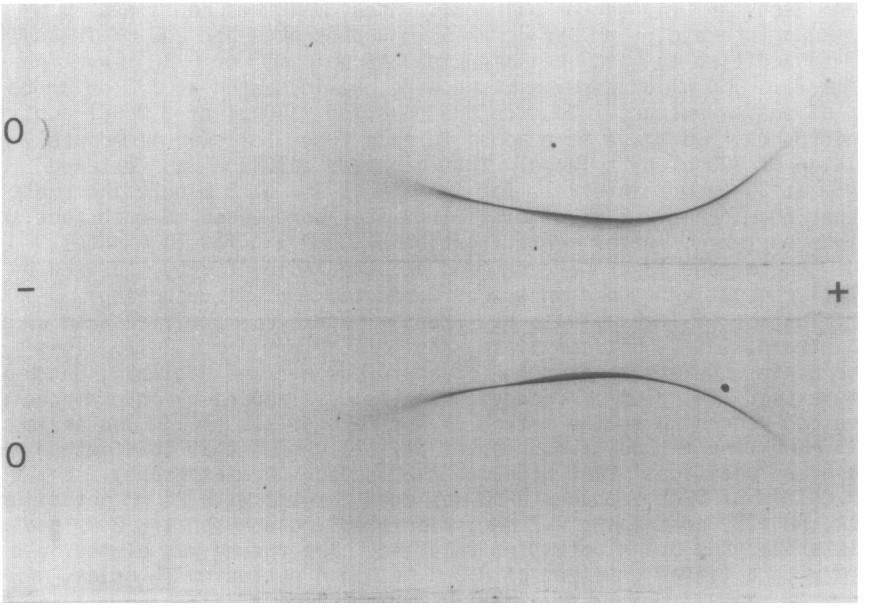

Fig. 1. Immunoelectrophoresis of purified transaminase B against whole immune serum. Transaminase B was loaded in well marked O (origin).

RESULTS

Criteria of Enzyme Purity. Transaminase B was judged pure by immunoelectrophoresis, SDS acrylamide gel electrophoresis, and amino terminal sequencing. The immunoelectrophoresis of the purified transaminase B against whole serum antisera results in a single band, indicating the presence of only one antigen (Fig. 1). SDS acrylamide gel electrophoresis (Fig. 2, lane 1) also indicates the enzyme preparation is homogeneous. The amino terminal sequence of transaminase B was determined to be:

$$\underset{\text{Gly Thr Lys Lys Ala Asp Tyr Ile Trp Phe-.}}{\overset{5 \qquad\qquad\qquad\qquad 10}{}}$$

The percent yield of amino acids from 20-25 nmoles of sample was greater than 40%. The sequence purity based on initial yield was determined to be greater than 95%.

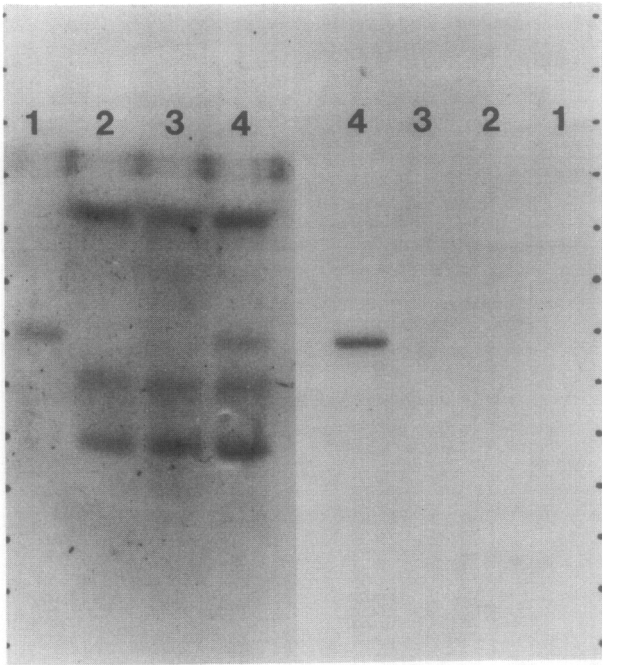

Fig. 2. SDS acrylamide gel electrophoresis of cellular proteins precipitated by antisera to transaminase B. Left: stained by Coomassie blue; Right: autoradiogram. Line 1: purified transaminase B marker; Line 2: proteins precipitated from an *ilvE* deletion (T31-4-505); Line 3: proteins precipitated from an *ilvE12* mutant (CU2); Line 4: proteins precipitated from an *ilvE* wild type strain (T31-4-4). Strains were grown in the presence of [^{35}S]-methionine and immunoprecipitates were prepared as described in Methods. Samples were electrophoresed in a slab gel containing an exponential gradient of 14 to 20% acrylamide (15). Unlabeled proteins which were stained by Coomassie blue are presumed to be serum components.

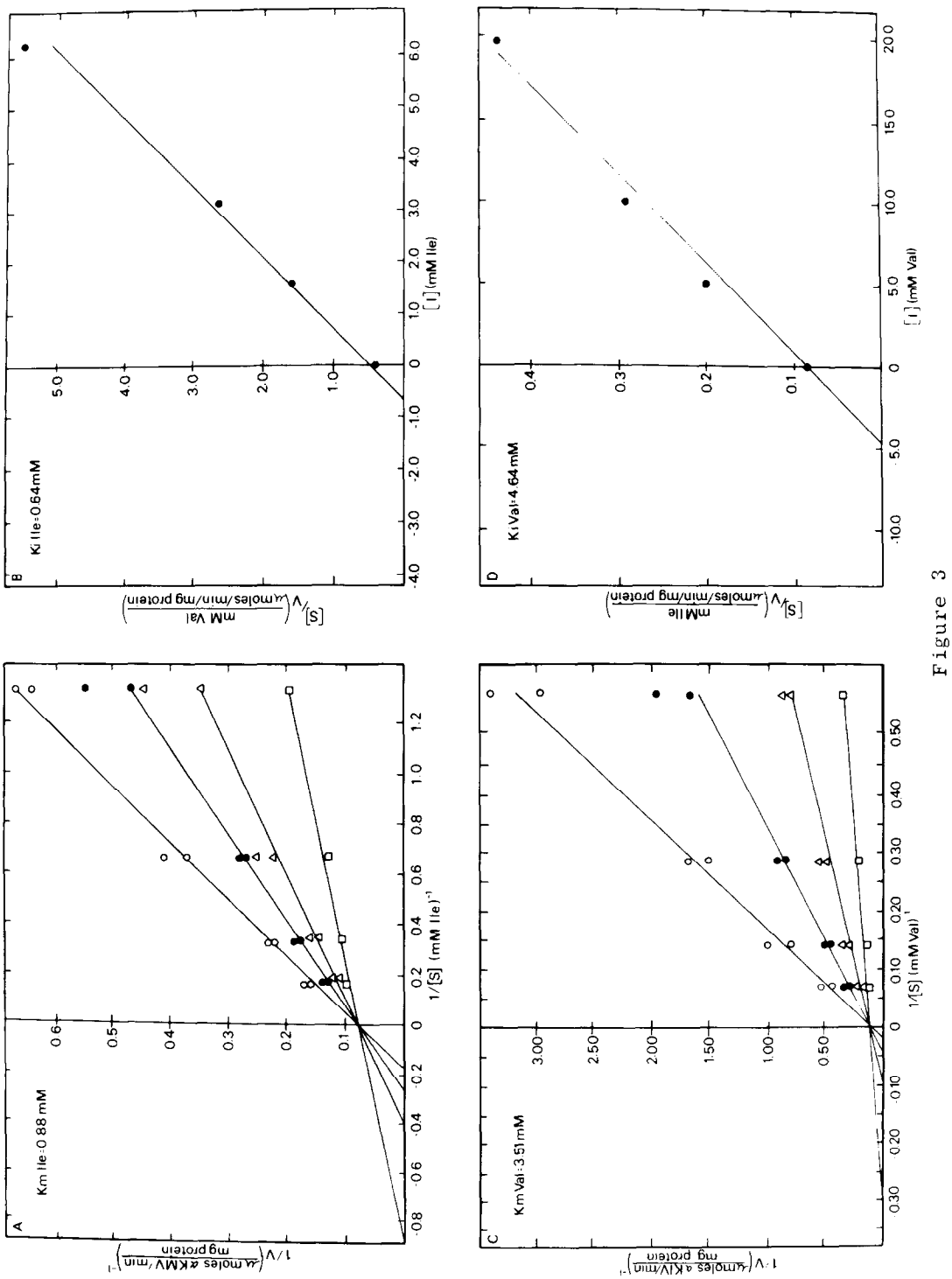
A
Km Ile=0.88 mM
1/V (μmoles αKMV/min / mg protein)⁻¹
1/[S] (mM Ile)⁻¹
B
Ki Ile=0.64mM
[S]/V (mM Val / μmoles/min/mg protein)
[I] (mM Ile)
C
Km Val=3.51 mM
1/V (μmoles αKIV/min / mg protein)⁻¹
1/[S] (mM Val)⁻¹
D
Ki Val=4.64 mM
[S]/V (mM Ile / μmoles/min/mg protein)
[I] (mM Val)

Figure 3

Kinetics of Isoleucine and Valine Binding to Transaminase B. The apparent K_m values of purified transaminase B for isoleucine (0.88 mM) and valine (3.51 mM) were calculated from the data shown in Figure 3. Based on these apparent K_m values, the affinity of this enzyme for isoleucine is four times greater than its affinity for valine. The double reciprocal plot of [^{14}C]-labeled isoleucine activity measured in the presence of various concentrations of unlabeled valine (Fig. 3A) shows that valine is a competitive inhibitor of the isoleucine activity. Likewise, the double reciprocal plot of [^{14}C]-labeled valine activity measured in the presence of various concentrations of unlabeled isoleucine (Fig. 3C) shows that isoleucine is also a competitive inhibitor of the valine activity. A replot of these data against inhibitor concentration (Fig. 3B,D) demonstrate that the apparent K_m for each substrate is very close to its apparent Ki. This result argues that a single enzyme activity transaminates both substrates.

Immunoprecipitation of Transaminase B. If the transamination of both valine and isoleucine is catalyzed by transaminase B, then immunoprecipitation of this single enzyme from a crude extract should result in the simultaneous loss of both activities. In order to test this hypothesis, it was necessary to demonstrate that the antisera used for the immunoprecipitation was directed specifically against transaminase B. Ouchterlony immunodiffusion (data not shown) and immunoelectrophoresis of purified transaminase B against the antisera, show a single precipitin band (Fig. 1). Furthermore, the immunoprecipitate of crude extracts from wild type *E. coli* cells grown in the presence of [^{35}S]-methionine results in the appearance of a single protein band on SDS electrophoresis gels (Fig. 2, lane 4). This protein, which comigrates with purified transaminase B (Fig. 2, lane 1) is not present in either the *ilvCADE2049* deletion strain, T31-4-505 (Fig. 2, lane 2), or the *ilvE12* mutant strain, CU2 (Fig. 2, lane 3).

Since these data clearly demonstrate that the antisera specifically removes only transaminase B from crude extracts, it is possible to examine the titration of transaminase B antisera against the α-ketoglutarate dependent isoleucine and valine transaminase activities in wild type extracts. The data in Figure 4 show a commensurate loss of both activities from a crude

Fig. 3. Lineweaver-Burk plots for (A), isoleucine- and (C) valine-α-ketoglutarate transaminase activity in the presence of increasing amounts of competitive substrate. (A) (□) no valine, (△) 5.0 mM valine, (●) 10.0 mM valine, (○) 20.0 mM valine. (C) (□) no isoleucine, (△) 1.6 mM isoleucine, (●) 3.2 mM isoleucine, (○) 6.4 mM isoleucine. Replots of Lineweaver-Burk slopes against (B) isoleucine and (D) valine concentrations. Assay mixtures were incubated for 15 minutes. K_m values are mM; V_{max} values are μmoles/min/mg protein.

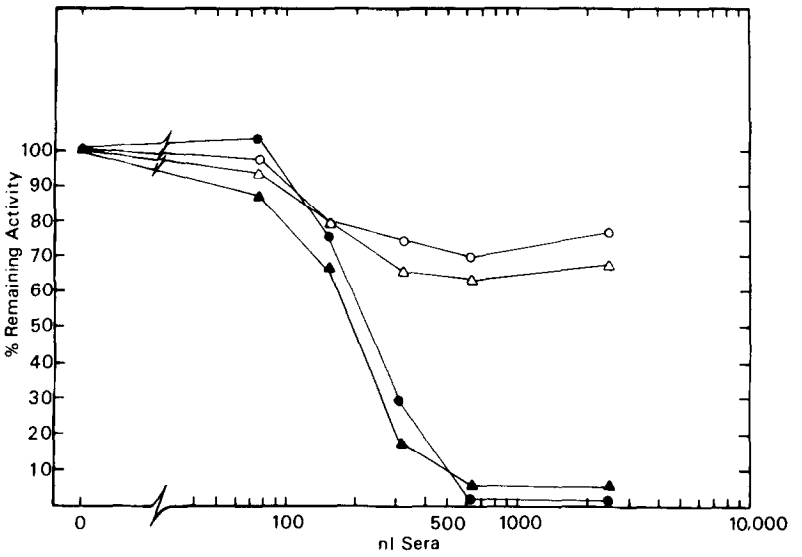

Fig. 4. Immunoprecipitation of transaminase B activity from crude extract. Valine activity following precipitation with (▲) immune and (△) nonimmune sera. Isoleucine activity following precipitation with (●) immune sera and (○) nonimmune sera. 150 µl of T31-4-4 crude extract (1.6 µg prot/ml) was incubated for one hour with 10 µls of increasing dilutions of antisera. To each tube 20 µls of protein-A-Sepharose (25 µls packed beads in 10 mls of TB buffer) was added and incubated for 5 minutes. Tubes were centrifuged at 15,000 x g for 2 minutes and the supernatant solutions were assayed for valine and isoleucine-α-ketoglutarate transaminase activity. All activities were standardized to a control sample containing no sera.

extract of T31-4-4 with increasing concentrations of antisera. These data, in conjunction with the immunoprecipitation of the [^{35}S]-methionine labeled crude extracts, demonstrate that a single protein, specifically removed by immunoprecipitation, comigrates with transaminase B on SDS electrophoresis gels; further, removal of transaminase B by immunoprecipitation causes a proportionate loss of both the valine- and the isoleucine-α-ketoglutarate dependent transaminase activities present in *E. coli* K12.

DISCUSSION

The organization of the isoleucine and valine genes at minute 83 on the *E. coli* K12 chromosome has been a subject of controversy for some time. The order of the *ilv* genes was originally described by Ramakrishnan and Adelberg (20) to be *rbs ilvEDACB cya*. They concluded that the direction of transcription of the *ilvEDA* genes was from *ilvA* to *ilvE*. More recent studies by Smith et al. (21), Cohen and Jones (22), and Patin and Calhoun

(23) have shown that the direction of transcription is, in fact, from ilvE to ilvA. In 1976, Favre et al. (24) reported the existence of an acetolactate synthase isoenzyme, the product of the ilvG gene, which they mapped between ilvE and ilvD. Subsequent work by Smith et al. (21) and Berg et al. (25) have clearly shown that the correct gene order is ilvGEDA.

This report concerns the work by Guardiola (12) who recently described yet another another α-ketoglutarate dependent transaminase activity in an ilvE12 strain of E. coli K12 (CU2) which required isoleucine but not valine for optimal growth. Subsequent mutagenesis of this strain yielded a second mutation resulting in valine auxotrophy. This new mutation was shown to lie between the ilvE12 mutation and another mutation in ilvD. On the basis of this genetic evidence, these workers proposed a new genetic locus, ilvJ. They mapped ilvJ between ilvE12 and ilvD and proposed that this gene coded for another valine transaminase activity in addition to those activities previously reported for transaminase B and transaminase C.

McGilvray and Umbarger (5) also investigated the transaminase activities in strain CU2, and showed by transduction analysis that the ilvE12 mutation caused a loss of both valine- and isoleucine-α-ketoglutarate transaminase activity. They further showed that the ability of this strain to grow well in the presence of isoleucine alone was due to a second mutation which mapped outside of the ilv gene cluster and caused a derepression of transaminase C. Monnier et al. (2), in agreement with McGilvray and Umbarger, showed by enzyme purification that this strain (CU2) lost both valine- and isoleucine-α-ketoglutarate transaminase activities. It is difficult to reconcile these findings with those of Guardiola (12).

We have shown by kinetic analyses that purified transaminase B catalyzes the α-ketoglutarate dependent transamination of both valine and isoleucine. In fact, we present kinetic studies which suggest that both substrates share a common site on transaminase B. The immunoprecipitation studies show that precipitation of only transaminase B removes both isoleucine- and valine-α-ketoglutarate dependent transaminase activities from crude extracts, disproving the existence of a separate valine-α-ketoglutarate transaminase. These results confirm the findings of McGilvray and Umbarger (5) and Monnier et al. (2) and rule out the existence of the enzyme described by Guardiola and, by inference, disprove the existence of the ilvJ gene.

ACKNOWLEDGMENTS

We have learned that Kohlhaw and coworkers have performed recent experiments which argue against the existence of a separate valine-α-ketoglutarate transaminase in E. coli K12. We thank G. B. Kohlhaw for communicating this result to us prior to publication. The authors are grateful to Paul Cartier, Mike W. Hunkapiller, and Leroy E. Hood for determining the amino terminal sequence of transaminase B.

REFERENCES

1. Umbarger, H. E., Amino Acid Biosynthesis, pp. 533-606, Ann. Review of Biochem. (eds. E. Snell et al.) Annual Reviews, Inc., California, 1978.
2. Monnier, S., Montmitonnet, A., and Pelmont, J. (1976) Biochemie 58, 663-675.
3. Gelfand, D. H., and Steinberg, R. A. (1977) J. Bacteriol. 130, 429-440.
4. Kline, E. L., Brown, C. S., Colman, W. G., Jr., and Umbarger, H. E. (1974) Biochem. Biophys. Res. Commun. 57, 1144-1151.
5. McGilvray, D., and Umbarger, H. E. (1974) J. Bacteriol. 120, 715-723.
6. Falkinham, J. O. III (1977) J. Bacteriol. 130, 566-568.
7. Chesne, S., Montmitonnet, A., and Pelmont, J. (1975) Biochemie 57, 1029-1034.
8. Collier, R. H., and Kohlhaw, G. (1972) J. Bacteriol. 112, 365-375.
9. Silbert, D. F., Jorgensen, S. E., and Lin, E. C. C. (1963) Biochim. Biophys. Acta 72, 232-240.
10. Wallace, B. J., and Pittard, J. (1969) J. Bacteriol. 97, 1234-1241.
11. Freundlich, M., Burns, R. O., and Umbarger, H. E. (1962) Proc. Nat. Acad. Sci. 48, 1804-1808.
12. Guardiola, J. (1977) Molec. Gen. Genet. 158, 157-164.
13. Lawther, R. P., and Hatfield, G. W. (1977) J. Bacteriol. 130, 550-557.
14. Duggan, D. E., and Wechsler, J. A. (1973) Anal. Biochem. 51, 67-79.
15. McGuire, J. C., Pene, J. J., and Barrow-Carraway, J. (1974) J. Virol. 13, 690-698.
16. Hersfeld, V., Boyer, H., Yanofsky, C., Lovett, M., and Helinski, D. (1974) Proc. Nat. Acad. Sci. 71, 3455-3459.
17. Hunkapiller, M. W., and Hood, L. E. (1978) Biochem. 17, 2124-2133.
18. Graber, P., and Williams, C. A. (1953) Biochem. Biophys. Acta 10, 193.
19. Ouchterlony, O. (1948) Acta Patha. Microbiol. Scand. 25, 186.
20. Ramkrishnan, T., and Adelberg, E. A. (1965) J. Bacteriol. 80, 661-664.
21. Smith, J. M., Smolin, D. E., and Umbarger, H. E. (1976) Molec. Gen.
22. Cohen, B. M., and Jones, E. J. (1976) Genetics 83, 201-225.
23. Patin, D. W., and Calhoun, D. H. (1979) J. Bacteriol. 137, 1234-1242.
24. Favre, R., Wiater, A., Puppo, S., and Iaccarino, M. (1976) Molec. Gen. Genet. 143, 243-252.
25. Berg, C. M., Shaw, K. J., Vender, J., and Boructa-Mankiewicz, M. Genetics, in press.