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Transaminase B from *Escherichia coli*: Quaternary Structure, Amino-Terminal Sequence, Substrate Specificity, and Absence of a Separate Valine-α-Ketoglutarate Activity†

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Transaminase B (branched-chain amino acid aminotransferase, EC 2.6.1.42), the *ilvE* gene product, was purified to apparent homogeneity from an *Escherichia coli* K-12 strain which carries the *ilvE* gene both on the host chromosome and on a plasmid. The oligomeric structure of the enzyme, as determined by analytical ultracentrifugation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was confirmed to be that of a hexamer with a molecular weight of about 182,000 and apparently identical subunits. Cross-linking with dimethylsuberimidate yielded trimers, dimers, and monomers, but essentially no species of higher molecular weight. These results are consistent with a double-trimer arrangement of the subunits in native enzyme. The amino-terminal sequence was found to be: Gly Thr Lys Lys Ala Asp Tyr Ile (Trp) Phe Asn Gly (Thr) (Met) Val. Purified transaminase B catalyzed transamination between α-ketoglutarate and L-isoleucine, L-leucine, L-valine, and, to a lesser extent, L-phenylalanine and L-tyrosine, the latter reacting very sluggishly. The enzyme was free of aspartate transaminase and of transaminase C. The apparent K_m values for the branched-chain α-ketoacids were smaller than those for the corresponding amino acids. The lowest K_m was recorded for DL-α-keto-β-methyl-*n*-valerate, and the highest was recorded for L-valine. The ratio of the valine- and isoleucine-α-ketoglutarate activities did not change significantly during purification, and both activities were quantitatively removed from crude extract by antibody raised against purified transaminase B. These observations argue against the existence of a separate valine-α-ketoglutarate transaminase. Anti-*E. coli* transaminase B antibody cross-reacted with crude extract from *Salmonella typhimurium*, but not with extract obtained from *Pseudomonas aeruginosa*.

In *Escherichia coli*, four major transaminases have been identified. They are the aromatic amino acid transaminase (7, 9, 10, 25, 26), the aspartate transaminase (4, 7, 9, 10, 26), transaminase B (22, 28), and transaminase C (21). The aromatic amino acid transaminase is the product of *tyrB*; it can be repressed by tyrosine and interacts not only with the aromatic amino acids and glutamate, but also with leucine, aspartate, and methionine. The aspartate transaminase is coded for by *aspC* and reacts with aspartate and glutamate and also with aromatic amino acids. The classification of the *tyrB* and *aspC* gene products as aromatic amino acid transaminase and aspartate transaminase, respectively, is justified on the basis of their substrate affinities (26). Transaminase B is coded for by *ilvE* (17, 27), is subject to multivalent repression (16, 17), and has been shown to interact with glutamate, leucine, isoleucine, valine, phenylalanine, and methionine (22, 28), although the relative affinities of the substrates have not been established. Transaminase C, finally, catalyzes the interconversion of valine, alanine, and α-aminobutyrate. It is repressed by either valine or leucine (20). The exact map location of the gene coding for this enzyme is not yet known.

Transaminase B has received renewed attention because of recent experimental results which suggest that the *ilvE* gene is located proximal to *ilvO*, a control region for the *ilvGOEDA* operon (1, 5, 29). Since the DNA sequence of this region is presently being studied (H. E. Umbarger and G. McCorkle, personal communication; G. W. Hatfield, personal communication), it seemed useful to establish at least a partial amino acid sequence of the enzymes whose structural genes are in the neighborhood

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of *ilvO*. We report here the sequence of 15 residues of the amino-terminal region of transaminase B. We also provide a more complete picture of the quaternary structure and the substrate specificity of this enzyme and address the recently raised question (12) of the existence of a separate valine-α-ketoglutarate transaminase.

MATERIALS AND METHODS

Organism. *E. coli* K-12 strain CU406(pGMM101) was obtained from H. E. Umbarger. CU406 has the genotype *ilvA454 galT12*; pGMM101 is a hybrid pBR313 plasmid containing the *ilvCADE* region and conferring ampicillin resistance upon its bacterial host (19). The specific activity of transaminase B in crude extracts of the plasmid-containing strain is 17 times greater than that of CU406, under repressing conditions (19). It is not clear whether this increase is strictly a gene dosage effect, since the plasmid copy number per cell has not been established. Cells were grown overnight in minimal medium (32) with 0.5% glucose. Ampicillin (5 μg/ml) was added to the culture 30 min after inoculation and again 2 to 3 h before harvesting.

Chemicals. Amino acids, α-ketoacids, coenzymes, and glutamic dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo. Ampicillin (Polycillin-N) was from Bristol Laboratories, Syracuse, N.Y. Dimethylsuberimidate dihydrochloride was from Pierce Chemical Co., Rockford, Ill. All other chemicals were of the best available grade.

Purification of transaminase B. All operations were performed at 0 to 4°C. Crude extract was prepared by suspending 110 g (wet weight) of cells in 760 ml of 50 mM potassium phosphate buffer (pH 7.2), passing the suspension through an Aminco French pressure cell, and centrifuging at 27,000 × *g* for 30 min. The supernatant solution (790 ml) was made 2.5% in streptomycin sulfate, left for 30 min with stirring, and centrifuged as before. Thereafter, the supernatant solution (1,010 ml) was made 0.1 mM in pyridoxal phosphate and fractionated with powdered ammonium sulfate. The precipitate obtained between 40 and 55% saturation (calculated for 25°C) was dissolved in 200 ml of 20 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal phosphate and 1.2 M ammonium sulfate (buffer A) and was loaded onto an L-leucine-Sepharose column (diameter, 4.4 cm; height, 24.5 cm) (30) equilibrated with buffer A. The enzyme was eluted with an ammonium sulfate gradient (decreasing linearly from 1.2 to 0 M) established in 1,840 ml of 20 mM potassium phosphate buffer (pH 7.2) and containing 0.1 mM pyridoxal phosphate (buffer B). The fractions with high specific activity of transaminase B were pooled. Enzyme-containing protein was precipitated with ammonium sulfate (55% saturation, calculated as described above), redissolved in 50 ml of 20 mM potassium phosphate buffer (pH 6.8) which contained 0.1 mM pyridoxal phosphate and 250 mM KCl (buffer C), and dialyzed overnight against the same buffer. It was then applied to a DEAE-Sepharose 4B column (diameter, 2.5 cm; height, 22 cm) equilibrated with buffer C. The column was eluted with 400 ml of a linear KCl gradient ranging from 250 to 500 mM and established in buffer B. The most active fractions were again pooled. Protein was precipitated with ammonium sulfate (65% saturation, calculated as described above), and the precipitate was redissolved in 5 ml of 20 mM potassium phosphate buffer (pH 6.8) which contained 0.1 mM pyridoxal phosphate, 1 mM α-ketoglutarate, and 100 mM KCl (buffer D). A 3-ml amount of this solution was applied to a Sephadex G-200 (superfine) column (diameter 1.5 cm; height, 80 cm) equilibrated with buffer D. Elution was performed with the same buffer. The two fractions with the highest specific activity of transaminase B (8 ml with a total of 22 mg of protein) constituted the purified enzyme referred to below. The enzyme was stored at −20°C in buffer D and was stable for at least several months.

Enzyme assays. For all activities, 1 U was defined as 1 μmol of product formed per min. Specific activities are given as units per milligram of protein. Transamination between the branched-chain amino acids and α-ketoglutarate was assayed by the method of Duggan and Wechsler (8). The standard assay mixture contained 0.1 M Tris-hydrochloride buffer (pH 8.0), 0.1 mM pyridoxal phosphate, 15 mM α-ketoglutarate, 25 mM amino acid, and enzyme. Transamination between the branched-chain α-ketoacids and L-glutamate was measured by the previously described coupled assay which uses glutamic dehydrogenase as auxiliary enzyme (7). Phenylalanine, tyrosine, and aspartate transamination (with α-ketoglutarate) were measured as previously described (7). Transaminase C was assayed by the method of McGilvray and Umbarger (20).

Amino-terminal sequence analysis. By using the methods of Hermodson et al. (13, 14), purified transaminase B (2 to 6 mg) was denatured, reduced, and derivatized with 4-vinylpyridine. The lyophilized, salt-free protein was dissolved in 1 ml of 50% acetic acid. A small sample (0.1 ml) was withdrawn for amino acid analysis, and a larger sample (0.8 ml) was subjected to automated Edman degradation in a Beckman 890C sequencer. The phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography on a Bio-Analytical Systems chromatograph, using the procedure of Zimmerman et al. (35).

Gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the procedure of Weber and Osborn (33), using gels with 6% acrylamide. Rapid denaturation of protein was achieved by heating samples for 3 min in a boiling water bath in the presence of 1% (wt/vol) sodium dodecyl sulfate and 1% (vol/vol) 2-mercaptoethanol.

Cross-linking. Enzyme which had been dialyzed overnight against 0.2 M triethanolamine-hydrochloride buffer (pH 8.5), either in the presence or in the absence of 0.1 mM pyridoxal phosphate, was incubated for 2.5 h at 37°C in dialysis buffer with 2 mg of dimethylsuberimidate per ml. The final enzyme concentration was 1.25 mg/ml. After cross-linking, the samples were denatured with 1% (wt/vol) sodium dodecyl sulfate and 1% (vol/vol) 2-mercaptoethanol and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as outlined above.

Protein determination. The protein concentration was determined by the biuret method (11), using lyophilized bovine serum albumin as a standard.

Analytical ultracentrifugation. The molecular weight of native transaminase B was determined in a Spinco model E analytical ultracentrifuge, using the long-column meniscus depletion method (2, 31). The experimental conditions were as follows: initial enzyme concentration, 1.25 mg/ml; buffer, 20 mM potassium phosphate (pH 6.8), containing 0.1 mM pyridoxal phosphate and 100 mM KCl; speed, 12,000 rpm (An-D rotor); and temperature, 4°C. The partial specific volume of the enzyme, calculated from the amino acid composition, was 0.732 cm^3/g at 20°C.

Immunological techniques. Antibody against purified transaminase B was raised in a group of three New Zealand rabbits (Antibodies Inc., Davis, Calif.), using 2 mg of protein per rabbit. Antigen mixed with Freund complete adjuvant was injected into the hind legs on days 0, 14, and 21, and serum was obtained on day 28. Immunoglobulin fractions were isolated from the serum by ammonium sulfate fractionation (15). Immunodiffusion experiments were conducted as described by Ouchterlony (24). Protein was stained with Light Green SF Yellowish stain (MCB Manufacturing Co., Norwood, Ohio).

RESULTS

Purification and analysis of the quaternary structure of transaminase B from strain CU406(pGMM101). Enzyme obtained after a 14-fold purification (Table 1) appeared to be homogeneous by the criterion of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1, gel D). Comparison with standards covering a molecular weight range from 18,400 to 67,000 yielded a molecular weight of 31,500 ± 1,000 for the subunit of transaminase B. When native enzyme was analyzed by sedimentation equilibrium centrifugation, the data yielded a straight line when plotted by the method of Yphantis (34), suggesting the presence of a monodisperse system. From the slope of the line, an apparent molecular weight of 182,300 was calculated. Cross-linking of native transaminase B with dimethylsuberimidate followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis resulted in the appearance of multiples of the 31,500-dalton subunit, which were identified as trimers and dimers (Fig. 1). The trimers predominated and were stabilized by the presence of pyridoxal phosphate (Fig. 1, gels B and C). They formed a relatively broad band corresponding to a molecular weight range of from 85,000 to 96,000. This result may have been brought on by reaction of the cross-linking reagent with lysine residues in different positions, thereby creating trimers of different shape and rigidity which then behaved differently in gel electrophoresis. A possible reason for the virtual absence of species larger than trimers in this experiment is discussed below.

Amino-terminal sequence and amino acid composition of transaminase B. Three separate Edman degradations were performed by using 40 to 60 nmol of protein (monomer) in each experiment. The following sequence was obtained: Gly Thr Lys Lys Ala Asp Tyr Ile (Trp) Phe Asn Gly (Thr) (Met) Val-. The quantities of the amino acids in the early cycles of the degradations were between 83 and 65% of those expected, based on the amount of protein used. The yields of Trp_9, Thr_{13}, and Met_{14} were less than half of the expected yields at those cycles and consequently can be considered tentative identifications only.

The amino acid composition of the enzyme (excepting tryptophan and cysteine) is shown in Table 2.

TABLE 1. *Purification of transaminase B*

Step	Activity (kU)		Amt of protein (mg)	Sp act		Ratio of sp act (Val/Ile)
	Valine[a]	Isoleucine[b]		Valine[a]	Isoleucine[b]	
Crude extract	17.63	28.44	14,220	1.2	2.0	0.60
Ammonium sulfate fractionation (40 to 55% pellet)	5.95	12.71	1,328	4.5	9.6	0.47
Leucine-Sepharose chromatography (pooled peak fractions)	2.48	4.82	245	10.1	19.7	0.51
DEAE-Sepharose 4B chromatography (pooled peak fractions)	0.68[c]	1.13[c]	45	15.1	25.1	0.60
Sephadex G-200 gel filtration (pooled peak fractions)	0.35[d]	0.61[d]	22	15.9	27.3	0.58

[a] Valine-α-ketoglutarate activity.
[b] Isoleucine-α-ketoglutarate activity.
[c] The low recovery in this step was due to accidental loss of enzyme.
[d] Only 60% of the material obtained after the previous step was carried through this step.

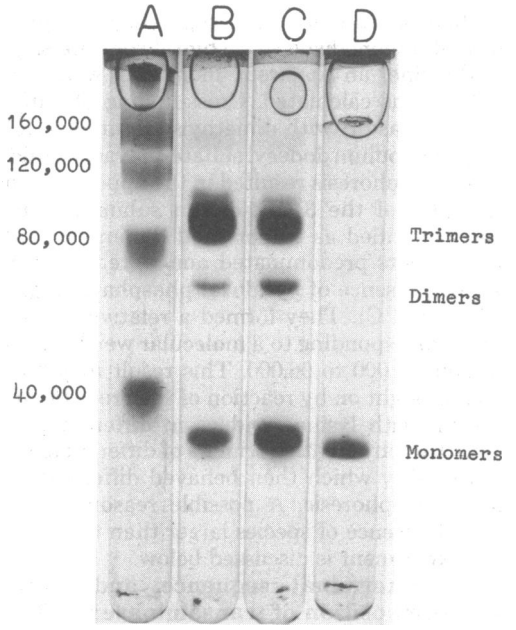

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cross-linked and untreated transaminase B. See text for details. Gel A, Cross-linked rabbit muscle aldolase, an internal standard (20 μg of protein applied to gel). Gel B, Transaminase B, cross-linked in the presence of 0.1 mM pyridoxal phosphate (10 μg applied to gel). Gel C, Transaminase B, cross-linked in the absence of pyridoxal phosphate (10 μg applied to gel). Gel D, Transaminase B, not cross-linked (4 μg applied to gel). Gel D was obtained in a separate experiment in which β-lactoglobulin, trypsinogen, pepsin, ovalbumin, and bovine serum albumin were included as standards.*

TABLE 2. *Amino acid composition of transaminase B based on 285 amino acids (estimated) per monomer of 31,500 daltons*[a]

Amino acid	Relative amt	Amino acid	Relative amt
Asx	25.7	Met	6.7
Thr	13.1	Ile	17.3
Ser	17.0	Leu	20.2
Glx	30.8	Tyr	10.3
Pro	13.4	Phe	11.8
Gly	29.8	His	7.3
Ala	27.4	Lys	11.2
Val	23.4	Arg	17.1

[a] The data are the combined results from three separate 24-h hydrolysates of 4-vinylpyridine-derivatized enzyme in 6 N HCl at 110°C. Tryptophan was not determined. Only a trace of *S*-pyridylethylcysteine which did not integrate was observed on the chromatogram. The analyses were performed with a Durrum D-500 amino acid analyzer. Asx, Asp plus Asn; Glx, Glu plus Gln.

Substrate specificity. To the degree that apparent K_m values reflect affinity, purified transaminase B had a greater affinity for branched-chain α-ketoacids than for branched-chain amino acids (Table 3). This was especially evident with the α-ketoacid analogs of isoleucine and valine. When it is assumed that only one stereoisomer of α-keto-β-methyl-*n*-valerate interacts with the enzyme, its apparent K_m value is reduced to 0.10 mM, or approximately 1/5 of that for isoleucine. A similar difference existed for α-ketoisovalerate and valine, albeit in a higher range of substrate concentrations. The apparent K_m values for valine and for α-ketoglutarate compared well with values that had been obtained with partially purified transaminase B (3).

Table 3 also shows that the highest specific activities were obtained with isoleucine, leucine, and valine (in the presence of saturating concentrations of α-ketoglutarate). The specific activities with phenylalanine and tyrosine were 4.6 and 0.7%, respectively, of the activity seen with isoleucine, under the conditions specified. The specific activities observed with the branched-chain α-ketoacids may only be compared among themselves, since they were obtained at 25°C, whereas the other values were measured at 37°C.

Purified transaminase B had no measurable aspartate-α-ketoglutarate activity and was also free of transaminase C activity. Both activities were detected in crude extract.

Immunodiffusion and immunoprecipita-

TABLE 3. *Substrate specificity of purified transaminase B*[a]

Variable substrate	Apparent K_m (mM)	Sp act
L-Isoleucine[b]	0.52	30.5[c]
L-Leucine[b]	0.58	27.7[c]
L-Valine[b]	3.13	20.3[c]
L-Phenylalanine[d]	ND[e]	1.4
L-Tyrosine[d]	ND	0.2
α-Ketoglutarate[f]	1.28	20.9[c]
DL-α-Keto-β-methyl-*n*-valerate[g]	0.20	9.0[c]
α-Ketoisocaproate[g]	0.56	17.9[c]
α-Ketoisovalerate[g]	0.57	9.3[c]

[a] All assays were performed in duplicate, and the results were averaged.
[b] Assayed in the presence of 15 mM α-ketoglutarate.
[c] Calculated from the corresponding V_{max} values.
[d] Assay mixtures contained 20 mM α-ketoglutarate and either 5 mM phenylalanine or 2 mM tyrosine.
[e] ND, Not determined.
[f] Assayed in the presence of 25 mM L-valine.
[g] Assayed in the presence of 40 mM L-glutamate.

tion experiments. When antibody raised against purified transaminase B was subjected to Ouchterlony double diffusion, using either purified enzyme or crude extract as antigen, a single line of precipitation was observed, indicating a high degree of immunological homogeneity in the purified enzyme preparation (Fig. 2). Anti-*E. coli* transaminase B antibody also reacted with crude extract from *Salmonella typhimurium* LT-2. The resulting precipitation line showed complete fusion with the lines arising from interaction of the *E. coli* system, suggesting the presence of identical antigenic determinants in the transaminase B enzymes of the two strains. Under the same conditions, no cross-reaction was seen with crude extract from *Pseudomonas aeruginosa* (Fig. 2).

To determine whether the valine-α-ketoglutarate and the isoleucine-α-ketoglutarate activities in crude extract of *E. coli* were immunologically distinguishable, crude extract was titrated with antibody (Fig. 3). Within experimental error, both activities were quantitatively removed from solution by the same amount of antibody.

DISCUSSION

The behavior of *E. coli* transaminase B in analytical ultracentrifugation and gel electrophoresis and the presence of only one amino terminus confirm the notion (22) that the enzyme is a hexamer with a molecular weight of about 185,000 and is composed of identical subunits. Some additional conclusions may be drawn from the cross-linking experiment with dimethylsuberimidate. First, this experiment corroborates the molecular weight of the monomer by revealing species whose molecular weights are approximate multiples of 31,500. Second, it suggests that the subunit interactions in the native hexamer are not all of the same type. This conclusion is based on the following considerations. Analytical ultracentrifugation (performed at pH 6.8) provided evidence for the presence of only one enzyme form, i.e. the hexamer, in a native enzyme preparation. In contrast, the cross-linking experiment (performed at pH 8.5) showed an overwhelming presence of trimers and significant amounts of dimers and monomers, but essentially no species with higher molecular weights. It is possible that the condi-

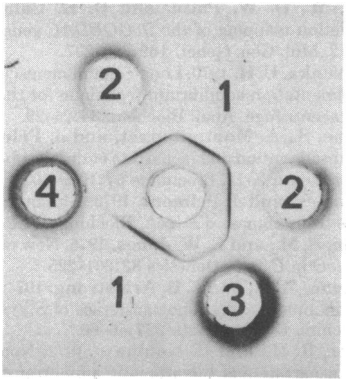

FIG. 2. *Ouchterlony double diffusion analysis of transaminase B. The center well contained partially purified anti-E. coli transaminase B antibody (135 μg of protein). Well 1, Purified transaminase B from E. coli (25 μg of protein). Well 2, Crude extract from E. coli (232 μg of protein). Well 3, Crude extract from S. typhimurium LT-2 (346 μg of protein). Well 4, crude extract from P. aeruginosa ATCC 10145 (325 μg of protein). The specific activities of valine-α-ketoglutarate transaminases in the crude extracts were as follows: E. coli, 1.20; S. typhimurium, 0.09; and P. aeruginosa, 0.17.*

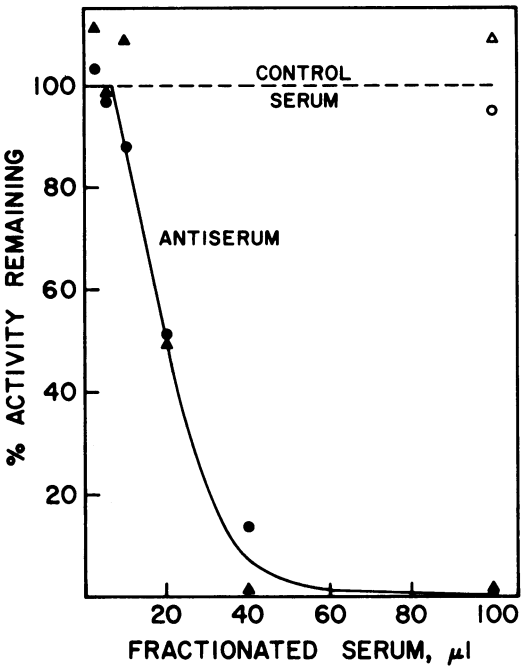

FIG. 3. *Immunoprecipitation of transaminase B in crude extract. Diluted crude extract (0.2 ml with 0.4 mg of protein) was incubated with the indicated amount of ammonium sulfate-fractionated serum. The volume was kept constant with 50 mM potassium phosphate buffer, pH 7.2. After 30 min at 30°C, the mixtures were centrifuged and the supernatant solutions were assayed for valine-α-ketoglutarate transaminase (○, ●) and for isoleucine-α-ketoglutarate transaminase (△, ▲). Incubation was with antiserum against transaminase B (closed symbols) or with control serum (open symbols). The protein concentrations of the ammonium sulfate-fractionated sera were 13 to 14 mg/ml.*

tions under which cross-linking was performed caused a dissociation of the hexamers. However, this should have led to a mixture of all species (or, for complete dissociation, to monomers only) if the subunit interactions were equivalent, as would be the case for a circular or linear arrangement of the subunits. Since trimers predominated and hexamers, pentamers, and tetramers were virtually absent, it is more likely that native enzyme consists of a "dimer of trimers," a configuration which would let intratrimer cross-linking occur more readily than intertrimer cross-linking. Dimers and monomers could easily arise from incomplete intratrimer cross-linking.

Not unexpectedly, there exists a close structural resemblance between the transaminase B from *E. coli* and that from *S. typhimurium*. The molecular weights of the subunits and of the native enzymes are practically identical, and the amino acid compositions are very similar (6, 18). The sequence of the five N-terminal amino acid residues is Gly Thr Lys Lys Ala for the *E. coli* enzyme and Thr Thr Lys Lys Ala for the enzyme from *S. typhimurium* (R. R. Randall, M. H. Wallis, G. J. Young, and F. B. Armstrong, Fed. Proc. **38**:325, 1979). Although it may be coincidental, it is interesting in this context that the amino acid analyses yielded 14 Thr and 29 Gly residues for the enzyme from *S. typhimurium* and 13 Thr and 30 Gly for the *E. coli* enzyme. The close relationship of the two enzymes is underscored by the immunological cross-reaction between anti-*E. coli* transaminase B antibody and crude extract from *S. typhimurium*. Little structural information is available on the branched-chain amino acid transaminase from *P. aeruginosa*, but its molecular weight has been estimated by gel filtration to be 130,000 (23), implying that at least its quaternary structure is different from that of the *E. coli* enzyme.

The kinetic studies reported here indicate that the most preferred substrate of transaminase B in the biosynthetic direction is the α-keto acid analog of isoleucine. Likewise, isoleucine itself is the best substrate in the direction of amino acid utilization. This selectivity may be related to the fact that, in *E. coli*, transaminase B is the only transaminase known to react with isoleucine and its α-keto acid analog. In contrast, both leucine and valine are acted upon by at least two of the four major transaminases.

Guardiola (12) has reported that the valine-α-ketoglutarate activity of *E. coli* K-12 could be partially separated from the isoleucine-α-ketoglutarate activity and that a strain defective in *ilvE* (CU2) had lost the latter but retained the former. He proposed that the valine-α-ketoglutarate activity was coded for by *ilvJ*, a gene located between *ilvE* and *ilvD*. The hypothesis of a distinct valine-α-ketoglutarate transaminase is in conflict with the finding of Monnier et al. (22) that this activity was absent from CU2. Our observations that the ratio of the valine-α-ketoglutarate and isoleucine-α-ketoglutarate activities remained practically constant during purification of transaminase B and that antibody raised against highly purified transaminase B completely removed both activities from crude extract also argue against the existence of a separate valine-α-ketoglutarate transaminase. It is possible, as noted by Guardiola (12), that isoleucine and valine bind to separate sites on transaminase B. However, no biochemical evidence is available at present to support this idea.

We have learned that Hatfield and co-workers have obtained an amino-terminal sequence for the first 10 residues of transaminase B which is identical to the one shown here (G. W. Hatfield, personal communication).

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