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Amino Acid Sequence of *Salmonella typhimurium* Branched-Chain Amino Acid Aminotransferase†

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ABSTRACT: The complete amino acid sequence of the subunit of branched-chain amino acid aminotransferase (transaminase B, EC 2.6.1.42) of *Salmonella typhimurium* was determined. An *Escherichia coli* recombinant containing the *ilvGEDAY* gene cluster of *Salmonella* was used as the source of the hexameric enzyme. The peptide fragments used for sequencing were generated by treatment with trypsin, *Staphylococcus aureus* V8 protease, endoproteinase Lys-C, and cyanogen bromide. The enzyme subunit contains 308 residues and has a molecular weight of 33 920. To determine the coenzyme-binding site, the pyridoxal 5-phosphate containing enzyme was treated with tritiated sodium borohydride prior to trypsin digestion. Peptide map comparisons with an apoenzyme tryptic digest and monitoring radioactivity incorporation allowed identification of the pyridoxylated peptide, which was then isolated and sequenced. The coenzyme-binding site is the lysyl residue at position 159. The amino acid sequence of *Salmonella* transaminase B is 97.4% identical with that of *Escherichia coli*, differing in only eight amino acid positions. Sequence comparisons of transaminase B to other known aminotransferase sequences revealed limited sequence similarity (24–33%) when conserved amino acid substitutions are allowed and alignments were forced to occur on the coenzyme-binding site.

The branched-chain amino acid aminotransferase [EC 2.6.1.42 (transaminase B, TmB[1])] of *Salmonella typhimurium* catalyzes the final step in the biosynthesis of isoleucine, leucine, and valine. The enzyme is essential only for isoleucine synthesis since valine and leucine can be synthesized by alternate aminotransferases. *Salmonella* TmB was purified, crystallized, and partially characterized by Coleman and Armstrong (1971). Lipscomb et al. (1974) identified the enzyme as a hexameric protein of 183 kDa composed of identical 31.5-kDa subunits. On the basis of this subunit size, amino acid analysis gave an estimated 288 amino acid residues per subunit. No sulfhydryls are required for activity, and the intact hexamer contains no disulfide bridges. One mole of pyridoxal 5-phosphate is bound per subunit. Serine was identified as the C-terminal residue. Randall et al. (1979) and Randall (1982) identified the first 20 residues of the amino terminus, with the N-terminal residue being threonine.

The present study on the *S. typhimurium* enzyme was undertaken to verify, by direct methods, the structure of TmB of *Escherichia coli* K-12, which was deduced from nucleotide sequence analysis (Kuramitsu et al., 1985). The study also identifies the coenzyme-binding site and reports a new procedure for the purification and crystallization of large quantities of the *Salmonella* TmB.

Materials and Methods

Transaminase B, the *ilvE* gene product of *S. typhimurium*, was isolated and crystallized from a recombinant organism, *E. coli* JA199 (pDU 11), a gift from the late Dr. R. O. Burns. The organism contains a deletion mutation of the *ilvGEDAYC* gene cluster, as well as a deletion mutation of the *trpE5* gene and a mutation in the *leuB6* gene. It harbors a multicopy plasmid, pDU11, a pBR322 derivative containing the *ilvGEDAY* gene cluster of *S. typhimurium*, and a gene for tetracycline resistance (*tet*[r]). When grown under derepressing conditions (limiting isoleucine), the organism produces up to 12% of its soluble protein as TmB.

For enzyme purification, the cells were grown in a modified Davis and Mingioli medium (1950) and contained 86 mM K_2HPO_4, 47 mM KH_2PO_4, 16 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 70 mM D-glucose, 0.85 mM L-valine, 0.76 mM L-leucine, 0.49 mM L-tryptophan, 0.076 mM L-isoleucine, and tetracycline hydrochloride at a concentration of 20 μg/mL and were incubated on a rotary shaker for 18 h at 37 °C. Cells were harvested by centrifugation; the resulting pellets were resuspended with 0.1 M potassium phosphate buffer, pH 7.4, and recentrifuged.

For the purification protocol, all steps were performed at 4 °C, and all buffers used were potassium phosphate. Cells were suspended in 0.1 M buffer, pH 6.5, and disrupted by

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[1] Abbreviations: TmB, transaminase B; AAT, aspartate:α-ketoglutarate aminotransferase; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; EDTA·4Na, ethylenediaminetetraacetate tetrasodium salt; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; CM, carboxymethyl; PE, pyridylethyl; UWGCG, University of Wisconsin Genetics Computer Group.

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sonication for 3 min. The suspension was centrifuged at 12000*g* for 30 min. The resulting supernatant was made 5 mM with respect to α-ketoglutarate and allowed to stand for 15 min. The supernatant was divided among 18 × 150-mm glass test tubes and heated to 60–62 °C for 5 min, then chilled in ice, and centrifuged. The supernatant was again divided among glass test tubes, heated to 71 °C for 5 min, chilled, and centrifuged. The supernatant was made 0.1 mM with respect to pyridoxal 5-phosphate. Then 100%-saturated $(NH_4)_2SO_4$ in 10 mM buffer, pH 7.4, was slowly added to bring the extract to 38% saturation. After sitting for 30 min, the extract was centrifuged for 15 min at 20000*g*. The supernatant was brought to 55% $(NH_4)_2SO_4$ saturation, allowed to sit 30 min, and then recentrifuged. The yellow pellet, containing the TmB, was redissolved in a minimum amount of 10 mM buffer, pH 7.8. The solution was adjusted to a protein concentration of 10 mg/mL with the same buffer and then brought to 31% $(NH_4)_2SO_4$ saturation using 100%-saturated $(NH_4)_2SO_4$ in 10 mM buffer, pH 6.8. The solution was centrifuged for 30 min at 20000*g* and the supernatant transferred to 15-mL glass centrifuge tubes. These were placed in a refrigerator for 2–5 days during which time crystallization occurred. The crystals were harvested by centrifugation, resuspended in 40%-saturated $(NH_4)_2SO_4$ in 10 mM buffer, pH 7.0, recentrifuged, and then refrigerated as a concentrated suspension in 40%-saturated $(NH_4)_2SO_4$ buffer. Protein concentration was determined by the method of Bradford (1976) and enzymatic activity by the method of Coleman and Armstrong (1971). The yield was approximately 100 mg of TmB crystals from 12 g of cells (wet weight).

Apoenzyme was produced by using a method described by Coleman and Armstrong (1971). Reduction of the Schiff base between pyridoxal 5-phosphate and TmB using NaB^3H_4 was performed by the method of Randall (1982) except that the reaction was performed at 0 °C. The proteins were reduced and S-carboxymethylated with sodium iodoacetate (Craven et al., 1965) or S-pyridylethylated with 4-vinylpyridine (Hawke & Yuan, 1987). Amino acid analysis was performed using phenyl isothiocyanate precolumn derivatization (Bidlingmeyer et al., 1984).

Digestion of TmB with TPCK–trypsin was performed by using the method of Randall (1982) or by using a 4% (w/w) trypsin concentration with TmB suspended at a concentration of 5 mg/mL in 0.1 M $(NH_4)HCO_3$, pH 7.81, and 2 M guanidine hydrochloride, at 37 °C for 2 h. TmB was treated with cyanogen bromide (CNBr) by using modifications of the procedure by Gross (1967). Conditions used were a 500-fold molar excess of CNBr with respect to methionyl residues at 20 °C. Digestion with *S. aureus* V8 protease was limited to glutamyl residues (Drapeau, 1977). Digestion of TmB with endoproteinase Lys-C, a lysine-specific endoproteinase, was performed by using a modified method of Steffens et al. (1982) by dissolving TmB in a buffer containing 1% SDS, 25 mM Tris, pH 8.51 with HCl, and 1 mM EDTA·4Na. The solution was heated to 60 °C for 10 min after which the SDS concentration was lowered to 0.1% with Tris buffer and then incubated at 37 °C. A protease to substrate ratio of 1:100 (w/w) was used, and the digestion period was 24 h. Digests were dissolved in appropriate starting solvents containing 6 M guanidine hydrochloride or 8 M urea prior to HPLC separations.

HPLC separations were performed with a Waters Associates Model 840 controller, Models 6000 and M-45 pumps, a Model U6-K injector, and a Model 440 detector with extended wavelength module. Peptide elution was monitored at 214 and 280 nm. Reverse-phase columns used contained C_{18}, pH-stable C_8, or C_4 bonded phases (Vydac, 4.6 × 250 mm, 5-μm particle size, 30-nm pore size). Some CNBr digest separations were performed using the C_4 and C_{18} reverse-phase columns linked in tandem. All separations were performed at room temperature (20–22 °C) using binary solvent systems. For reverse-phase separations, solvent A was 0.1% trifluoroacetic acid (TFA, w/v) in water, and solvent B was 0.1% TFA (w/v) in acetonitrile. The pH-stable C_8 column was also used with a solvent system containing 10 mM ammonium carbonate in water as solvent A and solvent B containing 80% acetonitrile and 20% solvent A (v/v). Flow rates ranged from 0.25 to 0.5 mL/min. Peaks eluted from the chromatograph were collected by a fraction collector.

Amino acid sequences of intact TmB and of digest peptides were determined according to the method of Hewick et al. (1981) using an Applied Biosystems (ABI) Model 470A gas-phase microsequenator with a Model 120 on-line HPLC PTH–amino acid analyzer and a Model 900 controller. Sequence information from long peptides containing prolyl residues was improved by sequencing the filter-bound peptides to the prolyl residues followed by modification with *o*-phthalaldehyde before continued sequencing (Brauer et al., 1984).

Sequence comparisons, performed on a Digital Equipment Corp. VAX 11/780 computer, relied on the Sequence Analysis Software Package (v.5) of the Genetics Computer Group (UWGCG, University of Wisconsin, Madison, WI) as developed by Devereux et al. (1984).

Results

The summary of peptide fragment overlaps is presented in Figure 1. The majority of the sequence was obtained by Edman degradation of *S*-carboxymethyl-TmB (CM-TmB) peptides generated by trypsin, CNBr, and *S. aureus* V8 protease digestion. The remaining overlaps were determined from *S*-pyridylethyl-TmB (PE-TmB) digested with endoproteinase Lys-C. Overlaps were obtained for all peptides at least once. The peptides are labeled according to the digestion performed and numbered according to their order of elution in the original HPLC separations. Some chymotrypsin activity in the TPCK-treated trypsin preparation was apparent as evidenced by cleavage at Met (T-6), Leu and Phe (lT-2/V-7b), and Trp (lT-2/V-6a).

Automated Edman degradation of intact CM-TmB yielded 32 amino acid residues. The sequence agrees with the 20 residues of the amino terminus determined by Randall (1982) except for the N-terminal and eleventh residues. The amino-terminal residue of TmB isolated from *S. typhimurium* is Thr while that of *S. typhimurium* TmB isolated from the recombinant *E. coli* is Gly. In *E. coli* (but not in *S. typhimurium*), there appears to be a posttranslational modification that converts the N-terminal Thr into Gly. The nucleotide sequence reported for the *ilvE* gene of *E. coli* K-12 encodes a Thr residue at this position (Kuramitsu et al., 1985; Lawther et al., 1979); however, Edman degradation of the protein product identifies Gly at the amino terminus (Lee-Peng et al., 1979). This conversion has been postulated to occur by a two-step enzymatic conversion involving threonine dehydrogenase and aminoacetone synthase coenzyme–A ligase (A. Mathais, unpublished results). Residue 11 was identified in this study as Asn instead of Asx.

Trypsin Peptides and the Coenzyme-Binding Site. The carboxymethylated forms of apoTmB and ^3H-pyridoxylated TmB were digested at lysyl and arginyl residues using TPCK–trypsin. The resulting peptides were fractionated by

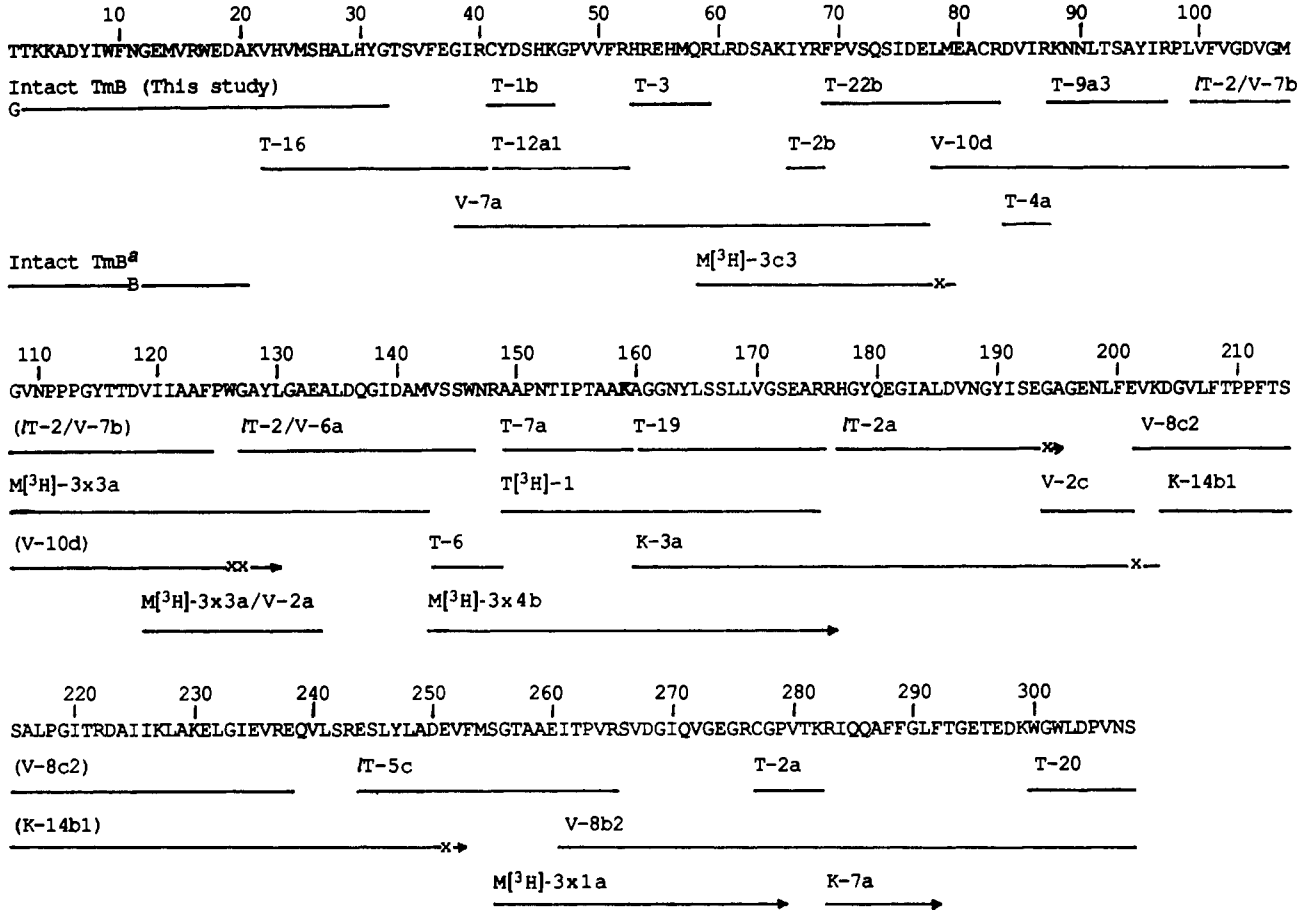

FIGURE 1: Summary of the sequence of *Salmonella typhimurium* TmB. The extent of sequences derived by Edman degradation is indicated by lines. Text above the lines indicates the type of cleavage from which the peptide was obtained. Sequences continued from the previous line are indicated as text enclosed in parentheses. The prefixes T, M, V, and K denote peptides derived by cleavage with trypsin, cyanogen bromide, *S. aureus* V8 protease, and endoproteinase Lys-C, respectively, and the prefix lT denotes a limited tryptic digestion. The next number indicates the peak from which the peptide was isolated in order of elution. Following this, alternating lower case letters and numbers refer to the successive rechromatography to isolate peaks from partially purified peptides. Subdigestion of selected peptides is identified by suffixes following a slash. ^3H refers to fragments obtained from tritiated holoenzyme. Unidentified residues are denoted by x. Incomplete sequences that continued further than what was sequencable (as determined by cleavage specificity or amino acid analysis) are terminated by arrows. The pyridoxal 5-phosphate binding lysyl residue is in bold type. Footnote *a*, from Randall et al. (1982).

C_{18} reverse-phase HPLC. A comparison of the resulting peptide maps allowed for preliminary identification of the peptide that binds pyridoxal 5-phosphate at the active site of TmB (Figure 2A). Since pyridoxal 5-phosphate binds to a specific lysyl residue, reduction of the resulting Schiff base with NaB^3H_4 destroys a potential trypsin cleavage site; hence, a difference in the maps should be observed. As compared with the ^3H-pyridoxylated holoenzyme peptide map (upper trace), the prominent peaks 1 and 2 in the lower trace are diminished, and peak 3 appears. Determination of the amount of ^3H incorporated into the various tryptic peptide fragments of the holoenzyme (Figure 2B) reveals that peak 3 contains the most significant amount of radioactivity incorporated (approximately 25% of total injected). Pooling these fractions and reinjecting them produced a single peak eluting with the incorporated radioactivity (Figure 2C). This peak (T[^3H]-1) was sequenced and found to contain 27 residues (Figure 3). The radioactivity was associated with residue 11 which contained no recognizable standard PTH–amino acid. Subsequent sequencing of peaks 1 and 2 (T-7a and T-19, respectively) verified them as the N- and C-terminal portions of T[^3H]-1, and residue 11 of the pyridoxylated peptide was indeed a lysine. This lysine is located at position 159 of the intact subunit.

S. aureus V8 Protease Peptides. CM-TmB was cleaved specifically at glutamyl residues by *S. aureus* V8 protease. The digest peptides were fractionated on a C_{18} reverse-phase HPLC column and eluted with an acetonitrile gradient. Many partially resolved absorbance peaks were identified. The peaks were separated into pooled fractions and further fractionated on the C_{18} column or on a C_4 column using modified acetonitrile gradients. The digest consisted mainly of large peptides, typically 30–50 residues in length, many of which could be isolated in sufficient quantity and purity for sequencing. From the sequences of the V8 protease generated peptides shown in Figure 1, it is apparent that not all Glu residues were susceptible to cleavage by the protease. A similar phenomenon was reported by Drapeau (1977). The largest V8 protease generated peptide sequenced was 48 residues in length and contained the C-terminus.

Cyanogen Bromide Generated Peptides. CNBr cleavage of CM-TmB (both apo- and ^3H-holoenzyme) yielded a number of peptide fragments that were poorly resolved by using any of the reverse-phase columns. To obtain separation of these fragments, two reverse-phase columns with different bonded phases were linked in tandem. The digest was injected onto a C_4 column which was linked directly to a C_{18} column and eluted with an acetonitrile gradient.

Endoproteinase Lys-C Generated Peptides. PE-apoTmB was digested at lysyl residues by using endoproteinase Lys-C. The resulting peptides proved to be insoluble at pH <6, even in the presence of 8 M guanidine hydrochloride. The digest was therefore kept at pH 8.5 which ruled out using any re-

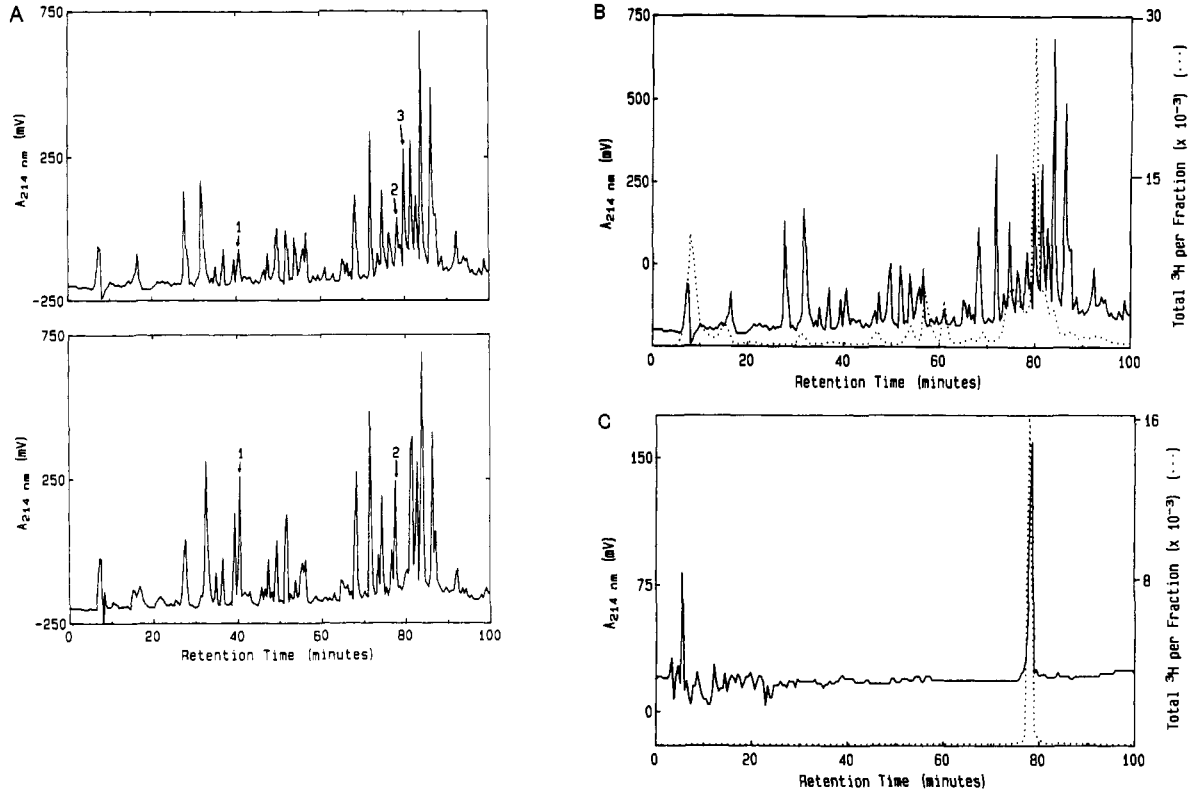

FIGURE 2: Tryptic peptide map comparison of CM-apoTmB with CM-[^{3}H]-pyx-TmB and identification of the coenzyme-binding peptide. (A) The upper trace denotes the HPLC chromatogram for the CM-[^{3}H]-pyridoxyl-TmB digest while the lower trace denotes the chromatogram for the CM-apoTmB digest. Identical sample loads (150 μg) were injected onto a C_{18} column at a flow rate of 0.5 mL/min. Eluate was monitored at 214 mn. Solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile. Peptides were eluted with an acetonitrile gradient. Initial conditions were 5% B. After a 2-min hold at initial conditions, a 100-min linear gradient was performed to 43% B and then a 3-min hold at 43% B, followed by a return to initial conditions. The numbers denote: (1) the amino-terminal half {T-7a} and (2) the carboxyl-terminal half {T-19} of (3) the intact ^{3}H-pyridoxylated peptide {T[^{3}H]-1}. (B) Display of the radioactivity incorporated into CM-[^{3}H]-pyx-TmB tryptic peptides. One-milliliter fractions were collected. One hundred microliters of each fraction was mixed with 3 mL of scintillation cocktail and counted by liquid scintillation. The total radioactivity per fraction (···) is superimposed over the absorbance at 214 nm (—). (C) Chromatogram of purified peak 3 (T[^{3}H]-1). Fractions from four digest separations containing peak 3 were pooled and reinjected onto the C_{18} column using the gradient in (A). The total amount of radioactivity per fraction (···) is superimposed over the absorbance at 214 nm (—). Fractions of 0.5 mL were collected, and 50-μL aliquots were counted by liquid scintillation.

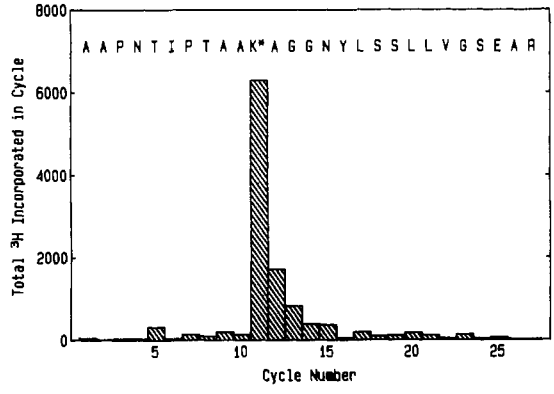

FIGURE 3: Sequence of the coenzyme-binding tryptic peptide of TmB. Approximately 1 nmol of peptide (60 kcpm) was applied to the gas-phase peptide sequenator. Identification of the PTH–amino acids was accomplished by on-line HPLC analysis described under Materials and Methods. The one-letter abbreviations of the identified amino acids are placed over the bar graph representing the total radioactivity obtained from each cycle as determined by liquid scintillation. Cycle 11 contained the highest radioactivity and no PTH–amino acid which corresponded to the 20 standard PTH–amino acids.

verse-phase column containing silica-based packing. A pH-stable C_8 column was used for these separations with an ammonium carbonate buffer system (pH 8.9) and eluted with an acetonitrile gradient. The protease treatment produced many large peptide fragments that were amenable to sequencing. Peptide K-3a was 44 residues in length, corresponding to residues 160–203, and linked the coenzyme-binding site to the C-terminal half of TmB.

Discussion

The branched-chain amino acid aminotransferase of *S. typhimurium* is a hexamer of identical subunits, and on the basis of the sequence obtained, each is a single polypeptide chain of 308 amino acids. The calculated molecular weight of the enzyme subunit is 33 920.74. The intact hexameric holoenzyme would have a mass (including 6 mol of pyridoxal 5-phosphate) of 204 916 daltons. These values differ from the subunit and hexameric molecular weights reported by Lipscomb et al. (1974) by 7.13 and 10.7%, respectively. The subunit molecular weight determined in this study is in close agreement with the 33 960 reported for TmB of *E. coli* K-12 (Kuramitsu et al., 1985).

A comparison of TmB's primary structure with other aminotransferase sequences in the current sequence databases using the UWGCG program GAP revealed that the only aminotransferase sequence with significant similarity was the inferred amino acid sequence of TmB from *E. coli* (Kuramitsu et al., 1985). The two enzymes are 97.4% indentical in sequence with only eight amino acid differences (Table I). Five of the eight residue differences can be accounted for by single-point mutations (residues 100, 113, 116, 140, and 305). As stated before, the N-terminal difference is probably the result of a posttranslational modification. The active-site residue of the *E. coli* enzyme has not been identified but is

Table I: Eight Amino Acid Differences between the Sequences of TmB of *S. typhimurium* and *E. coli*

residue	*S. typhimurium*	*E. coli*[a]
1	Thr	Gly
61	Arg	His
100	Val	Ile
113	Pro	Ala
116	Thr	Ser
140	Asp	Glu
305	Pro	Gln
308	Ser	Gln

[a] From Kuramitsu et al. (1985)

almost certainly the same Lys_{159} identified in this study.

Comparison of the sequence of *Salmonella* TmB with those of other aminotransferases (11 aspartate, 2 ornithine, 3 histidine, 2 tyrosine, and 1 succinyl diaminopimelate) revealed only limited similarity (24.3–33.3%) when allowing for conserved substitutions (data not presented). Forcing the alignment of sequences so that the coenzyme-binding sites are aligned changes the similarities slightly (27.5–31.9%). Disallowing conserved substitutions lowers similarities to less than 20%. The significance of the similarity was checked by varying the size and number of gaps allowed as well as by randomizing the sequences while keeping the amino acid composition unaltered. Similarity levels ranged from 15 to 32%. These results imply that the sequence similarity of *Salmonella* TmB with other known aminotransferases (except *E. coli* TmB) is probably not significant.

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