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Isoleucine Auxotrophy Due to Feedback Hypersensitivity of Biosynthetic Threonine Deaminase

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Isoleucine-deficient mutants of *Salmonella typhimurium* were isolated. Three groups of mutants can be discerned by their nutritional requirements and enzyme patterns. (i) Mutants which grow with isoleucine alone are devoid of biosynthetic threonine deaminase (TD). (ii) Mutants growing with isoleucine and valine are devoid of transaminase B. (iii) Mutants growing with either isoleucine or threonine have normal levels of TD. However, the sensitivity of this enzyme to feedback inhibition by isoleucine is greatly enhanced. The inhibitory effect of isoleucine can be counterbalanced by high concentrations of threonine. These results indicate that the production of isoleucine in the mutants is restricted to a low level not sufficient to support the growth of the cells. This hypothesis is confirmed by studies with revertants of an isoleucine-threonine mutant. In nine revertants, wild-type properties of TD have been restored. In four revertants, the hypersensitivity of TD is unchanged, but the strains produce a greatly enhanced quantity of threonine, which is excreted into the culture medium. It follows, that hypersensitivity of TD to inhibition by isoleucine is the cause of the nutritional requirement of isoleucine-threonine mutants.

Biosynthetic threonine deaminase (L-threonine hydrolyase, deaminating, EC 4.2.1.16; TD) is the first enzyme in the pathway of isoleucine biosynthesis. The activity of this enzyme is subject to feedback inhibition by isoleucine (9-12). In a previous study, we described mutants of *Escherichia coli* with a defect in the structural gene for TD resulting in the requirement for either isoleucine or pyridoxine (5). This character was shown to be due to a change in affinity of mutant TD to its coenzyme, pyridoxalphosphate. Recently, Guirard, Ames, and Snell described similar mutants of *Salmonella typhimurium* (6).

The present paper describes mutants of *S. typhimurium* which grow with either isoleucine or threonine. TD of these mutants exhibits an enhanced sensitivity to feedback inhibition by isoleucine. Thus, TD seems to be inhibited by isoleucine levels which do not allow the mutants to grow.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* LT 2 PLT 22 was obtained from U. Henning, Tübingen, Germany.

Chemicals. Dithioerythritol and Dowex 50 W × 8 were purchased from Serva AG, Heidelberg; L-amino acids were from Merck AG, Darmstadt; L-isoleucine-^{3}H was from Buchler Co., Braunschweig; and threonine assay medium was from Difco.

Media. Complete medium contained (per liter of deionized water): nutrient broth, 10 g; yeast extract, 5 g; and NaCl, 5 g. Minimal medium contained (per liter of deionized water): K_2HPO_4, 10 g; $NaNH_4HPO_4 \cdot 4\ H_2O$, 3.5 g; citric acid $\cdot H_2O$, 2 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; and glucose, 2 g. Cultures were incubated at 37 C on a rotary shaker.

Mutation. Exponentially growing cells were harvested and incubated in a solution containing 0.1 M citrate-phosphate buffer, *p*H 6, and 0.14 mM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at 37 C (1). The cells were harvested, washed twice, suspended in complete medium, and incubated for 3 hr at 37 C for phenotypic expression.

Isolation of revertants. Revertants of strain 741 were isolated by plating an average number of 10^8 cells on minimal plates supplemented with isoleucine (20 μg/ml).

Determination of growth rates. Erlenmeyer flasks (100 ml) containing 20 ml of minimal medium (supplemented as indicated) were inoculated with washed logarithmic-phase cells and incubated in a reciprocating shaker at 37 C. Turbidity was determined at appropriate intervals.

Accumulation of threonine. Cells grown in 500 ml of minimal medium with appropriate supplementation of leucine or leucine plus isoleucine (each 20

μg/ml) were pelleted in the logarithmic phase, suspended in 500 ml of minimal medium, and incubated for 5 hr in a reciprocating shaker at 37 C. The suspension was centrifuged. Threonine was determined in samples of the supernatant by microbiological assay with *Streptococcus faecalis* ATCC 8043.

Preparation of cell extracts. The bacteria were grown in flasks containing 500 ml of minimal medium supplemented with isoleucine (50 μg/ml) and leucine (12 μg/ml). This concentration of leucine is growth limiting and results in derepressed formation of TD (4).

Cells were harvested from exponentially growing cultures by centrifugation and disrupted by sonic treatment in 0.05 M potassium phosphate buffer (3).

Enzyme assays. Test solutions for the determination of TD contained 0.1 M tris(hydroxymethyl)-aminomethane buffer (*p*H 8), 10 mM ammonium chloride, 60 mM threonine, and protein in a total volume of 1 ml (3). Transaminase B activity was determined by the method of Szentirmai and Umbarger (8).

Protein was determined by the biuret method. Specific activities are expressed as micromoles of product formed per minute per milligram of protein.

RESULTS

Isolation of mutants. The parent strain used for the isolation of mutants was a leucine-deficient strain (ST 700) obtained after treatment of *S. typhimurium* with MNNG. The rationale was that in leucine-deficient mutants TD can be derepressed by growth with limiting concentrations of leucine.

Twenty mutants were isolated as isoleucine-requirers after treatment of strain ST 700 with MNNG. These mutants can be divided in three groups on the basis of growth requirements (Table 1). (i) Three mutants show full growth on isoleucine alone (ST 703, ST 730, ST 751). (ii) Four mutants grow with isoleucine, but growth is further enhanced by valine (ST 711, ST 732, ST 733, ST 753). (iii) Four mutants grow with either isoleucine or threonine (ST 710, ST 731, ST 741, ST 748). A 10-μg amount of isoleucine or 2-ketobutyric acid per ml allows full growth. Relatively high levels of threonine (50 μg/ml) are needed to satisfy the growth requirement of these mutants. Very little growth is observed in the absence of both isoleucine and threonine (Fig. 1).

Enzyme studies. Results of enzyme studies are shown in Table 1. Mutants growing with isoleucine (group i) are devoid of biosynthetic TD. A low residual enzyme activity (1%) found in all of these mutants is not susceptible to feedback inhibition by isoleucine.

Mutants growing with either isoleucine or isoleucine plus valine (group ii) exhibit a low activity of transaminase B. TD in these mutants has wild-type kinetic properties.

In mutants growing with either isoleucine or threonine (group iii), levels of TD are similar to that observed in the parent strain ST 700.

Figure 2 shows the inhibition by isoleucine of TD of parent strain ST 700 and mutants of group iii. The TD of the isoleucine-threonine mutants is inhibited by low levels of isoleucine which do not affect the enzyme of wild type.

The properties of the TD of the isoleucine-threonine mutant ST 741 have been studied more in detail. In 60 mM threonine solution, the isoleucine concentration necessary for 50% inhibition of TD is about 15-fold lower in mutant ST 741 than in the isoleucine-prototrophic strains ST 700 and LT 2. At a low substrate concentration (6 mM threonine), the mutant TD is 250 times more sensitive to inhibition (50%) by isoleucine than the enzyme of wild type.

Figure 3 shows the dependency of the initial velocity of enzymatic conversion of threonine on substrate concentration. Without addition of isoleucine, both wild-type and mutant TD show identical characteristics. The slightly sigmoidal form of the curves is probably caused by the presence of small amounts of isoleucine in the cell extract (3, 11). This sigmoidal character becomes more pronounced by addition of isoleucine to the assay mixture. Under these conditions, wild-type and mutant enzyme show different properties (Fig. 3). At a low concentration of isoleucine (3.3×10^{-5} M) only the mutant TD is inhibited. This inhibition is antagonized by increasing concentrations of threonine. We assume that this effect is responsible for the observed growth effect of threonine.

Leucine is another inhibitor of TD from *E. coli* and *S. typhimurium*, although less potent than isoleucine (12). Again TD of isoleucine-threonine mutants is much more sensitive to inhibition by leucine than wild-type TD (Fig. 4).

Low concentrations of valine exert an activating effect upon TD from *E. coli* and *S. typhimurium*, thus antagonizing the inhibitory effect of isoleucine (11). High concentrations of valine (10^{-2} M) inhibit wild-type TD. In contrast, TD of isoleucine-threonine mutants is not activated, but rather inhibited by small concentrations of valine (Fig. 5).

The effect of aspartic acid, lysine, methionine, and tryptophan on the activity of TD have been tested. TD from wild-type and isoleucine-threonine mutants of *S. typhimurium* is not significantly affected by any one of these compounds.

TABLE 1. *Properties of mutants of Salmonella typhimurium used in this study*[a]

Strain	Growth requirement	Derived from	TD[b]	TB[b]	Inhibition of TD by isoleucine[c] (M)
LT 2			0.15	0.15	3×10^{-4}
ST 700	Leu	LT 2	1.16	0.25	3×10^{-4}
ST 703	Leu + Ile	ST 700	0.01	0.26	N[d]
ST 730	Leu + Ile	ST 700	0.011	0.26	N
ST 751	Leu + Ile	ST 700	0.011	0.25	N
ST 711	Leu + Ile (+ Val)	ST 700	1.5	0.03	3×10^{-4}
ST 732	Leu + Ile (+ Val)	ST 700	1.33	0.04	3×10^{-4}
ST 733	Leu + Ile (+ Val)	ST 700	1.5	0.03	3×10^{-4}
ST 753	Leu + Ile (+ Val)	ST 700	1.33	0.03	3×10^{-4}
ST 710	Leu + Ile or Leu + Thr	ST 700	0.91	0.25	4×10^{-5}
ST 731	Leu + Ile or Leu + Thr	ST 700	0.73	0.26	6×10^{-5}
ST 741	Leu + Ile or Leu + Thr	ST 700	0.91	0.26	2×10^{-5}
ST 748	Leu + Ile or Leu + Thr	ST 700	0.41	0.26	2×10^{-5}

[a] Abbreviations: Leu, leucine; Ile, isoleucine; Thr, threonine; TD, threonine deaminase; TB, transaminase B.

[b] Specific activities of cells grown with limiting concentration of leucine (TD and TB derepressed).

[c] Isoleucine concentration required for 50% inhibition of threonine deaminase at a substrate concentration of 6×10^{-2} M threonine.

[d] No inhibition by isoleucine observed.

Revertants. Revertants were isolated by plating cells of the feedback hypersensitive mutant ST 741 on minimal agar plates containing leucine but not isoleucine. Among 13 revertants thus obtained, two groups can be distinguished on the basis of enzyme studies (Table 2). (i) TD of nine revertants shows a reduced feedback inhibition to isoleucine. A minimal concentration of 2×10^{-4} M isoleucine is required for 50% inhibition. Valine is an activator of TD of these revertants. Thus these revertants exhibit properties different from strain ST 741 and similar to strains ST 700 and LT 2. (ii) The kinetic properties of the TD of four revertants resembles that of mutant ST 741, being still hypersensitive to inhibition by isoleucine. These revertants produce greatly more threonine than strain ST 741 (Table 2).

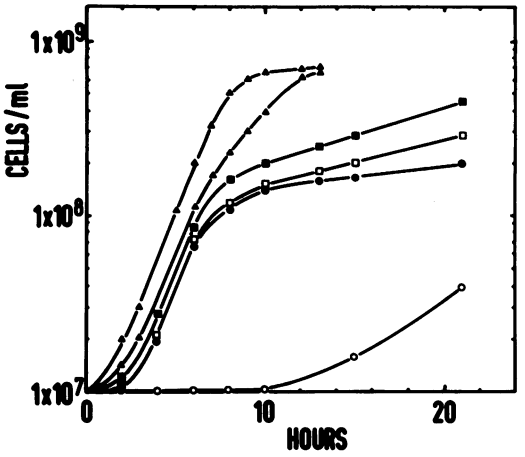

FIG. 1. *Growth characteristics of the feedback hypersensitive mutant ST 741. Symbols:* ○, *minimal medium;* ●, *threonine (50 μg/ml);* □, *threonine (100 μg/ml);* ■, *threonine (200 μg/ml);* △, *2-ketobutyrate (20 μg/ml);* ▲, *isoleucine (10 μg/ml). Minimal medium contained leucine (20 μg/ml).*

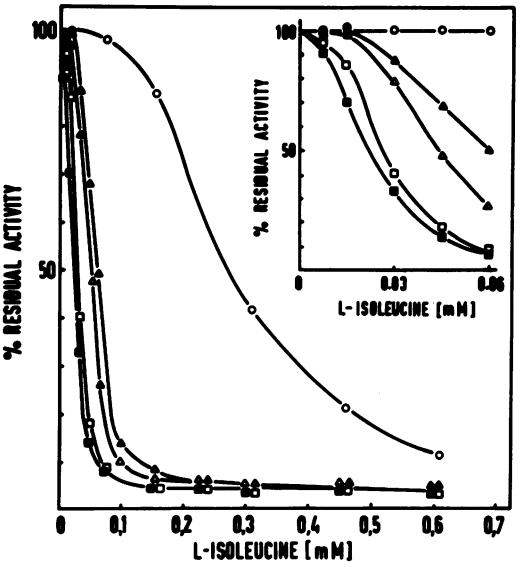

FIG. 2. *Feedback inhibition of threonine deaminase by isoleucine. Symbols:* ○, *LT2;* △, *ST 710;* ▲, *ST 731;* □, *ST 741;* ■, *ST 748. Concentration of threonine was* 6×10^{-2} *M.*

DISCUSSION

Three types of TD-mutants can be discerned on the basis of growth requirement and enzyme studies (Table 1). (i) Mutants devoid of TD require isoleucine for growth. It cannot be decided whether the low residual feedback resistant enzyme activity is really TD.

(ii) A few mutants are devoid of transaminase B. In these mutants, a limited amount of valine is still being produced by valine-α-aminobutyrate-alanine transaminase, thus enabling the mutants to grow with isoleucine alone (2, 7). However, full growth is only achieved with isoleucine and valine.

(iii) The auxotrophs of this group need iso-

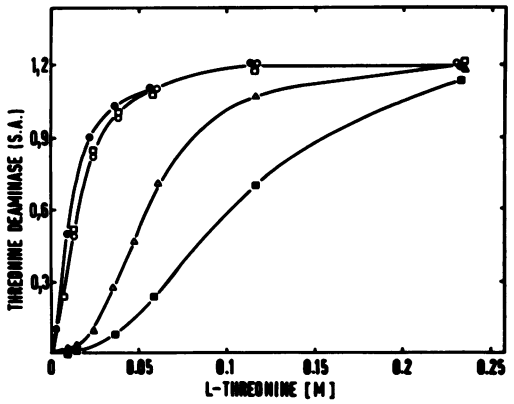

FIG. 3. *Dependence of reaction velocity of threonine deaminase on threonine and isoleucine. Symbols: LT 2, without isoleucine* (○); *LT 2, 3.3 × 10^{-5} M isoleucine* (□); *LT 2, 3 × 10^{-4} M isoleucine* (△); *ST 741, without isoleucine* (●); *ST 741, 3.3 × 10^{-5} M isoleucine* (■); *specific activity, S.A.*

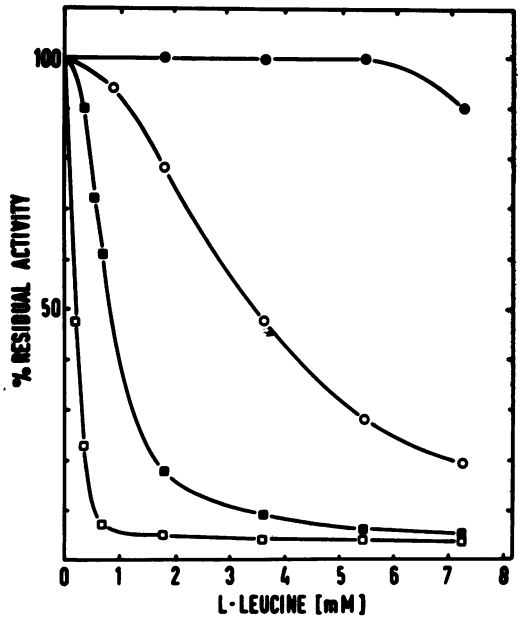

FIG. 4. *Effect of leucine on threonine deaminase of wild-type strain LT 2 and mutant ST 741. Symbols: LT 2, 1.2 × 10^{-2} M threonine* (○); *LT 2, 6 × 10^{-2} M threonine* (●); *ST 741, 1.2 × 10^{-2} M threonine* (□); *ST 741, 6 × 10^{-2} M threonine* (■).

leucine or threonine for growth. These mutants produce threonine at a normal rate. The growth response to the product of TD-action, 2-ketobutyrate, as well as to isoleucine, suggests that a defect in TD is responsible for the isoleucine deficiency. The activity of TD in these mutants is similar to that observed in the parent strain ST 700 or slightly less. The shape of substrate saturation curves is identical for TD of wild-type strain and mutants. Therefore the substrate affinity of the mutant TD seems not to be affected.

However, the TD of these mutants shows markedly increased sensitivity to feedback inhibition by isoleucine and leucine. Valine, which activates wild-type TD, inhibits the mutant TD. The inhibitory effect of isoleucine exceeds that of leucine and valine by a factor of 100 to 1,000. We suppose that the level of isoleucine which is normally present in the cells is sufficient to inhibit completely the activity of TD, thus hindering the mutants to grow.

In vitro experiments have shown that the inhibitory effect of isoleucine upon the mutant enzyme can be reversed, in part, by high concentrations of threonine. This is consistent with the finding that the mutants are able to grow in minimal medium with a high level of threonine.

The hypothesis that the isoleucine requirement of the mutants is a consequence of the feedback hypersensitivity of TD was further

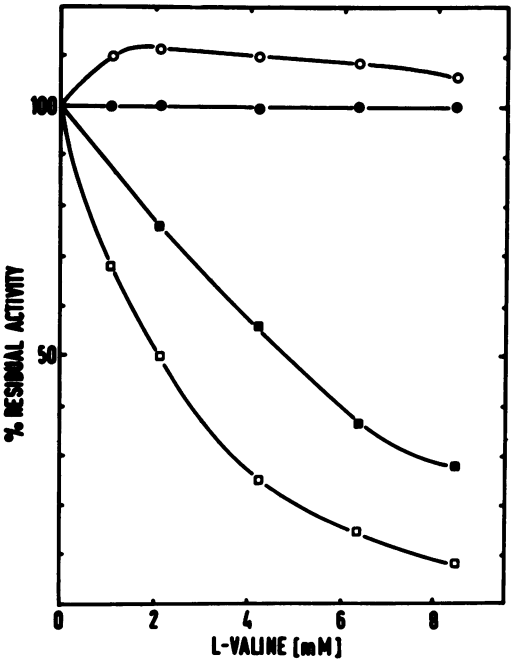

FIG. 5. *Effect of valine on threonine deaminase of wild-type strain LT 2 and mutant ST 741. Symbols: LT 2, 1.2 × 10^{-2} M threonine* (○); *LT 2, 6 × 10^{-2} M threonine* (●); *ST 741, 1.2 × 10^{-2} M threonine* (□); *ST 741, 6 × 10^{-2} M threonine* (■).

TABLE 2. *Properties of the feedback hypersensitive mutant ST 741 and of prototrophic revertants*[a]

Strain[b]	Growth requirement	TD[c]	Inhibition of TD by isoleucine[d] (M)	Effect of valine on TD	Accumulation of threonine (μmoles per liter)
ST 700	Leu	1.16	3×10^{-4}	Activation	30
ST 741	Leu + Ile or Leu + Thr	0.91	2×10^{-5}	Inhibition	30
ST 741/1	Leu	0.66	3×10^{-4}	Activation	30
ST 741/2	Leu	0.38	3×10^{-4}	Activation	30
ST 741/6	Leu	0.25	2×10^{-4}	Activation	30
ST 741/7	Leu	0.11	1.5×10^{-4}	Activation	30
ST 741/11	Leu	0.34	1×10^{-3}	Activation	30
ST 741/19	Leu	0.56	1×10^{-4}	Activation	30
ST 741/20	Leu	0.40	5×10^{-3}	Activation	30
ST 741/21	Leu	0.25	2×10^{-4}	Activation	30
ST 741/22	Leu	0.15	2×10^{-4}	Activation	30
ST 741/8	Leu	0.58	2×10^{-5}	Inhibition	700
ST 741/12	Leu	0.75	2×10^{-5}	Inhibition	800
ST 741/17	Leu	0.40	4×10^{-5}	Inhibition	400
ST 741/18	Leu	0.63	2×10^{-5}	Inhibition	300

[a] Abbreviations: Leu, leucine; Ile, isoleucine; Thr, threonine; TD, threonine deaminase.
[b] All strains except the first two are revertants derived from strain ST 741 by spontaneous reversion.
[c] Specific activities of TD.
[d] Concentration of isoleucine required for 50% inhibition of TD at a substrate concentration of 6×10^{-2} M threonine.

confirmed by experiments with revertants of strain ST 741. In nine revertants, the regulatory properties of TD are essentially the same as in wild type. In four other revertants, TD is still hypersensitive to inhibition by isoleucine, leucine, and valine. However, these strains produce considerably more threonine than the parent strain ST 741. As shown above, the inhibition of hypersensitive TD can be counterbalanced by high levels of threonine. Thus, the properties of the revertants are consistent with the view that the only metabolic defect of the isoleucine-threonine mutants is the observed alteration of sensitivity of feedback inhibition by isoleucine.

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