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CHANNELING BEHAVIOR AND ACTIVITY MODELS FOR *Escherichia coli* K-12 ACETOHYDROXY ACID SYNTHASES AT PHYSIOLOGICAL SUBSTRATE LEVELS

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SUMMARY. The channeling behavior of acetohydroxy acid synthases I and III (EC 4.1.3.18; AHAS) was studied by computer simulation of activities over a wide range of concentrations for the substrates pyruvate and 2-ketobutyrate. The ratios of reaction rates for both channels and three-dimensional plots of single-channel reaction rates versus substrate concentrations were introduced to compare the substrate channeling properties of the isozymes. Substrate ranges were identified in which AHAS I and III operated both channels, and in which they used only one. Kinetic constants were varied to simulate whether and how AHAS might be made channel-specific. Our study suggests that AHAS might be made channel-specific for acetolactate but not for acetohydroxybutyrate. We postulate specific physiological roles for AHAS I and III to support cell growth under conditions that vary the levels and balance of substrates.

The acetohydroxy acid synthases (EC 4.1.3.18; AHAS) are carboligase enzymes that partition a pyruvate molecule to condense with a second pyruvate to form 2-acetolactate (AL) or with 2-ketobutyrate (2-KB) to form 2-aceto-2-hydroxybutyrate (AHB) (1, Fig. 1). The wild-type *Escherichia coli* strain K-12 normally expresses two AHAS isozymes; AHAS I from *ilvBN* and AHAS III from *ilvIH* (2, 3, 4). Valine, at a concentration of 1.0 mM, causes 100% inhibition of AHAS I and 80% inhibition of AHAS III (2, 3, 4).

The purpose of multiple AHAS genes in bacteria has been subject to speculation for some time. The relative flow of pyruvate carbons toward isoleucine or valine has been characterized by a specificity ratio $R = (v_{AHB}/v_{AL})/([AHB]/[AL])$, where v represents the reaction velocity for AL or AHB biosynthesis (5, 6). AHAS III, with a high R-value, preferentially channels pyruvate carbons toward isoleucine biosynthesis and is consequently considered likely to be essential for growth in the presence of a rich carbon and energy source (6, 7). AHAS I, with a low R-value and nearly balanced channeling of pyruvate carbons toward isoleucine and valine, was reported to be required for growth on a poor carbon source (6, 7). The presence of multiple isozymes in other biosynthetic pathways, e.g. the aspartate kinases and deoxy-arabino-heptulosonate phosphate synthases, appears to ensure the supply of a common biosynthetic intermediate required for multiple endproduct pathways (8, 9). By analogy, it seems reasonable to propose that one purpose of AHAS I activity might be to ensure carbon flow into the pathway channel for valine biosynthesis, and at least one purpose for AHAS III activity might be to ensure the carbon flow toward isoleucine biosynthesis.

Abbreviations: AHAS, acetohydroxy acid synthase (EC 4.1.3.18); AL, 2-acetolactate; AHB, 2-aceto-2-hydroxy butyrate; 2-KB, 2-ketobutyrate; v_1 and v_2 are, respectively, the initial velocities for synthesis of AL and AHB; $K_{a2} = K_m$ for the recipient pyruvate going to AL; $K_b = K_m$ for AHB.

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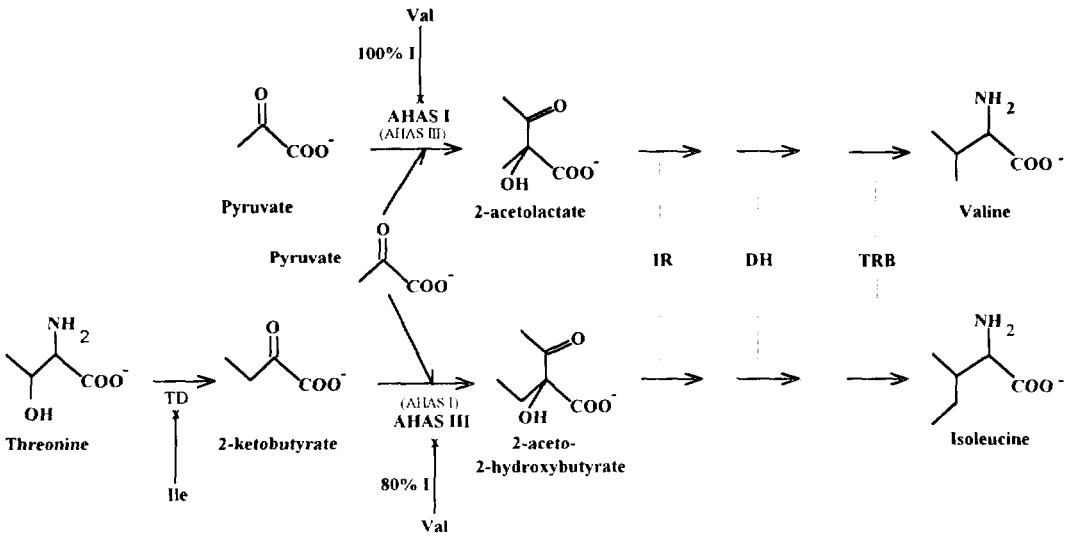

Fig. 1. Pathway for biosynthesis of isoleucine and valine. The pathway is pictured to emphasize the acetohydroxy acid synthase reactions. Abbreviations: threonine deaminase, TD; acetohydroxy acid synthase, AHAS; isomeroreductase, IR; dihydroxy acid dehydrase, DH; transaminase B, TRB.

We report the results of growth experiments and kinetic simulation analyses of the substrate channeling behavior of AHAS I and AHAS III at levels of pyruvate and 2-KB likely to occur in a variety of growth conditions. Our data analysis was focused to identify physiological conditions under which *a*) AHAS I and III were bifunctional and therefore each, alone, could support growth; *b*) a single isozyme activity would become monofunctional, and therefore not sufficient for cell growth; and *c*) cell growth might require AHAS activities with properties different than those of isozymes I and III.

MATERIALS AND METHODS

Bacterial strains used. Strains MJ6 (*rbs*), MJ170 [*ilvB2102 rbs-221 ara thi* Δ(*pro-lac*)], MJ171[*ilvI2202 rbs-221 ara thi* Δ(*pro-lac*)], and MJ133 (*ilvJ662 ilvB2102 ilvI2202 rbs 221 thi* Δ(*pro-lac*)] were used in this study. All strains were derivatives of *Escherichia coli* K-12 and previously described (5).

Growth methods and chemicals. Bacteria used for all physiological studies were grown in a mineral salts medium with 0.5% (w/v) glucose or 0.5% (w/v) acetate as the sole carbon source with required growth supplements provided as necessary (5). Methods to determine growth sensitivities of strains to specific reagents were previously described (10, 11). All chemicals were of reagent grade or the highest purity obtainable.

Reaction simulations. Equations used to simulate AHAS reactions were previously reported (5). The concentration ranges used were reported to occur from growth of *E. coli* under a variety of conditions (5, 6, 7, 12, 13).

Measurement of intracellular amino acid concentrations. Bacterial cultures were grown in appropriate media, harvested in mid-log phase by centrifugation, and rapidly resuspended in 3% sulfosalicylic acid containing L-norleucine as an internal control. Samples were processed and the intracellular amino acid content was determined according to procedures described elsewhere (14). Protein concentration was determined by the method of Bradford (15).

RESULTS

Growth of strains expressing a single AHAS isozyme. Strains MJ170 ($IlvIH^+$ $IlvBN^-$ $IlvGM^-$) and MJ171 ($IlvBN^+$ $IlvIH^-$ $IlvGM^-$) were tested for the ability to grow on minimal agar plates using glucose or acetate as the sole carbon and energy source. These strains were also tested for growth sensitivity to valine and to 2-KB.

Both strains grew with glucose or acetate as the sole carbon source and both strains were sensitive to growth inhibition by valine and by 2-KB.

Ratios of reaction rates. The ratios of reaction rates for synthesis of AL by AHAS I and AHAS III were determined for varying concentrations of pyruvate and 2-KB (Fig. 2A). At low pyruvate concentrations, 50 μM to 250 μM, AHAS I produced acetolactate at about 5 to 18 times the rate of AHAS III at all concentrations of 2-ketobutyrate. At the intermediate range of pyruvate levels, 0.5 mM to 2.5 mM, AHAS I made AL at 4 to 15 times the rate of AHAS III. At low 2-KB, the differences in rates of AL synthesis were lowest, and the reaction rate differences increased as a function of the 2-KB concentration. At the highest range of pyruvate concentrations, 5 mM to 15 mM, AHAS I showed only a slight rate advantage over AHAS III for AL synthesis at low concentrations of 2-KB. Increasing concentrations of 2-KB only slightly improved the AHAS I rate advantage over AHAS III for AL synthesis. Therefore, AHAS I appeared to have a higher reaction rate than AHAS III for AL synthesis at all levels of pyruvate and 2-KB which was consistent with predictions by the *R*-value.

The ratios of reaction rates for synthesis of 2-aceto-2-hydroxy butyrate revealed that AHAS I worked better than AHAS III at the low and intermediate ranges of pyruvate over all levels of 2-KB (Fig. 2B). However, this advantage for AHAS I was marginal at the lowest 2-KB concentrations. At the high pyruvate range, 5 mM to 15 mM, the value for the ratio of reaction rates was less than 1.0, indicating that AHAS III made AHB at a faster rate than AHAS I under these conditions.

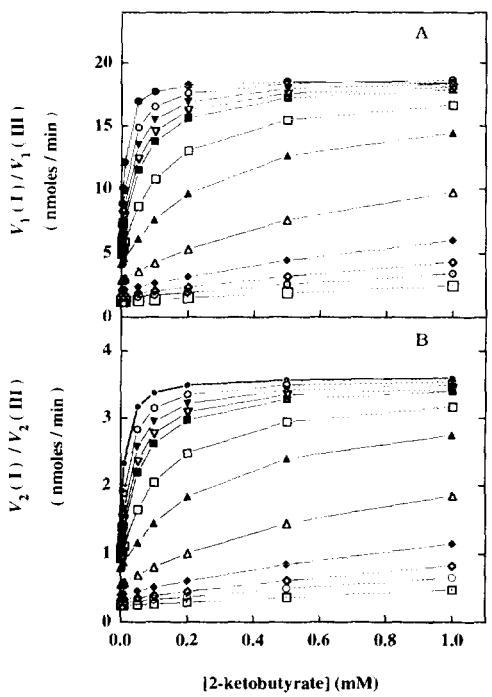

Fig. 2. Isozyme reaction rate comparisons for each channel. Panel A. The ratio of the rates of acetolactate synthesis by AHAS I and AHAS III. Symbols for pyruvate concentrations: ●, 0.05 mM; O, 0.10 mM; ▼, 0.15 mM; ▽, 0.20 mM; ■, 0.25 mM; □, 0.5 mM; ▲, 1.0 mM; △, 2.5 mM; ◆, 5.0 mM; ◇, 7.5 mM; ⊙, 10.0 mM; and ⊡, 15.0 mM. Panel B. The ratio of the rates of acetohydroxybutyrate synthesis by AHAS I and AHAS III. Symbols: same as panel A.

These results implied that various reaction conditions might exist in which: **a)** AL may be synthesized primarily by AHAS I activity; **b)** AL and AHB may be made by both AHAS I and III; and **c)** AHB may be synthesized primarily by AHAS III.

Kinetic behavior of AHAS I and AHAS III over a continuous, physiological range of pyruvate and 2-ketobutyrate concentrations. In order to examine the specific contribution of each isozyme to biosynthesis of valine and isoleucine precursors over a continuum of substrate concentrations, three dimensional (3-D) plots were made with the variables of reaction velocity, pyruvate concentration, and 2-KB concentration. The 3-D plots of AHAS I reaction simulations showed AL formation at the lowest concentrations of pyruvate and 2-KB, but not at high 2-KB and low pyruvate (Fig. 3A). Over the full range of pyruvate concentrations, AHB biosynthesis became appreciable only after the concentration of 2-KB exceeded 0.01 mM (Fig. 3B). At high 2-KB and low pyruvate concentrations, only AHB biosynthesis was apparent. The minimal conditions under which AHAS I synthesized both AL and AHB, were [pyruvate] > 0.10 mM with 0.01 mM < [2-KB] < 0.10 mM. Generally, AHAS I was bifunctional at [pyruvate] > 0.15 mM with [2-KB] > 0.01 mM. AHAS I appeared to be monofunctional for AL biosynthesis at [2-KB] < 0.01 mM, and monofunctional for AHB biosynthesis at [pyruvate] < 0.15 mM with [2-KB] > 0.1 mM.

The 3-D plots of AHAS III reaction simulations showed AL formation at [pyruvate] > 0.15 mM with [2-KB] < 0.10 mM (Fig. 3C). At higher concentrations of 2-KB with pyruvate < 0.15 mM, AL formation was marginal or absent but AHB was made over the same concentration ranges (Fig. 3D). Similar to the observation with AHAS I, AHAS III formed AHB at all pyruvate levels when the concentration of 2-KB exceeded 0.1 mM. For [pyruvate] > 0.5 mM, and 0.01 mM < [2-KB] < 10 mM, AHAS III made both AL and AHB (Fig. 3C, D). Thus, AHAS III appeared to be monofunctional for AL synthesis at 0.05 mM < [pyruvate] < 15.0 mM and 0 mM < [2-KB] < 0.01 mM. It was mono-functional for AHB synthesis at 0.5 mM < [pyruvate] < 0.25 mM with 0.1 mM < [2-KB] < 15.0 mM and at 0.25 < [pyruvate] < 5.0 mM with 2.5 mM < [2-KB] < 15.0 mM (Fig. 3C, D).

Growth conditions that influence pyruvate and 2-KB levels. Aerobic growth with glucose as the carbon and energy source has been reported to produce high, intracellular pyruvate (>10 mM) levels (12). Growth on acetate as a sole carbon and energy source has been reported to produce low pyruvate (< 0.4 mM) levels (7, 13). The level of 2-KB has been reported to rise following a shift of *E. coli* from anaerobic to aerobic growth (12), and in response to an increase in the valine concentration (5). A falling pyruvate level has been reported to be one consequence of rising 2-KB (21) and 2-KB appears to regulate the response of many cellular systems to changes in physiological conditions (12, 16-18). Based upon our results from AHAS reaction simulations, the range of pyruvate and 2-KB concentrations for cells growing on glucose minimal medium is such that either AHAS I or AHAS III could be bifunctional for AL and AHB biosynthesis (Figs. 3 A-D) under these conditions.

As previously noted, valine-inhibited K-12 strains lack AHAS I activity altogether, and are left with only 20% of the AHAS III activity to cope with the biosynthetic demands for growth. According to our reaction simulations, even when valine is supplied AHAS III should retain the capacity to make AHB at all pyruvate concentrations shown as long as the 2-KB level is above 10 μM (Fig. 3D). The simulations further suggested that even at high 2-KB levels and low pyruvate, AHAS III should retain the activity necessary to synthesize enough AHB to continue isoleucine biosynthesis in a valine-inhibited culture.

The intracellular concentration of isoleucine in a culture of strain K-12 grown in minimal medium was 90 pmol per mg total cell protein. In a valine-inhibited culture, the intracellular isoleucine concentration was 130 pmol per mg total cell protein. The maintenance or slight increase in the intracellular isoleucine concentration in

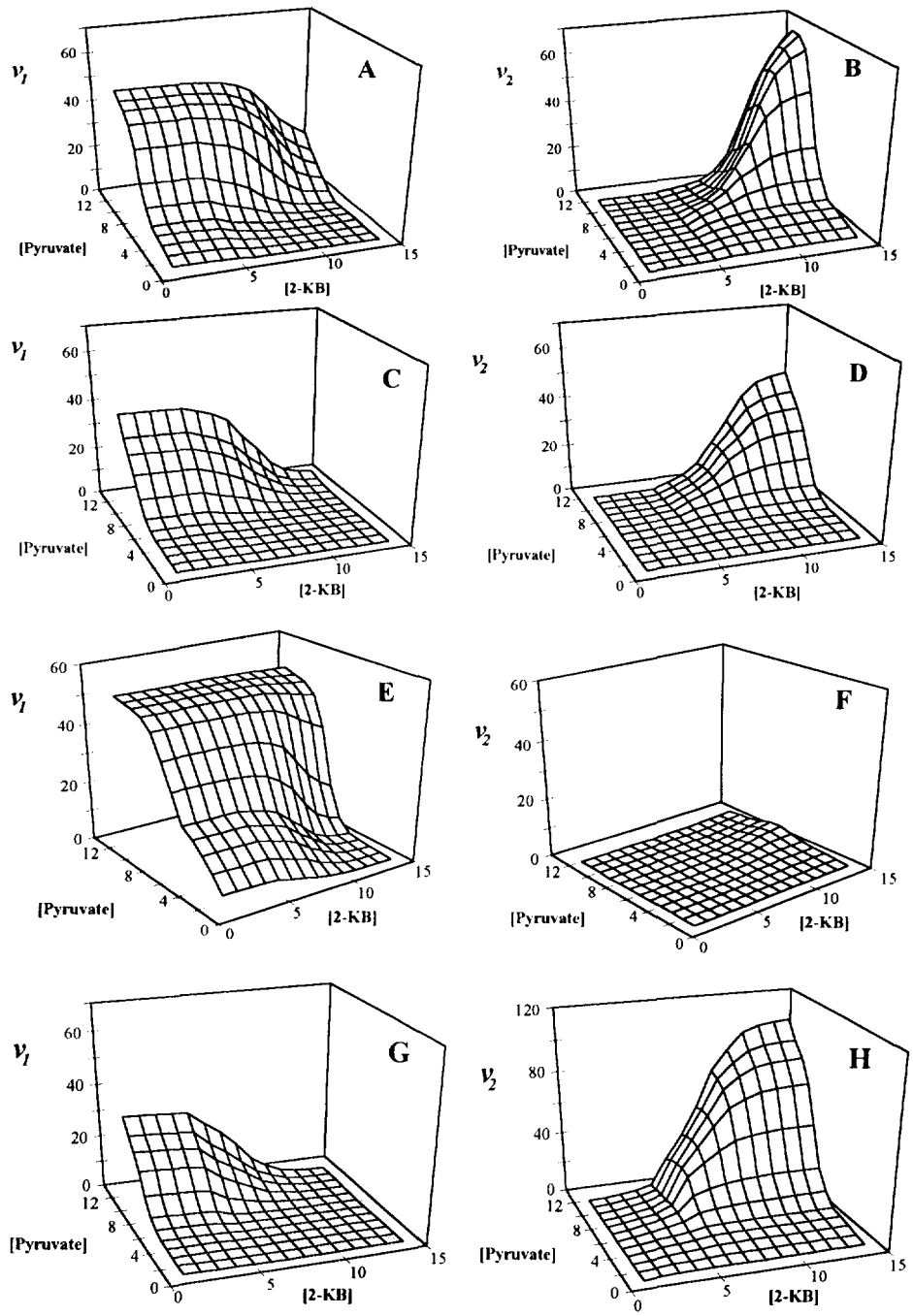

Fig. 3. **Reaction rate surface plot for each AHAS channel.** **A**, AL biosynthesis, AHAS I; **B**, AHB biosynthesis, AHAS I; **C**, AL biosynthesis, AHAS III; **D**, AHB biosynthesis, AHAS III; **E**, AL biosynthesis, hypothetical monofunctional AHAS; **F**, AHB, hypothetical monofunctional AHAS; **G**, AL biosynthesis, hypothetical monofunctional AHAS; **H**, AHB biosynthesis, hypothetical monofunctional AHAS. The scales for pyruvate and 2-KB are coded for convenience of plotting and correspond to concentrations. **Pyruvate scale: 1**, 0.05 mM; **2**, 0.1 mM; **3**, 0.15 mM; **4**, 0.2 mM; **5**, 0.25 mM; **6**, 0.5 mM; **7**, 1.0 mM; **8**, 2.5 mM; **9**, 5.0 mM; **10**, 7.5 mM; **11**, 10.0 mM; and **12**, 15.0 mM. **2-KB scale: 1**, 0.1 μM; **2**, 0.001 mM; **3**, 0.003 mM; **4**, 0.005 mM; **5**, 0.01 mM; **6**, 0.05 mM; **7**, 0.1 mM; **8**, 0.2 mM; **9**, 0.5 mM; **10**, 1.0 mM; **11**, 2.5 mM; **12**, 5.0 mM; **13**, 10.0 mM; **14**, 15.0 mM.

valine-inhibited cell cultures implied that the 20% residual AHAS III activity produced enough AHB to maintain the isoleucine pool. These results were consistent with the predictions from our reaction simulations.

Possibilities for single-function enzymes. The AHAS reaction mechanism does not appear to permit a single enzyme to produce both amino acid precursors in sufficient quantities to permit growth over the entire physiological range of pyruvate and 2-KB concentrations. Noting that AHAS I and III were largely monofunctional at certain combinations of substrate levels, we sought to learn how to change a single isozyme to make only one reaction product under all physiological levels of pyruvate and 2-KB. We found that the ratio of K_{a2} to K_b was the principal determinant to achieve monofunctional channeling. A change of K_{a2} to 0.1 and K_b to 10.0, for AHAS I, yielded a reaction rate surface that was essentially monofunctional for AL formation (Fig. 3E, F) However, a change of K_b to 0.1 and a variety of other combinations of values for K_{a2} and K_b failed to produce a reaction rate surface that was essentially monofuntional for AHB formation (Fig. 3G, H). It appeared that AHAS III may be Nature's attempt to produce an activity dedicated to AHB biosynthesis.

DISCUSSION

A unique feature for biosynthesis of the isoleucine and valine pathway intermediates is that two different, pathway-specific products are made by the AHAS activity. This study identified reaction conditions in which either AHAS I or AHAS III could catalyze formation of both reaction products, AL and AHB. We also defined reaction conditions under which each isozyme could make either AL or AHB but not both. Considering the growth conditions known to influence pyruvate and 2-KB levels, and that regulation of metabolite flow is achieved by valine, isoleucine and 2-KB, then neither AHAS I nor AHAS III could adequately make both reaction products under all conditions. The proposed reaction mechanism appears to preclude the development of a single enzyme capable of bifunctional activity for all physiologically relevant ranges of pyruvate and 2-KB concentrations. We have shown, however, that an enzyme with this proposed reaction mechanism could theoretically be altered to have monofunctional activity for AL synthesis but not for AHB under the substrate concentration ranges considered in this study.

Explanations offered for the normal function of and requirement for multiple AHAS isozymes to support cell growth (6, 7) have tended to emphasize the importance of pyruvate levels. Our study highlights the importance of 2-KB. It appears that cellular growth that depends solely upon AHAS I activity requires a threshold level above 50 μM pyruvate and 10 μM 2KB, while threshold levels for AHAS III-dependent growth are 250 μM pyruvate and 10 μM 2KB. Our estimate of the pyruvate level needed for AHAS III-dependent growth reasonably agrees with the level of 300 μM suggested by Barak et al (6) however our estimate for the intracellular concentration of 2-KB is about three times higher. If the pyruvate level during growth on acetate is about 400 μM as reported (13), our simulations suggest that the activity of either AHAS I or AHAS III should be sufficient to support growth. Experimentally, we confirmed these predictions. Based upon our reaction simulations, the intracellular concentration of 2-KB must exceed 10 μM to open the channel for isoleucine biosynthesis. Thus, the 2-KB concentration determines whether an AHAS is bifunctional or monofunctional and therefore whether cell growth in a minimal salts medium is possible.

A cascade of regulatory events depends upon the level of 2-KB which appears to regulate the phosphoenol pyruvate phosphotransferase system (PTS), pantothenate biosynthesis, coenzyme A utilization, methionine biosynthesis and pyruvate levels (12, 16-18, 19). A major means for metabolic regulation of the 2-KB level is the

feedback inhibition of threonine deaminase by isoleucine (1) and the level of AHAS activity to make the isoleucine precursor AHB from 2-KB.

Model for normal AHAS function. Based upon the computer modeling of AHAS reactions, we find that AHAS I activity functions best to make AL and AHB at low pyruvate concentrations and at all levels of 2-KB. At higher pyruvate concentrations AHAS III activity works best to make AHB, and AHAS I works best to make AL. High valine levels that result from the high production of AL, primarily from AHAS I activity, completely inactivate this enzyme while isoleucine biosynthesis continues from the 20% residual activity of AHAS III to regulate synthesis of 2-KB at an elevated level (5).

Increased 2-KB decreases glycolytic activity and thus the level of pyruvate. The valine concentration regulates carbon flow from pyruvate to valine and from 2-KB to isoleucine by feedback inhibition of the AHAS activities (1). The level of isoleucine regulates biosynthesis of 2-KB by feedback inhibition of threonine deaminase (TD). At high 2-KB and decreasing pyruvate AHAS III mostly makes AHB for isoleucine synthesis, and the isoleucine pool regulates 2-KB production at a level determined by the K_m of AHAS for 2-KB. Growth inhibition of strain K-12 by valine appears to be associated with elevated levels of 2-KB (5).

We propose that two AHAS isozymes are maintained by *E. coli* strain K-12 because AHAS I activity may ensure operation of the valine pathway channel, and AHAS III activity may ensure operation of the isoleucine pathway channel. By extension, this hypothesis may explain the tendency to find two AHAS activities in a single organism.

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