ISOLEUCINE AND VALINE METABOLISM IN *ESCHERICHIA COLI*.

XX. MULTIPLE FORMS OF ACETOHYDROXY ACID SYNTHETASE*

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Received June 12,1972

SUMMARY: Acetohydroxy acid synthetase (AHAS) from *Salmonella typhimurium* strain TU5006 (derived from LT2) and *Escherichia coli* strain M-42-11 (derived from W) consists of two activities. One (AHAS I) is sensitive to feedback inhibition by isoleucine, leucine and valine, and the other (AHAS II) is insensitive to these same effectors. *E. coli* strain CU1013, a derivative of strain K-12, lacks the ability to produce AHAS II, thus accounting for the valine-sensitive growth characteristic of derivatives of *E. coli* strain K-12. Production of the AHAS I activity appears to be under multivalent control by valine and leucine, while that of AHAS II, in those strains that produce it, appears to be under multivalent control by isoleucine, valine and leucine.

In recent years, many examples of multiple forms of an enzyme have been reported (1). We present evidence here for the existence of two forms of acetohydroxy acid synthetase (AHAS), the first shared enzyme in the branched-chain amino acid pathway in *Salmonella typhimurium* and *Escherichia coli*.

EXPERIMENTAL

Physiology experiments. The pattern of regulation of AHAS was examined in crude extracts of auxotrophs of *S. typhimurium* strain LT-2 and *E. coli* strains K-12 and W. The *S. typhimurium* pattern is shown in Fig. 1. The enzyme produced during isoleucine limitation was completely resistant to feedback inhibition by isoleucine, valine, or leucine (at 1 mM concentration). Valine limitation resulted in an enzyme that was more than 50% inhibited by 0.1 mM valine and was significantly inhibited by isoleucine and, to a lesser extent,

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*Supported by Grant GM12522 from the National Institute of General Medical Sciences, United States Public Health Service.

†Recipient of a United States Public Health Services Postdoctoral Fellowship, 1-F02-GM-30, 650-02, from the National Institute of General Medical Sciences.

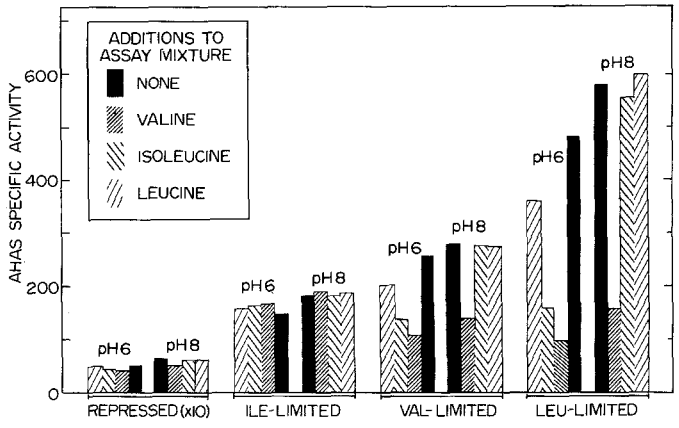

Figure 1. AHAS in crude extracts of *S. typhimurium* strain TU5006 (*ilvC853, leuB499*). Strain TU5006 was derived by John Wasmuth from strain TU5, formerly called *ilv-217*, (*ara-9, ilvC853*) by transduction using bacteriophage P_{LT22} H_4 grown on strain *leuB499*, selecting for Ara^+ Leu^-. Cultures were grown in the minimal salts medium of Davis and Mingioli (2), modified by the elimination of citrate.

Crude extracts were prepared from mid-log phase cultures (or from cultures that had been limited in their growth by restricting one amino acid). The cells were harvested by centrifugation and washed twice in a buffer consisting of the following: 0.05 M potassium phosphate, pH 8, 1.0 mM $MgCl_2$, 10 µg/ml flavine adenine dinucleotide (FAD), 0.5 mM dithiothreitol, and 0.1 mM thiamine pyrophosphate. The cells were suspended in eight volumes of the above buffer. The extracts were prepared by sonic oscillation and clarified by centrifugation at 25,000 x g for 40 min. They were dialyzed against the above buffer for two two-hour periods (using approximately 200 to 500 times their volume of buffer each time).

AHAS was assayed by the method described previously except that FAD was 20 µg/ml (3). Additions of L-isoleucine, L-valine, or L-leucine to the assay mixture were made at a concentration of 1.0 mM. Protein was assayed according to the biuret method (4). Specific activity is mµmoles acetolactate per min per mg protein.

Variations in growth conditions as follows: "repressed" - 0.4 mM L-isoleucine, 1.2 mM L-valine, and 0.4 mM L-leucine; "ile-limited" - 0.05 mM L-isoleucine, valine and leucine as above; "val-limited" - as in "repressed," except 0.1 mM glycyl-valine substituted for L-valine; "leu-limited" - as in "repressed," except 0.05 mM L-leucine.

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by leucine. Leucine limitation resulted in appearance of enzyme that had the greatest specific activity (in crude extracts) and also had the greatest sensitivity to inhibitors. The levels of residual, feedback-resistant activity with valine or leucine limitation were about two-thirds that obtained with isoleucine limitation.

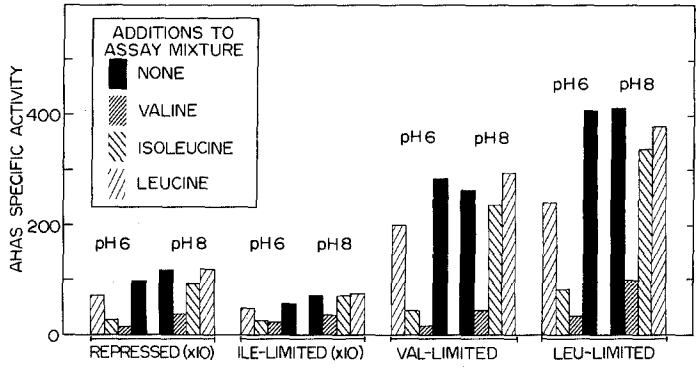

Figure 2. AHAS in crude extracts of *E. coli* strain CU1013 (K-12, *ilvC462*, *leu*). This strain was derived from CU1010 (K-12, *ilvC462*) by N-methyl-N-nitro-N-nitrosoguanidine mutagenesis and penicillin selection for Leu⁻. Culture and assay conditions as in Fig. 1.

Extracts from a derivative of *E. coli* strain K-12 gave strikingly different results. As Fig. 2 shows, growth of strain CU1013 on limiting isoleucine yielded AHAS activity that was less than that under repressing conditions (excess isoleucine, valine and leucine). These results are consistent with previous observations on K-12 strains of *E. coli* showing that valine inhibits growth in minimal medium (as a result of the lack of derepression of AHAS upon isoleucine limitation and of the extreme feedback sensitivity of the enzyme to valine). Feedback sensitivity of AHAS to valine was present under all conditions of growth, in *E. coli* strain K-12.

Fig. 3 shows results obtained with *E. coli* strain M-42-11, a derivative of strain W. The pattern of feedback sensitivity, as well as that of the relative levels of activity, was nearly identical to that of *S. typhimurium*. However, the absolute level of activity seen upon derepression by limiting isoleucine was about 30% lower than that in the *S. typhimurium* strain (Fig. 1). These results demonstrate a clear difference between *E. coli* strains W (valine-resistant) and K-12 (valine-sensitive).

Sucrose density gradient ultracentrifugation experiments. Crude extracts of the three strains examined above were subjected to sucrose density gradient

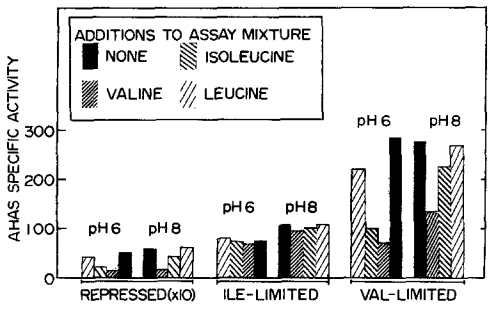

Fig. 3.

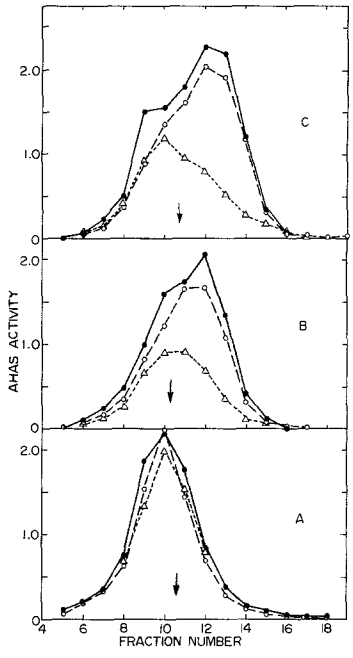

Fig. 4.

Figure 3. AHAS in crude extracts of *E. coli* strain M-42-11 (W, *ilvD*). This strain has been previously described (5). Culture and assay conditions as in Fig. 1.

Figure 4. Sucrose density gradient ultracentrifugation of AHAS in crude extracts of *S. typhimurium* strain TU5006. Culture conditions and extract preparation as in Fig. 1, except that cells were resuspended in three volumes of buffer.

Linear sucrose density gradients (5 to 20% w/v in the buffer described in Fig. 1, 4.5 ml total volume) were constructed using a Beckman Density Gradient Former. Samples (0.2 ml) of extract were mixed with approximately 0.2 µg of lactic acid dehydrogenase (LDH, Sigma, Type II from rabbit muscle), which served as a standard, and were placed on top of the gradients. The gradients were centrifuged in a Beckman/Spinco Ultracentrifuge Model L2 for 10 hours at 40,000 rpm in an SW50.1 rotor. Fractions, displaced from the tubes by injection of 60% sucrose, of approximately 0.2 ml (10 drops) were collected (ISCO Density Gradient Fractionator, Model D), divided into three 50 µl samples each (plus a small residue left for LDH assays), and analyzed for AHAS activity at pH 8, pH 6, and pH 8 in the presence of 1 mM valine, as well as for LDH activity. Incubation was for 30 min at 37° C before the addition of 0.1 ml of 50% H_2SO_4. An additional period of incubation was allowed (20 min at 50° C) for decarboxylation of acetolactate to acetoin. LDH was assayed by measuring the oxidation of NADH by pyruvate in 0.1 M potassium phosphate, pH 7. The position of the band of LDH activity is indicated by an arrow.

Cultures were grown under conditions of isoleucine limitation, A, valine limitation, B, and leucine limitation, C, as in Fig. 1.

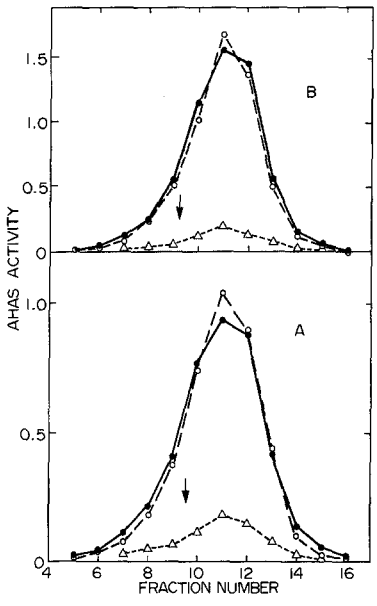

Fig. 5.

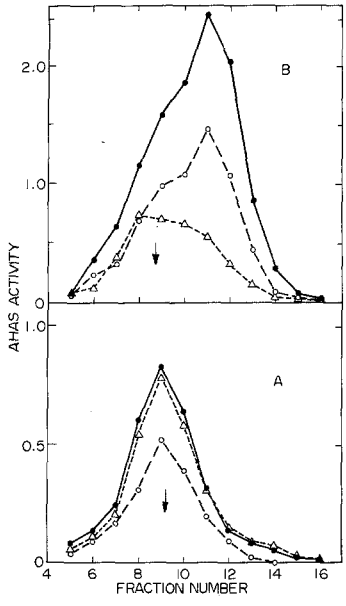

Fig. 6.

Figure 5. Sucrose density gradient ultracentrifugation of AHAS in crude extracts of *E. coli* strain CU1013. Culture, centrifugation, and assay conditions as in Fig. 4, except the results obtained from cultures grown under conditions of valine limitation are shown in A and under conditions of leucine limitation in B. AHAS was not produced in sufficient quantity under conditions of isoleucine limitation to be examined in this manner.

Figure 6. Sucrose density gradient ultracentrifugation of AHAS in crude extracts of *E. coli* strain M-42-11. Culture, centrifugation, and assay conditions as in Fig. 4 (A, isoleucine limitation; B, valine limitation).

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ultracentrifugation analysis. The results obtained with *S. typhimurium* (Fig. 4) clearly indicated the existence of two forms of AHAS. Isoleucine limitation led to the formation of an enzyme that was insensitive to feedback inhibition and sedimented more slowly than LDH and which we have termed AHAS II. Valine or leucine limitation resulted in a more complex picture, indicating more than one component. The valine-resistant activity sedimented more slowly than the bulk of the activity, and at a rate roughly corresponding to that of AHAS II. The faster sedimenting valine-sensitive activity has been termed AHAS I.

Fig. 5 shows the results of sucrose density gradient ultracentrifugation of extracts from *E. coli* strain CU1013. Isoleucine limitation of this strain resulted in extremely low levels of AHAS activity (Fig. 2). AHAS activity, formed under conditions of valine or leucine limitation, sedimented as a single component whose activity was strongly inhibited by valine. Moreover, the residual activity exhibited in the presence of valine was distributed exactly as was the activity in the absence of valine. The rate of sedimentation of this activity corresponded to that of AHAS I from *S. typhimurium*.

Fig. 6 shows that isoleucine limitation of strain M-42-11, derived from the valine-resistant W strain of *E. coli* resulted principally in AHAS II activity while limitation on valine resulted in both AHAS I and AHAS II activities, a situation similar to that in *S. typhimurium*.

DISCUSSION

From the data presented here we conclude that AHAS I is inhibited by valine, isoleucine and leucine and multivalently repressed by valine and leucine, and that AHAS II is insensitive to feedback inhibition by the above effectors and is multivalently repressed by isoleucine, valine and leucine (except in *E. coli* strain K-12, which lacks AHAS II). It is now possible to account for several observations made on AHAS activities in the past. For one, it was often noted that this enzyme was more sensitive to valine in derivatives of *E. coli* strain K-12 than in *E. coli* strain W or *S. typhimurium* derivatives (6, 7). AHAS activity remained low when isoleucine was limiting in strain K-12 whereas it was derepressed under these conditions in strain W and in *S. typhimurium*. These observations appeared to account for the inhibition of growth of the K-12 strain by valine. We now know that this behavior is due to the fact that AHAS I is the only activity in most K-12 derivatives and that AHAS II activity, which can be formed by the other strains examined, is insensitive to valine.

Another unexplained observation was the lack of mutants blocked in this enzymatic step in *S. typhimurium*. With the realization that there are two

forms of AHAS, it has been possible to isolate mutants lacking one or the other or both. These are presently being analyzed genetically.

Finally, these studies demonstrate that the distinction that has occasionally been made between enzyme activity at pH 6 and pH 8 (8) appears to have no relevance to molecular heterogeneity. Both enzyme forms had activity at pH 6 as well as at pH 8, the activity at pH 6 normally being 80 to 100% that at pH 8 in crude extracts. However, the ratio of one to the other was occasionally variable when AHAS I was present, especially in crude extracts of *E. coli* strains. Secondly, the differences in sensitivity to effectors at the two pH's were probably a result of differences in affinity as a function of pH, and not a direct result of any change in potential inhibitability of either enzyme form at either pH. That is, the apparent insensitivity to inhibition at pH 8 relative to pH 6 could often be overcome by increasing effector concentration. For example, the effect of 0.1 mM valine at pH 6 was roughly comparable to the effect of 1.0 mM valine at pH 8.

At the time that this work was being prepared for publication, we became aware of the results of O'Neill and Freundlich (9) who have come to a similar conclusion regarding the nature of AHAS activities.

ACKNOWLEDGEMENTS

The authors thank Dr. Carolyn S. Brown for communicating to us her method for preparation and dialysis of extracts containing AHAS activity and for participating in the early discussions that culminated in the concept of multiple AHAS activities.

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