TWO FORMS OF BIOSYNTHETIC ACETOHYDROXY ACID SYNTHETASE IN SALMONELLA TYPHIMURIUM

J. Patrick O'Neill and Martin Freundlich

Department of Biochemistry
State University of New York
Stony Brook, N.Y. 11790

Received June 12,1972

SUMMARY

The initial enzyme common to the isoleucine-valine biosynthetic pathway, acetohydroxy acid synthetase (AHAS), is inhibited by valine. Evidence is presented that there are two forms of this enzyme, one sensitive ($AHAS^s$) to inhibition by valine, and the other resistant ($AHAS^r$). $AHAS^r$ is under multivalent repression by isoleucine, valine and leucine, while $AHAS^s$ is repressed only by valine and leucine. Multiple forms of AHAS allow for the synthesis of isoleucine in the presence of excess valine.

The first step leading to the biosynthesis of valine in Escherichia coli and Salmonella typhimurium is mediated by acetohydroxy acid synthetase (AHAS). As has been found in numerous biosynthetic systems, this initial step is inhibited by the endproduct of the pathway, valine (1). However, AHAS is also required for the synthesis of isoleucine, catalyzing the second step in this pathway. Since it has been previously assumed that a single AHAS exists in these microorganisms, it has been unclear as to how isoleucine is normally formed when cells are grown in the presence of excess valine (2). In an unusual case, the growth of E. coli K-12 is inhibited by valine (3). It has been suggested that this growth inhibition is due to isoleucine starvation caused by the extreme sensitivity of AHAS in this strain to inhibition by valine (4). In the present report we present data for the existence of two forms of biosynthetic AHAS. One form of this enzyme is very sensitive to inhibition by valine ($AHAS^s$) and its formation is regulated by valine and leucine. The second form is not subject to feedback inhibition and it is repressed by isoleucine, valine, and leucine.

RESULTS

An initial indication of two forms of AHAS was found when valine inhibition of AHAS was examined in various strains of S. typhimurium. AHAS from wild type cells grown in minimal medium or under repressed conditions could be inhibited only 50% by valine (Table I). When strain ilv 217 leu 124 was starved for valine or leucine AHAS was more sensitive to inhibition by valine. However, the enzyme was refractory to inhibition when cells were starved for isoleucine. Strain POE-10 is a derivative of S. typhimurium ara-9 that is resistant to growth inhibition by the isoleucine analogue, cyclopentaneglycine (5). This strain is derepressed for the ilv ADE operon. AHAS in this mutant was not inhibited by 10mM valine but when this strain was starved for leucine approximately 70% inhibition of AHAS activity was observed (Table I). In contrast to these results, AHAS from S. typhimurium ilv 237 was extremely sensitive to feedback inhibition under all growth conditions. The growth of this strain is inhibited by valine (6) and the mutation site has been mapped in the ilv ADE operon (7).

A study of AHAS activity and inhibition over the pH range 5.7 - 9.0 gave a further indication of 2 forms of the enzyme in wild type cells (Fig. 1). Throughout the entire pH range a significant amount of AHAS activity was not inhibited by valine. Strain ilv 237 shows very little residual AHAS activity in the presence of valine at any pH. However, AHAS from cells starved for isoleucine was inhibited only slightly by valine at pH 5.8 - 9.0. In addition, AHAS activity from cells grown on limiting isoleucine was similar to the residual activity present in wild type extracts assayed in the presence of 10 mM valine (Fig. 1).

In order to study further the possibility that 2 forms of AHAS existed in S. typhimurium, extracts from wild type cells were chromatographed on hydroxylapatite. Two components with AHAS activity were separated by this method (Fig. 2). Enzyme activity in the first component was resistant to

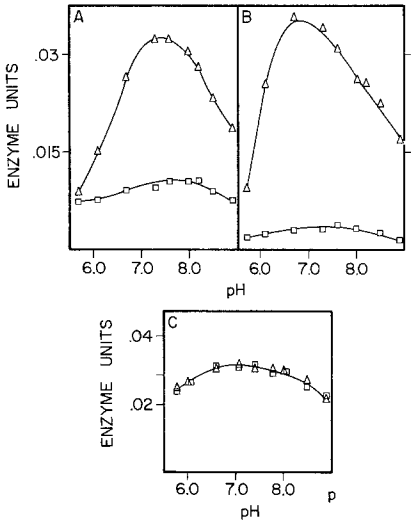

Fig. 1.

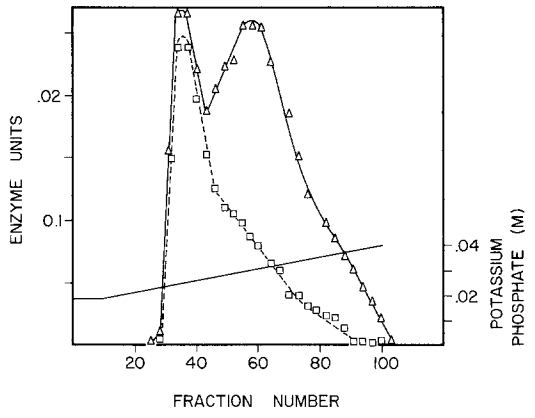

Fig. 2.

Fig. 1. Effect of pH on the activity of AHAS and on inhibition by valine. The strains of S. typhimurium were grown on the following media: wild type (A), minimal medium; ilv 237 (B) minimal plus 0.2 mM isoleucine; ilv 217 (C) minimal plus 0.08 mM isoleucine, 0.4 mM leucine and 0.8 mM valine. Crude extracts were prepared as described in Table I and the extracts passed through a sephadex G-25 column to remove small molecules. Enzyme units are μmoles acetolactate per min. Potassium phosphate was used for Ph 5.7 to 8.0 and Tris buffer from 8.2-9.0. Symbols: Δ , no valine; □ , 10 mM valine.

Fig. 2. Hydroxylapatite (HA) chromotography of AHAS from S. typhimurium grown on minimal medium. Crude extract from 2 liters of mid-exponential phase cells, in 5 ml of buffer, was layered on a 1x25mm column of HA (Bio rad Biogel HTP). The buffer contained 0.01 m potassium phosphate (pH 7.1), 0.005 M $MgCl_2$, 20 μg per ml flavin adenine dinucleotide, 200 μg/ml thiamine pyrophosphate and 20% glycerol. A linear gradient (200 ml) of 0.02 M to 0.04 M potassium phosphate containing the above additions was run after the column was washed with 25 ml of the 0.01 phosphate buffer. Fractions of 1.5 ml were collected and 0.2 ml samples were assayed, with (□) and without (Δ) 10 mM valine, for AHAS activity. No significant activity was obtained when the column was washed with 0.5 M potassium phosphate. Enzyme units are expressed as μmoles acetolactate per min per fraction.

inhibition by valine ($AHAS^r$) while that in the second component was strongly inhibited by valine ($AHAS^s$). Extracts made from strain ilv 217 starved for isoleucine revealed a single component of AHAS which was not inhibited by valine (Fig. 3A). The valine sensitive strain, ilv 237, contained a single activity which was highly sensitive to inhibition by valine (Fig. 3B). As was found in wild type, $AHAS^r$ from ilv 217 starved for isoleucine was eluted

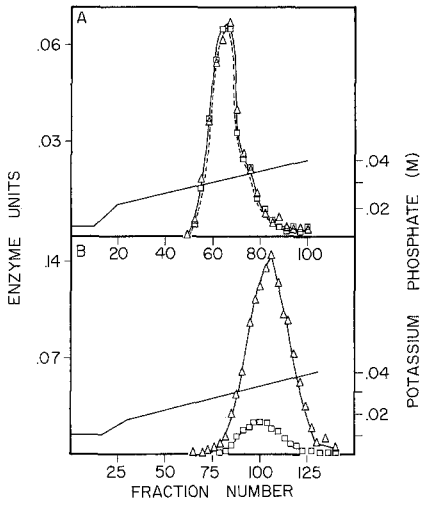

Fig. 3.

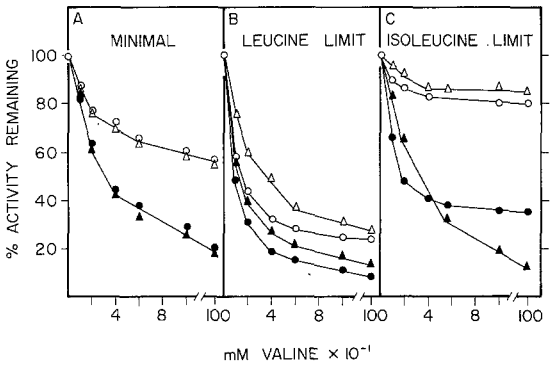

Fig. 4.

Fig. 3. Hydroxylapatite column chromotography of crude extracts of strains ilv 217 and ilv 237. Conditions were as in Fig. 2 except a gradient of 250 ml potassium phosphate was used. Strain ilv 217 (A) was grown under conditions of isoleucine limitation as described in Table I. Strain ilv 237 (B) was grown in minimal medium and 0.2 mM isoleucine.

Fig. 4. Effect of growth conditions on inhibition of AHAS by valine. Extract preparation and enzyme assay was done as described in Table I. Minimal (A): E. coli K-12 (●), E. coli W (○), S. typhimurium ara-9 (Δ) were grown in minimal medium. Strain ilv 237 (▲) was grown in minimal medium plus 0.4 mM isoleucine. Leucine limit (B): triple auxotrophs E. coli K-12 21B25 (●), E. coli W M4862 H-5(○), and S. typhimurium ilv 217 leu 124 (Δ), and strain ilv 237 leu 124 (▲) were grown under conditions of leucine limitation as described in Table I. Isoleucine limit (c): 21B25 (●), M4862 H-5 (○), ilv 217 leu 124 (Δ) and ilv 237 ilv 217 (▲) were grown under conditions of isoleucine limitation as described in Table I. E. coli K-12 strain 21B25 lacks transaminase B (ilv E). E. coli W strain M4862H-S lacks isomeroreductase (ilv C) and transaminase B. S. typhimurium strain ilv 217 leu 124 lacks isomeroreductase and β-isopropylmalate synthetase (leu A). ilv 237 was formally called ile 160.

---

at a lower phosphate concentration than AHAS^{s} found in ilv 237. Extracts prepared from auxotrophs grown on limiting amounts of valine or leucine were found to contain both AHAS^{r} and AHAS^{s} activities. The valine sensitive component was eluted as a more diffuse band than the AHAS^{s} found in wild type or in ilv 237. This behavior may indicate interactions between the various forms of the enzyme. This aspect is currently being investigated.

Table I. Effect of growth conditions on valine inhibition of acetohydroxy acid synthetase in strains of S. typhimurium

Strain	Amino acid limiting	Specific activity: Threonine deaminase	Specific activity: Acetohydroxy acid synthetase	Valine (10 mM) (% Inhibition)
wild	Minimal	12	2.4	52
wild	None	7.1	0.6	51
ilv 217 leu 124	Isoleucine	61.6	6.4	7
ilv 217 leu 124	Valine	64.6	13.6	67
ilv 217 leu 124	Leucine	67.4	14.4	71
POE-10 leu 124	None	73.7	6.2	5
POE-10 leu 124	Leucine	78.9	13.8	68
ilv 237 leu 124	None	6.6	0.4	92
ilv 237 leu 124	Leucine	65.2	10.7	94
ilv 237 ile 217	Isoleucine	-	1.4	91

The bacteria were grown with shaking at 37C in a glucose minimal salts medium (8) modified by the omission of citrate. L-Amino acids were added at the following concentrations: 0.4 mM leucine, 0.4 mM isoleucine, 0.8 mM valine. For growth limitation isoleucine or leucine were added at 0.08 mM and the other two amino acids added in excess as above. For growth limitation with valine, 0.2 mM glycycl-L-valine was substituted for valine, and leucine and isoleucine added at 0.4 mM. Crude extracts were prepared by sonic oscillation as described previously (5). Cells were grown overnight and diluted in the same medium to a Klett-Summerson colorimeter reading (blue filter) of about 60. The cells were grown for approximately 1 doubling (1-2 hr) and extracts prepared. Threonine deaminase (9) and AHAS (10) were assayed as described previously. Specific activity is μ moles product per hr per mg protein.

DISCUSSION

These results demonstrate the existence of two AHAS activities in S. typhimurium. The valine sensitive form $AHAS^s$, is repressed by valine and leucine and can be considered the initial enzyme in valine biosynthesis. The second activity, $AHAS^r$, is not inhibited by any of the branched chain amino acids and may be required for the second step in isoleucine biosynthesis. The loss, by mutation, of $AHAS^r$ in S. typhimurium ilv 237, causes a partial isoleucine requirement and growth inhibition by valine (6). This growth inhibition by valine of ilv 237 and its reversal by isoleucine is similar to the classic effects of valine on E. coli K-12 (3). A comparison of AHAS from

E. coli K-12 and ilv 237 shows an almost identical pattern of extreme sensitivity of enzyme activity to valine inhibition (Fig. 4A). In contrast, E. coli W and wild type S. typhimurium, strains that are not blocked in growth by valine, show only about 50% inhibition of AHAS activity by 10mM valine. Leucine auxotrophs of these strains were grown in limiting amounts of leucine (Fig. 4B), a condition which increases $AHAS^s$ (Table I). AHAS from all strains was more sensitive to valine inhibition and the enzyme from E. coli K-12 and ilv 237 had very similar inhibition curves. During isoleucine starvation, $AHAS^r$ is derepressed (Table I, Fig. 3A) and AHAS activity from E. coli W and S. typhimurium showed very little sensitivity to endproduct inhibition (Fig. 4C). However, ilv 237 which has only one peak of $AHAS^s$ activity, was inhibited by valine by almost 90%. AHAS of E. coli K-12 starved for isoleucine showed some residual activity not inhibited by valine (Fig. 4C). We cannot tell, as yet, whether this activity in E. coli K-12 is a small amount of $AHAS^r$ or a desensitized $AHAS^s$ activity. S. typhimurium ilv 237 appears to have no $AHAS^r$ enzyme and this renders the strain valine sensitive as in E. coli K-12. E. coli K-12 may also lack $AHAS^r$ (11). However, this would be difficult to reconcile with the valine-resistant mutant isolated in Adelberg's laboratory (12). This strain is an operator mutant constitutive for the ilv ADE operon. The structural gene for $AHAS^r$ has been mapped by transduction in S. typhimurium and appears to be in the ilv ADE operon (7). If the gene for $AHAS^r$ is similarly situated in E. coli K-12 then a residual level of $AHAS^r$ might account for valine resistance in an ilv ADE operator constitutive mutant.

The existence of multiple forms of biosynthetic AHAS has previously been suggested by the work of Varga and Horvath in Pseudomonas aeruginosa (13).

While preparing this report for publication we were advised of similar data found in E. coli and S. typhimurium by Blatt, Pledger, and Umbarger (11).

ACKNOWLEDGMENTS

We thank G. Childs for the initial observation that led to the use of

hydroxylapatite chromatography. This work was supported by USPHS grant GM17152. M.F is a Research Career Development Awardee of the USPHS (GM-14374). J.P.O. holds a predoctoral fellowship from the USPHS.

REFERENCES

1. Umbarger, H.E., and Brown, B., J. Biol. Chem., 233, 1156 (1958).
2. Cohen, G.N., in The Regulation of Cell Metabolism, Holt, Rinehart and Winston, New York (1968).
3. Bonner, D., J. Biol. Chem., 166, 545 (1946).
4. Leavitt, R.I., and Umbarger, H.E., J. Bacteriol., 83, 624 (1962).
5. O'Neill, J.P., and Freundlich, M., J. Bacteriol. (In Press).
6. O'Neill, J.P., and Freundlich, M., Bacteriol. Proc. 1971, 157 (1971).
7. O'Neill, J.P., and Freundlich, M., In Preparation.
8. Davis, B.D., and Mingioli, E.S., J. Bacteriol. 60, 17 (1950).
9. Freundlich, M., and Umbarger, H.E., Cold Spring Harbor Symp. Quant. Biol., 28, 505 (1963).
10. Stormer, F.C., and Umbarger, H.E., Biochem. Biophys. Res. Commun., 17, 587 (1964).
11. Blatt, J.M., Pledger, W.J., and Umbarger, H.E., BBRC, This issue, p. 444.
12. Ramakrishnan, T., and Adelberg, E.A., J. Bacteriol., 87, 566 (1964).
13. Varga, J.M., and Horvath, I., J. Mol. Biol. 13, 596 (1965).