Physiological Implications of the Substrate Specificities of Acetohydroxy Acid Synthases from Varied Organisms

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Acetohydroxy acid synthase (AHAS; EC 4.1.3.18) catalyzes the following two parallel, physiologically important reactions: condensation of two molecules of pyruvate to form acetalactate (AL), in the pathway to valine and leucine, and condensation of pyruvate plus 2-ketobutyrate to form acetohydroxybutyrate (AHB), in the pathway to isoleucine. We have determined the specificity ratio R with regard to these two reactions (where $V_{AHB}$ and $V_{AL}$ are rates of formation of the respective products) as follows:

$$\frac{V_{AHB}}{V_{AL}} = R \left( \frac{[2\text{-keto}]}{[\text{pyruvate}]} \right)$$

for 14 enzymes from 10 procaryotic and eucaryotic organisms. Each organism considered has at least one AHAS of $R > 20$, and some appear to contain but a single biosynthetic AHAS. The implications of this for the design of the pathway are discussed. The selective pressure for high specificity for 2-ketobutyrate versus pyruvate implies that the 2-ketobutyrate concentration is much lower than the pyruvate concentration in all these organisms. It seems important for 2-ketobutyrate levels to be relatively low to avoid a variety of metabolic interferences. These results also reinforce the conclusion that biosynthetic AHAS isozymes of low $R$ (1 to 2) are a special adaptation for heterotrophic growth on certain poor carbon sources. Two catabolic “pH 6 AL-synthesizing enzymes” are shown to be highly specific for AL formation only ($R < 0.1$).

In the pathway for the biosynthesis of branched-chain amino acids, the same set of enzymes catalyzes four consecutive reactions leading to two different sets of products, valine and leucine, on the one hand, and isoleucine on the other (40, 41) (Fig. 1). Acetohydroxy acid synthase (AHAS, also known as acetolactate synthase; EC 4.1.3.18) catalyzes the first of these reactions, the irreversible decarboxylation of pyruvate and its condensation with either pyruvate or 2-ketobutyrate (15). The relative amounts of the two possible products formed by AHAS thus determines the relative amounts of valine plus leucine and isoleucine synthesized in these pathways.

We have shown for a number of AHASs (3, 15) that the ratio of the relative rates of formation, $V$, of acetohydroxybutyrate (AHB) and acetolactate (AL) by a given enzyme is proportional to the ratio of the concentrations of the substrates 2-ketobutyrate and pyruvate and to a specificity ratio, $R$, characteristic of the enzyme

$$\frac{V_{AHB}}{V_{AL}} = R \left( \frac{[2\text{-kETO}]}{[\text{PYRUVATE}]} \right)$$

Such a fixed specificity is expected if the competition between 2-ketobutyrate and pyruvate occurs on the enzyme subsequent to the irreversible and rate-determining formation of an intermediate from the first pyruvate (15).

Pyruvate is a major intermediate in metabolism, whereas 2-ketobutyrate is a minor one, serving mainly as a precursor to isoleucine. Furthermore, 2-ketobutyrate may be toxic at high intracellular concentrations (7, 24). In Escherichia coli grown on glucose, the concentration of pyruvate is nearly 2 orders of magnitude higher than that of 2-ketobutyrate (7, 25). In order to synthesize similar quantities of each of the branched-chain amino acids under such circumstances, an organism must have at least one AHAS with a high specificity for 2-ketobutyrate, i.e., with an $R$ of 20 to 100. E. coli K-12 and Salmonella typhimurium LT2 each express such isozymes, AHAS III ($R = 40$) and AHAS II ($R = 65$), respectively (15). In addition, these bacteria express isozyme AHAS I ($R = 2$), which probably serves a special function when pyruvate levels are particularly low (3, 5, 6). We would predict that, if low 2-ketobutyrate-to-pyruvate ratios are a general phenomenon, every organism which normally synthesizes branched-chain amino acids will express an AHAS of high specificity for 2-ketobutyrate.

In this paper, we examine the above prediction, by determining the partitioning ratio $R$ for AHAS enzymes from a number of procaryotic and eucaryotic organisms, both heterotrophic and autotrophic. In addition, we also consider the substrate specificity of two enzymes that are believed to have purely catabolic roles and which have been called “pH 6 AL-synthesizing enzymes” (pH6 ALS) (17, 19, 35).

MATERIALS AND METHODS

Microorganisms and plasmids. The microorganisms and plasmids used in this study and their sources are listed in Table 1.

Media. The minimal medium for E. coli strains was that described by Vogel and Bonner (44) supplemented with the required amino acids (50 μg/ml each). LB was used as the rich medium. Tetracycline (15 μg/ml) and ampicillin (50 μg/ml) were added to the rich medium, when required. Saccharomyces cerevisiae was grown in the medium described by Zimmerman (48). Porphyridium sp. was grown in artificial seawater (22), and Chlorella emersonii was grown in medium N8 (33).

Cloning and transformation procedures. Cleavage with restriction enzymes and ligations were carried out in accordance with the recommendations of the suppliers. The preparation and isolation of DNA, restriction mapping, and

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transformation with plasmids were carried out by the methods of Maniatis et al. (26). Transformants were selected for the relevant antibiotic resistance and for the Ilv" phenotype. CU9090(pDK6) and MM294 transformants were selected for antibiotic resistance only.

The pNG047 plasmid was constructed in the course of attempts to clone the gene for the Klebsiella aerogenes pH6 ALS (17, 35). Chromosomal DNA from K. aerogenes AA-1 was subjected to partial cleavage by Sau3AI, and DNA fragments 4 to 6 kilobases in length were isolated by agarose gel electrophoresis with the Geneclean kit (45) according to the recommendations of the manufacturer (Bio 101 Inc., La Jolla, Calif.). These fragments were ligated into pBR322 cleaved with BamHI, and MM294 was transformed with the resulting plasmids. Colonies transformed with plasmids that contained inserted fragments were identified as AmpT TetT.

Such clones were then screened for a positive Voges-Proskauer test (acetoin production) (8). Plasmid pNG047 was isolated from one of the positive clones. MF2000 was transformed with this plasmid.

Enzymes. The purified isozymes AHAS II from S. typhimurium (32) and AHAS I from E. coli (A. Aulabaugh and J. V. Schloss, unpublished results) were gifts from J. V. Schloss. AHAS III was purified as described previously (2).

Crude enzymes were prepared from exponentially growing cultures of E. coli in a minimal medium containing glucose and harvested at a turbidity of about 50 Klett units (filter no. 66). Cells were washed and concentrated to approximately $10^{10}$ bacteria per ml in a disruption buffer (0.1 M potassium phosphate buffer, pH 7.6, containing 10 mg of flavin adenine dinucleotide (FAD) per ml, 0.5 mM dithiothreitol, and 10 mM EDTA) and lysed by ultrasonic disruption as previously described (14). Unbroken cells and debris were removed by centrifugation, and glyceral was added to the crude extract to 20% (vol/vol). The AHAS activities of similar extracts of host strains MF2000 and CU9090 were negligible under conditions which yield high activity for each of the cloned enzymes.

The pH6 ALS from K. aerogenes was prepared from a culture of K. aerogenes AA-1 grown to late stationary phase in minimal medium (36). Crude extracts containing AHAS were prepared from cultures of Porphyridium sp. (43) and C. emersonii [D. Landstein, D. M. Chipman, S. (Malis) Arad, and Z. Barak, unpublished data] as previously described.

Crude yeast AHAS was prepared from exponentially growing cultures (30°C, with vigorous mixing and aeration) of S. cerevisiae YT669. Washed cells were suspended in disruption buffer (see above) and were broken by ultrasonic
disruption (six successive 1-min treatments at 4°C with 1-min intervals between them). Debris was spun down, and glycerol was added to the supernatant to a final concentration of 33%. Extracts were stored at −70°C.

**Enzyme assay.** AHAS activity was measured either as AL formation in the presence of pyruvate by the standard colorimetric method (4) or by the gas chromatographic method, which determines the formation of both products (AL and AHB) simultaneously (12, 13). Unless otherwise stated, enzyme assays were carried out as previously described in reaction mixtures containing 10 mM MgCl₂, 20 μg of FAD per ml, and 30 μg of thiamine PP per ml (15). For the pH6 ALS from *K. aerogenes* and *Bacillus subtilis*, the reaction was carried out at pH 6 and pH 6.2, respectively (0.1 M phosphate or morpholineethanesulphonate [MES] buffer and 50 mM sodium acetate) in the absence of FAD.

**RESULTS**

The substrate specificity of AHAS activities from a variety of sources was examined by determining the amount of the two products (AL and AHB) formed in the presence of the two substrates (pyruvate and 2-ketobutyrate) by the gas chromatographic method (12, 13, 15) (Table 2). The first three lines in Table 2 are summaries of data obtained with purified enzymes from enteric bacteria (15). The remaining data were obtained by using crude extracts of the indicated strains.

**TABLE 1. Microorganisms and plasmids**

| Organism and strain                      | Description                          | Source or reference          |
|-----------------------------------------|--------------------------------------|------------------------------|
| *Klebsiella (Aerobacter) aerogenes*      | Wild type for AHAS + pH6 ALS         | Halpern and Umbarger (17)    |
| AA-1                                    | Wild type for AHAS                   |                              |
| *Escherichia coli*                      | Wild type for AHAS                   |                              |
| MM294                                   | D *ilv*800::Mu-1 Δ ara leu ilvIH*     | Newman et al. (28)           |
| MF2000                                  | Plasmid carries *ilvGME* from *Serratia marcescens* | Transformation (this work) with pPU129 (18) |
| MF2000(pNG047)                          | Plasmid carries *ilvGME* from *K. aerogenes* | Transformation (this work) with pPU137 (18) |
| MF2000(pPU129)                          | Plasmid carries *ilvGME* from *Edwardsiella tarda* | Transformation (this work) with pPU139 (18) |
| MF2000(pPU137)                          | AHAS⁻⁷ *ilvB2102* Δ(ilvIH) pro       | H. E. Umbarger, Purdue University |
| CU9090                                  | AHAS⁻⁷ *ilvGME*                      |                              |
| CU9090(pDK6)                            | Plasmid carries *alsS* from *Bacillus subtilis* | S. C. Falco (10)          |
| CU9090(pMAVxx)                          | Plasmid carries *ilvBN* from *B. subtilis* | University of Texas, Austin |
| *Saccharomyces cerevisiae*              | AHAS⁻⁷ strain transformed to *ilv*⁺ with pCP2-4 (carries ILV2) |                              |
| YT669                                   | Wild-type unicellular red alga       |                              |
| *Porphyridium* sp. (Rhodophyta)         | Wild-type unicellular green alga     |                              |
| UTEX637                                 |                                      |                              |
| *Chlorella emersonii* (Chlorophyta)     | Wild-type unicellular green alga     | Cambridge Culture Collection, Cambridge, United Kingdom |
| CCAP-211/11n                            |                                      |                              |

**TABLE 2. Specificities of some AHASs and AL synthases**

| Organism                        | Enzyme                       | R   | Reference |
|---------------------------------|------------------------------|-----|-----------|
| 1. *Escherichia coli* K-12      | AHAS I, purified (A. Aulabaugh and J. V. Schloss) | 2   | 15        |
| 2. *E. coli*                    | AHAS III, purified (2)       | 40  | 15        |
| 3. *Salmonella typhimurium*     | AHAS II, purified (32)       | 65  | 15        |
| 4. *Serratia marcescens*        | *ilvGM* product, crude extract of MF2000(pPU129) | 75  | This work |
| 5. *Edwardsiella tarda*         | *ilvGM* product, crude extract of MF2000(pPU137) | 65  | This work |
| 6. *Klebsiella aerogenes*       | *ilvGM* product, crude extract of MF2000(pNG047) | 80  | This work |
| 7. *K. aerogenes*               | AHAS III-like, crude extract of MF2000(pPU137) | 35  | This work |
| 8. *Bacillus subtilis*          | pHe ALS, crude extract of AA-1 | 0.08| This work |
| 9. *Bacillus subtilis*          | *ilvBN* product, crude extract of CU9090(pMAVxx) | 70  | This work |
| 10. *B. subtilis*               | *alsS* product (pHe ALS), crude extract of CU9090(pDK6) | 0.06| This work |
| 11. *Corynebacterium glutamicum*| Crude extract                 | 20  | Calculated from reference 9 |
| 12. *Saccharomyces cerevisiae*  | ILV2 product, crude extract of YT669 | 30  | This work |
| 13. *Chlorella emersonii*       | Crude extract                 | 22  | Landstein et al., unpublished data |
| 14. *Porphyridium* sp.          | Crude extract                 | 40  | This work |
single enzyme in question by the above criteria. The enzyme from *K. aerogenes* that is encoded by plasmid pNG047 (line 7) has properties that are similar, but not identical, to those of AHAS III of *E. coli*. These properties include pH optimum, $K_m$, valine sensitivity (data not shown), and $R$ (Table 2, line 7 compared with line 2), as well as similar restriction maps (34). The pNG047-encoded enzyme is different from the valine-insensitive *K. aerogenes* ilvGM product encoded by plasmid pPU137 (18) and from the pH6 AHAS (17, 35) of this bacterium (see below). The presence of both valine-sensitive and -insensitive biosynthetic AHASs in *K. aerogenes* has been previously suggested by Asada et al. (1).

The yeast enzyme studied (Table 2, line 12) was also encoded by a plasmid bearing the sole AHAS of *S. cerevisiae* in a host yeast strain free of background AHAS activity (10).

The pH6 ALS activity of *K. aerogenes* (Table 2, line 8) was studied in extracts of bacteria grown to the stationary phase to ensure maximal synthesis of this enzyme (17, 36). To minimize the contribution of residual biosynthetic AHAS enzymes to product formation, the reaction was carried out at pH 6 in the absence of FAD (37).

In contrast to the above cases, the AHAS activities in crude extracts from two algae, *Porphyridium* sp. and *C. emersonii* (Table 2, lines 13 and 14), could in principle be due to more than a single enzyme. However, as the $R$ values were also found to be essentially invariant with substrate concentration, the AHAS activities must be overwhelmingly due to a single enzyme or to two enzymes with similar $R$ values. Other properties of the AHAS activities in these organisms support the idea that each has a single AHAS (43; Landstein et al., unpublished data; D. van Moppes, unpublished data).

The $R$ value for the AHAS activity of *Corynebacterium glutamicum* (line 9) is based on the data reported by Eggeling et al. (9). These workers have shown that *C. glutamicum* has a single AHAS, and the calculated constant $R$ over a range of substrate ratios is consistent with this.

**DISCUSSION**

On the basis of their $R$ values, the enzymes listed in Table 2 can be separated into three major groups: (i) those with a strong preference for reaction with 2-ketobutyrate to form AHB ($R = 20$ to 80); (ii) those with a preference for reaction with pyruvate to form AL ($R < 0.1$); and (iii) AHAS I of enteric bacteria with almost equal preference for the two substrates ($R = 2$). In accord with our expectations, each of the organisms we have considered contains at least one AHAS of group one, with a high specificity for 2-ketobutyrate, i.e., $R > 20$. The same principle seems also to hold for the bacteria *Mycobacterium pellegrino*, *Streptomyces rimosus*, and *Pseudomonas aeruginosa*. Szentirmai and Horvath (38) have measured the rate of AHB formation as a function of the 2-ketobutyrate concentration in crude extracts of these bacteria, and despite the limitation of their method and the absence of information concerning the multiplicity of AHAS isozymes in these organisms, it is clear from an analysis of their data (not shown) that each of these organisms also has at least one AHAS with an $R$ of at least 10.

Many of the organisms we studied express only a single AHAS during active growth. The bacteria *B. subtilis* (42, 47) and *C. glutamicum* (9), the yeast *S. cerevisiae* (10), and in all probability, the two algae considered here each seems to have only a single biosynthetic AHAS, with an $R$ value...
between 20 and 40 (Table 2). In each of these cases, the single AHAS is also inhibited by valine (data not shown) and is thus similar in its properties to AHAS isozyme III of *E. coli*.

The above analysis suggests that low concentrations of 2-ketobutyrate relative to pyruvate are indeed the rule. It would seem to be advantageous to almost any prototrophic cell for 2-ketobutyrate levels to be relatively low, as a wide variety of metabolic complications are possible if 2-ketobutyrate levels rise too high (23, 24, 30). For example, 2-ketobutyrate can compete with 2-ketoisovalerate, which differs from it only by an additional methyl group, in the reactions catalyzed by isopropylmalate synthase and ketopantoate hydroxymethyltransferase (on the pathways to leucine and coenzyme A, respectively; Fig. 1). 2-Ketobutyrate can also be converted by transaminases to aminobutyric acid, which might compete with valine in protein synthesis. Studies by LaRossa and others (7, 23, 24, 30) have made it clear that 2-ketobutyrate can be toxic in bacteria. Pyruvate, on the other hand, is a central metabolite under a wide variety of metabolic regimes, including heterotrophic growth with carbohydrates as carbon sources, as well as photosynthetic growth. Its concentration would also be expected to be high and relatively constant in mitochondria of *Aerobacter aerogenes*, have additional enzymes that which also express such an enzyme.

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5,6), poor carbon sources that lead to low 2-ketobutyrate levels (25). One might expect that there will be other organisms (particularly other enterobacteria) which also express such an enzyme. However, a low internal pyruvate concentration is rather a special situation, with which many organisms do not seem to have to cope.

Finally, some organisms, such as *B. subtilis* and *K. aerogenes*, have additional enzymes that clearly belong to a separate class of PHb ALs on the basis of many properties. These specialized enzymes do not use FAD as a cofactor (19, 37), as do the biosynthetic AHASs. They are strictly catabolic enzymes, not expressed in exponentially growing cultures and induced, probably together with acetolactate decarboxylase, during the stationary phase of growth (17, 20, 36, 46, 47). Their pH optima are low (6.0 to 6.2), and they are thus well suited to function in a fermentation pathway leading to acetoin and butanediol (19, 20, 47) (Fig. 1). Presumably, this fermentation pathway prevents further acidification of the growth medium (20). The strong tendency of these enzymes towards AL synthesis (R < 0.1) is clearly appropriate to their physiological role.

We believe that an AHAS with a high preference for condensation of the active acetdehyde moiety with 2-ketobutyrate, rather than with pyruvate, is a major factor in the design of the biosynthetic pathway for the branched-chain amino acids. This preference presumably allows an organism to maintain 2-ketobutyrate at low concentrations. The ability of any enzyme to select one substrate in the presence of a competing substrate that is smaller by a single methyl group has physical limits (about 3 to 3.5 kcal/mol [12 to 14 kJ/mol]) (11). The selective pressure to maintain low 2-ketobutyrate concentrations seems to have been strong enough to push AHAS almost to these limits of selectivity (11, 15) in many cases. An alternative to an AHAS of high specificity would be the compartmentalization of 2-ketobutyrate, perhaps by its direct, channeled transfer from the enzyme that produces it (threonine deaminase) to the active site of an AHAS. If a prototrophic organism did not adopt one of these two strategies, it would have to deal with the limitations of enzyme specificity at another stage. If it were to maintain free 2-ketobutyrate levels close to those of pyruvate, it would require versions of all of the potentially sensitive enzymes (e.g., isopropylmalate synthase) with an extraordinary ability to select against 2-ketobutyrate or its undesirable metabolic products. On the other hand, if it were simply to produce an excess of valine precursors over isoleucine precursors, it would have to expend considerable metabolic energy on editing of mischarged aminoacyl tRNAs or on specific excretion of these compounds from cells. We thus suspect that all prototrophic organisms will either have a biosynthetic AHAS of high specificity for 2-ketobutyrate or will have some mechanism for channeling 2-ketobutyrate specifically to an AHAS.

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