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CHROMATOGRAPHIC DETECTION OF THE ACETOHYDROXY ACID SYNTHASE ISOENZYMES OF ESCHERICHIA COLI K-12

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SUMMARY: Two valine-sensitive acetohydroxy acid synthase activities were separable from Escherichia coli K-12 cells by virtue of their different affinities for DEAE-cellulose eluted with a KCl gradient. These activities appeared to be independent from a valine-resistant cryptic component expressed only in ilvO regulatory mutants. The properties of the first and second activity were coincident to those of extracts of ilvB and ilvHI mutants, respectively. These data prove that the ilvB and ilvHI gene products exist in the cell as physically distinct acetohydroxy acid synthase isoenzymes.

The synthesis of the two acetohydroxy acid precursors of isoleucine and valine is catalyzed by a common enzyme activity. In some enteric bacteria (1-4), this activity is composed of (a minimum of) two acetohydroxy acid synthase (EC 4.1.3.18) isoenzymes, one resistant and one sensitive to inhibition by valine. In Escherichia coli K-12 the valine-resistant enzyme (which has been designated as acetohydroxy acid synthase II isoenzyme) is not expressed, except in the presence of ilvO regulatory mutations (2). With regard to the valine-sensitive activity, a genetic analysis of E. coli K-12 mutants has suggested but not proven that it is composed of two distinct forms, tentatively designated as isoenzymes: acetohydroxy acid synthase I, coded for by the ilvB gene, and acetohydroxy acid synthase III, coded for by both the ilvI gene and the ilvH gene (5,6).

In the present report we show that, in appropriate conditions, it was possible to resolve the acetohydroxy acid synthase activity of E. coli K-12 into two physically separable forms, which provides direct proof for the

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Abbreviation: FAD, flavin adenine dinucleotide (disodium salt).

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existence of two isoenzymes. We report data suggesting that these isoenzymes are independent from each other and from the cryptic valine-resistant isoenzyme.

MATERIALS AND METHODS

Bacterial strains. The E. coli K-12 strains used in the present study are listed in Table 1.

Growth conditions. The minimal medium used was that described by Vogel and Bonner (8) with 0.4% glucose as the carbon source. For the experiments illustrated in Figures 1, 2 and 4, cells of each strain were grown at 32°C in minimal medium containing an excess of all the supplements required (10 µg/ml thiamine, 50 µg/ml uridine, 100 µg/ml arginine and 50 µg/ml other amino acids). Preparation of cells for the experiment illustrated in Figure 3 was accomplished by means of a protocol already described (9,10).

Chromatographic method. Sonic extracts, prepared as previously described (5) from exponentially growing cells and containing either 50 mg of protein (in the experiments of Figures 1, 2 and 4) or 75 mg of protein (in the experiment of Figures 3) in about 5 ml of Buffer A (5 mM Tris-HCl pH 8.3, containing 10% glycerol, 0.2 mg/ml thiamine pyrophosphate and 5 mM $MgCl_2$) were layered on a 0.6 x 18 cm column of DEAE-cellulose (Whatman DE52) equilibrated with Buffer A. The proteins which were not retained were washed from the column with 25 ml of Buffer A. The samples were then eluted with 120 ml of a linear gradient of KCl from 0 to 0.4 M in Buffer A, with a flow rate of 35 ml per hour. The eluates were collected in 50 fractions of 2.4 ml each. Further washing with 1.0 M KCl did not yield measurable residual enzyme activity. During the elution the conductivity increased linearly.

Enzyme and protein assay. Acetohydroxy acid synthase activity was assayed as previously described (11) in potassium phosphate buffer (at pH 6.6) and in Tris-HCl buffer (at pH 8.3 and 9.3). The pH values reported were measured in the final reaction mixtures. Enzyme units are expressed as nmoles of product formed per min. Proteins were assayed with the method of Groves et. al. (12).

Table 1

Bacterial strains

Genetic symbols are those used by Bachmann et al. (7)

Strain	Genotype	Origin or reference
AT739	HfrH thi-1 thr-10 car-53	A.T. Taylor
AW206	HfrH thi-1 thr-10 car-53 ilv0603	(2)
MI158a	HfrH thi-1	thr^+ car^+ transductant of AT739
PS1035	HfrH thi-1 bgl-20 glyA ilvB619	(9)
PS1036	HfrH thi-1 ara glyA ilvH612 ilvI614	(9)
PS1479	F^- xyl-7 lacY1 mglP1 ilvD530 leu	M. Levinthal

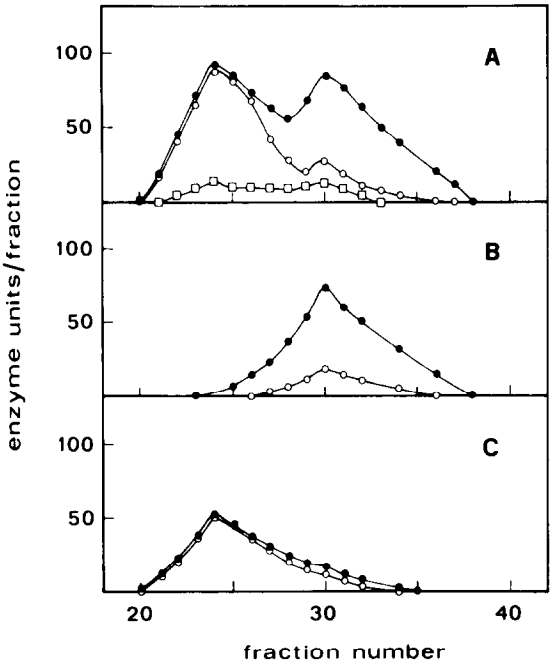

Figure 1. DEAE-cellulose chromatography of an extract of strain MI158a. The yield was about 35% at any pH. Experimental details are given in Materials and Methods. Acetohydroxy acid synthase activity was assayed at pH 8.3 (panel A), 6.6 (panel B) and 9.3 (panel C), in the presence (closed circles) and in the absence (open circles) of 2 µg/ml FAD. Squares refer to activities assayed in the presence of 1.5 mM valine in buffer containing FAD.

RESULTS

The DEAE-cellulose chromatography used throughout this work (see Materials and Methods for details) showed a partial but highly reproducible separation of distinct peaks of acetohydroxy acid synthase activity from extracts of *E. coli* K-12. The use of either different DEAE-cellulose protocols or different chromatographic materials (hydroxylapatite and valine-Sepharose) did not yield a better separation of activities than the one we show in this report.

The results obtained with an extract of strain MI158a (wild type) are reported in Figure 1. In panel A we show that when the enzyme assay was performed at pH 8.3, one FAD-independent component was eluted at a lower ionic strength than a second one, whose activity was about four-fold higher in the presence than in the absence of FAD. The relative size of the two

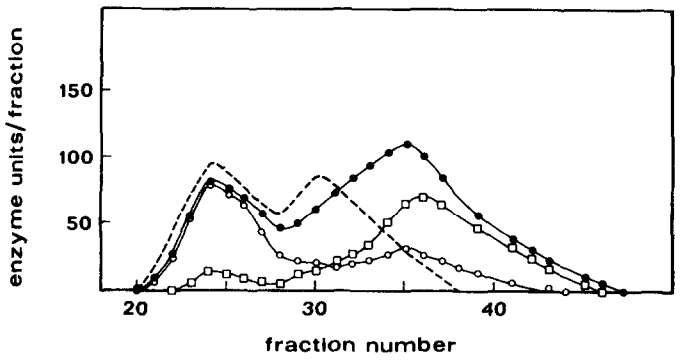

Figure 2. DEAE-cellulose chromatography of an extract of strain AW206. The yield was about 40%. Experimental conditions were identical to those used for strain MI158a (see Materials and Methods). Acetohydroxy acid synthase activity was assayed at pH 8.3 in the following conditions: 2 µg/ml FAD present and valine absent (closed circles); 2 µg/ml FAD and 1.5 mM valine present (squares); and FAD and valine absent (open circles). The dashed line is a projection of the MI158a elution profile with assay at pH 8.3 in the presence of FAD (curve of Figure 1A, closed circles).

peaks was different at different pH values. In panels B and C we show the results obtained in extreme conditions: at pH 6.6 (panel B) only the activity of the FAD-dependent component (second component) was detectable, while at pH 9.3 (panel C) only the activity of the component which did not require addition of FAD for full activity (first component) was detectable. The activity of the fractions of both peaks, as well as that of the extract, was severely inhibited by 1.5 mM valine (Figure 2A).

We determined the acetohydroxy acid synthase profile of strain AW206, in which the cryptic valine-resistant activity designated as acetohydroxy acid synthase II is expressed (2). In Figure 2 we show that the two valine-sensitive components deriving from strain MI158a were also found in strain AW206, which contained a further valine-resistant and FAD-dependent component, eluted at high ionic strength and evidently corresponding to acetohydroxy acid synthase II. This observation strongly suggests that the mutational appearance of acetohydroxy acid synthase II is not a consequence of a modification and subsequent disappearance of one (or both) of the two normally expressed activities.

We analyzed the effect of the three branched chain amino acids on the synthesis of each of the two activities which are separable from *ilvO*$^+$ strains.

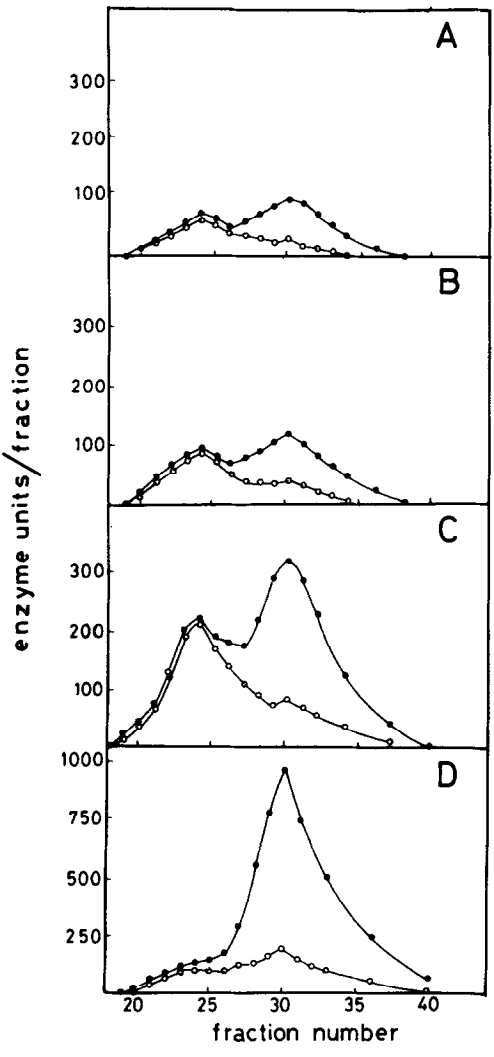

Figure 3. DEAE-cellulose chromatography of extracts of strain PS1479. Cells were grown in minimal medium with the following additions: A, excess of isoleucine (0.6 mM), leucine (0.6 mM) and valine (1.2 mM); B, excess of leucine and valine and limiting amount of isoleucine (0.02 mM glycyl-DL-isoleucine); C, excess of isoleucine and valine and limiting amount of leucine (0.02 mM glycyl-DL-leucine); D, excess of isoleucine and leucine and limiting amount of valine (0.04 mM glycyl-DL-valine). The various extracts were analyzed in identical experimental conditions (see Materials and Methods); the yield was constant and ranged between 30 and 40%. Acetohydroxy acid synthase activity was assayed at pH 8.3 in the presence (closed circles) and in the absence (open circles) of 2 µg/ml FAD.

Strain PS1479 (auxotrophic for isoleucine, leucine and valine) was grown in minimal medium containing either an excess of all three amino acids or a limiting amount of one of them in the presence of an excess of the other two. As we show in Figure 3, when isoleucine was limiting, the amount of

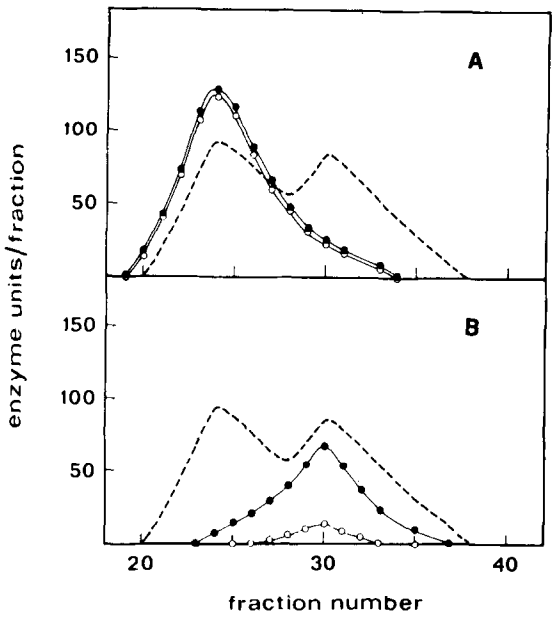

Figure 4. DEAE-cellulose chromatography of extracts of strains PS1035 (panel A) and PS1036 (panel B). The yield was about 30% in both cases. Experimental conditions were identical to those used for strain M1158a (see Materials and Methods). Acetohydroxy acid synthase activity was assayed at pH 8.3 in the presence (closed circles) and in the absence (open circles) of 2 μg/ml FAD. The dashed line is a projection of the MI158a elution profile with assay at pH 8.3 in the presence of FAD (curve of Figure 1A, closed circles).

each component did not change significantly as compared to excess conditions; when leucine was limiting, both components were derepressed (3.0- and 3.5-fold for the first and second component, detected separately at pH 9.3 and 6.6, respectively); and when valine was limiting, only the second component was derepressed (12-fold as detected separately at pH 6.6). These data permit us to conclude that the synthesis of the first component is regulated through repression by leucine and the synthesis of the second component is regulated through multivalent repression (13) by leucine and valine.

The properties of the two activities reported above were equivalent to those found in crude extracts of *ilvB* mutants (first component) and *ilvHI* mutants (second component). In fact, as reported previously, the activity of *ilvHI* mutants requires FAD addition for full expression, is detectable in the pH range of 6-9 with optimum at pH 7.5, and is synthesized through a mechanism involving multivalent repression by leucine and valine, while the

activity of ilvB mutants does not require FAD addition, is detectable in the pH range of 7-10 with optimum at pH 9.0, and in synthesized through a mechanism involving repression by leucine (9,14,15). Figure 4 shows that the acetohydroxy acid synthase activities of strains PS1035 (ilvB619) and PS1036 (ilvH612 ilvI614), analyzed with DEAE-cellulose, had chromatographic behaviours identical, respectively, to those of the first and second component deriving from their isogenic wild type strain MI158a.

DISCUSSION

The present paper fulfills the requirement for a direct demonstration that two valine-sensitive acetohydroxy acid synthase isoenzymes exist in wild type E. coli K-12. The coincidence of the properties of the two isoenzymes with those of the activities carried by ilvB and ilvHI mutant strains establishes the validity of the previous proposal (5,6) that these markers define the structural genes for acetohydroxy acid synthase I and III, respectively, and shows that the two isoenzymes are independent from each other with regard to those aspects of regulation of synthesis and catalytic activity which have been analyzed here.

The method described provides a useful tool for studying many unknown aspects of the regulation of synthesis of each acetohydroxy acid synthase. Such studies can be performed with strains containing both isoenzymes and therefore without introducing the complications associated with genetic manipulations involving ilvB and ilvHI mutations (16).

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