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THE EXISTENCE OF THREE TYPES OF ACETOHYDROXY ACID SYNTHETASE IN AN ISOLEUCINE-REQUIRING MUTANT OF *AEROBACTER AEROGENES*

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Summary

The synthesis of the three types of acetolactate synthase (EC 4.1.3.18) which are responsible for the biosynthesis of isoleucine and valine, was observed in *Aerobacter aerogenes* I-12, an isoleucine-requiring mutant, when grown on the four kinds of media. When the cells were grown on isoleucine-rich medium, acetolactate synthase sensitive to feedback inhibition and having an optimum pH at 8.0 was formed. By increasing the amount of potassium phosphate in the medium, the catabolite repression of the enzyme having an optimum pH at 6.0 and which is insensitive to feedback inhibition, was released. In contrast, acetolactate synthase having an optimum pH at 8.0 and insensitive to feedback inhibition was formed when isoleucine was limited, irrespective of phosphate concentrations. Two insensitive enzymes were not regulated by isoleucine, leucine and valine, although sensitive pH 8.0 enzyme was repressed by them. Thus, it may be assumed that the synthesis of insensitive pH 8.0 enzyme is derepressed by limiting the amount of isoleucine in the medium. The question how the insensitive pH 6.0 enzyme and sensitive pH 8.0 enzyme were repressed by limiting the amount of isoleucine is still open.

Introduction

Acetolactate synthase (EC. 4.1.3.18, Acetolactate pyruvate-lyase (carboxylating)) occupies an important regulatory site in the biosynthetic pathway for the synthesis of valine and isoleucine in many microorganisms. Two isozymes of acetolactate synthase have been found. The one has the optimum pH at 6.0 whose activity is insesitive to end-products while the other has optimum pH at 8.0 and is inhibited by end-products [1,2]. Recently, the presence of the insensitive form of the enzyme having an optimum pH at 8.0 has also been re-

ported, and this enzyme is a multiform of the sensitive enzyme [3,4]. As to the appearance of these enzyme activities in one strain, two strains are already known; the one produced the sensitive pH 8.0 enzyme or insensitive pH 6.0 enzyme depending on the conditions in which the cells were cultured [5] and the other produced the sensitive pH 8.0 and/or insensitive pH 8.0 enzyme [2]. However, there is no observation of the appearance of these three acetolactate synthases in one strain. The present paper describes a new type of strain *Aerobacter aerogenes* I-12, which produced either one of the three acetolactate synthases depending on the culture conditions. Some evidence was also presented that these three enzymes were regulated by isoleucine or potassium phosphate in the medium.

Materials and Methods

Organism

Aerobacter aerogenes I-12 was the isoleucine requiring mutant derived from *A. aerogenes* No. 19—35 which was the valine-producing bacterium [6].

Growth of bacteria and preparation of the enzyme

The basal media used were potassium phosphate deficient and sufficient ones. The former was composed of 7.5% glucose, 1.8% NH_4Cl, 0.05% K_2HPO_4, 0.035% $MgSO_4$ and 3% $CaCO_3$ (pH 7.2). The latter had the same composition except that it contained 0.2% of K_2HPO_4. The final concentration of isoleucine added was 5 μmol in both media. These media were designated as phosphate-deficient and phosphate-sufficient isoleucine-rich media, respectively. Phosphate-deficient and phosphate-sufficient isoleucine-poor media contained 0.5 μmol of isoleucine. In order to release catabolite repression, cells were grown in the basal medium containing 7.5% glycerol instead of glucose. The cells were cultured at 30°C for 2 days, then harvested by centrifugation and washed with 0.05 M of potassium phosphate buffer (pH 7.2) consisting of the following ingredients: 0.02 M potassium phosphate, 10 mM $MgCl_2$, 7 μM of flavin adenine dinucleotide (FAD) and 65 μM of thiamine pyrophosphate. Cells were resuspended in the same buffer. Then cells were disrupted by sonic oscillation and the crude extract was obtained by centrifugation at 20 000 × *g* for 20 min.

Enzyme assay

Acetolactate synthase was assayed by the method described previously, except that FAD was added to the reaction mixture [5]. One unit of the enzyme activity was defined as that amount which produces 1.0 μmol of acetohydroxy acid in 1 h. The specific activity is expressed as units per mg of protein which was determined by the micro-biuret method [7].

Results

1. Effect of pH on activities of acetolactate synthase from cells grown under various conditions

The activities of acetolactate synthase in crude extracts were assayed as a

function of pH in the range of 5.0–9.0. The results are given in Fig. 1. When cells were grown on the isoleucine-rich media, two acetolactate synthases differing in their optimum pH values appeared, depending on the phosphate sufficiency and deficiency. In the case of poor-isoleucine media, only the pH 8.0 enzyme appeared, irrespective of phosphate concentrations in the media.

2. Sensitivity of acetolactate synthase

As shown in Table I, the pH 6.0 enzyme was not inhibited by the end-products. The activity of the pH 8.0 enzyme prepared from cells grown on phosphate-deficient isoleucine-rich medium was markedly inhibited by valine, isoleucine and leucine. The activities of the enzymes prepared from cells grown both in isoleucine-poor phosphate-deficient and -sufficient media were not inhibited by the three above-mentioned amino acids. Furthermore, the activities of the two insensitive enzymes were not inhibited by these amino acids upto 10^{-2} M (Fig. 2). Thus, these acetolactate synthases might be called the sensitive pH 8.0 and insensitive pH 8.0 enzymes.

3. Effect of flavin adenine dinucleotide on the activity of acetolactate synthase

Table II shows that FAD stimulated the activity of the sensitive pH 8.0 enzyme. In contrast, no significant stimulation by FAD was observed in the insensitive pH 6.0 enzyme. When insensitive pH 8.0 enzymes were prepared in FAD-

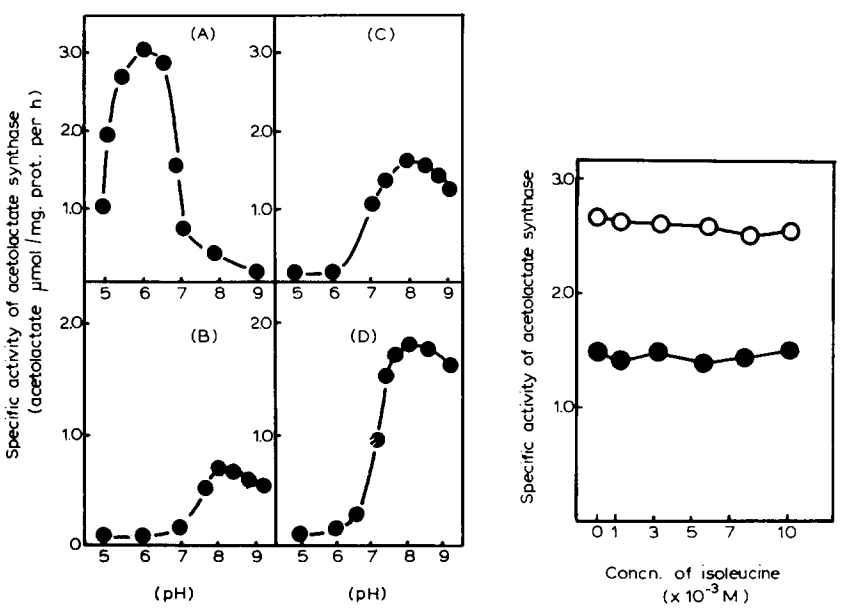

Fig. 1. Effect of pH on activities of acetohydroxy acid synthetase from *Ae. aerogenes* I-12 grown under various conditions. (A) Cells grown on phosphate-sufficient medium containing rich isoleucine ($5 \cdot 10^{-3}$ M). (B) Cells grown on phosphate-deficient medium containing a high concentration of isoleucine. (C) Cells grown on phosphate-sufficient medium containing limited isoleucine ($5 \cdot 10^{-4}$ M). (D) Cells grown on phosphate-deficient medium containing limited isoleucine.

Fig. 2. Effect of isoleucine concentration on the activity of acetohydroxy acid synthetase. Insensitive pH 6.0 enzyme, ○; insensitive pH 8.0 enzyme, ●.

TABLE I

THE SENSITIVITY OF ACETOLACTATE SYNTHASE TO ISOLEUCINE, LEUCINE AND VALINE

Enzyme was prepared from cells grown on	Optimum pH	Activity of acetolactate synthase * Amino acids ** added			
		None	Isoleucine	Leucine	Valine
Phosphate-suffieient isoleucine-rich medium	6.0	2.8	2.95	2.8	2.73
Phosphate-deficient isoleucine-rich medium	8.0	0.87	0.21	0.19	0.20
Phosphate-sufficient isoleucine-poor medium	8.0	1.98	1.92	1.72	1.96
Phosphate-deficient isoleucine-poor medium	8.0	1.85	1.81	1.70	1.90

* Acetolactate produced μmol/mg protein per h.
** $1 \cdot 10^{-3}$ M.

free phosphate buffer, no significant activity could be detected. If FAD was added into the enzyme solution immediately after preparation in FAD-free buffer, a weak enzyme activity was detectable. However, when FAD-containing phosphate buffer was used in the preparation of the enzyme, a high activity appeared but it was not stimulated by the further addition of FAD. Therefore, it is conceivable that FAD is not only an activator, but also a stabilizer for acetolactate synthases.

4. Hydroxyapatite column chromatography of acetolactate synthase

Acetolactate synthases were separated according to the method of Oneill and Freundlich [3]. Fig. 3 shows that extracts made from cells grown on the phosphate-deficient isoleucine-poor medium showed a single peak of insensitive pH 8.0 enzyme (Fig. 3A). From cells grown on the phosphate-deficient isoleucine-rich medium, the sensitive pH 8.0 enzyme was found (Fig. 3B). From cells grown on the phosphate-sufficient isoleucine-rich medium, only the insensitive pH 6.0 enzyme (Fig. 3C) was detected. Therefore, either one of the three acetolactate synthases, namely sensitive pH 8.0, insensitive pH 8.0 or insensitive

TABLE II

EFFECT OF FLAVIN ADENINE DINUCLEOTIDE ON THE ACTIVITY OF ACETOLACTATE SYNTHASE

Acetolactase synthase	Phosphate buffer used in preparation	Activity of acetolactase synthase * Flavin adenine dinucleotide **	
		Presence	Absence
Senstive pH 8.0	FAD-free	0.98	0.29
	FAD ***	1.02	0.73
Insensitive pH 6.0	FAD-free	3.20	3.28
	FAD ***	3.05	3.19
Insensitive pH 8.0	FAD-free	0.06	0
	FAD ***	2.00	1.87

* Acetolactate produced μmole/mg protein per h.
** 10 μg/ml.
*** FAD-phosphate buffer contained 5 μg/ml of flavin adenine dinucleotide.

pH 6.0 enzyme, was formed in *Ae. aerogenes* I-12 grown under various conditions.

5. Effect of potassium phosphate on the synthesis of acetolactate synthase

The synthesis of the insensitive pH 6.0 enzyme occurred in the cells grown in the medium containing a high concentration of phosphate (Fig. 4A). Weak activity was also observed at pH 8.0 although the sensitive pH 8.0 enzyme was not detected in this extract, as described in section 4. The synthesis of insensitive pH 8.0 enzyme was not affected by the concentration of phosphate in the medium (Fig. 4B).

6. Effect of leucine and valine on the synthesis of acetolactate synthase

As shown in Table III, the sensitive pH 8.0 enzyme was repressed by isoleucine, leucine and valine. However, the synthesis of the insensitive pH 6.0 enzyme was not repressed by these three amino acids. Similarly, these three amino acids did not affect the synthesis of the insensitive pH 8.0 enzyme.

7. Catabolite repression of acetolactate synthase

Previously, it was shown that in the parent strain, the insensitive pH 6.0 en-

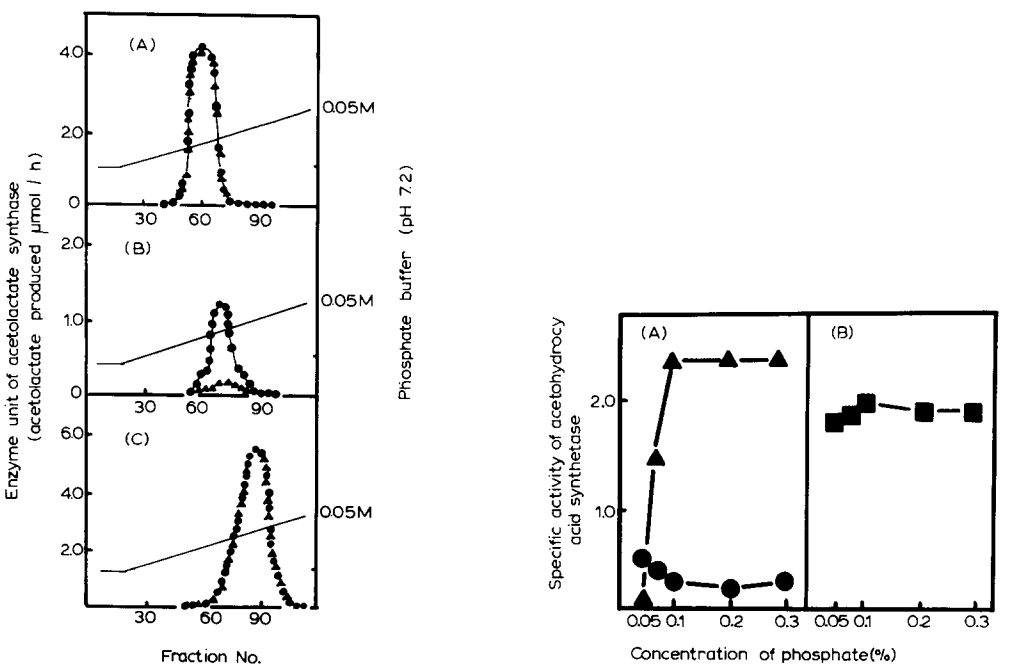

Fig. 3. Hydroxyapatite column chromatography of acetohydroxy acid synthetase. The crude extract (6 ml) was layered on a column of hydroxyapatite (1 × 20 cm). The buffer contained 0.02 M potassium phosphate (pH 7.2), 1 mM $MgCl_2$, 0.1 mM thiamine pyrophosphate and 10 μg per ml of flavin adenine dinucleotide. The column was eluted with a linear gradient (150 ml) of 0.02 M to 0.05 M potassium phosphate containing the above additions. Enzymatic activity was assayed with (▲) and without (●) valine. (A) Insensitive pH 6.0 enzyme; (B) sensitive pH 8.0 enzyme; (C) insensitive pH 6.0 enzyme.

Fig. 4. Effect of concentration of phosphate on the synthesis of acetohydroxy acid synthetase. (A) Cells were grown on isoleucine-rich medium; (B) cells were grown on isoleucine-poor medium. Sensitive pH 8.0 enzyme, ●; insensitive pH 6.0 enzyme, ▲; insensitive pH 8.0 enzyme, ■.

TABLE III

EFFECT OF LEUCINE AND VALINE ON THE SYNTHESIS OF ACETOLACTATE SYNTHASE

Acetolactate synthase	Added in medium *	Acetolactate synthase activity
Sensitive pH 8.0	None	0.96
	Leucine	0.85
	Valine	0.36
	Isoleucine	0.47
	Leu + Val + Ile	0.29
Insensitive pH 6.0	None	3.12
	Leucine	3.01
	Valine	3.12
	Isoleucine	2.96
	Leu + Val + Ile	3.00
Insensitive pH 8.0	None	2.13
	Leucine	2.09
	Valine	2.30
	Val + Leu	2.00

* $5 \cdot 10^{-3}$ M.

zyme was not formed in cells grown on glucose, due to the catabolite repression. It was investigated whether or not the mutant strain behaved similarly to the parent strain, using glycerol as the carbon source instead of glucose. The activity of the insensitive pH 6.0 enzyme increased almost linearly with cell growth on glycerol medium, while the activity of the enzyme was not observed when the cells were grown on glucose (Fig. 5). These results demonstrate that the synthesis of the insensitive pH 6.0 enzyme is repressed by glucose. Fig. 6 shows that the catabolite repression of the insensitive pH 8.0 enzyme did not occur.

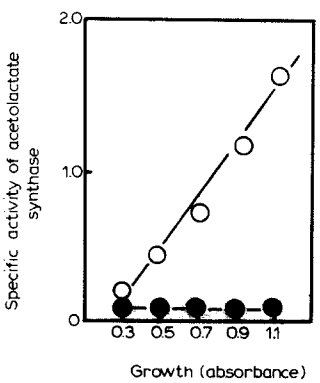

Fig. 5. Repression of insensitive pH 6.0 acetohydroxy acid synthetase by glucose. Cells were grown on phosphate-deficient isoleucine-poor medium containing glucose (●) or glycerol (○) as carbon source.

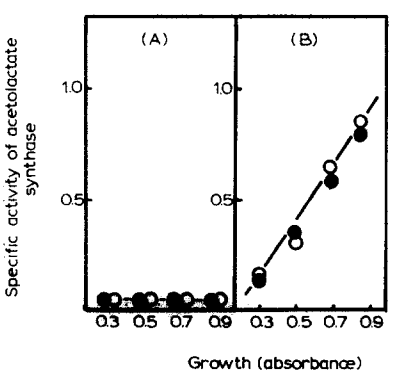

Fig. 6. Effect of glycerol and glucose on the synthesis of insensitive pH 8.0 acetohydroxy acid synthetase. (A) Cells were grown on phosphate-deficient isoleucine-rich medium containing glycerol (○) or glucose (●). (B) Cells were grown on phosphate-deficient isoleucine-poor medium containing glycerol (○) or glucose (●).

Discussion

It was shown in this paper that either one of three acetolactate synthases, insensitive pH 6.0 enzyme, sensitive pH 8.0 enzyme or insensitive pH 8.0 enzyme in one strain, *Ae. aerogenes* I-12, was detected in the cells grown on four kinds of culture media. It has been reported previously that the sensitive pH 8.0 enzyme was constitutive [1]. However, it is not the case in our experiments, since the sensitive pH 8.0 enzyme was formed only in cells grown on phosphate-deficient isoleucine-rich medium. Thus, the synthesis of the sensitive pH 8.0 enzyme was dependent on phosphate concentration in the medium. In the experiments with *Ae. aerogenes* No. 19—35, the parent strain, it had been found that the catabolite repression of insensitive pH 6.0 enzyme was released by increasing phosphate concentration in culture media [8]. Similar results were obtained with this mutant. Therefore, it may be concluded that the synthesis of the insensitive pH 6.0 enzyme occurred by the relaxation of catabolite repression that was caused by the presence of a sufficient amount of phosphate. However the dependence of the synthesis of insensitive pH 6.0 and sensitive pH 8.0 enzymes on phosphate concentration appeared only when isoleucine in the media was not limited.

On the other hand, when isoleucine was limited, sensitive pH 8.0 and insensitive pH 6.0 enzymes were completely repressed and the synthesis of the insensitive pH 8.0 enzyme occurred regardless of phosphate concentrations in the media. It has been reported in auxotrophs of *Escherichia coli* and *Salmonella typhimurium* that the synthesis of the insensitive pH 8.0 enzyme occurred in the medium containing a limiting amount of isoleucine [3,4]. However, various characteristics of strain I-12 differ from *Escherichia coli* and *Salmonella typhimurium*: (1) the multivalent repression by amino acids was not observed; (2) the coexistence of excess leucine and valine was not required for the synthesis of the insensitive pH 8.0 enzyme; (3) the synthesis of the insensitive pH 8.0 enzyme was dependent only on the presence of isoleucine; (4) in wild type strain, the insensitive pH 8.0 enzyme could not be formed under any of the conditions examined. Therefore, one might conclude that the synthesis of the insensitive pH 8.0 enzyme was derepressed by limiting isoleucine in the media. In the regulation of the synthesis of the insensitive pH 8.0 enzyme, isoleucine plays a more important role than phosphate. It is noticeable that derepression occurs in a manner of "on-off" type. However, insensitive pH 6.0 and sensitive pH 8.0 enzymes were repressed by limiting isoleucine. The mechanism is still unknown, because the release of enzyme repression was generally caused by decreased amounts of the suppressor.

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