SHORT COMMUNICATIONS

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Further evidence for two distinct acetolactate synthetases in *Aerobacter aerogenes*

Previous work[1] provided indirect evidence suggesting that *Aerobacter aerogenes* is capable of producing two distinct enzymes catalyzing the synthesis of α-acetolactate. One of these enzymes (pH 8 activity) is similar to the acetolactate synthetase of *Escherichia coli*[2,3] which is concerned with the biosynthesis of valine. The second enzyme (pH 6 activity) is similar to that found by JUNI[4] to catalyze the first step in acetoin formation from pyruvate, and serves to divert glucose catabolism from acidic to neutral products[5]. In this communication direct evidence will be presented to support our earlier suggestion that the two acetolactate synthetase activities of *A. aerogenes* are due to two separate enzymatic entities.

A. aerogenes 35 (wild type) was mutagenized with nitrosoguanidine[6] and valine–isoleucine-requiring mutants were selected by replica plating after penicillin treatment[7]. The selection was carried out on media at two pH values, 5.8 and 7.4, enabling us to detect mutants with a pH-dependent valine–isoleucine requirement. Twenty-four mutants, including two which required valine and isoleucine only when grown at low pH (5.8), were purified and tested for the acetolactate synthetase activity at pH 6.0 and the valine-sensitive acetolactate synthetase activity at pH 7.5. Three mutants were found to be deficient in the pH 6 activity and one (requiring valine and isoleucine at pH 5.8) had reduced valine-sensitive synthetase activity at pH 7.5. These four strains were further analyzed for their reductoisomerase[8], dihydroxy acid dehydrase[9] and transaminase B[10] activities (Table I). As can be seen from Table I, strain 9 which grew slowly in unsupplemented medium at pH 7.4 and not at all at pH 5.8, had normal levels of all the enzymes except for the biosynthetic valine-sensitive acetolactate synthetase. Among the three strains deficient in the pH 6 acetolactate synthetase, one (strain IV-2) also showed a 90% reduction in dehydrase activity, another mutant (strain III-45) was deficient in reductoisomerase, while the third (strain III-12) had a double lesion, in reductoisomerase and in dehydrase. It was of interest to see whether the pH 6 synthetase is required for the biosynthesis of valine and isoleucine and/or whether the deficiency of strains IV-2 and III-12 in the activity of this enzyme resulted from the mutations affecting dehydrase or reductoisomerase activities. Prototroph revertants of strains IV-2 and III-45 obtained spontaneously were tested for the activity of the biosynthetic enzyme deficient in the parent strain (reductoisomerase in the III-45 revertants and dihydroxy acid dehydrase in the revertants of mutant IV-2) and for the activity of the pH 6 acetolactate synthetase. No prototroph revertants were obtained from the double mutant III-12, either spontaneously or by treatment with ultraviolet light, ethyl methanesulfonate and nitrosoguanidine. The results obtained with 10 independently isolated prototroph revertants of strain IV-2 and with 8 such revertants of strain III-45 are given in Table II. All the revertants tested showed large increases in the activity of the biosynthetic enzyme which was impaired in their respective

TABLE I

ENZYME ACTIVITIES OF *A. aerogenes* WILD-TYPE AND MUTANT STRAINS

α-Acetolactate synthetase was determined in 1-ml reaction mixtures of the following composition (in μmoles): sodium pyruvate, 40; thiamine pyrophosphate, 0.188; $MgCl_2$, 10; L-valine (where indicated), 30; phosphate buffer (pH 6.0 or 7.5, as indicated), 100; cell-free extract equivalent to 2 mg protein. The mixture was incubated for 20 min at 37,° after which time 0.1 ml of 100% trichloroacetic acid was added and the mixture was autoclaved for 10 min at a pressure of 1 atm and centrifuged. Acetoin was determined on 0.1-ml aliquots of the supernatant by the colorimetric method of WESTERFELD[11], using pure acetoin as a standard. The pH 6 activity was tested on extracts of cells grown overnight in nutrient broth supplemented with 0.3% glucose and 0.06 M phosphate buffer (pH 6.0). For the pH 7.5 experiments the bacteria were grown overnight at 37° in the minimal medium of DAVIS AND MINGIOLI[12] adjusted to pH 8.0, from which citrate was omitted, with 0.3% glucose as the source of carbon. To cultures of the auxotroph mutants L-leucine, 12.5 μg/ml, DL-valine, 25 μg/ml, and DL-isoleucine, 25 μg/ml were also added. The extracts were prepared by treating cell suspensions in 0.067 M phosphate and 0.01 M β-mercaptoethanol in the M.S.E. ultrasonic disintegrator for 2 min. Reductoisomerase was determined according to ARMSTRONG AND WAGNER[8] with some modifications. The reaction mixture contained in 1 ml (in μmoles): α-acetohydroxybutyrate, 1.55; β-mercaptoethanol, 2.5; $MgSO_4$, 5; TPNH, 0.15; phosphate buffer (pH 7.5), 100; cell-free extract, 0.05–1.00 mg protein. α-Aceto-α-hydroxybutyrate was obtained by hydrolysis of ethyl acetoxy-α-acetohydroxybutyrate in 2 M NaOH at 37° for 1 h and subsequent neutralization with conc. HCl. Ultrasonicates of overnight cultures in minimal medium with amino acid supplementation where required, were centrifuged for 1 h at 100 000 × *g* and dialyzed overnight against 0.1 M phosphate buffer (pH 7.5), 0.002 M mercaptoethanol, and 0.0002 M $MgSO_4$. This treatment reduced the substrate-independent oxidation of TPNH to a negligible level. Dihydroxy acid dehydrase was estimated according to MYERS[9], with α,β-dihydroxyisovaleric acid as the substrate. The α-ketoisovalerate formed was determined by the method of FRIEDEMANN AND HAUGEN[13]. Transaminase B was measured by a modification of the method described by RAMAKRISHNAN AND ADELBERG[10]. The reaction mixture contained in 1 ml (in μmoles): α-ketoglutarate, 100; L-valine, 120; pyridoxal phosphate, 1; Tris buffer (pH 7.8), 200; cell-free extract, 1.2 mg protein. The bacterial extract was preincubated with pyridoxal phosphate at 37° for 30 min before the addition of the substrates; the complete system was further incubated for 30 min. The reaction was stopped with trichloroacetic acid, the mixture centrifuged and the α-ketoisovalerate formed was extracted with toluene and determined according to FRIEDEMANN AND HAUGEN[13].

Strain	*Enzyme activity (μmoles/mg protein per h)*						
	Acetohydroxy acid synthetase			*Ratio pH 6.0/ pH 7.5 activities*	*Reducto-isomerase*	*Dihydroxy acid dehydrase*	*Trans-aminase B*
	pH 6.0	*pH 7.5*					
	no valine added	*no valine added*	*plus valine*				
A. aerogenes 35 (wild type)	8.50	0.62	0.14	13.71	1.06	13.13	13.34
A. aerogenes 35/IV-2	0.13	3.05	0.32	0.04	2.10	1.26	25.09
A. aerogenes 35/III-45	0.08	2.24	0.22	0.04	0.05	22.03	13.36
A. aerogenes 35/III-12	0.12	0.48	0.13	0.25	0.00	1.81	22.79
A. aerogenes 35/9	11.98	0.22	0.08	54.45	5.98	11.03	13.23

parents. In none of the 18 revertants examined was any restoration of pH 6 acetolactate synthetase activity observed.

The results described here, showing that it is possible to separate the two acetolactate synthetase activities of *A. aerogenes* by mutation, substantiate our earlier conclusion[1] that the two activities are due to different enzymes. The enzymatic analysis of prototroph revertants (Table II) further shows that the pH 6 acetolactate synthetase is not required for the normal functioning of the valine biosynthetic pathway. Moreover, the behavior of strain 9 (see text and Table I) indicates that

TABLE II

ENZYME ACTIVITIES OF PROTOTROPH REVERTANTS OF *A. aerogenes* MUTANTS REQUIRING VALINE–ISOLEUCINE

For experimental details see text and Table I.

Strain	*Enzyme activity (μmoles/mg protein per h)*		*Strain*	*Enzyme activity (μmoles/mg protein per h)*	
	Acetohydroxy acid synthetase, pH 6.0	*Dihydroxy acid dehydrase*		*Acetohydroxy acid synthetase, pH 6.0*	*Reducto-isomerase*
A. aerogenes 35/IV-2	0.13	1.26	*A. aerogenes* 35/III-45	0.08	0.05
A. aerogenes 35/IV-2R1	0.16	2.90	*A. aerogenes* 35/III-45R1	0.12	2.52
A. aerogenes 35/IV-2R2	0.08	12.94	*A. aerogenes* 35/III-45R2	0.12	0.38
A. aerogenes 35/IV-2R3	0.10	7.32	*A. aerogenes* 35/III-45R3	0.07	1.21
A. aerogenes 35/IV-2R4	0.09	16.05	*A. aerogenes* 35/III-45R6	0.07	0.33
A. aerogenes 35/IV-2R5	0.12	5.46	*A. aerogenes* 35/III-45R7	0.13	8.47
A. aerogenes 35/IV-2R6	0.12	2.97	*A. aerogenes* 35/III-45R11	0.10	0.30
A. aerogenes 35/IV-2R10	0.09	4.18	*A. aerogenes* 35/III-45R12	0.11	11.83
A. aerogenes 35/IV-2R17	0.10	18.08	*A. aerogenes* 35/III-45R17	0.13	1.61
A. aerogenes 35/IV-2R28	0.07	7.47			
A. aerogenes 35/IV-2R31	0.12	12.50			

the pH 6 enzyme does not supply acetolactate for valine biosynthesis even during growth at low pH. This somewhat surprising finding may perhaps be due to compartmentalization of the two synthetases, or to channelling of the intermediates of the two pathways by some other mechanism. Further experiments are required to elucidate this point.

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