Taurocyamine-utilizing Mutant of *Pseudomonas aeruginosa* with Altered Induction of 3-Guanidinopropionate Amidinohydrolase

Takamitsu YORIFUJI, Katsuhiko SAWADA and Chikashi TOKUDA

Department of Agricultural and Biological Chemistry, Shinshu University,
Minamiminowa Kamiina, Nagano 399-45, Japan

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A mutant, strain M-6, capable of utilizing taurocyamine (2-guanidinoethanesulfonate) as a nitrogen source was isolated from the parent strain, *Pseudomonas aeruginosa* GB-4, a derivative of wild-type *P. aeruginosa* PAO1 lacking the ability to produce guanidinobutyrase (EC 3.5.3.7). 3-Guanidinopropionate amidinohydrolase (EC class 3.5.3), which acts slowly on taurocyamine, was induced effectively by only 3-guanidinopropionate in the parent strain, while the enzyme of strain M-6 was induced by taurocyamine, guanidinoacetate, 3-guanidinopropionate, 4-guanidinobutyrate, and guanidinosuccinate. Strain M-6 synthesized a slight amount of the enzyme constitutively. The enzyme partially purified from strain M-6 exhibited substrate specificity similar to that of the wild-type strain. The mutant could grow also on 4-guanidinobutyrate, unlike the parent strain. These results indicate that strain M-6 acquired the ability to grow on taurocyamine by virtue of a mutation at the regulatory gene for 3-guanidinopropionate amidinohydrolase, which led to alteration of the specificity of the regulatory protein.

A number of mutants of microorganisms that have acquired new metabolic capabilities have been studied.^{1~3)} These studies have indicated that the most common way for bacteria to acquire a new metabolic function is to synthesize constitutively an enzyme, which already has an activity toward the new growth substrate but is not induced by the substrate in the parent strain. For example, mutants of *Aerobacter* (*Klebsiella*) *aerogenes* that synthesized L-fucose isomerase constitutively could grow on D-arabinose; this isomerase was not induced by D-arabinose in the parent strain.⁴⁾

Pseudomonas aeruginosa PAO1 produces 3-guanidinopropionate amidinohydrolase (GPH, EC class 3.5.3) and guanidinobutyrase (GBH, EC 3.5.3.7).⁵⁾ GPH has been highly purified and characterized in our laboratory;^{6,7)} this enzyme is not induced by taurocyamine (2-guanidinoethanesulfonate), but catalyzes hydrolysis of its guanidino group to form urea at a rate of 11% of that toward 3-guanidinopropionate. Our previous paper reported a taurocyamine-utilizing mutant of

Pseudomonas sp. ATCC 14676; it was strongly suggested that the mutant produced GBH of altered substrate specificity to act on taurocyamine.⁸⁾ This paper deals with a taurocyamine-utilizing mutant from *P. aeruginosa* strain GB-4, a derivative of the wild-type strain PAO1 lacking the ability to synthesize GBH. In mutant strain M-6, there was little constitutive synthesis of GPH, and the enzyme was induced by some guanidino compounds including taurocyamine. This indicates that the mutant had a mutation at the regulatory gene of GPH, but it was not a typical constitutive mutant.

MATERIALS AND METHODS

Chemicals. Taurocyamine, 3-guanidinopropionate, 4-guanidinobutyrate, 5-guanidinovalerate, guanidinosuccinate, and 2-guanidinoethyl sulfate were synthesized from taurine, β -alanine, 4-aminobutyrate, 5-aminovallate, L-aspartate, and 2-aminoethyl sulfate, respectively, by amidination with S-methylisothiurea sulfate under the conditions described by Kimmel⁹⁾ and recrystallized from water. Amino acids and other chemicals were purchased

from Nakarai Chemicals, Ltd., unless specified otherwise.

Organism. *Pseudomonas aeruginosa* strain GB-4, which was used as the parent strain, was a kind gift of Dr. H. Matsumoto of the School of Medicine, Shinshu University. This strain, which required L-methionine for growth and lacked the ability to produce GBH, is a derivative of wild-type *P. aeruginosa* PAO1 (ATCC 15692). Strain GB-4 grew well on 3-guanidinopropionate as a nitrogen source, but could not grow on 4-guanidinobutyrate.

Media and culture conditions. Asparagine-succinate medium, which contained 0.1% asparagine and 0.5% sodium succinate, and taurocyamine-succinate medium, which contained 0.05% taurocyamine and 0.5% sodium succinate, were prepared by adding 0.01% L-methionine into the asparagine-succinate medium II⁸⁾ and the taurocyamine-succinate medium,⁸⁾ respectively, described in the previous paper; peptone medium was prepared as described.⁸⁾ Guanidinopropionate and guanidinobutyrate media were prepared by replacing taurocyamine in taurocyamine-succinate medium with 3-guanidinopropionate and 4-guanidinobutyrate, respectively. Solid media for plate cultures were prepared by adding 1.5% (w/v) of agar (AGAR PURIFIED, Difco Laboratories, Detroit, Mich.) into liquid media. For the isolation of mutants, cells were cultivated in a 20-ml L-shaped test tube or in a 500-ml culture flask with a side arm 15 mm in diameter (Wheaton Scientific, Millville, N. J.) at 28°C with aeration. Growth was monitored at 570 nm with a Hitachi EPO-B colorimeter.

Isolation of taurocyamine-utilizing mutants. All the cells of strain GB-4 grown in 15 ml (2 × 7.5 ml) of the asparagine-succinate medium for 8 hr were suspended in 16 ml of 0.85% NaCl to give 2.1×10^8 cells/ml, and the suspension was uv-irradiated for 10 sec under the conditions described previously;⁸⁾ 0.3% of the irradiated cells survived. To the cell suspension was added 5 ml of the sterile asparagine-succinate medium that was supplemented with additional L-asparagine to give a final concentration of 0.4%. The culture was grown in a culture flask at 28°C for 12 hr in the dark, and then 200 ml of the sterile taurocyamine-succinate medium was added into the culture. This diluted culture was incubated for 103 hr at 28°C with aeration. The cells grown were subcultured three times by transferring 0.2 ml of the culture into 200 ml of the fresh taurocyamine-succinate medium and incubating the new culture until the turbidity at 570 nm increased to about 0.3. The subcultured cells in 0.1-ml aliquots of the broth were plated on taurocyamine-succinate agar plates after appropriate dilution with 0.85% NaCl. After incubation of the plates at 41°C for 2 days, the colonies obtained were stocked on the taurocyamine-succinate agar slants.

Assay of enzymes and analytical methods. GPH was assayed by the method described in the previous paper,⁶⁾ by measuring urea formed from 3-guanidinopropionate. The rate of hydrolysis of taurocyamine or 4-guanidinobutyrate with GPH was measured similarly. Guanidino compounds were assayed colorimetrically by the method of Rosenberg *et al.*¹⁰⁾ Urea was assayed with diacetyl monoxime and antipyrine by the method of Prescott and Jones.¹¹⁾ Protein in the cell extracts was assayed by the method of Bradford¹²⁾ with Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Richmond, Calif.) using bovine serum albumin as the standard. Protein in purified enzyme fractions was assayed from the absorbance at 280 nm using an *E* (1%) value of 10.0.

Induction of GPH with guanidino compounds. The cells of strain M-6 or strain GB-4 were grown in a 2,000-ml shaking flask containing 350 ml of the asparagine-succinate medium at 30°C for 7 hr. Each of the 50-ml aliquots was placed in a 500-ml shaking flask and 50 ml of 20 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM MnCl₂ and 0.2% guanidino compound was added. The cell suspension was incubated at 30°C for 5 hr with aeration. The cells were harvested by centrifugation, washed once with 0.85% NaCl, and suspended in 1.0 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 1.0 mM 2-mercaptoethanol and 0.1 mM MnCl₂. The cell extract was prepared by disrupting the cells with a Kaijo Denki 19 kHz sonic disintegrator for 20 sec at 10°C or below, followed by centrifugation at $15,000 \times g$ for 10 min to remove cell debris. The enzyme activity was assayed as described above by use of 3-guanidinopropionate, taurocyamine, and 4-guanidinobutyrate as the substrates.

Purification of GPH from strain M-6. The cells of strain M-6 were grown in 15 liters of the taurocyamine-succinate medium under conditions described previously.⁶⁾ The methods for preparation of the cell extract, ammonium sulfate fractionation, and DEAE-cellulose column chromatography were similar to those described in the previous paper.⁶⁾ The cell extract contained 1,050 mg of protein and 2,320 units of GPH. The DEAE-cellulose fraction contained 47 mg of protein and 1,220 units of GPH. The enzyme was dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 1.0 mM 2-mercaptoethanol and 0.1 mM MnCl₂ (buffer TM), and put on a column of DEAE-Toyopearl (Toyo Soda Mfg. Co., Ltd., 1.2 × 45 cm) equilibrated with buffer TM containing 0.2 M KCl. After the column was washed with 150 ml of the equilibration buffer, the enzyme was eluted with a linear KCl gradient of 125 ml each of buffer TM containing 0.2 M KCl and buffer containing 0.6 M KCl. The active fractions contained 4.0 mg of protein and 840 units of GPH.

RESULTS

Growth of strain GB-4 on guanidino compounds

When the cells of *P. aeruginosa* strain GB-4, which was used as the parent strain in this study, were grown in the taurocyamine-succinate medium, they grew initially with a doubling time of 130 min for a short time; this burst of growth was also observed in the absence of a nitrogen source. Thereafter, the cells grew very slowly with a doubling time of 85 hr. In the guanidinopropionate medium, the cells grew rapidly with a doubling time of 88 min. This strain failed to grow on 4-guanidinobutyrate, unlike wild-type *P. aeruginosa* PAO1.

Isolation of taurocyamine-utilizing mutant

The apparent growth was observed after cultivation of the uv-irradiated cells of strain GB-4 for 103 hr in the taurocyamine-succinate medium. The cells grown were subcultured three times in the medium, and then thirty colonies were isolated and stocked as candidates of taurocyamine-utilizing mutant. After testing of these strains for L-methionine requirement and growth rate on taurocyamine, strain M-6 was selected for the further experiments. Strain M-6 required L-methionine for growth, grew well on taurocyamine, and produced GPH when grown in the guanidinopropionate medium. The minimum doubling time of the cells of strain M-6 grown in the taurocyamine-succinate medium was 130 min (Fig. 1). Taurocyamine was almost consumed during the cultivation. These results indicate that strain M-6 acquired the ability to grow rapidly on taurocyamine with a doubling time comparable to that observed with the parent strain growing on 3-guanidinopropionate.

Time study of GPH induction in strain M-6

The cells of the parent strain and strain M-6 were grown in the asparagine-succinate medium for 8 hr at 30°C, and then an equal volume of 20 mM potassium phosphate buffer, pH 7.5, containing 0.2% taurocyamine and 0.2 mM MnCl₂ was added, and the incubation

FIG. 1. Growth of Strain M-6 in Taurocyamine-Succinate Medium.

The cells of strain M-6 were cultivated at 30°C in 8.0 ml of the taurocyamine-succinate medium, which was inoculated by transferring 0.1 ml of a 8.0-ml culture in the asparagine-succinate medium. Each sample, 0.1 ml, was withdrawn at the time indicated and the concentration of taurocyamine was measured. (○), turbidity at 570 nm; (●), taurocyamine concentration.

FIG. 2. Induction of GPH in Strains M-6 and GB-4 with Taurocyamine.

The cells of strain M-6 or strain GB-4 were cultivated in a 2,000-ml shaking flask containing 200 ml of the asparagine-succinate medium at 30°C for 8 hr. To the culture was added 200 ml of 20 mM potassium phosphate buffer, pH 7.5, containing 0.2% taurocyamine and 0.2 mM MnCl₂, and incubation was continued with aeration. At the time indicated, a sample (50 ml) was withdrawn, the cells were corrected and washed, and the extract was prepared as described under MATERIALS AND METHODS. GPH activities of the extracts of the cells of strain M-6 (○) and those of the cells of strain GB-4 (△) were assayed. The activity toward taurocyamine was assayed but is not shown.

was continued with aeration. The samples were withdrawn at various times, the cell extracts were prepared, and the activities of the extracts to hydrolyze 3-guanidinopropionate and taurocyamine were assayed (Fig. 2). Unlike the parent strain, the extract of the cells of strain M-6 grown on asparagine showed a low GPH activity, and the GPH specific activity of the extract increased with an increase in the time of incubation with taurocyamine. Taurocyamine was hydrolyzed with relative rates of around 12% of those toward 3-guanidinopropionate with all the extracts of M-6 cells incubated with taurocyamine. These results indicate that taurocyamine induced GPH in the cells of strain M-6 but not in the cells of the parent strain, and that the cells of strain M-6 synthesized a slight amount of the enzyme constitutively.

Induction of GPH in strain M-6 by various guanidino compounds

The cells of strain M-6 and the parent strain grown on L-asparagine were incubated in 10mM potassium phosphate buffer, pH 7.5, containing 0.1 mM $MnCl_2$ and 0.1% guanidino compound. The enzyme activity of the extract of the cells was assayed with 3-guanidinopropionate, taurocyamine, or 4-guanidinobutyrate (Table I). GPH of the parent strain was induced strongly, by 3-guanidinopropionate and very weakly by guanidinoacetate. By contrast, GPH of strain M-6 was induced considerably by guanidinoacetate, taurocyamine, 4-guanidinobutyrate, guanidinosuccinate, and an unknown compound in peptone, as well as

3-guanidinopropionate. A slight constitutive synthesis of GPH in strain M-6 was also shown in this experiment. These results indicate that the mutation in strain M-6 led to a broadened inducer spectrum for GPH and to a slight constitutive synthesis of the enzyme.

Substrate specificity of GPH from strain M-6

GPH of strain M-6 was partially purified 94-

TABLE I. INDUCTION OF GPH IN STRAINS M-6 AND GB-4

Inducer	Strain	Activity ($\mu\text{mol}/\text{min}/\text{mg}$) ^a		
		GP	TC	GB
Guanidinoacetate	M-6	1.3	0.27	0.10
	GB-4	0.02	trace	trace
3-Guanidinopropionate	M-6	3.3	0.68	0.24
	GB-4	4.4	0.65	0.25
Taurocyamine	M-6	0.15	0.016	0.005
	GB-4	0.006	0	0
4-Guanidinobutyrate	M-6	0.16	0.016	0.006
	GB-4	trace	0	0
5-Guanidinovalerate	M-6	0.005	0	0
	GB-4	0	0	0
Guanidinosuccinate	M-6	0.11	trace	0
	GB-4	0	0	0
2-Guanidinoethyl sulfate	M-6	0.006	0	0
	GB-4	0	0	0
Creatine	M-6	trace	0	0
	GB-4	0	0	0
Peptone	M-6	0.047	trace	0
	GB-4	0	0	0
None	M-6	0.011	0	0
	GB-4	0	0	0

^a The value indicated is the activity toward 3-guanidinopropionate (GP), taurocyamine (TC), or 4-guanidinobutyrate (GB).

TABLE II. PURIFICATION OF GPH FROM STRAIN M-6

Step	Protein (mg)	Activity ($\mu\text{mol}/\text{min}/\text{mg}$) ^a (Relative activity, %)		
		GP	TC	GB
1. Sonic extract	1,050	2.2 (100)	0.29 (13)	0.11 (5)
2. Ammonium sulfate	511	7.0 (100)	1.0 (14)	0.20 (3)
3. DEAE-cellulose	47	26 (100)	3.8 (15)	1.2 (5)
4. DEAE-Toyopearl	4	210 (100)	35 (17)	12 (6)

^a The value indicated is the activity toward 3-guanidinopropionate (GP), taurocyamine (TC), or 4-guanidinobutyrate (GB).

FIG. 3. Growth of Strain M-6 Cells in Guanidinobutyrate Medium.

The experimental conditions were the same as those described in Fig. 1 except that the guanidinobutyrate medium was used. The growth of cells (O) and the concentration of 4-guanidinobutyrate (●) were measured similarly.

fold by a four-step procedure similar to that described in our earlier paper.⁶ Table II summarizes the purification. The activities of the individual fractions toward taurocyamine and 4-guanidinobutyrate, compared with those toward 3-guanidinopropionate, were close; about 15% for taurocyamine and about 5% for 4-guanidinobutyrate. The values obtained in our earlier experiment for highly purified GPH of the wild-type strain were 11% for taurocyamine and 3% for 4-guanidinobutyrate.⁶ These observations indicate that the substrate specificities of GPH produced by strain M-6 and the enzyme of the wild-type strain were closely similar.

Growth of strain M-6 on 4-guanidinobutyrate

Strain GB-4, the parent strain, which lacked the ability to produce GBH, could not grow on 4-guanidinobutyrate as the nitrogen source. The data in Table II show that 4-guanidinobutyrate, a poor substrate of GPH, became an inducer of GPH in strain M-6. Thus, it was expected that strain M-6 could grow on 4-guanidinobutyrate. The strain grew well in the guanidinobutyrate medium with a mini-

mum doubling time of about 200 min, and 60% of 4-guanidinobutyrate was consumed during cultivation for 24 hr (Fig. 3).

DISCUSSION

The parent strain GB-4 produces GPH, but does not produce GBH, which wild-type *Pseudomonas aeruginosa* PAO1 does produce. GPH of the wild-type strain hydrolyzes also taurocyamine and 4-guanidinobutyrate with relative rates of 11% and 3%, respectively, as fast as 3-guanidinopropionate.⁶ The growth of strain GB-4 on taurocyamine is very slow, probably due to the low level of GPH induced with taurocyamine; the doubling time is 85 hr. This strain cannot grow on 4-guanidinobutyrate. Strain M-6 derived from strain GB-4 grows rapidly on taurocyamine with a doubling time of 130 min, about forty times as fast as the parent strain. Thus, we think it is reasonable to refer to the strain as a taurocyamine-utilizing mutant. In strain M-6, a low level of GPH is synthesized constitutively, and taurocyamine, which is substantially not an inducer of GPH in the parent strain, elicits further synthesis of the enzyme. In addition, guanidinoacetate, 4-guanidinobutyrate, and guanidinosuccinate are the inducers of GPH in the mutant. These results indicate that the mutant produces an altered regulatory protein of lowered specificity for inducer, and this seems to enable the mutant to grow on taurocyamine, and also on 4-guanidinobutyrate. The transport system may be unchanged. 4-Guanidinobutyrate induces GBH and serves as a nitrogen source in wild-type strain PAO1. This implies that the compound permeates into the cell of the parent strain. It fails to induce the GPH of the parent strain, while it induces the enzyme of strain M-6. GPH may be unchanged; the substrate specificity of GPH purified from strain M-6 is close to that of the enzyme of the wild-type strain.

Although strain M-6 seems to bear a mutation at the regulatory gene for GPH, the regulation of GPH synthesis is almost con-

served in the strain; the constitutive GPH level in non-induced cells is very low. Mutants of similar type have been found in D-arabinose-utilizing mutants of *Klebsiella aerogenes*.¹³⁾ Many mutants of bacteria which acquired new catabolic activity gained the activity by virtue of a mutation at the regulatory gene for an existing enzyme that led to constitutive enzyme synthesis.^{1~3)} An inevitable view, as discussed by Hall,³⁾ is that constitutive enzyme synthesis may be wasteful and hence disadvantageous in the natural environment where the new resource is absent. The taurocyamine-utilizing mutant M-6, as well as the D-arabinose-utilizing mutants mentioned above, may not have such a disadvantage, since the ability to repress GPH synthesis is almost conserved.

The mutation in strain M-6 has allowed guanidinoacetate and guanidinosuccinate to induce GPH. These compounds are not the substrates of the enzyme, and hence they are gratuitous (non-substrate) inducers of the enzyme in this strain. Taurocyamine-utilizing mutants were obtained directly from wild-type *Pseudomonas* sp. ATCC 14676, in which taurocyamine was a gratuitous inducer of GBH.⁸⁾ One of the mutants appeared to produce altered GBH capable of hydrolyzing taurocyamine. The mutation at the regulatory gene of strain M-6 has, therefore, given rise to a possibility that a point mutation at the structural gene for GPH allows, in some cases, the enzyme to act on guanidinoacetate or guanidinosuccinate and it permits the organism to grow on the compound. Although our attempts to isolate such mutants have been unsuccessful, these types of mutation,

which cause alternate changes of inducer and substrate specificity of an enzyme and thereby provide new catalytic and regulatory functions, might possible to occur in nature, presumably accompanied with neutral and advantageous mutations.

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