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Demonstration of an Altered Aminoacyl Ribonucleic Acid Synthetase in a Mutant of *Escherichia coli**

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An analogue of phenylalanine, *p*-fluorophenylalanine, is known to inhibit the growth of many bacteria. A mutant strain of *Escherichia coli* has been isolated which is resistant to this analogue. Preliminary experiments (1) have indicated that the mutant is resistant because, unlike the parent strain, it is unable to activate *p*-fluorophenylalanine. This communication presents evidence that the mutant strain possesses an altered phenylalanyl ribonucleic acid synthetase. The companion paper considers the physiological consequences to the mutant of the altered enzyme (2).

EXPERIMENTAL PROCEDURE

Materials

All chemicals employed were commercial products and were of the purest grade available. Uniformly labeled L-phenylalanine-^{14}C (61.9 μc per μmole) was obtained from Nuclear-Chicago Corporation, and DL-*p*-fluorophenylalanine-3-^{14}C (3.5 μc per μmole) was purchased from Volk Radiochemical Company.

The glucose-salts medium consisted of basal salts Solution P (3) and 0.4% glucose. The enriched media contained 20% Solution P, 1% tryptone, 0.5% yeast extract, and 0.4% glucose.

E. coli KB was the wild strain used in these experiments. The resistant mutant, PFP-10, was isolated from it by selection in the presence of *p*-fluorophenylalanine after treatment with 8-azaguanine as a mutagen (4).

Assay of Aminoacyl RNA Synthetase

The enzymes measured in the following assay systems are called aminoacyl-RNA synthetases although only one of the three assays measures the function implied by this nomenclature.

Amino Acid Hydroxamate Formation—This assay is similar to that reported by Conway, Lansford, and Shive (5). The volume, unless noted, was 1.0 ml and contained, per ml, 1000 μmoles of $NH_2OH \cdot HCl$ (neutralized with KOH), 10 μmoles of $MgCl_2$, 3 μmoles of L-amino acid or 6 μmoles of DL-amino acid analogue, 10 μmoles of dipotassium ATP (pH 6.8), and a limiting amount of enzyme. The final pH, unless stated otherwise, was 7.3. After incubation at 37° for 1 hour, the reaction was terminated by the addition of 3 volumes of a solution containing 10% $FeCl_3$, 5% HCl, and 10% trichloroacetic acid. The precipitated protein was removed by centrifugation and filtration, and the hydroxamate complex was determined spectrophotometrically in a Zeiss model PMQ II instrument at 540 mμ. Phenylalanine hydroxamic acid (Sigma) was used as a standard in the determination of activity with phenylalanine and its analogues. In the hydroxamate assay system, phenylalanine hydroxamic acid produced an optical density change of 0.137 per μmole.

The assay yielded results proportional to enzyme concentration over a wide range (1 to 8 mg of protein in crude extracts). In the range employed, the formation of hydroxamate occurred at a constant linear rate for well over the hour chosen as a standard time of incubation.

Amino Acid-dependent Pyrophosphate Exchange—The procedure used has features commonly used by others (6–9). The reaction mixture contained 100 μmoles of potassium phosphate buffer (pH 7.3), 10 μmoles of $MgCl_2$, 10 μmoles of β-mercaptoethanol, 2 μmoles of KF, 3 μmoles of dipotassium ATP (pH 6.8), 1 μmole of L-amino acid or 2 μmoles of DL-amino acid analogue, 1 μmole of ^{32}P-pyrophosphate (10^5 to 2×10^6 c.p.m.), a limiting amount of enzyme, and water to a volume of 1.0 ml (final pH, 7.3). The mixture was incubated at 37° for 10 minutes, and the reaction was terminated by rapid chilling and addition of 0.2 ml of 25% trichloroacetic acid. Next, 0.3 ml of acid-washed Norit (Pfansteihl), 100 mg per ml, and 1.5 ml of 0.1 M sodium acetate were added with thorough mixing. After at least 10 minutes, the Norit was thoroughly resuspended and 0.5 ml of the suspension was rapidly pipetted onto a membrane filter (Schleicher and Schuell, No. B6). The filters were rinsed with 15 ml of 0.05 M sodium acetate buffer, pH 4.5, followed by 5 ml of water, and counted in a Nuclear-Chicago thin end window, gas flow counter.

^{32}P-Pyrophosphate was prepared according to the method of Spalding and Mueller (10). The pyrolysis yielded 98% ^{32}P-pyrophosphate, which was used without further purification. Pyrophosphate and orthophosphate were determined by colorimetric assay (11).

^{14}C-Amino Acid Attachment to Soluble RNA—This procedure is similar to that used by other workers (12, 13). The reaction mixture (final pH, 7.3) contained, in a volume of 0.5 ml, 50 μmoles of Tris-HCl buffer (pH 7.3), 5 μmoles of $MgCl_2$, 5 μmoles of KCl, 1 μmole of GSH (pH 7.0), 1 μmole of dipotassium ATP (pH 6.8), 0.01 μmole of ^{14}C-L-phenylalanine (10 μc per μmole) or 0.02 μmole of ^{14}C-DL-*p*-fluorophenylalanine (3.5 μc per μmole), 0.5 mg of soluble RNA, and a limiting amount of enzyme. In-

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cubation was for 15 minutes at 37°. The reaction was terminated by addition of 3 ml of cold (−10°) 67% ethanol containing 0.5 M NaCl and 1 mg of L-phenylalanine or 2 mg of DL-*p*-fluorophenylalanine per ml. The precipitates were centrifuged at 17,000 × *g* for 15 minutes, and the pellet was resuspended in 3 ml of the ethanol-NaCl-amino acid solution and filtered through a membrane filter. The RNA precipitate was washed on the filter with 10 ml of ethanol-NaCl-amino acid solution, followed by 5 ml of 67% ethanol (both at −10°). The filters were dried in air and counted in a thin end window, gas flow counter.

Soluble RNA was prepared from *E. coli* by a procedure already described (14). The concentration of soluble RNA was calculated on the assumption that a solution containing 1 mg of soluble RNA per ml has an optical density at 260 mμ of 24 with a 1-cm light path.

Other Assays—Protein was determined by the phenol method (15).

Enzyme Purification

Crude Extract—The purification scheme is similar to that reported by Conway *et al.* (5). Cells growing exponentially in the enriched medium were harvested with a Sharples centrifuge and washed once with 0.006 M potassium phosphate buffer, pH 7.3. DNase (4 μg per ml) and $MgSO_4$ (0.005 M) were added to the cells, which were resuspended in buffer before breakage in a French pressure cell. Unbroken cells and debris were removed by centrifugation at 18,000 × *g* for 1 hour. Before being assayed, a portion of the cell-free extract was filtered through a column (26 cm high and 1.2 cm in diameter) of coarse Sephadex G-25 (Pharmacia Fine Chemicals) with 0.006 M potassium phosphate, pH 7.3, as the eluent.

Centrifugation at 105,000 × g—The crude extract was centrifuged in the cold at 105,000 × *g* for 6 hours. The upper nine-tenths of the supernatant was used for further purification. A portion was treated with Sephadex gel before being assayed.

Acid Fractionation—The 105,000 × *g* supernatant was placed in an ice bath, and the pH was adjusted by addition of 0.2 N H_2SO_4 during constant stirring. Fractions were collected at pH 5.1, 4.9, 4.7, 4.5, and 4.2 by centrifugation at 27,000 × *g* for 15 minutes. Pellets were resuspended in 0.006 M potassium phosphate, pH 7.3, and assayed.

Ammonium Sulfate Fractionation—A saturated solution of ammonium sulfate (Mann, enzyme grade) was added to the most active fractions pooled from the previous step while the pH was held constant at 7.3. Precipitation was allowed to occur for 30 minutes at 0°. Fractions taken at 30, 40, 45, 50, and 55% ammonium sulfate saturation were collected by centrifugation at 27,000 × *g* for 20 minutes. The pellets were dissolved in 0.006 M β-mercaptoethanol-0.006 potassium phosphate, pH 7.3, and dialyzed overnight against 100 volumes of the same solution before being assayed.

Diethylaminoethyl Cellulose Chromatography—The most active fractions were pooled for column chromatography. Diethylaminoethyl cellulose (Schleicher and Schuell, type 40) was washed repeatedly with 0.006 M β-mercaptoethanol-0.006 potassium phosphate, pH 7.3, and used to make a column 24 cm high and 1 cm in diameter. The column was then packed under a pressure of 7 p.s.i. before addition of 50 mg or less of protein. Elution was performed with a linear gradient of potassium phosphate, pH 7.3, from 0.01 M to 0.15 M, containing 0.006 M β-mercaptoethanol. Approximately 40 5-ml fractions were collected. Portions of each tube were assayed directly.

RESULTS AND DISCUSSION

Properties of Crude Extracts

Crude extracts were prepared from mutant and wild cells harvested during exponential growth in a glucose-salts medium as described above for the enzyme purification, except that DNase and $MgSO_4$ were not added. After dialysis or Sephadex gel filtration, these crude extracts were assayed for phenylalanyl-RNA synthetase activity in three different assay systems. From Table I it is clear that the extract from the mutant has a markedly decreased ability to activate the phenylalanine analogue, *p*-fluorophenylalanine, as measured by hydroxamate formation, by attachment to soluble RNA, and by pyrophosphate-ATP exchange. The ability of crude extracts of a wild strain of *E. coli* to activate *p*-fluorophenylalanine has previously been shown by other workers (16).

To examine the specificity of the alteration in the mutant, extracts were assayed with other analogues of phenylalanine. The results given in Table II show that the alteration is, indeed, very specific. The extract from the mutant strain retains the ability to activate *o*- and *m*-fluorophenylalanine and β-2-thienylalanine.

Undialyzed extracts prepared from a mixture of wild and mutant cells give activities with *p*-fluorophenylalanine which are predictable from the amount of activity found in the separate extracts. There is no indication, then, that the mutant cells lack a stimulatory, or possess an inhibitory, factor involved in activation of the analogue. Purification of the phenylalanyl-RNA synthetase from the two strains was next undertaken to support the notion that a mutation had directly altered the substrate specificity of this enzyme.

Purification of Enzymes

The phenylalanyl-RNA synthetase preparations purified as described in "Experimental Procedure" contain only low levels

TABLE I

Comparison of mutant and wild extracts in activation of phenylalanine and p-fluorophenylalanine

Details of the three assay systems are given in "Experimental Procedure." The values are averages of duplicate determinations of ±10% precision.

Assay system	Specific activity*	
	Wild	Mutant
A. Hydroxamate formation		
Of phenylalanine	0.087	0.126
Of *p*-fluorophenylalanine	0.112	<0.010
B. Attachment to soluble RNA		
Of phenylalanine	0.059	0.030
Of *p*-fluorophenylalanine	0.019	0.0002
C. Pyrophosphate-ATP exchange		
With phenylalanine	0.800	0.490
With *p*-fluorophenylalanine	0.210	<0.050

* The data are expressed as micromoles of (A) phenylalanine hydroxamate, (B) ^{14}C-amino acid attached to RNA, or (C) ^{32}P-pyrophosphate exchanged per mg of protein per hour.

TABLE II

Comparison of mutant and wild extracts in activating phenylalanine and several of its analogues

The assay system is described in "Experimental Procedure." The values are averages of duplicate determinations of ±10% precision.

Substrate	Specific activity*		Ratio, (B × 100)/A
	Wild (A)	Mutant (B)	
Phenylalanine	0.086	0.180	209
β-2-Thienyl-L-alanine	0.091	0.140	154
o-Fluorophenylalanine	0.061	0.072	85
m-Fluorophenylalanine	0.170	0.141	83
p-Fluorophenylalanine	0.170	0.006	4

* The data are expressed as micromoles of hydroxamate per mg of protein per hour. In this experiment, the final pH of the assay system was 7.5. The increased activity of the wild-type extract with p-fluorophenylalanine relative to that with phenylalanine compared to Table I results from the increased pH. In all cases, the hydroxamate of phenylalanine was employed as a colorimetric standard.

TABLE III

Comparison of phenylalanine-dependent with p-fluorophenylalanine-dependent pyrophosphate-ATP exchange during purification of enzyme from wild strain

The assay conditions are described in "Experimental Procedure." The values are averages of duplicate determinations of ±10% precision.

Enzyme fraction	Total protein	Specific activity		Ratio, A:B	Yield
		Phenylalanine (A)	p-Fluorophenylalanine (B)		
	mg	*μmoles PP_i/mg protein/hr*			%
Crude	4500	0.80	0.21	3.8	100
Supernatant from centrifugation at 105,000 × *g* for 6 hours	1850	2.07	0.51	4.1	102
pH 4.9 to 4.7	364	5.52	1.46	3.8	54
50% ammonium sulfate	49	18.1	5.25	3.5	24
DEAE-cellulose, Fractions 26 to 28	2.9	164.0	39.2	4.2	13

of activity for some other amino acids as measured by pyrophosphate-ATP exchange. Preparations contain no detectable protease or ATPase activities. Table III compares the phenylalanine-dependent with the p-fluorophenylalanine-dependent pyrophosphate-ATP exchange activity during purification of enzyme from the wild strain. The ratio of activity stimulated by phenylalanine to that stimulated by p-fluorophenylalanine remains essentially constant, a good indication that the same enzyme is responsible for activation of both amino acids. The same comparison is shown in Table IV for purification of enzyme from the mutant strain. The difference in the elution position of the enzyme from the wild and that from the mutant strain is probably due to a difference in flow rates used with the DEAE-cellulose columns during the two purifications. The relation of the elution profile of enzyme activity to the profile of total protein in both instances was the same.

Assay of both purified enzyme preparations by hydroxamate formation and by attachment to soluble RNA also indicates a 200-fold purification of activity for phenylalanine and p-fluorophenylalanine. The purified enzymes activate o- and m-fluorophenylalanine and β-2-thienylalanine as measured by pyrophosphate-ATP exchange. The ability of purified preparations of the normal phenylalanyl-RNA synthetase to activate analogues of phenylalanine is in agreement with the results reported by Conway *et al.* (5).

Comparison of Properties of Purified Enzymes

The ability of the purified enzymes to activate p-fluorophenylalanine relative to phenylalanine in the three assay systems is presented in Table V. These results provide evidence that enzyme from the mutant has a greatly diminished ability to utilize p-fluorophenylalanine as a substrate when measured by hydroxamate formation, by attachment to soluble RNA, and by pyrophosphate-ATP exchange. An altered enzyme from

TABLE IV

Comparison of phenylalanine-dependent with p-fluorophenylalanine-dependent pyrophosphate-ATP exchange during purification of enzyme from mutant

The details are identical with those for the purification results shown in Table III.

Enzyme fraction	Total protein	Specific activity		Ratio, A:B	Yield
		Phenylalanine (A)	p-Fluorophenylalanine (B)		
	mg	*μmoles PP_i/mg protein/hr*			%
Crude	5000	0.49	<0.05		100
Supernatant from centrifugation at 105,000 × *g* for 6 hours	1870	1.22	<0.05		93
pH 4.7 to 4.5	284	2.85	<0.05		33
50% and 55% ammonium sulfate	42.2	13.5	0.2	68	23
DEAE-cellulose, Fractions 19 and 20	2.2	96.0	2.8	34	9

TABLE V

Comparison of mutant and wild purified enzymes in activation of phenylalanine and p-fluorophenylalanine

Enzyme preparations purified as recorded in Tables III and IV were assayed in the three systems described in "Experimental Procedure." The values are averages of duplicate determinations of ±10% precision.

Assay system	Activity with p-fluorophenylalanine*	
	Wild	Mutant
1. Hydroxamate formation	107	5
2. Attachment to soluble RNA	34	2
3. Pyrophosphate-ATP exchange	24	3

* The data are expressed as percentages of the activities obtained with phenylalanine.

the mutant was anticipated from the results obtained with mixed crude extracts. The significance of the finding that there is a difference in the ratio of activity of *p*-fluorophenylalanine to phenylalanine in the three assay systems (Table V) is not well understood. Perhaps the widely different assay conditions exert unequal effects on the apparent K_m for the two amino acids.

Next, other properties of the purified enzymes were measured in an attempt to detect additional consequences of the mutation.

Lineweaver-Burk plots of the effect of increasing concentrations of phenylalanine and ATP on the formation of phenylalanine hydroxamate by enzymes from both wild and mutant strains are shown in Fig. 1. Lines through the experimental points are drawn with their origins deliberately at the same point on the abscissa. This was done to demonstrate that no significant differences in the K_m values for phenylalanine or ATP between the enzymes from the two strains can be detected. The K_m for L-phenylalanine is approximately 4.4×10^{-4} M, and that for ATP, approximately 1.1×10^{-3} M. The K_m for DL-*p*-fluorophenylalanine with the enzyme from the wild strain is about 8.0×10^{-4} M.

The heat stabilities of the two enzymes, however, were found to differ significantly. Fig. 2 shows that the enzyme from the wild strain loses activity at 50° at 4 times the rate of the enzyme from the mutant strain. This result provides independent evidence that the mutant strain, PFP-10, possesses a structurally altered phenylalanyl-RNA synthetase.

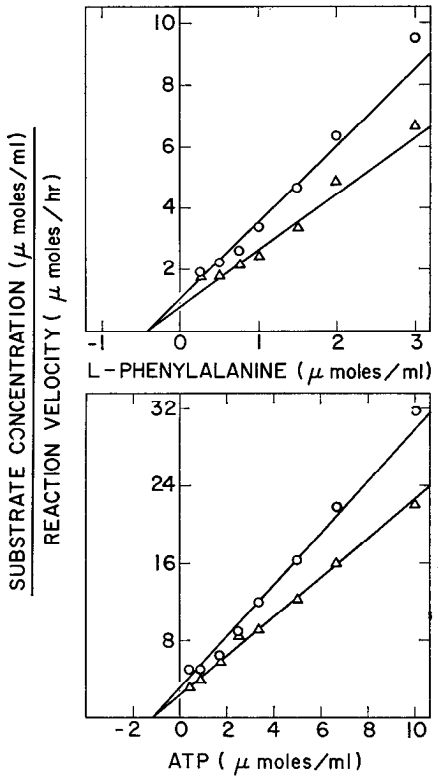

FIG. 1. Lineweaver-Burk plots for determination of K_m values for L-phenylalanine and ATP with purified enzymes from mutant and wild strains. Reaction mixtures were as described in "Experimental Procedure" except that the total volume was 0.3 ml and the final concentrations of L-phenylalanine and ATP were as indicated. The incubation mixtures contained 16.6 μg of enzyme from the wild strain (○) or 17.6 μg enzyme from the mutant strain (△).

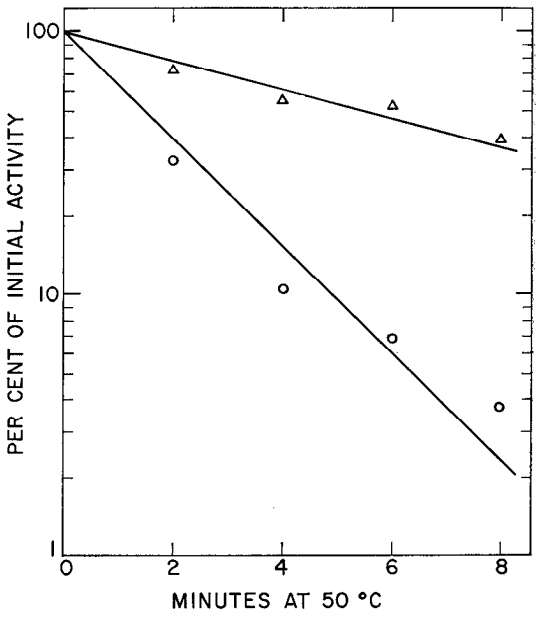

FIG. 2. Heat stabilities of enzymes from mutant and wild strains. Enzyme from the wild strain (1.84 μg) or the mutant strain (1.96 μg) in 0.1 ml of 0.015 M potassium phosphate, pH 7.3, and 0.006 M β-mercaptoethanol was incubated at 50° for the times indicated. Activity was determined with the pyrophosphate-ATP exchange assay described in "Experimental Procedure" with the use of phenylalanine. Values obtained with enzyme from wild strain (○) and mutant strain (△) are shown.

SUMMARY

A mutant of *Escherichia coli* has been isolated which is resistant to the growth-inhibitory effects of *p*-fluorophenylalanine, an analogue of phenylalanine. Assays with crude cell-free extracts suggested that the resistance was due to an inability to activate the analogue. To support the idea that the mutant strain possesses an altered phenylalanyl ribonucleic acid synthetase, purification of enzyme from wild and mutant strains was performed. The purified enzyme from the mutant strain, compared to that from the wild strain, has a greatly diminished ability to activate *p*-fluorophenylalanine. The alteration is very specific, since the enzyme from the mutant retains the ability to utilize *o*- and *m*-fluorophenylalanine and β-2-thienylalanine as substrates. Other properties of the enzymes were measured in an attempt to detect additional consequences of the mutation. No differences between the two enzymes in K_m values for phenylalanine and adenosine triphosphate could be detected. A large difference in the heat stabilities of the two enzymes, however, was easily demonstrated.

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