Protein and Ribonucleic Acid Synthesis in a Mutant of
Escherichia coli with an Altered Aminoacyl Ribonucleic Acid Synthetase*

WALTON L. FANGMAN† AND FREDERICK C. NEIDHARDT

From the Department of Biological Sciences, Purdue University, Lafayette, Indiana

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At present it is thought that adenosine triphosphate-mediated activation of the α-carboxyl group of amino acids is a necessary step in the synthesis of at least some bacterial proteins. Evidence for the obligatory role of this activation has been available mainly from studies in vitro of protein synthesis (e.g. Simpson (2)), but a mutant of Escherichia coli has recently been isolated (1) which allows supplementation of this information with physiological and genetic evidence.

For normal strains of E. coli, p-fluorophenylalanine is known to be bacteriostatic (3-5). Inhibition in these strains is accompanied by massive, random substitution of the analogue for phenylalanine in newly made protein (5, 6). This incorporation has been related to the ability of the phenylalanyl ribonucleic acid synthetase to utilize p-fluorophenylalanine (7).

In a companion paper (8), it has been shown that a mutant strain of E. coli, PFP-10, which we have isolated, possesses an altered phenylalanyl-RNA synthetase with a greatly diminished ability to activate p-fluorophenylalanine. It seemed worthwhile, therefore, to measure quantitatively the incorporation of analogue into protein by this strain. The results should allow an assessment of the role of activation by such enzymes in protein synthesis.

Furthermore, with this mutant it is possible to determine whether activation of the analogue is required to permit synthesis of RNA. Gros (9) has shown that amino acids are required in very small amounts for synthesis of RNA. Whether the requirement is fulfilled by amino acids per se, by one of their activated forms (10, 11), or by their actual incorporation into protein (12) has not been determined.

EXPERIMENTAL PROCEDURE

Materials

All chemicals used are commercially available and were of the purest grade available. DL-p-Fluorophenylalanine-3-14C (3.5 μC per μmole) was purchased from Volk Radiochemical Company.

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The glucose-salts medium consisted of 0.4% glucose in basal salts Solution P (13). When L-phenylalanine was used as a supplement, the concentration was 2.4 × 10⁻⁴ M. DL-p-Fluorophenylalanine was used in all experiments at a concentration of 10⁻³ M.

The wild strain used in these experiments was E. coli KB, and the p-fluorophenylalanine-resistant strain derived from it has been designated PFP-10 (8). Phenylalanine auxotrophs were isolated from KB and PFP-10 by the penicillin technique of Gorini and Kaufman (14) after mutagenesis by ethyl methane-sulfonate.

Methods

Method of Cultivation—Cells were grown aerobically on a rotary action shaker at 37°.

Measurement of 14C-p-Fluorophenylalanine Uptake—Exponentially growing cells were chilled and harvested from a glucose-salts medium and resuspended in the same medium to about 2 × 10⁹ cells per ml. Portions of the concentrated cultures were incubated for 1 minute at 37° with 50 μg of chloramphenicol (Parke-Davis) per ml. W-p-Fluorophenylalanine (0.8 μC per μmole) was then added to 0.001 M. After incubation for an additional 4 or 8 minutes, duplicate samples of 0.3 ml containing approximately 0.35 mg of cell protein were filtered (Schleicher and Schuell, No. B4) and washed with 25 ml of cold Solution P. At the same time, duplicate 0.3-ml samples were added to tubes containing 0.3 ml of cold 10% trichloroacetic acid. After standing at 0° for 30 minutes, these samples were filtered and washed with 15 ml of 5% trichloroacetic acid. Appropriate corrections were made for amino acid adsorbed to the filter.

Fractionation of Cells—After exposure of growing cells to 14C-p-fluorophenylalanine, chilled samples of cells were washed once with 0.001 M Tris-HCl solution, pH 7.3, containing 0.01 M MgCl₂. They were resuspended in a small volume of the same solution and DNase was added to 10 μg per ml. The cells were then broken in a French pressure cell at 3,200 to 4,100 p.s.i. This preparation was centrifuged at 15,900 × g for 20 minutes to sediment unbroken cells, cell walls, and membranes. The supernatant liquid was used for preparation of the soluble and ribosomal protein fractions.

The pellet obtained from the previous centrifugation was resuspended in 0.001 M Tris-HCl solution, pH 7.3, containing 0.01 M MgCl₂ and 0.001 M p-fluorophenylalanine, and centrifuged at 5,000 × g for 5 minutes. The pellet was discarded, and the
supernatant liquid was again centrifuged at 5,000 x g for 5 minutes. The supernatant from this centrifugation was then centrifuged at 30,000 x g for 20 minutes. The pellet, called the membrane fraction, was dissolved in 1 N NH₄OH and diluted, and portions were assayed for protein and radioactivity. Radioactivity was determined in a thin end window, gas flow Geiger counter.

The supernatant liquid obtained after clearing the extract of unbroken cells, walls, and membranes was centrifuged at 130,000 x g for 90 minutes. The top nine-tenths of the supernatant was carefully removed and used to obtain the soluble protein fraction. The remaining supernatant liquid was discarded, and the pellets were thoroughly resuspended in Tris-MgCl₂ solution and then centrifuged again at 130,000 x g for 90 minutes. The pellets were resuspended and used to obtain the ribosomal protein fraction. This procedure for separation of ribosomal and soluble components is based on that developed by Tissières and Watson (15).

The protein component of these two fractions was then purified (16) to yield the soluble and ribosomal protein fractions, which were assayed for radioactivity and protein.

**Assays**—Protein was determined by the phenol method (17) and RNA by the orcinol method (18).

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**RESULTS**

**Uptake of p-Fluorophenylalanine into Wild and Mutant Cells**—To ascertain whether there is impeded entry of analogue into the mutant, uptake of ¹⁴C-p-fluorophenylalanine was measured in the absence of protein synthesis as described in "Methods." Total accumulation of ¹⁴C-p-fluorophenylalanine by the wild and mutant strains under these conditions was essentially the same, 3.54 and 3.52 μmoles per g of cell protein, respectively. The accumulated analogue could be completely extracted with cold 5% trichloroacetic acid. These results, together with the fact that the analogue induces a transient but marked growth inhibition of the mutant strain (a phenomenon to be reported later), support the conclusion that p-fluorophenylalanine is rapidly taken up by the mutant.

**Effect of Altered Enzyme on Incorporation of Analogue into Phenylalanine-deprived Cells**—Incorporation of the analogue into protein was first measured under conditions which eliminate competition by endogenously synthesized phenylalanine. To accomplish this, phenylalanine auxotrophs derived from the wild (KB) and the mutant (PFP-10) strain were employed. Cultures of these auxotrophs were deprived of phenylalanine and provided with ¹⁴C-p-fluorophenylalanine. The analogue was rapidly incorporated into protein by the normal auxotroph, but incorporation into protein by the auxotroph with the altered enzyme occurred at a rate which was barely detectable. The rates of incorporation differed approximately 20-fold (Fig. 1).

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**Fig. 1.** Incorporation of ¹⁴C-p-fluorophenylalanine into phenylalanine auxotrophs of *E. coli* KB and PFP-10 deprived of phenylalanine. Cultures growing exponentially in a glucose-salts medium containing 2.4 X 10⁻⁸ M phenylalanine were chilled and centrifuged, then transferred to a glucose-salts medium containing 0.001 M p-fluorophenylalanine-³⁻¹⁴C (0.08 μc per μmole). Samples of cells taken at intervals were added to chilled tubes, and trichloroacetic acid was added to 5%. The protein fraction of each sample was extensively purified by a series of extractions (16), and a portion was measured for radioactivity. Incorporation into protein of the auxotroph derived from wild (KB) strain (○—○) and into protein of auxotroph derived from mutant (PFP-10) strain (△—△) is shown.

**Fig. 2.** Net protein and RNA synthesis by phenylalanine auxotrophs of *E. coli* KB and PFP-10 deprived of phenylalanine and supplemented with p-fluorophenylalanine. Cultures growing exponentially in a glucose-salts medium containing 2.4 X 10⁻⁸ M phenylalanine were chilled and centrifuged at zero time, then transferred to a glucose-salts medium containing 0.001 M DL-p-fluorophenylalanine. At intervals during the subsequent incubation, 5-ml samples were removed, chilled, and brought to 5% trichloroacetic acid. After extraction with cold acid, the cell pellets were analyzed for protein and RNA (17, 18). Protein (○—○) and RNA (●—●) synthesis by the wild strain (KB) and protein (△—△) and RNA (▲—▲) synthesis by the mutant strain (PFP-10) are indicated. (Control cultures of KB and PFP-10 unsupplemented with analogue at zero time show no synthesis of protein or RNA.)
Ability of p-Fluorophenylalanine to Replace Phenylalanine in Supporting Net Protein and RNA Synthesis in Wild and Mutant Strains—To measure total protein and RNA synthesis permitted by p-fluorophenylalanine when substituted for phenylalanine, the experiment with the auxotrophs was repeated. The normal auxotroph and the auxotroph with the altered enzyme were deprived of phenylalanine and provided with the analogue, and total protein and RNA synthesis was measured. p-Fluorophenylalanine allowed considerable net increase of protein and RNA in the normal auxotroph; Fig. 2 shows that protein and RNA synthesis occurred at a rapid rate for at least 80 minutes. The initial rate of protein synthesis on addition of the analogue is at least 95% of the initial rate on addition of phenylalanine. In the mutant with the altered enzyme, however, the analogue was completely ineffective in permitting net protein or RNA synthesis. Consistent with the specificity of the alteration of the phenylalanyl-RNA synthetase in the mutant strain, α- or m-fluorophenylalanine allowed a similar rate of increase in mass by auxotrophs derived from both KB and PFP-10.

Effect of Altered Enzyme on Incorporation of Analogue into Soluble, Ribosomal, and Membrane Protein—To measure incorporation of $^{14}$C-p-fluorophenylalanine under conditions of synthesis of a full complement of enzymatically active and structural protein, the prototrophic strains were used. $^{14}$C-p-Fluorophenylalanine (0.001 M) was added to mutant (PFP-10) and wild (KB) cells growing exponentially in an un supplemented glucose-salts medium. Samples of cells taken at intervals after addition of the analogue were fractionated into three components as described in “Methods.” Fig. 3 shows the incorporation of $^{14}$C-p-fluorophenylalanine into soluble, ribosomal, and membrane proteins by the wild and mutant strains. The rates of analogue incorporation into all three fractions by the mutant strain, which has the altered aminosy1-RNA synthetase, are decreased to a similar extent (92 to 95%) relative to the wild strain.

**Discussion**

The results reported in this paper show that a cell which possesses an aminosy1-RNA synthetase with a greatly decreased ability to activate a substrate has a greatly diminished ability to utilize that substrate for (a) incorporation into soluble and particulate proteins and (b) permission of net synthesis of protein and RNA.

To conclude that these properties of the mutant are a consequence of the alteration in the enzyme requires the assumption that PFP-10 differs from its parental wild strain only in this protein. This assumption is reasonable, but proof of its validity is difficult. There is evidence which suggests that a single protein change is involved. The mutant appears to possess a normal mechanism for analogue uptake. Furthermore, it has been shown that the mutant possesses a structurally altered phenylalanyl-RNA synthetase (8), and therefore the genetic lesion is more likely to be in a structural cistron than in an operator region which might control the synthesis of several proteins. Genetic mapping of the lesion is being carried out to demonstrate that only one cistron has been altered in the mutant strain. (Support for the single nature of the genetic lesion is provided by the fact that mutants with properties identical with those described here have been isolated in this laboratory by short selective steps without induced mutagenesis.) Demonstration of a single cistron difference, however, cannot be taken as proof that there is only one protein different in the two strains.

If the mutant does differ from its parent only in the one enzyme, then the results reported here directly indicate that some one or more steps in the conversion of free p-fluorophenylalanine to aminosy1-RNA is obligatory for the incorporation of this analogue into the bulk of the soluble and particulate protein of E. coli and for permission of RNA synthesis. Furthermore, it one assumes that the analogue is utilized in a way identical with phenylalanine, this conclusion is also valid for natural amino acids.

Actually, there is good evidence which indicates that E. coli utilizes p-fluorophenylalanine for protein synthesis in the same manner as phenylalanine. Richmond (6) has discussed the evidence that p-fluorophenylalanine is very similar to phenylalanine in size, shape, and ionic properties. p-Fluorophenylalanine has been found to be incorporated into E. coli only at the expense of phenylalanine and not of any other amino acids in protein (5). After incorporation of $^{14}$C-p-fluorophenylalanine by a wild strain of E. coli, Cowie et al. (19) chromatographed the soluble proteins on diethylaminoethyl cellulose. Measurement of the specific radioactivities of the different fractions obtained showed a similar degree of incorporation of analogue. These results indicated little, if any, discrimination against the analogue for incorporation into many classes of soluble protein. In the
case of a specific protein, alkaline phosphatase of *E. coli*, p-fluorophenylalanine has been found to substitute randomly for phenylalanine residues (6).

An efficient and essentially normal utilization of p-fluorophenylalanine is indicated also by the high degree of replacement (75%) of phenylalanine by the analogue (5), by the high absolute rate of analogue incorporation into both soluble and particulate proteins by the wild strain, and by the ability of the analogue to permit a doubling of protein in a normal auxotroph starved of phenylalanine.¹

**SUMMARY**

Synthesis of protein and ribonucleic acid has been examined in a mutant of *Escherichia coli* possessing a phenylalanyl ribonucleic acid synthetase with a decreased ability to utilize p-fluorophenylalanine. The mutant, which appears to have an unaltered mechanism for uptake of p-fluorophenylalanine, was found to have a greatly reduced ability to incorporate the analogue into ribosomal, soluble, and membrane proteins. In addition, an auxotrophic derivative of the mutant, unlike a similar derivative of the wild strain, was incapable of utilizing the analogue in place of phenylalanine to allow net protein or RNA synthesis.

If one assumes that the mutant differs from the wild strain only in this one enzyme, it may be concluded that adenosine triphosphate-dependent activation of p-fluorophenylalanine is requisite for its incorporation into the bulk of the soluble and particulate proteins of *E. coli* and for permission of RNA synthesis. Evidence which indicates that *E. coli* utilizes p-fluorophenylalanine in a manner identical with phenylalanine suggests that this conclusion is valid for the utilization of natural amino acids as well.

**REFERENCES**


¹ Note: After submission of this paper we received a report (20) of the isolation of a mutant of *Coprinus lagopus* which appears to have an altered activation and incorporation of ethionine.
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