INCORPORATION OF MONOFLUOROTRYPTOPHANS INTO PROTEIN DURING THE GROWTH OF *ESCHERICHIA COLI*

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Received January 12, 1970

SUMMARY: DL-Tryptophan substituted with a fluorine in either the 4, 5, or 6 position is a potent inhibitor of the growth of wild type *E. coli* and is capable of supporting some growth in a strain that lacks both tryptophanase and tryptophan synthetase. In both situations, the fluorotryptophan is incorporated into bacterial protein in place of tryptophan. 4-Fluorotryptophan is superior to the 5- and 6-isomers with respect to ability to support growth of the mutant which cannot synthesize tryptophan and to allow synthesis of active β-galactosidase in this strain.

Tryptophan residues are located at or near catalytic or binding sites in a variety of enzymes (1). It is therefore of considerable interest to be able to attach "reporter groups" (2) to tryptophan residues in intact proteins. In order to determine the feasibility of studying the environment of tryptophan residues in enzymes by means of ^{19}F nmr spectroscopy, we have studied the *in vivo* incorporation of 4-, 5-, and 6-fluorotryptophan into the protein of *E. coli*. Some aspects of the *in vivo* incorporation of 5- and 6-fluorotryptophan into *E. coli* protein have been investigated previously (3). The biological effects of 4-fluorotryptophan are described for the first time.

MATERIALS AND METHODS

4-, 5-, and 6-Fluorotryptophan were purchased from Sigma Chemical Co. and 3-methylanthranilic acid from Aldrich Chemical Co. *E. coli* Strain TIR-8 (4) was obtained from Dr. Paolo Truffa-Bacchi. A strain of *E. coli* lacking tryptophanase (*tna*$^-$) and with an absolute requirement for tryptophan for growth was prepared by transduction using bacteriophage P1kc. A *cysB*$^+$, tryptophan synthetase deletion mutant (A-C9) served as donor and a *tna*$^-$ *cysB*$^-$ strain was the recipient; both were derived from strain W1100 and obtained from Dr. C. Yanovsky. A transductant was selected that grew on mineral-glucose medium supple-

mented only with tryptophan but that did not grow in the absence of tryptophan.

Cultures were grown aerobically at 37°C in Vogel-Bonner medium (5) with 1% glucose and 1% Vitamin-free Casamino acids (Difco). This medium was otherwise supplemented as indicated for various experiments. Growth was estimated turbidimetrically using a Klett-Summerson colorimeter. The incorporation of glycine-1-^{14}C into cellular material was determined by removing samples of cells from growing cultures at intervals, collecting the cells on 0.45 μ Millipore filters, and subsequently washing them with ^{14}C-free medium.

Tryptophan and the fluorotryptophans were obtained non-destructively from cellular protein by the alkaline hydrolytic method described by Stahl (6), except that the tubes were evacuated before sealing. Two methods were used for the analysis of the basic amino acids. The first involved chromatography on a 0.9×55-cm. column of Beckman AA-15 resin. For elution at 70 ml/hr and 55°C with pH 5.26, 0.35*N* citrate buffer, the following elution times (min) were observed: tryptophan, 133; 4-fluorotryptophan, 167; 5-fluorotryptophan, 178; 6-fluorotryptophan, 205. A second system employed a 0.9×18-cm. column of AA-27 resin eluted at 70 ml/hr with pH 4.25, 0.4*N* citrate buffer for 125 min at 32.5°C, then with the pH 5.26 buffer at 55°C. In this case, the following elution times (min) were observed: tryptophan, 167; 4- and 5-fluorotryptophan, 185; 6-fluorotryptophan, 191.

β-Galactosidase was assayed by measuring the enzyme-catalyzed rate of release of *o*-nitrophenol from 0.01 *M* *o*-nitrophenyl-β-D-galactoside in 0.1 *M* phosphate buffer (pH 8.0) at 410 nm. Protein was determined with the biuret reagent using bovine serum albumin as standard.

RESULTS

A strain of *E. coli* lacking both tryptophanase and tryptophan synthetase was prepared by the transduction procedure described in Methods and Materials. When cells of this strain which had been grown in a medium supplemented with L-tryptophan were harvested by centrifugation and resuspended in a medium lacking tryptophan, no growth was observed. However, addition of DL-4-, 5-,

or 6-fluorotryptophan to the medium permitted a limited amount of growth (estimated turbidimetrically) to occur (see Figure 1 for growth curve in the presence of Dl-4-fluorotryptophan). DL-5-Fluorotryptophan ($10^{-4}M$) present in the medium supported growth for 1.3 generations; DL-6-fluorotryptophan at the same concentration for 1.6 generations; and DL-4-fluorotryptophan for 2.0 generations. When ^{14}C-glycine was present in the medium containing DL-4-fluorotryptophan, radioactivity was incorporated into the cells in a manner which paralleled the increase in Klett units (Figure 1), indicating that the increase in turbidity reflected protein synthesis.

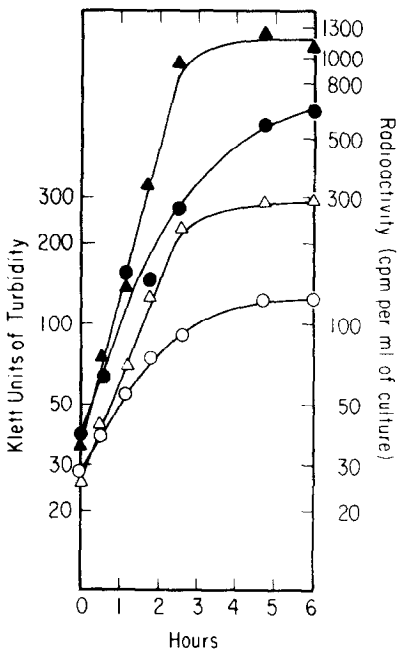

Figure 1. A comparison of growth curves (open dots) and time courses for the uptake of ^{14}C-glycine into cells (solid dots) for the *E. coli* strain unable to synthesize tryptophan grown in the presence of tryptophan (triangles) or 4-fluorotryptophan (circles).

Cells grown for at least one generation in the presence of one of the monofluorotryptophans exhibited swelling, odd shapes, and irregular contrast (Figure 2). Similar swelling has been observed for cells grown in the presence of D_2O (7) or trifluoroleucine (8) and is possibly a consequence of the increased synthesis of various enzymes whose efficiencies are lowered by incorporation of the amino acid analogs.

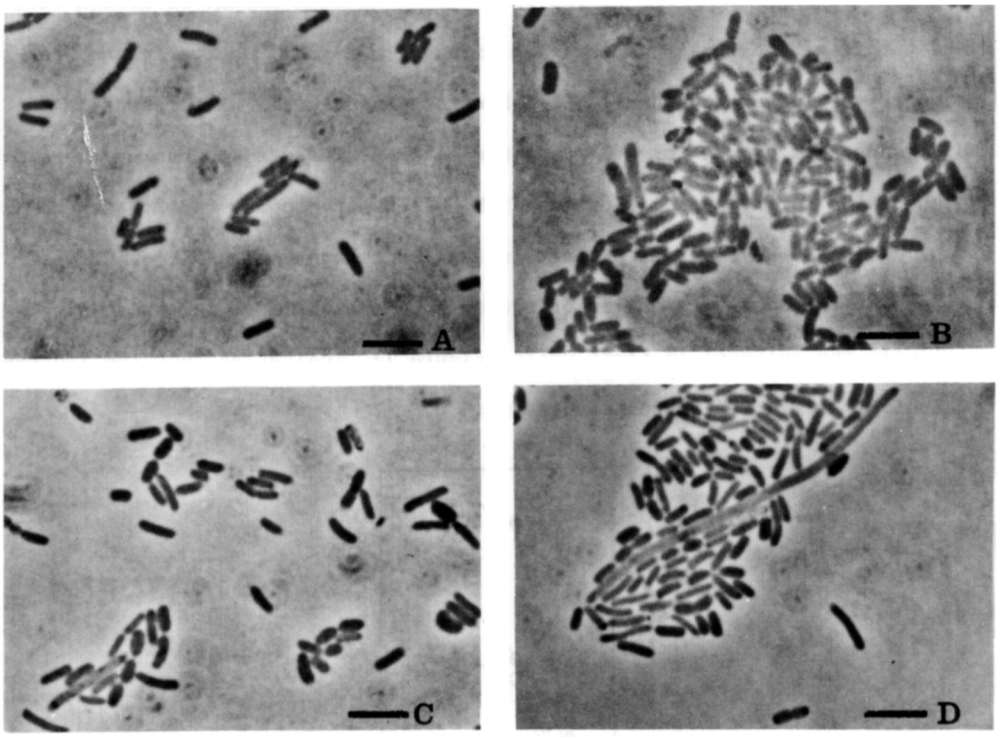

Figure 2. Phase-contrast photomicrographs of cells of *E. coli* unable to synthesize tryptophan grown for at least one generation in the presence of $10^{-4}M$ DL-tryptophan or the same concentration of DL-4-, 5-, or 6-fluorotryptophan, respectively. The line in each case equals 5 μ. A, tryptophan; B, 4-fluorotryptophan; C, 5-fluorotryptophan; D, 6-fluorotryptophan.

Incorporation of 4-, 5-, or 6-fluorotryptophan into bacterial protein was demonstrated by amino acid analyses performed on barium hydroxide hydrolysates (see Methods and Materials). In all three cases, chromatograms of protein isolated from cells grown in a medium supplemented by L-tryptophan were very similar to those of protein from cells grown in media supplemented with a monofluorotryptophan and lacking tryptophan, except that in the latter cases the tryptophan peak was reduced in area and a new peak having the elution time characteristic of the appropriate monofluorotryptophan appeared. The extent of incorporation of the monofluorotryptophans into the protein was close to that expected on the basis of the increase in turbidity that occurred in the presence of the analog. For example, protein isolated from cells grown two

generations in the presence of DL-4-fluorotryptophan had only 25% (±10%) of the normal tryptophan content, the other 75% being replaced by 4-fluorotryptophan.

The monofluorotryptophans were potent inhibitors of the growth of *E. coli* Strain TIR-8 (wild type with respect to tryptophan biosynthesis). Even greater inhibition of growth by the monofluorotryptophans was observed when tryptophan biosynthesis was partially inhibited (9) by the addition of 3-methylanthranilic acid (375 μg/ml) to the medium. Incorporation of 4-, 5-, or 6-fluorotryptophan into the protein of this strain was demonstrated directly by amino acid analysis. In these cases, no more than 40-50% of the tryptophan was replaced by a fluorotryptophan before growth ceased. In analogy to the growth patterns observed in the case of the mutant lacking tryptophanase and tryptophan synthetase, the 5-fluorotryptophan was the most potent growth inhibitor of the wild type bacteria and the 4-fluoro isomer the least potent.

The ability of the monofluorotryptophans to permit synthesis of β-galac-

TABLE 1

β-Galactosidase Activity of Extracts of *E. coli* Grown in the Presence of Monofluorotryptophans

Additive (DL, $10^{-4}M$)	% Specific Activity*
none	0.3
4-fluorotryptophan	28
5-fluorotryptophan	5.6
6-fluorotryptophan	0.7

*One hundred percent specific activity (units of β-galactosidase activity/mg protein ×100) is defined as the specific activity of an extract of the strain lacking tryptophanase and tryptophan synthetase which was grown one generation in the presence of DL-tryptophan and isopropylthio-β-D-galactopyranoside.

tosidase in the mutant unable to synthesize tryptophan was determined. In these cases, the bacteria were grown until they had depleted the tryptophan in the medium. The β-galactosidase inducer isopropylthio-β-D-galactopyranoside ($10^{-3}M$) (10) was then added, along with additional DL-tryptophan or one of the monofluorotryptophans ($10^{-4}M$), and the suspension was incubated for six hours at 37°C. As seen in Table 1, addition of 4-, 5-, or 6-fluorotryptophan allowed production of significant amounts of β-galactosidase activity. The 4-isomer was superior to the 5- and 6-isomers in ability to allow formation of active enzyme. Our observation that 5-fluorotryptophan is superior to the 6-isomer in this respect is in qualitative agreement with the findings of Munier *et al.* (3).

DISCUSSION

4-, 5-, and 6-Fluorotryptophan support a limited amount of growth in a strain of *E. coli* which cannot synthesize tryptophan and are inhibitors of the growth of a strain of *E. coli* which is capable of tryptophan biosynthesis. In all six cases, the monofluorotryptophan is incorporated into bacterial protein. This incorporation presumably yields proteins with altered properties, thus altering the growth characteristics of the bacteria. The observations that 4-fluorotryptophan is superior to the 5- and 6-isomers in supporting growth of the mutant unable to synthesize tryptophan and is a less potent growth inhibitor of a wild-type *E. coli* than 5-fluorotryptophan or 6-fluorotryptophan suggest that, on the average, enzymes in which tryptophan is replaced by 4-fluorotryptophan are more similar to the corresponding native enzymes than enzymes in which tryptophan is replaced by either 5-fluorotryptophan or 6-fluorotryptophan. Observations on the ability of the monofluorotryptophans to allow formation of β-galactosidase activity also tend to support this view for a particular enzyme.

Our results on incorporation of 5- and 6-fluorotryptophan into *E. coli* protein and on inhibition of bacterial growth by these analogs differ substantially from those of Munier *et al.* (3), who reported essentially complete replacement of tryptophan in wild-type bacteria by 5- or 6-fluorotryptophan

supplied as the DL mixture at 2.5×10^{-3} *M* and who did not observe a cessation of growth of wild-type and mutant strains after several generations of growth in the presence of the tryptophan analogs. One possible explanation for this discrepancy is that different cultivation conditions and strains of bacteria were employed in the two studies.

We are currently engaged in isolation of enzymes in which tryptophan residues are replaced by monofluorotryptophans.

ACKNOWLEDGMENTS: We wish to thank Dr. C. Yanovsky and Dr. P. Truffa-Bacchi for generously supplying some of the strains used in these experiments. We also wish to acknowledge the excellent technical assistance of Mr. Vazken Tashinian with the amino acid analyses. This research was supported in part by USPHS grants HD-02448 from NICHD to George D. Hegeman and AM-13529 from NIAMD to George L. Kenyon and by unrestricted grants from Research Corporation, E. I. duPont de Nemours, and Merck and Co. to Douglas T. Browne.

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