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Suppression of fluorescence of tryptophan residues in proteins by replacement with 4-fluorotryptophan

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The tryptophan-auxotrophic *Bacillus subtilis* LC33 mutant strain utilizes either tryptophan or 4-fluorotryptophan for growth. Proteins therefore could be isolated from these cells in either tryptophan-containing or 4-fluorotryptophan-containing forms. Since 4-fluorotryptophan is non-fluorescent, tryptophan fluorescence would be suppressed in the 4-fluorotryptophan-containing proteins, facilitating the investigation of other chromophores either on the proteins or interacting with the proteins. This approach, potentially applicable to any protein endogenous to or clonable into *B. subtilis*, was illustrated by an examination of the fluorescence of *B. subtilis* ribosomal proteins.

INTRODUCTION

Fluorine-containing amino acid analogues are useful tools in the investigation of protein structure and function. Especially with the more bulky amino acids, replacement of a hydrogen atom by fluorine brings about only a limited change in chemical properties. Accordingly, fluorophenylalanine (Cohen & Munier, 1959; Yoshida, 1960), fluorotyrosine (Sykes *et al.*, 1974) and fluorotryptophan (Browne *et al.*, 1970; Pratt & Ho, 1975) have been incorporated into cellular proteins, partially substituting for the natural amino acids and thereby permitting ^{19}F-n.m.r. analysis of proteins (Dettman *et al.*, 1985). Trifluoroleucine could replace leucine totally in the growth of adapted but apparently unmutated cells of *Escherichia coli* (Rennert & Anker, 1963). Such total replacement of an amino acid by its analogue eliminates difficulties in interpretation arising from the simultaneous presence of both compounds in the same proteins. In recent years we have obtained from the tryptophan-auxotrophic *Bacillus subtilis* QB928 the mutant strains LC33 and HR15 (Wong, 1983, 1984). Strain LC33 grows nearly as well on 4-fluorotryptophan as on tryptophan. Strain HR15 is de-adapted towards tryptophan; it grows well only on 4-fluorotryptophan but not on tryptophan. For protein investigations, strain LC33 is particularly useful in allowing the ready preparation of proteins containing exclusively either tryptophan or 4-fluorotryptophan. The present study finds that, besides its utility for ^{19}F-n.m.r., 4-fluorotryptophan also does not display the intrinsic fluorescence typical of tryptophan. Its replacement of tryptophan therefore provides a means to suppress tryptophan fluorescence in cellular proteins.

MATERIALS AND METHODS

Materials

The 4-fluoro-, 5-fluoro-, 6-fluoro- and 5-methyl-DL-tryptophan were supplied by Sigma Chemical Co., and trifluoroacetic acid was from Pierce Chemical Co.

General methods

Absorption spectra were obtained with a Kontron Unikon spectrophotometer, and corrected fluorescence spectra with a Spex Fluorolog F2B spectrofluorimeter equipped with DM1B data-processing unit. Protein determination was performed with the Lowry reagent, with bovine serum albumin as standard.

Cell growth

Bacillus subtilis strain LC33, derived from strain QB928, was grown in medium G as described previously (Wong, 1983) and supplemented with 5 μg of either tryptophan or DL-4-fluorotryptophan/ml. Cells grown to mid-exponential phase at 37 °C were harvested by centrifugation, washed twice with 50 mM-Tris/HCl buffer, pH 7.2, frozen in liquid N_2 and stored at −80 °C.

Ribosomal proteins

Preparation of ribosomes and ribosomal proteins was performed as described previously (Milne *et al.*, 1975). The extracted proteins in 66% (v/v) acetic acid containing 30 mM-magnesium acetate were injected directly into a Synchropak RP-P C_{18} column as suggested by Ferris *et al.* (1984). H.p.l.c. was carried out at room temperature in accordance with Kerlavage *et al.* (1983), except for the use of substantially shortened elution times to limit exposure to acidic conditions. Gradient solvents were aq. 0.1% (v/v) trifluoroacetic acid, pH 2.1 (solvent A), and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). A convex gradient (curve 5, Waters Programmer) of 15% to 45% (v/v) solvent B was run for 50 min, 45% (v/v) solvent B was further run isocratically for 5 min, and finally a linear gradient of 45% to 100% (v/v) solvent B was run for 25 min. The flow rate was held constant at 0.7 ml/min. Peaks from the chromatograms were collected, and dialysed overnight against 6% (v/v) acetic acid at 4 °C before fluorimetry.

RESULTS

Tryptophan and its derivatives 4-fluoro-, 5-fluoro- and 6-fluoro-tryptophan as well as 5-methyltryptophan all displayed u.v. absorbance near 280 nm (Fig. 1). In the excitation and emission fluorescence spectra presented in Fig. 2 4-fluorotryptophan distinguished from the other compounds by a surprising lack of significant fluorescence.

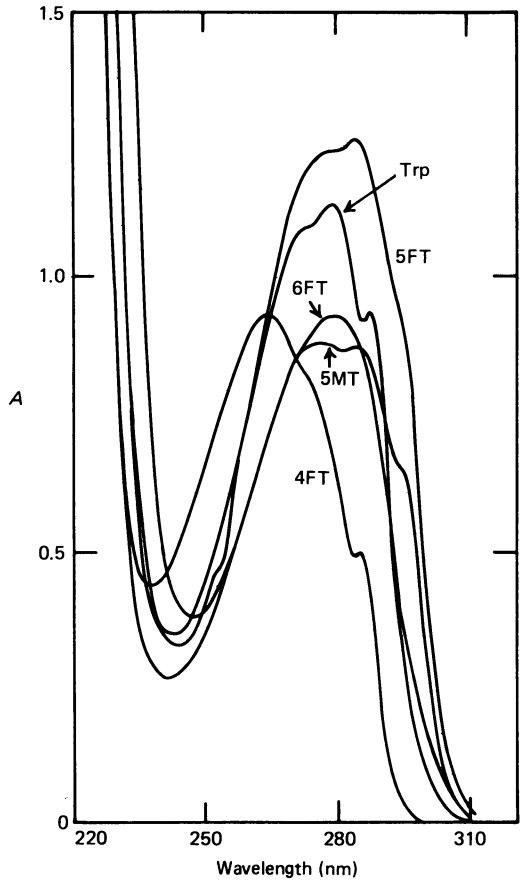

Fig. 1. Absorption spectra of tryptophan (Trp) and derivatives

All concentrations were 0.20 mM. Abbreviations: 4FT, 4-fluorotryptophan; 5FT, 5-fluorotryptophan; 6FT, 6-fluorotryptophan; 5MT, 5-methyltryptophan.

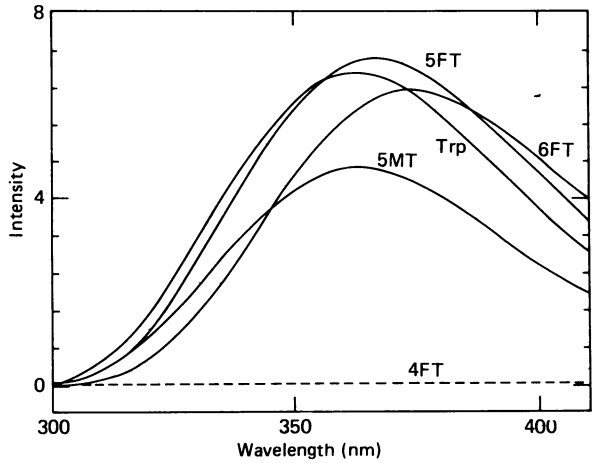

Fig. 2. Fluorescence spectra of tryptophan (Trp) and derivatives

Excitation was at 280 nm. Abbreviations are as defined in Fig. 1 legend.

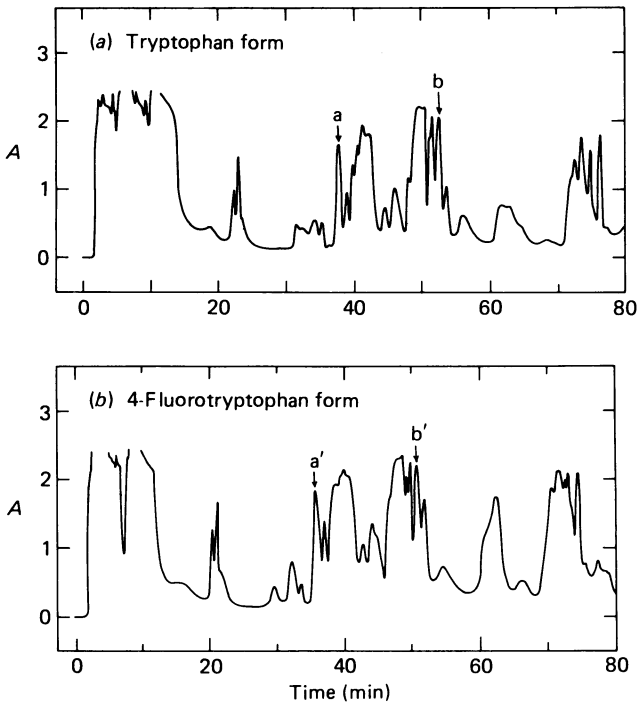

Fig. 3. H.p.l.c. profiles of tryptophan and 4-fluorotryptophan forms of ribosomal proteins

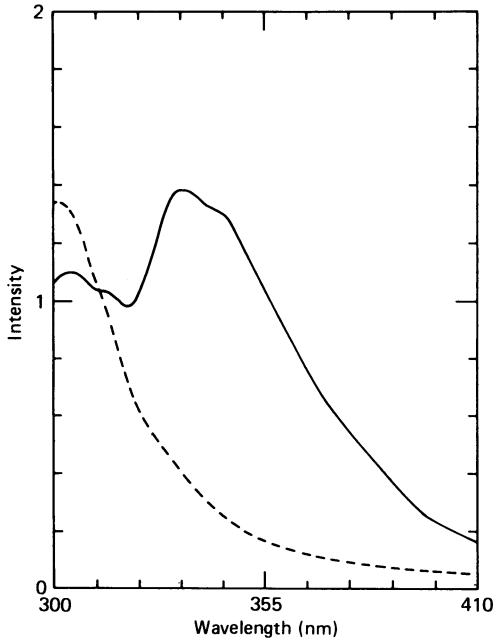

Fig. 4. Fluorescence of tryptophan (——) and 4-fluorotryptophan (– – – –) forms of total ribosomal proteins, normalized to the same protein content

The contrast between tryptophan and 4-fluorotryptophan with respect to fluorescence persisted when these amino acids were incorporated into proteins. By growing *B. subtilis* LC33 cells on either tryptophan or 4-fluorotryptophan, their proteins were obtainable in either tryptophan- or 4-fluorotryptophan-containing forms. Since the ribosome is characterized by a constant and well-defined complement of protein components, these components would be identical in the two types of *B. subtilis* LC33 cells. This expectation was confirmed by the similar chromatograms of ribosomal proteins pre-

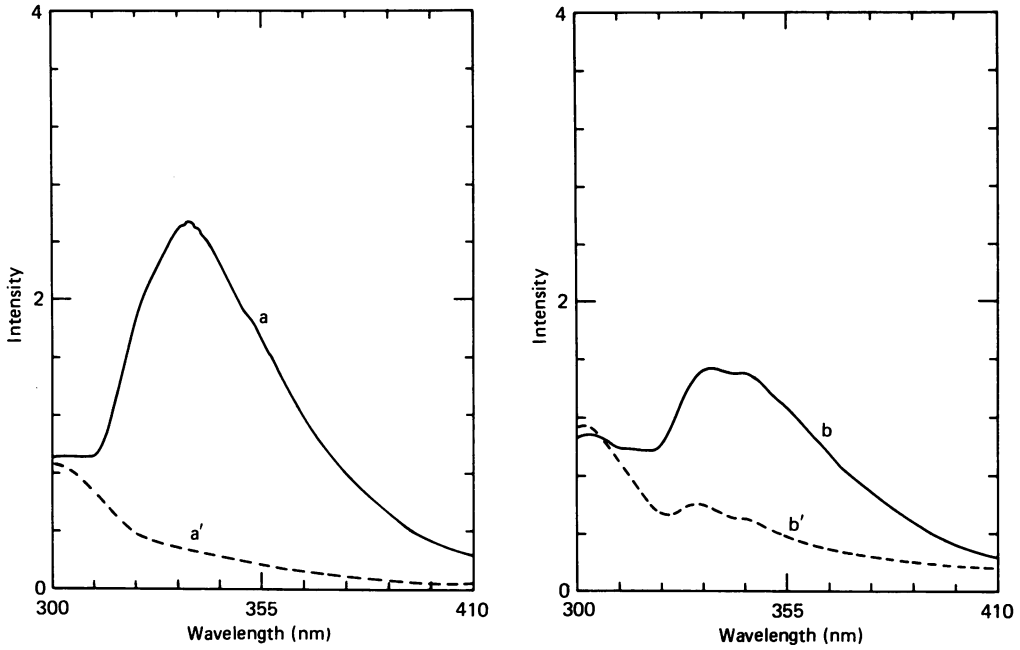

Fig. 5. Fluorescence of corresponding fractions of ribosomal proteins, normalized to the same protein content

Peaks a and b (tryptophan form) and a′ and b′ (4-fluorotryptophan form) were from the chromatograms shown in Fig. 3.

pared from tryptophan- and 4-fluorotryptophan-grown cells (Fig. 3). When total ribosomal proteins (Fig. 4) or corresponding peaks from the two chromatograms (Fig. 5) were compared for fluorescence, the fluorescence peak near 355 nm characteristic of tryptophan was evident in the tryptophan-containing proteins, but clearly suppressed in the 4-fluorotryptophan-containing proteins. The fluorescence peak shown by the 4-fluorotryptophan-containing proteins near 300 nm probably stemmed from tyrosine and phenylalanine residues, which are known to fluoresce in that region.

DISCUSSION

Halogenation is known to perturb the fluorescence of aromatic systems, but the effect increases with the atomic number of the halogen (Becker, 1969). The spectra of 5-fluoro-, 6-fluoro- and 5-methyl-tryptophan in Fig. 1, exhibiting some change in fluorescence intensity and peak position relative to tryptophan, are therefore not unexpected. The extreme behaviour of 4-fluorotryptophan is clearly exceptional. Although the mechanistic basis for its loss of fluorescence is unclear, the striking dependence of fluorescence on the position of indole ring substitution is consistent with observations on the monoazaindoles: the relative fluorescence intensities of 4-, 5-, 6- and 7-azaindoles are respectively 1.09, 0.27, 2.16 and 0.38, thus varying over an 8-fold range (Adler, 1962). The loss lends itself to application in protein investigations. Tryptophan fluorescence has long provided an intrinsic probe for monitoring protein structure and conformation. A number of experimental situations would benefit, however, from a suppression of this fluorescence. Thus suppression would facilitate the observation of (*a*) fluorescence by tyrosine or phenylalanine residuess in proteins, which is often masked by the stronger fluorescence of tryptophan, (*b*) fluorescence of exogenous probes that are covalently attached to defined residues on a protein, and overlap spectrally with tryptophan, and (*c*) protein interactions with fluorescent ligands that overlap spectrally with tryptophan residues in the protein, including the interactions of tryptophyl-tRNA synthetase, elongation factor or peptidases with free tryptophan or tryptophan ester as a ligand. In these systems the 4-fluorotryptophan in the protein will be deficient in fluorescence, but could still suppress the fluorescence of tyrosine or other fluorescent groups if resonance transfer is able to operate through proximity and spectral overlap (Stryer, 1978).

Constancy of ribosome structure in tryptophan-grown and 4-fluorotryptophan-grown cells is suggested by the similar h.p.l.c. profiles of the two sources of ribosomal proteins (Fig. 3). This constancy in turn ensured the comparability of these proteins from the two types of cells. Upon comparison, the suppressed fluorescence of the 4-fluorotryptophan-containing proteins becomes evident. Furthermore, since the bacterial ribosome contains more than 50 protein components, the observed suppression of fluorescence of total ribosomal proteins (Fig. 4) indicates a phenomenon general to different cell proteins rather than a phenomenon restricted to a small number of proteins. H.p.l.c. separates the ribosomal proteins into multiple peaks, some but not all of which would be individual proteins (Kerlavage *et al.*, 1983). Spectral analysis of the two most sharply defined peaks from the chromatograms in Fig. 3 indicates that suppression was observable with separated ribosomal protein fractions as much as with total unfractionated ribosomal proteins. Fig. 5 shows, however, that these separated fractions from 4-fluorotryptophan-grown cells exhibited significant residual fluorescence in the neighbourhood of 330 nm. Since proteins from 4-fluorotryptophan-grown cells contained little unfluorinated tryptophan (Wong, 1983), the residual fluorescence could be due to fluorescence of tyrosine or phenylalanine in these particular proteins. That one or more special 4-

fluorotryptophan residues within these proteins might have acquired significant fluorescence on account of environmental modulation within the molecules is also not ruled out, along with the possibility of some defluorination of such special 4-fluorotryptophan residues. In any event, through suppression of tryptophan fluorescence the way is now open to detailed investigations of the usually hidden chromophores in proteins.

Growth of amino acid auxotrophs on fluorinated amino acid analogues previously has made possible the introduction of fluorine atoms into proteins to enable ^{19}F-n.m.r. investigations (Sykes *et al.*, 1974; Pratt & Ho, 1975). Such introduction is performed readily on non-essential proteins, the synthesis of which could be turned on only after the cells are placed in a growth medium constituted with a fluorinated analogue. Labelling with the fluorinated analogue as a rule could not be complete in the case of proteins that are essential to cell growth, since with the notable exception of trifluoroleucine bacterial cells have resisted indefinite propagation on a fluorinated analogue. That LC33 cells may be propagated on 4-fluorotryptophan thus facilitates a complete, and therefore uniform, fluorine labelling of tryptophan residues in essential proteins such as DNA polymerases, RNA polymerases, aminoacyl-tRNA synthetases and ribosomal proteins. Furthermore, by means of cloning into LC33 cells, protein sequences originating from organisms other than *B. subtilis* will become equally obtainable in a uniformly fluorine-labelled fluorescence-suppressed form for either ^{19}F-n.m.r. or fluorescence analysis.

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